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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Manuscript received October 9, 1995
Accepted for publication February 27, 1996

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 *trans*-activation 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 coordinately modulates the activities of both the Sec14p/Golgi pathway and the pathway through which transcription of phospholipid biosynthetic genes is derepressed.

THE *Saccharomyces cerevisiae SEC14* gene product (Sec14p) is a phosphatidylinositol (PI)/phosphatidylcholine (PC) transfer protein that localizes as a peripheral Golgi membrane protein and is required for secretory protein transport from a late Golgi compartment (BANKAITIS *et al.* 1989, 1990; CLEVES *et al.* 1991b). Mutations in any one of at least seven genes effect an efficient bypass of Sec14p, and studies have established that genetic inactivation of PC biosynthesis via the CDP-choline pathway represents one mechanism by which bypass suppression of Sec14p function can be realized (CLEVES *et al.* 1991b; Figure 1). Indeed, three of the seven known classes of "bypass Sec14p" mutations define structural genes for enzymes of the CDP-choline pathway (CLEVES *et al.* 1991b; MCGEE *et al.* 1994a). Biochemical and genetic evidence suggests that the PC-bound form of Sec14p functions on Golgi membranes to down-regulate the activity of the rate-determining enzyme of the CDP-choline pathway, thereby creating a sensitive feedback loop that couples the activity of the CDP-choline pathway to Golgi PC levels (MCGEE *et al.* 1994a; SKINNER *et al.* 1995). The precise basis for why CDP-choline pathway activity might be toxic to the function of yeast Golgi membranes, however, remains obscure.

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"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 initially 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 effects a tight inositol auxotrophy in yeast. These phenotypes are reminiscent of those observed for *sac1* mutants of yeast (CLEVES *et al.* 1989; WHITTERS *et al.* 1993), and these phenotypic similarities suggest the possibility that *sac1*- 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

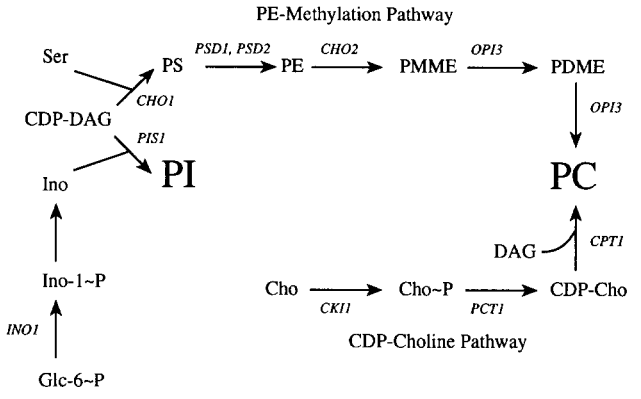


FIGURE 1.—The CDP-choline and PE-methylation pathways for PC biosynthesis are shown along with the pathway for phosphatidylinositol (PI) and phosphatidylserine (PS) biosynthesis in yeast. Serine, diacylglycerol, CDP-diacylglycerol, glucose-6-phosphate, inositol, inositol-6-phosphate, choline, choline-phosphate, CDP-choline, phosphatidylethanolamine, phosphatidylmono- and dimethylethanolamine are indicated as Ser, DAG, CDP-DAG, Glc-6~P, Ino, Ino-1~P, Cho, Cho~P, CDP~Cho, PE, PMME, and PDME, respectively. Genetic designations for the structural genes that encode relevant enzymes of these pathways are also indicated at their corresponding execution points. Lipid metabolism in yeast has been reviewed (CARMAN and HENRY 1989).

contribute to the "bypass Sec14p" phenotype of these strains. Finally, the data indicate that PI is subject to accelerated turnover in *BSD2-1* mutants and that both this accelerated rate of PI metabolism and *BSD2-1*-mediated suppression of *sec14* growth and secretory defects are negated by overproduction of the *SAC1* gene product (Sac1p).

The collective data suggest that the *BSD2-1* gene product executes its function at the divergence of two distinct cellular pathways: a Sec14p-dependent pathway for yeast Golgi secretory function and an Ino2p-dependent pathway through which yeast signal the transcriptional derepression of phospholipid biosynthetic genes. We propose that these effects are mediated through accelerated PI turnover that is, either directly or indirectly, the result of *BSD2-1* gene product activity. We suggest that a product(s) generated by such accelerated PI turnover imposes an independent regulation of the activities of the Sec14p- and Ino2p-dependent pathways, respectively.

MATERIALS AND METHODS

Yeast strains, plasmids, media and reagents: The genotypes of the yeast strains used in this study are provided in Table 1. YP, YPD and yeast minimal media have been described (SHERMAN *et al.* 1983). Yeast minimal media that either lack or contain inositol (1 mM) and choline (1 mM) have been described (KLIG *et al.* 1985). [^{14}C]-chloramphenicol, [^{32}P]-orthophosphate, [methyl-^{14}C]-methionine, and [^{3}H]-inositol were purchased from Amersham Co. (Arlington Heights, IL). *myo*-Inositol, choline chloride, acetyl-CoA, and reagents for invertase assays were obtained through Sigma Chemical Co. (St. Louis, MO).

YCp(*INO2, URA3*) and YEp(*INO2, URA3*) plasmids were constructed by subcloning *INO2* as a 2.7-kb *Pst*I-*Hind*III restriction fragment from pSC1E (HOSAKA *et al.* 1994) into the YCp*lac*33 and YEp*lac*195 vectors, respectively (GIETZ and SUGINO 1988). The YCp(*SAC1, URA3*) and YEp(*SAC1, URA3*) plasmids employed in these studies were described as pCTY101 and pCTY134, respectively (WHITTERS *et al.* 1993). The YEp(*INO4, URA3*) plasmid employed carries a 1.35-kb *INO4* restriction fragment in YEp352 (HILL *et al.* 1986) and was generously provided by S. HENRY (Carnegie Mellon University, Pittsburgh, PA). The *P*INO2::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 olionucleotides 5′-CCGAGCTCAATATTGCTCCAATCACC-3′ and 5′-CCGCATGCCTTTAAGAACGAAGTGAC-3′ as forward and reverse primers, respectively. Codons 3 and 4 (GAAGAT) of *INO1* in the forward primer were converted to an *Sst*I site (underlined) in a manner that did not alter Ino1p primary sequence, and an *Sph*I site (GCATGC) was engineered at the 5′ end of the reverse primer. The *INO1* PCR product was digested with *Sst*I and *Sph*I, and the resultant fragment was inserted into pTZ19R vector to yield pRE91.

A 381-bp SEC14 promoter region was amplified by the PCR using pRE71 including the entire *SEC14* gene as a template, and oligonucleotides 5′-GGGAATTCACGCGTGAATATCTTCCTC-3′ and 5′-CCGAGCTC**TGT**CATTGTGTTTTACCCGC-3′ as forward and reverse primers, respectively. An *Eco*RI site (GAATTC) was engineered at the 5′ end of the forward primer. In the reverse primer, codon 2 (GTT) 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 *Sst*I site (GAGCTC) engineered adjacent to codon 2 to facilitate construction of the fusion. The PCR product was digested with *Eco*RI and *Sst*I, and the resultant fragment was inserted into the corresponding sites of pRE91 to yield pRE90. The 2.2-kb *Eco*RI-*Sph*I fragment of pRE90 was then subcloned into YCp*lac*33 and YEp*lac*195 to yield the YCp(*PSEC14::INO1*) and YEp(*PSEC14::INO1*) plasmids; pCTY174 and pCTY175, respectively. The translational fusion resulted in expression of Ino1p under Sec14p 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 H_2O, washed once with minimal medium lacking inositol and choline (I^-C^- medium), and resuspended in the same I^-C^- medium at a density of 1×10^6 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°. Total cell numbers were determined by direct microscopic counting using a hemacytometer.

Phospholipid analyses: For steady-state [^{32}P]-radiolabeling experiments, the appropriate yeast strains were grown overnight in minimal medium lacking choline and subcultured into the same medium. The cultures were subsequently presented with [^{32}P]orthophosphate (10 μCi/ml) and incubated for 20–24 hr at 26° with shaking. For pulse-radiolabeling experiments, cells were cultured in choline- and methionone-free minimal medium and challenged with [*methyl*-^{14}C]-methionine (1 μCi/ml) for 30 min at 26°. Procedures for

TABLE 1

Yeast strains

Yeast strain	Genotype	Source
CTY1-1A	*MAT***a** *ura3-52 Δhis3-200 lys2-801 sec14-1^{ts}*	BANKAITIS *et al.* (1989)
CTY182	*MAT***a** *ura3-52 Δhis3-200 lys2-801*	BANKAITIS *et al.* (1989)
CTY212	*MATα BSD2-1 sec14-1^{ts} ade2-101 his3-200 ura3-52*	This study
CTY214	*MAT***a** *sec14-1^{ts} ade2-101 his4-519 leu2-3, 112 ura3-52*	This study
CTY417	*MATα ino1-13*	S. HENRY
CTY424	*MAT***a** *opi1::LEU2 his3-11*	This study
CTY459	*MAT***a** *ino2::TRP1*	This study
CTY479	*MATα ura3-52 Δhis3-200 ade2-101 BSD2-1*	MCGEE *et al.* (1994b)
CTY483	*MATα ura3-52 sec14-1^{ts} ade2 ade3 Δhis3 leu2-3,112 ura3-52*	This study
CTY811	CTY182/YCp*lac33*	This study
CTY813	*MAT***a** *ura3-52 Δhis3-200 lys2-801 sec14-1^{ts}*/YCp*lac33*	This study
CTY832	CTY479/YCp(*INO2*)	This study
CTY834	*MAT***a** *sec14-1^{ts} ade2-101 his4-519 leu2-3, 112 ura3-52 ino2::LEU2*	This study
CTY856	*MAT***a** *ura3-52 Δhis3-200 leu2-3, 112 ino2::LEU2*	This study
CTY857	CTY417/YEp(*PSEC14::INO1*)	This study
CTY858	CTY479/YEp(*PSEC14::INO1*)	This study
CTY859	CTY479/YEp(*SAC1*)	This study
CTY860	CTY479/YCp*lac33*	This study
CTY865	*MAT***a** *ade2-101 Δhis3-200 BSD2-1 leu2-3, 112 opi1::LEU2*	This study
CTY866	*MATα ade2-101 ura3-52 Δhis3-200 leu2-3,112 opi1::LEU2 BSD2-1*	This study
CTY878	CTY479/YCp(*INO4*)	This study
CTY879	CTY479/YCp(*PSEC14::INO1*)	This study
CTY880	CTY479/YCp(*SAC1*)	This study
CTY881	CTY417/YCp(*PSEC14::INO1*)	This study
CTY882	CTY212/YCp(*SAC1*)	This study
CTY883	CTY212/YEp(*SAC1*)	This study
CTY884	CTY212/YCp(*INO2*)	This study
CTY903	CTY832/YEp(*SAC1*)	This study
CTY904	CYT182/YCp(*INO2*)	This study
CTY905	CTY904/YEp(*SAC1*)	This study
CTYD162	*MAT***a**/*MATα ura3/ura3Δhis3/Δhis3 lys2/+ +/ade2 +/BSD2-1*	This study

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

phospholipid extraction and thin-layer chromatography have been described (MCGEE *et al.* 1994a). Resolved phospholipids were visualized and quantitated by phosphorimaging using the PhosphorImager 425 instrument marketed by Molecular Dynamics (Sunnyvale, CA).

Northern blot hybridization: The appropriate yeast strains were grown overnight in I^+C^+ minimal medium. The cells were washed three times with I^-C^- minimal medium and incubated in I^-C^- medium for 4 hr at 30° to permit derepression of transcription of phospholipid biosynthetic genes. Total RNA extraction, hybridization conditions, and probes used for specific detection of the *CHO2, OPI3, PIS1, CHO1* and *INO1* genes have been described in detail elsewhere (HOSAKA *et al.* 1994).

Measurement of *PINO2::CAT* activity: Yeast strains carrying the *PINO2::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 26°. 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 [^{14}C]-chloramphenicol (GORMAN *et al.* 1982). Radiolabeled chloramphenicol species were resolved by thin-layer chromatography using a chloroform:methanol (95:5) solvent system, and the two radiolabeled chloramphenicol species were quantitated using a PhosphorImager 425 instrument (Molecular Dynamics). CAT activity was expressed as percent conversion of [^{14}C]-chloramphenicol substrate to the acetylated product/microgram protein/hour.

***ino2::LEU2* strain construct:** A 4.9-kb *Pst*I-*Hin*dIII fragment derived from pSC1EL, a plasmid that harbors an *ino2::LEU2* disruption allele where *LEU2* has been inserted into the unique *Hpa*I site within the *INO2* coding region (HOSAKA *et al.* 1994), was introduced into the appropriate yeast strains (CTY214 and CTY483) by selection for Leu^+ using the lithium acetate transformation method (ITO *et al.* 1983). The resulting Leu^+ transformants were confirmed to have experienced the expected recombinational events by their acquisition of a YCp(*INO2*)-remedial Ino^- phenotype and by genomic Southern blot analysis.

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 26°. Cells were washed and shifted to YP + 0.1% glucose medium for 2 hr at 37°. Total and extracelluar 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 (I^- medium). The cells were concentrated in a 1 ml volume and presented with [^{3}H]-inositol (8 mCi/ml; Amersham Co.) for 30 min at 26° with shaking. The radiolabeled cells were then washed three times in the same I^- 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 [^{3}H]-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, [^{3}H]-PI was quantitated by direct liquid scintillation counting of the organic extracts.

Incorporation of [^{3}H]-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° for 2 days, and counting of colony forming units.

RESULTS

Characterization of Ino$^-$ phenotype of *BSD2-1* mutants: We had previously reported that Δ*sac1* 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 Δ*sac1* strains also exhibit an inositol auxotrophy (Whitters *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 *BSD2-1* mutants also exhibited a strict requirement for exogenous inositol for growth. A comparison of the growth characteristics of wild-type, *ino1-13* and *BSD2-1* strains on inositol-replete and inositol-free media is depicted in Figure 2A. The *ino1-13* strain is defective in the activity of Ino1p, 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 *ino1-13* and *BSD2-1* strains exhibited clear Ino$^-$ phenotypes (Figure 2A). Interestingly, most heterozygous *BSD2/BSD2-1* diploid strains tested were Ino$^+$, indicating that the Ino$^-$ phenotype associated with *BSD2-1* most often behaved as a recessive trait (see Figure 2A). However, we have always found *BSD2-1* to score as a dominant trait with regard to suppression of *sec14* growth and secretory defects (Cleves *et al.* 1991b; not shown).

To further characterize the Ino$^-$ phenotype of *BSD2-1* strains, we compared the effects of inositol starvation on the viability of *ino1-13* and *BSD2-1* strains. As shown in Figure 2B, both *ino1-13* and *BSD2-1* strains maintained viability for ≥4 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 *ino1-13* strain. Whereas the *ino1-13* mutant suffered a 1000-fold reduction in viablity after a 24-hr period of inositol starvation, the *BSD2-1* strain experienced a 40- to 50-fold reduction in viability. The kinetics of inositol-less death for *ino1-13* and *BSD2-1* 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 *ino1-13* 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 *BSD2-1* mutant (not shown). These comparative pool measurements demonstrated that the more rapid kinetics of inositol-less death in *ino1-13* strains (relative to *BSD2-1* mutants) correlated with a considerably sharper decline in intracellular inositol pool size in the *ino1-13* strain.

Specific duplication of *INO2* effects a suppression of the *BSD2-1* inositol auxotrophy: Characterization of the *BSD2* gene has been precluded by our failure to recover *BSD2-1* clones on the basis of suppression of *sec14* growth defects. The recessive inositol auxotrophy of *BSD2-1* strains provided the alternative strategy of recovering *BSD2* clones by complementation of the Ino$^-$ phenotype of *BSD2-1* strains. Strain CTY479 (*ura3-52, BSD2-1*) 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 *BSD2-1* 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 Δ*ino2*.

The possibility that *INO2* and *BSD2* represented the same gene was tested both by attempts to genetically inactivate *BSD2-1* by introduction of Δ*ino2* alleles into *BSD2-1 sec14-1*^{ts} strains and by meiotic segregation analy-

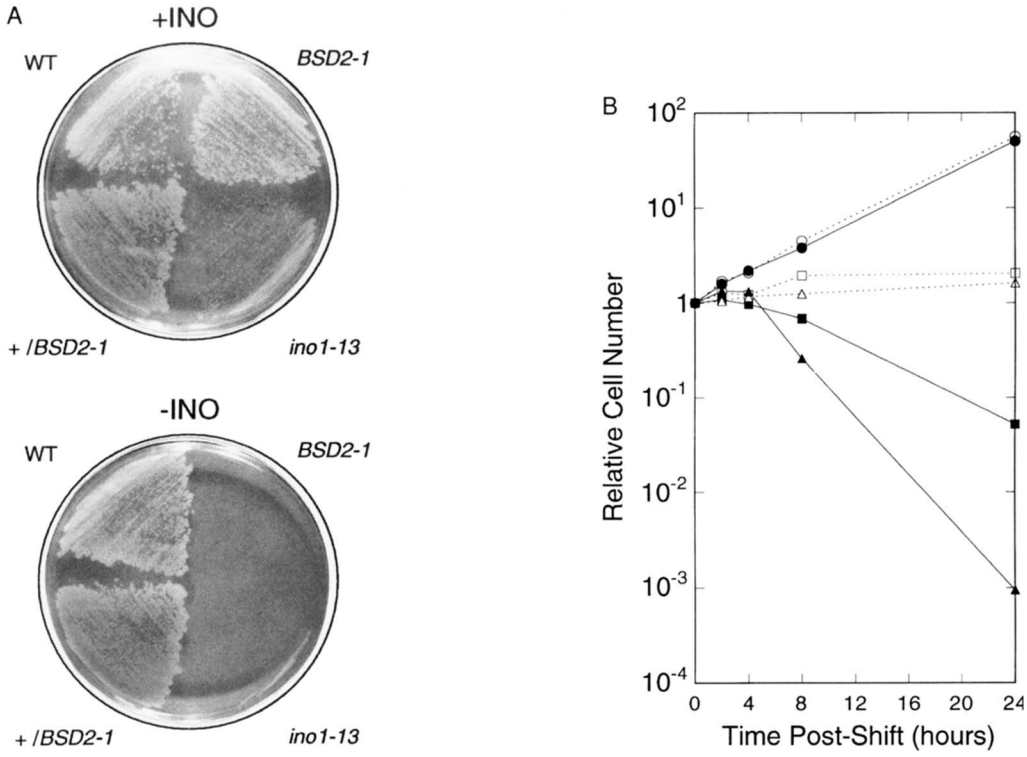


FIGURE 2.—Inositol auxotrophy of *BSD2-1* mutants. (A) Yeast strains with the indicated relevant genotypes were streaked for isolation on either inositol-containing (+INO), or inositol-free minimal medium (−INO), and incubated at 26° for 96 hr. (B) Effects of inositol starvation on cell viability. Yeast 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 ■, *BSD2-1* strain. Yeast strains employed in these experiments included: CTY182 (wild type); CTY479 (*BSD2-1*); CTY417 (*ino1-13*); CTYD162 (*MATα/MAT***a,** BSD2-1/+).

ses. With regard to the former approach, we were unable to eliminate the dominant "bypass Sec14p" phenotype in *BSD2-1*/+ heterozygous diploids that had been transformed with an *ino2*Δ allele, as would be expected if *INO2* and *BSD2* were allelic (not shown). These data suggested a nonallelism between *BSD2* and *INO2*. To confirm these results, integrative genetic mapping analyses were performed. A 2.5-kb *Hin*dIII-*Eco*RI fragment derived from the *INO2* locus was subcloned into the yeast integration vector YIplac211. The resultant plasmid (pCTY211) was linearized at a unique *Bgl*II site within *INO2* and integrated by homologous recombination into strain CTY1-1A (**a** *BSD2 sec14-1ts ura3-52*) with selection for Ura$^+$ transformants. The resulting strain was mated to strain CTY212 (*α BSD2-1 sec14-1*^{ts} *ura3-52*), the diploid sporulated, and meiotic progeny analyzed for linkage of Ura$^+$ to the *BSD2-1* locus. The *BSD2-1* 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 nonparental ditype:eight tetratype asci was recorded, and this lack of cosegregation of the Ura$^+$ and Ino$^-$/"bypass Sec14p" phenotypes was indicative of nonlinkage between the *BSD2-1* and *INO2* loci.

Neither increases in *INO4* gene dosage nor *opi1* mutations suppress the *BSD2-1* inositol auxotrophy: In addition to Ino2p, transcriptional regulation of yeast phospholipid (PL) biosynthetic genes is also responsive to the action of the *INO4* and *OPI1* gene products (HOSHIZAKI *et al.* 1990; WHITE *et al.* 1991). The *INO4* 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(*INO4*) plasmid nor of an *opi1::LEU2* allele restored an Ino$^+$ phenotype to *BSD2-1* strains. This was in contrast to the ability of a YCp(*INO2*) plasmid to confer an Ino$^+$ phenotype to the same *BSD2-1* strain. These collective data demonstrated a specificity of suppression of the *BSD2-1* inositol auxotrophy by duplication of *INO2* that was neither recapitulated by substantial increases in *INO4* dosage nor by genetic inactivation of the Opi1p-mediated repression of PL-biosynthetic gene expression. The *opi1::LEU2* result was unexpected as *opi1* mutations have the effect of upregulating Ino2p expression (ASHBURNER and LOPES 1995), an effect also realized

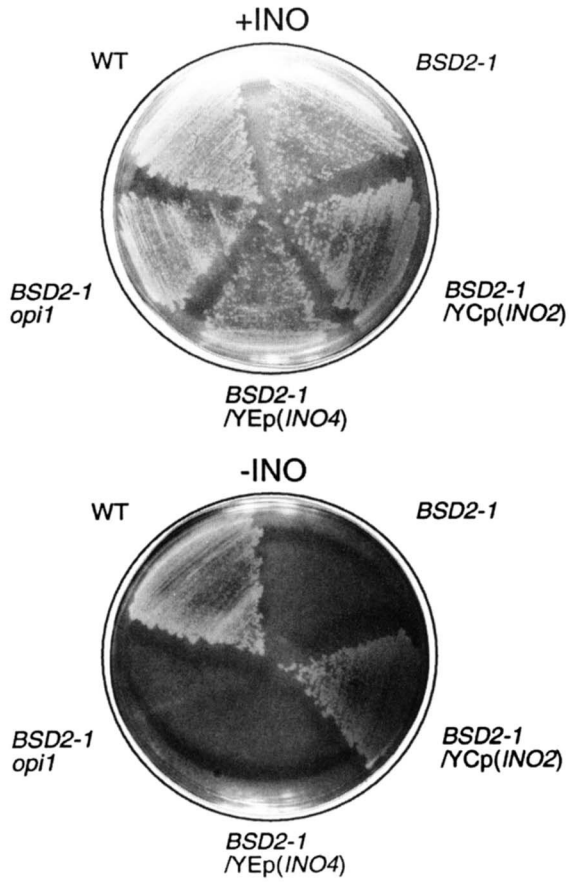


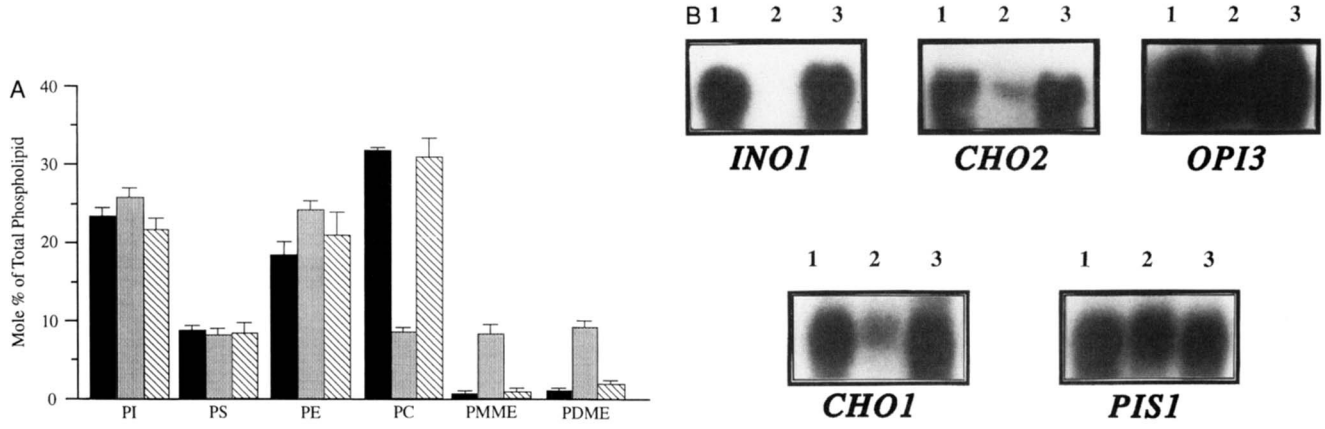
FIGURE 3.—*INO2* overexpression suppresses the inositol auxotrophy associated with *BSD2-1*. The appropriate yeast strains were streaked for isolation on uracil-deficient minimal medium that was either inositol-replete (+INO) or inositol-free (−INO), and incubated at 26° for 96 hr. Yeast strains employed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

by increased *INO2* dosage. This apparent paradox is resolved below.

***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 (*CHO2* and *OPI3* gene 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 Opi1p-dependent fashion (WAECHTER *et al.* 1969; KODAKI and YAMASHITA 1987; CARMAN and HENRY 1989). As shown in Figure 4A, the undermethylated PC precursors phosphatidylmonomethylethanolamine (PMME) and phosphatidyldimethylethanolamine (PDME) were detected only at very low steady-state levels in wild-type 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 abberant 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, CHO2, OPI3, CHO1,* 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, CHO2, OPI3* and *CHO1* 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 *ino1-13* 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 *ino1-13* and *BSD2-1* strains. This Ino$^+$ phenotype was dependent on the dosage of *PSEC14::INO1,* since neither *ino1-13* nor *BSD2-1* strains carrying YCp(*PSEC14::INO1*) were able to grow on inositol-free medium (Figure 4C). We pre-



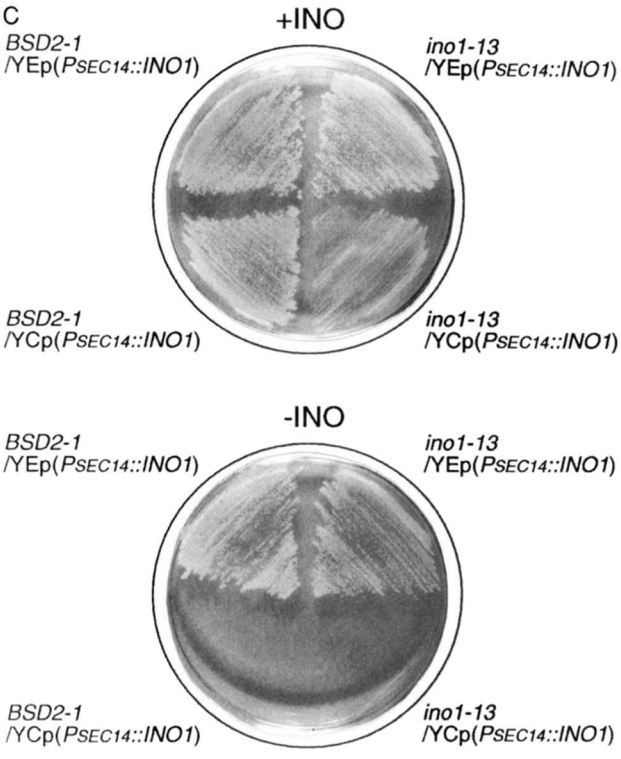


FIGURE 4.—*BSD2-1* mutants are defective in PL-biosynthetic activities whose expression is regulated by the *INO2* gene product. (A) Phospholipid composition of bulk yeast membranes. The appropriate wild-type (solid bars), *BSD2-1* (stippled bars), and *BSD2-1*/YCp(*INO2*) strains (striped bars) were grown for 20–24 hr at 26° in choline-free minimal medium supplemented with [^{32}P]-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°, 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° 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*)]; CTY858 [*BSD2-1*/YEp(*PSEC14::INO1*)]; CTY881 [*ino1-13*/YCp(*PSEC14::INO1*)]; CTY857 [*ino1-13*/YEp(*PSEC14::INO1*)].

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 (*PINO2::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^-C^-) and repressing (I^+C^+) 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-

TABLE 2

Regulation of *PINO2::CAT* gene expression

Genotype	CAT activity		*INO2* derepression ratio
	I^-C^-	I^+C^+	
Wild type	2.88 ± 0.26	0.24 ± 0.05	12.0
BSD2-1	0.0125 ± 0.0003	0.0053 ± 0.0016	2.4
BSD2-1 opi1::LEU2	0.0128 ± 0.0009	0.0036 ± 0.0016	3.6
ino2::TRP1	0.0067 ± 0.0005	0.0067 ± 0.0007	1.0

The *PINO2::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 (I^-C^-), or in inositol and choline-replete medium (1 mM final concentration for inositol and choline; I^+C^+). *INO2* transcriptional derepression ratios for these strains were calculated as the quotient of averaged CAT activities measured in I^-C^- medium divided by averaged CAT activities measured in I^+C^+ medium.

sis of *BSD2-1* over *opi1::LEU2* with regard to *INO2* expression accounted for the somewhat paradoxical finding that the *opi1::LEU2* mutation failed to suppress the Ino^- phenotype associated with *BSD2-1* (see above; Figure 3).

The Ino^- and "bypass Sec14p" phenotypes 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 *INO2* restored a Golgi secretory block to *BSD2-1 sec14-1*^{ts} strains. As shown in Figure 5, introduction of a YCp(*INO2*) plasmid into a *BSD2-1 sec14-1*^{ts} strain did not compromise the ability of such a strain to grow at 37° relative to an isogenic strain that was not subject to increased *INO2* dosage. Quantitation of the invertase secretory efficiencies of these strains at 37° 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 *sec14-1*^{ts} strains at 37° (Figure 5A) and that this *ino2* disruption allele did not significantly alleviate the *sec14-1*^{ts} 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 *sec14-1*^{ts} *ino2::LEU2* strain carrying YEp(*PSEC14::INO1*). As this strain expresses *INO1* in an Ino2p/Ino4p-independent fashion, it can be grown on either I^+C^+ or I^-C^- media to assess any effects of growth medium on suppression.

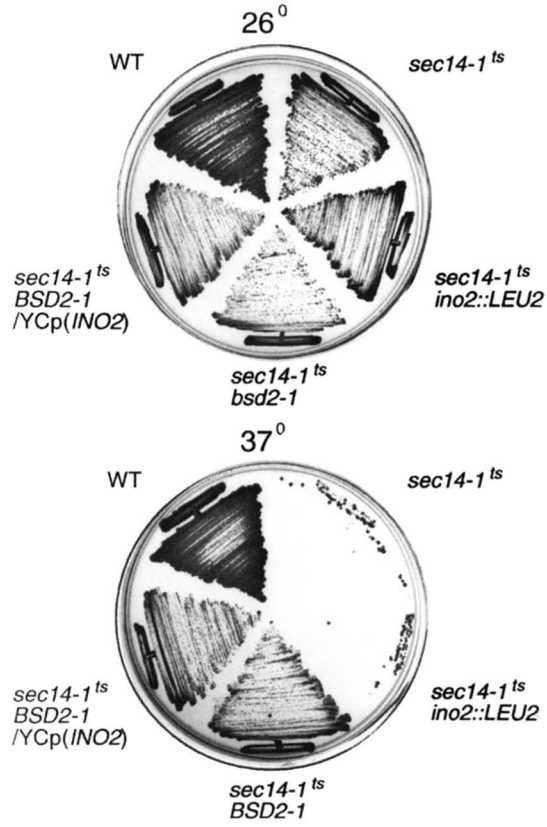


Figure 5.—*INO2* is irrelevant to the bypass Sec14p phenotype of *BSD2-1* mutants. Yeast strains of the indicated genotypes were streaked and incubated on YPD medium at 26° or 37°, as indicated, for 48 hr.

TABLE 3

Efficiency of invertase secretion

Relevant genotype	Secretion index
Wild type	0.93 ± 0.06
sec14-1^{ts}	0.31 ± 0.07
sec14-1^{ts} *BSD2-1*	0.87 ± 0.04
sec14-1^{ts} *BSD2-1*/YCp(*INO2*)	0.93 ± 0.07
sec14-1^{ts} *ino2::LEU2*	0.32 ± 0.08
sec14-1^{ts} *BSD2-1*/YCp(*SAC1*)	0.91 ± 0.07
sec14-1^{ts} *BSD2-1*/YEp(*SAC1*)	0.35 ± 0.08

The appropriate yeast strains (relevant genotypes indicated) were grown at 26° in YPD and shifted to YP + 0.1% glucose at 37° for 2 hr. Total and extracellular invertase were subsequently determined as described in the MATERIALS AND METHODS. The secretion index is expressed as the ratio of extracellular invertase to total invertase (SALAMA *et al.* 1990), and the values given represent averages from at least three independent experiments. The growth phenotypes of the corresponding strains at 37° are also indicated. Strains employed for these experiments included: CTY182 (wild type); CTY214 (*sec14-1*^{ts}); CTY834 (*sec14-1*^{ts} *ino2::LEU2*); CTY212 (*sec14-1*^{ts} *BSD2-1*); CTY884 [*sec14-1*^{ts} *BSD2-1*/YCp(*INO2*)]; CTY882 [*sec14-1*^{ts} *BSD2-1*/YCp(*SAC1*)]; CTY883 [*sec14-1*^{ts} *BSD2-1*/YEp-(*SAC1*)].

Such a strain was unable to grow at 37° regardless of which growth medium was employed (not shown); indicating that the failure of *ino2::LEU2* to suppress *sec14* 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 4.3 ± 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 [^{14}C]-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 Sac1p functions to prevent inappropriate turnover of inositol phospholipids (particularly PI) in cells (CLEVES *et al.* 1991a; WHITTERS *et al.* 1993). As loss of Sac1p function results in phenotypes that mimic those associated with the dominant *BSD2-1* mutation (*i.e.*, bypass suppression of *sec14* defects and an Ino$^-$ phenotype; CLEVES *et al.* 1989; WHITTERS *et al.* 1993), we considered the possibility that overproduction of Sac1p 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 Sac1p overproduction (ca. 15-fold; WHITTERS *et al.* 1993) was driven by a YEp-(*SAC1, HIS3*) plasmid. As shown in Figure 6A, overproduction of Sac1p 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 Sac1p in otherwise wild-type cells did not significantly affect the stability of bulk membrane PI, however (Figure 6A).

Overproduction of *SAC1* antagonizes suppression of *sec14-1*^{ts} by *BSD2-1*, but not the associated Ino$^-$ phenotype: Our finding that Sac1p overproduction precluded the accelerated turnover of PI in *BSD2-1* strains predicted that Sac1p overproduction would reimpose *sec14*-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 *sec14* defects. The data demonstrating phenotypic fulfillment of this prediction are shown in Figure 6B. A *BSD2-1* *sec14-1*^{ts} strain transformed with a YEp(*SAC1*) plasmid failed to grow at the *sec14-1*^{ts}-restrictive temperature of 37° whereas the same strain transformed with a YCp-(*SAC1*) plasmid exhibited wild-type growth characteristics at this temperature. As expected, the *BSD2-1* *sec14-1*^{ts}/YEp(*SAC1*) strain exhibited reestablishment of the *sec14* secretory block at its restrictive temperature of 37° whereas the isogenic YCp(*SAC1*) partner exhibited wild-type secretory capacity (Table 3). These collective data suggest that Sac1p and the *BSD2-1* gene product have the capacity to interface with the same pathway for suppression of *sec14* defects and that this pathway likely involves accelerated PI turnover. The antagonistic effect of Sac1p overproduction on suppression of *sec14* growth and secretory defects was specific to *BSD2-1* mutants as introduction of YEp(*SAC1*) into *sec14-1*^{ts} strains carrying each of the other five classes of "bypass Sec14p" mutations (*i.e.*, *cki1, pct1, cpt1, bsr3,* and *BSD1;* CLEVES *et al.* 1991b; MCGEE *et al.* 1994a) did not measurably affect the efficiency with which these suppressed *sec14-1*^{ts} growth and secretory defects (not shown). Simi-

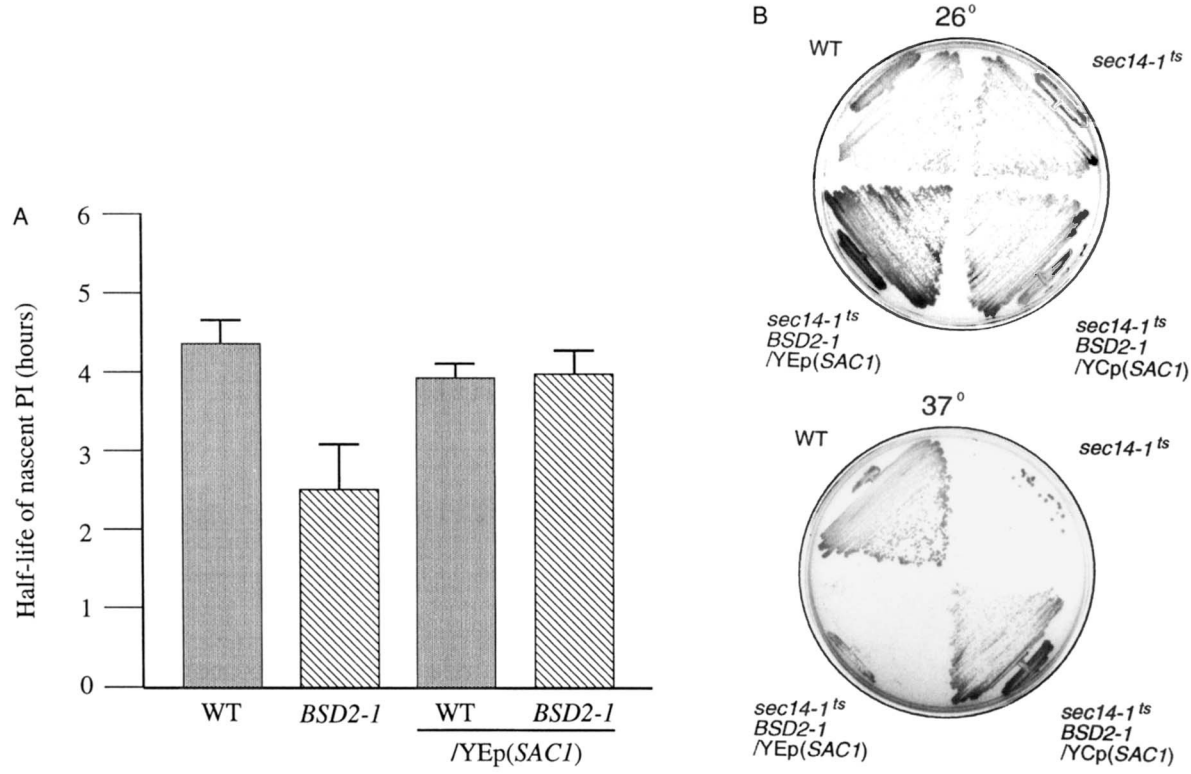


FIGURE 6.—Overproduction of Sac1p antagonizes the "bypass Sec14p" phenotype of *BSD2-1* mutants. (A) The *BSD2-1* mutation evokes an accelerated and Sac1p-remedial turnover of nascent bulk membrane PI. The appropriate strains were cultured in inositol-free medium, pulse-radiolabeled with [^{3}H]-inostol for 30 min and samples were harvested after various times of chase for extraction of phospholipid and quantitation of radiolabeled PI (see MATERIALS AND METHODS). PI half-lives were determined by plotting the logarithm of the relative amounts of radiolabeled PI remaining after 0, 1, 2, 3, and 4 hr of chase (0 time point set to 100%) as a function of time. Pulse-labeled PI exhibited uniform decay kinetics under the experimental time course employed (not shown), and turnover kinetics were determined from the slope of the decay curve. The PI half-lives given for each strain (relevant genotypes given at bottom) represent the averages obtained from at least three independent determinations. The Sac1p-overproducing strains are identified by the YEp(*SAC1*) designation that represents that overproducing plasmid vector employed. All of the strains employed in these experiments were genotypically wild type with respect to *SEC14* carried a YCp(*INO2*) plasmid, and these strains included: CTY904 (wild type), CTY832 (*BSD2-1*), CTY905 [wild type, YEp(*SAC1*)], and CTY903 [*BSD2-1* YEp(*SAC1*)]. (B) The appropriate strains were streaked for isolation on uracil-deficient minimal medium, and incubated at 26° or 37°, as indicated, for 48 hr. (C) Inositol auxotrophy of *BSD2-1* mutants carrying YCp(*SAC1*) or YEp(*SAC1*) plasmids. The appropriate strains (relevant genotypes indicated) were streaked for isolation on uracil-deficient minimal medium supplemented with inositol (+INO), or left unsupplemented (−INO), and incubated at 26° for 96 hr. Strains used for the experiments in B and C included: CTY811 (wild type); CTY813 (*sec14-1*^{ts}); CTY882 [*sec14-1*^{ts}, *BSD2-1*/YCp(*SAC1*)]; CTY883 [*sec14-1*^{ts}, *BSD2-1*/YEp(*SAC1*)]; CTY880 (*BSD2-1*/YCp(*SAC1*); CTY859 [*BSD2-1*/YEp(*SAC1*)].

larly, 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 *BSD2-1* 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 *BSD2-1* 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 *BSD2-1* strains is related to neither (not shown).

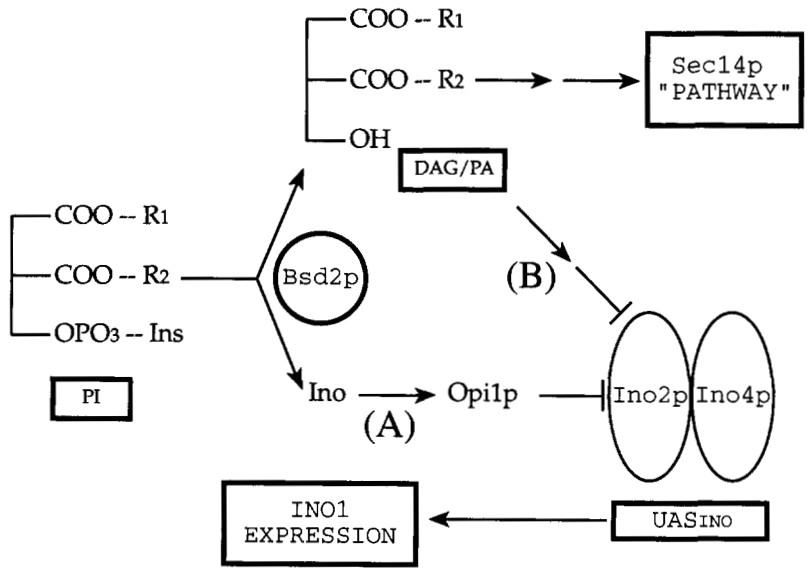


FIGURE 7.—The involvement of the *BSD2-1* gene product in both the Sec14p pathway and the pathway for transcriptional derepression of phospholipid-biosynthetic genes. In model A, the *BSD2-1* gene product functions to generate two signals each specific for the Sec14p and transcriptional signaling pathways, 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/Ino4p-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 effects 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 excude 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 *cis*-acting element through which Ino2p/Ino4p-mediated regulation of transcription is exerted, is designated as UAS_{INO}.

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 *opi1* 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 *BSD2-1* 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 *BSD2-1* is not executed through a global misregulation of PL-biosynthetic genes.

The demonstration that *BSD2-1* strains exhibited accelerated PI turnover (Figure 6A), when coupled with the observation that both the accelerated turnover of PI and *BSD2-1*-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 *BSD2-1* 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 *sec14-1*^{ts} 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 turnover coordinately influences the activities of the Sec14p- and Ino2p-dependent pathways (Figure 7). With regard to the Sec14p pathway, some pharmacological support for this idea comes from our preliminary data indicating that, while addition of phorbol esters to *sec14-1*^{ts} strains of yeast fails to effect rescue of the *sec14-1*^{ts} secretory block, challenge of such strains with a short chain diacylglycerol does evoke a partial suppression of the *sec14-1*^{ts} secretory block (not shown). This pharmacological rescue is not sufficiently powerful to phenotypically rescue *sec14-1*^{ts} 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 *BSD2-1* mutants to a common event influenced by the *BSD2-1* gene product (Figure 7). First, the data are consistent with a model where *BSD2-1* 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, *BSD2-1* 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 *BSD2-1* 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 postranslational modulation of transcription factor function (*e.g.,* through postranslational 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; LISCOVITCH and CANTLEY 1995).

We thank SUSAN HENRY and JOHN LOPES for yeast strains and plasmids and an anonymous referee for helpful comments. This work was supported by National Institutes of Health grant GM-44530 to V.A.B. S.K. and B.G.K. were supported by a postdoctoral fellowship from the Human Frontier Science Program (LT-396-93) and a National Science Foundation predoctoral traineeship (NSF-BIR-9256853), respectively.

LITERATURE CITED

ALB, J. G., JR., A. GEDVILAITE, R. T. CARTEE, H. B. SKINNER and V. A. BANKAITIS, 1995 Mutant rat phosphatidylinositol/phosphatidylcholine transfer proteins specifically defective in phosphatidylinositol transfer: implications for the regulation of phospholipid transfer activity. Proc. Natl. Acad. Sci. USA **92:** 8826–8830.

AMBROZIAK, J., and S. A. HENRY, 1994 *INO2* and *INO4* gene products, positive regulators of phospholipid biosynthesis in *Saccharomyces cerevisiae,* form a complex that binds to the *INO1* promoter. J. Biol. Chem. **269:** 15344–15349.

ASHBURNER, B. P., and J. M. LOPES, 1995 Autoregulated expression of the yeast *INO2* and *INO4* helix-loop-helix activator genes effects cooperative regulation on their target genes. Mol. Cell. Biol. **15:** 1709–1715.

BANKAITIS, V. A., D. E. MALEHORN, S. D. EMR and R. GREENE, 1989 The *Saccharomyces cerevisiae SEC14* gene encodes a cytosolic factor that is required for transport of secretory proteins from the yeast Golgi complex. J. Cell Biol. **108:** 1271–1281.

BANKAITIS, V. A., J. R. AITKEN, A. E. CLEVES and W. DOWHAN, 1990 An essential role for a phospholipid transfer protein in yeast Golgi function. Nature **347:** 561–562.

BROWN, H. A., S. GUTOWSKI, C. R. MOOMAW, C. SLAUGHTER and P. C. STERNWEIS, 1993 ADP-Ribosylation factor, a small GTP-dependent regulatory protein, stimulates phospholipase D activity. Cell **75:** 1137–1144.

CARMAN, G. M., and S. A. HENRY, 1989 Phospholipid biosynthesis in yeast. Annu. Rev. Biochem. **58:** 635–669.

CLEVES, A. E., P. J. NOVICK and V. A. BANKAITIS, 1989 Mutations in the *SAC1* gene suppress defects in yeast Golgi and yeast actin function. J. Cell Biol. **109:** 2939–2950.

CLEVES, A. E., T. P. MCGEE and V. A. BANKAITIS, 1991a Phospholipid transfer proteins: a biological debut. Trends Cell Biol. **1:** 30–34.

CLEVES, A. E., T. P. MCGEE, E. A. WHITTERS, K. M. CHAMPION, J. R. AITKEN *et al.*, 1991b Mutations in the CDP-choline pathway for phospholipid biosynthesis bypass the requirement for an essential phospholipid transfer protein. Cell **64:** 789–800.

COCKROFT, S., G. M. H. THOMAS, A. FENSOME, B. GENY, E. CUNNINGHAM *et al.*, 1994 Phospholipase D: a downstream effector of ARF in granulocytes. Science **263:** 523–526.

FERNANDEZ, S., M. J. HOMANN, S. A. HENRY and G. M. CARMAN, 1986 Metabolism of the phospholipid precursor inositol and its relationship to growth and viability in the natural auxotroph *Schizosaccharomyces pombe*. J. Bacteriol. **166:** 779–786.

GIETZ, R. D., and A. SUGINO, 1988 New yeast-*Escherichia coli* shuttle vectors constructed with *in vitro* mutagenized yeast genes lacking six-base pair restriction sites. Gene **74:** 527–534.

GORMAN, C. M., E. F. MOFFAT and B. H. HOWARD, 1982 Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell Biol. **2:** 1044–1051.

HAY, J. C., P. L. FISETTE, G. H. JENKINS, R. A. ANDERSON and T .J. F. MARTIN, 1995 ATP-dependent inositide phosphorylation required for Ca^{2+}-activated secretion. Nature **374:** 172–177.

HILL, J. E., A. M. MYERS, T. J. KOERNER and A. TZAGALOFF, 1986 Yeast/*E. coli* shuttle vectors with multiple unique restriction sites. Yeast **2:** 163–167.

HOSAKA, K., J. NIKAWA, T. KODAKI and S. YAMASHITA, 1994 Cloning and characterization of the *SCS1* gene required for the expression of genes in yeast phospholipid synthesis. J. Biochem. **115:** 131–136.

HOSHIZAKI, D. K., J. E. HILL and S. A. HENRY, 1990 The *Saccharomyces cerevisiae INO4* gene encodes a small, highly basic protein required for derepression of phospholipid biosynthetic enzymes. J. Biol. Chem. **265:** 4736–4745.

ITO, H., Y., FUKUDA, K., MURATA and A. KIMURA, 1983 Transformation of intact yeast cells treated with alkaline cations. J. Bacteriol. **153:** 163–168.

KLIG, L. S., M. J. HOMANN, G. M. CARMAN and S. A. HENRY, 1985 Coordinate regulation of phospholipid biosynthesis in *Saccharomyces cerevisiae*: pleiotropically consititutive *opi1* mutant. J. Bacteriol. **162:** 1135–1141.

KODAKI, T., and S. YAMASHITA, 1987 Yeast phosphatidylethanolamine methylation pathway: cloning and chracterization of two distinct methyltransferase genes. J. Biol. Chem. **262:** 15428–15435.

LISCOVITCH, M., and L. C. CANTLEY, 1995 Signal transduction and membrane traffic: the PITP/phosphoinositide connection. Cell **81:** 659–662.

MCGEE, T. P., H. B. SKINNER, E. A. WHITTERS, S. A. HENRY and V. A. BANKAITIS, 1994a A phosphatidylinositol transfer protein controls the phosphatidylcholine content of yeast Golgi membranes. J. Cell Biol. **124:** 273–287.

MCGEE, T. P., H. B. SKINNER and V. A. BANKAITIS, 1994b Functional redundancy of CDP-ethanolamine and CDP-choline pathway enzymes in phospholipid biosynthesis: ethanolamine-dependent effects on steady-state membrane phospholipid composition in *Saccharomyces cerevisiae*. J. Bacteriol. **176:** 6861–6868.

NIKOLOFF, D. M., and S. A. HENRY, 1994 Functional chracterization of the *INO2* gene of *Saccharomyces cerevisiae*: a positive regulator of phospholipid biosynthesis. J. Biol. Chem. **269:** 7402–7411.

NIKOLOFF, D. M., P. MCGRAW and S. A. HENRY, 1992 The *INO2* gene of *Saccharomyces cerevisiae* encodes a helix-loop-helix protein that is required for activation of phospholipid synthesis. Nucleic Acids Res. **20:** 3253.

SALAMA, S. R., A. E. CLEVES, D. E. MALEHORN, E. A. WHITTERS and V. A. BANKAITIS, 1990 Cloning and characterization of *Kluyveromyces lactis SEC14*, a gene whose product stimulates Golgi secretory function in *Saccharomyces cerevisiae*. J. Bacteriol. **172:** 4510–4521.

SHERMAN, F., G. R. FINK and J. B. HICKS, 1983 pp. 1–120 in *Methods in Yeast Genetics*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

SKINNER, H. B., J. G. ALB, JR., E. A. WHITTERS, G. M. HELMKAMP, JR. and V. A. BANKAITIS, 1993 Phospholipid transfer activity is relevant to but not sufficient for the essential function of the yeast *SEC14* gene product. EMBO J. **12:** 4775–4784.

SKINNER, H. B., T. P. MCGEE, C. R. MCMASTER, M. R. FRY, R. M. BELL *et al.*, 1995 The *Saccharomyces cerevisiae* phosphatidylinositol-transfer protein effects a ligand-dependent inhibition of cholinephosphate cytidylyltransferase activity. Proc. Natl. Acad. Sci. USA **92:** 112–116.

WAECHTER, C. J., M. R. STEINER and R. L. LESTER, 1969 Regulation of phosphatidylcholine biosynthesis by the methylation pathway in *Saccharomyces cerevisiae*. J. Biol. Chem. **244:** 3419–3422.

WHITE, M. J., J. P. HIRSCH and S. A. HENRY, 1991 The *OPI1* gene of *Saccharomyces cerevisiae*, a negative regulator of phospholipid biosynthesis, encodes a protein containing polyglutamine tracts and a leucine zipper. J. Biol. Chem. **266:** 863–872.

WHITTERS, E. A., A. E. CLEVES, T. P. MCGEE, H. B. SKINNER and V. A. BANKAITIS, 1993 SAC1p is an integral membrane protein that influences the cellular requirement for phospholipid transfer protein function and inositol in yeast. J. Cell Biol. **122:** 79–94.

Communicating editor: E. JONES