

SEC14-dependent Secretion in *Saccharomyces cerevisiae*

NONDEPENDENCE ON SPHINGOLIPID SYNTHESIS-COUPLED DIACYLGLYCEROL PRODUCTION*

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The *SEC14* gene in *Saccharomyces cerevisiae* encodes a phosphatidylinositol transfer protein required for secretory protein movement from the Golgi. Mutation of *SAC1*, a gene of unknown function, restores secretory flow in *sec14-1^{ts}* strains. The existing model for the bypass of the *sec14-1^{ts}* defect by *sac1-22* involves stimulation of sphingolipid biosynthesis and, in particular, the synthesis of mannosyl-diinositolphosphoryl-ceramide with concomitant increases in Golgi diacylglycerol levels. To test this model, we disrupted *IPT1*, the mannosyl-diinositolphosphoryl-ceramide synthase of *S. cerevisiae*. Disruption of the *IPT1* gene had no effect on the ability of *sac1-22* to bypass *sec14-1^{ts}*. Furthermore, sphingolipid analysis of *sec14-1^{ts}* and *sec14-1^{ts} sac1-22* strains showed that mannosyl-diinositolphosphoryl-ceramide synthesis was not stimulated in the bypass mutant. However, the *sec14-1^{ts}* strain had elevated mannosyl-monoinositolphosphoryl-ceramide levels, and the *sec14-1^{ts} sac1-22* strain showed an 8-fold increase in phosphatidylinositol 4-phosphate along with a decrease in phosphatidylinositol 4,5-bisphosphate. Cellular diacylglycerol levels, measured by [¹⁴C]acetate incorporation, did not differ between the *sec14-1^{ts}* and the *sec14-1 sac1-22* bypass strains, although disruption of *IPT1* in the bypass strain resulted in reduced levels. These data indicate that phosphatidylinositol 4-phosphate, rather than mannosyl-diinositolphosphoryl-ceramide, accumulates in the *sec14-1^{ts} sac1-22* bypass strain, and that Golgi diacylglycerol accumulation is not required for bypass of the *sec14-1^{ts}* growth and secretory phenotypes.

The yeast *Saccharomyces cerevisiae* is an important model system for determining the molecular mechanisms of eukaryotic intracellular protein transport. Of particular interest is the yeast secretory pathway in which *SEC* genes essential for protein secretion by *S. cerevisiae* have been identified (1). These genes were discovered as conditional lethal mutations that interrupt secretory flow at specific points along the secretory pathway. Biochemical and functional identification of the *SEC* gene products has increased the understanding of mechanisms involved in the yeast secretory pathway.

The yeast *SEC14* gene is required for the formation of transport vesicles from the Golgi (2). However, the mechanism by which its encoded protein, Sec14p, facilitates this process is not precisely known. When conditional lethal mutant *sec14-1^{ts}*

strains are shifted to nonpermissive temperatures, protein secretion halts (1). Sec14p is a phosphatidylinositol transfer protein with the ability to transfer both phosphatidylinositol and phosphatidylcholine between membranes (2). A unique protein in *S. cerevisiae*, Sec14p, is set apart from other phospholipid transfer proteins by its ability to interact specifically with the Golgi (3), an association that is required for secretory competence in yeast (4). Sec14p is proposed to have a sensor function that maintains a critical phosphatidylinositol to phosphatidylcholine ratio required for vesicle formation at the Golgi (3).

To further understand the mechanism of *SEC14* action in the secretory pathway, suppressor mutants of *sec14-1^{ts}* were identified (3). Characterization of the genes defective in these mutants revealed that genes of three of six gene complementation groups identified were involved in the CDP-choline pathway for phosphatidylcholine biosynthesis. Apparently, inhibition of phosphatidylcholine synthesis through the CDP-choline pathway leads to efficient bypass of *sec14-1^{ts}*, probably by altering phospholipid composition of the Golgi membrane.

Other suppressors of *sec14-1^{ts}* are mutations of the *SAC1* gene (5). Originally identified as a suppressor of *act1* alleles when mutated, *SAC1* encodes an integral membrane protein that is located in the endoplasmic reticulum and Golgi (6). Generally, *SAC1* mutations lead to a cold-sensitive phenotype and inositol auxotrophy. However, mutants with the *sac1-22* allele do not display inositol auxotrophy, despite an inositol requirement for its bypass of *sec14-1^{ts}*. Sac1p has significant homology with the noncatalytic domains of the yeast and mammalian polyphosphoinositide 5-phosphatases (7), but its function is not known.

Phospholipase D activity was recently shown to be essential for bypass of the *sec14-1^{ts}* phenotypes in several bypass mutants, but not for normal secretion (8, 9). The formation of phosphatidic acid from phospholipase D-mediated hydrolysis of phosphatidylcholine has been implicated in this process. This is supported by results showing that phosphatidic acid promotes Golgi vesicle formation in a mammalian cell system (10).

Kearns *et al.* (11) suggested that *sec14-1^{ts}* bypass by *sac1-22* occurs by increasing the amount of diacylglycerol (DAG)¹ in the Golgi through stimulated sphingolipid biosynthesis. This model was based on an apparent 6-fold increase in the cellular levels of mannosyl-diinositolphosphoryl-ceramide (M(IP)₂C). M(IP)₂C synthesis occurs in the Golgi with the transfer of phosphorylinositol from phosphatidylinositol to mannosyl-monoinositolphosphoryl-ceramide (MIPC) to yield a molar equivalent of DAG (12). The *sac1-22* bypass was perturbed by heterologous expression of *Escherichia coli* DAG kinase, pre-

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¹ The abbreviations used are: DAG, diacylglycerol; M(IP)₂C, mannosyl-diinositolphosphoryl-ceramide; MIPC, mannosyl-inositolphosphoryl-ceramide; PtdIns, phosphatidylinositol; PtdIns(4)P, PtdIns 4-phosphate; PtdIns(3)P, PtdIns 3-phosphate; PtdIns(4,5)P₂, PtdIns, 4,5-bisphosphate; SC, synthetic complete; HPLC, high performance liquid chromatography.

sumably due to the conversion of DAG to phosphatidic acid.

The recent identification of *IPT1* as M(IP)₂C synthase (13) allowed us to test the role of M(IP)₂C production in the bypass of *sec14-1^{ts}* by *sac1-22*. *IPT1* was also recently found to be identical to *SYR4*, a gene necessary for yeast growth inhibition by the bacterial metabolite syringomycin E.² In this work, we report that disruption of *IPT1* has no effect on the bypass, indicating that M(IP)₂C production is not important in the *sac1-22* suppression of *sec14-1^{ts}*. Instead, phosphatidylinositol 4-phosphate (PtdIns(4)P) is shown to increase in the *sec14-1^{ts}* *sac1-22* bypass mutants, suggesting a role for this lipid in Golgi-localized secretory pathway events.

EXPERIMENTAL PROCEDURES

Strains and Media—*S. cerevisiae* strains used in this work were CTY182 (*MATa ura3-52 Δhis3-200 lys2-801*) (2), CTY1-1A (*MATa ura3-52 Δhis3-200 lys2-801 sec14-1^{ts}*) (2), CTY165 (*MATa ura3-52 Δhis3-200 ade2-101 sec14-1^{ts} sac1-22*) (11), and CTY-SR (CTY165 *Δipt1::URA3*, this work). Cells were typically grown in synthetic complete (SC) media as described by Kaiser *et al.* (15). For lipid analyses, cells were grown in synthetic minimal medium (16).

Disruption of *IPT1* in CTY165—The *IPT1* disruptant strain CTY-SR was constructed by the one-step disruption method (17). The *IPT1* disruption construct described elsewhere² was made by replacing a 1-kilobase *PvuII-PvuII* fragment, which included the 5' portion of *IPT1*, with a 1.1-kilobase *URA3* fragment. This construct was linearized and used to transform CTY165. Disruptants were selected on SC-ura, and *IPT1* disruption was confirmed by Southern blot analysis.

Liquid Secretion Assay—Ten-ml cultures grown in SC medium at 28 °C were harvested at an optical density between 0.5 and 1 at 600 nm, washed once in SC low-glucose medium (1 mM glucose), resuspended into 10 ml of low-glucose medium, and split into two 5-ml cultures. Low-glucose cultures were incubated at either 28 °C or 37 °C for 1 h. Secretory indices were determined by assaying the total and secreted invertase activity as described by Westphal *et al.* (18).

Quantification of Sphingolipids—Sphingolipid extractions were modified from the methods described by Smith and Lester (19). Steady-state measurements of sphingolipid levels were conducted by growing 20-ml cultures for 18–24 h at 28 °C in the presence of 200 μCi of H₃³²PO₄. Growth was terminated by the addition of trichloroacetic acid to a final concentration of 5%. Cells were washed twice with 1 ml of H₂O and freeze-dried overnight. Dried cells were resuspended in 1 ml of H₂O and extracted by the addition of 1.4 ml of ethanol:diethyl ether:pyridine (15:5:1) for 30 min at 57 °C. Debris was pelleted by centrifugation, and the supernatant was removed to a fresh tube. After drying under N₂, lipids were resuspended in 1 ml of solvent A (chloroform:methanol:water (16:16:5 v/v/v)), and glycerophospholipids were deacylated by the addition of 1 ml of 0.2 N NaOH in methanol and incubation at 30 °C for 45 min. One ml of 5% EDTA was added, and samples were neutralized by the addition of 0.2 ml of 1 N acetic acid. Lipids were then extracted with 1 ml of chloroform and dried under N₂. Lipids were resuspended in 0.1 ml of solvent A and analyzed by thin layer chromatography on 1-mm Silica Gel G plates (Analtech, Newark, DE) treated with 2.5% EDTA (pH 7.2) and developed in chloroform:methanol:4.2 N ammonium hydroxide (9:7:2 v/v/v). Radiolabeled lipids were identified by autoradiography and quantified by scraping relevant spots followed by liquid scintillation counting. The identities of inositolphosphoryl-ceramide, MIPC, and M(IP)₂C were confirmed by electrospray ionization mass spectroscopy (Utah State University Biotechnology Center).

Quantification of Diacylglycerol—Steady-state and pulse analyses of DAG levels were measured by labeling cells with [¹⁴C]acetate (ICN Pharmaceuticals, Costa Mesa, CA). Steady-state labeling was conducted by adding 5 μCi of [¹⁴C]acetate to 20-ml cultures and growing the cells for 18–24 h at 28 °C. Pulse labeling was done by adding 40 μCi of [¹⁴C]acetate to 20-ml cultures, with an optical density at 600 nm between 0.7 and 0.8, for 20 min at 28 °C. DAG extraction was done as described by Buttke and Pyle (20). The total lipid fractions from ¹⁴C pulse-labeled and steady-state-labeled cells were extracted with 2 ml of methanol under reflux for 1 h, followed by the addition of 4 ml of chloroform and incubation at room temperature for 18 h. Phases were separated by the addition of 1.2 ml of 0.1 M potassium chloride. The chloroform phase was removed to a fresh tube and dried under N₂.

Labeled lipids were suspended in 100 μl of chloroform and separated by thin layer chromatography with the solvent petroleum ether:diethyl ether:acetic acid (85:15:1 v/v/v). ¹⁴C-labeled lipids were detected by autoradiography, scraped, and quantified by liquid scintillation counting. Nonlabeled DAG and other lipid standards were detected by charring after spraying with 50% sulfuric acid.

Phospholipid Analysis—Cells were grown at 26 °C for 18 h in synthetic defined minimal media supplemented with 1 mM *myo*-inositol in the presence of 40 μCi/ml H₃³²PO₄ (ICN Pharmaceuticals) as performed in the work of Kearns *et al.* (11). Total lipids were extracted from 20 A₅₉₀ units of each cell culture as described for sphingolipid analysis without deacylation. Dried lipids were suspended in 400 μl of solvent A and separated by two-dimensional thin layer chromatography as described above and visualized by autoradiography. The relevant lipid spot was scraped from plates and extracted three times with 500 μl of solvent A, dried under N₂, and processed for high performance liquid chromatography (HPLC) analysis as described below.

Phosphoinositide Analysis—Yeast cells were grown at 26 °C in synthetic minimal media supplemented with 100 μM *myo*-inositol and 5 μCi/ml *myo*-[³H]inositol (23 Ci/mmol; NEN Life Science Products, Boston, MA) to a mid-log phase. Lipids were extracted as described under "Quantification of Sphingolipids." Deacylation was carried out by the method of Serunian *et al.* (21). Dried glycerophosphoinositols were subjected to anion exchange chromatography using a Whatman Partisil 5 SAX column (25 cm × 4.6 mm; Whatman Inc., Clifton, NJ) on a Beckman System Gold HPLC system. The column was pre-equilibrated with 10 mM ammonium phosphate, pH 3.8. A portion of each sample (2.5 × 10⁶ cpm) was applied to the column, washed with 5 ml of 10 mM ammonium phosphate (pH 3.8), and eluted with a 40-ml linear gradient from 10 mM to 0.7 M ammonium phosphate (pH 3.8) at a flow rate of 1 ml/min. [³²P]Glycerophosphoinositol 3-phosphate and [³²P]glycerophosphoinositol 4-phosphate standards were generated by *in vitro* phosphorylation of phosphatidylinositol with α-[³²P]ATP followed by deacylation. For the production of PtdIns(3)P, an extract enriched in PtdIns 3-kinase was prepared from a 25–30% ammonium sulfate precipitate of a yeast cytosolic fraction of strain TVY614 (22) carrying a multicopy plasmid of the *VPS15* gene (*pJSY324.15*) (23) and the *VPS34* gene (*pPHY52*) (24). The PtdIns 4-kinase source was a crude cell extract from a yeast strain (PHY102) that lacks the *VPS34* gene (24). The glycerophosphoinositol 3,5-bisphosphate peak was identified as a species that increased 16-fold by osmotic stress (1 M NaCl for 20 min; data not shown) (25). [³H]Glycerophosphoinositol 4,5-bisphosphate was generated by deacylation of [³H]PtdIns(4,5)P₂ (NEN Life Science Products).

RESULTS

IPT1* Disruption Does Not Affect *sac1-22* Bypass of *sec14-1^{ts}—The proposed involvement of M(IP)₂C synthesis in the *sac1-22* bypass of *sec14-1^{ts}* prompted us to disrupt *IPT1*, the structural gene for M(IP)₂C synthase, in strain CTY165 (*sec14-1^{ts} sac1-22*). If increased M(IP)₂C synthesis in this strain promotes bypass, then disruption of *IPT1* should alleviate the bypass. A disruption strain was obtained by transforming CTY165 with a linearized *ipt1::URA3* construct. After transformation, disruptants were selected on SC-ura plates. Three Ura⁺ transformants were randomly selected and found to be resistant to syringomycin E (1.0 μg/ml), which is characteristic of mutants that lack M(IP)₂C.² Disruption was confirmed by Southern blot analysis. One strain, CTY-SR, was selected and used for the remaining studies. To examine the effect of *IPT1* disruption, strains CTY182 (wild type), CTY1-1A (*sec14-1^{ts}*), CTY165 (*sec14-1^{ts} sac1-22*), and CTY-SR (*sec14-1^{ts} sac1-22 Δipt1*) were streaked onto minimal medium plates with or without *myo*-inositol (0.1 mM) and incubated at permissive (28 °C) and nonpermissive (37 °C) temperatures (Fig. 1). All strains grew with and without inositol at 28 °C. CTY1-1A (*sec14-1^{ts}*) did not grow in either case at 37 °C, as expected, whereas CTY182 (wild type) CTY165 (*sec14-1^{ts} sac1-22*), and CTY-SR (*sec14-1^{ts} sac1-22 Δipt1*) grew at 37 °C with inositol, but only CTY182 was able to grow at 37 °C on medium lacking inositol. These phenotypes are characteristic of the *myo*-inositol requirement for *sac1-22* bypass of *sec14-1^{ts}* and demonstrate that disruption of *IPT1* does not abolish the bypass.

Secretory indices were determined for each strain by assaying

² S. D. Stock, D. A. Young, J. A. Radding, and J. Y. Takemoto, manuscript in preparation.

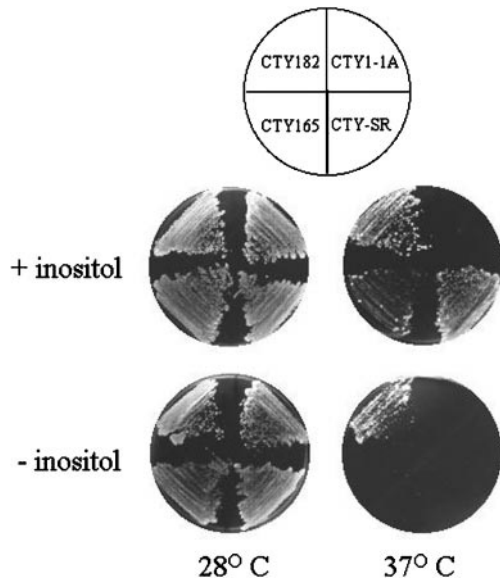


FIG. 1. Disruption of *IPT1* does not affect suppression by *sac1-22*. Strains CTY182 (wild type), CTY165 (*sec14-1^{ts} sac1-22*), CTY-SR (*sec14-1^{ts} sac1-22 Δipt1*), and CTY1-1A (*sec14-1^{ts}*) were streaked onto synthetic minimal media with 0.1 mM inositol or without inositol at permissive (28 °C) or nonpermissive (37 °C) temperatures.

whole cell and secreted invertase activities at 28 °C and 37 °C (Fig. 2). All strains showed similar levels of invertase secretion at 28 °C. At 37 °C, strains CTY182 (wild type) and CTY165 (*sec14-1^{ts} sac1-22*) had similar secretory indices, secretion was impeded in strain CTY1-1A (*sec14-1^{ts}*), and strain CTY-SR (*sec14-1^{ts} sac1-22 Δipt1*) showed a slightly enhanced secretory index. The results confirm the growth phenotypes of these strains, showing that *IPT1*-encoded M(IP)₂C synthesis is not involved in the *sac1-22* bypass of the *sec14-1^{ts}* secretory defect.

Sphingolipid Levels—The above observation that *IPT1* disruption did not influence *sac1-22* suppression of *sec14-1^{ts}* prompted a re-examination of the sphingolipid levels in the relevant strains. Strains CTY182 (wild type), CTY1-1A (*sec14-1^{ts}*), CTY165 (*sec14-1^{ts} sac1-22*), and CTY-SR (*sec14-1^{ts} sac1-22 Δipt1*) were steady-state labeled with H₃³²PO₄ to quantify sphingolipids. Quantification of M(IP)₂C levels in strains CTY182 (wild type), CTY1-1A (*sec14-1^{ts}*), and CTY165 (*sec14-1^{ts} sac1-22*) revealed similar degrees of ³²P incorporation into M(IP)₂C (in cpm per mg dry weight of cells: 836 ± 356 (*n* = 3), 1080 ± 516 (*n* = 3), and 853 ± 341 (*n* = 3), respectively), and, as expected, CTY-SR (*sec14-1^{ts} sac1-22 Δipt1*) did not produce M(IP)₂C (Fig. 3). Differences were observed in the relative MIPC levels. CTY1-1A (*sec14-1^{ts}*) had higher cellular levels of MIPC (29% of total sphingolipids) compared with the isogenic wild type strain CTY182 (15% of total sphingolipids) and the *sac1-22* bypass strain CTY165 (7.5% of total sphingolipids) (Fig. 3). These results show that the *sac1-22* bypass of *sec14-1^{ts}* does not involve increased M(IP)₂C production.

Phosphatidylinositol 4-Phosphate Levels Are Elevated in the *sac1-22* Mutant—The above finding that M(IP)₂C levels are unchanged in the CTY165 (*sec14-1^{ts} sac1-22*) bypass strain prompted us to revisit the effects of the *sac1-22* mutation on the cellular levels of inositol-containing lipids. Strains CTY1-1A (*sec14-1^{ts}*) and CTY165 (*sec14-1^{ts} sac1-22*) were grown at permissive temperature in the presence of [³²P]phosphate, and the total lipids were extracted and analyzed by two-dimensional thin layer chromatography (Fig. 4). A preferential increase in the level of a phosphate-containing lipid that resembled a diphosphoinositide (26) was observed in extracts of strain CTY165 (*sec14-1^{ts} sac1-22*). To identify this lipid, it was isolated from the silica matrix of the thin layer chromatographic

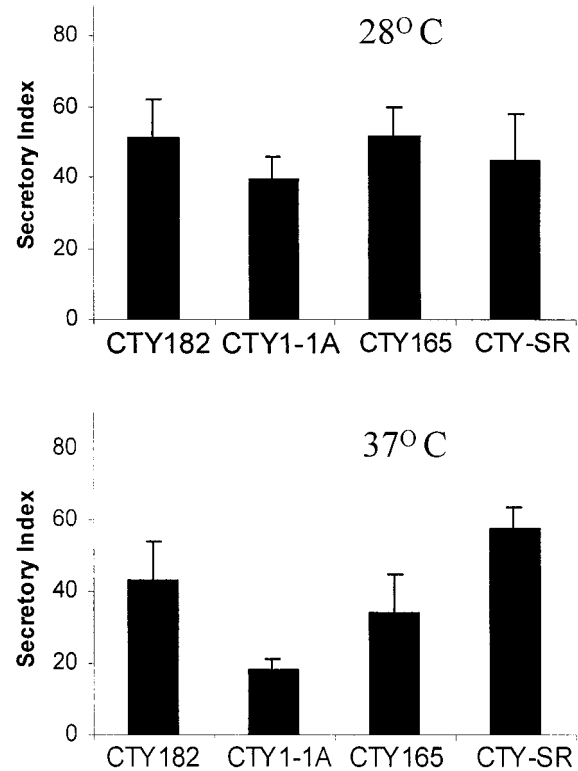


FIG. 2. Secretory indices of CTY strains. Secretory indices were determined for strains CTY182 (wild type), CTY1-1A (*sec14-1^{ts}*), CTY165 (*sec14-1^{ts} sac1-22*), and CTY-SR (*sec14-1^{ts} sac1-22 Δipt1*) at a permissive temperature (28 °C; top panel) and a nonpermissive temperature (37 °C; bottom panel). The secretory index is the percentage of total cellular invertase activity that is secreted. Data presented are averages of three experiments, with standard deviations shown as error bars.

plates, extracted, deacylated, and subjected to anionic exchange HPLC analyses (21) (Fig. 4). Comparisons to authentic glycerophosphoinositol standards indicated that the lipid was PtdIns(4)P. To confirm this identification, lipid extracts were prepared from cultures of strains grown in the presence of *myo*-[³H]inositol and deacylated, and the glycerophosphoinositols were identified by anionic exchange HPLC analyses (Fig. 5). A HPLC peak that co-eluted with glycerophosphoinositol derived from PtdIns(4)P was 8-fold more abundant in the CTY165 (*sec14-1^{ts} sac1-22*) samples as compared with the CTY1-1A (*sec14-1^{ts}*) samples. The only other significant difference detected between the two strains was a 60% reduction in the glycerophosphoinositol derived from PtdIns(4,5)P₂ in strain CTY165.

DAG Production—In the current model for *sac1-22* suppression of *sec14-1^{ts}*, bulk DAG levels are hypothesized to increase as a result of elevated M(IP)₂C synthesis (11). However, the results presented above show that M(IP)₂C levels do not increase with *sac1-22* suppression. To reconcile this discrepancy, the effects of *IPT1* disruption and *sac1-22* suppression of *sec14-1^{ts}* on DAG production were determined. Cultures of strains CTY182 (wild type), CTY1-1A (*sec14-1^{ts}*), CTY165 (*sec14-1^{ts} sac1-22*), and CTY-SR (*sec14-1^{ts} sac1-22 Δipt1*) were incubated with [¹⁴C]acetate continually (steady-state labeling) or for a 20-min interval (pulse labeling) before harvesting the cells. Total lipid extracts were separated by thin layer chromatography, and DAG was identified by comparison to an authentic standard. With steady-state labeling, no significant differences in DAG levels were observed between the four strains (data not shown). With pulse labeling, strains CTY182 (wild type), CTY1-1A (*sec14-1^{ts}*), and CTY165 (*sec14-1^{ts} sac1-22*) showed

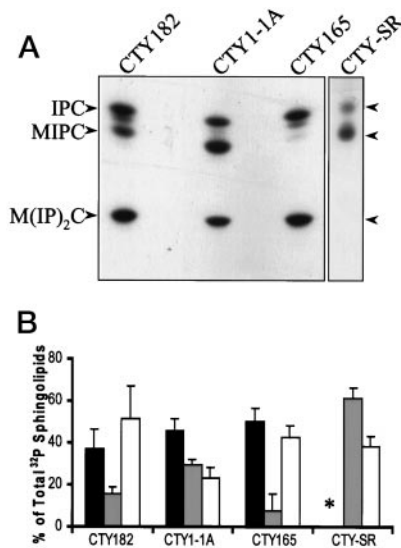


FIG. 3. M(IP)₂C levels of strains CTY1-1A (*sec14-1^{ts}*) and CTY165 (*sec14-1^{ts} sac1-22*) are similar. Twenty-ml cultures of strains CTY182 (wild type), CTY1-1A (*sec14-1^{ts}*), CTY165 (*sec14-1^{ts} sac1-22*), and CTY-SR (*sec14-1^{ts} sac1-22 Δipt1*) were radiolabeled with 200 μ Ci of H₃³²PO₄. Sphingolipids were extracted (see "Experimental Procedures"), and extracts equivalent to 2–3 mg dry weight of cells were subjected to one-dimensional thin layer chromatography and autoradiography (A). Individual sphingolipids (M(IP)₂C, ■; MIPC, ▒; inositol-phosphoryl-ceramide, □) were quantified by scraping off the radioactive spots and estimating the radioactivity by scintillation counting (B). *, M(IP)₂C was not detected in lipid extracts of strain CTY-SR. The results shown are the averages of three experiments, with standard deviations shown as error bars.

similar rates of net DAG production, but CTY-SR (*sec14-1^{ts} sac1-22 Δipt1*) produced approximately one-third less DAG (Fig. 6).

DISCUSSION

The main conclusion of our work is that M(IP)₂C synthesis, which is coupled to DAG production, is not involved in the bypass of *sec14-1^{ts}* by *sac1-22*. This was determined by assessing the consequences of disrupting *IPT1*, the gene that encodes the M(IP)₂C synthase, and also by examining the cellular levels of M(IP)₂C and DAG as a function of the bypass. These conclusions differ from those of Kearns *et al.* (11), who previously reported increases in both M(IP)₂C and DAG in the *sec14-1^{ts} sac1-22* bypass mutant. It is clear that in the previous report, M(IP)₂C was misidentified. A re-evaluation in the present work of alterations in lipid composition caused by the *sac1-22* bypass mutation revealed that the amount of a single phosphoinositide-containing lipid species, PtdIns(4)P, was preferentially elevated rather than M(IP)₂C.

Our analyses, however, did reveal an increase in the relative level of MIPC in CTY1-1A (*sec14-1^{ts}*) at permissive temperature (Fig. 3). This could be due to product inhibition of Ipt1p by accumulated M(IP)₂C in the Golgi compartment caused by a defect in the secretory pathway that is nonetheless partially functional at permissive temperature. Alleviation of this defect by *sac1-22* would account for the lowered MIPC levels observed in strain CTY165 (*sec14-1^{ts} sac1-22*). However, why the MIPC levels of strain CTY165 were lower than those of strain CTY182 (wild type) (Fig. 3) remains unexplained.

It is premature to assign a specific role to PtdIns(4)P in the SEC14-dependent protein secretory pathway. The observed increased amounts of this lipid (Figs. 4 and 5) are only correlated with the *sec14-1^{ts} sac1-22* bypass. Nevertheless, the potential importance of PtdIns(4)P in the bypass is consistent with several observations that link phosphatidylinositol transfer pro-

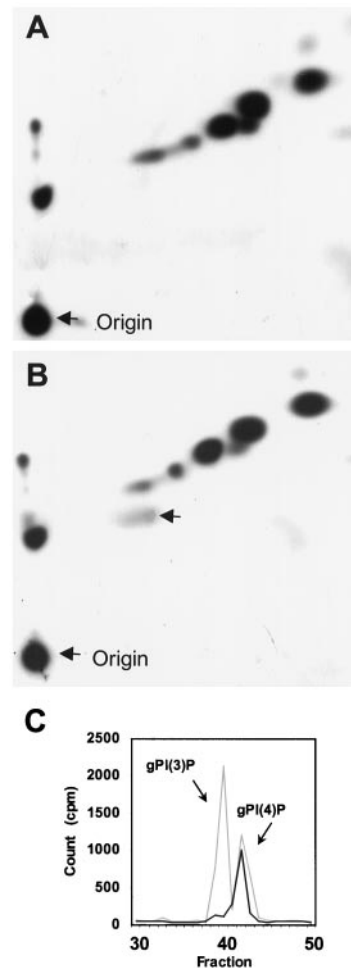


FIG. 4. PtdIns(4)P levels are elevated in the CTY165 (*sec14-1^{ts} sac1-22*) bypass strain. The autoradiographs of two-dimensional thin layer chromatograms of total ³²P-labeled lipid extracts from (A) CTY1-1A (*sec14-1^{ts}*) and (B) CTY165 (*sec14-1^{ts} sac1-22*) are shown. The elevated spot in B is indicated by an arrow. C, HPLC chromatogram of deacylated lipids isolated from the thin layer chromatography media. Lipids were extracted from the spot indicated by an arrow in B, deacylated, and analyzed by HPLC as described under "Experimental Procedures" (black line). Fractions (0.5 ml each) were collected from 0 to 40 ml. Counts in the fractions from 15 to 25 ml are shown. No radioactivity above the background level was detected in the rest of the fractions. The chromatogram with ³²P-labeled deacylated phosphatidylinositol 3-phosphate and PtdIns(4)P is superimposed (gray line). *gPI(3)P*, glycerophosphoinositol 3-phosphate; *gPI(4)P*, glycerophosphoinositol 4-phosphate.

tein function and phosphoinositide production. For example, it has been demonstrated that mammalian phosphatidylinositol transfer proteins are co-factors for phosphoinositide production by PtdIns kinases that participate in signaling and membrane traffic (27). In assays for certain signaling or trafficking processes, Sec14p can substitute for mammalian phosphatidylinositol transfer proteins (28–31). It is worth noting that Sac1p has significant similarity to a noncatalytic domain of mammalian polyphosphoinositide 5-phosphatases (7), and Sac1p may conceivably be a novel phosphatase that regulates PtdIns(4)P levels. If so, Sac1p inactivation would lead to elevated levels of PtdIns(4)P, which, in turn, may compensate for insufficient PtdIns(4)P production due to a defective Sec14p. In numerous studies of phosphoinositide roles in membrane traffic, PtdIns(4)P is typically regarded as a precursor to PtdIns(4,5)P₂ (32). However, the present finding that PtdIns(4,5)P₂ levels are lowered in the *sec14-1^{ts} sac1-22* bypass emphasizes the potential importance of PtdIns(4)P, rather

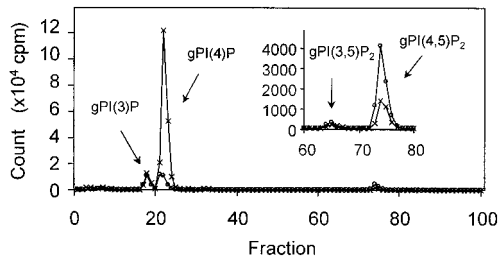


FIG. 5. Phosphoinositide levels of strains CTY1-1A and CTY165. Ten-ml cultures of strains CTY1-1A (○) and CTY165 (x) were labeled with 5 $\mu\text{Ci/ml}$ [^3H]inositol at 26 °C overnight. Lipids were extracted and deacylated, and extracts containing 2.5×10^6 cpm were subjected to anion exchange chromatography as described under "Experimental Procedures." Lipid extracts from 10-ml cultures of strains CTY1-1A and CTY165 contained 4×10^6 and 5.6×10^6 cpm, respectively. Fractions (0.3 ml each) were collected from 15 to 45 ml. Counts of fractions 61–80 are shown in a different scale in the inset. *gPI(3)P*, glycerophosphoinositol 3-phosphate; *gPI(4)P*, glycerophosphoinositol 4-phosphate; *gPI(3,5)P₂*, glycerophosphoinositol 3,5-bisphosphate; *gPI(4,5)P₂*, glycerophosphoinositol 4,5-bisphosphate. The chromatograms of deacylated lipids from CTY182 were indistinguishable from CTY1-1A (data not shown).

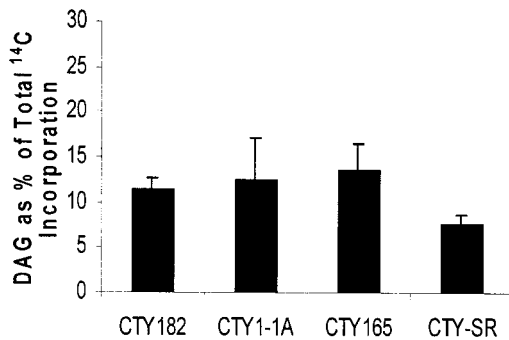


FIG. 6. Rates of diacylglycerol production in strains CTY1-1A (*sec14-1^{ts}*) and CTY165 (*sec14-1^{ts} sac1-22*) are similar. Exponentially growing cells (20-ml cultures) of strains CTY182 (wild type), CTY1-1A (*sec14-1^{ts}*), CTY165 (*sec14-1^{ts} sac1-22*), and CTY-SR (*sec14-1^{ts} sac1-22 Δ ipt1*) were pulse-labeled (20 min) with [^{14}C]acetate before lipid extraction, and the amounts of ^{14}C recovered in diacylglycerol are shown as a percentage of the total amount of [^{14}C]acetate incorporated into the cells. Five μl of each lipid extract (equal to one-twentieth of the total lipids extracted from cells of a 20-ml culture) were subjected to thin layer chromatography (see "Experimental Procedures"), and the radioactivity in diacylglycerol was determined. The average ($n = 3$) amounts of total radioactivity (in cpm) in the extracted cells (from 20-ml cultures) were 177,757 (CTY182), 160,386 (CTY1-1A), 89,492 (CTY165), and 149,608 (CTY-SR). Results shown are the averages of three separate experiments, with standard deviation shown as error bars.

than PtdIns(4,5)P₂, in *SEC14*-dependent secretion. In support of the significance of PtdIns(4)P in trafficking, Matsuoka *et al.* (14) reported that either PtdIns(4)P or PtdIns(4,5)P₂ promoted the binding of coat proteins to liposomes in an *in vitro* assay for COPII-coated vesicle formation.

Roles for phospholipase D and phosphatidic acid in the bypass have been suggested recently (8, 9). How the presently observed increases in PtdIns(4)P relate to phospholipase D function and phosphatidic acid production in the Golgi secretory machinery is not clear. However, it is conceivable that all of these elements are coordinately regulated in yet unknown ways to allow efficient operation of the *SEC14*-dependent secretory pathway.

We also examined the effect of *IPT1* disruption on DAG production after both steady-state and pulse labeling of cells with [^{14}C]acetate. The pulse-labeling experiments revealed that rates of DAG production were reduced by one-third in the strain CTY-SR (*sec14-1^{ts} sac1-22 Δ ipt1*) compared with the CTY182 (wild type), CTY1-1A (*sec14-1^{ts}*), or CTY165 (*sec14-1^{ts}*)

sac1-22) strains. The decrease in DAG production with *IPT1* disruption is plausible because elimination of M(IP)₂C synthase, which is coupled to phosphorylinositol transfer from PtdIns, would eliminate a source of cellular DAG. Steady-state and pulse labeling of DAG did not reveal an increase in DAG levels in strain CTY165 as reported previously (11). This discrepancy between the previous work of Kearns *et al.* (11) and the present work remains unresolved. However, the present findings are consistent with results reported by Sreenivas *et al.* (9), who found no difference in the DAG levels of other bypass mutants of *sec14-1^{ts}*. Furthermore, because *IPT1* disruption leads to lower DAG production levels despite the retention of the *sec14-1^{ts}* bypass phenotype, our results suggest that an increase in DAG levels is not essential for suppression by *sac1-22*.

Taken together, the present findings raise concerns about certain features of the proposed mechanisms of *sac1-22*-mediated bypass of *sec14-1^{ts}* (11). DAG production through sphingolipid metabolism and M(IP)₂C biosynthesis does not appear to play a role in the bypass. Instead, another lipid, PtdIns(4)P, is revealed to be of potential importance to *SEC14*-dependent secretion. However, insight into the mechanisms of the role of PtdIns(4)P in the secretion pathway will require further investigation.

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REFERENCES

- Novick, P., Field, C., and Schekman, R. (1980) *Cell* **21**, 205–215
- Bankaitis, V. A., Malehorn, D. E., Emr, S. D., and Greene, R. (1989) *J. Cell Biol.* **108**, 1271–1281
- Cleves, A. E., McGee, T. P., Whitters, E. A., Champion, K. M., Aitken, J. R., Dowhan, W., Goebel, M., and Bankaitis, V. A. (1991) *Cell* **64**, 789–800
- Skinner, H. B., Alb, J. G., Jr., Whitters, E. A., Helmkamp, G. M., Jr., and Bankaitis, V. A. (1993) *EMBO J.* **12**, 4775–4784
- Cleves, A. E., Novick, P. J., and Bankaitis, V. A. (1989) *J. Cell Biol.* **109**, 2939–2950
- Whitters, E. A., Cleves, A. E., McGee, T. P., Skinner, H. B., and Bankaitis, V. A. (1993) *J. Cell Biol.* **122**, 79–94
- Woscholski, R., and Parker, P. J. (1997) *Trends Biochem. Sci.* **22**, 427–431
- Xie, Z., Fang, M., Rivas, M. P., Faulkner, A. J., Sternweis, P. C., Engebrecht, J. A., and Bankaitis, V. A. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 12346–12351
- Sreenivas, A., Patton-Vogt, J. L., Bruno, V., Griac, P., and Henry, S. A. (1998) *J. Biol. Chem.* **273**, 16635–16638
- Siddhanta, A., and Shields, D. (1998) *J. Biol. Chem.* **273**, 17995–17998
- Kearns, B. G., McGee, T. P., Mayinger, P., Gedvilaite, A., Phillips, S. E., Kagiwada, S., and Bankaitis, V. A. (1997) *Nature* **387**, 101–105
- Becker, G. W., and Lester, R. L. (1980) *J. Bacteriol.* **142**, 747–754
- Dickson, R. C., Nagiec, E. E., Wells, G. B., Nagiec, M. M., and Lester, R. L. (1997) *J. Biol. Chem.* **272**, 29620–29625
- Matsuoka, K., Orci, L., Amherdt, M., Bednarek, S. Y., Hamamoto, S., Schekman, R., and Yeung, T. (1998) *Cell* **93**, 263–275
- Kaiser, C., Michaelis, S., and Mitchell, A. (1994) *Methods in Yeast Genetics*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
- Sherman, F. (1991) in *Guide to Yeast Genetics and Molecular Biology* (Guthrie, C., and Fink, G. R., eds) Vol. 194, pp. 3–21, Academic Press, San Diego, CA
- Rothstein, R. J. (1983) *Methods Enzymol.* **101**, 202–221
- Westphal, V., Marcusson, E. G., Winther, J. R., Emr, S. D., and van den Hazel, H. B. (1996) *J. Biol. Chem.* **271**, 11865–11870
- Smith, S., and Lester, R. L. (1974) *J. Biol. Chem.* **249**, 3395–3405
- Buttke, T. M., and Pyle, A. L. (1982) *J. Bacteriol.* **152**, 747–756
- Serunian, L. A., Auger, K. R., and Cantley, L. C. (1991) *Methods Enzymol.* **198**, 78–87
- Vida, T. A., and Emr, S. D. (1995) *J. Cell Biol.* **128**, 779–792
- Stack, J. H., Herman, P. K., Schu, P. V., and Emr, S. D. (1993) *EMBO J.* **12**, 2195–2204
- Herman, P. K., and Emr, S. D. (1990) *Mol. Cell Biol.* **10**, 6742–6754
- Dove, S. K., Cooke, F. T., Douglas, M. R., Sayers, L. G., Parker, P. J., and Michell, R. H. (1997) *Nature* **390**, 187–192
- Talwalkar, R. T., and Lester, R. L. (1973) *Biochim. Biophys. Acta* **306**, 412–421
- Wirtz, K. W. (1997) *Biochem. J.* **324**, 353–360
- Jones, S. M., Alb, J. G., Jr., Phillips, S. E., Bankaitis, V. A., and Howell, K. E. (1998) *J. Biol. Chem.* **273**, 10349–10353
- Ohashi, M., Jan de Vries, K., Frank, R., Snoek, G., Bankaitis, V., Wirtz, K., and Huttner, W. B. (1995) *Nature* **377**, 544–547
- Cunningham, E., Tan, S. K., Swigart, P., Hsuan, J., Bankaitis, V., and Cockcroft, S. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93**, 6589–6593
- Panaretou, C., Domin, J., Cockcroft, S., and Waterfield, M. D. (1997) *J. Biol. Chem.* **272**, 2477–2485
- Martin, T. F. J. (1998) *Annu. Rev. Cell Dev. Biol.* **14**, 231–264