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 sec14-1ts strains. The existing model for the bypass of the sec14-1ts 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 sec14-1ts. Furthermore, sphingolipid analysis of sec14-1ts and sec14-1ts sac1-22 strains showed that mannosyl-diinositolphosphoryl-ceramide synthesis was not stimulated in the bypass mutant. However, the sec14-1ts strain had elevated mannosyl-monoinositolphosphoryl-ceramide levels, and the sec14-1ts 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 sec14-1ts and the sec14-1ts 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 sec14-1ts sac1-22 bypass strain, and that Golgi diacylglycerol accumulation is not required for bypass of the sec14-1ts 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 sec14-1ts strains are shifted to nonpermissive temperatures, protein secretion halts (1). Sec14p is a phosphatidylinositol transfer protein with the ability to transfer both phosphatidylinositol and phosphatidylinositol 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 phosphatidylinositol ratio required for vesicle formation at the Golgi (3).

To further understand the mechanism of SEC14 action in the secretory pathway, suppressor mutants of sec14-1ts 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 phosphatidylinositol biosynthesis. Apparently, inhibition of phosphatidylinositol synthesis through the CDP-choline pathway leads to efficient bypass of sec14-1ts, probably by altering phospholipid composition of the Golgi membrane.

Other suppressors of sec14-1ts 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 sec14-1ts. 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 sec14-1ts phenotypes in several bypass mutants, but not for normal secretion (8, 9). The formation of phosphatidic acid from phospholipase D-mediated hydrolysis of phosphatidylinositol 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 sec14-1ts 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)₃)₆. M(IP)₃₆ 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 sec1-22 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)₃₆, mannosyl-diinositolphosphoryl-ceramide; MIPC, mannosyl-inositolphosphoryl-ceramide; PtdIns, phosphatidylinositol; PtdIns4P, 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<sup>ts</sup> 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. 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<sup>ts</sup>. Instead, phosphatidylinositol 4-phosphate (PtdIns(4)P) is shown to increase in the sec14-1<sup>ts</sup> 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 (MATα ura3-52 Δhis3-200 lys2-801 sec14-1<sup>ts</sup>), CTY165 (MATα ura3-52 Δhis3-200 ade2-101 sec14-1<sup>ts</sup> 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 disrupted strain CTY-SR was constructed by the one-step disruption method (17). The IPT1 disruption construct described elsewhere was made by replacing a 1.1-kilobase 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 indices for each strain by assaying for each strain by assaying the total and secreted indices for each strain.

**Quantification of Diacylglycerol—** Diacylglycerol was quantified by thin layer chromatography as described. Deacylation was carried out by the bacterial metabolite syringomycin E. 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<sup>ts</sup>. Instead, phosphatidylinositol 4-phosphate (PtdIns(4)P) is shown to increase in the sec14-1<sup>ts</sup> sac1-22 bypass mutants, suggesting a role for this lipid in Golgi-localized secretory pathway events.

**RESULTS**

**IPT1 Disruption Does Not Affect sac1-22 Bypass of sec14-1<sup>ts</sup>—** The proposed involvement of M(IP)_2C synthase in the sec1-22 bypass of sec14-1<sup>ts</sup> prompted us to disrupt IPT1, the structural gene for M(IP)_2C synthase, in strain CTY165 (sec14-1<sup>ts</sup> sac1-22). If increased M(IP)_2C synthase 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<sup>+</sup> 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. 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<sup>ts</sup>), CTY165 (sec14-1<sup>ts</sup> sac1-22), and CTY-SR (sec14-1<sup>ts</sup> 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<sup>ts</sup> Δipt1) did not grow in either case at 37 °C, as expected, whereas CTY182 (wild type) CTY165 (sec14-1<sup>ts</sup> sac1-22), and CTY-SR (sec14-1<sup>ts</sup> 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<sup>ts</sup> and demonstrate that disruption of IPT1 does not abolish the bypass.

Secretory indices were determined for each strain by assaying for each strain by assaying the total and secreted indices for each strain.
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" sac1-22) had similar secretory indices, secretion was impeded in strain CTY1-1A (sec14-1"), and strain CTY-SR (sec14-1" 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" secretory defect.

Sphingolipid Levels—The above observation that IPT1 disruption did not influence sac1-22 suppression of sec14-1" prompted a re-examination of the sphingolipid levels in the relevant strains. Strains CTY182 (wild type), CTY1-1A (sec14-1"), CTY165 (sec14-1" sac1-22), and CTY-SR (sec14-1" sac1-22 Δipt1) were steady-state labeled with H32P04 to quantify sphingolipids. Quantification of M(IP)2C levels in strains CTY182 (wild type), CTY1-1A (sec14-1"), and CTY165 (sec14-1" sac1-22) revealed similar degrees of 32P incorporation into M(IP)2C (Fig. 3). Differences were observed in the relative MIPC levels. CTY1-1A (sec14-1") had higher cellular levels of MIPC (29% of total sphingolipids) compared with the isogenic strain CTY165 (7.5% of total sphingolipids) and the CTY1-1A (sec14-1") bypass strain CTY165 (7.5% of total sphingolipids) (Fig. 3). These results show that the sac1-22 bypass of sec14-1" 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" 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") and CTY165 (sec14-1" 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" sac1-22). To identify this lipid, it was isolated from the silica matrix of the thin layer chromatographic 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" sac1-22) samples as compared with the CTY1-1A (sec14-1") 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", 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" on DAG production were determined. Cultures of strains CTY182 (wild type), CTY1-1A (sec14-1"), CTY165 (sec14-1" sac1-22), and CTY-SR (sec14-1" sac1-22 Δipt1) were incubated with [3H]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"), and CTY165 (sec14-1" sac1-22) showed...
similar rates of net DAG production, but CTY-SR (sec14-1ts sac1-22 Ipt1p) 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 sec14-1ts 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 alterations in lipid composition caused by the sec14-1ts bypass mutant. It is clear that in the previous report, M(IP)2C was misidentified. A re-evaluation in the present work emphasizes the potential importance of PtdIns(4)P, rather than M(IP)2C levels of strains CTY1-1A (sec14-1ts) and CTY165 (sec14-1ts sac1-22) are similar. Twenty-ml cultures of strains CTY182 (wild type), CTY1-1A (sec14-1ts), CTY165 (sec14-1ts sac1-22), and CTY-SR (sec14-1ts sac1-22 Ipt1p) were radiolabeled with 200 μCi of H3-32P-O4. 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, MIP, inositolphosphoryl-ceramide, [1]) 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.

Our analyses, however, did reveal an increase in the relative level of MIPC in CTY1-1A (sec14-1ts) 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 sec14-1ts would account for the lowered MIPC levels observed in strain CTY165 (sec14-1ts 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 sec14-1ts sec14-1ts bypass. Nevertheless, the potential importance of PtdIns(4)P in the bypass is consistent with several observations that link phosphatidylinositol transfer protein function and phosphoinositol production. For example, it has been demonstrated that mammalian phosphatidylinositol transfer proteins are co-factors for phosphoinositol 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 4-phosphate and PtdIns(4)P is superimposed (gray line). gPI(3,4)P2, glycerophosphoinositol 3-phosphate; gPP(3,4)P2, glycerophosphoinositol 4-phosphate.
Fig. 5. Phosphoinositide levels of strains CTY1-1A and CTY165. Ten-ml cultures of strains CTY182 (wild type), CTY165 (a), and CTY1-1A (Ts) were labeled with 5 μCi/ml [3H]inositol at 26 °C overnight. Lipids were extracted and deacylated, and extracted 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 (data not shown).

Fig. 6. Rates of diacylglycerol production in strains CTY1-1A (sec14-1ts) and CTY165 (sec14-1ts sac1-22) are similar. Exponentially growing cells (20-ml cultures) of strains CTY182 (wild type), CTY1-1A (sec14-1ts), CTY165 (sec14-1ts sac1-22), and CTY-SR (sec14-1ts sac1-22 Δipt1) were pulse-labeled (20 min) with [14C]acetate before lipid extraction, and the amounts of 34C recovered in diacylglycerol are shown as a percentage of the total amount of [14C]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), 99,492 (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. (11) 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 COPI-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 facilitate 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 [14C]acetate. The pulse-labeling experiments revealed that rates of DAG production were reduced by one-third in the strain CTY-SR (sec14-1ts sac1-22 Δipt1) compared with the CTVY182 (wild type), CTY1-1A (sec14-1ts), or CTY165 (sec14-1ts sac1-22) strains. The decrease in DAG production with IPT1 disruption is plausible because elimination of M(IP)3C 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 sec14-1ts. Furthermore, because IPT1 disruption leads to lower DAG production levels despite the retention of the sec14-1ts 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 sec14-1ts (11). DAG production through sphingolipid metabolism and M(IP)3C 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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