

Direct Involvement of Phosphatidylinositol 4-Phosphate in Secretion in the Yeast *Saccharomyces cerevisiae**

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The *SEC14* gene encodes an essential phosphatidylinositol (PtdIns) transfer protein required for formation of Golgi-derived secretory vesicles in yeast. Suppressor mutations that rescue temperature-sensitive *sec14* mutants provide an approach for determining the role of Sec14p in secretion. One suppressor, *sac1-22*, causes accumulation of PtdIns(4)P. *SAC1* encodes a phosphatase that can hydrolyze PtdIns(4)P and certain other phosphoinositides. These findings suggest that PtdIns(4)P is limiting in *sec14* cells and that elevation of PtdIns(4)P production can suppress the secretory defect. Correspondingly, we found that PtdIns(4)P levels were decreased significantly in *sec14-3* mutants shifted to 37 °C and that *sec14-3* cells could grow at an otherwise non-permissive temperature (34 °C) when carrying a plasmid overexpressing *PIK1*, encoding one of two essential PtdIns 4-kinases. This effect is specific because overexpression of the other PtdIns 4-kinase gene (*STT4*) or a PtdIns 3-kinase gene (*VPS34*) did not rescue *sec14-3* cells. To further address *Pik1p* function in secretion, two different *pik1^{ts}* mutants were examined. Upon shift to restrictive temperature (37 °C), the PtdIns(4)P levels dropped by about 60% in both *pik1^{ts}* strains within 1 h. During the same period, cells displayed a reduction (40–50%) in release of a secreted enzyme (invertase). However, similar treatment did not effect maturation of a vacuolar enzyme (carboxypeptidase Y). These findings indicate that, first, PtdIns(4)P limitation is a major contributing factor to the secretory defect in *sec14* cells; second, Sec14p function is coupled to the action of *Pik1p*, and; third, PtdIns(4)P has an important role in the Golgi-to-plasma membrane stage of secretion.

In eukaryotic cells, secreted proteins are synthesized on ribosomes targeted to the endoplasmic reticulum (ER),¹ translo-

cated into the ER lumen, and transported through the secretory pathway (1). From the ER, secretory proteins are transported to the Golgi apparatus, through the subcompartments of the Golgi, and then to the cell surface or to certain intracellular organelles, all via small membrane-bound vesicles (transport vesicles) (2–4). Cargo proteins are packaged into transport vesicles that bud from one compartment and fuse with another. Mechanisms of vesicle budding and fusion are conserved from yeast to mammalian cells (3, 5).

Because of its tractability for genetic analysis, bakers' yeast (*Saccharomyces cerevisiae*) has proven to be a useful organism to identify gene products required for various events in secretion. Genetic screens, first applied by Schekman and co-workers (6), resulted in the isolation of temperature-sensitive *sec* mutants that displayed defects in different stages of secretion at the nonpermissive temperature. Characterization of the corresponding normal (*SEC*) genes has pinpointed many proteins necessary for secretory processes; and, a large number of gene products are now known to function at various steps in the secretory pathway (reviewed in Ref. 7). The *SEC14* gene encodes a phosphatidylinositol (PtdIns) transfer protein (PITP) (8). This phospholipid exchange protein is relatively specific for PtdIns *in vitro*; for example, Sec14p does not interact detectably with PtdIns (4, 5)P₂ (9) and, although Sec14p will mediate transfer of phosphatidylcholine (PtdCho) from one liposome to another, it does so at only 5% of the rate of PtdIns (10). Sec14p is a cytoplasmic protein (11) that associates preferentially with Golgi membranes (12, 13) and is required for the formation of Golgi-derived vesicles (14) and for protein export from the Golgi (11). In addition, it has been reported that Sec14p also acts as a sensor of the PtdIns/PtdCho ratio in Golgi compartments by serving as a direct regulator of an enzyme in PtdCho biosynthesis (15). Nonetheless, the precise mechanism by which Sec14p action contributes to the process of protein secretion has remained obscure.

It has been proposed that diacylglycerol (DAG) plays a key role in the budding of vesicles from the Golgi compartment (16). This conclusion was reached on the basis of analysis of the lipid composition in a mutant that does not require Sec14p function for growth and secretion. The strain examined carries both the *sec14-3* mutation² and a suppressor mutation, *sac1-22*, and was reported to have elevated levels of both DAG and a sphingolipid, mannosyldiinositolphosphorylceramide (M(IP)₂C) (16).

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¹ The abbreviations used are: ER, endoplasmic reticulum; DAG, diacylglycerol; HPLC, high performance liquid chromatography; M(IP)₂C, mannosyldiinositolphosphorylceramide; PITP, phosphatidylinositol transfer protein; PtdCho, phosphatidylcholine; PtdIns, phosphatidylinositol; PtdIns(3)P, phosphatidylinositol 3-phosphate; PtdIns(4)P,

phosphatidylinositol 4-phosphate; PtdIns(4,5)P₂, phosphatidylinositol 4,5-bisphosphate; PtdIns(3,5)P₂, phosphatidylinositol 3,5-bisphosphate.

² In many publications, including our own (19), the genotype of CTY1-1A has been designated as *sec14-1*. However, the *sec14* allele in CTY1-1A was derived from strain SF292-2A and was originally designated *sec14-3* (11). Hence, the latter designation (*sec14-3*) has been adopted here.

M(IP)₂C is the terminal sphingolipid in *S. cerevisiae* (17), and its biosynthesis by Ipt1p is coupled to PtdIns consumption and DAG production (18). One of our laboratories recently re-examined the properties of the *sec14-3 sac1-22* double mutant and found that the species attributed to M(IP)₂C had been misidentified and that suppression did not require either Ipt1p function or an elevated DAG level (19). Moreover, analysis of phospholipid composition showed that the *sec14-3 sac1-22* cells contained 8-fold more PtdIns(4)P than either *sec14-3 SAC1* or *SEC14* strains (19). The fact that a *sac1* loss-of-function mutation caused an elevation in PtdIns(4)P was readily explained by the contemporaneous demonstration that Sac1p is a phosphoinositide phosphatase (20). Taken together, these findings suggested that, instead of DAG, PtdIns(4)P might have a key role in constitutive secretion in yeast. In mammalian cells, there is some evidence for involvement of a derivative, PtdIns(4,5)P₂, in regulated secretion, especially in neurons (21, 22).

S. cerevisiae possesses two essential genes (*PIK1* and *STT4*) that encode PtdIns 4-kinase isoforms (23, 24). Pik1p is a cytosolic enzyme (25) that can be targeted to membranes via its interaction with a small Ca²⁺-binding protein (26). One report described Pik1p in the nucleus and suggested that loss of *PIK1* function interferes with cytokinesis through effects on the actin cytoskeleton (27). The *STT4* gene was uncovered in a screen for mutants defective in the pathway upstream of the protein kinase, Pkc1p (28). Stt4p apparently produces substrate for a PtdIns(4)P 5-kinase, Mss4p (29, 30), and also appears to be involved in phosphatidylethanolamine biosynthesis, possibly by regulating lipid movement between the ER and Golgi (and vacuole) (31). We investigated whether either PtdIns 4-kinase serves as the source of the PtdIns(4)P accumulated in the *sec14-3 sac1-22* cells. The results presented here clearly implicate Pik1p in generating this pool of PtdIns(4)P and support a direct role for PtdIns(4)P in secretion.

EXPERIMENTAL PROCEDURES

Strains, Plasmids, and Media—The *S. cerevisiae* strains used in this study were: CTY182 (*MATa ura3-52 his3-Δ200 lys2-801 SEC14*) (11), CTY1-1A (*MATa ura3-52 his3-Δ200 lys2-801 sec14-3*)² (11), YES32 (*MATa ade2-101 his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ1 ura3-52 PIK1::TRP1*) (32), YES95 (*YES32 pik1-63::TRP1*) (32), and YES102 (*YES32 pik1-83::TRP1*) (26, 32). Yeast strains were grown in YPD medium (1% yeast extract, 2% peptone, 2% glucose), SC-low glucose medium (0.1% glucose), or in SC medium (yeast nitrogen base, without amino acids; Difco) supplemented with 2% glucose and nutrients as appropriate for the maintenance of plasmids or integrated markers. Multicopy plasmids YEp-*PIK1(URA3)* (23), YEp352-*STT4* (31), and pDD-34.426 were used to overexpress *PIK1*, *STT4*, and *VPS34*, respectively. pDD-34.426 was constructed by ligating the ~3.9-kilobase *Clal-KpnI* fragment of pPHY34 (33) into *Clal*- and *KpnI*-digested pRS426 (34). pHHYS14-5 (plasmid-borne *SEC14*) was derived from a genomic DNA clone in a *URA3*-marked multicopy (2- μ m DNA-based) vector, pSEY18 (35). This plasmid was isolated by complementation of the temperature-sensitive phenotype of CTY1-1A; restriction enzyme cleavage site mapping and amplification by the polymerase chain reaction with specific primers (GenePair; Research Genetics) confirmed the presence of the *SEC14* gene. The plasmid was digested with *SphI* and re-ligated, which removed an 8-kilobase segment of the original 13-kilobase insert, yielding pHHYS14-5.

Assay for Secretion of Invertase—Yeast cells were grown at 29 °C to $A_{600\text{ nm}} = \sim 0.8$ in SC medium. The culture was resuspended in an equivalent amount of SC-low glucose medium (to induce expression of the *SUC2* gene), split into two equal portions, one of which was incubated at 29 °C and the other at 37 °C for 1 h. The extracellular invertase activity and total cellular invertase activity were then measured as described in detail elsewhere (36). Secretion index was calculated as the ratio of the secreted activity to the total activity.

Assay for Vacuolar Delivery of Carboxypeptidase Y—Cells were pulse-labeled and chased as described in detail elsewhere (33). In brief, cells were grown at 25 °C to mid-exponential phase and used to inoculate two separate cultures, which were incubated with shaking at 25 and 37 °C, respectively, for 10 min before addition of [³⁵S]cysteine and

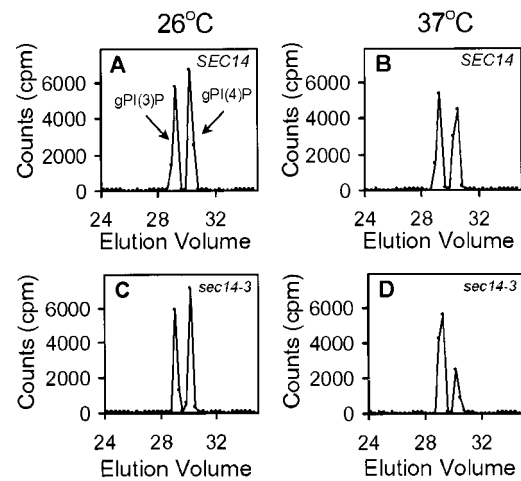


FIG. 1. Phosphoinositide levels in temperature-sensitive *sec14-3* mutant. Liquid cultures of yeast strains CTY182 (*SEC14*, *A* and *B*) and CTY1-1A (*sec14-3*, *C* and *D*) were grown at 26 °C, labeled with *myo*-[2-³H]inositol, and split into two equal portions. One-half was maintained at 26 °C (*A* and *C*) and the other was incubated at 37 °C for 1 h (*B* and *D*) prior to analysis. Lipids were extracted, deacylated, subjected to anion exchange HPLC, and labeled species identified as described under "Experimental Procedures." Equal amounts of total radioactivity from each sample (1×10^6 cpm) were loaded. *gPI(3)P*, glycerophosphoinositol 3-phosphate; *gPI(4)P*, glycerophosphoinositol 4-phosphate.

[³⁵S]methionine (20 μ Ci/ $A_{600\text{ nm}}$ unit; Express³⁵S Label; NEN Life Science Products) and 1 mg/ml bovine serum albumin (final concentration). After addition of label, cells were incubated for 15 min, whereupon nonradioactive methionine (5 mM), cysteine (1 mM), and yeast extract (0.2%) were added at the indicated final concentrations. After the chase period (25 min), trichloroacetic acid was added (6% final concentration), and the samples were chilled on ice. Cells were collected by centrifugation, washed *in vacuo*, and lysed. The cleared supernatant solution was removed to a fresh tube for immunoprecipitation of carboxypeptidase Y (CPY), and mixed with rabbit polyclonal anti-CPY antibodies (gift of Scott Emr, University of California, San Diego, CA) and incubated overnight at 4 °C on a rocker platform. After addition of protein A-Sepharose (Amersham Pharmacia Biotech) and incubation for 1 h at 4 °C, bead-bound immune complexes were collected by brief centrifugation at 12,000 \times g, and washed extensively as described earlier (33). The samples were resuspended in SDS-urea sample buffer (1% SDS, 6 M urea, 50 mM Tris-HCl, pH 6.8, 2% β -mercaptoethanol), heated at 60–65 °C, and analyzed by electrophoresis on a 10% polyacrylamide slab gel containing SDS and autoradiography.

Analysis of Phosphoinositides—Yeast cells were labeled with 5 μ Ci/ml *myo*-[2-³H]inositol (16.5 Ci/mmol, Amersham Pharmacia Biotech) in SC medium for 16–20 h at 26 °C (20 ml each for CTY strains) or 29 °C (10 ml each for YES strains). When cultures reached mid-exponential phase ($A_{600\text{ nm}} = \sim 0.8$), one-half of each culture was shifted to 37 °C for 1 h prior to termination of growth by addition of trichloroacetic acid (5% final concentration). Extraction of lipids and their analysis by high pressure liquid chromatography (HPLC) were carried out as described in detail previously (19, 37).

RESULTS

Sec14p Function Is Required to Maintain PtdIns(4)P Levels—The observation that elevation of PtdIns(4)P overcomes the secretory defect of *sec14-3* cells is consistent with a requirement for this lipid in efficient formation of Golgi-derived transport vesicles. If the primary role of Sec14p in secretion is to stimulate PtdIns(4)P production, then *sec14-3* mutants should show a decrease in PtdIns(4)P levels at restrictive temperature. To test this prediction, we explored the effect of the *sec14-3* mutation on PtdIns(4)P production by labeling the *sec14-3* strain (CTY1-1A) and an otherwise isogenic *SEC14* wild-type strain (CTY182) with *myo*-[2-³H]inositol at both permissive and nonpermissive temperature and measuring the phosphoinositide levels in these cells (Fig. 1). At 26 °C, the amount of PtdIns(4)P in *sec14-3* cells was equivalent to the

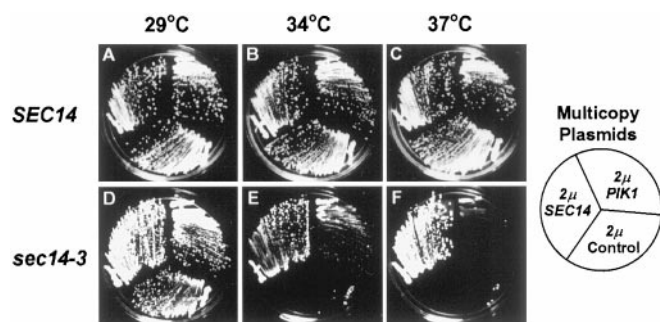


FIG. 2. Overexpression of *PIK1* partially suppresses the temperature sensitivity of *sec14-3* cells. Yeast strains CTY182 (*SEC14*, A–C) and CTY1-1A (*sec14-3*, D–F) were transformed with either pHYS14-5 (2- μ m *SEC14*), YEp352(*URA3*)-*PIK1* (2- μ m *PIK1*), or an empty multicopy vector, pRS426 (2- μ m control). The transformants were streaked onto agar plates selective for maintenance of the plasmids (SC-uracil) and incubated at the indicated temperatures, 29 °C (A and D), 34 °C (B and E), or 37 °C (C and F) for 3 days.

level in *SEC14* cells, and the ratio of PtdIns(4)P to PtdIns(3)P was essentially identical to that in the normal cells. One hour after shift to restrictive temperature (37 °C), the level of PtdIns(4)P in wild-type cells decreased only slightly (25%) and the ratio of PtdIns(4)- to PtdIns(3)P was essentially unaffected. In contrast, in the mutant cells, the level of PtdIns(4)P fell by about 60% and the ratio of PtdIns(4)P to PtdIns(3)P also fell precipitously. No significant differences were observed between these strains in the relative levels of other phosphoinositides, including PtdIns(3,5)P₂ and PtdIns(4,5)P₂, at either temperature (data not shown). These findings are consistent with the conclusion that Sec14p function is required to maintain normal cellular levels of PtdIns(4)P. Because synthesis of PtdIns(4)P requires catalysis by a PtdIns 4-kinase, we examined the relationship between Sec14p function and the action of Pik1p and Stt4p.

Pik1p Overproduction Partially Suppresses *sec14-3* Growth Defects—Yeast strains carrying the *sec14-3* mutation exhibit defective growth and secretion at temperatures above 33 °C (12, 14, 38). If the primary impairment in *sec14-3* cells at restrictive temperature is the inability to deliver PtdIns for PtdIns(4)P production, then it might be anticipated that, at a temperature where Sec14p function is severely compromised (but not totally absent), overexpression of the responsible PtdIns 4-kinase might increase the rate of PtdIns(4)P production to a level sufficient to restore viability. To test this prediction, multicopy plasmids overexpressing either *SEC14*, *PIK1*, *STT4*, *VPS34*, or appropriate vector controls were introduced by transformation into a *sec14-3* strain (CTY1-1A) or into an otherwise isogenic *SEC14* strain (CTY182) and the ability of these transformants to grow on agar plates at different temperatures was assessed (Fig. 2). Wild-type cells carrying all of these plasmids grew readily at all temperatures tested. As expected, the *sec14-3* strain carrying a control plasmid grew at 29 °C, but failed to grow at 34 °C or 37 °C; however, the mutant cells were able to grow at 34 °C and 37 °C when they carried plasmid-borne *SEC14*. Similarly, *sec14-3* cells expressing *PIK1* from a multicopy plasmid were able to grow reasonably well at 34 °C. Overexpression of the other PtdIns 4-kinase gene, *STT4*, or the PtdIns 3-kinase gene, *VPS34*, was unable to rescue the temperature-sensitive phenotype of *sec14-3* cells, even at 34 °C (data not shown). The fact that Pik1p-mediated PtdIns(4)P production suppresses the growth defect of *sec14-3* cells provides an independent demonstration that elevation of this lipid overcomes the secretory defect caused by *sec14* deficiency. These findings also provide further support for the conclusion

TABLE I
Temperature-sensitive growth of *pik1* mutants

Strain	Allele	Temperature	SC-Trp	YPD	YPD + 1 M sorbitol
		°C			
YES32	<i>PIK1</i>	30	+	+	+
		32	+	+	+
		34	+	+	+
		35	+	+	+
		37	+/-	+/-	+
YES95	<i>pik1-63</i>	30	+	+	+
		32	+	+	+
		34	-/+	-/+	+/-
		35	-	-	+/-
		37	-	-	-
YES102	<i>pik1-83</i>	30	+	+	+
		32	+	+	+
		34	-/+	-/+	+/-
		35	-	-	+/-
		37	-	-	-

Phenotype: +, wild-type growth; +/-, reduced growth rate; -/+ , weak growth; -, no detectable growth.

that PtdIns(4)P is important for some step required for protein transport from the Golgi.

PtdIns(4)P Level Is Reduced in *pik1* Mutants—It has been demonstrated previously that, like *SEC14*, *PIK1* is an essential gene (23, 27). If the pool of PtdIns(4)P in Golgi membranes is supplied by Pik1p and is important for secretion, then cells carrying temperature-conditional *pik1* alleles should show a reduction in PtdIns(4)P at restrictive temperature and should manifest a corresponding defect in secretion. As a prelude to labeling and phosphoinositide analysis, the growth properties of strains carrying two different *pik1*^{ts} alleles, YES95 (*pik1-63*) and YES102 (*pik1-83*), which were generated by random polymerase chain reaction mutagenesis (26, 32), were examined on a variety of different media to evaluate the minimum non-permissive temperature. Regardless of the medium, both *pik1*^{ts} mutants failed to grow above 34 °C, whereas the otherwise isogenic parental strain (YES32) grew well at all temperatures tested, but perhaps less robustly at the highest temperature examined (37 °C) (Table I). Addition of 1 M sorbitol to the medium improved the growth of the *pik1*^{ts} mutants only slightly, as observed for other *pik1*^{ts} alleles (27).

These same strains were labeled with *myo*-[2-³H]inositol, shifted from permissive (29 °C) to restrictive (37 °C) temperature for 1 h, and then lipids were extracted and analyzed by HPLC as above (Fig. 3). At permissive temperature, both mutant strains contained levels of PtdIns(4)P equivalent to or exceeding that observed in the wild-type control cells. After incubation at restrictive temperature for 1 h, the levels of PtdIns(4)P decreased substantially (by 60%) in both mutants, but not in the normal cells. These results demonstrate that inactivation of two different temperature-sensitive Pik1p derivatives causes the PtdIns(4)P pool to be depleted rather rapidly. Thus, Pik1p is responsible for production of a significant fraction of the cellular content of PtdIns(4)P. Presumably, at least some of the residual PtdIns(4)P present is supplied by Stt4p (24) and/or is inaccessible to Sac1p (20) and/or other *SAC1*-like domain-containing phosphoinositide phosphatases (39, 40). Thus, as observed in *sec14* mutants (Fig. 1), reduction in PtdIns(4)P level correlates with the inability of *pik1*^{ts} cells to grow at restrictive temperature.

Pik1p Function Is Required for Secretion—A standard assay for secretory proficiency in *S. cerevisiae* is release of the extracellular enzyme, invertase (*SUC2* gene product) (6, 7). Therefore, the amount of invertase secreted by *pik1*^{ts} mutants was measured at permissive temperature and after a shift to restrictive temperature for 1 h, under which conditions the mu-

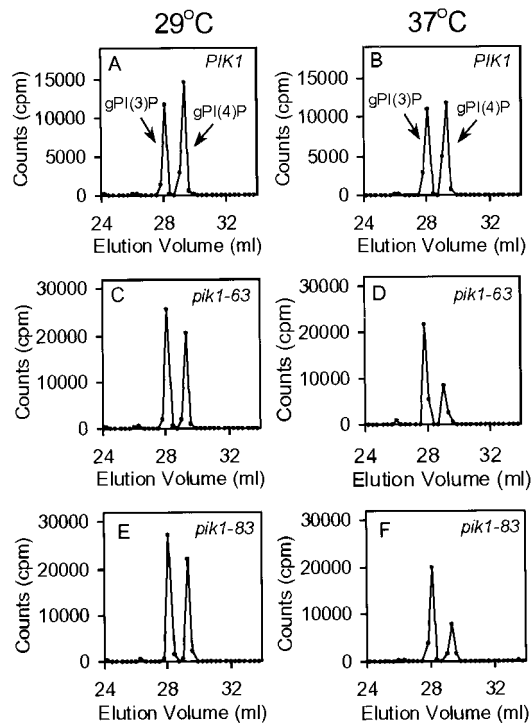


FIG. 3. **Phosphoinositide levels in temperature-sensitive *pik1* mutants.** Phosphoinositides in yeast strains YES32 (*PIK1*, A and B), YES95 (*pik1-63*, C and D), and YES102 (*pik1-83*, E and F) were labeled and analyzed at permissive and restrictive temperature, as described in the legend to Fig. 1. Equal amounts of total radioactivity from each sample (2×10^6 cpm) were loaded.

tants displayed a pronounced decrease in PtdIns(4)P level (Fig. 3). Both the amount of secreted (extracellular) and total invertase activities were measured in each sample. Similar levels of total activity rule out the possibility that any reduction in secreted invertase is simply the result of a lower rate of enzyme synthesis. The ratio of the amount of secreted invertase relative to the total cellular activity provides a “secretory index” that is a reflection of the secretory capacity of the cells (blocks in secretion result in lower secretory indices). At permissive temperature (29 °C), both *pik1^{ts}* mutants displayed a secretory index indistinguishable from the otherwise isogenic wild-type cells (Fig. 4). In contrast, after shift to restrictive temperature for 1 h (37 °C), each mutant showed a marked reduction in the secretory index (down to 40–50% of wild-type), whereas the normal cells showed a slight increase in their secretion index. These results indicate that Pik1p function is required for the efficient release of a secretory protein. Moreover, the rather remarkable correlation between the extent of the drop in PtdIns(4)P level and the degree of reduction in the efficiency of secretion provides further evidence for a direct role of PtdIns(4)P in secretory transport.

Delivery of CPY to the Vacuole Is Not Blocked in *pik1^{ts}* Mutants—In addition to the transport vesicles that deliver secretory products from the Golgi to the plasma membrane for release to the external milieu, discrete Golgi-derived vesicles also transport proteins to an internal compartment, the vacuole (41, 42). If the effect of the reduction of PtdIns(4)P level caused by *sec14* and *pik1* mutations is specific for the production of Golgi-derived vesicles destined for the plasma membrane, then these mutations should have little or no effect on the delivery of products to the vacuole. Indeed, *sec14* mutations have minimal effects on the sorting of proteins to the vacuole

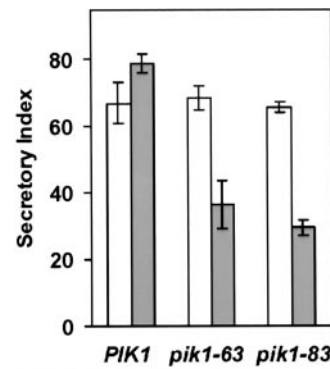


FIG. 4. **Defect in invertase secretion at restrictive temperature in *pik1* mutants.** Liquid cultures of yeast strains YES32 (*PIK1*), YES95 (*pik1-63*), and YES102 (*pik1-83*) were grown to $A_{600\text{ nm}} \sim 0.8$ at 29 °C, resuspended in low glucose medium to induce invertase expression, and divided into two equal portions. One-half was maintained at 29 °C (open bars) and the other was incubated at 37 °C for 1 h (filled bars) before assay of total and secreted invertase as described under “Experimental Procedures.” Secretory index is the ratio of secreted (extracellular) activity to the total activity. Values shown are the averages of three independent experiments (error bars represent S.D.).

(43)³; and, conversely, there is strong evidence that Vps34p-dependent PtdIns(3)P production is critical for the delivery of vacuolar hydrolases to the vacuole (44). Given the effect of *pik1* mutations on PtdIns(4)P formation and on secretion of invertase, it was of interest to test the effect of these mutations on the transport of a vacuolar constituent. A standard assay for protein progression through the Golgi and targeting to the vacuole is to follow the appearance of two precursor forms of CPY and their conversion to the final mature enzyme (41, 42). When the first precursor (so-called p1), which is the ER-modified form, travels from the ER to the Golgi, addition of mannose residues yields the second precursor (so-called p2). Cleavage of p2 by a specific protease in the lumen of the vacuole yields the mature (m) form. Therefore, we examined CPY synthesis and maturation by a pulse-chase protocol in the *pik1-83* mutant, and in an otherwise isogenic *PIK1* control strain. After shift to restrictive temperature (37 °C) for 10 min, followed by a 15-min labeling period and a 25-min chase (both at 37 °C), there was no significant difference in the efficiency that the precursor forms were converted to mature CPY compared with wild-type cells or the mutant itself at 25 °C (Fig. 5), indicating that delivery to the vacuole was occurring essentially normally at the nonpermissive temperature. The amount of mature CPY observed in the mutant was much greater than that seen in bona fide *vps* mutants that are defective in Golgi-to-vacuole transport (41, 42). These results suggest that, first, the secretory defect observed in *pik1* mutants is specific for the branch of the secretory pathway that leads from the Golgi to the cell surface and, second, PtdIns(4)P has a vital and specific role in this phase of secretion.

Pik1p Functions Downstream of Sec14p—If Sec14p serves as a P1TP to supply PtdIns as a substrate for Pik1p, then Sec14p acts biochemically “upstream” of Pik1p. Consistent with this view, overexpression of *PIK1* rescued *sec14-3* cells at an otherwise nonpermissive temperature (Fig. 2). If the order is *SEC14* → *PIK1* in a formal genetic sense, then *pik1* mutations should be epistatic to *SEC14* function, which we tested by the converse experiment. Indeed, in agreement with this prediction, overexpression of *SEC14* from a multicopy plasmid was unable to

³ E. Harsay and R. W. Schekman, personal communication.

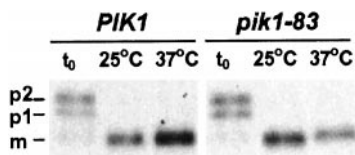


FIG. 5. Maturation of vacuolar enzyme, CPY, is normal in *pik1* mutants. Processing and maturation of CPY was assessed by pulse-chase analysis in strains YES32 (*PIK1*) and YES102 (*pik1-83*) as described under "Experimental Procedures." Cultures grown at 25 °C were split into two equal portions and incubated at either 25 ° or 37 °C for 10 min, labeled at the same temperatures for 15 min, and chased at the same temperatures for 25 min. The t_0 time point was taken from the 25 °C samples prior to initiation of the chase.

rescue the growth defect of either the *pik1-63* mutant or the *pik1-83* mutant, even under the least stringent nonpermissive condition (34 °C) (Table I), whereas a *PIK1* plasmid restored growth to both mutants, as expected (Fig. 6).

DISCUSSION

We recently demonstrated that a mutation, *sac1-22*, that suppresses the growth and secretion defect of *sec14-3* mutants causes a specific and pronounced elevation in the cellular level of PtdIns(4)P (19). It was also demonstrated by others that *SAC1* encodes a novel multifunctional phosphoinositide phosphatase that can use PtdIns(4)P, among other phosphoinositides, as a substrate (20). The fact that elevated PtdIns(4)P rescues the *sec14-3* mutant suggests that the cause of the secretion defect in Sec14p-deficient cells involves limitation for this lipid. Consistent with this supposition, we have now shown (Fig. 1) that the level of PtdIns(4)P drops markedly when *sec14-3* cells are shifted to nonpermissive temperature. These observations raised an important question. If inactivation of Sec14p lowers the rate of PtdIns(4)P production, what PtdIns 4-kinase is responsible for the Sec14p-dependent synthesis of this lipid? The evidence documented here indicates that formation of the Sec14p-dependent pool of PtdIns(4)P is specifically catalyzed by the PtdIns 4-kinase encoded by the *PIK1* gene (23, 27).

As we have demonstrated here, expression of *PIK1* from a multicopy plasmid was able to rescue growth of *sec14-3* cells at a temperature that is otherwise nonpermissive (Fig. 2). Overexpression of *STT4*, the other known PtdIns 4-kinase in *S. cerevisiae* (24), or of *VPS34*, a PtdIns 3-kinase (45), did not rescue the growth defect of *sec14-3* cells. It has been documented that expression of *PIK1* from a multicopy plasmid raises the level of this enzyme (23, 26) and its lipid product (32). Thus, two different conditions (loss of Sac1p and elevation of Pik1p) that cause an increase of the cellular content of PtdIns(4)P ameliorate the temperature sensitivity of *sec14-3* cells. As observed for *sec14-3* cells, PtdIns(4)P level falls dramatically when *pik1* mutants are shifted to restrictive temperature (Fig. 3), again consistent with the view that reduction of this lipid contributes to cell inviability.

Taken together, these results suggest that Sec14p is required to maintain PtdIns(4)P production by Pik1p for secretion. There are two plausible mechanisms by which Sec14p could contribute to Pik1p-dependent PtdIns(4)P synthesis. First, Sec14p and Pik1p may interact directly by physical association. Mammalian PITPs can associate directly with PtdIns 4-kinases or PtdIns 3-kinases and stimulate activity (46–49). In one case, *in vitro* activation of PtdIns 3-kinase was observed by adding yeast Sec14p (48). At present, however, there is no evidence that Sec14p and Pik1p physically associate. A second model is indirect enhancement of Pik1p-dependent PtdIns(4)P production. Sec14p may transfer PtdIns from its sites of synthesis, thought to be the ER and mitochondria in yeast (reviewed in Ref. 50), to other membranes, including the Golgi,

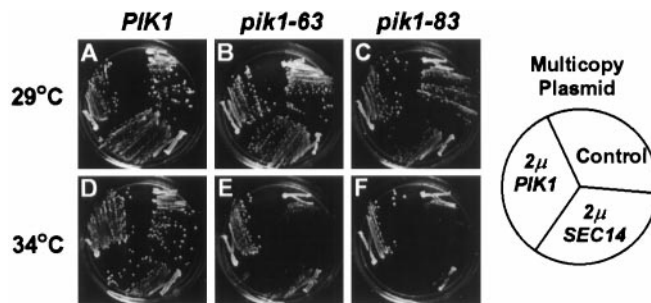


FIG. 6. Overexpression of *SEC14* does not suppress the temperature sensitivity of *pik1* mutants. Yeast strains YES32 (*PIK1*, A and D), YES95 (*pik1-63*, B and E), and YES102 (*pik1-83*, C and F) were transformed with the same plasmids described in the legend to Fig. 2. The transformants were grown at either 29 °C (A–C) or 34 °C (D–F) as described in the legend to Fig. 2.

where it can be converted to PtdIns(4)P by the action of Pik1p. This mechanism accounts for the enhancement of phosphoinositide levels at the plasma membrane by at least one mammalian PITP (51). A rat PtdIns/PtdCho exchange protein that bears little sequence similarity to Sec14p is nonetheless able to complement a *sec14* temperature-sensitive mutant *in vivo* (52), suggesting that the phospholipid exchange activity of this heterologous PITP is responsible for the phenotypic rescue rather than its specific contacts with any yeast protein. In any event, whether Sec14p and Pik1p interact and are localized to the Golgi in yeast are important issues for future study. One intriguing possibility is that Frq1p, which was shown recently to physically associate with Pik1p (26), may have a critical role in localization of Pik1p. Overexpression of *FRQ1* suppresses the temperature-sensitivity of certain *pik1* alleles (26), whereas overexpression of *SEC14* does not rescue any *pik1* mutant tested (Fig. 6), suggesting that Sec14p and Frq1p contribute to Pik1p function via different mechanisms.

Most significantly, we also demonstrated here that *pik1* cells are defective in the Golgi-to-plasma membrane phase of secretion, as judged by a dramatic reduction in the release of extracellular invertase at restrictive temperature (Fig. 4). Under similar conditions, the *pik1* mutants are not defective in the Golgi-to-vacuole pathway, as judged by essentially normal rates of maturation of CPY (Fig. 5). This observation is reminiscent of *sec14* mutants that exhibit nearly wild-type CPY maturation under conditions where invertase secretion is highly defective (43). This specific effect supports the hypothesis that transport vesicles destined for the cell surface require the Sec14p/Pik1p-dependent generation of PtdIns(4)P and that this lipid is necessary for some aspect of the formation, stability, movement, targeting, and/or fusion of secretory vesicles. In this regard, it should be noted that there is no evidence that the PtdIns(4)P generated by Pik1p is subsequently converted to PtdIns(4,5)P₂, in contrast to what has been observed for the other yeast PtdIns 4-kinase, Stt4p (29, 30).

What steps in the Golgi-to-plasma membrane stage of secretion may require PtdIns(4)P? It was shown nearly two decades ago (6, 53) that, at nonpermissive temperature, *sec14* mutants accumulate unusual membrane structures ("Berkeley bodies") that are thought to represent hypertrophy of the Golgi cisternae due to blockade of the exit of transport vesicles from this compartment. Indeed, secretory proteins, like invertase and acid phosphatase, accumulate in Golgi cisternae in *sec14* mutants (reviewed in Ref. 7). It has been reported that ER- and Golgi-modified and immature (unprocessed) forms of the vacuolar enzyme, proteinase A, also accumulate in *sec14* mutants, but the modest precursor accumulation observed could be the indirect result of the protracted incubation time at restrictive

temperature (43). These observations indicate that the formation of secretory vesicles and/or the packaging of their cargo is defective in *sec14* mutants, suggesting further that PtdIns(4)P may have a direct role in biogenesis of Golgi-derived secretory vesicles. In an *in vitro* reconstituted vesicle coating reaction, association of COPII coat components with liposomes, the presence of PtdIns(4)P (or PtdIns(4,5)P₂) promoted coat formation (54). In an analogous way, PtdIns(3)P has an important function in the formation of transport vesicles necessary for delivery of proteins to the vacuole (reviewed in Ref. 55). If the PtdIns(4)P generated by Pik1p has a similar role in vesicle budding from the Golgi, then *pik1* mutants should also accumulate Berkeley body-like membranes at the restrictive temperature. Indeed, when shifted to restrictive temperature, *pik1* mutants display a dramatic accumulation of internal membranes (32), which, at the electron microscopy level, appear morphologically similar to the membrane structures accumulated in *sec14* cells.⁴ Alternatively, PtdIns(4)P may affect exit of secretory vesicles from the Golgi less directly, for example, by providing contact sites between the vesicles and phosphoinositide-binding proteins thought to be associated with the actin cytoskeleton (reviewed in Ref. 56). Transport of secretory vesicles from the Golgi to the plasma membrane is an actin-dependent process (57, 58).

Although PtdIns(4)P degradation by Sac1p clearly modulates the PtdIns(4)P pool (19, 20), it cannot be the only phosphatase that regulates the level of this lipid, since Sac1p function is dispensable for cell survival under most growth conditions (57). In this regard, it is important to note that *S. cerevisiae* contains other phosphoinositide phosphatases (39, 40). Two of these enzymes are involved in membrane trafficking pathways: Sjl3p/Inp53p in recycling of Golgi proteins from early endosomes (59) and Sjl1p/Inp51p in endocytosis (60). It will be interesting to explore the influence of these proteins on the Sec14p- and Pik1p-dependent pool of PtdIns(4)P, since these phosphatases contain two catalytic domains, one of which is a SAC1-like domain (20, 40).

In addition to *PIK1* and *SAC1*, several other genes interact with *SEC14* in secretion. Mutations in several genes (*CKI1*, *PCT1*, *CPT1*, *KES1*, *BSD1*, *BSD2*) also suppress the secretory defects of *sec14* mutants (12, 14). Three genes (*CKI1*, *PCT1*, *CPT1*) encode enzymes in a PtdCho biosynthesis pathway (61–63) and *KES1* encodes an oxysterol-binding protein (64). Furthermore, Pld1p (a phospholipase D), which is activated in *sec14* mutants (65), is required for these mutations to suppress *sec14* defects (65, 66), suggesting that the *sec14* suppression by these mutations takes place only when basal PtdCho levels are decreased or phosphatidic acid levels are elevated. Currently, it is unclear whether any of the proteins encoded by these *sec14* suppressor genes interact with Pik1p. It is possible that defects in these genes increase the PtdIns 4-kinase activity of Pik1p or decrease the PtdIns(4)P phosphatase activity of Sac1p (or both). Alternatively, these suppressor gene products may act independently of Pik1p activities and PtdIns(4)P levels, to restore secretion in *sec14* mutants. It will be interesting to explore the influence of these proteins on the *PIK1*-dependent pool of PtdIns(4)P, thereby adding to our understanding of Sec14p and Pik1p regulation of secretion.

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