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The Yeast *SLY* Gene Products, Suppressors of Defects in the Essential GTP-Binding Ypt1 Protein, May Act in Endoplasmic Reticulum-to-Golgi Transport

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It has been shown previously that defects in the essential GTP-binding protein, Ypt1p, lead to a block in protein transport from the endoplasmic reticulum (ER) to the Golgi apparatus in the yeast *Saccharomyces cerevisiae*. Here we report that four newly discovered suppressors of *YPT1* deletion (*SLY1-20*, *SLY2*, *SLY12*, and *SLY41*) to a varying degree restore ER-to-Golgi transport defects in cells lacking Ypt1p. These suppressors also partially complement the *sec21-1* and *sec22-3* mutants which lead to a defect early in the secretory pathway. Sly1p-depleted cells, as well as a conditional lethal *sly2* null mutant at nonpermissive temperatures, accumulate ER membranes and core-glycosylated invertase and carboxypeptidase Y. The *sly2* null mutant under restrictive conditions (37°C) can be rescued by the multicopy suppressor *SLY12* and the single-copy suppressor *SLY1-20*, indicating that these three *SLY* genes functionally interact. Sly2p is shown to be an integral membrane protein.

Evidence is accumulating that small GTP-binding proteins are essential regulatory elements in the secretory pathway of all eukaryotic cells. Initially, GTP-binding proteins were implicated in secretion by conditional lethal yeast mutants defective in two *ras*-like genes, *SEC4* (45) and *YPT1* (48, 51), as well as by the inhibition of defined steps in protein transport upon the addition of nonhydrolyzable GTPγS to a mammalian cell-free system (31). These findings offer a possible solution to the long-standing problem of how vesicular transport of proteins destined to reach the plasma membrane, secretory granules, or lysosomes (vacuoles) proceeds unidirectionally. It was postulated (11) that by cycling between a GTP- and a GDP-bound state, specific proteins might function in vesicle budding and/or fusion events between different compartments of the secretory pathway.

The specificity of intracellular transport and of sorting vesicle-enclosed proteins in the exo- and endocytic pathways could indeed be due to a multitude of different GTP-binding proteins. This hypothesis is supported by the recent findings that in *Saccharomyces cerevisiae*, in addition to the previously identified Ypt1p and Sec4p, small GTP-binding proteins encoded by *SAR1*, *ARF1*, and *ARF2* seem to play a role in endoplasmic reticulum (ER)-to-Golgi and in intra-Golgi transport (36, 53). Furthermore, different members of the ypt/rab family of *ras*-like proteins in mammals appear to be specifically localized in different cellular organelles belonging to the exo- and endocytic pathways (13, 18, 23, 32).

The formation, transport, and fusion of vesicles may each require multisubunit protein complexes (30). Indeed, the large number of yeast genes that have been shown to be involved in protein secretion (39) may code for various multicomponent complexes. Wilson et al. (59) and Clary et al. (14) recently showed that two of these genetically identified proteins, Sec18p and Sec17p, which are known to be essential for ER-to-Golgi transport, are interchangeable with the mammalian proteins NSF (for *N*-ethylmaleimide-sensitive fusion protein) and α-SNAP (for soluble NSF attachment protein). A functional role for NSF and α-SNAP in the fusion of ER-derived vesicles with Golgi membranes was established biochemically. Functional equivalence of yeast and mammalian Ypt1p has also been documented (24), indicating that essential components of the protein secretion machinery are highly conserved throughout evolution.

By a combination of molecular and classical genetics, we have recently isolated and characterized four yeast genes (*SLY*) that act as suppressors of the *YPT1* deletion. From the nucleotide sequences of these genes, the protein products of three multicopy suppressors, *SLY2*, *SLY12*, and *SLY41*, are predicted to be integral membrane proteins, whereas a single-copy suppressor, *SLY1-20*, encodes a mutant protein of hydrophilic character (15).

In this report, we show that the *SLY* gene products most likely act early in secretion, like the GTP-binding Ypt1 protein, whose defects they are able to suppress.

MATERIALS AND METHODS

Yeast strains and growth conditions. Yeast strains are described in Table 1. Genetic techniques were performed as described by Sherman et al. (52). Yeast cells were transformed by the Li acetate treatment described by Ito et al. (26). The transformants were selected and maintained on minimal media (SD and SGal medium containing either glucose or galactose, respectively) lacking either leucine or uracil. The Glu^+/Glu^- and Ts^+/Ts^- phenotypes were checked after replica plating onto rich medium (YEPD) containing 8% glucose and incubation at 25°C or replica plating onto rich medium (YEPD) containing 2% glucose and incubation at different temperatures, respectively. For growth curves, the preparation of cell extracts, and ultrastructural inspections, liquid media and the same growth conditions were used. Media used for invertase induction and sulfur starvation are specified below.

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TABLE 1. Yeast strains used in this study

Strain	Genotype	Parental strains	Source[a]
AG430-1A	*MATa leu2 his3*		
HLR3	*MATa leu2 his3 GAL10-YPT1-HIS3*		48
TSU3-5D	*MATα ura3 ypt1* (Ts)-*LEU2*		
NTSU3-1B	*MATa leu2 ura3 his3 ypt1-44-LEU2*		J. Becker and H. D. Schmitt
HMSF176	*MATa sec18-1*		Yeast Genetic Stock Center, Berkeley, Calif.
RH220-7D	*MATα sec4-2 ura3 leu2 his4 lys2*		H. Riezman (Basle)
RH235-4C	*MATa sec13-1 ura3 leu2 his4 lys2*		H. Riezman (Basle)
RH238-1B	*MATa sec16-2 leu2 his4*		H. Riezman (Basle)
RH233-3A	*MATα sec20-1 ura3 leu2 his4*		H. Riezman (Basle)
RH239-5A	*MATα sec21-1 ura3 leu2 his4 lys2*		H. Riezman (Basle)
RH227-3A	*MATa sec23-1 ura3 leu2 his4*		H. Riezman (Basle)
SEL12-3C	*MATa sec12-4 leu2*		
SEL18-10C	*MATα sec18-1 leu2*		
SEL21-5B	*MATa sec21-1 leu2*		
SEL22-7D	*MATα sec22-3 ura2 leu2 lys2*		
MB7	*MATα sec7-1 leu2 his4 ura3 suc2-Δ9*		M. Bielefeld (Düsseldorf)
LSY20-1A	*MATa leu2 his3 GAL10-YPT1-LEU2 SLY1-20*		15
GFUII-2B	*MATα leu2 his3 GAL10-YPT1-HIS3*		15
INT1[b]/INT2[c]	Same as GFUII-2B with plasmid YRp5L-*SLY1/5* integrated at the *SLY1* locus		15
SD104-1A	*MATα leu2 his3 sly2::HIS3* (Cs^- Ts^-)		
SD104-1B	*MATa leu2 his3 sly2::HIS3* (Cs^- Ts^-)		
SD104-1C	*MATa leu2 his3 SLY2*		
SD104-1D	*MATα leu2 his3 SLY2*		
SD10-10B	*MATα leu2 his3 sly2::His3* (Cs^-)		
20B-12	*MATα trp1 pep4-3*		E. W. Jones (Pittsburgh)
GSF1	*MATa leu2 his3 sly1-(YIp51-GAL10)-SLY1*		
Diploid strains derived from haploid strains listed above			
WM3		HLR3 × INT1	
WM21		HLR3 × INT2	
WM210		LSY20-1A × INT1	
WM12		LSY20-1A × INT2	

[a] Unless indicated otherwise, the strains listed are from this study.
[b] This transformant is phenotypically Glu^-, presumably *SLY1(wt)*-YRp5L-*SLY1(wt)*.
[c] This transformant is phenotypically Glu^+, presumably *SLY1(wt)*-YRp5L-*SLY1-20* or *SLY1-20*-YRp5L-*SLY1(wt)*.

Plasmids and nucleic acid techniques. To test the suppression of various *ypt1* and *sec* defects, we transformed *leu2* yeast strains with the 2μm vector YEp511 (15) containing the *SLY2*, *-12*, or *-41* gene as a 1.5-kb *Eco*RV-*Bam*HI, a 2.7-kb *Hin*dIII-*Sau*3A, or a 1.6-kb *Hin*dIII-*Sau*3A fragment, respectively. (The *Sau*3A site marks the end of the original insert. Actually, *Hin*dIII fragments were subcloned which carry 352 bp derived from pBR322 sequences in vector YEp13.) YEp24 (12) subclones containing the *SLY2*, *-12*, or *-41* gene were used for transformation of *ura3* strains (e.g., strains TSU3-5D and NTSU3-1B) or for double transformations of *leu2/ura3* strains (strains RH239-5A and SEL22-7D). The single-copy plasmid YCp5LU-*SLY1/5* (15) was used to introduce the mutant *SLY1-20* allele into *ura3* or *leu2* strains.

The chromosomal *SLY1* gene was replaced by the *GAL10-SLY1* fusion as follows. First, a 2.1-kb *Spe*I fragment (including parts of the promoter region and the first 1.8 kb of the *SLY1* coding sequence) derived from plasmid YRp5L-*SLY1/5(wt)* (15) was inserted into the unique *Sma*I site of the M13 vector. Site-directed mutagenesis (34) was used to introduce a *Sal*I site 21 bp upstream of the first initiation codon of the *SLY1* coding region. A 1.2-kb *Sal*I-*Hpa*I fragment of this construct was subcloned into the yeast vector YEp51 which was cut with *Sal*I and *Nru*I to delete the 2μm origin of replication, thereby creating the yeast integration vector YIp51-*SLY1*. This plasmid carries the *GAL10* promoter fused to a truncated *SLY1* coding sequence which ends at the *Hpa*I site in codon 390. Plasmid YIp51-*SLY1* was linearized by *Bgl*II cleavage to direct integration into the chromosomal *SLY1* locus of strain AG430-1A. This integration should lead to a truncated *SLY1* copy in tandem with a complete *SLY1* allele under control of the *GAL10* promoter. Southern analysis of two transformants confirmed integration at the *SLY1* locus (data not shown).

Analysis of invertase by gel electrophoresis under nondenaturing conditions and by immunoblotting. Synthesis of the periplasmic invertase was induced by transferring cells to YEPD medium containing 0.1% glucose. Secreted and intracellular invertase were isolated from cells grown at different temperatures or for different times in glucose- or galactose-containing medium as described by Schauer et al. (47). Briefly, spheroplasts were generated by treatment with lyticase (Sigma) and pelleted. The supernatant contained the periplasmic invertase. The spheroplasts were broken by vortexing with lysis buffer (47), and cell debris was removed by centrifugation. Aliquots of both the periplasmic and intracellular fractions were loaded on 6.5% nondenaturing polyacrylamide gels. After electrophoresis, invertase was stained as described previously (17).

For immunoblot analysis of the invertase secreted by cells deficient in Ypt1p and expressing the different suppressors (Fig. 1B), aliquots of the periplasmic fraction obtained after lyticase treatment were mixed with sodium dodecyl sulfate (SDS) loading buffer and subjected to SDS-8% polyacryl-

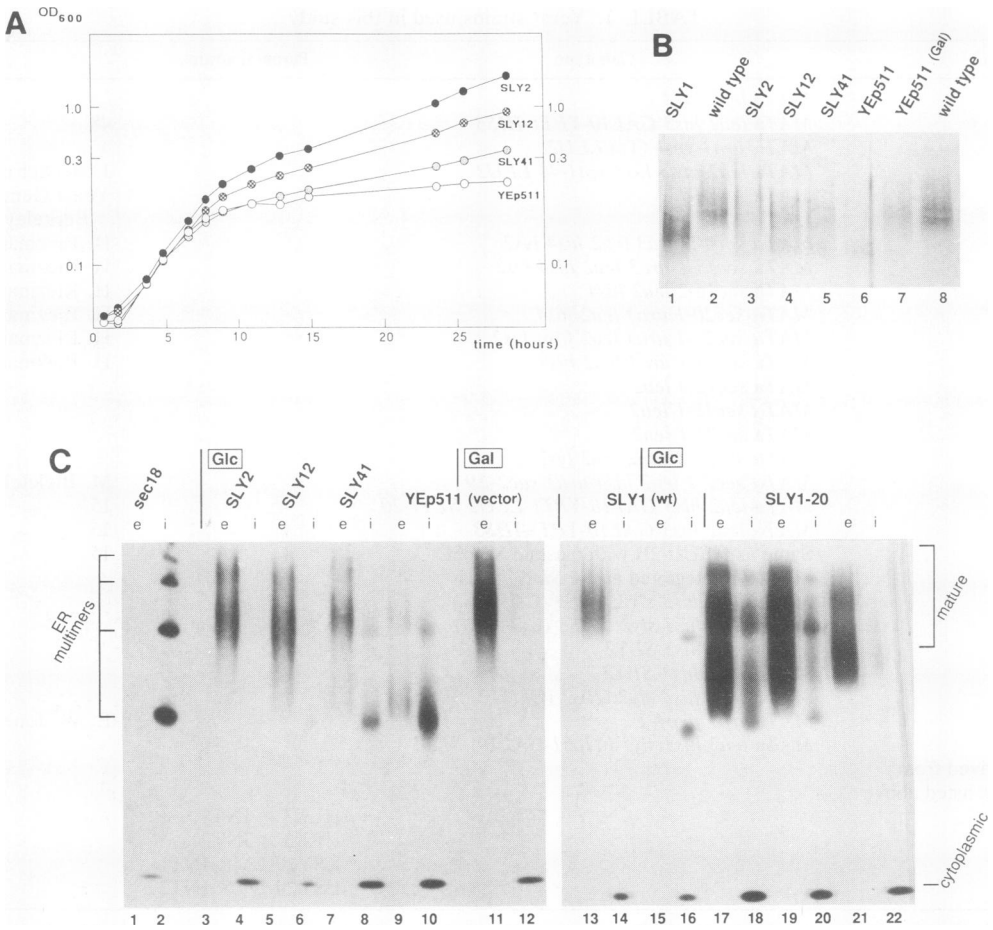

FIG. 1. *SLY* suppressor genes improve the proliferation and invertase secretion of Ypt1p-depleted cells. (A) Strain HLR3 (*GAL10-YPT1*) was transformed with the vector YEp511 or *SLY2*, *SLY12*, or *SLY41* sequences carried by this multicopy vector. Cells growing in selective medium containing galactose were shifted to rich medium containing either galactose or glucose and incubated at 25°C. Growth of transformants was observed by measuring the OD_{600}. (B) These transformants as well as different *SLY1-20* strains were analyzed for secreted invertase. At 15 h after the shift from SGal medium to rich medium containing glucose or galactose, cells were derepressed for synthesis of secreted invertase for 1 h, spheroplasts were prepared and pelleted by centrifugation, and the supernatant was subjected to SDS-PAGE and immunoblot analysis. (C) Aliquots derived from both periplasmic (e) and cytoplasmic (i) fractions were analyzed on a 6.5% nondenaturing polyacrylamide gel followed by activity staining of the invertase. As a control, extracts from the ER-accumulating Ts^- *sec18-1* mutant shifted to 37°C during the invertase induction were analyzed. Periplasmic and intracellular invertase was also analyzed from various diploid and haploid cells carrying the *GAL10-YPT1* fusion gene(s) and different numbers of *SLY1(wt)* and *SLY1-20* alleles. These four strains express three *SLY1(wt)* alleles (strain WM3; lanes 13 to 16), two wild-type and one mutant *SLY1-20* allele (strain WM210; lanes 17 and 18), one of each (strain INT2; lanes 19 and 20), or one wild-type and two mutant alleles (strain WM12; lanes 21 and 22). See Tables 1 and 2 for a description of these strains. The faster mobility of invertase secreted by these cells could also be shown in extracts from the *SLY1-20* mutant not expressing *YPT1* (panel B, lane 1).

amide gel electrophoresis (PAGE). The same results were obtained when whole cells were treated for 2 min at 100°C with SDS loading buffer. This treatment was sufficient to release the periplasmic invertase from the cells (data not shown).

The defect in intracellular transport of invertase in the galactose-dependent *SLY1* mutant was examined by immunoblot analysis of whole-cell extracts. Strain GSF1 was transferred from permissive minimal medium (SGal) to rich medium containing 2% glucose and incubated at 30°C for different times as indicated in Fig. 4B. Cells (10 units at an optical density of 600 nm [OD_{600}]) were washed with YEPD medium containing 0.1% glucose and shaken for 1 h at 30°C in the same medium to derepress the synthesis of invertase. As a control, whole-cell extracts from a *sec18-1* mutant strain shifted to 37°C during induction of external invertase were prepared. Cells were collected by brief centrifugation, washed with ice-cold 10 mM NaN_3, and broken with glass beads. Samples (20 μl, 1.3 OD_{600} units of cell extract) were resolved by SDS-8% PAGE and electrophoretically transferred to nitrocellulose filters which were then incubated in buffer A (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 0.05% Tween 20) for 1 h at room temperature. Antibody binding was done in buffer A with 30 μl of invertase antiserum (a generous gift of L. Lehle, Regensburg, Federal Republic of Germany) and a crude extract obtained from 150 OD_{600} units of cells of the Suc^- strain MB7 to reduce unspecific background (27). Filters were washed with buffer A, two times with buffer B (0.2% SDS, 0.9% NaCl, 0.5% Triton X-100), and again with buffer A for 10 min each. Staining was

performed with peroxidase-conjugated anti-rabbit immunoglobulin and the ECL Western blotting (immunoblotting) detection system (Amersham) according to the supplier's recommendations.

Radiolabeling of cells, immunoprecipitation, and endo H treatment. Growth conditions for immunoprecipitation of carboxypeptidase Y (CPY) varied depending on which mutant strain was used (for details, see the legends to Fig. 2, 4, and 7). For sulfur starvation, cells were incubated in semiminimal medium (41) which contained either 4% glucose or galactose in order to repress or induce the expression of genes fused to the *GAL10* promoter. It could be shown that the growth characteristics of strain GSF1 in rich medium and in the sulfur starvation medium are identical. Cells (3 OD_{600} units) were harvested and resuspended in 0.5 ml of fresh medium containing 150 μCi of [^{35}S]methionine (Amersham). After 60 min of radiolabeling, the cells were chased for 30 min by the addition of 0.01 volume of a solution containing 100 mM $(NH_4)_2SO_4$ and 0.4% L-methionine (42). Incubation was terminated by the addition of 5 mM NaN_3 and 0.5 mM phenylmethylsulfonyl fluoride (final concentration). Radiolabeled cells were collected and resuspended in 0.15 ml of spheroplasting buffer (47) containing 150 U of lyticase. Spheroplasts generated during a 60-min incubation at 30°C were sedimented by centrifugation for 5 min at 2,000 × *g*. The spheroplast pellet was lysed in 100 μl of 1% SDS and heated for 3 min at 100°C. The following immunoprecipitation was performed by the method of Raymond et al. (40). CPY antiserum was a generous gift of A. Finger and D. Wolf, Stuttgart, Federal Republic of Germany.

For endo-β-*N*-acetylglucosaminidase H (endo H) treatment, aliquots of radioactively labeled CPY were acetone precipitated for 30 min at −80°C. Protein pellets were dried under vacuum, resuspended in 0.2% SDS, and heated at 100°C for 2 min. Samples were incubated in 50 mM K phosphate buffer (pH 5.4)–0.7% mercaptoethanol–0.5 mM phenylmethylsulfonyl fluoride–20 mU of endo H (Boehringer GmbH, Mannheim, Federal Republic of Germany) at 37°C for 48 h. Protein samples were lyophylized and resuspended in SDS loading buffer.

For immunoprecipitation of invertase, cells were grown to the exponential phase in YEPD medium containing 2% glucose. A total of 3×10^7 cells were harvested, washed with 1:2 diluted YEP medium containing 0.05% glucose and 0.5% sucrose, and preincubated in 1 ml for 30 min at 25 or 37°C to derepress synthesis of invertase. Radiolabeling of cells after the addition of 150 μCi of [^{35}S]methionine was allowed to proceed for 60 min. After labeling, cells were harvested and resuspended in prewarmed fresh medium and the chase was continued for 30 min. Cells were maintained at 25 or 37°C during pulse and chase as specified. Incubation was terminated by the addition of 10 mM NaN_3. Labeled cells were washed with 10 mM NaN_3 containing 10 OD_{600} units of cells of the MB7 (*suc2-Δ9*) strain and broken with glass beads (42). Immunoprecipitation was performed as described above for CPY. All samples were resolved by SDS-8% PAGE. After electrophoresis, the gels were fixed, prepared for fluorography by incubation for 20 min with Amplify (Amersham), dried, and exposed to Kodak X-Omat AR films at −80°C.

Sly2p antiserum and identification of Sly2p. A 0.8-kb *Hin*cII-*Eco*RV fragment, ranging from codon 5 of the *SLY2* sequence to 175 bp downstream of the stop codon, was subcloned into the expression vector pUR288 (44), thereby fusing almost the entire coding region of the *SLY2* gene to the C terminus of the *lacZ* gene. The β-galactosidase–Sly2 fusion protein was overexpressed in exponentially growing *Escherichia coli* cells (strain JM101) by incubation with 1 mM isopropylthio-β-D-galactoside. Cells were harvested after 4 h of induction, treated with lysozyme, and broken with detergent and by sonication. The inclusion bodies containing the fusion protein were washed with 1 M urea and suspended in 7 M urea. This extract, consisting of about 50% fusion protein, was either subjected to a preparative SDS-8% PAGE (for the first four injections) or dialyzed against phosphate-buffered saline (50 mM potassium phosphate (pH 7.2), 150 mM NaCl) and used directly for immunization of two rabbits. For the primary injection, the extract (about 1 mg of protein) was mixed with complete Freund adjuvant, and boosts with incomplete Freund adjuvant were given at 6-week intervals.

Extracts from wild-type yeast cells (SD104-1C), Sly2p overproducer cells (AG430-1A transformed with YEp511-*SLY2*), and *sly2* cells (SD104-1A) were prepared as follows. Exponentially growing cells (40 ml) were harvested and washed with ice-cold water and suspended in lysis buffer (0.2 M Tris-HCl [pH 8.0], 6 mM $MgCl_2$, 1 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 15 μg of trasylol per ml, 5 μg of leupeptin per ml, 20 μg of trypsin inhibitor per ml, 1 mM benzamidine, 5 μM E-64). Cells were broken by vortexing for 20 s six times with 2 ml of glass beads per g of cells. Unbroken cells were removed by a 10-min centrifugation at 500 × *g*. The supernatant was centrifuged at 100,000 × *g* for 1 h, the pellet was suspended in water, and both the pellet and the supernatant fraction were mixed with SDS loading buffer, boiled for 2 min, and separated by SDS-12.5% PAGE for immunoblot analysis. To determine the membrane localization of Sly2p, the following reagents (final concentrations are given) were added individually to the extracts prior to the centrifugation at 100,000 × *g*: lysis buffer, 5 M urea, 1 M NaCl, 1 M potassium acetate, 1% Triton X-100 (in lysis buffer), or 0.1 M sodium carbonate (pH 11) (in water). After mixing and incubation at 0°C for 30 min, extracts were centrifuged at 100,000 × *g*, and pellet and supernatant were separated, subjected to SDS-12.5% PAGE, and electrophoretically transferred to nitrocellulose. For immunoblot analysis of Sly2p, the nitrocellulose filters were treated with polyclonal anti-Sly2p antisera and stained with biotinylated anti-rabbit immunoglobulin from donkey and streptavidin-conjugated horseradish peroxidase (Amersham) according to the supplier's recommendations.

Electron microscopy. For electron microscopy, samples were taken from yeast cultures grown in different media or at different temperatures as indicated in the legend to Fig. 5. Cells were prepared by the permanganate fixation technique (54). Fixation was achieved by washing 50 OD_{600} units of cells (1 to 2 OD_{600} units/ml) once with distilled water and then resuspending the cells in 10 ml of fresh 2% $KMnO_4$ and incubating them for 2 h at room temperature. Fixed cells were washed twice with distilled water, dehydrated in a graded series of ethanol washes, and embedded in Epon. Silver to pale-gold sections (about 60 nm) were cut and stained with uranyl acetate and lead citrate for 5 min each. Sections were examined in a Philips CM12 electron microscope at 80 kV.

RESULTS

Multicopy suppressors *SLY2*, *SLY12*, and *SLY41* restore ER-to-Golgi transport in cells lacking Ypt1p. The four *SLY* genes that we have recently isolated and characterized were selected by their ability to suppress the deletion of the

otherwise essential *YPT1* gene (15). As Ypt1p seems to be required for vesicular protein transport from the ER to the Golgi apparatus, the *SLY* gene products may act by suppressing the secretion defects observed in *ypt1* mutants (48, 51). To prove this, we monitored the processing and secretion of invertase in a *GAL10-YPT1* strain transformed with plasmids carrying the multicopy suppressor *SLY2*, *SLY12*, or *SLY41*. Synthesis of the secreted form of invertase can be induced by reducing the glucose concentration of the growth medium (12). After cell wall and spheroplasts are separated, the extent of secretion as well as the glycosylation state of the enzyme can be monitored by activity staining in nondenaturing polyacrylamide gels (17). Under nonpermissive conditions, different temperature-sensitive *ypt1* mutants accumulate multimeric, core-glycosylated invertase intracellularly (6, 48), a phenotypic alteration characteristic for mutants, like *sec18* (17), that are blocked in protein transport from the ER to the Golgi apparatus. Likewise, preventing the expression of the *GAL10-YPT1* fusion gene leads to an intracellular accumulation of core-glycosylated invertase (48, 51) (Fig. 1B).

Fifteen hours after the *YPT1* gene was silenced by transfer to a glucose-containing medium, at which time cellular proliferation had stopped and more than 90% of the cells were still viable (49), invertase synthesis was induced in different *SLY* transformants and intra- and extracellular enzyme patterns were analyzed. As can be seen in Fig. 1A, the three multicopy suppressors differed in their ability to rescue cells from growth repression caused by Ypt1p depletion. *SLY41* was the weakest and *SLY2* the most efficient of the multicopy suppressors. The improvement of invertase secretion perfectly correlated with the degree of suppression (Fig. 1C). In cells transformed with control vector YEp511, intracellular core-glycosylated forms of invertase dominated besides the cytoplasmic, unglycosylated invertase which is constitutively expressed in all cells. In contrast, cells transformed with *SLY2* and *SLY12* secreted incompletely glycosylated enzyme. This is seen by a broad smear in gels stained for activity (Fig. 1C) and in immunoblots of denaturing gels (Fig. 1B), due to heterogeneous glycosylation of invertase that normally takes place in different compartments of the Golgi apparatus (17, 20). Although *SLY41*-transformed cells also secreted incompletely glycosylated invertase, they accumulated significant amounts of partially processed enzyme intracellularly (Fig. 1C, lane 8). In the denaturing polyacrylamide gels (Fig. 1B), only the secreted invertase from wild-type and *SLY*-transformed, Ypt1p-depleted cells is shown.

A partial rescue by the multicopy suppressors of protein transport through the Golgi complex in Ypt1p-depleted cells was also observed by tracking the fate of the vacuolar hydrolase CPY. CPY is core glycosylated in the ER (precursor p1), is further modified by carbohydrate addition during transit through the Golgi complex (precursor p2), and finally loses its propeptide sequence by proteolytic cleavage within the vacuole to adopt its mature form (m) (29, 55). As shown in Fig. 2A, Ypt1p-depleted cells (lane 8) accumulated the ER form of CPY (p1) like the *sec18* mutant at the nonpermissive temperature (lane 12). Expression of the vector-borne multicopy suppressors, however, led to the generation of mature enzyme to some extent in rich medium (Fig. 2A, lanes 9 to 11) but to a significant proportion in minimal medium (Fig. 2B, lanes 1 to 3) used to select against plasmid loss. We noted repeatedly that the mature form of CPY in *SLY*-suppressed, Ypt1p-depleted cells had a slightly faster electrophoretic mobility than that of wild-type cells (Fig. 2A, lanes 9 to 11). This seems to be caused by an incomplete glycosylation of the enzyme during its passage through the Golgi complex since endo H treatment resulted in an identical electrophoretic mobility of mature CPY in wild-type and *SLY*-suppressed cells (Fig. 2C).

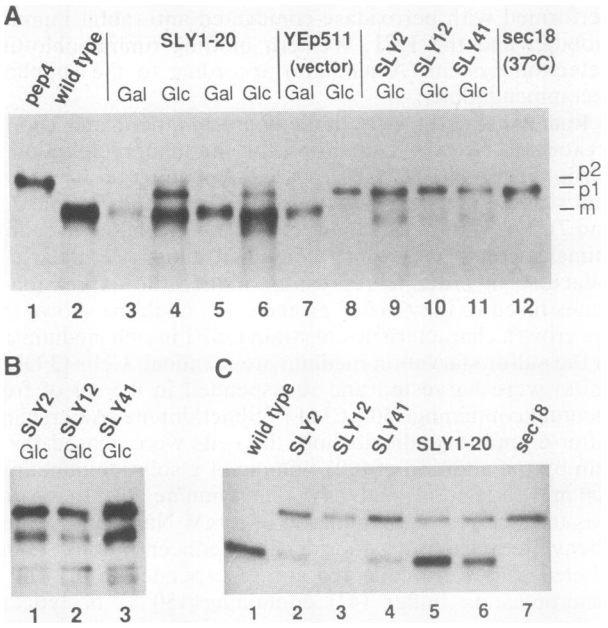

FIG. 2. Processing of the vacuolar enzyme CPY in cells depleted of Ypt1p and expressing *SLY1-20*, *SLY2*, *SLY12*, or *SLY41*. (A) Strains INT2 [*SLY1-20*::*SLY1(wt)*, lane 4], LSY20-1A (*SLY1-20*, lane 6), and HLR3 transformed with vector DNA (lane 8) or the multicopy suppressors *SLY2*, *SLY12*, and *SLY41* (lanes 9, 10, and 11) were radiolabeled after shifting these cells carrying *GAL10-YPT1* to YEPD medium containing 2% glucose for 20 h. As controls, extracts from wild-type cells (AG430-1A, lane 2), *GAL10-YPT1* cells growing in galactose medium (lane 3, 5, and 7), and strains accumulating particular precursor forms of CPY, 20B-12 (*pep4*, lane 1) and HMSF176 (*sec18*, lane 12), were also included in this experiment. Radiolabeling was performed by first shifting the cells to sulfur starvation medium for 30 min, followed by a 1-h pulse with [^{35}S]methionine and then by a 30-min chase with an excess of sulfate and L-methionine. All incubations (with the exception of the *sec18* strain) were done at 25°C. Preparation of cell lysates, immunoprecipitation of CPY, and gel electrophoresis were performed as described in Materials and Methods. The relative positions of the core-glycosylated form (p1), the Golgi form (p2), and the vacuolar mature form (m) of CPY are indicated at the right. (B) The same transformants used in panel A, lanes 9, 10, and 11, were incubated in minimal medium containing glucose and lacking leucine. This should prevent loss of the multicopy plasmids while the cells are depleted of Ypt1p. The cells were pulse-labeled 26 h after the transfer to this medium. (C) Aliquots of samples analyzed in panel A, lanes 2, 4, 6, and 12, and panel B were treated with endo H and loaded on an SDS-8% polyacrylamide gel. Lane 5 shows endo H-digested CPY from strain LSY20-1A (*SLY1-20*) growing in glucose, while the radioactive CPY running in lane 6 is derived from strain INT2, which expresses one mutant and one wild-type *SLY1* allele.

These results indicate that in the absence of Ypt1p, high expression of other yeast genes (*SLY2*, *SLY12*, *SLY41*) partially overcomes the ER-to-Golgi transport block caused by the depletion of the GTP-binding protein.

***SLY1-20* mutant allele suppresses growth inhibition and secretion defects in a semidominant manner.** The processing of invertase was also observed in order to analyze the way in

TABLE 2. *SLY1-20* mutant allele suppresses the growth inhibition of Ypt1p-deficient cells in a semidominant fashion

Strain	No. of *SLY1(wt)*/*SLY1-20* alleles	YEP medium +:	Doubling time[a] (h)
WM3 (diploid)	3/0	Galactose	1.5
WM3 (diploid)	3/0	Glucose	>30
WM21 (diploid)	2/1	Glucose	7.0
INT2 (haploid)	1/1	Glucose	2.3
WM12 (diploid)	1/2	Glucose	2.3
LSL20-1A (haploid)	0/1	Glucose	2.1

[a] The doubling time of *GAL10-YPT1* cells containing different numbers of *SLY1(wt)* and *SLY1-20* alleles was determined by monitoring the OD_{600} at 25°C between 15 and 19 h after the transfer to YEPD medium. For comparison, *SLY1(wt)* cells growing in YEP medium containing galactose were also included (first row).

which *SLY1-20* acts as a suppressor. Although this allele is dominant and suppresses the deletion of the *YPT1* gene in the presence of a wild-type *SLY1* gene, *SLY1-20* is not able to compete with two wild-type genes present in the same cell. As described previously (15), tandem insertion of *SLY1* sequences at their normal chromosomal site can be used to construct haploid and diploid cells with different numbers of *SLY1-20* and *SLY1(wt)* alleles. Cells expressing one mutant and two wild-type alleles grew poorly on glucose plates, regardless of whether the two wild-type genes were in a tandem array or whether the mutant allele was in tandem with a wild-type gene. These cells accumulated significant amounts of incompletely glycosylated invertase (Fig. 1C, lane 18). In contrast, cells expressing equal numbers of mutant and wild-type genes or a surplus of *SLY1-20* alleles were able to grow with almost the same generation time in the absence or presence of an expressed *YPT1* gene (Table 2). Under those conditions, the *SLY1-20* allele was able to efficiently suppress the invertase secretion defect even in the presence of two wild-type *SLY1* genes, and there was no significant intracellular accumulation of invertase in cells with two *SLY1-20* mutant alleles and one *SLY1(wt)* wild-type allele (Fig. 1C, lane 22). This suggests that the cells containing the suppressor can deliver invertase to the Golgi compartment, where mannose chains are added to the enzyme. However, the extent of glycosylation in *SLY1-20* cells devoid of Ypt1p did not reach that of wild-type cells. This is most convincingly shown in the immunoblot of Fig. 1B (lanes 1 and 2) that compares the electrophoretic mobility under denaturing conditions of periplasmic invertase from Ypt1p-depleted cells expressing one *SLY1-20* allele (strain LSY20-1A; Table 1) and that of wild-type cells. We would like to stress that *SLY1-20* mutants expressing Ypt1p secrete completely glycosylated invertase and that the differences in the mobility of invertase in SDS-PAGE were observed when fractions containing only periplasmic invertase were analyzed (Fig. 1B) or when whole-cell extracts were subjected to immunoblot analysis or immunoprecipitation (data not shown). It is unknown, however, whether the observed difference in electrophoretic mobility is due to a change in the size or a change in the number of oligosaccharide side chains (4).

The ability of the *SLY1-20* single-copy suppressor to efficiently restore the protein transport defect caused by the loss of *YPT1* gene function is also shown by the generation of mature CPY in the absence of Ypt1p. Whereas Ypt1p-deficient cells accumulated core-glycosylated CPY exclusively (Fig. 2A, lane 8), a major fraction of the enzyme was correctly processed to its mature form in cells expressing the *SLY1-20* suppressor (Fig. 2A, lanes 4 and 6). Similar to the results obtained with invertase described above, the ER-to-Golgi transport defect in the absence of Ypt1p was more efficiently corrected in cells expressing one *SLY1-20* mutant allele only (Fig. 2A, lane 6, and 2C, lane 5) than in cells with one *SLY1(wt)* and one *SLY1-20* gene (Fig. 2A, lane 4, and 2C, lane 6).

Thin-section micrographs of Ypt1p-depleted cells expressing *SLY1(wt)* (strain HLR3) or in addition the *SLY1-20* mutation (strain INT2) showed that the presence of the mutant *SLY1-20* allele prevents the accumulation of membrane material in these cells (data not shown).

***SLY1-20*, *SLY2*, and *SLY12* can partially suppress *sec21-1* and *sec22-3* defects.** As *ypt1* defects lead to a block early in secretion (3, 48, 51), we investigated whether the different *SLY* genes are also able to complement other known secretion defects (39). Temperature-sensitive mutants accumulating ER (*sec12-4*, *sec13-1*, *sec16-2*, *sec18-1*, *sec20-1*, *sec21-1*, *sec22-3*, and *sec23-1*), Golgi structures (*sec7-1*), or post-Golgi vesicles (*sec4-2*) were included in this analysis. The mutants were transformed with *SLY2*, *SLY12*, or *SLY41* on multicopy plasmids or with the *SLY1-20* mutant gene on a single-copy vector. Transformants were isolated, spread onto plates, and grown at different temperatures. It was found that all *SLY* genes, except *SLY41*, could partially suppress the growth defect of *sec21-1* and *sec22-3* only (Fig. 3). *SLY41* was unable to suppress any of these defects. The permissive temperature was raised from 33 to 34.5°C; for the *SLY12*-transformed *sec21-1* mutant, it was elevated to 36°C (Fig. 3, lower panel). The resistance to elevated temperatures was only slightly improved by simultaneously transforming *sec21-1* or *sec22-3* mutants with either *SLY2* and *SLY12* or *SLY2* and *SLY41*. The apparent slight improvement of growth observed with the *sec7* mutant (Fig. 3, upper panel) was not reproducible and may be due to the fact that the transformant is prototrophic for uracil.

As the *SLY* genes are unable to suppress the *YPT1* deletion at 37°C (15), it was important to exclude the possibility that ER-to-Golgi transport defects observed at elevated temperatures are generally not compensated by these suppressors at high temperatures. This was also indicated by the finding that the *SLY* genes were not able to suppress the temperature-sensitive growth defect of the *ypt1*(Ts) mutant (48). Although this mutation is recessive, the gene product may be toxic in a haploid at elevated temperatures, since the protein carries the dominant lethal N121I mutation in addition to an intragenic suppressor mutation. However, a new temperature-sensitive mutant, *ypt1-44*, was recently identified among a series of mutants with single-amino-acid substitutions within the putative effector domain of Ypt1p (6). This mutant is unable to grow at temperatures higher than 33°C. It was found that the multicopy suppressors *SLY2*, *SLY12*, and *SLY41* as well as the single-copy suppressor *SLY1-20* allowed *ypt1-44* mutant cells to grow even at 37°C. This suggests that the *SLY* suppressors can function at elevated temperatures in the presence of either certain mutant Ypt1 proteins or wild-type Ypt1p but not in the absence of this GTP-binding protein.

Since both *YPT1* and *SEC4* encode structurally related GTP-binding proteins (21, 45) and the *SLY* genes do not suppress the *sec4-2* mutation, it can be concluded that the *SLY* genes are not general suppressors of defects in small GTP-binding proteins. They seem to act specifically in those steps of the secretion pathway in which Ypt1p, Sec21p, and Sec22p exert their function, i.e., in ER-to-Golgi transport.

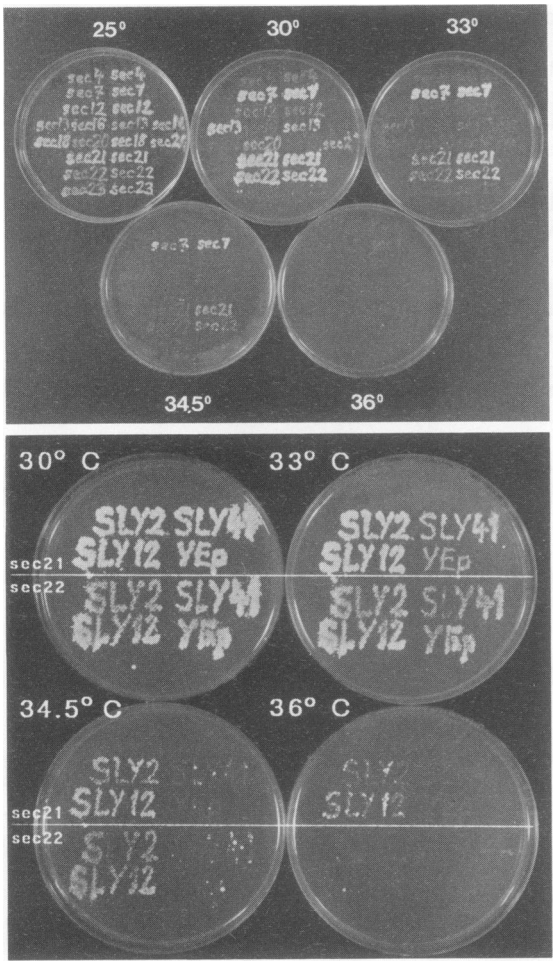

FIG. 3. Partial suppression of *sec21-1* and *sec22-3* defects by *SLY1-20*, *SLY2*, and *SLY12*. Different *sec* mutants carrying suitable markers were transformed with plasmid YCp5LU-*SLY1/5* containing the *SLY1-20* suppressor gene. Nontransformed *sec* mutants growing on YEPD plates and transformants growing on selective medium were replica plated onto YEPD plates and incubated for 2 days at the temperatures indicated (upper panel). When transforming the same set of *sec* mutants with multicopy plasmids containing *SLY2*, *SLY12*, or *SLY41*, partial suppression was observed only for *sec21-1* and *sec22-3* mutants (lower panel). *SLY2* and *SLY12* but not *SLY41* or the vector without insert (YEp) were able to improve the growth of these mutants at restrictive temperatures. *sec21-1* (top) and *sec22-3* (bottom) mutants were transformed with the *SLY* genes indicated.

Sly1p-depleted cells are defective in protein secretion and accumulate ER. We have shown that the Sly1 protein itself is essential for cell viability (15). The fact that the *SLY1-20* mutant protein is able to suppress the deletion of *YPT1* and the conditional lethal *ypt1-44* mutation and to partially complement the *sec21-1* and *sec22-3* mutations indicates that the *SLY1* gene product also acts early in the secretion pathway.

In an attempt to prove this assumption, *SLY1(wt)* was brought under transcriptional control of the regulatable *GAL10* promoter to allow an investigation of phenotypic alterations caused by depleting cells of the essential gene product. It was found that cells slowed down growth about 15 h after the expression of *SLY1(wt)* was shut off by transferring the cells to glucose-containing medium and that they completely stopped proliferation after 20 h. Glycosylation and secretion of invertase were analyzed following induction of the secreted form at different time points after repression of the *SLY1(wt)* gene. As shown in gels stained for activity (Fig. 4A), the secretion of apparently incompletely glycosylated invertase, in combination with an intracellular accumulation of underglycosylated invertase and of multimeric core-glycosylated forms of the enzyme, was observed during the final stage of logarithmic growth (cells grown for 14 h in glucose medium). After 16 h in glucose-containing medium, a time point at which more than 50% of cells were still viable, invertase secretion was hardly detectable but the ER form of the core-glycosylated enzyme was readily observed to accumulate within the cells.

Incomplete glycosylation of invertase and the appearance of its core-glycosylated forms were also clearly observed by using denaturing SDS-polyacrylamide gels. As shown in an analysis of total cellular invertase (Fig. 4B), the same heterogeneous population of differently glycosylated enzyme molecules, typical for wild-type cells, was also seen in cells induced to synthesize secreted invertase either 5 or 10 h after the shutdown of *SLY1(wt)* gene expression. At 12 h, however, incompletely glycosylated invertase that had obviously reached (or passed) the Golgi complex and core-glycosylated enzyme could be detected. The ER form of invertase, the characteristic end product of the ER-to-Golgi transport block in *sec18* mutants, prevailed in Sly1p-deficient cells 14 h after the shift from galactose- to glucose-containing medium.

A block in protein transport between the ER and the Golgi apparatus following Sly1p depletion is also evident from the data presented in Fig. 4C. In cells 12 h after repression of Sly1p function, the core-glycosylated precursor form p1 was the predominant form of CPY.

Sly1p-depleted cells accumulate ER as well as incompletely glycosylated invertase and CPY, a phenotype shared with other mutants defective in ER-to-Golgi transport, including *ypt1* mutants (6, 48, 51). Concomitant with the arrest of cell proliferation resulting from the depletion of Sly1p, an enrichment of intracellular membranes could be observed by electron microscopy when arrested cells had been fixed with permanganate (Fig. 5d and e). These membranes were often associated with the plasma membrane or the nuclear envelope, indicating that they represent ER. The morphological alterations were studied in arrested cells 20 h after *SLY1(wt)* gene repression. At this time, roughly 50% of the cells were still viable. It therefore seems rather unlikely that the accumulation of ER membranes can be simply explained by a loss of cell viability.

Taken together, these results suggest that Sly1p indeed performs an essential function in protein transport between the ER and the Golgi compartments.

A *sly2* null mutant is Cs^- and Ts^-, is defective in protein transport, and accumulates ER. Disruption of the *SLY2* multicopy suppressor gene proved not to be lethal but conferred on the mutant cells a conditional lethal phenotype (15). Compared with the wild type, cells of an isogenic *sly2* mutant strain (*sly2::HIS3*) exhibited a somewhat prolonged generation time (2.7 h opposed to 2.1 h) at 25°C but ceased to proliferate at 15°C as well as at 37°C (Fig. 6). As discussed previously (15), *sly2* mutants were always Cs^-, whereas their Ts^- phenotype depended on the genetic background of the strain harboring the disrupted *SLY2* gene.

To examine whether *SLY2*, like *SLY1*, is an integral element of the secretion pathway, the processing and trans-

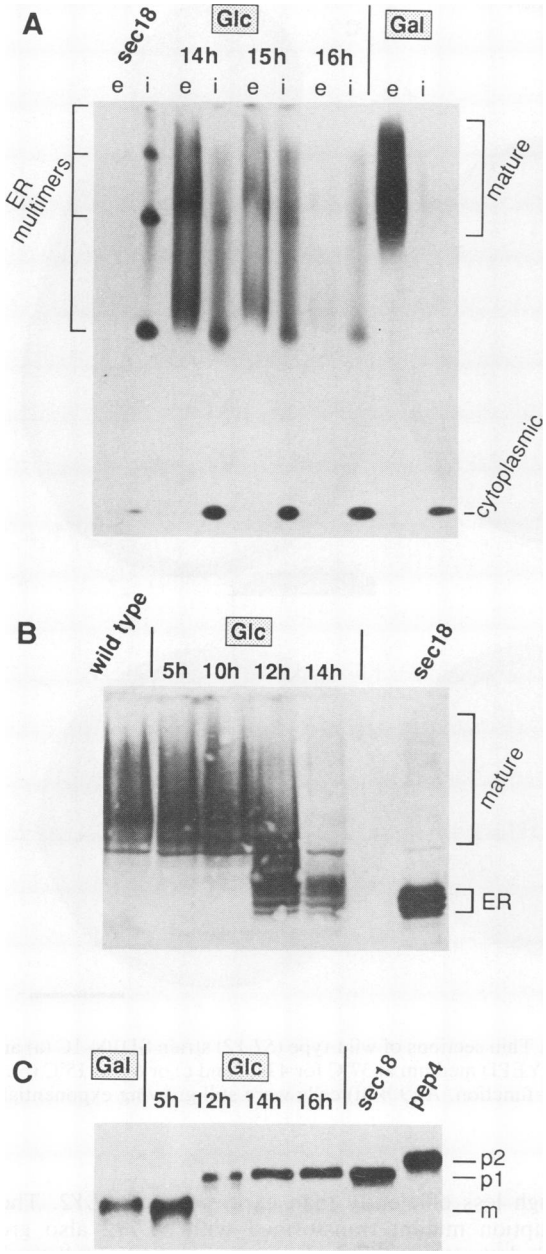

FIG. 4. Defects in intracellular transport of invertase and CPY in Sly1p-depleted cells. (A) Mutant strain GSF1 carrying the *GAL10-SLY1* fusion was grown to the late exponential phase in SGal medium, and the cells were washed with sterile water and transferred to either YEPGal or YEPD medium. At the times indicated, aliquots were taken, synthesis of invertase was induced, and intracellular and periplasmic fractions were prepared and analyzed as described in the legend to Fig. 1 and Materials and Methods. (B) Whole-cell extracts used for immunoblotting analysis of invertase were prepared by lysis of cells in SDS loading buffer and vortexing with glass beads. After SDS-PAGE and blotting to a nitrocellulose filter, anti-invertase antiserum was used to detect invertase. The ER form and highly glycosylated mature form of invertase are indicated at the right. (C) Pulse-chase labeling and immunoprecipitation were used to detect the accumulation of the p1 precursor form of CPY in GSF1 cells depleted of Sly1p. GSF1 mutant cells were cultured at 30°C in sulfur starvation medium containing galactose or glucose for the times indicated. Cell lysis and immunoprecipitation were performed as described in Materials and Methods. The relative mobility of the ER form (p1), the highly glycosylated Golgi form (p2), and the vacuolar mature form (m) of CPY are indicated at the right. Strains which accumulate particular precursor forms of CPY were included in this experiment: p2 (strain 20B-12, *pep4*); p1 (strain HMSF176, *sec18*).

port of the secreted form of invertase and the vacuolar enzyme CPY were observed in *sly2* mutant cells under nonpermissive conditions. Cells derived from the four spores of a complete tetrad of a heterozygous *sly2*::*HIS3*/*SLY2* strain were grown at 25°C. At 1.5 h after a shift to 37°C, invertase synthesis was induced. Fractions of secreted and intracellularly located invertase were separated on nondenaturing gels and stained for invertase activity. As can be seen in Fig. 7A, in contrast to wild-type cells (lanes C and D), *sly2* mutant cells (lanes A and B) accumulated significant amounts of core-glycosylated invertase intracellularly. However, the block of invertase secretion was not as severe as that observed in a *sec18-1* mutant, and some apparently incompletely glycosylated enzyme was still secreted under these conditions. To verify the defect of invertase processing in *sly2* mutant cells, total cellular invertase of haploids derived from the same tetrad described above and grown at either 25 or 37°C was radiolabeled with [^{35}S]methionine and, after immunoprecipitation, was analyzed by SDS-PAGE (Fig. 7B). Wild-type and mutant cells, derepressed for invertase induction, were shifted to 37°C and 30 min later were labeled with [^{35}S]methionine for 1 h, followed by a 30-min chase. It was found that at 25°C invertase of *sly2* cells already exhibited a slightly faster electrophoretic mobility than that derived from wild-type cells, suggesting a partial defect in glycosylation. This phenotypic alteration might be related to the somewhat prolonged generation time of *sly2* mutants observed at 25°C. However, at 37°C, *sly2* mutant cells contained significant amounts of core-glycosylated and variable amounts of incompletely glycosylated invertase (Fig. 7B), emphasizing the validity of the data obtained with nondenaturing gels stained for activity (Fig. 7A).

Further evidence for the role of Sly2p at an early step in the secretion pathway was obtained by observing the processing of CPY. For this purpose, *SLY2* and *sly2* cells were grown at 15, 25, or 37°C for 2 h, labeled with [^{35}S]methionine for an additional 2 h, and chased with sulfate and methionine for either 1 h (cells at 15°C) or 30 min (cells at 25 and 37°C). Electrophoretic separation of immunoprecipitated CPY (Fig. 7C) revealed that at 15°C a significant proportion of the labeled enzyme was in the core-glycosylated ER form (p1). The defect in CPY processing seemed somewhat more pronounced in mutant cells that were Cs$^-$ and Ts$^-$ than in cells that were cold sensitive only (15). Accumulation of core-glycosylated CPY in *sly2* mutants was likewise seen at 37°C and to a small extent at 25°C. Fully glycosylated, unprocessed CPY precursor p2, typical for the protease-deficient *pep4* strain (Fig. 7C), was not detected in *sly2* mutant cells. Although the block in ER-to-Golgi transport was not complete in cells lacking Sly2p, we noted that, similar to the situation in Ypt1p-depleted cells suppressed by different *SLY* genes, the mature form of CPY had a slightly faster electrophoretic mobility than that of wild-type cells.

These transport defects indicate a specific, although not exclusive, role for Sly2p early in the secretion pathway, i.e., in ER-to-Golgi transit. This assumption is strengthened by the observation that *sly2* null mutants at nonpermissive temperatures (15 and 37°C) accumulated ER membranes like Sly1p-depleted cells. ER membranes often developed into an

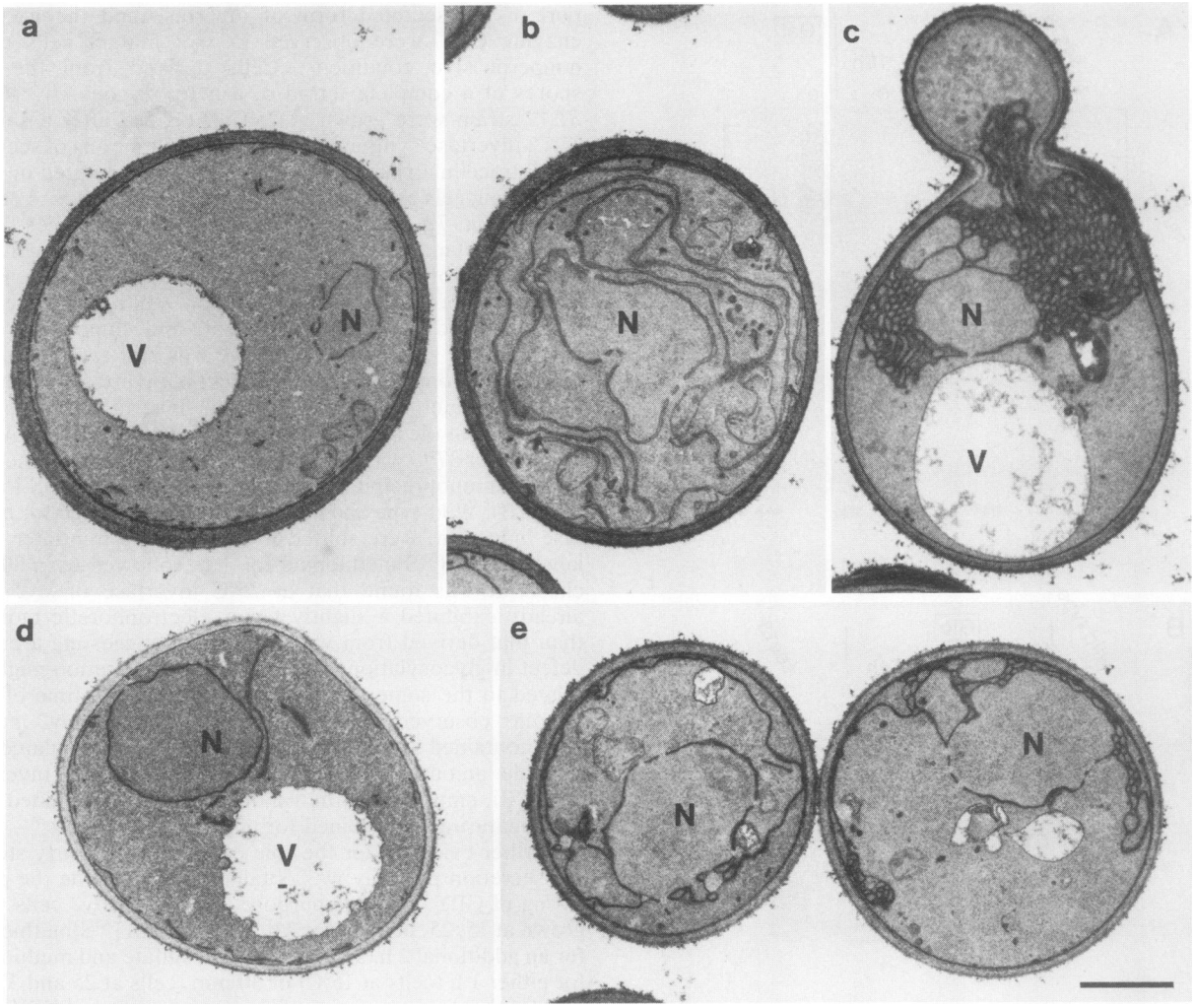

FIG. 5. Thin-section micrographs of *sly2* mutants and Sly1p-depleted cells. Thin sections of wild-type (*SLY2*) strain SD104-1C (a) and *sly2* mutant strain SD104-1A (b and c) were prepared after the cells were grown in YEPD medium at 37°C for 4 h (a and c) or 6 h at 15°C (b). GSF1 cells (*GAL10-SLY1*) were harvested 9 h (d) or 20 h (e) after repressing *SLY1* function. At 9 h (d) cells were still growing exponentially. N, nucleus; V, vacuole. Bar, 1 μm.

extensive network (Fig. 5a to c). As with the core-glycosylated ER forms of CPY and invertase, a moderate accumulation of ER was seen in *sly2* mutant cells grown at 25°C. All the phenotypic alterations described cannot be ascribed to a loss of cell viability; a viability test revealed that about 80% of the *sly2* cells that had been kept at 37°C for 4 h or at 15°C for 6 h were still able to proliferate.

Genetic interaction between *SLY2* and other *SLY* genes. The suppression of *ypt1* mutants and partial complementation of *sec21-1* and *sec22-3* mutants by different *SLY* genes suggests a functional connection between these newly discovered genes. To explore this possibility, we transformed the *sly2* null mutant either with *SLY2*, *SLY12*, or *SLY41* on a multicopy plasmid or with *SLY1-20* on a single-copy vector. Transformants were grown on selective SD plates, replica plated onto YEPD plates, and incubated at 15, 25, or 37°C. As shown in Table 3, *sly2* mutant cells transformed with the multicopy vector without an insert did not grow at 15 and 37°C, but as expected, transformants expressing the *SLY2* gene from a plasmid did. Expression of *SLY1-20* also rescued the *sly2* mutant at the restrictive temperatures, although less efficiently than expression of *SLY2*. The *sly2* disruption mutant transformed with *SLY12* also grew at 37°C, but not at 15°C. In contrast, *SLY41* allowed *sly2* mutant cells to grow only very slowly at 37°C and not at all at 15°C.

These findings suggest that there are genetic interactions between *SLY2*, *SLY1*, and *SLY12*.

Sly2p is an integral membrane protein. The DNA sequence of the *SLY2* gene predicts a protein of 214 amino acids (25 kDa) with a hydrophobic tail long enough to span a membrane. Since a membrane-bound protein is a likely candidate for interactions with Ypt1p, we raised antibodies to Sly2p in order to test this hypothesis. Rabbits were immunized by the injection of a β-galactosidase–Sly2p fusion protein, and the resulting polyclonal antibodies were used for immunoblot analysis. As shown in Fig. 8A, these antibodies detected a protein of about 25 kDa in total cellular extracts from wild-type cells and from wild-type cells transformed with a multicopy vector carrying the cloned *SLY2* gene. This 25-kDa protein was missing, however, in *sly2* mutant cells. Cell fractionation studies established that Sly2p is present

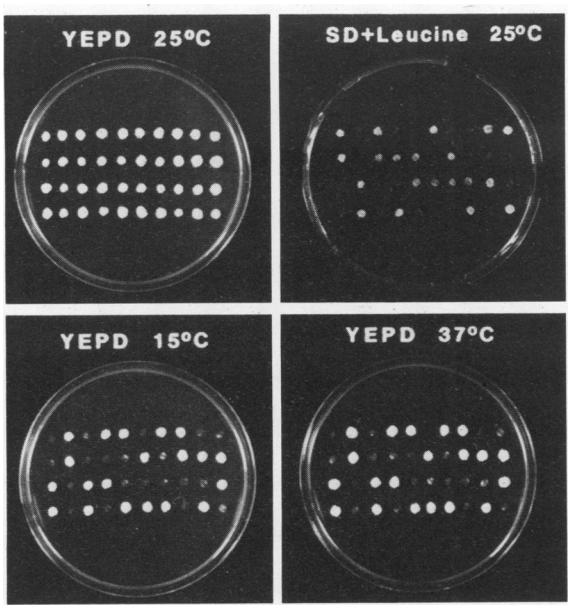

FIG. 6. Diploid cells with one *SLY2* allele disrupted by insertion of the *HIS3* gene were sporulated and subjected to tetrad analysis. Spore viability was 94%. Colonies representing the four spores of complete tetrads were transferred to YEPD plates, grown at 25°C, and replica stamped to either SD plates lacking histidine (SD + Leucine) or to YEPD plates and grown at the indicated temperatures. Note that *sly2* cells (His^+) do not grow at 15 or 37°C.

exclusively in a particulate fraction sedimenting at 100,000 × *g*. As can be seen in Fig. 8B, Sly2p could be solubilized only by treating the protein extracts with the detergent Triton X-100. Other treatments (5 M urea, 1 M sodium chloride, 1 M potassium acetate, or 0.1 M sodium carbonate [pH 11]) were unable to convert Sly2p to a soluble form, indicating that Sly2p is an integral membrane protein.

DISCUSSION

We have shown that suppressors allowing the deletion of the normally essential *YPT1* gene can be readily identified (15). None of the suppressors identified so far encodes a GTP-binding protein that would simply substitute for the missing Ypt1p. The fact that several different suppressors can bypass the requirement for a functional *YPT1* gene argues for a defined execution point of Ypt1p. Another, less likely, possibility would be that several related processes involving the same group of proteins are governed by the action of Ypt1p. Therefore, the functional analysis of the *SLY* gene products sheds new light on the function of Ypt1p itself.

In the budding yeast *S. cerevisiae*, *ypt1* defects result in many different phenotypic alterations. Besides abnormal nuclear morphology, disorganization of microtubules, and sensitivity to nitrogen starvation (49, 50), Ypt1p-depleted cells and conditional *ypt1* mutants are typically blocked in ER-to-Golgi protein transport (48, 51). Initially, we interpreted the partial rescue of the temperature-sensitive *ypt1*(Ts) mutant by elevated Ca^{2+} concentrations in the growth medium to indicate that a defective regulation of intracellular Ca^{2+} might be the primary lesion causing secretion defects in Ypt1p-deficient cells (48). What would have been consistent with this interpretation is the observation

TABLE 3. Some suppressors of the *YPT1* deletion can also complement the disruption of the *SLY2* gene[a]

Plasmid	Growth at:		
	15°C	25°C	37°C
YEp511	−	++	−
YEp511-*SLY2*	+++	+++	+++
YCp5LU-*SLY1/5* (*SLY1-20*)	+	++	++
YEp511-*SLY12*	−	++	++
YEp511-*SLY41*	−	++	±

[a] *sly2*::*HIS3* cells (Cs^- Ts^-) were transformed with vector YEp511 or plasmids containing either the *SLY2* gene or other suppressors of the *YPT1* deletion. Growth was tested by replicating the different transformants from selective SD plates onto YEPD plates which were incubated at 25 or 37°C for 2 days or at 15°C for 4 days. Growth of colonies was scored as follows: +++, wild type; ++, almost wild type; +, intermediate; ±, poor; −, no growth.

that *pmr1* mutant alleles encoding a defective Ca^{2+} ATPase suppress the lethality of the cold-sensitive *ypt1-1* mutant (43). However, in vitro experiments showed that Ca^{2+} addition could not restore protein transport in extracts from *ypt1* mutants (2) and that the Ypt1p-requiring step precedes the Ca^{2+}-dependent one (3). Moreover, several other yeast genes encoding small GTP-binding proteins, *SEC4* (45), *SAR1* (36), and *ARF1* and *ARF2* (53), fulfill essential functions at distinct steps of the secretory pathway. To sum up, these findings indicate that the primary role of Ypt1p is in protein transport as originally suggested by Segev et al. (51).

The phenotypes of the suppressors of the *YPT1* deletion present additional clues regarding structural elements required for those steps in protein secretion that also involve Ypt1p. Since Sly1p-depleted cells and a *sly2* null mutant at restrictive conditions accumulate ER membranes and ER forms of core-glycosylated enzymes, it is possible that Sly1p and Sly2p are themselves important participants in the regulation of ER-to-Golgi transport. The same may be true for Sly12p, but this has not yet been examined. It is very likely, however, that *SLY12* is identical with *BET1*, a gene identified by Newman and Ferro-Novick (37) to be required for ER-to-Golgi transport. Both genes are suppressors of the *sec21-1* and *sec22-3* defects (38; this work), and the restriction maps are remarkably similar (15, 38). As the *ypt1* suppressors *SLY12* and *SLY1-20* are likewise able to partially suppress the growth arrest of a *sly2* null mutant at restrictive conditions, it might well be that Ypt1p, Sly1p, Sly2p, and Sly12p (or some of these proteins) interact directly or as an ensemble transiently.

As discussed before (15), both the suppression of a mutant phenotype by overproduction and the suppression of a deletion often indicate that a new pathway or a short circuit around the mutant block has been activated (10). Several lines of evidence suggest that the overexpression of *SLY2*, *SLY12*, or *SLY41* or expression of the *SLY1-20* mutant gene is specific for the suppression of *YPT1* defects. If these genes would act to bypass the Ypt1p-controlled pathway, this bypass must be a direct one, allowing transit of secreted proteins through most of the normal pathway. Evidence supporting this specificity is as follows. First, the *SLY* suppressor genes did not cause secretion of the vacuolar enzyme CPY (19a). Such a missorting has been observed in cells carrying a *pmr1* mutation able to suppress a conditional *ypt1-1* mutant, indicating that *pmr1* rescue is the result of a major bypass (43); second, the *ypt1* suppressors to a varying degree restored ER-to-Golgi transport as indicated by the reestablishment of outer-chain glycosylation of secreted

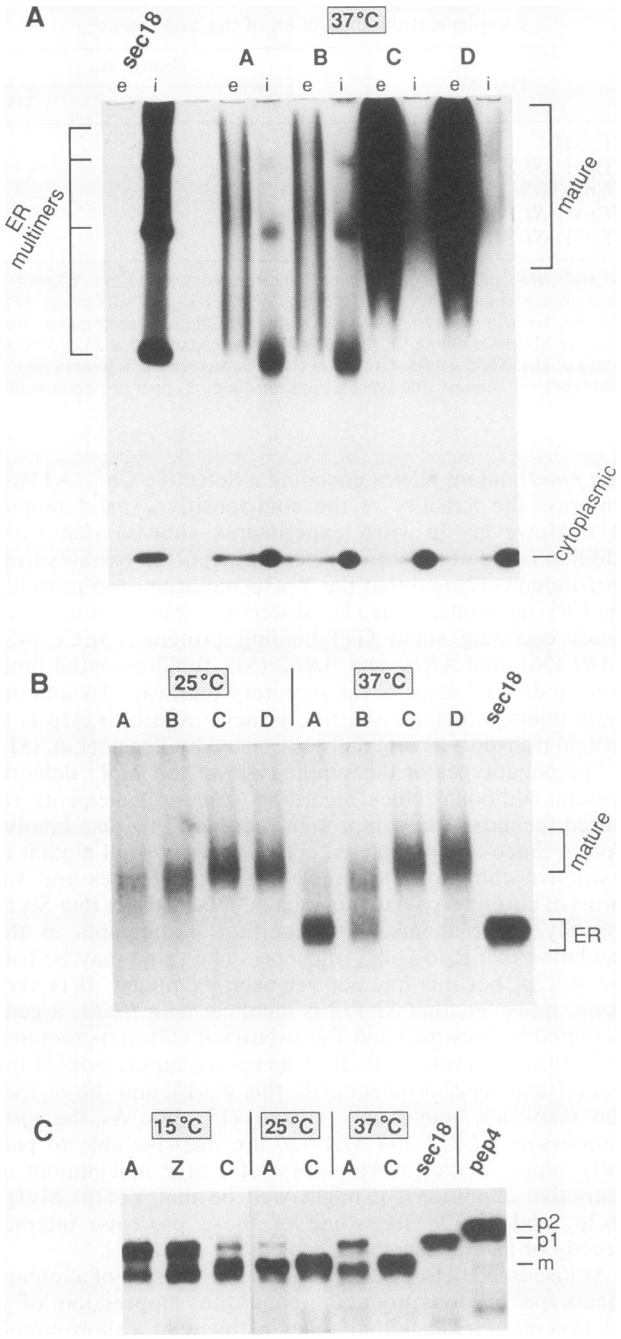

FIG. 7. Defects in intracellular transport of invertase and CPY in *sly2* cells. Wild-type (SD104-1C and -1D; lanes C and D) and *sly2*::*HIS3* mutant cells (SD104-1A and -1B; lanes A and B) growing exponentially at 25°C in YEPD medium were shifted to the temperatures indicated. (A) Synthesis of the periplasmic invertase was induced 1.5 h after the temperature shift. After another 2.5 h of incubation at 37°C, cells were analyzed for intracellular (i) and periplasmic (e) invertase as described in the legend to Fig. 1. Note the characteristic pattern of multimeric, core-glycosylated forms in *sec18-1* and *sly2* mutant cells. (B) For immunoprecipitation of invertase from whole-cell extracts, synthesis of secreted invertase was derepressed for 30 min at 25 or 37°C. Radiolabeling and immunoprecipitation were performed as described in Materials and Methods. (C) For the pulse-chase labeling of CPY, a *sly2* strain (SD10-10B; lane Z) was also included which did not grow at 15°C but did grow at 37°C. Cells were shifted to the indicated temperatures 2 h before they were labeled with [^{35}S]methionine. After an additional 2 h of incubation, cells were transferred to fresh medium and chased for 30 min at 25 or 37°C or for 1 h at 15°C, respectively. Note the occurrence of the core-glycosylated ER form (p1) in *sly2* and *sec18* mutant cells and the fully glycosylated, unprocessed precursor (p2) in a *pep4-3* strain.

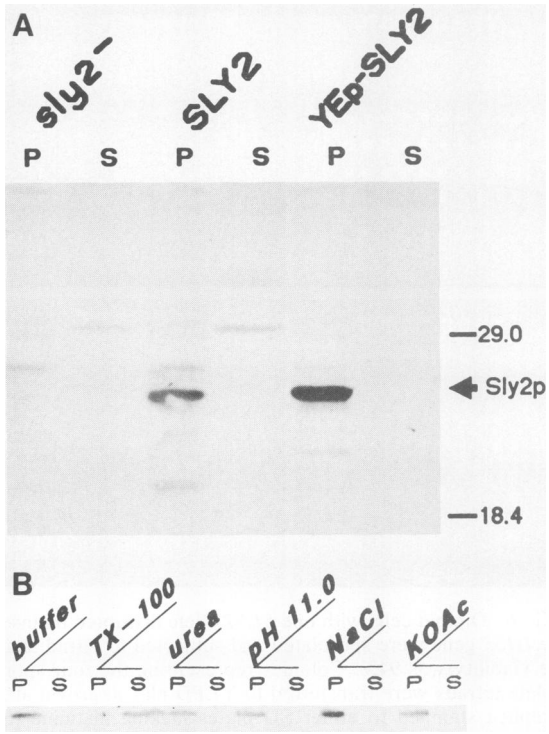

FIG. 8. Immunoblot analysis of Sly2p. Total proteins of a crude membrane (P) and a soluble (S) fraction derived from an *sly2* strain, a *SLY2* strain, and a wild-type strain transformed with a multicopy vector carrying the cloned *SLY2* gene were separated by SDS-PAGE (A). Crude extracts from the Sly2p overproducer were treated with different reagents as indicated prior to separation of particulate and soluble fractions (lysis buffer: 1% Triton X-100, 5 M urea, 0.1 M sodium carbonate [pH 11], 1 M sodium chloride, 1 M potassium acetate) and then subjected to SDS-PAGE (B). The proteins were transferred to nitrocellulose filters. The filters were treated with anti-Sly2p antisera and then stained as described in Materials and Methods. Numbers on right show size in kilodaltons.

invertase and the appearance of mature CPY in Ypt1p-depleted cells; third, among many secretion-defective mutants tested, only *sec21-1* and *sec22-3* were partially suppressible by *SLY1-20*, *SLY2*, and *SLY12*. In different *SLY*-transformed *sec21-1* or *sec22-3* mutants, the growth-restrictive temperature was raised by about 3°C. This was clearly a minor effect, in contrast to the effective suppression of the *ypt1-44* mutant by all four *SLY* genes. Nevertheless, Sec21p and Sec22p are required for vesicular transport from ER to Golgi (17, 28), as are Ypt1p, Sly1p, Sly2p, and possibly Sly12p. The partial suppression of the *sec21-1* and *sec22-3* mutants by *SLY1-20*, *SLY2*, and *SLY12* may be viewed as genetic evidence for related functions between the two known *SEC* and the newly discovered *SLY* genes.

Considering the extremely different primary structures of the *SLY*-encoded proteins, it can be assumed that mechanis-

tically they act quite differently. Sly2p and Sly12p are structurally related to a class of small synaptic vesicle proteins (15, 56, 57), and therefore, they seem to belong to a group of *SEC* gene products likely to be integral membrane proteins, like Sec11p (9), Sec12p (35), and Sec59p (8). As shown by subcellular fractionation and immunoblot analysis, Sly2p is indeed an integral membrane protein. In contrast, *SLY1* encodes a hydrophilic protein (15) which may be transiently or peripherally associated with membranes as has been discussed for other *SEC* gene products (1, 5, 7, 16, 25, 33, 46).

In this regard, the interesting finding that *ypt1* mutant cells transformed with any one of the four *SLY* genes secreted incompletely glycosylated invertase may indicate a short bypass in the secretory pathway activated by the Sly1-20 mutant protein or by an overproduction of *SLY2*, *SLY12*, or *SLY41* gene products. The detection in *SLY*-suppressed *ypt1* cells of presumably underglycosylated mature CPY (shown by its slightly faster electrophoretic mobility) is in line with this argument. In yeast and mammalian cells, glycosylation of secretory proteins proceeds in a sequential fashion, presumably in different compartments of the Golgi complex (20). The short bypass induced or activated by the *SLY* suppressors may concern a particular Golgi compartment and result in the exclusion of secreted proteins from the addition of some mannose residues to the outer carbohydrate chains. Similar glycosylation defects, even at permissive conditions, have been observed in the cold-sensitive *ypt1-1* mutant (51) but not in Ypt1p-depleted or *ypt1*(Ts) mutant cells (49). In our assays, the multicopy suppressors are not as effective as *SLY1-20*, but this may be due to plasmid loss and variations in the copy number of the multicopy plasmids.

As we discussed previously (15), based on its sequence similarity with chloroplast phosphate translocators (19), Sly41p could be a channel protein acting in the translocation of ions or metabolites. Hypothetically, high expression of Sly41p in Ypt1p-depleted cells could lead to changes in intracellular flow of ions or metabolites which, perhaps unspecifically, might result in the weak suppression of the secretory defect. It is worth mentioning in this context that high expression of Sly41p does not complement any of the tested secretion-defective mutants, including *sec21-1* and *sec22-3* and *sly2* null mutants at nonpermissive temperature.

Evidence suggesting the participation of Sly1p and Sly2p in ER-to-Golgi protein transport was obtained by analyzing phenotypic alterations resulting either from the shutdown of gene expression (*SLY1*) or from gene disruption (*SLY2*). In Sly1p-depleted cells, the transport block appears to be tight: arrested cells accumulated the core-glycosylated forms of invertase and CPY exclusively, and an enrichment of ER was easily observed. In contrast, *SLY2* gene disruption proved not to be lethal, but *sly2* mutants were strictly Cs^-. Depending on the genetic background, they were also Ts^-, suggesting some kind of modifier gene (mutant or wild type) that seems to be responsible for generating this type of conditional lethality (15). Nevertheless, under nonpermissive conditions, a partial block of ER-to-Golgi protein transport was evident from the accumulation of core-glycosylated enzymes and the generation of an elaborate network of ER membranes. From the fact that incompletely glycosylated invertase was still secreted from *sly2* cells and presumably underglycosylated mature CPY was formed at the restrictive temperatures, it is tempting to speculate that a short bypass at the entry into the Golgi complex prevented the addition of some mannose residues to the outer-chain carbohydrates of the traversing proteins. It may be that the presence of proteins having properties similar to Sly2p can partially compensate for the loss of *SLY2* function. The *SLY12* gene product which is structurally related to Sly2p appears to be one such candidate.

It is a question of general interest whether related GTP-binding proteins have a similar mode of action. Sec4p is more than 50% identical to Ypt1p, and it is clearly involved in vesicle transport between the Golgi complex and the plasma membrane (22, 45). This makes the differences between the *YPT1* and the *SEC4* results intriguing. Interestingly, there are several suppressors of defects in the *YPT1* gene (43; this work), whereas Walworth et al. (58) did not succeed in isolating extragenic suppressors of the *sec4-8* defect. Walworth et al. (58) interpreted the failure to obtain such revertants as an inherent property of a system that operates in a cyclic fashion. On the other hand, *SEC4* is an effective suppressor of several secretion defects (45), while *YPT1*, even when highly overexpressed, acts as a very poor suppressor of defects in the secretory pathway (2). It remains to be seen whether the reason for these observations is the different mechanisms by which these regulators act.

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