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Structure-based Functional Analysis Reveals a Role for the SM Protein Sly1p in Retrograde Transport to the Endoplasmic Reticulum

Yujie Li, Dieter Gallwitz, and Renwang Peng

Max Planck Institute for Biophysical Chemistry, Department of Molecular Genetics, 37077 Göttingen, Germany

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Sec1p/Munc18 (SM) proteins are essential for membrane fusion events in eukaryotic cells. Here we describe a systematic, structure-based mutational analysis of the yeast SM protein Sly1p, which was previously shown to function in anterograde endoplasmic reticulum (ER)-to-Golgi and intra-Golgi protein transport. Five new temperature-sensitive (ts) mutants, each carrying a single amino acid substitution in Sly1p, were identified. Unexpectedly, not all of the ts mutants exhibited striking anterograde ER-to-Golgi transport defects. For example, in cells of the novel *sly1-5* mutant, transport of newly synthesized lysosomal and secreted proteins was still efficient, but the ER-resident Kar2p/BiP was missorted to the outside of the cell, and two proteins, Sed5p and Rer1p, which normally shuttle between the Golgi and the ER, failed to relocate to the ER. We also discovered that in vivo, Sly1p was associated with a SNARE complex formed on the ER, and that in vitro, the SM protein directly interacted with the ER-localized nonsyntaxin SNAREs Use1p/Slt1p and Sec20p. Furthermore, several conditional mutants defective in Golgi-to-ER transport were synthetically lethal with *sly1-5*. Together, these results indicate a previously unrecognized function of Sly1p in retrograde transport to the endoplasmic reticulum.

INTRODUCTION

Membrane fusion in eukaryotic cells is of fundamental importance for exo- and endocytosis and for maintaining the integrity of intracellular organelles. Essential components of the machinery that mediates lipid bilayer mixing or fusion are members of three protein families that are conserved from unicellular yeast to man: the soluble *N*-ethylmaleimide-sensitive fusion (NSF)-attachment protein receptors (SNAREs), the Sec1/Munc18 (SM) proteins and the Ypt/Rab GTPases. So far, evidence favors the view that SNAREs catalyze membrane fusion (Sutton *et al.*, 1998; Weber *et al.*, 1998; Schuette *et al.*, 2004), whereas Ypt/Rab GTPases and SM proteins regulate upstream processes, termed "tethering," to program the membranes to be fused (Jahn *et al.*, 2003; Pfeffer, 2001). Being first identified in screens for membrane-trafficking mutants in *Caenorhabditis elegans* (Brenner, 1974), all the SM family members are soluble, peripherally membrane-associated proteins with molecular masses of 60–90 kDa. There are four SM proteins in the budding yeast and seven in mammalian cells. Although the primary sequence homology within SM proteins is rather low, their three-dimensional structures are highly conserved, suggesting that SM proteins fulfill a general role in membrane fusion (Halachmi and Lev, 1996; Rizo and Sudhof, 2002; Gallwitz and Jahn, 2003).

The first evidence regarding SM protein function came from the finding that the mammalian N-Sec1/Munc18 protein binds with high affinity to the SNARE syntaxin1 exclusively in its closed conformation, therefore precluding syntaxin1 from SNARE complex assembly (Hata *et al.*, 1993; Pevsner *et al.*, 1994; Garcia *et al.*, 1994; Dulubova *et al.*, 1999; Yang *et al.*, 2000). In agreement with this, overexpression of N-Sec1/Munc18 in *Drosophila* neurons inhibits exocytosis, but this effect is rescued by concomitant overexpression of syntaxin 1 (Wu *et al.*, 1998). These findings have led to a model according to which SM proteins inhibit SNARE complex formation. However, not all the function of SM proteins can be explained by this model. First, deletions of SM genes in yeast, invertebrates, and vertebrates invariably lead to a block of intracellular fusion steps (Novick and Schekman, 1979; Ossig *et al.*, 1991; Verhage *et al.*, 2000), whereas overexpression of SM proteins does not inhibit membrane trafficking as the inhibitory model would predict. For instance, in chromaffin cells, overexpression of Munc18 increases rather than decreases exocytosis (Voets *et al.*, 2001); and not all syntaxins are capable of adopting closed conformations (Dulubova *et al.*, 2001, 2002). Importantly, the binding of SM proteins to their respective syntaxins does not necessarily preclude formation of fusogenic SNARE complexes. For example, yeast Sly1p, which in solution binds to the Golgi syntaxin Sed5p with high affinity (Grabowski and Gallwitz, 1997), also associates with a fully assembled SNARE complex and promotes the assembly of physiologically relevant SNARE complexes (Peng and Gallwitz, 2002), and yeast Sec1p binds to the exocytotic SNARE complex and promotes membrane fusion in vivo and in vitro (Carr *et al.*, 1999; Scott

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Address correspondence to: Renwang Peng (rpeng@gwdg.de) or Dieter Gallwitz (Dieter.Gallwitz@mpi-bpc.mpg.de).

Abbreviations used: ER, endoplasmic reticulum; SM, Sec1/Munc18 proteins; SNARE, soluble *N*-ethylmaleimide-sensitive fusion (NSF)-attachment protein receptors; CPY, carboxypeptidase; ALP, alkaline phosphatase.

Table 1. Yeast strains used in this study

Strain	Genotype	Source
MSUC1A3D	MATa/α *ura3/ura3 leu2/leu2 his3/his3 trp1/trp1 LYS2/lys2 ade8/ADE8*	This laboratory
MSUC1A	MATa *ura3 leu2 his3 trp1 ade8*	This laboratory
MSUC3D	MATα *ura3 leu2 his3 trp1 lys2*	This laboratory
RPY135	MATa/α *ura3/ura3 leu2/leu2 his3/his3 trp1/trp1 LYS2/lys2 ade8/ADE8 SLY1/sly1::KanMX$_4$*	Peng *et al*. (2002)
RPY18	MATa *sec24-11 ura3 leu2 his3 trp1 ade8*	Peng *et al*. (2000)
RPY152	MATα *GFP-SLY1 sec12-4 ura3 leu2 his3* lys2	Peng *et al*. (2004)
RH236-3A	MATα *sec20-1ura3 his4 leu2*	H. Riezman, Geneva
RH227-3A	MATa *sec23-1 ura3 his4 leu2*	H. Riezman, Geneva
Myc-Sec20	MATα *his3 leu2 ura3::TPI-SEC20-MYC*	H. D. Schmitt, Göttingen
dsl1-22	MATα *dsl1-22 ura3 leu2 his3 lys2 suc2*	H. D. Schmitt, Göttingen
tip20-5	MATα *tip20-5 ura3 leu2 his3*	H. D. Schmitt, Göttingen
sec22-3	MATα *sec22-3 ura3 his3 lys2 suc2-Δ9*	H. D. Schmitt, Göttingen
bos1-1	MATα *bos1-1 ura3-52 leu2-3112 his3-Δ200*	H. D. Schmitt, Göttingen
YLY1	MATa *sly1-1::LEU2 ura3 leu2 his3 LYS2 trp1ade8*	This study
YLY2	MATa *sly1-2::LEU2 ura3 leu2 his3 lys2 trp1 ade8*	This study
YLY3	MATa *sly1-3::LEU2 ura3 leu2 his3 lys2 trp1ade8*	This study
YLY4	MATa *sly1-4::LEU2 ura3 leu2 his3 lys2 trp1 ADE8*	This study
YLY5-1	MATα *sly1-5::LEU2 ura3 leu2 his3 LYS2 trp1ADE8*	This study
YLY5-2	MATa *sly1-5::LEU2 ura3 leu2 his3 LYS2 trp1 ADE8*	This study
YLY6	MATα *sly1-6::LEU2 ura3 leu2 his3 LYS2 trp1 ADE8*	This study
YLY17	MATa *ura3::TPI-SEC20-MYC his3 leu2 HA-UFE1, CEN, LEU2*	This study
PS42-1	MATa *ura3 leu2 his3 trp1 lys2 suc-Δ9 pep4::HIS3*	H. D. Schmitt
PS42-1A	MATα *ura3 leu2 his3 trp1 lys2 suc-Δ9 pep4::HIS3*	H. D. Schmitt
YLY23	MATa *sly1-5::LEU2 ura3 leu2 his3 trp1 pep4::HIS3*	This study
YLY24	MATa *sly1-6::LEU2 ura3 leu2 his3 trp1 pep4::HIS3*	This study
RP174	MATα *GFP-SED5 sec23-1 ura3 leu2 his3 trp1 lys2*	This study
YLY25	MATα *GFP-SED5 sly1–5::LEU2 sec23-1 ura3 leu2 his3 trp1 lys2*	This study
YLY26	MATα *sly1–5::LEU2 his3 leu2 ura3::TPI-SEC20-MYC*	This study

et al., 2004). Similarly, Vps45p binds to a very short N-terminal peptide of the syntaxin Tlg2p, and from cell lysates it coprecipitates with syntaxin Tlg2p and its SNARE partners. This indicates that Vps45p, like Sly1p and Sec1p, not only binds to syntaxin but also to syntaxin-containing SNARE complexes (Nichols *et al.*, 1998; Coe *et al.*, 1999; Dulubova *et al.*, 2002). Although the mechanism of SM/syntaxin binding is diverse, the structures of members of both protein families are conserved. This suggests that all the SM proteins might share a common role but the bimolecular binding of SM/syntaxin per se is not relevant or important for their function. In support of this, we have recently shown that in yeast, mutant Sly1p and Sed5p incapable to bind to each other were fully functional in vivo (Peng and Gallwitz, 2004). A similar observation has also been reported for N-Sec1/Munc18 in mammalian cells (Ciufo *et al.*, 2005).

To look more closely into the structure-function relationship of SM proteins, we took advantage of the recently solved crystal structure of the yeast Sly1p and performed a thorough mutational analysis. In this study, we report the identification of five novel *sly1* temperature-sensitive mutants. Extensive phenotypic analysis of these ts mutants leads to the conclusion that Sly1p plays a vital and previously unrecognized role in retrograde transport to the endoplasmic reticulum (ER).

MATERIALS AND METHODS

Yeast Strains, Genetic Methods, and Plasmids

Yeast strains used in this study are listed in Table 1. Yeast transformation, mating, sporulation, and tetrad analysis were performed by using standard techniques (Sherman and Hicks, 1991; Gietz *et al.*, 1992).

Yeast strains expressing GFP-Sed5p was constructed as follows: The coding region of *SED5* and the 5′ and 3′ adjacent sequences were amplified by the PCR from genomic DNA of *Saccharomyces cerevisiae*, subcloned, and sequenced to prevent possible errors introduced by PCR. A *Bam*HI site was created after the start codon of translation (ATG) by Quick Change Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA), and an enhanced version of *GFP* (Stratagene) amplified as a *Bam*HI fragment was then inserted into the *Bam*HI of *SED5*, which was on an integrating vector pRS306 (*CEN*, *URA3*). The resulting pRS306-GFP-SED5 was used for yeast transformation after linearizing by digestion with *Bsu*36I. Transformants on SC-Ura dropouts were further grown on media containing 5′-FOA, which selected against *URA3*. Yeast strains losing wild-type *SED5* but containing *GFP-SED5* as the sole source for Sed5 protein was finally chosen for further study. The plasmid expressing GFP-Rer1p was kindly provided by A. Nakano (Tokyo, Japan).

pRS316-SLY1-LEU2 was constructed by subcloning the 2.2-kb *Sal*I-*Xho*I fragment from YEp13 (New England Biolabs, Beverly, MA) containing the *LEU2* gene into the *Sal*I site created right behind the stop codon of *SLY1*. The DNA fragments encoding the entire cytoplasmic regions of Use1p (1–218), Sec20p (1–275) and Ufe1p (1–329) were amplified from genomic DNA prepared from MSUC3D by standard PCR. The PCR products were then subcloned into pGEX-KG or pGEX-KT vectors (Hakes and Dixon, 1992) for standard bacterial expression of GST-fusion proteins. Constructs expressing His_{10}-tagged Sly1p (full length), GST-Vti1p, and GST-Sec22p without their transmembrane domain were made as previously described (Peng and Gallwitz, 2002).

The point mutations in the *SLY1* gene were generated with pRS316-SLY1-LEU2 by site-directed mutagenesis using the QuickChange Site-Directed Mutagenesis Kit (Stratagene). The plasmids carrying *sly1* mutant genes, verified by DNA sequence analysis, were transformed into a diploid strain (RPY135) in which one copy of *SLY1* was replaced by *KanMX4*. After selected on kanamycin-containing plates, transformants were sporulated and the tetrad progeny was dissected. The Leu^+/Kan^+ haploids with the genomic *SLY1* disruption by *KanMX4* but containing different mutant *sly1* genes on plasmids were selected, and growth was followed at temperatures of 14, 25, and 37°C. The *sly1* alleles that conferred temperature sensitivity were integrated into the genome for further study. Briefly, an *Xba*I I-*Xho*I fragment bearing a mutant *sly1* allele, and the adjacent *LEU2* gene was introduced into RPY135, which was resistant to G418. The resulting Leu^+ transformants, in which the *sly1::KanMX4* was replaced by a certain *sly1::LEU2* mutant allele and became sensitive to G418, were identified. After sporulation and tetrad analysis, the

Leu$^+$ haploids bearing the mutant *sly1* allele were finally selected, and their phenotypes were further characterized.

Cell Labeling and Invertase Assay

Cell labeling and immunoprecipitations were performed as described previously (Tsukada and Gallwitz; 1996). Newly synthesized CPY or alkaline phosphatase (ALP), precipitated with affinity-purified antibodies, was resolved by SDS-PAGE, followed by autoradiography. Invertase activity staining was carried out as described (Peng *et al.,* 2000).

Protein Expression, Purification, and GST Pulldown Assay

Expression in *Escherichia coli* and purification of GST- and His-tagged proteins as well as GST pulldown experiments were performed as described (Peng and Gallwitz, 2002). GST pulldown experiments involved the following modifications: 10 μl of 50% glutathione Sepharose slurry (Pharmacia, Piscataway, NJ) were mixed with excessive amounts of GST fusion proteins at 4°C for 60 min to saturate the available binding sites on the beads. The beads were then washed three times with buffer F (50 mM Tris-HCl, pH 7.5, 150 mM KCl, 10% glycerol, 0.5% Triton X-100, 2 mM β-mercaptoethanol) and mixed with purified Sly1p-His$_{10}$ in a total volume of 100 μl in buffer F adjusted to a final KCl concentration of 375 mM. Incubation proceeded for 60 min at 4°C before washing six times with buffer F (500 μl each per washing). Proteins bound on beads were eluted with SDS sample buffer, analyzed by SDS-PAGE, and viewed by Coomassie blue staining or Western blotting.

Antibodies, Immunoprecipitation, and Western Blot Analysis

Antibodies against Sly1p, Sec22p, and Bos1p have been described (Peng and Gallwitz, 2002). Antisera against Kar2p/BiP were raised in rabbits. The c-Myc antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA) Polyclonal antiserum against Use1p and Ufe1p was kindly provided by G. Fischer von Mollard (University Goettingen) and S. Jentsch (MPI for Biochemistry, Munich), respectively. Immunoprecipitation experiments and Western blotting analysis were performed as previously described (Peng and Gallwitz, 2002).

The quantification of the relative amounts of Sly1p on immunoblots was performed using a LumiImager (Boehringer, Mannheim, Germany).

Kar2p/BiP Secretion Assay

Cells were grown in YEPD at 25°C to midlog phase before harvested. Around 0.1 OD of cells were spotted onto YEPD plates and incubated at 25°C for 8–10 h. Nitrocellulose filters were directly placed onto the plates, and the plates were incubated for additional 2 h at 25°C or 37°C. The filters were then carefully peeled off, washed three times in Western blot buffer A (10 mM Tris-HCl, pH 7.4, 0.9% NaCl), and treated subsequently with antiserum against Kar2p.

Fluorescence Microscopy

Cells expressing GFP-fused proteins were grown in YPG or SC medium under the appropriate conditions. The fluorescence was viewed in living or fixed cells as indicated in the figure legends. For viewing living cells, the midlog phase cells were collected by centrifugation and washed once with sterile, distilled H_2O, and three times with ice-cold phosphate-buffered saline (PBS) buffer. The washed cells were then resuspended in appropriate volume of ice-cold PBS buffer. Cell suspension (1.5 μl) was dropped onto a 76 × 26-mm microscope slide, covered with a coverslip, and subjected to immediate viewing. For analyzing cells at specific temperatures, midlog phase cells grown at either 25°C or nonpermissive temperature (35 or 37°C) were collected by centrifugation, washed once in ice-cold PBS buffer, and fixed at −20°C in absolute methanol for 15 min. Cells were then washed once with acetone (precooled to −20°C) and three times with ice-cold PBS buffer. Further treatment was essentially as described (Carr *et al.,* 1999). Fluorescence microscopy was performed with an Axioplan microscope equipped with a 100 × oil-immersion objective (Carl Zeiss, Thornwood, NY) and with a FITC filter (excitation 485 nm, emission 535 nm). Images were recorded with a photometric CCD camera and processed with the help of Photoshop software (Adobe, San Jose, CA).

RESULTS

Directed Mutagenesis of SLY1 Yields Five New Temperature-sensitive Mutants

We undertook a systematic, genetic approach to explore the structure-function relationship of the yeast SM protein Sly1p. The available structures of the yeast Sly1p (Bracher and Weissenhorn, 2002) and the neuronal homologue N-Sec1/Munc18 (Misura *et al.,* 2000) allowed for targeted mutagenesis by altering charged and highly conserved amino acids in order to identify residues or structural elements of physiological importance. Of the 666 amino acids of the whole protein, around seventy were selected for substitutions. As outlined in detail in *Materials and Methods*, *sly1* mutant genes on a single-copy vector that were able to support the growth of haploid cells lacking the genomic *SLY1* gene were identified. Growth properties of the different viable mutant strains (n = 62) were investigated at temperatures between 14 and 37°C. None of the *sly1* mutants generated caused cold sensitivity, but five new temperature-sensitive alleles with a single amino acid substitution each (*sly1-2*, D59A; *sly1-3*, I153K; *sly1-4*, H276A; *sly1-5*, R452A; *sly1-6*, E618A) were found (Figure 1). These were used to replace the chromosomal *SLY1* gene of a haploid wild-type strain and subjected to further study.

The six point mutations conferring temperature sensitivity to yeast cells, including the amino acid substitution of the previously isolated *sly1-1* mutant (R266K), are evenly scattered through the entire Sly1 protein (Figure 1A and Table 2). Mutations analogous to D59A in *sly1-2* have previously been described in *Drosophila* Rop/N-Sec1 (Harrison *et al.,* 1994) and in rat Sly1p, the substitution in the latter apparently interfering with syntaxin 5 binding in vitro (Dulubova *et al.,* 2003). The substitution I153K (*sly1-3*), which maps to the conserved hydrophobic pocket for Sed5p-binding, also conferred temperature sensitivity to haploid cells (Peng and Gallwitz, 2004). Two mutations in domain II, R266K (*sly1-1*) and E618A (*sly1-6*), concern amino acid residues engaged in a conserved salt bridge between their side chains (Bracher and Weissenhorn, 2002). They showed pronounced growth defects already at 35°C (Figure 1B). Of the pairs of amino acids that interact through conserved hydrogen bonds in domain III of Sly1p, H276/D449, and D286/R452, only one substitution each generated temperature-sensitive mutants: *sly1-4* (H276A) and *sly1-5* (R452A). In contrast, alanine substitutions of neither of the amino acid residues showing conserved hydrogen bonds in the crystal structure of Sly1p domain III, D325/H400 and K394/D398, led to a detectable growth retardation. Likewise, alanine substitutions of amino acids forming conserved salt bridges between domain II (E167) and domain III (W328), or within domain II (R258 and D586), did not exhibit temperature-dependent growth phenotypes (Table 2).

The five newly identified *ts* mutants (*sly1-2, sly1-3, sly1-4, sly1-5, sly1-6*), together with the previously identified *sly1-1* allele (Mizuta and Warner, 1994; Cao *et al.,* 1998), exhibited growth arrest at different temperatures when grown on rich media (Figure 1B). Cells of *sly1-4* and *sly1-6* exhibited the most severe growth defect and almost failed to proliferate already at 30°C. Cells of *sly1-1* and *sly1-2* grew well at 30°C, but extremely slow at 35°C. Although the growth of all *ts* mutants ceased at 37°C, the mutant proteins in *sly1-3*, *sly1-4*, and *sly1-5* cells were stable for at least 2 h at the nonpermissive temperature (Figure 1C). However, Sly1 mutant proteins decreased with time in *sly1-1*, *sly1-2*, and *sly1-6* cells, but 4 h after cell growth at 37°C 25% (*sly1-1*), 15% (*sly1-2*), and 32% (*sly1-6*) of the protein level seen at 25°C was still detectable (Figure 1C).

Protein Traffic Defects in Newly Identified sly1 Temperature-sensitive Mutants

Sly1p acts early in the secretory pathway, downstream of the GTPase Ypt1p and the vesicle tethering factor Uso1p (Dascher *et al.,* 1991; Ossig *et al.,* 1991; Sapperstein *et al.,* 1996; Cao and Barlowe, 2000) and has been shown to promote the assembly of ER/Golgi SNARE complexes (Kosodo *et al.,*

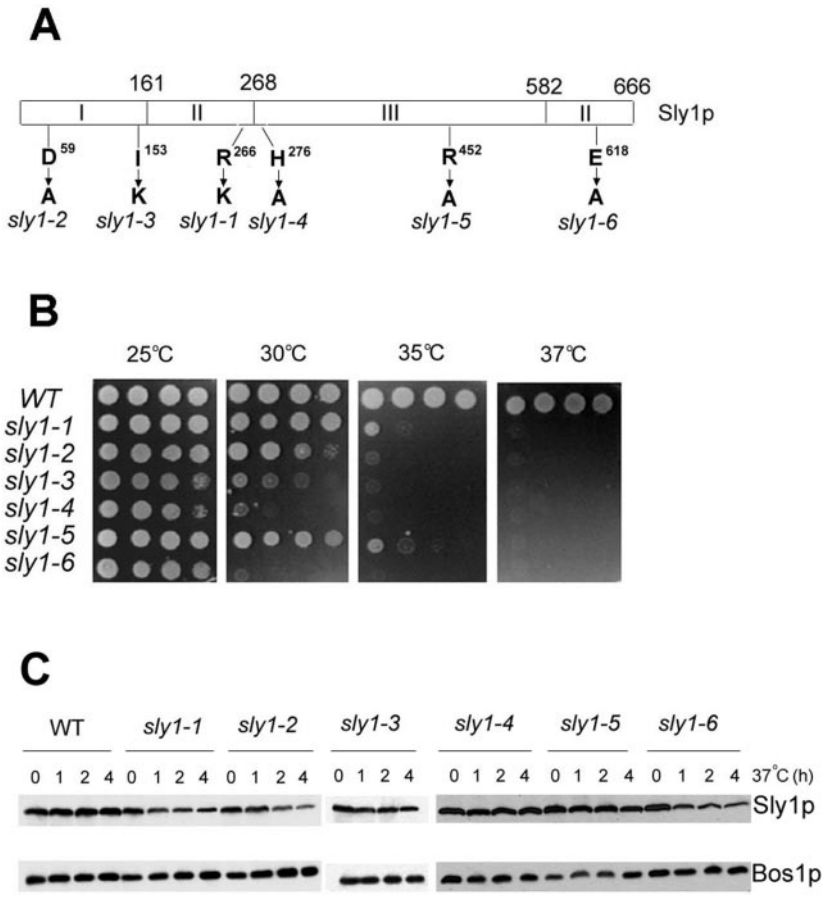

Figure 1. The growth phenotype of temperature-sensitive (ts) *sly1* mutants and the stability of mutant Sly1 proteins. (A) Location of single amino acid substitutions in six *sly1* ts mutants. I, II, III, domain I (a.a.1–161), domain II (a.a.162–268 and a.a.582–666) and domain III (a.a.269–581) of Sly1p. (B) Three μl of 10-fold diluted log-phase cultures, starting with 10^6 cells/ml, were spotted onto YPD plates and incubated for 3 d at the indicated temperatures. (C) Cells of wild-type and mutant *sly1* strains grown at the permissive temperature (25°C) to midlog phase were shifted to and incubated at 37°C for 1, 2, 3, and 4 h. Total proteins were prepared from these cells by lysis in NaOH/β-mercaptoethanol and precipitated by trichloroacetic acid. After separation of proteins by SDS-PAGE, Sly1p was detected by anti-Sly1p antibodies. The Sly1 protein amounts from immunoblots were calculated by LumiImager analysis, and the loading materials were evaluated by Bos1p signals on the same blot.

2002; Peng and Gallwitz, 2002; Peng and Gallwitz, 2004). The newly identified temperature-sensitive *sly1* mutants were first tested for their defects in protein transport by following the fate of newly synthesized lysosomal enzymes carboxypeptidase Y (CPY) and ALP, and of the secreted invertase. The three enzymes are N-linked glycoproteins that undergo core glycosylation in the ER and outer chain elongation in the Golgi before being transported to their final destination. Different glycosylated forms, and mature lysosomal enzymes generated by proteolytic cleavage, can be

Table 2. Summary of *SLY1* mutations generated in this study

Mutation	Domain	Significance of amino acid	Growth phenotype	Allele
D59A	I	Residue conserved in SM proteins	ts	*sly1-2*
I153K	I	Part of Sed5p binding pocket	ts	*sly1-3*
E167A	II	Conserved salt bridge with W328	Inconspicuous	
R258A	II	Conserved salt bridge with D586	Inconspicuous	
R266K	II	Conserved salt bridge with E618	ts	*sly1-1*
H276A	III	Conserved H bond with D449	ts	*sly1-4*
D286A	III	Conserved H bond with R452	Inconspicuous	
D325A	III	Conserved H bond with H400	Inconspicuous	
W328A	III	Conserved salt bridge with E167	Inconspicuous	
K394E	III	Conserved H bond with D398	Inconspicuous	
K394R	III	Conserved H bond with D398	Inconspicuous	
K394A	III	Conserved H bond with D398	Inconspicuous	
D398A	III	Conserved H bond with D394	Inconspicuous	
H400A	III	Conserved H bond with D325	Inconspicuous	
D449A	III	Conserved H bond with H276	Inconspicuous	
R452P	III	Conserved H bond with D286	Lethal	
R452E	III	Conserved H bond with D286	Inconspicuous	
R452A	III	Conserved H bond with D286	ts	*sly1-5*
D586A	II	Conserved salt bridge with R258	Inconspicuous	
E618A	II	Conserved salt bridge with R266	ts	*sly1-6*

easily identified by gel electrophoretic analysis. Surprisingly, at the nonpermissive temperature of 37°C, not all of the *sly1* mutants exhibited striking anterograde ER-to-Golgi transport defects as expected. In *sly1-1* and *sly1-2*, and especially in *sly1-5* mutant cells, only a slight delay of CPY and ALP maturation was observed (Figure 2A). However, most of the newly synthesized enzymes were properly and efficiently processed in *sly1-5* within 30 min at 37°C, indicating that they reached their final destination. In sharp contrast, ER-to-Golgi protein transport in *sly1-3*, *sly1-4*, and *sly1-6* mutant cells was completely blocked as in *sec23-1* mutant cells that are defective in vesicle formation at the ER (Figure 2A). Similar results were obtained with invertase, whose intracellular and periplasmic portions were assessed in nondenaturating acrylamide gels. Whereas *sly1-3*, *sly1-4*, and *sly1-6* mutant cells were almost completely defective in invertase secretion and accumulated the ER core-glycosylated enzyme intracellularly at 37°C, *sly1-1*, and especially *sly1-2* and *sly1-5* mutant cells secreted a significant portion of the newly synthesized invertase, although in underglycosylated form (Figure 2B).

According to the transport defects observed, the *sly1* mutants could be teamed into two subgroups: one group (*sly1-3*, *sly1-4*, and *sly1-6*) exhibited striking inhibition of ER-to-Golgi transport, whereas the other, particularly *sly1-5*, showed only mild anterograde transport defects, resembling mutants whose gene products are directly involved in Golgi-to-ER transport. This suggested that Sly1p might also have a function in retrograde protein transport.

Golgi-to-ER Retrograde Traffic Is Defective in Some sly1 Mutants

Anterograde and retrograde traffic between the ER and the Golgi is tightly coupled. A block in the retrograde transport has an indirect but rapid effect on anterograde transport (Lewis and Pelham, 1996). Several assays have been developed to distinguish these transport steps. Kar2p/BiP is a soluble ER protein, which is being retrieved from the Golgi when escaped from the ER (Semenza *et al.*, 1990). However, Kar2p is secreted into the medium when retrograde traffic is blocked. We therefore searched for Kar2p secretion of the newly identified *sly1* mutants using a qualitative immunological detection method with cells grown on filters both at permissive and nonpermissive temperatures. As shown in Figure 3A, Kar2p was missorted in *sly1-5* cells at 25°C, but much more pronounced at 37°C. Kar2p was also secreted from *sly1-1* and *sly1-2* cells, especially at 37°C, but less efficiently than from *sly1-5* mutant cells. Low amounts of the ER-resident protein appeared to also reach the outside of the *sly1-3* and *sly1-6* mutant cells at both temperatures, whereas *sly1-4* behaved like wild-type cells. Weak Kar2p signals on filters might have been caused by cell breakage, although the test for cytoplasmic hexokinase in the same filter assay did not indicate that this was a serious problem.

As an independent approach to substantiate the apparent retrograde transport defect in the *sly1-5* mutant, we investigated the localization of the Golgi syntaxin Sed5p. At stead state, Sed5p is localized to the Golgi compartments, but it also actively cycles through the endoplasmic reticulum (Wooding and Pelham, 1998). When expressed in COPII mutant cells (like *sec23*) that at nonpermissive temperature are defective in vesicle budding from the ER, Sed5p will be trapped on ER membranes because ER-to-Golgi transport is blocked, whereas the retrograde Golgi-to-ER transport is still intact. In contrast, when expressed in mutants defective in retrograde Golgi-to-ER route, Sed5p will not reach the ER. Sed5p localization was therefore studied in *sec23-1* cells and

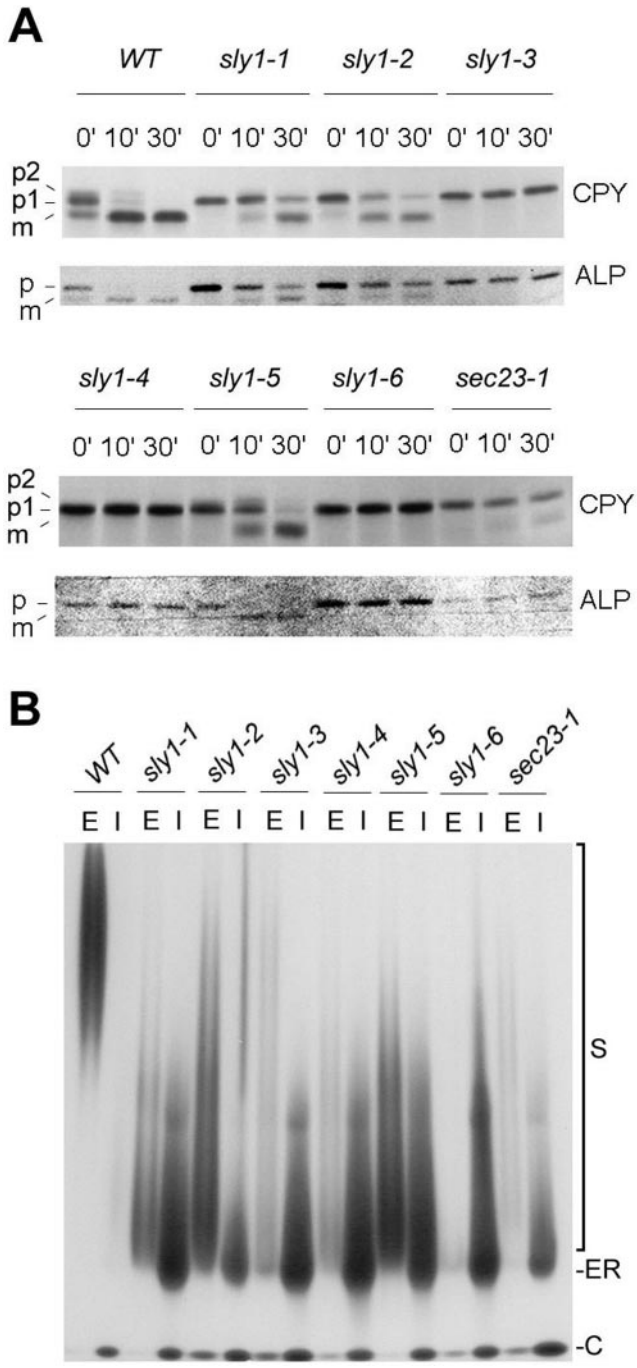

Figure 2. Analysis of CPY transport and invertase secretion in temperature-sensitive *sly1* mutants. (A) Wild-type (MUSC3D) and various mutant cells were grown at 25°C, shifted to 37°C for 30 min, and then labeled with Tran-^{35}S-label at 37°C for 7 min and chased for 0, 10, and 30 min. CPY was immunoprecipitated from cell lysates, analyzed by SDS-PAGE, and viewed by autoradiography. ER core-glycosylated (p1), Golgi-modified (p2), and the mature form (m) of CPY are indicated. (B) Cells of wild-type and mutant strains grown at 25°C were transferred to 0.1% glucose medium to induce invertase synthesis for 60 min at either 25 or 37°C. Spheroplasts were prepared and pelleted to obtain the periplasmic (E) and intracellular (I) fractions. Proteins of both fractions were separated on a nondenaturing polyacrylamide gel and subjected to activity staining of invertase. S, secreted invertase; ER, ER core-glycosylated invertase; C, cytoplasmic form of invertase.

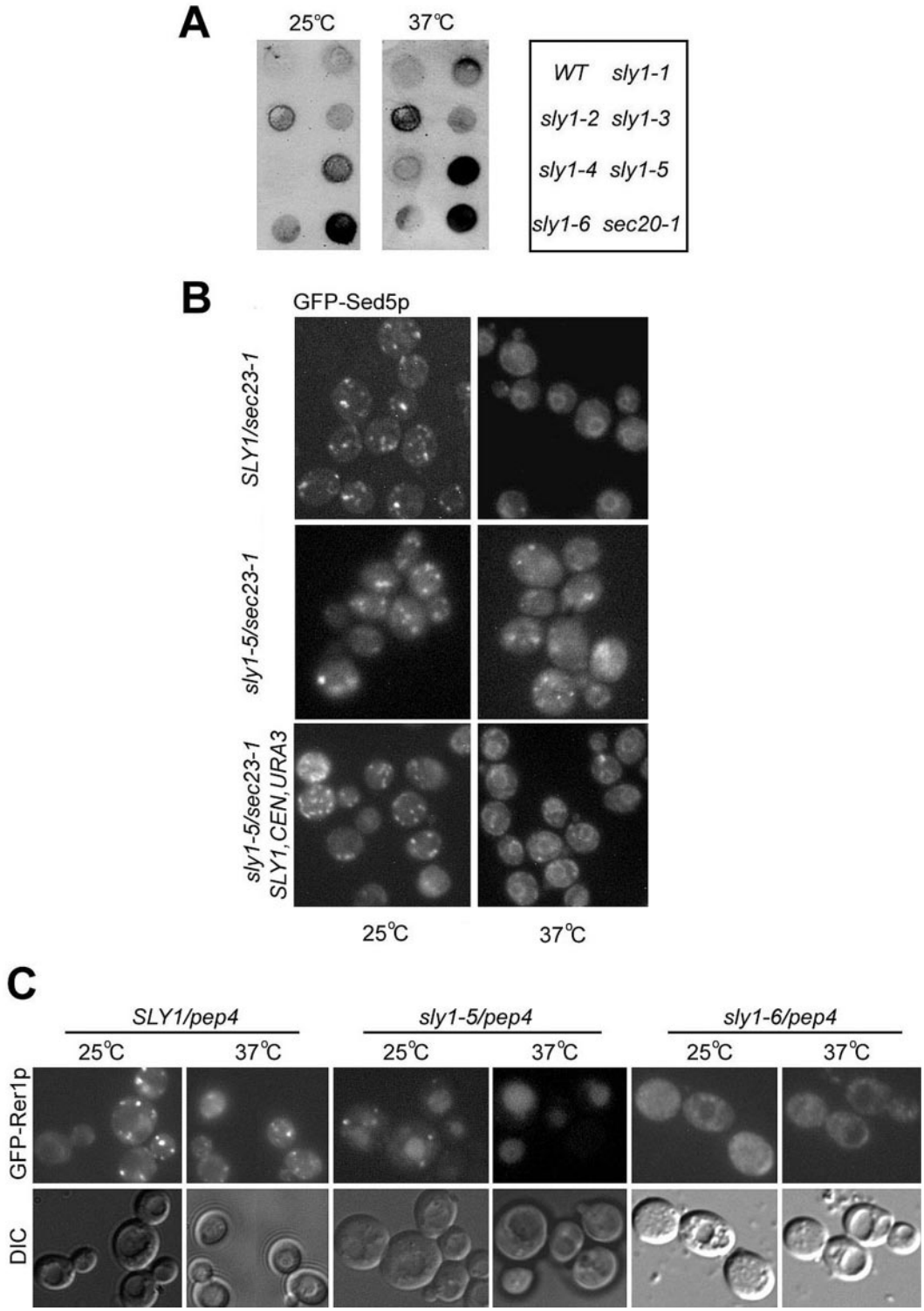

Figure 3. Golgi-to-ER trafficking is defective in some *sly1* mutant cells. (A) Cells of wild-type, *sly1* and *sec20-1* (RH236–3A) mutant strains were grown at 25°C to midlog phase before they were spotted onto YPD plates and incubated at 25°C for 8 h. The plates were then overlaid with nitrocellulose filters, and cells were allowed to grow at 25 or 37°C for additional 2 h. The Kar2p secreted and bound to the nitrocellulose filter was detected by immunoblotting with anti-Kar2p antibodies. (B) Cells of *sec23-1* and double *sly1-5/sec23-1* mutant cells stably expressing GFP-Sed5p (strain RPY174 and YLY25, respectively) were grown at 25°C and then shifted to and incubated at 37°C for 1 h. Samples of the cells were fixed before viewing the fluorescence under the microscope. To see whether the wild-type *SLY1* gene can rescue the defect of Sed5p cycling between the Golgi and the ER, a single copy vector bearing the wild-type *SLY1* was introduced into the *sly1-5/sec23-1* double mutant, and the GFP-Sed5p signal was viewed as above. (C) The dynamics of Rer1p localization in *sly1* mutants. The *SLY1/pep4* (PS42-1), *sly1-5/pep4* (YLY23), and *sly1-6/pep4* (YLY24) strains expressing GFP-Rer1p from a single copy vector were grown, treated, and observed microscopically as in B. DIC images are shown underneath the fluorescently labeled cells.

in *sec23-1/sly1-5* double mutant cells that expressed GFP-Sed5p from the fusion gene replacing the genomic *SED5* wild-type gene. As shown in Figure 3B, in *sec23-1* mutant cells at permissive temperature (25°C), GFP-Sed5p showed the Golgi-typical punctate staining, whereas at nonpermissive conditions of 37°C, staining of the nuclear rim, typical for ER membranes, was observed. A remarkably different pattern of fluorescence was observed when GFP-Sed5p was expressed in *sly1-5/sec23-1* double mutant cells: at both 25 and 37°C, fluorescently labeled punctate structures but no ER staining was observed (Figure 3B). This indicated that the retrograde Golgi-to-ER transport was blocked in *sly1-5/sec23-1* mutant cells at nonpermissive temperature. Importantly, with the introduction of a plasmid-borne wild-type *SLY1* gene copy into *sly1-5/sec23-1* mutant cells, transport of GFP-Sed5p to the ER was reestablished (Figure 3B), clearly demonstrating that *sly1-5* mutant cells are defective in retrograde transport.

The same conclusion could be reached from experiments in which we followed the dynamics of Rer1p localization in *sly1-5* mutant cells. Rer1p is a Golgi-localized receptor capturing and relocalizing several ER membrane proteins, and itself cycles through to the ER (Boehm *et al*., 1997; Sato *et al.,* 2001). A construct expressing GFP-Rer1p from a single-copy vector (Sato *et al.,* 2001) in yeast strains lacking vacuolar Pep4 protease (Δ*pep4*) was used to follow the localization of the receptor protein. As shown in Figure 3C, when expressed in cells in the presence of wild-type *SLY1 (SLY1/*Δ*pep4*), GFP-Rer1p exhibited normal Golgi localization at both permissive (25°C) and nonpermissive temperature (37°C). In sharp contrast, when expressed in *sly1-5/*Δ*pep4* mutant cells, typical Golgi staining was only scarcely seen, but instead the fluorescence was mostly confined to vacuoles. This indicated that GFP-Rer1p was missorted to the vacuole as a result of the blockage of the retrograde Golgi-to-ER pathway. Vacuolar staining was already visible at 25°C, but it was much more pronounced at 37°C. Importantly, vacuolar staining by missorted GFP-Rer1p was observed only in *sly1-5/pep4* but not in *sly1-6/pep4* mutant cells, which are primarily defective in anterograde ER-to-Golgi transport. However, in the latter mutant, the number and brightness of punctate Golgi structures decreased at nonpermissive temperature, and a diffuse fluorescence of the entire cytoplasm increased. This might have been the result of an accumulation of anterograde COPII vesicles formed at the ER but unable to dock to and fuse with Golgi membranes.

Together, these findings are in accord with the observed protein transport defects (Figure 2) and support the conclusion that in *sly1-5* mutant cells, defects in retrograde transport outweigh those in anterograde transport between the ER and the Golgi complex. This clearly indicates that Sly1p has a function in both trafficking steps.

Sly1p Directly Binds to Nonsyntaxin ER-SNAREs and Is Associated with SNARE Complexes on ER Membranes

It has been reported that in vitro, Sly1p binds to the ER-syntaxins Ufe1p from yeast and syntaxin18 from vertebrate cells and that Sly1p coimmunoprecipitates with ER-SNAREs from mammalian cell lysates (Yamaguchi *et al.,* 2002; Hirose *et al.,* 2004). These studies suggested a possible role for Sly1p in retrograde transport although direct evidence remained to be established. Our recent work demonstrated that Sly1p not only binds to the Golgi syntaxin Sed5p but also directly and specifically interacts with nonsyntaxin SNAREs on Golgi membranes (Peng and Gallwitz, 2004).

To address whether Sly1p also binds to nonsyntaxin SNAREs on ER membranes, we screened with purified proteins for possible interactions between Sly1p and the three nonsyntaxin ER-SNARES, Sec20p, Sec22p, and Use1p (Lewis *et al.,* 1997; Burri *et al.,* 2003; Dilcher *et al.,* 2003). For this purpose, the cytosolic regions of the three nonsyntaxin SNAREs and of the syntaxin Ufe1p were fused to the C-terminus of GST. The fusion proteins were immobilized on glutathione Sepharose beads and incubated with N-terminally His_{10}-tagged Sly1p at salt concentration of 375 mM. After extensive washing of the beads, significant binding of Sly1p was observed with the nonsyntaxin SNAREs Sec20p and Use1p and, as expected from a previous study (Yamaguchi *et al.,* 2002), with Ufe1p. As previously seen (Peng and Gallwitz, 2004), Sly1p did not bind to Sec22p. Vti1p, a nonsyntaxin SNARE that acts in the transport from Golgi to endosomes and to the vacuole, did not interact with Sly1p, indicating that the observed interactions were specific (Figure 4A).

We also tested for Sly1p/ER-SNARE interaction in vivo. Detergent lysates from cells of a strain expressing Myc-tagged Sec20p and HA-Ufe1p were subjected to immunoprecipitation with anti-HA antibodies so that the syntaxin Ufe1p was completely precipitated. As shown in Figure 4B, a fraction of Sec20p, Sec22p, and Use1p, the nonsyntaxin SNAREs involved in Golgi-to-ER retrograde trafficking, were coprecipitated with HA-Ufe1p and with Sly1p. Importantly, the vacuolar SNARE Nyv1p (Nichols *et al.,* 1997) did not coprecipitate with Ufe1p. This indicates that Sly1p association with Ufe1p-containing SNARE complexes in vivo was specific.

Inefficient Coprecipitation of Sly1-5p with Ufe1p and ER-Nonsyntaxin SNAREs from sly1-5 Mutant Cell Lysates

As described above, the most severe defects of retrograde transport were observed in *sly1-5* mutant cells. Because Sly1p also binds to ER-nonsyntaxin SNAREs (Figure 4, A and B), we reasoned that this binding might be affected in *sly1-5* mutant cells. Therefore, immunoprecipitates with anti-Sly1p antibodies were prepared from detergent lysates of wild-type and *sly1-5* mutant cells that had been grown for 60 min at 37°C. Coprecipitation of Sly1 proteins with Ufe1p and with several ER- and Golgi-nonsyntaxin SNAREs was analyzed by immunoblotting. As shown in Figure 5, the Golgi syntaxin Sed5p as well as nonsyntaxin SNAREs specific for anterograde ER-to-Golgi traffic (Bos1p and Bet1p) were coprecipitated with the SM protein with similar efficiency from extracts of wild-type and *sly1-5* cells. However, compared with Sly1p from wild-type cell extracts, coprecipitation with Sly1-5p of the ER syntaxin Ufe1p and of nonsyntaxin SNAREs specific for retrograde transport (Use1p and Sec20p) was significantly reduced, indicating a specific defect in the formation of ER SNARE complexes. As can be seen from Figure 5, the significant reduction of ER-SNAREs coprecipitating with Sly1-5p from mutant cell lysates is not the result of lower intracellular levels of these SNAREs. As Ufe1p appears to be a permanent ER resident (Sato and Nakano, 2004), a hypothetical defect of *sly1-5* mutant cells in sorting of the ER syntaxin into COPI vesicles would be highly unlikely to account for its highly reduced coprecipitation with Sly1-5p and other ER SNAREs.

This finding again is in support of the conclusions drawn from the experimental results described above that Sly1p has a function in retrograde Golgi-to-ER transport and that Sly1-5p is specifically defective in this trafficking step.

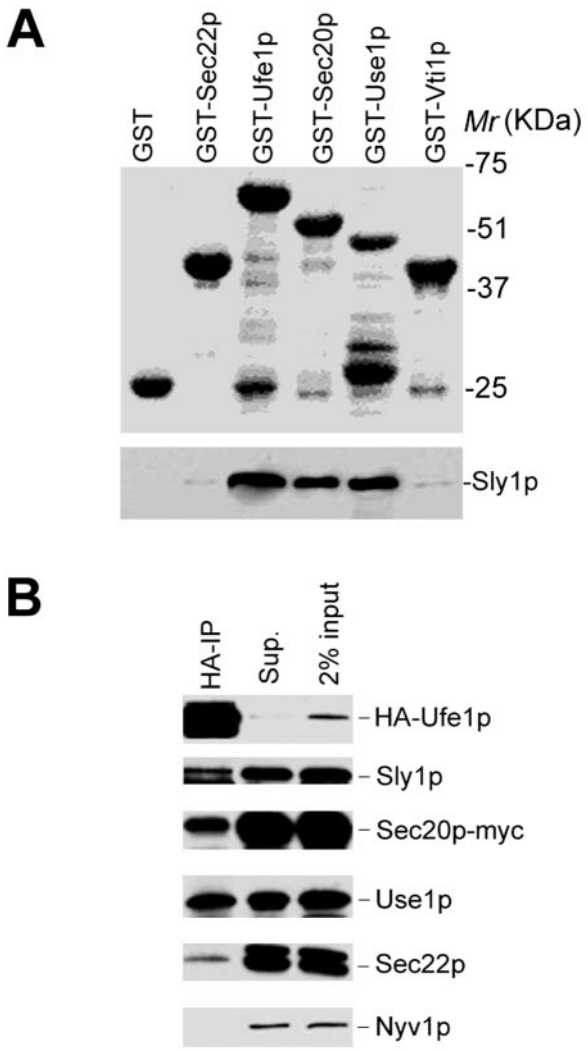

Figure 4. Sly1p directly binds to nonsyntaxin ER-SNAREs and is associated with a SNARE complex on the ER. (A) Bacterially produced GST fusions of SNAREs involved in Golgi-to-ER retrograde transport were immobilized on glutathione Sepharose beads and incubated with purified Sly1p. After washing, proteins bound to beads were analyzed by SDS-PAGE and Coomassie blue staining (top panel) or by immunoblotting using anti-Sly1p antibodies (bottom panel). (B) Cells of a HA-Ufe1p-expressing strain (YLY17) were grown at 25°C to midlog phase. Detergent lysates prepared from the strain were subjected to immunoprecipitation by using anti-HA antibodies. HA-Ufe1p and its coimmunoprecipitated proteins were analyzed on immunoblots.

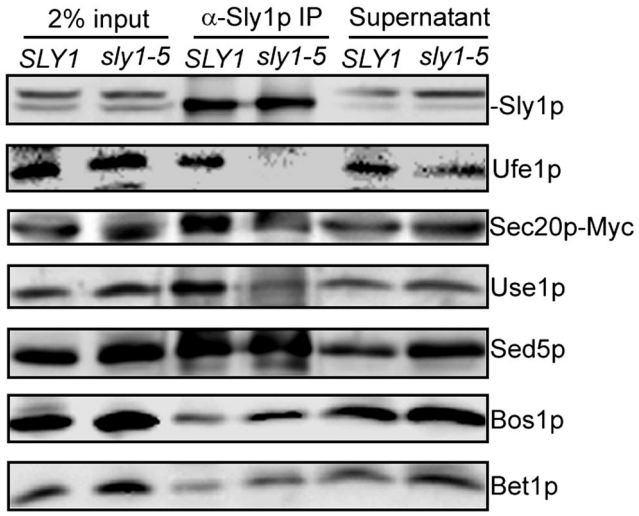

Figure 5. Inefficient coprecipitation of Sly1-5p with SNARE proteins of the retrograde Golgi-to-ER trafficking. Cells of wild-type (MSUC3D) and of *sly1-5* (YLY26) were grown at 25°C before transfer to and incubation at 37°C for 60 min. Detergent lysates were prepared from the cells, and immunoprecipitation was performed with affinity-purified Sly1p antibodies. Proteins of the precipitates were separated by SDS-PAGE, followed by immunoblotting with antibodies against Sly1p, Bet1p, Bos1p, Sed5p, Ufe1p, Use1p, and Myc (Sec20p). Compared with wild-type, 23, 34, and 24% of Ufe1p, Sec20p, and Use1p, respectively, coprecipitated with Sly1 protein from lysates of *sly1-5* mutant cells (LumiImager analysis, corrected for different amounts of lysates; see below). Note that coprecipitation with Sly1 protein of Golgi SNAREs was not impaired in the mutant cells. Also note that the material above Sly1p that cross-reacted with anti-Sly1p antibodies (input and supernatant) is absent from immunoprecipitates and that, according to LumiImager analysis, ~40% more lysates from *sly1-5* as compared with wild-type cells was used for immunoprecipitation in the experiment shown (compare intensity of bands in *WT* and *sly1-5* lanes of input and supernatant).

Genetic Interactions between sly1-5 and Mutants Defective in Golgi-to-ER Retrograde Transport

Genetic interactions provide a means to identify components of a common pathway. A functional relationship of gene products is likely if the combination of two conditional mutations aggravates the growth defect of a single mutant. We applied this method to analyze if components of the retrograde transport pathway have close functional connections with Sly1p. To observe such synthetic effects, we created heterozygous diploids by mating haploid *sly1-5* cells with other single mutants defective in Golgi-to-ER retrograde transport: *sec20-1* and *sec22-5*, both expressing mutant nonsyntaxin ER-SNAREs (Lewis *et al.*, 1997; Ballensiefen *et al.*, 1998), *tip20-5*, expressing defective Sec20p-interacting protein (Cosson *et al.*, 1997), and *dsl1-22*, encoding a defective COPI-interacting protein (Andag *et al.*, 2001; Reilly *et al.*, 2001). The resulting diploids were sporulated, tetrads were dissected, and the growth properties of the resulting spores were analyzed. As shown in Table 3, spores carrying both *sly1-5* and either *sec20-1*, *sec22-5*, *tip20-5*, or *dsl1-22* were not viable, demonstrating that the double mutants were synthetically lethal. In contrast, combinations of *sly1-5* either with *sec23-1* or with *sec24-11*, genes encoding COPII components, or with *bos1-1*, encoding a Golgi-SNARE protein defective in anterograde ER-to-Golgi transport, were viable. These re-

Table 3. Genetic interactions between *sly1-5* and other temperature-sensitive transport mutants

Double mutant with	Protein encoded	Growth phenotype at 25°C
sec20-1	ER SNARE	Inviable
sec22-5	ER/Golgi SNARE	Inviable
tip20-5	Sec20-interacting protein	Inviable
dsl1-22	COP I-binding protein	Inviable
bos1-1	Golgi SNARE	Viable
sec23-1	COP II component	Viable
sec24-11	COP II component	Viable

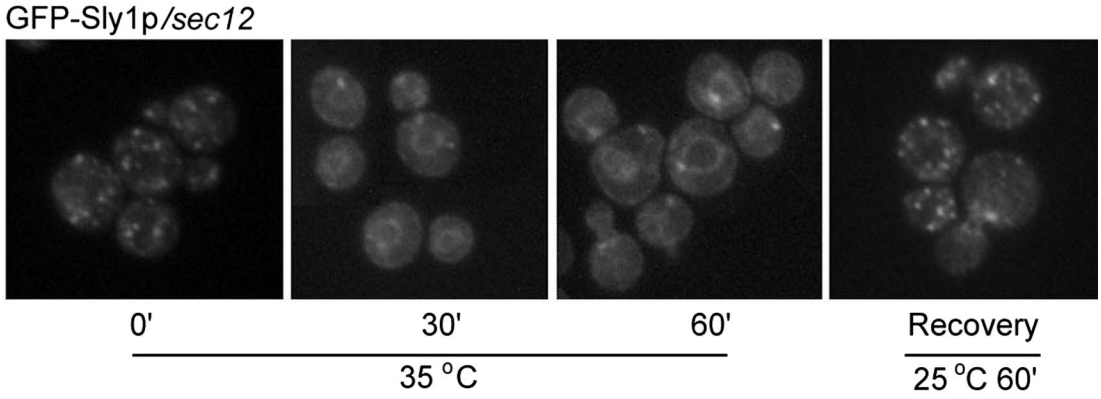

Figure 6. Sly1p cycles between the Golgi and the ER. Cells of *sec12* stably expressing GFP-Sly1p (strain RPY152) were grown at 25°C and then shifted to and incubated at 35°C for the indicated time periods. Samples of cells were fixed before viewing under the microscope. To see if the temperature effect was reversible, samples treated at 35°C for 60 min were quickly cooled to 25°C and further incubated at 25°C for 60 min. Cells were then fixed and viewed by fluorescence microscopy.

sults also support the conclusion that Sly1p also plays a vital role in retrograde transport to the ER.

Sly1p Cycles through the ER

Sly1p binds to the Golgi syntaxin Sed5p with high affinity, and to the ER syntaxin Ufe1p more weakly (Grabowski and Gallwitz, 1997; Yamaguchi *et al.,* 2002). In our present study, several lines of evidence point to a hitherto unrecognized function of Sly1p in membrane fusion at the ER membrane. Sly1p is peripherally membrane-associated, and it mainly localizes to Golgi compartments at steady state (Peng and Gallwitz, 2004). However, for a retrograde Golgi-to-ER transport function, the SM protein is expected to also associate with ER membranes. This in fact was seen by its coprecipitation with ER-SNARE complexes (see above).

In a further attempt to prove this, we followed the fate of GFP-tagged Sly1p in a *sec12* mutant that is unable to form transport vesicles at the ER but still allows retrograde Golgi-to-ER transport at nonpermissive conditions (Schröder *et al.,* 1995). As shown in Figure 6, cells at permissive conditions exhibited the Golgi-typical dot-like fluorescence, but after a shift to 35°C, Sly1p accumulated in the ER, which is easily seen by fluorescence of the nuclear rim and adjacent to the plasma membrane. Full recovery of Golgi localization was observed within minutes after shifting cells back to 25°C. This clearly demonstrates that Sly1p in yeast, like its homologue in mammalian cells (Rowe *et al.,* 1998), cycles between the Golgi apparatus and the ER, and it suggests that the SM protein is likely to be available at any given time to act as a component of the machinery needed to fuse Golgi-derived vesicles with the ER membrane.

DISCUSSION

The purpose of our study was to shed new light on the functioning of SM proteins in vesicular trafficking by directed mutagenesis of the Sly1 protein that according to present knowledge acts close to membrane fusion in anterograde transport of vesicles between the ER and Golgi. Selected amino acid substitutions of the Sed5p-binding region of yeast Sly1p have already shown the value of such an approach because it was discovered that 1) the high-affinity binding of the syntaxin Sed5p to Sly1p does not serve an essential function and 2) that Sly1p does also bind nonsyntaxin SNAREs in a specific manner and, apparently, at region(s) of the SM protein different from the Sed5p binding pocket (Peng and Gallwitz, 2004). Furthermore, a single amino acid change within domain III in Sly1-20p was previously shown to make yeast cells independent of the need of the GTPase Ypt1p (Dascher *et al.,* 1991; Ossig *et al.,* 1991) and the tethering protein Uso1p (Sapperstein *et al.,* 1996), strongly suggesting that the essential Sly1 protein interacts functionally with other components of the membrane fusion machinery.

Because of the lack of known interaction partners of Sly1p and of critical regions of the protein for functional integrity, we focused on amino acid substitutions, single or in combination, of conserved residues in potentially important secondary structure elements, and of residues forming conserved salt bridges or hydrogen bonds (Misura *et al.,* 2000; Bracher and Weissenhorn, 2002).

Substitutions of the highly conserved aspartic acid located as single residue between β-strand 1 and the following α-helix in N-Sec1 (Misura *et al.,* 2000) as well as in Sly1p (Bracher and Weissenhorn, 2002; Arac *et al.,* 2005) have previously been found to interfere with the binding of cognate syntaxins and with the function of the mutant proteins in vesicular transport (Harrison *et al.,* 1994; Dulubova *et al.,* 2003). As we have shown here, mutation of the analogous aspartate (D59A) in yeast Sly1p generated a conditional mutant, *sly1-2*, with a relatively unstable protein at elevated temperatures. In contrast to the analogous mutation (D44N) in rat Sly1p, which completely abolished binding of syntaxin 5 (Dulubova *et al.,* 2003), yeast Sly1p domain I carrying the D59A substitution still interacted with Sed5p in vitro, although less efficiently than the same fragment of the wild-type protein (our unpublished observation). As D59A is not in direct contact with the bound syntaxin, less efficient binding of Sly1(D59A) to Sed5p in vitro and destabilization of the mutant protein in vivo may have been caused by an alteration of the conserved five-stranded β-sheet and its adjacent helices, of which helix 6 and β-strand 5 form the hydrophobic pocket accommodating Sed5p (Bracher and Weissenhorn, 2002). Likewise, mutation I153K (*sly1-3*) in β-strand 5 may significantly alter the structural arrangement of the 5-stranded β-sheet by generating a patch of three adjacent lysine residues and resulting in protein instability. Because our recent work disclosed that high-affinity Sed5p binding to Sly1p is not essential for the functioning of the two proteins (Peng and Gallwitz, 2004), the loss of Sed5p binding

resulting from the I153K substitution within the hydrophobic binding pocket cannot explain the conditional defect of the *sly1-3* mutant.

Amino acid residue R266 when mutated to lysine (*sly1-1* allele) was previously shown to result in temperature-sensitive cell growth (Cao *et al.*, 1998). R266, which is located in a short α-helix (η^2) near the N-terminal insertion point of Sly1p domain III into domain II, forms a conserved salt bridge with E618 that interconnects the two parts of domain II. Our finding that the substitution E618A also causes temperature sensitivity (*sly1-6* allele) underlines the importance of this salt bridge for structural integrity of the protein. This is further illustrated by the relative lability of both mutant proteins whose intracellular level after 1 h at restrictive temperature dropped to <50% compared with the level at 25°C. However, the two proteins were stable at permissive temperature (Figure 1C), and even after 4 h at 37°C, 25 and 32% of Sly1-1p and Sly1-6p, respectively, were still detectable on protein blots, indicating that loss of function was not simply caused by protein degradation. Although the E618A substitution in *sly1-6* is expected to interrupt the salt bridge with R266, the conservative mutation in Sly1-1p (R266K) might interfere primarily with other polar interactions that the guanidinium group of R266 is engaged in with several backbone carbonyl groups (Bracher and Weissenhorn, 2002). Different structural alterations of the SM protein induced by the amino acid substitutions in Sly1-1p and Sly1-6p may explain the different functional alterations exhibited by *sly1-1* and *sly1-6* mutant cells at restrictive conditions.

A second conserved salt bridge between R258 and D586 that interconnects the N- and C-terminal parts of domain II appears to be less important for the arrangement of the protein's domain structure because substitutions with alanine of neither of the two amino acid residues impaired cell growth at temperatures ranging from 14 to 37°C. Likewise, interference with a conserved salt bridge by mutating E167 of domain II and W328 of domain III proved functionally insignificant, and disruption of conserved hydrogen bonds discovered in the Sly1p crystal structure between D325 and H400, and between K394 and D398 of domain III, were functionally neutral (see Table 2).

On the other hand, substitutions of some amino acid residues involved in conserved hydrogen bonds within domain III of Sly1p (H276/D449 and D286/R452) resulted in conditional mutants, or even in lethality. The H276A mutation caused temperature-sensitive growth already at 30°C and a complete block in ER-to-Golgi forward transport. In vivo, the mutant protein, Sly1-4p, was perfectly stable for at least 4 h at 37°C. As the substitution D449A, which should affect hydrogen bond formation with H276, did not impair cell growth at various temperatures, it seems most likely that the side chain of H276, a residue within the loop between the structurally conserved α-helices 10 and 11 (Bracher and Weissenhorn, 2002), serves an important role for functional interactions of the Sly1 protein.

Previous studies have shown that the yeast Sec1 protein with a R432P substitution (*sec1-1* allele) causes temperature sensitivity and an accumulation of Golgi-derived vesicles at restrictive temperature (Novick *et al.*, 1979; Brummer *et al.*, 2001). The arginine analogous to R432 in yeast Sec1p is conserved in other yeast, in plant and in mammalian SM proteins. A comparison of crystal structures of Sly1p with those of neuronal Sec1 proteins from rat (Misura *et al.*, 2000) and squid (Bracher and Weissenhorn, 2001) revealed that this highly conserved arginine is positioned in the middle of an α-helix (α17 in Sly1p; α15 in N-Sec1p) in a larger region of domain III that shares the same topology in both proteins. Our study showed that the substitution R452P in Sly1p, which is analogous to the mutation in Sec1-1p, is lethal. As in general, proline residues are not tolerated in the central part of α-helices in globular proteins (Presta and Rose, 1988), this mutation may have destabilized Sly1p and caused its fast degradation. Significant destabilization of Sec1-1p (R432P substitution) and very high reversion frequency of *sec1-1* underlines the severity of this mutation (Brummer *et al.*, 2001). However, Sly1p can obviously accommodate glutamate instead of R452 without impairment of its function, suggesting that the α-helical arrangement might not be disturbed in such a mutant. In contrast, removal of the large hydrophilic side chain through the substitution of alanine for R452 generated a viable, but temperature-sensitive mutant (*sly1-5*). The Sly1-5 protein was rather stable, and after 2 h following the shift of mutant cells to the restrictive temperature of 37°C, the level of the mutant protein was still ~80% of that of wild type.

The *sly1-5* mutant turned out to be of special interest. At nonpermissive conditions, ER-to-Golgi transport of newly synthesized proteins was only slightly affected, as shown by the efficiency with which lysosomal enzymes reached the vacuole or secreted invertase arrived in the periplasmic space, the latter, however, in underglycosylated form. Importantly, *sly1-5* mutant cells heavily secreted the ER-resident Kar2p, like the *sec20* mutant, which is known to be defective in retrograde transport from the Golgi to the ER. Indications for defects in Golgi-to-ER transport were also obtained with *sly1-1* and *sly1-2* mutants, but less pronounced than with *sly1-5*. We further inquired into a possible role of Sly1p in retrograde protein transport by following the intracellular localization of the Golgi protein Rer1p, which is known to cycle through the ER and which was previously shown to be missorted to the vacuole via the multivesicular body (MVB)-sorting pathway in COPI mutants (Sato *et al.*, 2001). Mislocalization of a GFP-Rer1p fusion protein to the vacuole in the *sly1-5* mutant at nonpermissive conditions, but not in the forward transport-defective mutant *sly1-6*, strongly supported the conclusion that Sly1p has an essential function in retrograde transport too. Likewise, disruption of cycling through the ER of the Golgi syntaxin Sed5p in *sly1-5* mutant cells led to the same conclusion. As Sly1p was shown to bind to the ER syntaxin Ufe1p (Yamaguchi *et al.*, 2002), although less efficiently than to the Golgi syntaxin Sed5p (Grabowski and Gallwitz, 1997; Peng and Gallwitz, 2004), an additional role of the SM protein in retrograde Golgi-to-ER protein transport was not unexpected but had not been demonstrated until now. In strong support of this newly discovered functional role of Sly1p are the synthetically lethal interactions observed when the *sly1-5* allele was combined specifically with various mutant alleles of genes having a clearly defined function in retrograde transport (Table 3).

Another significant outcome of our study is the finding that, similar to the interaction of Sly1p seen with nonsyntaxin SNAREs involved in ER-to-Golgi and intra-Golgi transport (Peng and Gallwitz, 2004), Sly1p could also be demonstrated to bind specifically to nonsyntaxin SNAREs essential for retrograde transport (Sec20p and Use1p) and to be part of assembled SNARE complexes at the ER. Importantly, we observed that compared with wild-type cells, the fraction of the ER-nonsyntaxin SNAREs (Use1p and Sec20p) that coprecipitated with Sly1p from lysates of *sly1-5* mutant cells was significantly reduced, whereas complexes of ER-to-Golgi SNAREs (Bet1p and Bos1p) with Sly1p in wild-type and *sly1-5* cell lysates were almost indistinguishable. Because coprecipitation with Sly1-5p of the ER syntaxin Ufe1p

was similarly diminished, it seems likely that in the *sly1-5* mutant, a failure in the efficient generation of SNARE complexes at ER membranes might have caused the rather specific inhibition of retrograde vesicular transport between Golgi compartments and the ER. We have not yet investigated with purified proteins whether the direct binding of Sly1-5 mutant protein to ER-SNAREs might be impaired. However, because it has been demonstrated previously that Ufe1p, like Sed5p, binds to a short N-terminal peptide region of Sly1p domain I (Yamaguchi *et al.*, 2002), it is highly unlikely that Ufe1p binding to Sly1-5p with its single amino acid substitution in domain III could be affected. The significant reduction of ER-SNARE complexes in *sly1-5* cells again is in support of a role of Sly1p in retrograde transport from the Golgi to the ER, which appears to be specifically affected by the R452A substitution of the SM protein. Given the observation that R432P in yeast Sec1-1p apparently affects the interaction of the SM protein with other binding partners (Brummer *et al.*, 2001), one might assume that, especially at elevated temperature, the R452A substitution in Sly1p alters the α-helical arrangement of this structurally conserved region in such a way that the interaction with protein(s) of the fusion machinery at the ER is no longer possible.

It is widely accepted that SM proteins are essential components of the membrane fusion machineries at all steps of vesicular transport. In yeast, there are only four SM proteins, Sly1p, Sec1p, Vps45p, and Vps33p. Vps33p has been suggested to act between Golgi and endosomes as well as in vacuole fusion (Subramanian *et al.*, 2004). Our finding that the function of Sly1p is not only connected to fusion of ER-derived vesicles with Golgi membranes but also of Golgi-derived vesicular carriers with the ER is an instructive example for a single SM protein acting in more than one fusion step through specific interaction with subsets of single SNAREs, and, possibly, their assembled complexes.

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