Binding of Sly1 to Sed5 enhances formation of the yeast early Golgi SNARE complex

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Summary

SLY1 is an essential gene for vesicular transport between the ER and the early Golgi apparatus in Saccharomyces cerevisiae. It encodes a hydrophilic Sec1/Munc18 family protein that binds to the t-SNAREs. The amount of Sly1 protein that coprecipitated with the t-SNARE Sed5 was much reduced in a temperature-sensitive sly1ts mutant yeast compared with the wildtype. The mutant Sly1ts protein was shown to have a reduced binding activity to Sed5. In the wildtype, a detectable amount of Sly1 was found in the complex between Sed5 and the v-SNARE Bet1. In vitro formation of this complex on different membranes in yeast lysate was enhanced by the addition of recombinant Sly1. These results indicate that binding of Sly1 to Sed5 enhances trans-SNARE complex formation.

Key words: Sec1 family protein, SNARE complex, Vesicle fusion, Sly1 protein, Saccharomyces cerevisiae

Introduction

In eukaryotic cells, major intracellular protein trafficking is mediated by vesicular transport, and this has been well studied in the past decade. Docking and fusion of the transport vesicles to the target organelle are mediated by several proteins. In vitro studies using liposomes showed that the membrane proteins SNAREs (soluble N-ethylmaleimide-sensitive factor attachment protein receptors) form the central physical fusion machinery, and the combination of SNAREs determines the compartmental specificity (Weber et al., 1998; McNew et al., 2000). In addition to SNAREs, other proteins are also needed to fulfill membrane fusion in vivo. In Saccharomyces cerevisiae, proteins of the Ypt, Uso1 and Sec1 families have important roles (Nichols and Pelham, 1998; Yoda and Noda, 2001).

Members of the Sec1 family have strong physical interactions with target membrane (t-)SNAREs. Munc18/nSec1 binds to syntaxin 1a, a neural plasma membrane t-SNARE (Hata et al., 1993), and Sly1 binds to Sed5, a yeast Golgi t-SNARE (Søgarrd et al., 1994). Recently, the X-ray crystal structure of the Munc18-syntaxin 1a complex was reported (Misura et al., 2000). However, experimental data indicate that the interaction between Munc18 and Sed5 is not interfered by the interaction between Sed5 and the v-SNARE Bet1 (Misura et al., 2000). However, Sly1 does not interact with uncomplexed Sso1, a yeast plasma membrane t-SNARE (Carr et al., 1999).

Genetic studies in S. cerevisiae have indicated that a number of ER-Golgi secretory genes have interactions with SLY1. A single copy of the gain-of-function mutation sly1-20 was originally identified as a suppressor mutation that enables the ER-Golgi vesicular transport to occur in the absence of YPT1 (Dascher et al., 1991). Interestingly, it could also suppress the temperature-sensitive defects caused by mutations in many other ER-Golgi secretory genes including uso1-1 and bet1-1 (Ossig et al., 1991; Sapperstein et al., 1996; Kito et al., 1996). The sly1ts allele was identified in a screen for a mutant that arrests transcription of the genes for ribosomal proteins and RNAs at the restrictive temperature (Mizuta and Warner, 1994). The sly1ts mutant grows normally at 25°C but arrests growth when the temperature is shifted to 37°C and accumulates the ER-form precursor of vacuolar enzymes. Multicopy USO1, BET1 or HSD1 genes could suppress the temperature sensitivity of sly1ts (Kosodo et al., 2001).

Barlowe and colleagues have reproduced the ER-Golgi transport in vitro by using semi-intact cells and temperature-sensitive mutants. They proposed that membrane fusion of the transport vesicles can be classified into tethering, docking and fusion steps. Mutant proteins of Sly1, Bet1, Bos1, Sed5 and a yeast NSF Sec18 do not interfere with tethering, but block docking and fusion steps (Barlowe, 1997; Cao et al., 1998). They have also showed that Sly1, Sed5 and Ypt1 are required on the acceptor Golgi membrane (Cao and Barlowe, 2000).

Thus the role of Sec1 family proteins, including Sly1, in membrane fusion has not been fully understood yet (Jahn, 2000). Although the binding between t-SNAREs and Sec1 family proteins has an important role, the function of the binding is
### Table 1. Yeast strains used in this study

| Strain      | Genotype                                                                 |
|-------------|---------------------------------------------------------------------------|
| W303        | MATa ura3 leu2 ade2 his3 can1                                              |
| 312         | MATa ade2 can1 sly1<sup>ts</sup>                                           |
| YKY201      | MATa leu2 sly1::SLY1-6MYC-TRP1 ade2 his3 can1                              |

### Table 2. Plasmids used in this study

| Plasmid      | Description                              | Expression in          |
|--------------|------------------------------------------|------------------------|
| pKD5         | GST-Sed5H1 (aa 1-78) in pGEX4T-3         | E. coli                |
| pKD35        | MBP-Sed5H1 (aa 1-78)-myc in pMALc2       | E. coli                |
| pKD37        | MBP-Sed5H3 (aa 208-324) in pMALc2       | E. coli                |
| pKD38        | MBP-Sed5ATMD (aa 1-324) in pMALc2       | E. coli                |
| pKD11        | GST-Sly1 (aa 1-666) in pGEX4T-3         | E. coli                |
| pKD26        | GST-Sly1<sup>ts</sup> (aa 1-666) in pGEX4T-3 | E. coli                |
| pKD211       | Streptagged-Sly1 (aa 1-666) in pASK-IBA2 | E. coli                |
| pKD212       | Streptagged-Sly1<sup>ts</sup> (aa 1-666) in pASK-IBA2 | E. coli                |
| pKD47        | Sly1 (aa 1-666)-6myc under SLY1 promoter in pRS416 | S. cerevisiae          |
| pKD43        | Sly1-20 (aa 1-666)-6myc under SLY1 promoter in pRS416 | S. cerevisiae          |
| pKD48        | Sly1<sup>ls</sup> (aa 1-666)-6myc under SLY1 promoter in pRS416 | S. cerevisiae          |
| pKD201       | 3HA-Bet1 (aa 1-142) under GALI promoter in pYES2.0 | S. cerevisiae          |
| pYN110       | 6myc-Sed5 (aa 1-340) under SED5 promoter in pRS416 | S. cerevisiae          |

### Materials and Methods

#### Strains and media

The yeast strains used in this study are listed in Table 1. They were grown in a YEPD (1% Bacto yeast extract (Difco), 2% Bacto peptone (Difco), 2% glucose) or in an SD (0.5% (NH₄)₂SO₄, 0.17% Bacto yeast nitrogen base without amino acids (Difco), 2% glucose and appropriate supplements) medium. An SG (2% galactose and 1% nitrogen base without amino acids (Difco), 2% glucose) or in an SD (0.5% (NH₄)₂SO₄, 0.17% Bacto yeast nitrogen base without amino acids (Difco), 2% glucose and appropriate supplements) medium. An SG (2% galactose and 1% raffinose instead of glucose in SD) medium was used to induce genes appropriate supplements) medium. An SG (2% galactose and 1% raffinose instead of glucose in SD) medium was used to induce genes.

#### Plasmids

The plasmids used in this study are listed in Table 2 and were constructed as follows. The DNA fragments encoding various proteins were amplified either from genomic DNA or from plasmid DNAs harboring the gene by polymerase chain reaction (PCR) with Pfu DNA polymerase (Stratagene). The myc-encoding sequence was from pYN168 (Kosodo et al., 2001). The subcloned from pKT10mycC (Matsui et al., 1996), and the HA-encoding sequence was from pKD43 Sly1-20 (aa 1-666)-6myc under GALI promoter in pRS416 (Pharmacia), as maltose-binding protein (MBP) fusion proteins in pMalc2 (New England Biolabs) or as Streptagged fusion proteins in pAKS-IBA2 (Genosys) in E. coli. For protein production in yeast, a low-copy vector pRS416 or multicopy vectors pRS426 (Sikorski and Hieter, 1989) and pYES2.0 (Invitrogen) were used.

#### Protein purification and protein-protein binding assays

Production and purification of the GST- and MBP-tagged proteins were done as described previously (Kosodo et al., 1998). Streptagged Sly1/Sly1<sup>ls</sup> was produced in E. coli, extracted and bound to StreptTactin-Sepharose (Sigma) and then eluted with 2.5 mM desthiobiotin after extensive washing. The concentration of all recombinant proteins was determined by a protein assay kit (BioRad), and proteins were stored with 40% glycerol at –20°C.

#### Binding assays of recombinant proteins

Binding assays of recombinant proteins were carried out as described previously (Kosodo et al., 1998).

#### Preparation of yeast lysate

Yeast cells were grown to an OD₆₀₀ of 0.6 to 0.8 in YEPD or SD medium. They were harvested by centrifugation, washed in distilled water and resuspended in an ice-cold B88 buffer (150 mM KOAc, 5 mM Mg(OAc)₂, 20 mM HEPES, 200 mM sorbitol, pH 6.8) with PIC (1 mM phenylmethylsulfonyl fluoride (PMSF), 1 µg/ml 1,10-phenanthroline, 1 µg/ml pepstatinA, 1 µg/ml aprotime, 1 µg/ml leupeptin). One-half volume of acid-washed glass beads was added to the cell suspension in a glass tube, which was then vortexed eight times for 30 seconds, with a 30 second incubation on ice between each burst. The crude lysate was centrifuged at 500 g for 3 minutes at 4°C to remove unbroken cells. The supernatant was used as a yeast lysate in this study.

#### Differential centrifugation

The yeast lysate was centrifuged at 10,000 g for 10 minutes at 4°C to generate the supernatant (S10) and pellet (P10) fractions. The S10 was centrifuged at 100,000 g for 60 minutes at 4°C to generate the high-speed supernatant (S100) and pellet (P100) fractions. The same amount of each fraction was resuspended in the Laemmli sample buffer (Laemmli, 1970) and applied to SDS-polyacrylamide gel electrophoresis (PAGE). Immunodetection of western blotting was performed using indicated primary antibodies followed by horseradish-peroxidase-coupled goat anti-mouse or anti-rabbit IgG as secondary antibodies at a 1:5000 dilution. The bound antibodies were visualized by enhanced chemiluminescence (Pierce).

#### Immunoprecipitation from yeast lysate

Affinity-purified rabbit anti-Sed5 or the monoclonal anti-myc antibody 9E10 was used for immunoprecipitation. Triton X-100 was added to the yeast lysate to a 1% final concentration. The lysate was incubated on ice for 5 minutes to solubilize membranes and centrifuged at 10,000 g for 5 minutes to remove debris. After the lysate was mixed with an antibody and incubated at 4°C for 1 hour, Protein A-Sepharose beads (Amersham) were added, and the mixture was incubated at 4°C for 2 hours. Beads were washed five times with B88 buffer with 0.5% Tween 20 and boiled in the laemmli sample buffer for 1 minute. The supernatants were analyzed by SDS-PAGE and western blotting.

#### Binding assay of SNARE proteins in yeast lysate

The sly<sup>ls</sup> strain was transformed with pYN110 (6myc-SED5 under SED5 promoter on a CEN plasmid) or with pKD201 (3HA-BET1 under GALI promoter on a 2µ plasmid). The cells were grown to an OD₆₀₀ of 0.5 in 800 ml SD medium and, as for the sly<sup>ls</sup> strain, they were further incubated in 200 ml SG medium to produce 3HA-Bet1 protein for 2 hours. After harvesting the cells by centrifugation, lysates were prepared by vortexing with glass beads in 8 ml ice-cold B88 buffer. The protein concentration of both lysates was adjusted to 8.0 mg/ml, and the aliquots (850 µl each) were stored at –80°C. The lysates from both strains were defrosted before the reaction and centrifuged at 5000 g for 5 minutes to remove the
cytoskeleton and aggregates before the reaction. The cleared lysates of two strains (600 µl) were mixed and then divided into eight aliquots (150 µl, each including 1.2 mg protein) quickly on ice. The recombinant Strep-tagged Sly1 or Sly1ts protein (2.4 µg) was added to each mix. The reaction was started by transferring the mix to a 35°C bath and incubated up to the indicated time. In case of the experiments described in the figure legends. At the end of the reaction, the mixtures were diluted by adding 850 µl of B88 buffer with 1% Triton X-100 and subjected to immunoprecipitation by the anti-myc antibody. Anti-Sly1, anti-myc and anti-HA antibodies were used for western blotting. Quantification of the signals was performed by a luminoimage analyzer (LAS-1000plus, Fuji film).

Results

Binding affinity of Sly1 and Sly1ts proteins to the t-SNARE Sed5

Sly1 strongly binds to the yeast Golgi t-SNARE Sed5 (Sogarri et al., 1994), and therefore this interaction is believed a priori to be crucial for their function. However, this needs to be confirmed by experimental evidence. A temperature-sensitive sly1ts mutant with a single amino-acid substitution (R266K in Sly1) arrests ER-Golgi transport at the restrictive temperature (Mizuta and Warner, 1994; Cao et al., 1998). We sought to determine whether the interaction between Sed5 and Sly1 was altered in the mutant or not. The SLY1 alleles from the wild-type, sly1ts mutant and gain-of-function SLY1-20 mutant (Dacher et al., 1991) were cloned, ligated with the sequence encoding a hexameric myc tag at their C-termini and introduced into the sly1ts mutant. The tagged products were functional because the sly1ts cells carrying SLY1-6myc or SLY1-20-6myc did not show temperature sensitivity. We prepared the lysate containing Sly1-6myc, Sly1-20-6myc or Sly1ts-6myc at the permissive temperature of 25°C. After solubilizing the membrane with 1% Triton X-100, the myc-tagged protein was collected by immunoprecipitation, and the amount ofocoprecipitated Sed5 was examined by SDS-PAGE and western blotting using anti-Sed5 antiserum. The amount of Sed5 bound to Sly1ts-6myc was much less than that bound to Sly1-6myc or to Sly1-20-6myc (Fig. 1A). This result suggests that the Sly1ts protein has a reduced binding affinity for Sed5. However, it may be claimed that the reduction in the amount of Sed5 bound to Sly1ts was a result of the decreased frequency of ER-Golgi transport in the sly1ts mutant.

To demonstrate the protein interaction directly, we adopted a coprecipitation assay using recombinant proteins. Glutathione S-transferase (GST) was fused at the N-terminus of SLY1 and Sly1ts protein, and maltose-binding protein (MBP) was fused at the N-terminus of Sed5ΔTMD (amino acids 1-324 of Sed5), Sed5H1 (amino acids 146-169) or Sed5H2 (amino acids 252-311), each of which carried a myc tag at the C-terminus. The fusion proteins were produced in E. coli, purified by affinity resins and used in the binding assay at 4°C. As shown in Fig. 1B, a considerable amount of GST-Sly1 was recovered in the immunoprecipitate of MBP-Sed5ΔTMD-mycc (lane 1), whereas much less GST-Sly1ts could be recovered (lane 2). GST-Sly1 was coprecipitated with MBP-Sed5H1-myc, which contains the Sly1-binding helix of Sed5 (lane 3; Kosodo et al., 1998), but a detectable amount of GST-Sly1ts was not coprecipitated (lane 4). Neither GST-Sly1 nor GST-Sly1ts bound to MBP-Sed5H3-myc (lanes 5 and 6). From these results, we conclude that Sly1ts has a defect in binding to Sed5.

Binding of Sly1 proteins to the membrane fraction

The Sly1 protein is not predicted to have a transmembrane domain, and the recombinant Sly1-fusion proteins produced in E. coli were soluble. By contrast, nearly 60% of Sly1 localized on the Golgi membrane in yeast cells (Lupashin et al., 1996). This membrane localization of Sly1 has been explained by its interaction with Sed5 (Grabowski and
Sly1tsproteins were produced in the type Sly1 protein. The C-terminally myc-tagged Sly1, Sly1-20 and Sly1ts were subjected to centrifugation at 1,000 g for 1 hour at 4°C. Aliquots of the fractions were subjected to SDS-PAGE and western blot analysis with antibodies against the localization marker proteins shown to the left.

Using an in vitro assay, it was shown that a combination of v-SNARE(V AMP2) was excluded from the n-SNARE(Sec1(Munc-18)/t-SNARE(syntaxin1)) complex (Pevsner et al., 1994). This observation suggests that Sly1 binds only to free Sed5 or whether it also binds to Sed5 as Bos1 and Sec17. In case of exocytosis in neuron, it was suggested to be susceptible to the action of dissociating ATPase, because Bet1 was not detected in similar experiments when 0.5 mM ATP was added to the immunoprecipitation mix (data not shown).

Fig. 2. Sly1ts protein localizes on the membrane as does the wild-type Sly1 protein. The C-terminally myc-tagged Sly1, Sly1-20 and Sly1ts proteins were produced in the sly1ts strain from CEN plasmids. Cells containing Sly1-6myc (lanes 1-3), Sly1-20-6myc (lanes 4-6) and Sly1ts-6myc (lanes 7-9) were lysed by vortexing with glass beads and were subjected to centrifugation at 1,000 g for 5 minutes to remove unbroken cells and debris. The supernatant was fractionated by differential centrifugation at 10,000 g for 10 minutes and 100,000 g for 1 hour at 4°C. Aliquots of the fractions were subjected to SDS-PAGE and western blot analysis with antibodies against the localization marker proteins shown to the left.

Gallwitz, 1997; Cao and Barlowe, 2000). Because Sly1ts turned out to be defective in binding to Sed5, we examined its intracellular localization by differential centrifugation. The lysate with myc-tagged Sly1, Sly1-20 or Sly1ts was separated into the membrane fractions, P10 or P100, and the soluble fraction S100. Sly1, Sed5, Scs2 [an ER membrane protein (Kagiwada et al., 1998)], Van1 [a Golgi membrane protein (Hashimoto and Yoda, 1997)] and Pgk1 [a soluble cytosolic protein (Schneiter et al., 1999)] were similar. It is unlikely that this distribution was caused simply by contamination because Pgk1 was only detected in one fraction – S100. This result suggests that the yeast membrane has other factors than Sed5 to localize more than half of the total Sly1 protein on the membrane.

Sly1 was included in the Sed5-Bet1 SNARE complex

Using an in vitro assay, it was shown that a combination of the Q-SNAREs (Sed5, Bos1 and Sec22) and an R-SNARE (Bet1) constitute a potential minimum fusion machinery in the ER-Golgi transport, although other combinations may exist (McNew et al., 2000). Thus, it was important to see whether Sly1 binds only to free Sed5 or whether it also binds to Sed5 in the SNARE complex. We found that the sly1ts mutation is partially suppressed by introduction of multicyc BET1 (Kosodo et al., 2001). This observation suggests that Bet1 may have an important role among v-SNAREs, in concert with Sly1. Therefore, we focused our study on the interaction with Bet1 and Sly1. First, we examined whether Sly1 is in the Bet1-Sed5 v-t-SNARE complex in the normal yeast cells. The 20 S complex found in the sec18-1 cell at the nonpermissive temperature contained Sed5, Bet1 and Sly1 proteins as well as Bos1 and Sec17. In case of exocytosis in neuron, it was shown that v-SNARE(VAMP2) was excluded from the n-Sec1(Munc-18)/t-SNARE(syntaxin1) complex (Pevsner et al., 1994).

The genomic SLY1 was replaced with SLY1-6myc in the wild-type strain. The cells grew well without any detectable defect. The lysate was prepared from them, with 1% Triton X-100 added to solubilize the membrane, and then divided into three parts. Immunoprecipitation was performed using an anti-myc monoclonal antibody, an affinity-purified polyclonal anti-Sed5 antibody or, as a control, preimmune serum from the rabbit from which we raised the anti-Sed5 antiserum. As shown in Fig. 3, Bet1 was detected in the immunoprecipitate of anti-Sed5 (lane 2). This indicates the presence of the Bet1-Sed5 SNARE complex in the lysate. Sly1-6myc was also detected in this immunoprecipitate. In accordance with this, both Bet1 and Sed5 were detected in the immunoprecipitate of Sly1-myc (lane 3). Because Sly1 does not bind to Bet1 directly, this indicates the presence of a complex containing these three proteins. Preimmune serum precipitated none of these proteins (lane 1). These results strongly suggest that Sly1 still binds to the Sed5-Bet1 SNARE complex. As the H1 helix of Sed5 binds to Sly1 and the H3 helix binds to Bet1 (Kosodo et al., 1998; Sacher et al., 1997), simultaneous binding of Sed5 to both Sly1 and Bet1 would be possible. This Bet1-Sed5 interaction was suggested to be susceptible to the action of dissociating ATPase, because Bet1 was not detected in similar experiments when 0.5 mM ATP was added to the immunoprecipitation mix (data not shown).

Fig. 3. Sly1 is included in the Bet1-Sed5 v-t-SNARE complex in vivo. The SLY1 gene of the wild-type strain W303 was substituted with an epitope-tagged SLY1-6myc. Cells incubated in YEPD medium at 30°C were lysed by vortexing with glass beads on ice. After adding 1% Triton X-100, the lysate was divided into three parts, and each was immunoprecipitated at 4°C by a monoclonal anti-myc antibody, by an affinity-purified polyclonal anti-Sed5 antibody or by the preimmune rabbit serum from which the anti-Sed5 antibody had arisen. Anti-myc, anti-Sed5 and anti-Bet1 antibodies were used to detect Sly1-6myc, Sed5 and Bet1 proteins in the immunoprecipitates (lanes 1-3) and one fifth of their supernatants (lanes 4-6). One fifth of starting lysate is shown in lane 7.
Almost all endogenous Sly1ts protein was recovered in S4 (data for 5 minutes and the supernatants (S4) were stocked at –80°C. Disrupted by vortexing with glass beads, centrifuged at 4000 g, induced in SG medium for 2 hours. The cells were harvested, transformed with a recombinant 6myc-SED5 plasmid (strain I) or with a 3HA-BET1 plasmid (strain II). Preparation of the yeast lysates and recombinant proteins are described in Materials and Methods. The reaction time was fixed at 20 minutes, but the reaction temperature was 0°C (lanes 1 and 2), 15°C (lanes 3 and 4), 25°C (lanes 5 and 6) or 35°C (lanes 7 and 8). At the end of the reaction, the mixture was diluted and solubilized with Triton X-100 and subjected to immunoprecipitation using an anti-myc antibody. Western blotting was done to detect 6myc-Sed5, 3HA-Bet1 and Sly1/Sly1ts-Strep and endogenous Sly1ts protein in lysate I and II. The bands of Sly1/Sly1ts-Strep, 6myc-Sed5 and 3HA-Bet1 are indicated by arrowheads. * indicates the endogenous Sly1ts protein in lysate I and II.

Sly1 stimulated the trans-SNARE complex formation between the membranes

As a complex containing Sly1, Sed5 and Bet1 was detected, Sly1 may somehow be involved in the formation of the Bet1-Sed5 SNARE complex. We constructed an in vitro assay system to evaluate the effect of Sly1 in this reaction. The lysate of the sly1ts mutant was selected as the basal condition, because Sly1 is an essential protein and its depletion by other means have obvious shortcomings. The sly1ts mutant was transformed either with 6myc-SED5 on a CEN plasmid (strain I) or with a GAL1p-3HA-BET1 construct on a 2μ plasmid (strain II). These strains were grown at 25°C, and the expression of 3HA-BET1 in strain II was induced in SG medium for 2 hours. The cells were harvested, disrupted by vortexing with glass beads, centrifuged at 4000 g for 5 minutes and the supernatants (S4) were stored at –80°C. Almost all endogenous Sly1ts protein was recovered in S4 (data not shown). Sly1 or Sly1ts protein with Strep tag at the C-terminus was produced in E. coli and purified by affinity resin to a single band in SDS-PAGE. The lysates of strains I and II were mixed and incubated with or without adding a purified recombinant protein. Then, the mixture was diluted 6.7-fold by BB8 buffer on ice, and Triton X-100 was added to 1% of the final concentration to dissolve the membranes. The monoclonal anti-myc antibody was used to precipitate 6myc-Sed5, and 3HA-Bet1, exogenous Sly1/Sly1ts-Strep and endogenous Sly1ts in the precipitate were detected by western blotting.

First, we measured the effect of temperature on the cell-free system. 0°C serves as a control for basal reactions. At 15°C, both wild-type and sly1ts strains grew slowly without apparent difference (data not shown). At 25°C, the wildtype grew slightly faster than the sly1ts mutant (data not shown). 35°C is a restrictive temperature for the sly1ts strain and the vesicle transport between ER-Golgi stops at this temperature (Mizuta and Warner, 1994). The reaction mix was kept at these temperatures for 20 minutes. At 0°C, no increase of the amount of Bet1 was found when either Sly1-Strep or Sly1ts-Strep was added (Fig. 4, lanes 1 and 2). At 15°C, a slight increase in the amount of 3HA-Bet1 was detected in the precipitates by adding either protein (Fig. 4, lanes 3 and 4). At 25°C, either protein gave a considerable increase, but Sly1-Strep had a stronger effect (Fig. 4, lanes 5 and 6). At 35°C, Sly1ts-Strep had little effect (Fig. 4, lanes 7 and 8). The activity of recombinant proteins in the assay system may have a good accordance with yeast growth.

The temperature dependence of the binding of 3HA-Bet1 to 6myc-Sed5 (Fig. 4) also indicates that the Bet1-Sed5 complex was formed during the incubation at each temperature and not in the later process after the dilution and addition of Triton X-100. When Triton X-100 was added before mixing the lysates, we could not find any increase in the amount of 3HA-Bet1 (data not shown).

We further evaluated the time course of the change in the amount of coprecipitated proteins after the temperature shift from 0°C to 35°C. During the incubation at 35°C, the amount of coprecipitated 3HA-Bet1 clearly increased by adding Sly1-Strep protein to the mixture (Fig. 5A, lanes 1-3). Addition of the same amount of Sly1ts-Strep did not give such an increase (Fig. 5A, lanes 4-6). The exogenous Sly1-Strep protein was found in the precipitates, but Sly1ts-Strep was hardly detected in them (lanes 1-6). We detected a faint band of endogenous Sly1ts protein in all lanes (compare with Fig. 4). Most of the endogenous Sly1ts protein bound to 6myc-Sed5 before cell disruption was not released in the present condition and might prevent exogenous Sly1ts-Strep from replacing its position.

The amount of protein in the immunoprecipitate was measured using a luminoimage analyzer. At 0°C, no difference was found in the amount of 3HA-Bet1 irrespective of whether Sly1-Strep or Sly1ts-Strep was added. The SNARE-mediated membrane fusion was temperature dependent and did not occur at 0°C (Weber et al., 1998). At 35°C, the increased amount of 3HA-Bet1 with Sly1-Strep was 17 times of that with Sly1ts-Strep (Fig. 5B). These indicate that Sly1-Strep stimulated formation of the Bet1-Sed5 trans-SNARE complex in a time- and temperature-dependent manner.

Next, we addressed the question of whether the trans-SNARE complex is formed from the cis-SNARE complex or whether its dissociation by NSF/Sec18 ATPase is required to prime complex formation. If 1 mM ATP was added into the reaction mix, the amount of coprecipitable 3HA-Bet1 decreased (Fig. 6, lanes 2 and 6), and it was almost under the limit of detection in the case of Sly1ts-Strep. This indicates that ATPase Sec18 that dissociates the cis-SNARE complex was active in our reaction mix. If the unhydrolyzable analog AMPPNP was added instead of ATP, the result was the same as the result without its addition (compare lane 1 with 3, and lane 5 with 7). This indicates that trans-SNARE complex formation in our system did not require ATP hydrolysis. However, if the lysate was incubated in the presence of ATP with Sly1-Strep or Sly1ts-Strep and then AMPPNP was added, the amount of 3HA-Bet1 significantly increased (lane 4 or 8). This result indicates that the dissociated SNAREs present at the time of lysate preparation participated in the formation of...
the trans-SNARE complex under the standard conditions. Probably ATP in the lysate was soon exhausted by reactions that consumed ATP. We guess that the pre-incubation of lysate with ATP increased the amount of dissociated SNAREs by the action of the NSF/Sec18 ATPase and thus the amount of the newly formed SNARE complex increased. AMPPNP probably inhibited further action of Sec18 ATPase, which would also dissociate the newly formed SNARE complex after vesicle fusion. We conclude that dissociation of pre-formed SNARE complex is also a prerequisite for trans-SNARE complex formation in the presence of Sly1 protein.

Discussion

In this report, we have demonstrated that less Sly1ts protein binds to Sed5 than wild-type Sly1, and this is because of the reduced binding affinity of the mutant Sly1ts protein for Sed5. We have also shown that Sly1 is included in a SNARE complex containing Bet1 and Sed5 in vivo. Finally, we have shown that addition of purified Sly1 enhances the formation of the trans-SNARE complex in a cell-free system. This is the first demonstration that Sly1 stimulates SNARE complex formation.

Sly1 and the other membrane fusion machinery

GLUT4 vesicle trafficking is abolished at the restrictive temperature in a 3T3L1 mutant cell that has the same point mutation in Munc18c as the yeast sly1ts allele (Thurmond and Pessin, 2000). Thurmond and Pessin showed the mutant Munc18c had a reduced affinity for the t-SNARE syntaxin 4. On the basis of our results and their report, we conclude that a single amino-acid substitution R266K in Sly1 renders its binding activity to t-SNARE much lower and temperature labile, and consequently vesicle transport is abolished at a nonpermissive temperature. Therefore, the binding of the Sec1 family protein to t-SNARE would be necessary to form the correct SNARE complex.

Sly1 is one of the Sec1 family proteins in S. cerevisiae. The Sec1 family proteins have long been implied as regulators of the membrane fusion process in vesicular transport because of their high affinity for t-SNAREs that trigger membrane fusion (Halachmi and Lev, 1996; Nichols and Pelham, 1998). The Munc18/n-Sec1 protein was first identified and purified from mammalian brain owing to its high binding affinity to the t-SNARE syntaxin 1 (Hata et al., 1993). Munc18 was thought to be a negative regulator of membrane fusion, since it prevented
synaptobrevin and SNAP from binding to syntaxin 1 (Pevsner et al., 1994). However, the negative regulator model does not fully explain why the protein is essential for neurotransmitter release and intracellular vesicle transport (Ossig et al., 1991; Verhage et al., 2000). By contrast, it has also been proposed that the Sec1 family proteins have a positive or chaperone-like role rather than a negative role (Grabowski and Gallwitz, 1997; Yang et al., 2000; Jahn, 2000). Our results strongly suggest that Sly1, at least, has a positive role in SNARE complex formation, which is followed by membrane fusion.

We have previously reported that Sly1 had no effect on binding of Bet1 to Sed5 using recombinant protein without a transmembrane domain (Kosodo et al., 1998) (K.Y., N.Y., A.H. and Y.K., unpublished). Recently, it has been reported that the transmembrane domain of syntaxin 1 is critical for its proper interaction with other proteins (Lewis et al., 2001). Actually, Sly1 did not have an effect on the binding after membrane solubilization by Triton X-100 in our cell free system (data not shown). The lipid bilayer and transmembrane domain of SNAREs therefore are probably indispensable for the correct SNARE complex formation and functional role of Sly1. To confirm this, we have shown for the first time the direct role of Sec1/Munc18 family in trans-SNARE complex assembly by a novel in vitro assay system using recombinant Sly1 proteins and intact SNAREs with a transmembrane domain embedded in the membrane in the yeast lysate. This method will be applied to address the role of other fusion-mediating proteins in yeast ER-Golgi vesicle transport such as Uso1, Ypt1, Yip1, and Y.K., unpublished). Recently, it has been reported that the Sec1 family may function at two different stages. The Sec-1 family may function at two different stages. The

The Sec-1 family may function at two different stages. The Sec1 family may function at two different stages. The Sec1 family protein may act upon t-SNAREs before SNARE complex formation followed by the interaction of v-SNARE and t-SNARE. In this case, the Sec1 family protein would change or fix t-SNAREs into an effective conformation. The other is that the Sec1 family protein may bind to t-SNARE after SNARE complex formation, as is the case in yeast exocytosis. It has been reported that yeast Sec1 does not bind to a single Sso1 molecule, a t-SNARE of plasma membrane, but binds to the v-t-SNARE complex (Grote et al., 2000). We suggest that Sly1 acts before SNARE complex formation because the amount of Sly1 binding to Sed5 is almost the same before and after SNARE complex formation in our cell-free assay system (Fig. 5A, lane 1 or 3). Probably, Sly1 binds to Sed5 before SNARE complex formation, which would, consequently, induce Sed5 into an efficient binding state for Bet1.

SNAREs themselves are the minimal fusion machinery, and trans-SNARE complex formation drives membrane fusion. Thus formed cis-SNARE complexes must be dissociated by the action of NSF/Sec18 ATPase in combination with SNAP/Sec17 before they are used in the next round of membrane fusion. Our data (Fig. 6) implies that the dissociation of cis-SNARE complex is a pre-requisite for stimulation of trans-SNARE complex assembly by Sly1.

SNAREs are considered to be in a cycle of assembly and disassembly in the cell. Solubilization of membrane by detergent stopped trans-SNARE complex assembly, although disassembly could occur if ATP was supplied. The amount of Bet1-Sed5 complex reflects the amount of SNARE complex present at the time of the detergent extraction. During this study, we found that the amount of Bet1 in the Sed5 immunoprecipitate was significantly less when Sly1 is fully active than when it is less active [the sly1ts mutant was compared with the parent, and the sly1ts/vector was compared with the sly1ts/SLY1 plasmid (K.Y., N.Y., A.H. and Y.K., unpublished)]. Apparently, the amount of Bet1-Sed5 complex in detergent extract decreases when Sly1 is more active. So, Sly1 may stimulate disassembly of the cis-SNARE complex by the action of NSF/Sec18 ATPase as well as the assembly of trans-SNARE complex in the presence of the membrane. To examine this biochemically, it will be necessary to develop a cell-free system in which SNAREs are at a specific stage of the assembly/disassembly cycle so that the reaction can be started simultaneously. Genetic data may support the idea that Sly1 may be involved in both assembly and disassembly of SNARE complex. Synthtic lethality of mutations generally suggests that processes that the mutant gene products concern are closely related. When the sec18-1 and sly1ts mutants were crossed and the progeny was processed for tetrad analysis, the cells that have both mutant alleles did not grow even at the permissive temperature for each single mutation (K.Y., N.Y., A.H. and Y.K.). Thus, the mutant alleles sec18-1 and sly1ts were synthetic lethal.

**Role of Sec1 family proteins at the membrane fusion step**

Bryant and James recently reported that Vps45, one of the yeast Sec1-family proteins, is essential for ternary SNARE complex formation (Bryant and James, 2001). During vesicle transport between the late Golgi and the endosome, the t-SNARE Tlg2 and its binding partners Tlg1 and Vti1 form a ternary complex. In the absence of Vps45, which binds to Tlg2,
Tlg2 suffers rapid degradation, and even if the degradation is prevented through abolition of proteasome activity Tlg2 cannot form a ternary complex. Bryant and James suggest that Sec1 family proteins have chaperone-like activity on t-SNAREs and play an essential role in the activation process that allows t-SNARE to participate in ternary complex formation. Although their observations are mainly made on the basis of immunoprecipitation from cell lysates of null mutants, they are essentially consistent with our results in that Sec1 family proteins enhance ternary SNARE complex formation.

There are four Sec1 family genes in S. cerevisiae (Aalto et al., 1992). Sec1 acts in vesicular transport to the plasma membrane, Vps33 or Vps45 to the vacuole or endosome and Syl1 to the Golgi apparatus (Halachmi and Lev, 1996). Although Sec1 family proteins have no transmembrane domain, a part of them localizes on the organelle membranes as a part of protein complexes. Vps33 is a component of ‘C-Vps complex’ on the vacuolar membrane with Vps11, Vps16, Vps18, Vps39 and Vps41 (Wurmser et al., 2000). Vps45 interacts with Vps21 and Vac1 and localizes on the endosomal membrane (Tall et al., 1999). As shown in Fig. 2, in spite of different binding affinities for Sed5, both Syl1 and Syl116, Sec1 proteins similarly localize on the membrane. This suggests that Syl1 localization is not only dependent on Sed5, but also that other factors have a crucial role. Syl1 might be a part of a certain protein complex, as other Sec1 family proteins are, and might settle on Golgi membrane through this complex.

Our findings with Syl1 and observations of other investigators suggest that Sec1 family proteins generally localize on the target membranes independently from the SNARE proteins that they bind to. SNARE proteins cycle between organelle membranes. For example, Sed5 mainly localizes on the Golgi membrane, but it cycles between the ER and Golgi membranes (Wooding et al., 1998; Cho et al., 2000). There are probably some mechanisms to induce SNARE-dependent membrane fusion and to mix the content of cargo exactly on the Golgi membrane. When Syl1 regulates trans-SNARE complex formation as we have described, Syl1 should localize on the area where membrane fusion is expected to occur. In other words, the area of membrane fusion would not be determined without the correct localization of Sec1 family proteins on the proper membranes.

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