Both Svp26 and Mnn6 are required for the efficient ER exit of Mnn4 in Saccharomyces cerevisiae

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Incorporation of membrane and secretory proteins into COPII vesicles are facilitated either by the direct interaction of cargo proteins with COPII coat proteins, or by ER exit adaptor proteins which mediate the interaction of cargo proteins with COPII coat proteins. Svp26 is one of the ER exit adaptor proteins in the yeast Saccharomyces cerevisiae. The ER exit of several type II membrane proteins have been reported to be facilitated by Svp26. We demonstrate here that the efficient incorporation of Mnn4, a type II membrane protein required for mannose phosphate transfer to glycoprotein-linked oligosaccharides, into COPII vesicles is also dependent on the function of Svp26. We show that Mnn4 is localized to the Golgi. In addition to Mnn4, Mnn6 is known to be also required for the transfer of mannose phosphate to the glycans. We show, by indirect immunofluorescence, that Mnn6 localizes to the ER. As in the case with Svp26, deletion of the MNN6 gene results in the accumulation of Mnn4 in ER. In vitro COPII vesicle budding assays show that Svp26 and Mnn6 facilitate the incorporation of Mnn4 into COPII vesicles. In contrast to Svp26, which is itself efficiently captured into the COPII vesicles, Mnn6 was not incorporated into the COPII vesicles. Mnn4 and Mnn6 have the DXD motif which is often found in the many glycosyltransferases and functions to coordinate a divalent cation essential for the reaction. Alcian blue dye binding assay shows that substitution of the first D in this motif present in Mnn4 by A impairs the Mnn4 function. In contrast, amino acid substitutions in DXD motifs present in Mnn6 did not affect the function of Mnn6. These results suggest that Mnn4 may be directly involved in the mannosyl phosphate transfer reaction.

Key Words: endoplasmic reticulum; Golgi; mannosyl phosphate; membrane traffic; Saccharomyces cerevisiae

Introduction

N-glycans are important for the folding process of newly synthesized proteins and for their solubility and stability (Aebi et al., 2010). At the cell surface where glycosylated proteins are exposed to the cell exterior, they have a protective function and an important role in recognition by other cells. S. cerevisiae has two types of N-glycans (Munro, 2001). ‘Core type’ is composed of a structure built in the ER and a few mannoses extended at the early Golgi and is found on the proteins destined for the internal organelles. ER type glycans is further elongated with α-1,6-linked mannoses by the sequential actions of Och1, mannan-polymerase I (M-Pol I) and mannan-polymerase II (M-Pol II) protein complexes to build a large ‘mannan’ structure (Stolz and Munro, 2002). This long α-1,6-linked backbone is further branched with α-1,2-mannoses by Mnn2 and Mnn5. (Rayner and Munro, 1998) and α-1,3-mannoses by Mnn1 (Romero et al., 1999; Wiggins and Munro, 1998). Mannan is usually found in the cell wall proteins and secreted proteins. A mechanism by which one of these two types of glycan is selectively added to a certain protein is not yet fully understood. To some of the
side chains of yeast mannan, a phosphomannose is attached by the inherited mutations in the genes encoding the lysosomal enzymes which normally degrade glycans, is important not only to advance our basic knowledge about the role of N-glycans in yeast but also to accelerate the development of clinically applicable reagents.

In this paper, we show that Mnn4 is a Golgi-localized protein and its ER exit is dependent on Svp26 (Noda and Yoda, 2010). Meanwhile, it was reported that Mnn6 has a positive regulatory role in the phosphomannosyl transfer (Odani et al., 1997). However, Mnn4 has a homologous domain to several enzymes known to utilize a nucleotide sugar as a substrate to transfer sugar-phosphate and has the DXD signature sequence found in many glycosyltransferases (Aravind and Kooning, 1999; Corbacho et al., 2005). So, the precise individual roles of Mnn4 and Mnn6 may not be fully understood.

Lysosomal storage diseases such as GM2-gangliosidosis are caused by the inherited mutations in the genes encoding the lysosomal enzymes which normally degrade glycolipids, glycoproteins, or glycolgen in the lysosome. It has been demonstrated that enzyme replacement therapy, in which an externally administered recombinant enzyme is aimed to replace the deficient enzyme and degrade the accumulated glycolipids in the lysosome of the patient, could be a promising treatment of such diseases (Chiba et al., 2002; Desnick and Schuchman, 2012). To achieve an efficient enzyme replacement therapy, a recombinant therapeutic enzyme is required to contain a high amount of phosphorylated N-glycan, as an enzyme needs to be endocytosed and delivered to the lysosome through interaction with the mannose 6-phosphate receptor of the target cells. An initial attempt by Chiba and Jigami revealed that a recombinant enzyme produced in yeast O. minuta contained only a low level of phosphorylated N-glycan (Akeboshi et al., 2007). They subsequently overexpressed the MNN4 gene cloned from O. minuta (OmMNN4) in the yeast producing the recombinant enzyme and found that the enzyme with increased phosphorylation in its N-glycans, as expected, showed the higher therapeutic potential when tested in the cultured patient fibroblasts (Akeboshi et al., 2009).

Therefore, studying the biological functions and localization mechanisms of Mnn4 and Mnn6, both of which are involved in the addition of mannosyl phosphate to N-glycans, is important not only to advance our basic knowledge about the role of N-glycans in yeast but also to accelerate the development of clinically applicable reagents.
Materials and Methods

**Strains, plasmids, media, and reagents.** *S. cerevisiae* strains used in this study are listed in Table 1. All strains are derivatives of KA31a. All DNA constructs were verified by DNA sequencing.

For the chromosomal tagging of *SVP26* with 6 tandem copies of myc epitope at its C-terminus, an appropriate DNA fragment of the 3'-region was amplified by PCR and cloned in pYN495 (*HIS3* marker). This plasmid carries a coding sequence of 6 myc followed by *TDH3* terminator. The sequences of primers used are available upon request. This resulting plasmid was linearized by cutting at a unique restriction site (MunI) within a cloned region of the *SVP26* ORF, and was transformed into the appropriate yeasts to obtain strains with chromosomally-tagged genes by homologous recombination. For the integration of *SVPAC2-6MYC* construct, an *SVP26* sequence, with a 195 nucleotides region from the 3'-end of its ORF deleted, was amplified and similarly subcloned in pYN495. The resulting plasmid was linearized by MunI digestion and tagging was similarly done by homologous recombination. Chromosomal tagging of *MNN6* with a single copy of FLAG sequence was similarly performed using pH1106 as an integration vector.

To create a 2µ plasmid expressing Mnn4-xGFP2, a DNA fragment containing a promoter region and the ORF of the *MNN4* was amplified by PCR, and the resulting product was ligated into the high-copy expression vector, which was created based on SGFP2-N (Suzuki et al., 2012) This plasmid, pYN883, was used to transform KA31a (wild-type), HIY22 (Δsyp26) or YNY1057 (Δmnn6) and the localization was observed by fluorescent microscopy as described.

For high-copy expression of *MNN6-MYC* from its own promoter, we created the plasmid pYN538 by subcloning the PCR-amplified fragment containing 5′-promoter regions and ORF of the *MNN6* gene in pKT10mycC (*URA3* marker). KA31a (wild-type) or HIY22 (Δsyp26) was transformed with pYN538 and the localization of Mnn6-myc was observed by indirect immunofluorescent microscopy using the anti-myc antibody.

Yeast cells were grown in YPD [1% Bacto yeast extract (BD Biosciences, Franklin Lakes, NJ, USA), 2% Bacto peptone (BD Biosciences), and 2% glucose] or SD [0.17% yeast nitrogen base without amino acids (BD Biosciences), 0.5% ammonium sulphate, 2% glucose, and appropriate supplements] medium at 30°C. *Escherichia coli* DH5 (F-, supE44 lacU169 80lacZD15 hsdR17 recA1 endA1 gyrA96 thi-1 relA1) was used in plasmid preparation. *E. coli* was grown in an LB [1% Bacto tryptone (BD Biosciences), 0.5% Bacto yeast extract (BD Biosciences) and 1% NaCl] medium. Protein A sepharose resin was purchased from GE Healthcare UK Ltd (UK). Digitonin, Chymostatin, aprotinin, leupeptin, pepstatin A, and antipain were purchased from MilliporeSigma (St. Louis, MO, USA). Phenylmethylsulfonyl fluoride (PMSF) was purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan).

**Preparation of the total cell lysate using trichloroacetic acid (TCA).** Preparation of the total cell lysate using TCA was performed as described with minor modifications (Cheong and Klionsky, 2008). Logarithmically-growing yeast culture (1.8 ml, OD₆₀₀ = ~1.0) was mixed with 108 µl of TCA and incubated on ice for 15 min. Precipitated proteins were pelleted at 15,000 × g for 10 min at 4°C. The pellet was suspended in 1 ml of cold 70% ethanol and stored at −80°C, usually overnight but could be stored for a longer period. When ready to proceed, the sample was centrifuged at 15,000 × g for 10 min at 4°C and the supernatant was discarded by decantation. The pelleted cells were resuspended in the 200 µl urea buffer (50 mM Tris, Cl, pH 7.5, 5 mM EDTA, 6 M urea, 1% SDS, 50 mM NaF) supplemented with 1 mM PMSF; and disrupted by vortex with 200 µl acid-washed glass beads for 5 min. The sample was then heated at 65°C for 10 min and insoluble debris was removed by centrifugation at 15,000 × g for 10 min at room temperature. The supernatant (90 µl) was mixed with 30 µl 4xSDS-PAGE sample buffer and heated at 65°C for 5 min.

**Antibodies, Immunoblotting and Indirect Immunofluorescence.** Rabbit antiseria against Erv46 and Mnn1 were gifts from Dr. Charles Barlowe (Dartmouth Medical School, USA) and Dr. Todd Graham (Vanderbilt University, USA), respectively. An anti-Tcm1 monoclonal antibody was a gift from Dr. Jonathan Warner (Albert Einstein College of Medicine, USA). Anti-Van1 antiseria was produced in our laboratory (Hashimoto and Yoda, 1997). The peptide CLVPGFSDLM, which corresponds to the C-terminal amino acid sequence of Sec61, was chemically synthesized and coupled to keyhole limpet hemocyanin (KLH). Two rabbits were injected with KLH-peptide conjugate to produce the anti-Sec61 antiserum. Anti-HA (12CA5) and anti-FLAG (M2) monoclonal antibodies were purchased from MilliporeSigma (St. Louis, MO, USA). Anti-myc monoclonal antibody (9E10) was purchased from Berkeley Antibody. For immunoblotting, each antibody was used at a dilution of 1/1000. The intracellular localizations of myc-tagged proteins were observed by indirect immunofluorescence as previously described (Noda and Yoda, 2010). Anti-myc mouse monoclonal antibody was used as a primary antibody. Alexa 488-conjugated goat antibody to mouse immunoglobulin G (Thermo Fisher Scientific, Waltham, MA, USA) was used as a secondary antibody.

**Fluorescence microscopy.** Exponentially growing yeast cells were fixed for 15 min in the phosphate buffer (pH 7.5) containing 3.6% (w/v) paraformaldehyde, and washed 3× with the phosphate buffer. Fluorescence confocal images were captured with an A1R microscope, using a ×100 oil-immersion objective (numeric aperture 1.49) (Nikon Instech, Tokyo, Japan).

**Subcellular fractionation.** Subcellular fractionation in a sucrose density gradient was performed basically as described previously (Noda and Yoda, 2010). Briefly, cell lysates were prepared by suspending frozen yeast spheroplasts corresponding to the 25 OD unit cells by several strokes in a Dounce homogenizer in an ice-cold sucrose solution (10 mM HEPES, 12.5% (w/v) sucrose, 1 mM EDTA, pH 7.4 containing protease inhibitors (1 µg/ml each of chymostatin, aprotinin, leupeptin, pepstatin A,
antipain, 1 mM benzamidine and 1 mM phenylmethylsulfonyl fluoride), and unlysed cells were removed by a centrifugation at 400 × g for 3 min. An aliquot of the supernatant was diluted in a 2% SDS solution and absorbance at 280 nm was measured. After diluting the supernatant to A$_{280}$ = 0.45, 0.2 ml was loaded onto a sucrose step gradient, which was generated using the following steps [all sucrose solutions were made (w/v, %) in 10 mM HEPES-KOH, pH 7.4, 1 mM MgCl$_2$]: 0.5 ml 60%, 1.25 ml 48%, and 0.25 ml 18% sucrose. After 2.5 h of centrifugation in a Beckman TLS55 rotor at 100,000 × g, 6 fractions of 0.35 ml were collected from the top of the gradient. Aliquots of each fraction were mixed with an SDS sample buffer, and proteins were resolved by SDS-PAGE and detected by immunoblotting using anti-HA, anti-Van1 and anti-Sec61 antibodies. Bands were visualized using the LI-COR Odyssey system and subsequently analyzed with LI-COR Odyssey software. Data represent the mean and SD of triplicate experiments.

In vitro COPII vesicle generation assay. Purification of COPII coat components Sar1, Sec23/24 and Sec13/31, and the vesicle budding assay were performed as previously described (Noda and Yoda, 2010; Shimoni and Schekman, 2002). Microsomal membranes were prepared from YNY1055 (MNN4-3HA::LEU2 SVP26), YNY1059 (MNN4-3HA::LEU2 Δsvp26), and YNY1168 (MNN4-3HA::LEU2 Δmnn6), and YNY1164 (MNN4-3HA::LEU2 Δsvp26 Δmnn6), were used as donor membranes in the budding reactions and incubated either in the absence or presence of purified COPII coat components. For the measurement of Mnn6-HA packaging efficiency, microsomes from THY25 (Mnn6-HA::LEU2) were used. After the reaction, samples were separated by SDS-PAGE followed by immunoblotting anti-HA, anti-Erv46 (a positive control), and anti-Sec61 (a negative control) and packaging efficiency was quantified from band intensities in a total membrane fraction and in an MSS (medium speed supernatant) fraction, in which COPII vesicles generated in a reaction were found, using the LI-COR Odyssey system. Because western signals of Mnn4-3HA in this assay were variable between experiments, which is a problem often associated with high molecular weight proteins, for the detection of Mnn4-3HA signals, samples were applied to the membrane by dot blotting. Briefly, fractions generated in the in vitro COPII vesicle generation assay were concentrated by vacuum centrifuge, and then spotted onto a nitrocellulose membrane (PALL, 0.2 μM). The membrane was processed for immunoblotting with the anti-HA antibody following the normal western blotting procedure. Samples derived from the assay using the microsomes of the wild-type strain were spotted onto the same nitrocellulose membrane, and their signals were subtracted as backgrounds from those of the Mnn4-3HA samples, and packaging efficiencies were calculated. The signal obtained in the negative control reactions (–COPII components) was subtracted from the signal found in COPII vesicle fractions (+COPII components) and then the incorporation efficiencies of Mnn6-HA or Mnn4-3HA normalized to those of Erv46 (Otte et al., 2001) were calculated and graphed. Averages from 3 independent experiments were plotted with standard deviations.

Cell lysates derived from the wild-type (YNY1055), Δsvp26 (YNY1059), Δmnn6 (YNY1168) and Δsvp26 Δmnn6 (YNY1164) strains were prepared using TCA as described in the section Materials and Methods. They were analyzed by SDS-PAGE and immunoblotting with anti-HA monoclonal antibody. Tcml was also detected as an internal control using an anti-Tcm1 monoclonal antibody. Bands were visualized using the LI-COR Odyssey system and subsequently analyzed with LI-COR Odyssey software. The migration of molecular weight markers is indicated at the side of the blots.

Co-immunoprecipitation. Prior to the start of the experiment, a 5% digitonin stock solution was prepared by adding 50 mg of digitonin to 1 ml of water, which was then heated at 95°C for 10 min, as described in Anderson et al. (2017).

Logarithmically-growing yeast cells (100 OD$_{600}$) units) grown in YPD were collected, washed in water, and resuspended in 750 μl B88 buffer (20 mM HEPES, 150 mM potassium acetate, 5 mM magnesium acetate, 250 mM sorbitol, pH 6.8) supplemented with protease inhibitors. Cell lysates were prepared by rigorously agitating with glass beads using a Multi-beads shocker (Yasui Kikai, Osaka, Japan) three times for 1 min with 1-min intervals at 4°C between each burst and the supernatant was recovered. The remaining beads were washed in 550 μl B88 buffer supplemented with protease inhibitors and combined with the initial supernatant. Unbroken cells were removed by centrifugation at 4,000 × g for 5 min. The supernatant (900 μl) was mixed with 100 μl of 5% (w/v) digitonin (final 0.5%) and kept on ice for 4 h. Unsolubilized material was next removed by centrifugation at 100,000 × g for 45 min. A small portion of the clarified lysates was transferred to new tubes as ‘Input’ fractions. The remaining clarified lysates were incubated with anti-FLAG antibody or anti-myc antibody for 30 min at 4°C with gentle rotation. Then, Protein A Sepharose beads (10 μl bed volume/sample) were added and incu-
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bated o/n at 4°C with gentle rotation. After the brief centrifugation, a small portion of the supernatant was transferred to new tubes as ‘Unbound’ fractions. Sedimented beads were washed four times with B88 containing 0.5% digitonin and bound proteins were eluted by heating in 20 μl of 1× SDS sample buffer [0.125 M Tris-HCl, pH 6.8, 1% SDS, 10% glycerol, 5% (v/v) 2-mercaptoethanol] at 95°C for 2 min. The Input and Unbound fractions were mixed with 4× SDS sample buffer [0.5 M Tris-HCl, pH 6.8, 4% SDS, 40% glycerol, 20% (v/v) 2-mercaptoethanol] and heated at 65°C for 5 min prior to SDS-PAGE.

**Alcian blue dye binding experiment.** The Alcian blue dye binding experiment was performed basically as described in Ballou (1990). Fully grown overnight culture (5 ml) in an appropriate medium was centrifuged and pelleted cells were washed once in 0.5 ml of water. Cells were then suspended in 0.5 ml of 0.1% alcian blue solution, which was prepared by 10× dilution of 1% alcian blue in 3% acetic acid (Fujifilm Wako Pure Chemical Corporation, Japan) with 0.02 N HCl. The suspension was allowed to stand at room temperature for 15 min. Cells were collected by brief centrifugation and washed 3 times with 0.02 N HCl. Then the color of the cell pellets was visually compared with that of the positive control (wild-type, blue) and the negative control (Δmnn4, white), and the effects of amino acid substitutions in DXD motifs on the functions of Mnn4 and Mnn6 were determined.

**Results and Discussion**

**Deletion of either the SVP26 gene or the MNN6 gene leads to the accumulation of Mnn4 in the ER**

Mnn6 belongs to the Kre2 family proteins, and, unlike other members of the Kre2 family which localize to the Golgi, probably localizes to the ER as previously suggested by our sucrose density fractionation experiment (Noda et al., 2014). It has been shown that in addition to Mnn6, Mnn4, which is probably also a type II membrane protein, is required for mannosyl phosphate transfer to the glycans (Odani et al., 1996). Although Mnn4 has been reported to function as a putative positive regulator of the Mnn6 protein (Odani et al., 1997), neither its subcellular
The precise molecular function of Svp26 has not been clearly established as far as a search of the literature revealed. Because Svp26 functions as an ER exit adaptor protein for Golgi mannosyltransferases involved in either O-glycosylation or N-glycosylation (Anand et al., 2009; Bue et al., 2006; Inadome et al., 2005; Noda and Yoda, 2010; Noda et al., 2014), we considered the possibility that Svp26 is involved also in the ER exit of Mnn4. We first examined if deletion of the SVP26 gene or the MNN6 gene affects the localization of Mnn4. Figure 1 shows a western blot of Mnn4-3HA in the total cell lysates of the wild type, Δsvp26, Δmnn6 and Δsvp26Δmnn6 strains. A faster-migrating band of Mnn4 in SDS-PAGE became more abundant when SVP26, MNN6, or both, were deleted than Mnn4 in the wild-type cells. We predicted that this increased mobility was probably due to a decrease in the size of N-glycans and indicated an impaired exit of Mnn4 from the ER in these strains. By Endoglycosidase H treatment, the difference in their electrophoretic mobilities disappeared and they co-migrated more rapidly than the position of the fast-migrating band of untreated Mnn4 in Δsvp26, Δmnn6 and Δsvp26Δmnn6 strains. A faster-migrating band of Mnn4 in SDS-PAGE became more abundant when SVP26, MNN6, or both, were deleted than Mnn4 in the wild-type cells. We predicted that this increased mobility was probably due to a decrease in the size of N-glycans and indicated an impaired exit of Mnn4 from the ER in these strains. By Endoglycosidase H treatment, the difference in their electrophoretic mobilities disappeared and they co-migrated more rapidly than the position of the fast-migrating band of untreated Mnn4 in Δsvp26 cells (data not shown), supporting our prediction. To further confirm this possibility, we examined the distribution of Mnn4-3HA by subcellular fractionation on sucrose density gradients. In wild-type cells, Mnn4-3HA showed a typical distribution pattern of a Golgi-localized protein as was shown by the Golgi marker Van1 (Fig. 2). When MNN6 or SVP26 was deleted, although Golgi distribution of Mnn4-3HA was still observed, more Mnn4-3HA was co-fractionated with the ER marker Sec61. When both MNN6 and SVP26 were deleted, distribution of Mnn4-3HA shifted very close to that of Sec61. These results indicate that both Mnn6 and Svp26 are required for the Golgi-localization of the Mnn4 protein.

To further examine the subcellular localization of Mnn4, we microscopically analyzed the localization of Mnn4. As seen in Fig. 3, Mnn4-sGFP2 was found as a dot-like structure in the wild-type. In contrast, in Δsvp26 strain, Mnn4-sGFP2 showed ring-like and cortical staining, a typical pattern obtained with ER-localized proteins, with some Golgi dot signals in Δsvp26 cells. Localization of Mnn6-myc was also observed by indirect immunofluorescent microscopy. Mnn6-myc showed an image of ER-localized proteins, both in the wild-type and Δsvp26 cells, which is consistent with our previous result shown by sucrose density fractionation (Noda et al., 2014).

**In vitro COPII vesicle generation assay**

Accumulation of the Golgi membrane protein in the ER suggests the possibility that the incorporation of Mnn4 into the COPII vesicle is decreased in these deletion mutants. To directly measure COPII packaging efficiency, in vitro COPII budding assay was performed using ER-enriched membranes prepared from the wild-type, Δsvp26, Δmnn6,
Both Svp26 and Mnn6 are required for the efficient ER exit of Mnn4 in *Saccharomyces cerevisiae*.

**Fig. 4.** *In vitro* COPII vesicle generation assays using membranes from the wild-type, Δsvp26, Δmnn6, or Δsvp26 Δmnn6 cells. The ER-enriched membrane fractions prepared from the indicated strains were incubated in the presence of purified COPII coat components and the incorporation of Mnn4-3HA into the COPII vesicles was analyzed by immunoblotting. Percentages of individual proteins packaged into vesicles from a total reaction were determined from signal intensities of immunoblots and graphed. Averages from 3 independent experiments were plotted with standard deviations.

...and Δsvp26 Δmnn6 strains. As Mnn4 is a relatively large protein (139 K) and transferring the protein from the gel to the PVDF membrane by a regular blotting apparatus yielded experiment-to-experiment variations in western signal intensities, samples for the detection of Mnn4-3HA in the COPII budding assay were directly spotted onto the nitrocellulose membranes, which were subsequently processed for the detection by anti-HA antibody. As can be seen in Fig 4, compared with the wild-type, when microsomal membranes were prepared from the Δsvp26 and Δmnn6 strains, packaging of Mnn4 into the COPII vesicles was severely impaired. When the membrane from the Δsvp26 Δmnn6 strain was used, the packaging efficiency was almost as low as that obtained when Δmnn6 membrane was used.

These results suggest that Svp26 and Mnn6 function to facilitate the packaging of Mnn4 into the COPII vesicles. Although Svp26 was reported to be incorporated into the COPII vesicle efficiently probably with its clients, it was unknown whether Mnn6, an ER-resident protein, functions at the ER without ever leaving the ER, or whether it leaves the ER during the process of assisting the exit of Mnn4 out of the ER. *In vitro* COPII vesicle generation assay demonstrated that the packaging efficiency of Mnn6 normalized to that of Erv46 was 0.0076 (n = 3), which is even lower than that of Sec61 (0.0294, n=3), a negative control of packaging. This result indicates that, unlike Svp26, Mnn6 exclusively stays, and functions, in the ER.

**Co-immunoprecipitation experiments of Mnn4 with Mnn6 or Svp26**

Next, to test the possible interaction of Mnn4 with Svp26, co-immunoprecipitation experiments were similarly performed from strains expressing Mnn4-3HA and Svp26-FLAG. From digitonin-solubilized membranes, Mnn4-3HA was not co-immunoprecipitated with Svp26-FLAG, either in the presence or absence of Mnn6 (data not shown). Deletion of the C-terminal tail region of Svp26 (Svp26ΔC2) was reported to lead to reduced packaging efficiency of itself into the COPII vesicles (Bue and Barlowe, 2009). We hypothesized that this may allow more chance or time for Svp26 to interact with Mnn4, thus facilitating the detection of the interaction between these proteins. As expected, Mnn4 was co-immunoprecipitated with Svp26ΔC2-6myc (Fig. 5). Mnn1, a Golgi-localized membrane protein whose ER exit is not dependent on Svp26 (Noda and Yoda, 2010, see also the Introduction), was not present in the immunoprecipitates, supporting the specificity of the co-immunoprecipitation. Ktr3, a known cargo of Svp26, was also co-immunoprecipitated better with Svp26ΔC2-6myc than with Svp26-6myc (data not shown). These results further support the function of Svp26 as an ER-exit adaptor of Mnn4.

**Mnn4 is co-immunoprecipitated with Mnn6 only in the absence of Svp26**

We next tested whether Mnn4 interacted with Mnn6 by co-immunoprecipitation experiments. Whole cell extracts were solubilized with 0.5% digitonin and a C-terminally FLAG-tagged version of Mnn6 was immunoprecipitated with anti-FLAG immunoaffinity resin. A C-terminally 3xHA tagged version of Mnn4 was co-immunoprecipitated with Mnn6 only in the Δsvp26 background (Fig. 6). Mnn1, a Golgi-localized membrane protein, was not present in the immunoprecipitates, supporting the specificity of the co-immunoprecipitation of Mnn4 with Mnn6.

**Amino acid substitutions in the DXD motifs in Mnn4 and Mnn6**

Although Mnn4 has been reported to be a putative positive regulator of Mnn6 (Odani et al., 1997), Mnn4 has an Asp-X-Asp (DXD) motif which is found in most glycosyltransferases and is used to coordinate a divalent cation and/or a ribose. Amino acid substitutions in this motif often result in the loss of the transferase activity. So we tested if the mutation of first Asp to Ala would influence the function of Mnn4. The function of Mnn4 was assessed by the degree of affinity of the cationic dye, alcian blue. In the process of creating the Δmnn4 strain, we noticed that our wild-type strain showed an affinity for alcian blue. In the wild-type strain used in our lab as a standard strain, KA31a, has two copies of the MNN4 gene (YNY1130) exhibited a clear reduction of the transferase activity. So we tested if the mutation of first Asp to Ala would influence the function of Mnn4. The function of Mnn4 was assessed by the degree of affinity of the cationic dye, alcian blue. In the process of creating the Δmnn4 strain, we noticed that our wild-type strain showed an affinity for alcian blue. In the wild-type strain used in our lab as a standard strain, KA31a, has two copies of the MNN4 gene (YNY1130) exhibited a clear reduction of the affinity to alcian blue. In the wild-type strain, KA31a, has two copies of the MNN4 gene. Deletion of the single copy of the MNN4 gene (YNY1115) retained dye binding at the same level with the wild-type cells, but simultaneous deletion of both copies of the MNN4 genes (YNY1130) exhibited a clear reduction of the affinity to alcian blue (Table 2). Δmnn6 (YNY1057) cells also completely lost dye binding activity. In contrast to this result, the Δsvp26 cells showed an alcian blue affinity indistinguishable from that shown by the wild-type, indicating that despite the localization shift of Mnn4 from the Golgi to the ER in the Δsvp26 cells, mannosyl phos-
Fig. 5. Co-immunoprecipitation of Mnn4-3HA with Svp26ΔC2-6myc.

Results using strains co-expressing Mnn4-3HA with either non-tagged Svp26 (a negative control), endogenously C-terminally 6myc-tagged Svp26, or endogenously C-terminally 6myc-tagged Svp26ΔC2, were shown. The total cell lysates of these strains were prepared by agitation with glass beads and were solubilized with 0.5% digitonin. After centrifugation, Svp26-6myc or Svp26ΔC2-6myc was precipitated with anti-myc monoclonal antibody (9E10) from the supernatants. Proteins in the immunoprecipitates (B), in the input (I) and the unbound (U) were separated by SDS-PAGE and analyzed by immunoblotting with an anti-HA and anti-Mnn1 antibodies. Signals were detected using the LI-COR Odyssey system. Each input lane contains 2% of the total material used for the precipitation. The migration of molecular weight markers is indicated at the side of the blots.

Fig. 6. Co-immunoprecipitation of Mnn4-3HA with Mnn6-FLAG.

Results using strains co-expressing Mnn4-3HA with either non-tagged Mnn6 (a negative control) or endogenously C-terminally FLAG-tagged Mnn6, either in the wild-type (SVP26) or in the svp26 deletion strain (Δsvp26), were shown. The total cell lysates of these strains were prepared by agitation with glass beads and were solubilized with 0.5% digitonin. After centrifugation, Mnn6-FLAG was precipitated with anti-FLAG M2 antibody from the supernatants. Proteins in the immunoprecipitates (B), in the input (I) and the unbound (U) were separated by SDS-PAGE and analyzed by immunoblotting with an anti-HA and anti-Mnn1 antibodies. Signals were detected using the LI-COR Odyssey system. Each input lane contains 2% of the total material used for the precipitation. The migration of molecular weight markers is indicated at the side of the blots.

Mnn6 also has DGD and DIDHD, which corresponds to residues 219–221 and 260–264 of Mnn6, respectively. To determine whether the presence of DXD motifs of Mnn6 are required for its function, a plasmid encoding Mnn6D219A or Mnn6D262A was introduced into the Δmnn6 strain, and the functions of the mutant forms of Mnn6 were assessed by the alcian blue dye binding assay.
As shown in Table 2, the DXD motif mutants showed dye binding indistinguishable from that of the wild-type strain, indicating that the DXD motifs present in the sequence of Mnn6 are dispensable for its function related to mannosyl phosphate transfer. Among the samples tested in this experiment, the depth of the blue color was clearly divided into staining plus and staining minus and no intermediate staining was observed. Accordingly, the result was indicated as “+” or “–” in Table 2.

In this paper, we show that Mnn4 is a Golgi-localized protein and its ER exit is dependent on Svp26 and Mnn6. ER adaptor proteins in S. cerevisiae, including Svp26, facilitate ER exit of many proteins destined for the intracellular organelles or periplasmic space (Dancourt and Barlowe, 2010; Noda and Yoda, 2013). ER exit of the 7 proteins, Ktr3, Ktr1, Kre2, Mnn2, Mnn5, Pho8 and Gda1, has been previously reported to be dependent on Svp26 (Anand et al., 2009; Bue et al., 2006; Inadome et al., 2005; Noda and Yoda, 2010; Noda et al., 2014). In addition, we recently identified Mnt2 and Mnt3 proteins as new cargos of Svp26 (in press). Mnn4, as we report here, is the tenth cargo whose ER exit is promoted by Svp26. Moreover, we show that Mnn6 is an ER-localized protein and demonstrated by in vitro COPII vesicle generation assay that the efficiency of ER exit of Mnn6 is even lower than that of Sec61, which is widely used as a negative control in this assay. We also demonstrated by alcian blue dye binding assay that, while mutation in the DXD motif in Mnn4 impaired the function of Mnn4, the DXD motifs in Mnn6 are dispensable for its function.

It has been suggested that Mnn6 functions as a transferase of mannosyl phosphate and its activity is positively regulated by Mnn4 (Odani et al., 1997). However, as Mnn4 has a region (~130 amino acids) homologous to a set of proteins considered to function as transferases of sugar-phosphate and the DXD motif of Mnn4 is also conserved in the homologous region, it has been suggested that Mnn4 may function also as a transferase (Aravind and Kooning, 1999; Corbacho et al., 2005). Fukutin, which localizes to the Golgi and secretory granules, is a member of homologous proteins, including Mnn4, and it was reported that the mutations in the gene encoding fukutin could cause Fukuyama congenital muscular dystrophy (Kobayashi et al., 1998). It was recently reported that fukutin is an enzyme that transfers ribitol-5-phosphate to the glycan portion of dystroglycan using CDP-ribitol as a substrate and the mutation in its DXD motif completely diminished the enzymatic activity (Kanagawa et al., 2016).

Considering our study here, and reported functions of Fukutin family proteins, we propose a model in which Mnn4 functions in the Golgi as a mannosyl phosphate transferase and Mnn6 facilitates the ER exit of Mnn4. Although this model is not mutually exclusive from the previous model in which Mnn6 is a mannosyl phosphate transferase and Mnn4 is a positive regulator of Mnn6, as Mnn6 is exclusively ER localized, it seems likely that Mnn4 needs to function not only as a regulator but also as a transferase that adds mannosyl phosphate to the mannian side chains in the Golgi.

As to how Mnn6 and Svp26 facilitate the ER exit of Mnn4, two models can be imagined. The first possibility is that Mnn4 first interacts with Mnn6, and then is handed over to Svp26, which facilitates the incorporation of Mnn4 into the COPII vesicles. Co-immunoprecipitation of Mnn4 with Mnn6 in the absence of Svp26, but not in the presence of Svp26, seems to be consistent with this idea. Another possibility is that Svp26 and Mnn6 independently assist the ER exit of Mnn4 and this is consistent with the fact that no interaction was observed between Svp26 and Mnn6. As svp26 deletion and mnn6 deletion has an additive effect on the behavior of Mnn4, both in a fractionation experiment, it seems unlikely that only the former mechanism is operating in the ER exit of Mnn4. This implies that the latter or both mechanisms are probably functioning to promote the ER exit of Mnn4.

As previously reported, the deletion of MNN4 or MNN6 led to reduction or loss of mannosyl phosphate on the cell surface. In contrast, a Δsvp26 deletion mutant showed a

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Dye binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>KA31a</td>
<td>wild-type</td>
<td>+</td>
</tr>
<tr>
<td>YNY1130</td>
<td>Δmnn4::CgURA3 Δmnn4::KANMX4</td>
<td>–</td>
</tr>
<tr>
<td>HIY22</td>
<td>Δsvp26::KANMX4</td>
<td>+</td>
</tr>
<tr>
<td>YNY1057</td>
<td>Δmnn6::KANMX4</td>
<td>–</td>
</tr>
<tr>
<td>YNY1115</td>
<td>Δmnn4::CgURA3</td>
<td>+</td>
</tr>
<tr>
<td>YNY1364</td>
<td>YNY1130::pRS314 (empty vector)</td>
<td>+</td>
</tr>
<tr>
<td>YNY1365</td>
<td>YNY1130::pYN852 (MNN4-HA, low-copy TRP1)</td>
<td>+</td>
</tr>
<tr>
<td>YNY1372</td>
<td>YNY1130::pYN861 (MNN4D519A-HA, low-copy TRP1)</td>
<td>–</td>
</tr>
<tr>
<td>YNY1404</td>
<td>YNY1057::pRS316 (empty vector)</td>
<td>–</td>
</tr>
<tr>
<td>YNY1405</td>
<td>YNY1057::pYN714 (MNN6, low-copy URA3)</td>
<td>+</td>
</tr>
<tr>
<td>YNY1406</td>
<td>YNY1057::pYN881 (MNN6D219A, low-copy URA3)</td>
<td>+</td>
</tr>
<tr>
<td>YNY1407</td>
<td>YNY1057::pYN882 (MNN6D262A, low-copy URA3)</td>
<td>+</td>
</tr>
</tbody>
</table>

Table 2. Alcian blue binding was assessed in the indicated strains. As mutant strains showed either dark blue indistinguishable from the color of the wild-type cell pellet or very light blue indistinguishable from the color of the Δmnn4 cell pellet (a negative control), the result was indicated either ‘+’ (dye binding level equal to the wild-type) or ‘–’ (dye binding level equal to Δmnn4).
result indistinguishable from that seen for the wild-type strain in the alcin blue dye binding assay (Table 2). As shown in Figs. 1 and 3, although Mnn4 accumulates in the ER in the absence of Svp26, a significant amount of Mnn4 still resides at the Golgi. This fraction of Mnn4 in the Δsvp26 strain may transfer mannosyl phosphate to a sufficient level to give a positive result in alcin blue staining.

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References