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Immunoisolaton of the Yeast Golgi Subcompartments and Characterization of a Novel Membrane Protein, Svp26, Discovered in the Sed5-Containing Compartments

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The Golgi apparatus consists of a set of vesicular compartments which are distinguished by their marker proteins. These compartments are physically separated in the *Saccharomyces cerevisiae* cell. To characterize them extensively, we immunoisolated vesicles carrying either of the SNAREs Sed5 or Tlg2, the markers of the early and late Golgi compartments, respectively, and analyzed the membrane proteins. The composition of proteins was mostly consistent with the position of each compartment in the traffic. We found six uncharacterized but evolutionarily conserved proteins and named them Svp26 (*S*ed5 compartment *v*esicle *p*rotein of *26* kDa), Tvp38, Tvp23, Tvp18, Tvp15 (*T*lg2 compartment *v*esicle *p*roteins of *38*, *23*, *18*, and *15* kDa), and Gvp36 (*G*olgi *v*esicle *p*rotein of *36* kDa). The localization of Svp26 in the early Golgi compartment was confirmed by microscopic and biochemical means. Immunoprecipitation indicated that Svp26 binds to itself and a Golgi mannosyltransferase, Ktr3. In the absence of Svp26, a considerable portion of Ktr3 was mislocalized in the endoplasmic reticulum. Our data suggest that Svp26 has a novel role in retention of a subset of membrane proteins in the early Golgi compartments.

The Golgi apparatus of the eukaryotic cell plays a central role in intracellular vesicular transport as the main secretory protein-processing factory (39, 45, 47, 76). Secretory proteins are translocated in the lumen or membrane of the endoplasmic reticulum (ER), and some receive primary glycosylation from dolichol-P(P)-sugars. They are then transferred to the early (*cis*) Golgi network by COPII transport vesicles, and some receive further additions of carbohydrates and proteolytic processing during their traffic to the late (*trans*) Golgi network before sorting to their final destination, such as the vacuole/lysosome or cell surface.

The Golgi apparatus is composed of several compartments that are different in function and composition. This compartmentalization is widely recognized as the nature of the Golgi apparatus, although there are intensive debates regarding whether the compartments are fixed in existence or are in the transient process of cisternal maturation. In mammalian secretory cells, and also in several yeasts such as *Pichia pastoris*, these compartments form a polarized stack and give us a more complicated situation (54, 61). On the other hand, in *Saccharomyces cerevisiae*, Golgi compartments are dispersed in the cytosol, and similar Golgi compartment-specific maturation occurs as in the stacked Golgi apparatus. Several Golgi glycosyltransferases stay mainly in certain compartments and have therefore been regarded as the markers of those compartments (15). Strictness of localization or possible distribution among compartments in various physiological conditions may depend on the marker proteins.

Marker proteins practically define a certain compartment, and double immunofluorescence staining and cell fractionation experiments indicate a similar or different distribution in the cell. However, the composition of each defined compartment remains mostly unclear. To examine this issue, it is important to isolate enough vesicles derived from each specific compartment. Yeast Golgi subcompartments have been separated by differential centrifugation and density gradient centrifugation, depending on the difference in size and/or density of the vesicles (13, 71, 78). But the fractions have not yet been subjected to proteome analysis. Affinity purification is another method for isolating compartments that have a specific marker molecule on the surface, and immunoisolation using specific antibodies has been effectively used in several studies (9, 12, 66).

The yeast tSNAREs Sed5 and Tlg2 have been reported to localize in the early and late Golgi compartments, respectively (1, 21, 24). Although the SNAREs move dynamically in the process of vesicle transport (32, 74), their main localization is restricted to guarantee the fidelity of vesicle targeting and fusion, and therefore, they are also acceptable as markers of the subcompartments. We previously reported the immunoisolation and characterization of the vesicles that carry Sed5 on their surface and demonstrated the usefulness of immunoisolation in analytical preparation of Golgi apparatus-derived membrane vesicles (12).

In this paper, we extended immunoisolation to a large-scale separation of the compartments which have either Sed5 or Tlg2, the early and late Golgi markers, respectively. Immunological and proteomic analyses of the isolated compartments show distinct profiles of the membrane proteins in each compartment. Six previously uncharacterized membrane proteins were discovered as novel residents in the specific compartment of the yeast Golgi apparatus. We studied one of them, Svp26, in detail and found that it has an important and novel role in

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TABLE 1. Plasmids used in this study

Plasmid	Description	Source and/or reference
pRS314	*CEN6-TRP1*	65
pRS316	*CEN6-URA3*	65
pYN222	pRS314-*3HA-TDH1* terminator	This study
pNT125-2	pRS316-*6myc-TDH1* terminator	This study
pHI128	Svp26-3HA (*CEN6-TRP1*)	This study
pHI129	Svp26-Myc_6 (*CEN6-URA3*)	This study
pHI258	Myc_6-Ktr3 (*CEN6-URA3*)	This study
pHI218	Ktr3-Myc_6 (*CEN6-URA3*)	This study
pHI274	Svp26 (*CEN6-TRP1*)	This study
pHI282	GFP-Gos1 (*CEN6-URA3*)	This study
pYN348	Myc-Emp47 (*CEN6-URA3*)	This study, reference 60

retaining a subset of Golgi membrane proteins in the early Golgi compartment.

MATERIALS AND METHODS

Yeast strains and media. *S. cerevisiae* KA31a (*MATa Δhis3 Δleu2 Δtrp1 Δura3*), CJY119 (*MATa Δhis3 Δleu2 Δtrp1 Δura3 Δsed5*::[*6myc-SED5 URA3*]), and HIY1 (CJY119 *Δtlg2*::[*6myc-TLG2 HIS3*]) were used (12, 25). The *sec12* temperature-sensitive (*sec12^{ts}*) mutant (*MATa sec12-4 ura3*) was obtained from Randy Schekman (University of California, Berkeley). Yeast cells were grown in yeast extract-peptone-dextrose or synthetic dextrose (SD) medium at 30°C (12).

Plasmid construction. The plasmids used in this study are listed in Table 1. The epitope tagging was made by PCR amplification of the sequence encoding each open reading frame and its 5′ region from the genomic DNA using the primers shown (Table 2) and cloning into pYN222 or pNT125-2.

Antibodies. Antiserum was raised against the glutathione-*S*-transferase fusion protein having a fragment of Kex2 (amino acids 700 to 814), Vti1 (amino acids 3 to 188), Gos1 (amino acids 3 to 198), or Sft1 (amino acids 15 to 73). Antiserum against Mnn9 was described previously (22). Antisera against Scs2, Snc1, Sec22, Kar2, and Kre9 were kindly provided by S. Kagiwada (Nara Woman's University, Japan), Jeffrey Gerst (Weizmann Institute of Science, Israel), Dieter Gallwitz (Max Planck Institute, Göttingen, Germany), Masao Tokunaga (Kagoshima University, Japan), and Howard Bussey (McGill University, Montreal, Quebec, Canada), respectively. Anti-hen egg white lysozyme was purchase from Rockland. For immunoblotting, these were diluted at 1:1,000. The anti-Pep12 mouse (2C3-G4; Molecular Probes), anti-Myc mouse (9E10; Berkeley Antibody), and anti-hemagglutinin (HA) rat (3F10; Roche Diagnostics) monoclonal antibodies (MAbs) were purchased.

Immunoisolation of the Golgi compartments. Immunoisolation of the Sed5 or Tlg2 compartments was performed essentially as described previously (12), with some modifications. Strains CJY119 (Myc_6-Sed5), HIY1 (Myc_6-Tlg2), and KA31a (wild type, as a negative control) were grown in 100 ml (for immunoblot analysis) or 4 liters (for proteome analysis) of yeast extract-peptone-dextrose medium at 30°C to an optical density at 600 nm (OD_{600}) of 1.0. Cells were converted to spheroplasts by incubation with recombinant lyticase (63) in 0.1 M potassium phosphate, pH 7.5, 1 mM $MgCl_2$, 1.2 M sorbitol, and 0.1% 2-mercaptoethanol for 30 min at 30°C. The spheroplasts were suspended in ice cold B88-500 (20 mM HEPES, pH 6.8, 500 mM potassium acetate, 5 mM magnesium acetate, 200 mM sorbitol) buffer containing protease inhibitors (1 μg/ml each of chymostatin, aprotinin, leupeptin, pepstatin A, and antipain and 1 mM phenylmethylsulfonyl fluoride) and homogenized by seven strokes of a Dounce homogenizer (tight fitting; Wheaton). Following a short centrifugation (5 min at 1,000 × *g*) to remove unlysed cells, the lysate was centrifuged at 10,000 × *g* for 20 min to remove the nuclei, ER, and mitochondria. The anti-Myc MAb 9E10 was added to the supernatant and incubated for 1 h at 4°C. Pansorbin cells (Calbiochem) were washed with B88-500 buffer and incubated with B88-500 buffer containing 10 mg/ml bovine serum albumin at 4°C for 1 h for blocking. Pansorbin cells were then added to the mixture and incubated at 4°C for 1 h. Pansorbin cells were collected by centrifugation at 2,000 × *g* for 5 min. The pellets were washed five times with ice-cold B88-500 buffer. The compartments that precipitated with Pansorbin cells were solubilized by the addition of B88-500 buffer containing 1% Triton X-100 or 1% Triton X-114 and incubation on ice for 15 min. Pansorbin cells were removed by centrifugation at 2,000 × *g* for 5 min. The supernatant was collected as a Sed5 compartment, a Tlg2 compartment, or a control fraction.

Triton X-114 phase separation. Triton X-114 phase separation was performed essentially as described previously by Bordier (6), with some modifications. In brief, the vesicles on Pansorbin cells were solubilized in B88-500 buffer containing 1% Triton X-114 by incubating them on ice for 15 min. The solution was kept at 30°C for 5 min and centrifuged at 5,000 × *g* for 5 min at room temperature. After removing the aqueous phase, B88-500 buffer containing 0.5% Triton X-114 was added to the detergent phase. The detergent phase was collected after incubation on ice for 5 min and at 30°C for 5 min, and centrifugation was performed at 5,000 × *g* for 5 min at room temperature.

Protein identification by mass spectrometer analyses. Identification of proteins by mass spectrometer analyses was performed as described previously by Fujiyama et al. (18). In brief, the protein bands in the sodium dodecyl sulfate (SDS)-polyacrylamide gel were cut out, reduced, alkylated, and subjected to in-gel digestion by trypsin. The peptides were extracted, and their mass was determined by Voyager DE-STR (PerSeptive Biosystems). The protein was identified by a database search in PeptIdent (http://ca.expasy.org/tools/peptident.html) and MS-Fit (http://prospector.ucsf.edu/ucsfhtml4.0/msfit.htm). The criteria adapted for identification of the yeast membrane proteins were that the coverage of peptide should exceed 7% without including a missed cleavage, the mass accuracy should be within 50 ppm, and the observed molecular mass of the protein should be within a 20% deviation from the calculated mass.

Subcellular fractionation and solubility test of membrane proteins. Cells were converted to spheroplasts as described above and burst in B88 (20 mM HEPES, pH 6.8, 150 mM potassium acetate, 5 mM magnesium acetate, 200 mM sorbitol) buffer containing protease inhibitors. Unbroken cells were removed by centrifugation at 850 × *g* for 5 min. For subcellular fractionation, the cleared lysate was sequentially centrifuged to generate a 10,000 × *g* pellet (P10), a 100,000 × *g* pellet (P100), and a 100,000 × *g* supernatant (S100). Each fraction was adjusted to the original volume of the lysate, and the same amount was applied for SDS-polyacrylamide gel electrophoresis (PAGE) and Western blotting. For a solubility test, a portion of lysate was mixed with the same amount of B88 buffer containing 2% Triton X-100, 0.2 M Na_2CO_3, or 4 M urea and kept on ice for 15 min. After removing a sample as the total, the mixture was centrifuged at 100,000 × *g* for 60 min. The precipitate was suspended in the same volume of the same buffer as the supernatant.

Indirect immunofluorescence microscopy. Fixation and permeabilization of the yeast cells for indirect immunofluorescence were performed as described previously by Wooding and Pelham (74). Subsequent steps were performed as described previously by Vashist et al. (70), with some modifications. Primary antibodies anti-Myc mouse MAb 9E10 and anti-HA rat MAb 3F10 were used at 2 μg protein/ml and 1 μg protein/ml in phosphate-buffered saline (PBS) block (1% skim milk and 0.1% bovine serum albumin in PBS), respectively, for 1 h. Slides were washed four times with PBS block. Secondary antibodies rhodamine-

TABLE 2. Primers used in this study

Primer	Gene	Tagging position	Direction	Sequence
HI51	*SVP26*	C	Forward	5′-ATTCTGGCAGGATCCATTATACCG-3′
HI52	*SVP26*	C	Reverse	5′-AAAAAAGGCCTCGAGAAACAGCCA-3′
HI135	*KTR3*	N	Forward	5′-TCCAGCGTGGATCCATGTCTGTGC-3′
HI136	*KTR3*	N	Reverse	5′-GGTGACTAGTAATATGAACACC-3′
HI117	*KTR3*	C	Forward	5′-CTGCCATATTCGGATCCAAGAGAAA-3′
HI118	*KTR3*	C	Reverse	5′-TTGTAGTTTGCTCGAGAAATTCCTGGTG-3′
HI146	*GOS1*	N	Forward	5′-CAAGCAACCAGGATCCATGAGCTCAC-3′
HI147	*GOS1*	N	Reverse	5′-TCTGTTCTTGAATTCAACTAGTGTAAAG-3′

conjugated goat anti-mouse immunoglobulin G antibody and Alexa 488-conjugated goat anti-rat immunoglobulin G antibody (Molecular Probes) were used at 10 mg protein/ml in PBS block for 30 min in the dark. Slides were washed four times with PBS block and two times with PBS. Five microliters of mounting solution (90% glycerol and 0.1 mg/ml *p*-phenylendiamine in PBS) was added to the sample, and the slide was sealed with a glass coverslip. Samples were viewed on a Fluoview FV500 digital confocal scanning laser microscope equipped with a PlanApo ×60 1.4-numerical-aperture objective (Olympus). For each field of cells, multiple images were recorded from focal planes spaced 0.5 μm apart; four to five of these images were deblurred using a simple deconvolution algorithm and then combined to form a single projected image.

Immunoprecipitation. The yeast cell lysate in B88 buffer containing protease inhibitors and a 1/10 volume of 10% Triton X-100 were mixed and kept on ice for 15 min. The supernatant of a centrifugation at 100,000 × *g* for 1 h was mixed with anti-Myc MAb 9E10 and kept gently shaking at 4°C for 1 h. Protein A-Sepharose beads (Pharmacia) washed with B88 buffer containing 1% Triton X-100 were added, and incubation was continued at 4°C for 1 h more. The beads were washed five times with B88 buffer containing 1% Triton X-100 and then boiled in the SDS sample buffer for 3 min to obtain the samples.

Secretory invertase studies. Secretory invertase was detected by activity staining or immunoprecipitation and blotting as described previously (22). Kinetic analysis of invertase secretion was basically done as described previously (73). Yeast cells were grown overnight to an OD_{600} of 0.5 in SD-Ura medium. Cells were collected and washed once with SD-Ura medium lacking glucose. The cells were resuspended in synthetic sucrose-Ura medium (2% sucrose) at 1 OD_{600} unit of cells per milliliter. At several time points (0, 10, 20, 30, 40, and 60 min), samples were transferred to a tube containing sodium azide (20 mM), and samples were kept on ice. At the end of the time course, cells were collected and washed three times with ice-cold water containing 10 mM sodium azide. External invertase was prepared from cells of 2 OD_{600} units at each time point by zymolyase treatment (250 mg/ml) in the sorbitol buffer (50 mM Tris-Cl, 1.24 M sorbitol, 28 mM 2-mercaptoethanol, pH 8.0), and the invertase activity was measured using a Glucose C2 kit (Wako) (mutarotase-glucose oxidase method).

G49N version of hen egg white lysozyme. Yeast strain KA31a (wild type) or HIY22(Δ*svp26*) transformed with pVT100-U-HELG49N (a kind gift of S. Munro and T. Yoko-o [77]) was grown in SD-Ura medium for 3 days. The medium was dialyzed against 10 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] (pH 6.5)–50 mM NaCl for 1 h. Ten microliters of SP Sepharose (Amersham Biosciences) was added to 1 ml of the medium and stirred for 4 h at 4°C. The beads were washed once with 500 μl of 10 mM PIPES (pH 6.5)–50 mM NaCl, and the protein was eluted by boiling for 1 min in 20 μl SDS sample buffer. Five microliters of the eluted protein fraction was mixed with 10 μl of 0.1 M sodium citrate buffer (pH 5.5) and incubated either in the presence or in the absence of 10 mU endoglycosidase H (Roche) overnight at 37°C. Proteins were separated in 7.5% (without endoglycosidase H) or 10% (in the presence of endoglycosidase H) SDS-polyacrylamide gels, and immunoblotting was performed using a rabbit anti-lysozyme (Rockland) antibody. The signals were detected by chemiluminescence (ECL; Amersham Biosciences).

Subcellular fractionation by sucrose density gradient centrifugation. Subcellular fractionation by sucrose density gradient centrifugation was performed as described previously by Antebi and Fink (2) and Powers and Barlowe (50), with modifications. Cells were grown in 100 ml selective SD medium to mid-log phase (OD_{600} of 0.8) and converted to spheroplasts by lyticase treatment. Spheroplasts were lysed in a 1-ml ice-cold sucrose solution (10 mM HEPES, pH 7.5, 12.5% sucrose, 1 mM EDTA) supplemented with a cocktail of protease inhibitors. The lysate was centrifuged at 850 × *g* for 5 min to remove unlysed cells. The resulting pellet was extracted again with 0.5 ml of sucrose solution and centrifuged at 850 × *g* for 5 min. The combined supernatant was centrifuged, and 1.2 ml of the resultant supernatant was layered onto gradients containing 1-ml steps of 18, 22, 26, 30, 34, 38, 42, 46, 50, 54, and 60% sucrose (wt/vol) in 10 mM HEPES (pH 7.5)–1 mM $MgCl_2$. Gradients were centrifuged at 35,000 rpm (model SW41 Ti rotor; Beckman Coulter) for 2.5 h at 4°C. Twelve fractions of 1 ml each were taken sequentially from the top of the gradient. Aliquots of each fraction were mixed with SDS sample buffer and boiled for 1 min, and proteins were resolved by SDS-PAGE and immunoblotted for anti-Gos1, anti-Sed5, anti-Van1, anti-Pep12, or anti-Scs2 antiserum. Anti-Myc MAb 9E10 was used to detect Myc_6-Ktr3. Enhanced chemiluminescence signals were captured by an image analyzer equipped with a cooled charge-coupled-device camera (LAS-1000plus; Fuji Film), and digital images were quantified using image analysis software (Image Gauge; Fuji Film). The intensity of each band was normalized by adjusting the maximum value to 100%.

"Secretion block" in the *sec12* temperature-sensitive cell. "Secretion block" in the *sec12* temperature-sensitive cell was performed as described previously by Schröder et al. (60) and Lewis and Pelham (33). The *sec12*^{ts} cells producing Myc-tagged Emp47 or HA-tagged Svp26 were grown at 25°C until the OD_{600} reached 0.5. Cycloheximide was added to 20 μg/ml, and the cells were shaken at either 25 or 35°C for 1 h. Cells were pelleted, washed in 20 mM sodium azide, and then prepared for indirect immunofluorescence microscopy. Cells were viewed with an Axiovert 200 MOT inverted microscope with a ×100 1.4-numerical aperture oil immersion objective and captured with a AxioCam MRm charge-coupled-device camera (Carl Zeiss).

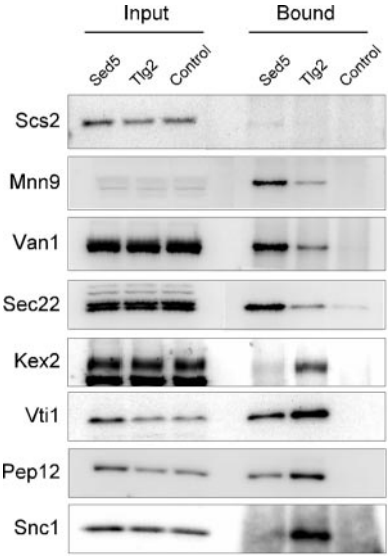

FIG. 1. The early and late Golgi markers in the Sed5 and Tlg2 compartments. Each fraction was collected from wild-type yeast KA31a (control), Myc_6-Sed5-producing yeast CJY119 (Sed5 compartment), and Myc_6-Tlg2-producing yeast HIY1 (Tlg2 compartment). The proteins of these compartments were extracted by 1% Triton X-100 from the immunoadsorption beads and analyzed by Western blotting against anti-Scs2, anti-Mnn9, anti-Sec22, anti-Van1, anti-Kex2, anti-Vti1, anti-Pep12, or anti-Snc1 antibody. Samples derived from 1 and 10 ml of the yeast culture were loaded in the input and bound lanes, respectively.

RESULTS

Isolation of the early Golgi and late Golgi/endosome compartments. We chose Sed5 and Tlg2 proteins as the probes to collect the early Golgi and late Golgi/endosome compartments, respectively. Sed5 and Tlg2 are syntaxin family tSNAREs in *S. cerevisiae* (46). Their chromosomal genes were replaced with tagged genes which encode modified SNAREs with the Myc_6 epitope at the N terminus facing the cytosol. The tagged proteins have the same function and localization as the wild-type proteins (12, 74; our unpublished results). After mild cell disintegration, the membrane vesicles which are derived from the Golgi compartment and which have Myc epitope on their surface were collected, as described in Materials and Methods. Hereafter, the vesicles isolated from the cell lysate producing Myc_6-Sed5 and those producing Myc_6-Tlg2 are referred to as the Sed5 compartment and Tlg2 compartment, respectively.

To characterize the isolated compartments, we examined the detergent extract of the compartments by immunoblotting (Fig. 1). None of the marker proteins were detected from the control lysate without the Myc epitope. Mnn9, Van1, and Sec22 were present in the Sed5 compartment, whereas they

were hardly detected in the Tlg2 compartment. Mnn9 and Van1 are subunits of mannosyltransferase complexes in the early Golgi compartment (22, 27), and Sec22 is a vSNARE of the transport vesicles of the ER-to-early-Golgi transport (4). On the other hand, Vti1, a vSNARE of the late-Golgi prevacuolar compartment (72), is enriched in the Tlg2 compartment but hardly detected in the Sed5 compartment. Kex2, a processing protease of the late Golgi compartment (9), was detected only in the Tlg2 compartment and was not detected in the Sed5 compartment, even after prolonged exposure. This suggests that a small amount of Vti1 detected in the Sed5 compartment is present in the compartment that also contains Myc-Sed5. It is consistent with the current understanding that most of the Golgi proteins recycle dynamically by vesicular transport (32, 74). Detection of a late-endosome protein, Pep12, and a secretory vesicle/plasma membrane protein, Snc1, in the Tlg2 compartment is likely to indicate recycling of those proteins. Similarly, Mnn9, Van1, and Sec22 in the Tlg2 compartment are likely to indicate their transient stay in those later compartments. In contrast, an ER membrane protein, Scs2 (29), was not detected in either compartment, as in the case of Sec71 (12). We concluded that the Sed5 compartment and the Tlg2 compartment are representatives of the early and late Golgi compartments, respectively.

Identification of membrane proteins in the Sed5 and Tlg2 compartments. We sought to identify the proteins that localize in the Sed5 and Tlg2 compartments comprehensively to characterize each of the Golgi compartments. However, several attempts to identify the spot in two-dimensional gel electrophoresis suggested that a large number of cytosolic proteins had bound to the immunoadsorbant Pansorbin cells and interfered with detection of the Golgi compartment-specific proteins. To overcome this difficulty, we adopted Triton X-114 phase separation (6). The cytosolic proteins were removed in the aqueous phase, while the marker membrane proteins remained in the detergent phase as confirmed by immunoblotting (data not shown).

As shown in Fig. 2, most of the proteins detected by silver staining in a 4 to 20% gradient gel of SDS-PAGE were clearly different between the Sed5 and Tlg2 compartments. We found 29 and 32 proteins in the Sed5 and Tlg2 compartments, respectively, using peptide mass fingerprint analysis (Tables 3 and 4). Reproducible data were obtained in at least two independent experiments. The proteins are classified according to their reported functional remarks and the compartments where they were detected in the present study (Table 5).

Sequence characteristics of the newly identified Golgi membrane proteins. We found six proteins encoded by previously uncharacterized genes in either of the immunoisolated compartments. We termed the product of *YHR181w* found in the Sed5 compartment as Svp26 (*S*ed5 compartment *v*esicle *p*rotein of *26* kDa), the products of *YKR088c*, *YDR084w*, *YMR071c*, and *YDR100w* found in the Tlg2 compartment as Tvp38, Tvp23, Tvp18, and Tvp15 (*T*lg2 compartment *v*esicle *p*roteins of *38*, *23*, *18*, and *15* kDa), respectively, and the product of *YIL041w* found in both Sed5 and Tlg2 compartments as Gvp36 (*G*olgi *v*esicle *p*rotein of *36* kDa). The number of amino acids, the calculated molecular mass, and the number of hydrophobic stretches long enough to span the membrane are listed in Table 6.

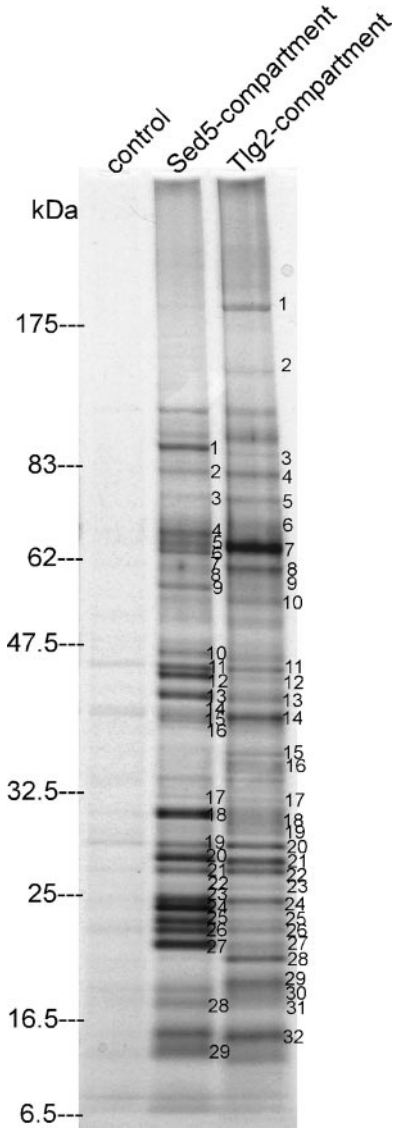

FIG. 2. Membrane proteins of the Sed5 or Tlg2 compartments. Each fraction was collected from KA31a (control), CJY119 (Sed5 compartment), or HIY1 (Tlg2 compartment). The proteins were extracted by 1% Triton X-114 from the immunoadsorption beads and subjected to phase separation. Membrane proteins enriched in the detergent phase were separated by SDS-PAGE on a 4 to 20% gradient gel and detected by silver staining. Protein bands indicated with numbers were identified by mass spectrometric analysis. The identified proteins are shown in Tables 3 and 4.

Homologous proteins of all these novel Golgi proteins are found in the other yeast species *Candida albicans* and *Schizosaccharomyces pombe* (Table 6). Homologues of some Tvp proteins are found in nematode or in plant. A homologous protein of Tvp38 is even found in *Escherichia coli*, although its significance is not clear at present. Homologues of Svp26, Tvp38, Tvp23, and Tvp18 with 20 to 40% sequence identity are found in the fly, mouse, and human genome databases. The human homologue of Tvp23 (FAM18B [GenBank accession number NP_057162]) is reported to be present in most tissues examined (26), and the mouse homologue of Svp26 (Tex261

TABLE 3. Proteins identified in the Sed5 compartment[a]

No.	Name	Description	Localization	MM (kDa)	TMD	% Coverage	Reference or source
1	Pmr1	Ca^{2+}, Mn^{2+}-transporting P-type ATPase	Medial Golgi compartment	104.6	9	9.5	16
2, 3	Akr1	Palmityol transferase	Golgi compartment or endosome	85.8	6	9.3, 6.5	55
4	Van1	Mannosyltransferase	Early Golgi compartment	61.1	1	11	67
5, 6	Mnn2	Putative α-1,2-mannosyltransferase	Early Golgi compartment	67.8	2 (1)	13.1, 15.4	52
7,10	Kre2	α-1,2-Mannosyltransferase	Medial Golgi compartment	51.4	1	17.4, 12.7	36
8	Ktr4	Putative α-1,2-mannosyltransferase	Golgi compartment	54.6	1	7.1	36
9	Anp1	Mannosyltransferase	Early Golgi compartment	58.2	1	18.0	27
11	Eht1	Alcohol acyl transferase	Lipid particles	51.3	1	21.5	3
11	Erv46	COPII vesicle protein	ER	46.2	2	10.4	43
12	Mnn11	α-1,6-Mannosyltransferase	Early Golgi compartment	47.8	1	22.7	28
12	Ktr1	α-1,2-Mannosyltransferase	Medial Golgi compartment	46.0	1	19.3	36
12	Emp47	COPII vesicle protein	ER-Golgi compartment	50.3	2 (1)	36.9	57
13	Ktr3	α-1,2-Mannosyltransferase	Golgi compartment	47.5	1	9.4	36
13	Mnn10	α-1,6-Mannosyltransferase	Early Golgi compartment	46.7	1	12.2	28
14	Gvp36	Peripheral membrane protein	Golgi compartment	36.7	0	31.0	This study
15	Erv41	COPII vesicle protein	ER	40.7	3	19.3	43
16	Gcs1	ARF GAP	ER-Golgi compartment	39.3	0	23.9	49
17	Sec17	α-SNAP	Transport vesicles	32.8	0	45.7	20
18	Yif1	Yip1-interacting factor	ER-Golgi compartment	35.5	5	25.8	38
18	Ssp120	Secretory protein	Extracellular	27.3	1 (0)	26	64
19	Etf1	Effector of the Vps34	Prevacuolar compartments	21.6	1	13.3	75
20	Gos1	vSNARE	Golgi compartment	25.4	1	22.9	46
20	Ypt52	Rab GTPase	Early to late endosome	26.1	GG	21.4	31
21	Ypt31	Rab GTPase	Golgi compartment	24.5	GG	39.0	31
21	Ypt32	Rab GTPase	Golgi compartment	24.5	GG	22.1	31
22	Sec4	Rab GTPase	Golgi compartment to plasma membrane	23.5	GG	37.2	31
23	Ykt6	vSNARE	Golgi compartment	22.7	F	17.0	46
23	Ypt1	Rab GTPase	ER-Golgi compartment	23.2	GG	21.8	31
24	Erp1	p24 protein	ER-Golgi compartment	24.7	2 (1)	35.2	37
25	Erp2	p24 protein	ER-Golgi compartment	24.1	2 (1)	36.3	37
25	Svp26	Membrane protein	Early Golgi compartment	26.3	4	19.3	This study
26	Sar1	ARF GTPase	ER-Golgi compartment	21.5	0	37.4	68
26	Rer1	Retrieval receptor for ER protein	Golgi compartment	22.3	3	13.3	59
27	Erv25	p24 protein	ER	24.1	2 (1)	52.1	5
27	Emp24	p24 protein	ER	23.3	2 (1)	46.4	5
28	Yip3	Function unknown	ER-Golgi compartment	19.4	2	27.3	11
29	Erv14	COPII vesicle protein	ER and Golgi compartment	15.9	3	10.9	50

[a] Numbers correspond to those indicated at the bands in Fig. 2. TMD represents the number of predicted transmembrane domains. GG, F, and M indicate anchorage by geranylgeranyl, farnesyl, and myristyl anchors, respectively. Numbers in parentheses indicate TMD after processing of the signal sequence. MM, molecular mass.

[accession number NP_653183]) is found in the gonads and testis (34). However, any information on the functional properties of these homologues is not available so far. *S. cerevisiae* strains with the disrupted gene encoding each of the six proteins were obtained from the EUROSCARF consortium (http://www.uni-frankfurt.de/fb15/mikro/euroscarf). All strains are viable, and therefore, each of the novel Golgi proteins is dispensable for growth under laboratory culture conditions. By detailed examination of the intracellular alterations of the null mutants and the interacting proteins, we found that Svp26 has distinguishably novel characteristics in the cell. We report these important results in the rest of this paper.

Membrane association and early-Golgi compartment-specific localization of Svp26 protein. Svp26 has four putative transmembrane domains (Fig. 3B), and the amino acid sequences of these and the N-terminal region are well conserved in higher eukaryotes (Fig. 3A). To confirm that Svp26 is an integral membrane protein, an HA-tagged protein was tested for solubility. Svp26-HA_3 was recovered in the pellet after centrifugation. It was solubilized in the presence of 1% Triton X-100 but not in 0.1 M Na_2CO_3 or 2 M urea (Fig. 3C). Therefore, Svp26 is an integral membrane protein.

To see the compartment-specific localization, we isolated the Sed5 and Tlg2 compartments from the lysate containing Svp26-HA_3. As shown in Fig. 4A, Svp26-HA_3 was enriched in the Sed5 compartment, while only a small amount was detected in the Tlg2 compartment, in accordance with the fact that Svp26 was originally found only in the Sed5 compartment. The HA tag at the C terminus apparently does not affect the main localization of Svp26.

Next, we examined the intracellular localization of Svp26 by double staining of indirect immunofluorescence microscopy (Fig. 4B). In *S. cerevisiae*, the Golgi apparatus is dispersed in the cytoplasm and is observed as a punctate pattern by immunofluorescent staining of their resident marker proteins. The signals of Sed5 and Tlg2 were in a similar punctate pattern, but most of Sed5 and Tlg2 were present in different structures (24; our unpublished results). The fluorescence of Svp26-HA_3 was in a punctate pattern, which overlapped with the fluorescence of Myc_6-Sed5 but was distinct from the signals of Myc_6-Tlg2. These results indicate that Svp26 mainly localizes in the early Golgi compartment.

Proteins that interact with the Svp26 protein. To obtain clues concerning the biological function of Svp26, we sought to

TABLE 4. Proteins identified in the Tlg2 compartment[a]

No.	Name	Description	Localization	MM (kDa)	TMD	% Coverage	Reference or source
1	Vps10	Sorting receptor for vacuolar hydrolases	Late Golgi compartment	177.8	1	8.5	14
2	Vps18	Vacuolar protein sorting	Vacuole and endosome	107.4	0	13.0	48
3	Stv1	V-ATPase V0 domain subunit a	Late Golgi compartment	101.7	9	10.9	41
4, 5	Akr1	Palmitoyl transferase	Golgi compartment or endosome	85.8	6	10.2, 5.6	55
6	Mnn5	α-1,2-Mannosyltransferase	Golgi compartment	67.3	2	12.5	52
7	Ptm1	Function unknown	Unknown	61.0	8 (7)	11.9	
8	Kre2	α-1,2-Mannosyltransferase	Medial Golgi compartment	51.4	1	11.8	36
9	Ktr4	Putative α-1,2-mannosyltransferase	Golgi compartment	54.6	1	19.2	36
10	Gnt1	GlcNAc transferase	Golgi compartment	57.6	1	8.1	77
11	Eht1	Alcohol acyl transferase	Lipid particles	51.3	1	11.8	3
12	Ktr1	α-1,2-Mannosyltransferase	Medial Golgi compartment	46.0	1	21.4	36
13	Ktr3	α-1,2-Mannosyltransferase	Golgi compartment	47.5	1	7.9	36
14	Gvp36	Peripheral membrane protein	Golgi compartment	36.7	0	27.0	This study
15	Vma6	V-ATPase V0 domain subunit d	Vacuole	39.8	0	28.4	41
16	Tvp38	Membrane protein	Late Golgi compartment/endosome	38.3	5	20.2	This study
17	Sec17	α-SNAP	Transport vesicles	32.8	0	13.7	20
18	Trs31	TRAPP subunit	Golgi compartment	31.7	0	13.4	56
19	Dpm1	Dolichyl-P-mannosyltransferase	ER	30.4	1	19.1	51
20	Etf1	Effector of the Vps34	Prevacuolar compartments	21.6	1	27.0	75
21	Gos1	vSNARE	Golgi compartment	25.4	1	24.2	46
21	Ypt52	Rab GTPase	Early to late endosome	26.1	GG	16.7	31
22	Ypt31	Rab GTPase	Golgi compartment	24.5	GG	20.6	31
22	Ypt32	Rab GTPase	Golgi compartment	24.5	GG	27.0	31
23	Sec4	Rab GTPase	Golgi compartment to plasma membrane	23.5	GG	25.1	31
24	Ykt6	vSNARE	Golgi compartment	22.7	F	33.5	46
24	Ypt1	Rab GTPase	ER-Golgi compartment	23.2	GG	44.7	31
25	Erp2	p24 protein	ER-Golgi compartment	24.1	2 (1)	29.5	37
26	Sar1	ARF GTPase	ER-Golgi compartment	21.5	0	52.1	68
27	Arf1	ARF GTPase	Golgi compartment	20.5	M	23.8	68
28	Tvp23	Membrane protein	Late Golgi compartment/endosome	23.1	3	26.1	This study
29	Tvp18	Membrane protein	Late Golgi compartment/endosome	18.7	3	32.9	This study
30	Sna4	Homology to Pmp3/Sna1	Vacuole	15.7	2	17.1	53
31	Yip3	Rab GTPase-interacting protein	ER-Golgi compartment	19.4	2	21.0	11
32	Tvp15	Membrane protein	Late Golgi compartment/endosome	15.9	3	12.6	This study

[a] Numbers correspond to those indicated at the bands in Fig. 2. TMD represents the number of predicted transmembrane domains. GG, F, and M indicate anchorage by geranylgeranyl, farnesyl, and myristyl anchors, respectively. Number in parentheses indicates TMD after processing of the signal sequence. MM, molecular mass.

determine which proteins interact with Svp26 in the cell. We examined the anti-HA immunoprecipitate from the Triton X-100 lysate of wild-type yeast producing Svp26-HA_3 by SDS-PAGE and silver staining and found that several protein bands were distinct from the control gel (Fig. 5A). Band *b* was Svp26-HA_3 itself, because it was detected by anti-HA immunoblotting. According to peptide mass fingerprinting, band *c* below the 25-kDa size marker was predicted to be the endogenous Svp26. To confirm that Svp26-HA_3 binds to Svp26, immunoprecipitation was performed using anti-Myc antibody from the Triton X-100 lysate of cells producing both Svp26-HA_3 and Svp26-Myc_6. As shown in Fig. 5C, Svp26-HA_3 was coprecipitated with Svp26-Myc_6, which indicates that the Svp26 protein binds to itself. Band *a* of approximately 43 kDa was predicted to be Ktr3, a Kre2 family α-1,2-mannosyltransferase in the Golgi compartment by using peptide mass fingerprinting. To confirm that Ktr3 binds to Svp26, a cleared lysate prepared from cells producing Myc_6-Ktr3 and Svp26-HA_3 was subjected to immunoprecipitation using anti-Myc antibody. Immunoblotting in Fig. 5B indicated that Svp26 binds to Ktr3.

Requirement of Svp26 protein for normal glycosylation and cell wall integrity. Ktr3 mannosyltransferase is reported to have a redundant function with Kre2 and Ktr1, which transfer the second and third α-1,2-linked mannose residues in *N*- and *O*-glycan chains of glycoprotein. Physical interaction between Ktr3 and Svp26 suggests that Svp26 may have some role in protein glycosylation. It has been shown that cells lacking Ktr3 have a defect in O glycosylation of the Kre9 protein (35). We compared the mass of Kre9 produced in the Δ*svp26* mutant and wild-type yeast by SDS-PAGE and immunoblotting, but a reproducible large difference was not obtained.

In contrast, the secretory invertase (17) produced by the Δ*svp26* mutant migrated much slower than that of the control (Fig. 6A). Endoglycosidase H digestion indicated that the difference in migration was caused by the degree of N-glycosylation (Fig. 6B). The time course of invertase secretion after the induction was not different between the wild type and the Δ*svp26* mutant (Fig. 6C). Therefore, it is unlikely that the more extensive glycosylation in the Δ*svp26* mutant was caused by a defective transport and longer stay of invertase in the Golgi compartment. An increase of the number of oligosaccharide chains actually attached to the possible Asn-X-Ser/Thr glycosylation sites of invertase, and an increase of the length of each polysaccharide chain will result in extensive glycosylation. We examined the mobility of a mutant hen egg white lysozyme, G49N, which has only a single N-glycosylation site (77). The

TABLE 5. Proteins found in the Sed5 and Tlg2 compartments

Compartment	Protein						
	COPII component	Mannosyltransferase	Vesicle transport	Small GTPase	H^+-ATPase	Other	Function unknown
Sed5 (early Golgi proteins)	Emp24 Erv25 Erp1 Emp47 Erv46 Erv41 Erv14	Van1 Anp1 Mnn10 Mnn11 Mnn2	Gcs1 Rer1 Yif1			Pmr1	Svp26 Ssp120
Sed5 and Tlg2 (medial Golgi or broadly localized Golgi proteins)	Erp2	Kre2	Etf1	Sar1		Akr1	Gvp36
		Ktr1 Ktr3 Ktr4	Gos1 Sec17 Ykt6 Yip3	Sec4 Ypt1 Ypt31 or 32 Ypt52		Eht1	
Tlg2 (late Golgi/early endosome proteins)		Mnn5	Trs31	Arf1	Stv1	Dpm1	Tvp15
			Vps10 Vps18		Vma6	Gnt1	Tvp18 Tvp23 Tvp38 Ptm1 Sna4

lysozyme produced by the Δ*svp26* mutant was significantly larger than that produced by the wild type (Fig. 6D), and endoglycosidase H digestion eliminated the difference (Fig. 6E). This suggests that the elongation of the *N*-glycosy chain is stimulated in the absence of Svp26.

The Δ*ktr3* mutant also shows a higher sensitivity to calcofluor white and Congo red, similar to a variety of mutants with defects in the cell wall integrity (35). The Δ*svp26* mutant also showed higher sensitivity to these chitin-binding dyes (Fig. 6B). However, because the Δ*ktr3* Δ*svp26* double disruptant was more sensitive to them than the Δ*ktr3* mutant, the lack of Svp26 has some effects in addition to the damage of Ktr3 activity.

Requirement of Svp26 protein for localization of a subset of Golgi membrane proteins. To gain further insight into the relationship between the Ktr3 and Svp26 proteins, we examined the intracellular localization of Ktr3 in the presence or absence of Svp26. In the wild-type cell, the immunofluorescent image of Myc_6-Ktr3 is a punctate pattern, like those of the other Golgi compartment-resident proteins and distinct from the ER-characteristic staining pattern of Kar2/BiP (Fig. 7A). In the Δ*svp26* cell, however, a significant part of Myc_6-Ktr3 is

TABLE 6. Novel Golgi proteins and their homologues[a]

Characteristic	Protein					
	Svp26 (Yhr181w)	Tvp38 (Ykr088c)	Tvp23 (Ydr084w)	Tvp18 (Ymr071c)	Tvp15 (Ydr100w)	Gvp36 (Yil041w)
AA (no.)	288	337	199	167	143	326
MW	26,283	38,306	23,151	18,692	15,875	36,654
TMD (no.)	4	5	3	3	3	0
% Identity of homologues (accession no.)						
E. coli		24.7 (NP_416264)				
C. albicans (orf no.)	53.2 (orf6.3135)	35.3 (orf6.1549)	35.9 (orf6.7349) 33.6 (orf6.689)	49.4 (orf6.7366)	33.6 (orf6.1633)	41.9 (orf6.710) 40.4 (orf6.5081)
S. pombe	44.0 (NP_588034)	24.2 (NP_595882)	37.3 (NP_596214)	42.9 (NP_596156)	29.0 (NP_593290)	24.2 (NP_593220)
Caenorhabditis elegans			35.8 (NP_501122)	30.7 (NP_510484)		
Arabidopsis thaliana		21.9 (NP_564182)	34.6 (AAO44038)			
D. melanogaster	35.1 (AAL68029)	22.4 (NP_649412)	36.3 (NP_648270)	27.7 (NP_730072)		
Mus musculus	38.3 (NP_033383)	23.1 (AAH30341)	34.9 (NP_080486)	24.0 (XP_194006)		
H. sapiens	37.2 (NP_653183)	22.5 (CAD39028)	34.2 (NP_057162) 31.7 (NP_660344)	23.5 (NP_060056)		

[a] The total number of amino acids (AA), the calculated molecular weight (MW) (in thousands), and the number of predicted transmembrane domains (TMD) are shown. The name of the homologue is shown by GenBank accession number, except for *C. albicans*, which is shown by its orf number. The percentage of sequence identity was calculated by PIR pairwise alignment (http://pir.georgetown.edu/pirwww/search/pairwise.html) against the full-length sequence.

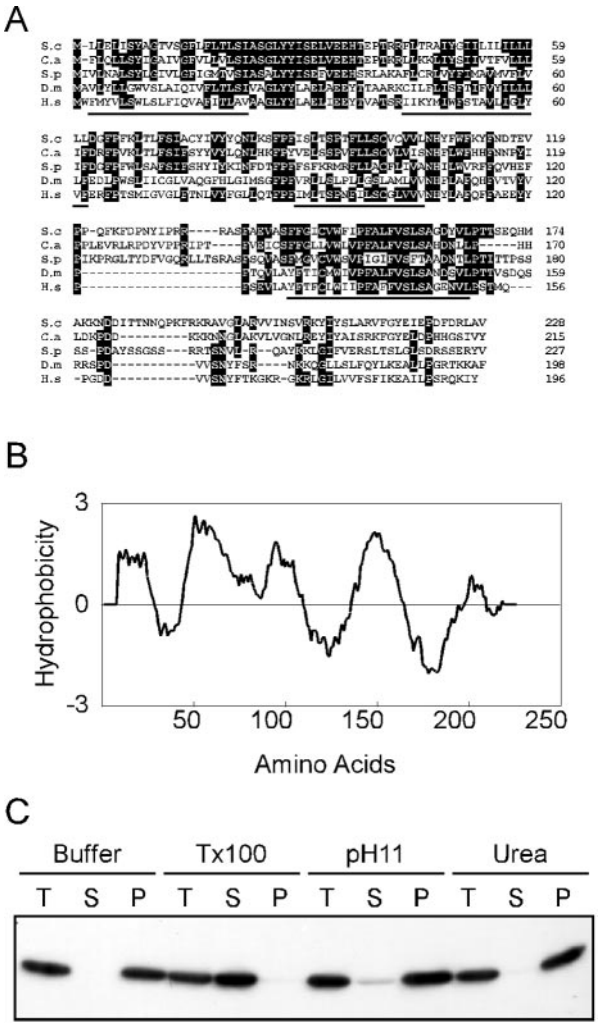

FIG. 3. Sequence homology, hydropathy profiles, and membrane association of Svp26 protein. (A) Amino acid sequence alignment of the Svp26 homologues listed in Table 6. Data are from *S. cerevisiae* (S.c), *Candida albicans* (C.a), *Schizosaccharomyces pombe* (S.p), *Drosophila melanogaster* (D.m), and *Homo sapiens* (H.s). Identical amino acids are shown in reverse characters, and predicted transmembrane domains are indicated by bars. (B) The hydrophobicity index was calculated according to a method described previously by Kyte and Doolittle (30), with a window size of 19 amino acids. (C) Cells producing Svp26-HA_3 were converted to spheroplasts and osmotically lysed. The lysate was subjected to either 1% Triton X-100 (Tx100), 0.1 M Na_2CO_3 (pH11), 2 M urea (Urea), or mock (Buffer) treatment on ice for 15 min. Lysate (T) was then centrifuged at 100,000 × *g* to yield a high-speed membrane pellet (P) and supernatant (S). Proteins were analyzed by immunoblotting against anti-HA antibody.

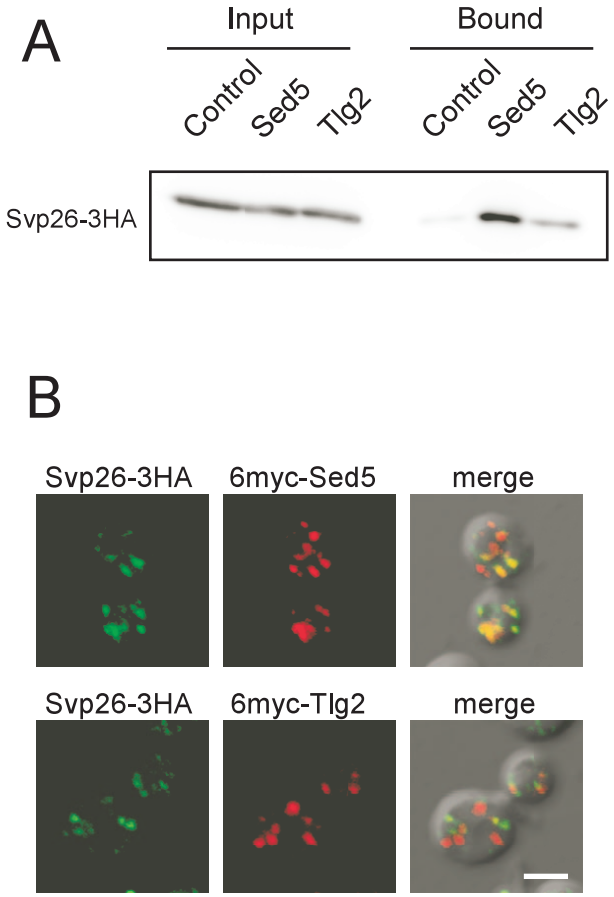

FIG. 4. Svp26-HA_3 (Svp26-3HA) protein in the immunoisolated compartments and in the cell detected by immunofluorescent microscopy. (A) The centromeric plasmid that produces Svp26-HA_3 was introduced into KA31a (control), CJY119 (Sed5 compartment), and HIY1 (Tlg2 compartment). Immunoisolation of the Golgi compartments and protein extraction were done as described in the legend of Fig. 1. Proteins were analyzed by immunoblotting with an anti-HA monoclonal antibody. Samples derived from 1 and 2.5 ml of the yeast culture were loaded in the input and bound lanes, respectively. (B) Svp26-HA_3 (green) and Myc-tagged Golgi tSNAREs (red) were visualized as described in Materials and Methods. The immunofluorescent images were merged with the Nomarski image. The bar represents 5 μm.

detected in the ring structures which overlap with the Kar2 signal. By examining 200 cell images, the partial overlap of Kar2 and Myc_6-Ktr3 signals was found in only 18% of wild-type cells, but it was found in 76% of the cells without Svp26 protein. To confirm this by biochemical means, each cell lysate was applied to a differential centrifugation (Fig. 7B). In the presence of Svp26, Ktr3 was mainly recovered in the P100 fraction that contains Golgi, endosome, and other small compartments. In the absence of Svp26, the amount of Ktr3 in P10, which contains the ER-resident Kar2 protein, significantly increased. This is consistent with the microscopic observation that the localization of the Ktr3 protein shifted from the Golgi compartment to the ER in the absence of Svp26. Therefore, Svp26 interacts with Ktr3 and helps the residence of Ktr3 in the early Golgi compartment.

Because the loss of Svp26 had an influence on the state of a membrane protein, other Golgi proteins may also be affected. Gos1 was found in both Sed5 and Tlg2 compartments (Fig. 2 and Table 5), and green fluorescent protein (GFP)-Gos1 is functional as it complements the temperature sensitivity of the Δ*gos1* null mutant. In the wild-type cell, GFP-Gos1 exclusively localized in a punctate Golgi pattern. In contrast to this, a part of the GFP-Gos1 signal in the Δ*svp26* cell was also found in the ER, although the overlaps were much less frequently found than in the case of Ktr3 (Fig. 8). We concluded that the absence of Svp26 slightly affects the localization of Gos1.

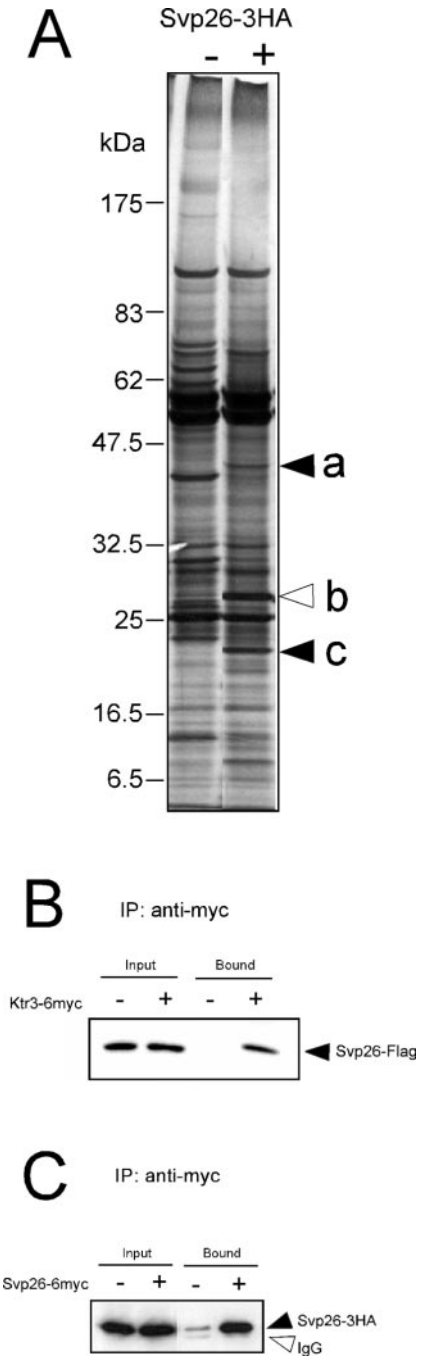

FIG. 5. Identification of the proteins that bind to the Svp26 protein. (A) A cleared lysate was solubilized with 1% Triton X-100 in the presence or absence of Svp26-HA_3. It was subjected to immunoprecipitation using anti-HA antibody, and the precipitate was analyzed by SDS-PAGE and silver staining. Bands indicated by arrowheads (a, b, and c) were cut out, and the protein was identified by peptide mass fingerprinting as described in the legend to Fig. 2. (B) Immunoprecipitates (IP) using anti-Myc antibody from the cleared cell lysate having Svp26-Flag in both the presence and absence of Ktr3-Myc_6 were examined by immunoblotting using anti-Flag antibody. (C) Immunoprecipitates using anti-Myc antibody from the cleared cell lysate having Svp26-HA_3 in both the presence and absence of Svp26-Myc_6 were examined by immunoblotting using anti-HA antibody. IgG, immunoglobulin G. In panels B and C, samples derived from 1 and 10 ml of the yeast culture were loaded in the input and bound lanes, respectively.

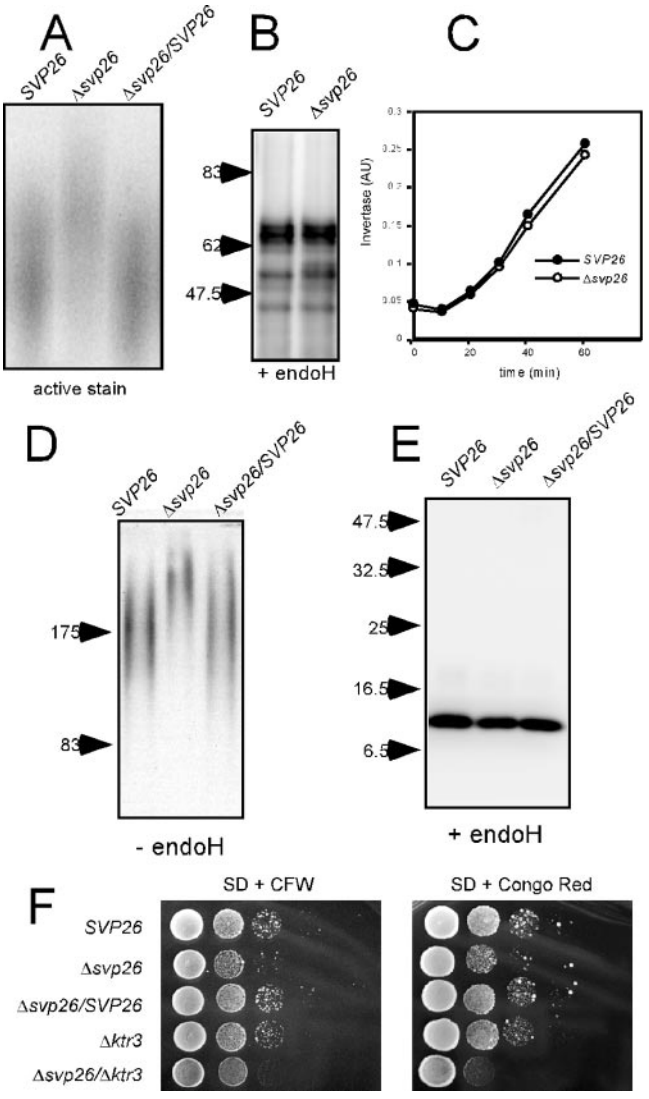

FIG. 6. Altered protein glycosylation and sensitivity to chitin-binding dyes in the absence of Svp26 protein. (A) Secretory invertase produced by KA31 (wild-type)/pRS314, HIY22 (Δ*svp26*)/pRS314, or HIY22 (Δ*svp26*)/pHI274 (a low-copy-number plasmid carrying *SVP26*) was subjected to PAGE and detected by activity staining. (B) The secretory invertase of the wild-type or Δ*svp26* cells was detected by immunoprecipitation and blotting after endoglycosidase H (endoH) digestion. (C) Kinetics of invertase secretion by wild-type and Δ*svp26* cells. (D) Hen egg white lysozyme G49N produced by wild-type, Δ*svp26*, or Δ*svp26*/*SVP26* cells was immunoblotted with the anti-lysozyme antibody. (E) The samples in panel D were digested with endoglycosidase H before SDS-PAGE. (F) Serial 10-fold dilutions of a fresh log-phase yeast culture (OD_{600} of 1) were spotted (5 μl) on solid SD medium containing 100 μg/ml of calcofluor white (CFW) or Congo red and incubated at 30°C for 2 days.

Effect of the absence of Svp26 protein in the Golgi compartments and the ER. We examined Myc_6-Sed5 by immunofluorescent microscopy in the wild-type and Δ*svp26* cells to see if this Golgi tSNARE showed any change in its localization. However, we found no differences in the microscopic images (Fig. 9). Therefore, no extensive alteration in the early Golgi compartment is likely to occur in the Δ*svp26* cells, and the localization of a part of a certain subset of Golgi proteins

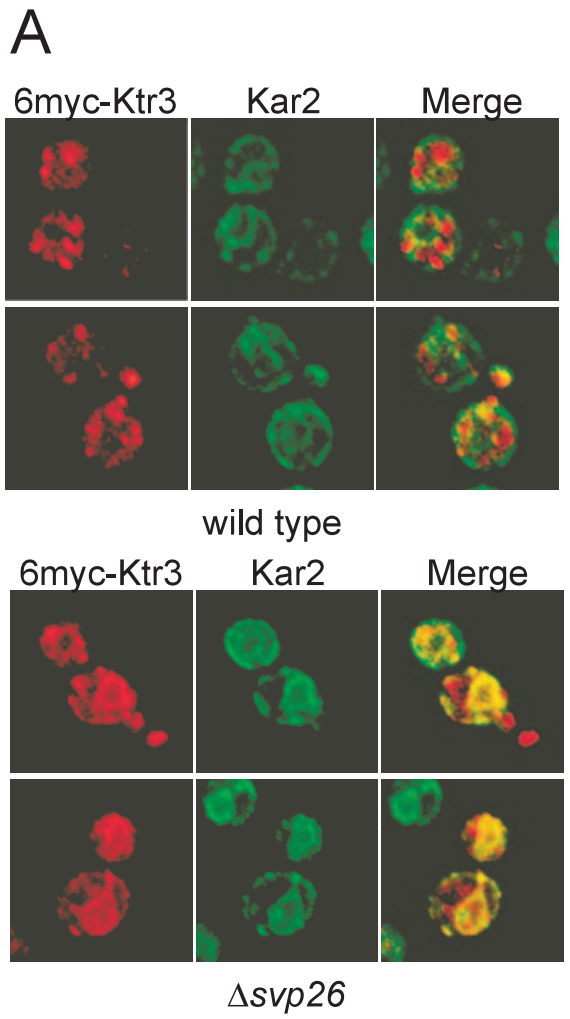

FIG. 7. Alteration of intracellular localization of Ktr3 protein in the absence of Svp26 protein. (A) Immunofluorescent double staining of Myc_6-Ktr3 and Kar2 proteins in wild-type and Δ*svp26* cells. (B) The lysate of wild-type, Δ*svp26*, or Δ*svp26*/*SVP26* cells (T) was fractionated by differential centrifugation at 10,000 × *g* (P10) and 100,000 × *g* (S100 and P100). Aliquots of fractions were subjected to SDS-PAGE and immunoblot analysis with antibodies against marker proteins shown to the left.

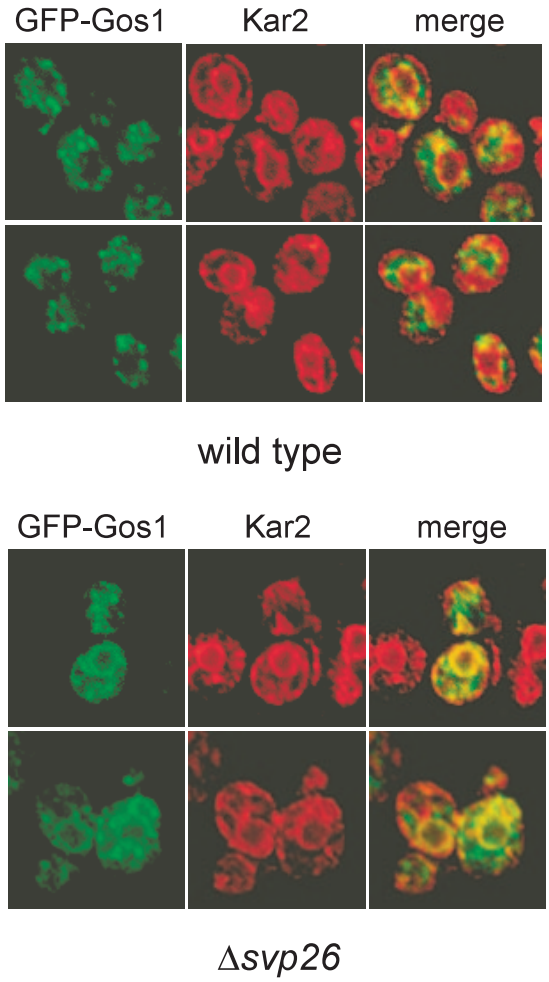

FIG. 8. Localization of Gos1 protein in both the presence and absence of Svp26 protein. The wild-type and Δ*svp26* cells producing GFP-Gos1 were examined by indirect immunofluorescence microscopy using anti-GFP mouse and anti-Kar2 rabbit antisera. Images of the same field were merged in the right panel. The images of the wild-type cells are mostly similar, but those of Δ*svp26* cells are significant representatives.

including Ktr3 and Gos1 is shifted to the ER. To see this issue biochemically, we did subcellular fractionation experiments using lysates of the Δ*svp26* and Δ*svp26*/*SVP26* cells by sucrose density gradient centrifugation. The distributions of various marker proteins were determined by immunoblotting. As shown in Fig. 10, the distribution of Sed5, Van1, and Pep12 had peaks in the middle of the gradient (fractions 5 to 7) and was almost the same between the Δ*svp26* and Δ*svp26*/*SVP26* cells. This is consistent with the observation that the immunofluorescent profile of Sed5 did not change significantly in the Δ*svp26* or Δ*svp26*/*SVP26* cells. Scs2, an ER membrane protein, was distributed much closer to the bottom than most of the Golgi proteins, with its peak at fraction 10 in the Δ*svp26*/*SVP26* cells. The position of Scs2 slightly shifted to the top of the gradient, with its peak at fraction 9 in the absence of Svp26 protein. In contrast to Sed5 and Van1, while the signal peak of Ktr3 was at fraction 6 in the Δ*svp26*/*SVP26* cell, it was at fraction 9 with a shoulder at fractions 6 to 8 in the Δ*svp26* cell. A similar shift was also found in the case of Gos1. These results are consistent with the immunofluorescence data that suggested that a portion of Ktr3 and Gos1 moved from the Golgi compartments to the ER in the absence of Svp26. Although the peaks of Sed5, Van1, and Pep12 did not alter in the gradients, the band intensities of these proteins in fraction 9 were higher in the absence of Svp26 than in its presence. These shifts of distribution in the gradient were repeatedly observed in independent experiments. It may be possible that some

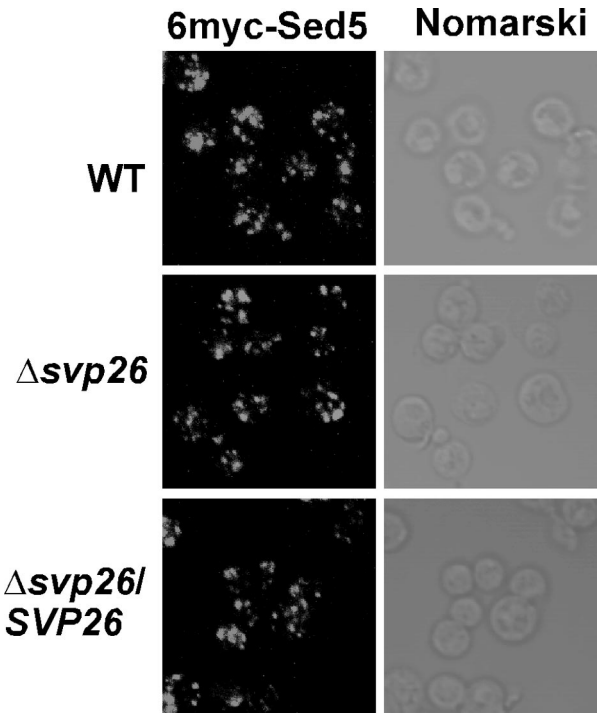

FIG. 9. Intracellular localization of the Sed5 protein in both the presence and absence of the Svp26 protein. A Δ*svp26*::*KanMX4* derivative (Δ*svp26*) of CJY119 (wild type [WT]) in which *SED5* was replaced with *6myc-SED5* was constructed and used for indirect immunofluorescence microscopy using anti-Myc MAb 9E10 to detect Myc_6-Sed5. A plasmid rescue control (Δ*svp26*/*SVP26*) was also examined. Immunofluorescence and Nomarski images of the same field are shown.

minor alterations occurred in the organelle structure and composition that were not detectable by immunofluorescence microscopy analysis.

Stable localization of Svp26 in the early Golgi compartment. There are two possible explanations for the fact that Svp26 is required for Ktr3 to stay in the early Golgi compartment. Many Golgi proteins recycle between their major resident compartments and the ER. Ktr3 is likely to recycle between the early Golgi compartment and the ER. So Svp26 may help the efficient transport of Ktr3 from the ER to the Golgi compartment, or it may help the retention in the Golgi compartment against the retrieval to the ER. In the former case, although Svp26 is mainly found in the Sed5 compartment, it may transiently move to the ER, interact with Ktr3, and help Ktr3 to leave the ER and go to the Golgi compartment. To examine this possibility, we did a "secretion block" experiment. In a *sec12*^{ts} mutant, the budding of transport vesicles from the ER was arrested at a nonpermissive temperature, causing the recycling proteins to gradually disappear from the Golgi compartment and accumulate in the ER (60, 74). As shown in Fig. 11, most of the Myc-Emp47 signal was found in the circle and ribbon structures which represent the ER within 60 min at 35°C (60). Under the same conditions, the signal of Svp26 was still found only in the punctate pattern as at 24°C, and no signal in the ER pattern was found. Similar results were obtained using a *sec23*^{ts} mutant. Therefore, we conclude that the Svp26 protein stays mainly in the Golgi compartments and does not frequently recycle between the ER and early Golgi compartment. This suggests that Svp26 plays a role in the retention of Ktr3 in the Golgi compartment rather than in its recycling.

DISCUSSION

Vesicles that have a marker membrane protein on their surface can be effectively collected from the total cell lysate by immunoadsorption. We adapted this strategy to isolate the early and late Golgi compartments using tSNAREs Sed5 and Tlg2, respectively. The presence or absence of other marker proteins guaranteed the quality of isolated compartments (Table 5). All components of the COPII vesicle reported by Otte et al. (44) except Erv29 were found in the Sed5 compartment. Erv29 should have been found near bands 19 to 21 (Fig. 2), but the other abundant proteins might have prevented its detection. In contrast to this, none of these proteins except Erp2 was found in the Tlg2 compartment. The presence of COPII components in the Sed5 compartment is consistent with the fact that the Sed5 compartment is very close to the ER.

A number of mannosyltransferases were found in the isolated compartments. The glycosylation of N-linked carbohydrates occurs sequentially in the Golgi compartment; Och1 initiates α-1,6-mannose elongation, and complexes M-Pol I (Van1 and Mnn9) and M-Pol II (Anp1, Mnn9, Mnn10, Mnn11, and Hoc1) mediate the α-1,6- and α-1,2-mannose addition (67). Subsequently, Mnn2, Mnn5, Kre2, Ktr1, Ktr2, Ktr3, and Yur1 are supposed to add α-1,2-mannose, Mnn6 adds mannosylphosphate, and Mnn1 transfers terminal α-1,3-mannose, which prevents further mannose addition (15, 36). Each of the sequential modifications is catalyzed in a distinct Golgi compartment (8). In our present results, Van1, Anp1, Mnn10, Mnn11, and Mnn2 were found only in the Sed5 compartment; Kre2, Ktr1, Ktr3, and Ktr4 were found in both compartments; and Mnn5 was found only in the Tlg2 compartment. This distribution is in good accordance with the action sequence of glycosyltransferases localized in the different compartments (15).

In the Tlg2 compartment, we found proteins related to vacuolar protein sorting, Vps10 (14) and Vps18 (48), and subunits of V-ATPase, Stv1 and Vma6 (41). It is consistent with the current understanding that a part of the vacuolar protein temporarily stays in the late Golgi/endosome compartment.

Akr1, Mnn2, and Kre2 were detected in pairs of different protein bands. These proteins are reported to receive a post-translational modification: Akr1 is palmitoylated (55), and Mnn2 and Kre2 are glycosylated (23, 52). Therefore, the two bands are likely to correspond to the modified and unmodified forms of a protein.

Unexpectedly, except for Arf1, the small GTPases were found in both the Sed5 and Tlg2 compartments. The small GTPases participate in the control of various processes in vesicle transport. The binding of the GTP form Sar1 to the ER membrane initiates the formation of the COPII-coated vesicles for anterograde transport. Ypt1 is necessary in the vesicle fusion to the Golgi compartment. Ypt31 and Ypt32 function in the intra-Golgi transport, and Ypt52 functions in the Golgi-to-vacuole trafficking. Sec4 is required for the fusion of the secretion vesicle membrane with the plasma membrane (68). The immunofluorescence signal of Ypt1 and Sec4 was reported to coincide with the place of their function, while nearly half of

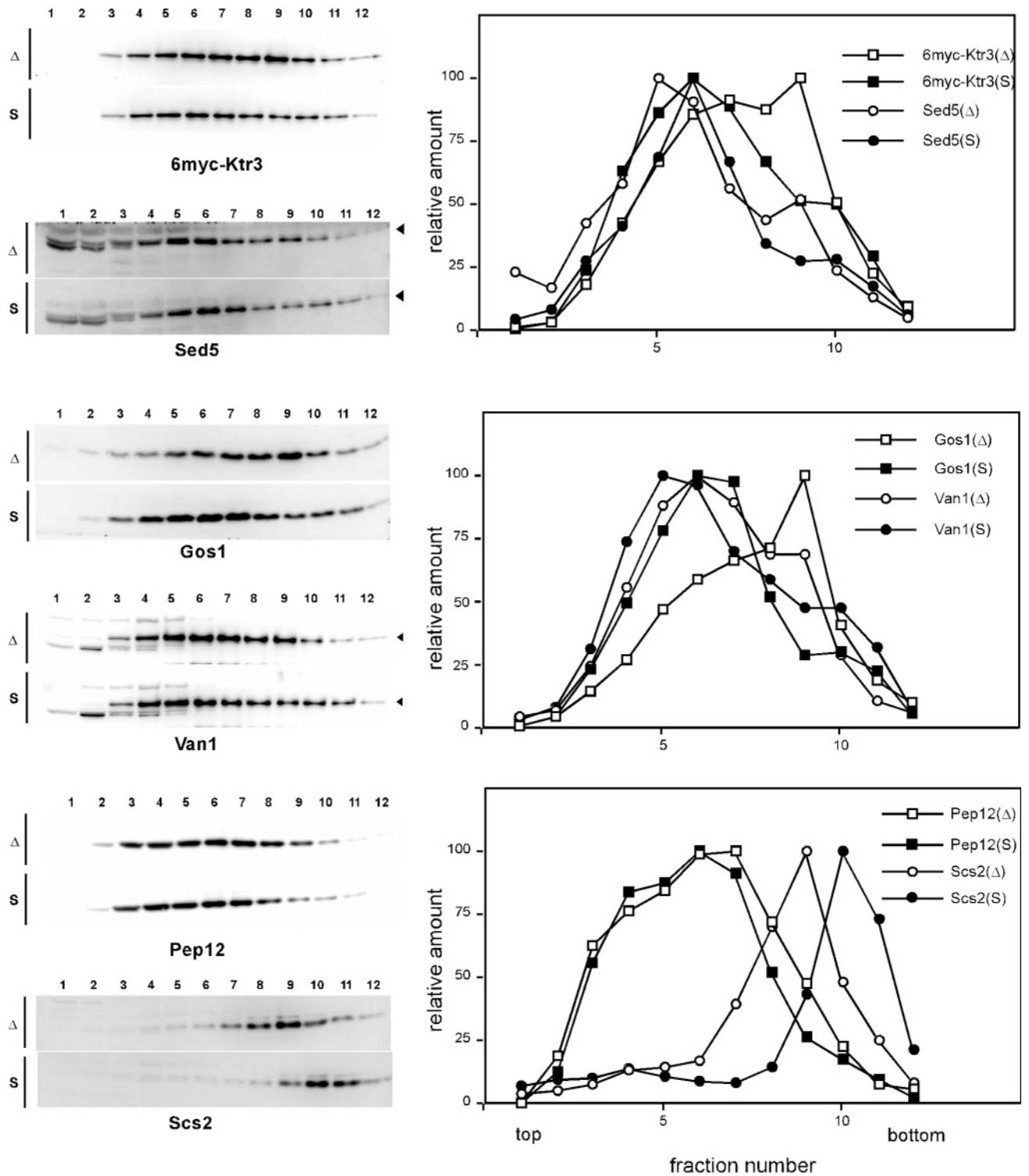

FIG. 10. Subcellular fractionation of the ER and Golgi proteins in both the presence and absence of Svp26 protein. The Δ*svp26* and Δ*svp26*/*SVP26* cells both carrying pHI258 (*6myc-KTR3 CEN6 URA3*) were converted into spheroplasts and lysed. The lysate was layered onto an 18 to 60% (wt/vol) sucrose step gradient, and subcellular organelles were separated by centrifugation as described in Materials and Methods. Proteins in each fraction were resolved on SDS-polyacrylamide gels and immunoblotted for antibodies to detect Myc_6-Ktr3, Sed5, Gos1, Van1, Pep12, or Scs2. Δ, the Δ*svp26* cells; S, Δ*svp26*/*SVP26* cells. Fraction 1, top of the gradient; fraction 12, bottom of the gradient. Arrowheads at the right of the panels of Sed5 and Van1 indicate the positions of the Sed5 or Van1 band to discriminate them from cross-reacting bands. Enhanced chemiluminescence signals were captured by an image analyzer and quantified using image analysis software. The intensities of bands were normalized by adjusting the maximum value to 100%.

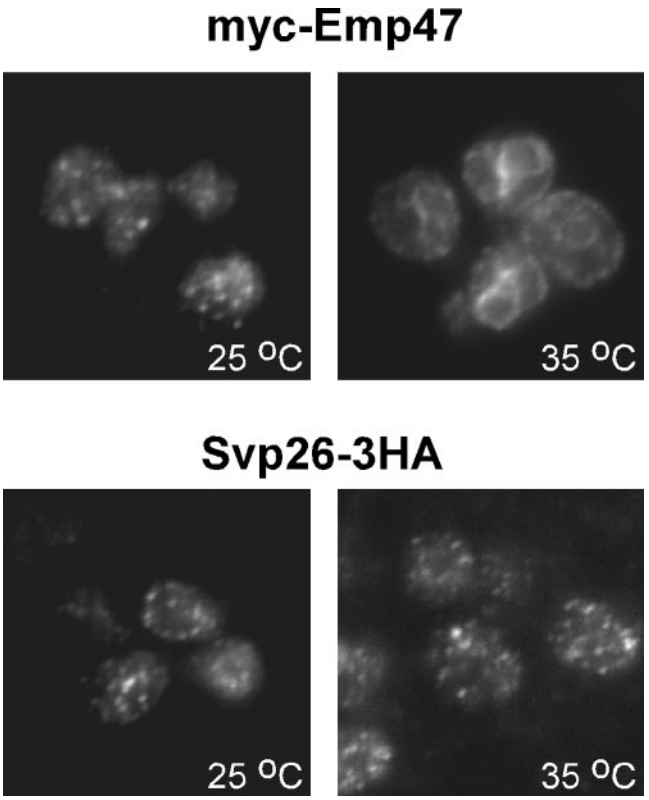

FIG. 11. Effect of "secretion block" on Svp26 in the *sec12* temperature-sensitive cells. Myc-Emp47 was used as a control protein that recycles between the ER and Golgi compartment. The *sec12-4* cells producing Svp26-HA_3 or Myc-Emp47 were incubated in the presence of 20 mg/ml cycloheximide for 1 h at either 25°C or 35°C and then processed for indirect immunofluorescence microscopy using anti-HA or anti-Myc MAb.

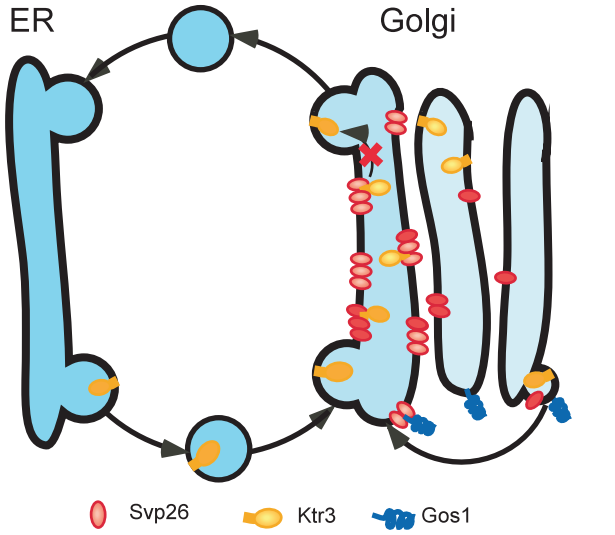

FIG. 12. Our current working hypothesis of the function of Svp26 protein in the early Golgi compartment. The integral membrane protein Svp26, having four transmembrane domains, binds to itself, forms oligomeric structures, and stays mainly in the early Golgi membrane. It binds to Ktr3 mannosyltransferase and probably also interacts with other Golgi proteins. These interactions interfere with their incorporation into the retrograde transport vesicles and thus make the interacting proteins stay in the early Golgi compartment.

them were found in the cytosol (19, 62). It is unlikely that they bound to Pansorbin cells nonspecifically. A possible explanation is that those GTPases are abundant and their localizations are not strongly restricted to the places where they function. Also, because only the activated GTPase plays its role, the localization of the proteins that regulate GTPase activity, such as the guanine-nucleotide exchange factor and the GTPase activation protein, is more important than the localization of GTPase itself. Brennwald and Novick (7) showed that by using Ypt1 and Sec4, chimera GTPases in which the hypervariable C-terminal regions that are important to determine their localization were mutually exchanged could function normally to support the growth of the mutant cell. Their results are consistent with the above-mentioned explanation that the restricted localization of GTPase itself is not so important.

Among the novel membrane proteins, Svp26 was selected for further study. As it has been reported that a mammalian homologue of Svp26 is actually produced in animal tissue (34), it is likely that it plays some functional role in the cell. Svp26 is a relatively small membrane protein, with four predicted transmembrane domains, and mainly localizes in the early Golgi compartment. Svp26 binds to itself and to Ktr3 mannosyltransferase. Ktr3 shares sequence homology with Kre2 and Ktr1 and is proposed to have redundant activity in the cell. Mislocalization of Ktr3 to the ER in the absence of Svp26 is consistent with abnormality in protein glycosylation and increased sensitivity to growth inhibition by the chitin-binding dyes calcofluor white and Congo red. However, as the Δ*ktr3* mutant did not show hyperglycosylation of invertase and was less sensitive to the dyes, the loss of Svp26 is likely to cause other abnormalities in the cell. We examined proteins in the secretory pathway extensively and found that a small portion of a Golgi SNARE, Gos1, was mislocalized in the ER. No serious change in the organization of Golgi compartments is likely to occur, because we could not find any alteration in the immunofluorescent microscope images of Sed5. In addition, an overall efficiency of transport and processing of carboxypeptidase Y was not affected by Δ*svp26* mutation (our unpublished result). However, it was suggested that a moderate structural alteration occurred in the absence of Svp26, because there was a detectable shift of distribution of the ER and Golgi proteins in our sucrose density gradient fractionation experiments (Fig. 10).

Recently, a comprehensive synthetic lethality screening found that *SVP26* showed synthetic growth defects with *EMP24* or *VPS38*, whose products work in vesicular transport (69). Emp24 is a p24 family protein that is considered to work as a receptor in the ER to incorporate the selected cargo proteins in the COPII vesicles (40) and also has a role in the retrograde transport from the Golgi compartment to ER, because a portion of Kar2 is secreted in the medium from the Δ*emp24* cell (37). Vps38 is a component of phosphatidylinositol 3-kinase complex II, which plays an important role in the endosome-to-Golgi retrograde transport (10). It is possible that the defect of these genes results in an abnormal distribution of membrane proteins in the vesicular transport compart-

ments, which is incompatible with the abnormal distribution caused by the loss of Svp26.

Golgi proteins Emp46 and Emp47 recycle between the ER and Golgi compartment and help the sorting of cargo proteins into the COPII vesicle and their exit from the ER (57, 58). In contrast to Emp46 and Emp47, our "secretion block" experiments showed that the Svp26 protein does not recycle back to the ER. Therefore, it is unlikely that Svp26 helps the exit of the early Golgi residents from the ER. Figure 12 shows our current working hypothesis. Svp26 mainly stays in the early Golgi compartment and is prevented from recycling to the ER by an as-yet-unknown mechanism. Svp26 protein binds to itself and may form oligomeric structures in the membrane, which may be responsible for the resistance to recycling as proposed in the "kin recognition" hypothesis (42). Svp26 interacts with a mannosyltransferase, Ktr3, and probably also interacts with some other Golgi proteins (Fig. 5). These interactions somehow interfere with the retrograde transport of these early Golgi compartment-resident proteins to the ER. Svp26 may keep these proteins from moving to the region where packaging of the COPI-coated vesicles occurs. In the absence of Svp26, the Ktr3 protein is transported from the early Golgi compartment to the ER more efficiently than the transport back from the ER to the Golgi compartment. Gos1 and several other Golgi proteins are also apt to be retrieved back to the ER. Therefore, Svp26 has a role in retaining a group of proteins in the early Golgi compartment. A Golgi membrane protein, Rer1, recognizes a subset of the ER membrane proteins, including Sec12 and Sec71, and helps them retrieve back to the ER when they are mistakenly transported to the early Golgi compartment (59). Svp26 is likely to work in a complementary way to prevent a subset of the early Golgi compartment-resident membrane proteins from being mistakenly transported back to the ER. The details of this retention mechanism will be uncovered by molecular dissection of the Svp26 protein and its interacting molecules.

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