Localization of GDP-mannose transporter in the Golgi requires retrieval to the endoplasmic reticulum depending on its cytoplasmic tail and coatomer

Masato Abe, Yoichi Noda, Hiroyuki Adachi and Koji Yoda*

Department of Biotechnology, University of Tokyo, Yayoi, Bunkyo-Ku, Tokyo 113-8657, Japan

*Author for correspondence (e-mail: asdfg@mail.ecc.u-tokyo.ac.jp)

Accepted 16 August 2004
Journal of Cell Science 117, 5687-5696 Published by The Company of Biologists 2004
doi:10.1242/jcs.01491

Summary

The *Saccharomyces cerevisiae* GDP-mannose transporter (GMT) encoded by the essential gene *VRG4/VIG4* is a member of the nucleotide-sugar transporter family in the Golgi apparatus. We examined GMT in the secretory mutant cells to investigate the mechanism of its localization in the Golgi. At the nonpermissive temperature, most GMT was found in the endoplasmic reticulum of *sec23*^{ts} cells, which have defective COPII, and in the vacuole of *sec21*^{ts} cells, which have defective COPI. The C-terminal hydrophilic peptide of GMT that is exposed to the cytosol binds to Ret2p, a subunit of the COPI coat. Mutant peptide derivatives that have lost a cluster of lysine in the vicinity of the transmembrane domain had reduced binding activity to Ret2p and the GMT with this sequence was delivered to the vacuole. Our results indicate that GMT escapes from delivery to the vacuole by recycling to the endoplasmic reticulum and retrieval requires the lysine-rich C-terminal tail that can bind to the COPI coat.

Key words: Yeast, Golgi, GDP-mannose transporter, COPI, Recycling

Introduction

In the eukaryotic cell, the secretory proteins are transported to the cell surface through the endoplasmic reticulum (ER) and Golgi apparatus. During the transport process, most of the secretory proteins are modified by oligosaccharides. The initial transfer of carbohydrate to the polypeptide from dolichol-sugar intermediates occurs in the ER and further addition and modification of oligosaccharides occurs mainly in the Golgi. The Golgi glycosylation is conducted by a variety of glycosyltransferases and the donor substrate is nucleotide-sugar. The nucleotide-sugar is synthesized in the cytoplasm and incorporated in the Golgi lumenal space by a specific nucleotide-sugar transporter (NST).

In the Golgi of *Saccharomyces cerevisiae*, the glycosylation occurs solely by mannose and GDP-mannose is the essential substrate of various mannosyltransferases. Several Golgi mannosyltransferases have been identified. Och1p is the crucial α-1,6-mannosyltransferase, which initiates the elongation of outer mannosyl chain of the *N*-oligosaccharide core (Nakanishi-Shindo et al., 1993). Two hetero-oligomeric complexes that contain Mnn9p as a common subunit, Mnn9p-Van1p and Mnn9p-Anp1p-Hoc1p-Mnn10p-Mnn11p [named MTase I and II by Jungmann et al. (Jungmann et al., 1999) or the V and A complexes by Kojima et al. (Kojima et al., 1999), respectively], transfer either α-1,6- or α-1,2-mannose to the acceptor *N*-glycosyl groups (Jungmann and Munro, 1998). The latter complex might also participate in elongation of *O*-mannosyl groups (Kojima et al., 1999). Mnt1p, Mnn2p and Mnn3p function as α-1,2-mannosyltransferase (Hausler and Robbins, 1992; Rayner and Munro, 1998). The elongation of α-1,6-mannose outer chain with α-1,2-mannose branches is terminated by the addition of α-1,3-mannose by α-1,3-mannosyltransferases Mnt2p, Mnt3p, Mnt4p and Mnn1p (Romero et al., 1999; Yip et al., 1994). All these mannosyltransferases are type-II membrane proteins and their catalytic domains localize in the Golgi lumen. The GDP-mannose transporter (GMT) moves GDP-mannose into the Golgi lumen from the cytosol and moves GMP out. GMT is a member of the NST family (Berninsone and Hirschberg, 2000), which has similar multiple transmembrane domains (TMD) and functions as an antiporter of nucleotide-diphosphate–sugar and nucleotide-monophosphate in the organelle membrane (Abeijon et al., 1996; Eckhardt et al., 1996; Goto et al., 2001; Ma et al., 1997; Miura et al., 1996; Poster and Dean, 1996; Tabuchi et al., 1997).

The mannosyltransferases and GMT localize to the Golgi apparatus for precise glycosylation but the mechanism is still not clear. Two models have been proposed for stable localization of membrane protein – the 'kin recognition' model (Nilsson et al., 1994; Nilsson et al., 1993) and the 'lipid bilayer' model (Bretscher and Munro, 1993; Munro, 1995). The kin recognition model proposes that the resident proteins form a large hetero-oligomer by protein-protein interaction in the organelle membrane and are consequently prevented from entering the budding vesicles destined to another organelle. The lipid bilayer model proposes that the fit between the length of TMD and the thickness of lipid bilayer of each organelle membrane determines the localization because each organelle has its specific lipid composition. In contrast to the elaborative mechanism for stable localization, proteins in the route of

traffic are inevitably subject to the dynamic vesicle transport. Therefore, retrieval or recycling is important for ensuring localization, as reported for the p24-family proteins (Fiedler et al., 1996), the t-SNARE Sed5p (Wooding and Pelham, 1998), the glycosyltransferase Mnn9p complexes (Todorow et al., 2000) and the retrieval receptor Rer1p (Sato et al., 2001).

Here, we examined the mechanism for Golgi localization of GMT in *S. cerevisiae*. Our results indicate that GMT also recycles between the ER and Golgi, although it apparently localizes to the Golgi. Especially, a cluster of lysine residues in the C-terminal tail of GMT is important for the recognition of GMT by COPI coat and for GMT to escape from delivery to the vacuole.

Materials and Methods

Strains and media

Escherichia coli DH5α (F⁻, *ϕ80lacZΔM15 supE44 ΔlacU169 hsdR17 recA1 endA1 gyrA96 thi-1 relA1*) was used in plasmid construction, and BL21 (F⁻, *ompT hsdS gal dcm*) in production of glutathione-*S*-transferase (GST) fusion proteins. *S. cerevisiae* KA31-1A (*MATα his3 leu2 trp1 ura3*) was used. The *sec* mutant strains S18 (*MATα sec18-1 ura3*), S23 (*MATα sec23-1 ura3*) and S21 (*MATα sec21-1 ura3*) were kindly provided by R. Sheckman (University of California, Berkeley). K2-1C (*MATα vig4-1 ura3-52 lys2-801 his3-Δ200 leu2-Δ1*) was used as the mutant with defective GMT.

Yeast cells were grown in YPD [1% Bacto yeast extract (Difco), 2% Bacto peptone (Difco), 2% glucose] or SD [0.67% Bacto yeast nitrogen base without amino acids (Difco), 2% glucose] medium containing required supplements or 0.2% yeast extract where indicated. Solid media were made with 2% agar.

Plasmid

The plasmid used in this study is listed in Table 1. The DNA fragment containing the *VRG4* open reading frame was obtained by PCR amplification of the yeast genomic DNA using LA Taq polymerase (Takara, Kyoto, Japan). Primers were designed to place a *Bam*HI site at the 5′ end and an *Xho*I site at the 3′ end. The amplified DNA was ligated into the pUC119 or pBluescript II SK for subcloning. The *VIG4* and *vig4-1* mutant derivative were cut out and subcloned in expression vectors, having the *VIG4* promoter in pMA82 [*CEN ARS URA3*, N-terminal green fluorescent protein (GFP) tagging], pMAV420 (*CEN ARS URA3*, N-terminal Myc_6 tagging) and pMA71 (*CEN ARS URA3*, C-terminal Myc_6 tagging), if necessary.

GST fusion protein was produced by using the expression vector pGEX4T-3 (Pharmacia). The oligonucleotides encoding appropriate peptides were prepared so as to have a *Bam*HI site at the 5′ end and an *Xho*I site at the 3′ end, and were inserted in the expression vector.

Immunoprecipitation

Immunoprecipitation of tagged proteins was done as follows. Log-phase yeast cells (10 ml) were collected and broken by shaking with glass beads in 1 ml lysis buffer [20 mM HEPES-KOH, pH 6.8, 150 mM potassium acetate, 200 mM sorbitol, 2 mM EDTA, 1 mM

Table 1. Plasmids used in this study

Plasmid	Description	Source
pRS416	*URA3 CEN6* yeast vector	
pRS426	*URA3 2μ* yeast vector	
pMA12	Vig4-6myc in pRS416	Abe et al., 1999
pMA50	Vig4Δ12-6myc in pRS416	Abe et al., 1999
pMAV426	6myc-Vig4 in pRS416	This study
pMAV425	6myc-Vig4Δ12 in pRS416	This study
pMAV424	6myc-Vig4Δ4 in pRS416	This study
pSV128	Van1-6myc in pRS416	Hashimoto and Yoda, 1997
pSV314	Anp1-6myc in pRS416	Hashimoto and Yoda, 1997
pMAV502	Ret2-3HA in pRS416	This study
pMA89	GFP-Vig4 in pRS416	This study
pMA86	GFP-Vig4-Δ12 in pRS416	This study
pMAV594	GFP-Vig4-Δ12-[AQPLRK] in pRS416	This study
pMAV587	GFP-Vig4-[SRAAAS] in pRS416	This study
pMAV595	GFP-Vig4-Δ12-[QQQQQQAQPLRK] in pRS416	This study
pMAV593	GFP-Vig4-Δ12-[KQKKQQAQPLQQ] in pRS416	This study
pMAV596	GFP-Vig4-Δ12-[KQQQQQAQPLRK] in pRS416	This study
pMAV597	GFP-Vig4-Δ12-[QQKQQQAQPLRK] in pRS416	This study
pMAV598	GFP-Vig4-Δ12-[QQQKQQAQPLRK] in pRS416	This study
pMAV599	GFP-Vig4-Δ12-[QQKKQQAQPLRK] in pRS416	This study
pMAV600	GFP-Vig4-Δ12-[KQKQQQAQPLRK] in pRS416	This study
pMAV601	GFP-Vig4-Δ12-[KQQKQQAQPLRK] in pRS416	This study
pMAV592	GFP-Vig4-Δ12-[KKLETPKKTN] in pRS416	This study
pMAV494	GST-KKLETPKKTN (Wbp1)	This study
pMAV510	GST-KKLETPKKTN-SRAAAS (Wbp1)	This study
pMAV515	GST-KQKKQQAQPLRK-SRAAAS	This study
pMAV518	GST-KQKKQQAQ	This study
pMAV520	GST-KQKKQQAQPLRK	This study
pMAV524	GST-KQQQQQAQPLRK	This study
pMAV525	GST-QQKKQQAQPLRK	This study
pMAV526	GST-KQKQQQAQPLRK	This study
pMAV527	GST-KQQKQQAQPLRK	This study
pMAV561	GST-KQKKQQAQPLQQ	This study
pMAV562	GST-AQPLRK	This study
pMAV563	GST-QQQQQQAQPLRK	This study
pMAV564	GST-QQQKQQAQPLRK	This study
pMAV565	GST-QQKQQQAQPLRK	This study

phenylmethylsulfonyl fluoride (PMSF), 1/500 volume of a protease inhibitor cocktail (1 mg ml^{-1} each of leupeptin, chymostatin, pepstatin, aprotinin and antipain)]. The lysate was centrifuged at 500 ***g*** for 5 minutes to remove debris. The 900 µl supernatant was collected and 0.5% (w/v) CHAPS was added to solubilize the membrane. After the lysate was incubated at 4°C for 30 minutes, it was centrifuged at 500 ***g*** for 10 minutes. The supernatant was collected ('Total') and mixed with 1 µl antibody and incubated at 4°C for 1 hour. Protein-A/Sepharose beads were added and the mixture was incubated at 4°C overnight. After the mixture was centrifuged at 500 ***g*** for 1 minute and the supernatant was collected ('Unbound'). The beads were washed ten times with lysis buffer containing 0.5% (w/v) CHAPS. Finally, sodium dodecyl sulfate (SDS) sample buffer [50 mM Tris-HCl, pH 6.8, 10% (v/v) glycerol, 1% (w/v) SDS, 4% (v/v) 2-mercaptoethanol] was added to analyse by 10% SDS-PAGE and western blotting with anti-Myc monoclonal antibody (9E10; Berkeley Antibody) or anti-Ret2p antiserum (a kind gift of F. Letourneur, University of Lyon, France).

Immunofluorescence

Cycloheximide (0.1 mg ml^{-1}) was added to the log-phase cells grown in 10 ml of YPD and incubation was continued at 37°C for 20 minutes or 40 minutes, if necessary. Then, cells were fixed by adding 1 ml 37% formaldehyde and 330 µl 2% NaN_3 directly to the medium. The cells were collected and fixed with 3.7% formaldehyde in buffer A (100 mM potassium phosphate, pH 7.3, 10 mM NaN_3) for 90 minutes. The cells were washed twice with buffer A and once with buffer B (1.2 M sorbitol, 100 mM potassium phosphate, pH 7.3) before being resuspended in five volumes of buffer B containing 25 mM 2-mercaptoethanol and 0.1 mg ml^{-1} Zymolyase 100-T (Seikagaku Kogyo, Tokyo, Japan). Samples were incubated for 30 minutes at 37°C, washed with buffer B, resuspended in buffer B containing 0.1% Triton X-100 and then incubated for 10 minutes on ice. After being washed with buffer B, cells were pipetted onto polylysine-coated microscope slides. Anti-GFP and anti-Myc sera were diluted to 1/500 in buffer C (1 mg ml^{-1} bovine serum albumin, 0.1% Tween 20 in PBS) and applied to the slide. Incubation was carried out for 60 minutes at room temperature. Slides were washed and incubated with appropriate secondary antibodies (FITC-conjugated goat anti-mouse IgG, diluted to 1/200; Cappel) for 30 minutes, washed with buffer C and mounted. Images were obtained using an AX-80 microscope (Olympus).

Subcellular fractionation

Cells were suspended in sorbitol buffer (0.9 M sorbitol, 10 mM HEPES-KOH, pH 7.4) containing 10 mM sodium azide and 25 mM 2-mercaptoethanol, and incubated with lyticase at 30°C for 30 minutes. Spheroplasts were collected, washed twice with sorbitol buffer containing sodium azide, and lysed in the ice-cold lysis buffer. After unbroken cells were removed by centrifugation at 500 ***g*** for 5 minutes, the lysate was centrifuged at 7000 ***g*** for 3 minutes to obtain the pellet (P7) fraction. The supernatant was further centrifuged at 100,000 ***g*** for 1 hour to generate the pellet (P100) and supernatant (S100) fractions.

Invertase staining

The log-phase cells in 10 ml of YPD were collected and incubated in 10 ml YPS medium for 2 hours at 30°C. Then, the cells were washed with sorbitol buffer before being resuspended in 200 µl sorbitol buffer containing 25 mM 2-mercaptoeathanol and 0.1 mg ml^{-1} Zymolyase 100-T. Cells were incubated at 37°C for 30 minutes and then removed by centrifugation. The supernatant was mixed with 20% volume of ECB buffer (10 mM HEPES-KOH, pH 7.4, 20% glycerol) and heated at 50°C for 10 minutes. Samples were subjected to 7.5% SDS-PAGE. The running gels were washed five times with distilled water and incubated in the prewarmed sucrose solution (0.1 M sucrose, 0.1 M sodium acetate, pH 5.2) for 60 minutes at 37°C. After being washed twice with distilled water, the reducing sugar was detected with TTC solution [0.1 M NaOH, 0.2% (w/v) triphenyltetrazolium chloride]. The gel was heated in a microwave oven to develop colour and then washed with 10% acetic acid.

GST pull-down assay

GST chimera proteins were prepared as follows. Oligonucleotide fragments encoding the authentic sequence of C-terminal peptide of GMT (12 residues: KQKKQQAQPLRK) or its mutant variations were inserted into the bacterial expression vector pGEX4T-3. *E. coli* transformants were grown in 100 ml nutrient broth [1% peptone, 0.3% meat extract, 0.5% NaCl; 1.8% (w/v) dry mix (Eiken Kagaku, Tokyo, Japan)] at 25°C until the optical density at 550 nm reached 0.2. Isopropylthio-β-galactoside (0.25 mM) was added and incubation was continued at 25°C for 4 hours. The cell lysate was prepared by sonication in 1 ml STB buffer [10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 10 mg ml^{-1} lysozyme, 1 mM PMSF, 1/500 volume protease inhibitors cocktail (1 mg ml^{-1} each of leupeptin, chymostatin, pepstatin, aprotinin and antipain)], and was centrifuged at 100,000 ***g*** for 10 minutes. The 900 µl supernatant was collected and 1% (w/v) Triton X-100 was added. The GST fusion proteins were absorbed to 50 µl glutathione/Sepharose-4B beads (Pharmacia) at 4°C for 1 hour. After washing five times with 1 ml ice-cold PBS, the beads and absorbed proteins were stored at 4°C.

Yeast producing Ret2p-3HA was broken by shaking with glass beads in HEPES buffer (50 mM HEPES-HCl, pH 7.3, 90 mM KCl) containing 0.5% (v/v) Triton X-100 and the lysate was precleared with 50 µl glutathione/Sepharose-4B beads. The precleared lysates were applied to the GST fusion proteins bound to beads. After incubation at 4°C for 16 hours, beads were washed six times in HEPES buffer containing 0.1% (v/v) Triton X-100. The bound proteins were eluted by boiling in SDS sample buffer and separated on a 10% SDS-PAGE gel followed by western blotting with anti-haemagglutinin (anti-HA) monoclonal antibody (12CA5; Berkeley Antibody).

Results

GMT is recycling between the ER and Golgi apparatus

Yeast GMT predominantly localizes to the Golgi apparatus as detected by indirect immunofluorescent staining (Abe et al., 1999). When the mechanism of the localization of a Golgi-resident protein in the stream of vesicular traffic is considered, retrieval from the later compartment to the earlier one with the aid of retrograde transport vesicles is often important. Recent works showed that yeast Golgi proteins including Emp47p and Rer1p undergo recycling between the ER and Golgi compartments (Sato et al., 2001; Schroder et al., 1995). Therefore, we sought to examine whether GMT also follows this mechanism. The yeast temperature-sensitive secretion mutants of vesicular traffic are useful to examine the intracellular movement of a particular protein by seeing its localization when the vesicular traffic is arrested at various stages.

We visualized GMT in the *sec18*^{ts} mutant, in which the yeast homologue of NSF is inactivated and the vesicle fusion at all stages is blocked at the nonpermissive temperature (Graham and Emr, 1991). A control experiment using a wild-type yeast showed that temperature shift from 25°C to 37°C had little effect on the punctate localization of GMT (Fig. 1A, WT). When the *sec18*^{ts} mutant was incubated at 37°C for 20 minutes, the bright spots of GMT observed at 25°C disappeared and

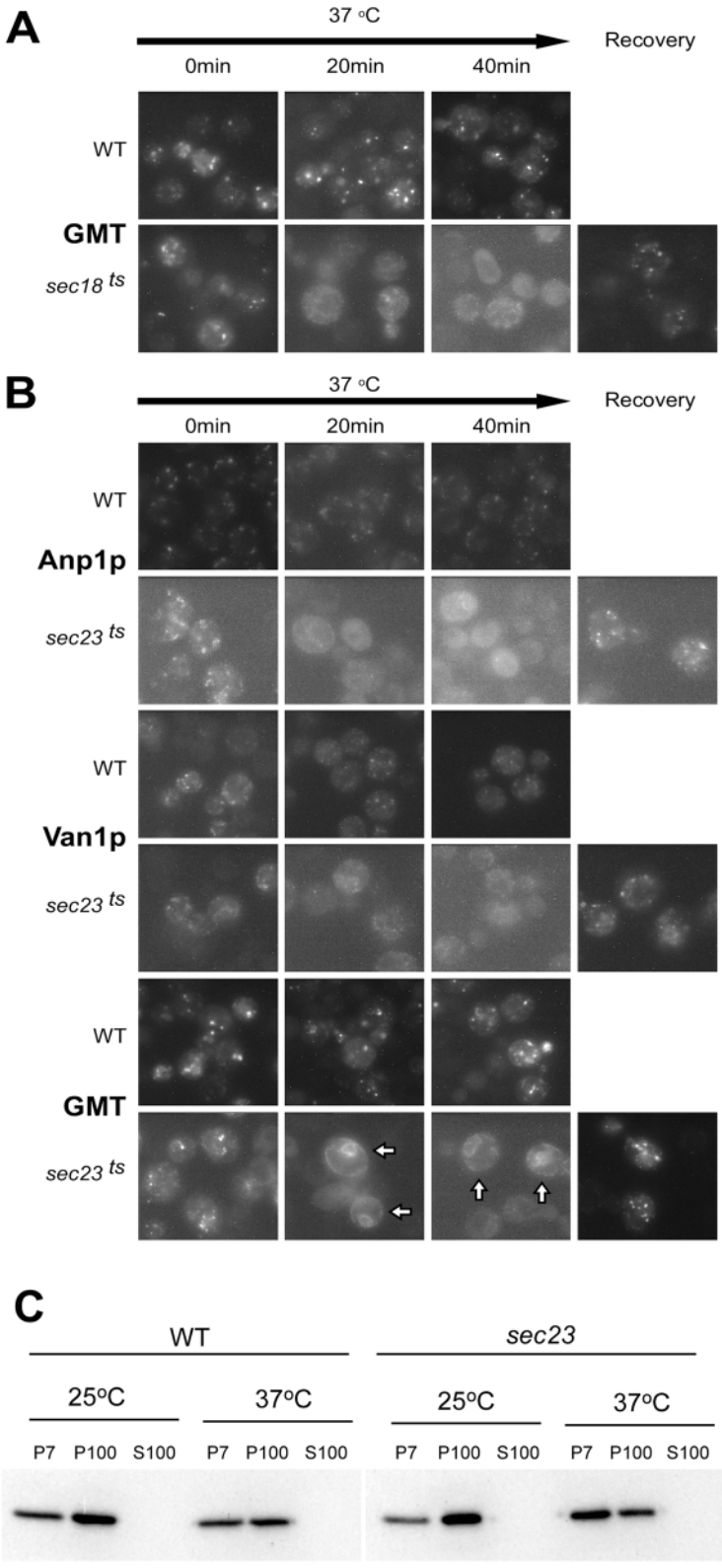

Fig. 1. GMT recycles between the ER and Golgi apparatus. (A,B) Yeast cells producing the Myc_6-tagged GMT, Anp1p or Van1p from CEN plasmid were fixed with formaldehyde at the time indicated after the shift to 37°C and prepared for indirect immunofluorescence staining using anti-Myc monoclonal antibody 9E10 and FITC-conjugated affinity-purified goat anti-mouse-IgG. Cells were treated with cycloheximide (0.1 mg ml^{-1}) before being shifted to 37°C. After 20 minutes at 37°C, the culture was divided in two; one portion was kept at 37°C and the other was shifted back to 25°C and incubated for 20 minutes to see the reversibility of localization (Recovery). (A) Wild-type (WT) and *sec18*^{ts} mutant cells expressing Myc_6-GMT. (B) Wild-type and *sec23*^{ts} mutant cells expressing Myc_6-GMT, Myc_6-Anp1p and Myc_6-Van1p. (C) Subcellular fractionation of the wild type and *sec23*-mutant-expressing Myc_6-GMT. Medium-speed pellet (P7), high-speed pellet (P100) and high-speed supernatant (S100) were prepared, and GMT was detected by immunoblotting.

finely punctate fluorescence was dispersed in the cytoplasm (Fig. 1A, *sec18*^{ts} 20 minutes). When the temperature was returned to 25°C after 20 minutes at 37°C, GMT exhibited the punctate Golgi pattern again within 20 minutes (Fig. 1A, *sec18*^{ts} recovery). In the *sec18*^{ts} mutant at 37°C, although the small vesicles are still produced by budding from the donor compartments, they are unable to fuse with the target organelles. The change of the localization pattern of GMT from distinct dots to many very fine structures in the *sec18*^{ts} mutant is consistent with the idea that GMT leaves the Golgi compartments and enters many smaller vesicles.

Then, we examined the localization of GMT in the *sec23*^{ts} mutant. *SEC23* encodes a component of the coat protein II (COPII) complex and, when it is inactivated, generation of the COPII vesicles is arrested and the cargo accumulates in the ER. If the recycling molecules return to the ER, their localization shifts from the Golgi to the ER in the *sec23*^{ts} mutant at 37°C. Cycloheximide was added in the culture to block protein synthesis and entry of new GMT to the ER during the temperature-shift experiment. GMT localized to the punctate structures at the time of shift in this mutant (Fig. 1B). After 20 minutes at 37°C, GMT was detected predominantly in the central ring structure and strings at the periphery of the cell, indicating that the localization of GMT was shifted from the Golgi to ER. When the temperature was shifted back to 25°C, GMT returned to the punctate structures. To confirm this shift of localization, we examined GMT by subcellular fractionation and immunoblot analysis. When the *sec23*^{ts} mutant cells were incubated at 37°C, more GMT was found in the P7 fraction that contains large vesicles (Fig. 1C). From an average of two experiments, the GMT in P7 of the *sec23*^{ts} cell was estimated to be 24% at 25°C and 55% at 37°C, or it increased 2.3 times after 40 minutes at the nonpermssive temperature. These results demonstrate that GMT cycles between the ER and Golgi.

We also examined two subunit proteins of glycosyltransferases, Anp1p and Van1p, to see whether other Golgi-localized proteins related to glycosylation behave in the same way. These proteins are components of the elongating mannosyltransferase complexes that localize to the early Golgi compartment (Hashimoto and Yoda, 1997; Jungmann and Munro, 1998) and co-localized with GMT by double immunofluorescence staining (data not shown). In the *sec23*^{ts} mutant cell at 37°C, both Anp1p and Van1p were detected as finely punctate fluorescence distinct from the dots of the Golgi staining at 24°C (Fig. 1B), but they were not observed in the ring and strings of the ER. These results indicate that

the localization mechanism is different between the mannosyltransferase complexes and GMT.

Physical interaction of GMT with COPI coatomer

The retrograde transport vesicles are generated with the aid of coat protein I (COPI) complex (coatomer). Because it was suggested that GMT undergoes retrograde transport, we examined whether the coatomer binds to GMT by immunoprecipitation analysis. Ret2p is an essential subunit of coatomer, the yeast homologue of mammalian δ-COP. A cleared lysate was prepared from the yeast cells producing Myc_6-tagged GMT by solubilizing the membrane with CHAPS. The Myc epitope was added at either the N- or the C-terminus (Myc_6-GMT or GMT-Myc_6) and immunoprecipitation was done using anti-Myc monoclonal antibody. Western blot indicated that Ret2p was present in both immunoprecipitate and that more Ret2p was found with Myc_6-GMT than with GMT-Myc_6 (Fig. 2, lanes 3 and 12). In contrast to the result with GMT, Ret2p was not detected in the similar immunoprecipitate from the cleared lysate of the cells producing Myc_6-tagged Anp1p (Fig. 2, lane 18). These results indicate that coatomer binds to GMT but not to the Anp1p mannosyltransferase complex in this experimental condition.

C-terminal tail of GMT binds to COPI coatomer

The COPI coatomer is recruited to the Golgi membrane to produce the retrograde transport vesicles and the binding of coatomer to cytoplasmic peptides of membrane proteins is a known mechanism to collect the cargo molecules in the COPI vesicles. The C-terminal KKxx or KxKxx is generally accepted as the consensus sequence for this binding (Cosson and Letourneur, 1994). Although the C-terminal 12 amino acid peptide of GMT (KQKKQQAQPLRK) does not have the sequence, it does contain four lysine residues. We previously reported that, although a mutant GMTΔ12 lacking the peptide was inactive, the Myc signal was detected in punctate Golgi pattern (Abe et al., 1999). Because the Myc epitope is susceptible to digestion in the vacuole, the localization of GMTΔ12 was re-examined using stable GFP as a tag. We found that GFP-GMTΔ12 localizes to both Golgi and vacuole (Fig. 4C). Therefore, the C-terminal tail of GMT plays a role in restriction of its localization in the Golgi.

We examined whether the truncated GMT binds to Ret2p by immunoprecipitation assay. We could not detect Ret2p in the precipitate of Myc_6-GMTΔ12 and GMTΔ12-Myc_6 (Fig. 2, lanes 9 and 15). Because Ret2p was hardly detected in the precipitate of Myc_6-GMTΔ4, which has a smaller deletion, (Fig. 2, lane 6), the C-terminal peptide of GMT plays an important role for binding to coatomer.

C-terminal peptide binds to coatomer in vitro

Physical interaction has been demonstrated between peptides containing a dilysine motif (KKxx or KxKxx) and coatomer. We next examined whether the GMT tail peptide also interacts with coatomer using an in vitro binding assay. The chimeric GST proteins were produced in *E. coli* and purified using glutathione-Sepharose beads. As a positive control, the C-terminal peptide of Wbp1p was used. Wbp1p is an ER-resident membrane protein as a component of the yeast *N*-oligosaccharyltransferase complex (te Heesen et al., 1992), and its cytoplasmic domain contains a dilysine motif that was shown to bind to COPI coatomer directly (Cosson and Letourneur, 1994). The beads bound with a GST chimera were incubated with lysate prepared from the yeast producing Ret2p-3HA. The bound materials were eluted and Ret2p-3HA was detected by immunoblotting with anti-HA antibody. A similar amount of Ret2-3HA was recovered from the beads carrying GST-Wbp1p and GST-GMT (Fig. 3, lanes 2 and 3). This indicates that the C-terminal peptide of GMT binds to coatomer as efficiently as that of Wbp1p.

To characterize the GMT tail sequence that binds to coatomer, we examined the GST fusions of a series of peptides that had an additional hexapeptide (SRAAAS) at the C-terminus as a remnant of removal of the Myc tag sequence. It was reported that the dilysine motif should be at the exact C-terminus to bind to coatomer and an extension with four serine residues inhibited the binding (Cosson and Letourneur, 1994). In the case of Wbp1p, the SRAAAS extension also abolished the binding activity to Ret2p (Fig. 3, lane 16). However, in the case of GMT, GST-KQKKQQAQPLRKSRAAAS bound to Ret2p-3HA (lane 17) as effectively as the authentic sequence (lane 15). This indicates that the essential sequence of GMT to bind to Ret2p does not have a strict position limitation at the C-terminus as the dilysine motif.

We focused on the role of the basic residues in the C-terminal peptide of GMT. As the basic amino acids are clustered, we constructed deletions of the lysine-rich region (KQKK) and the arginine-lysine region (PLRK). In both

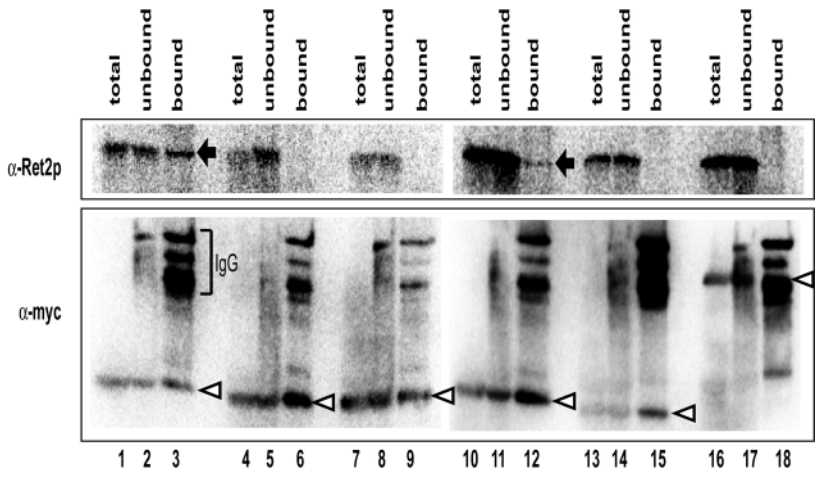

Fig. 2. GMT is precipitated with coatomer. The cleared lysate was prepared from yeast producing the Myc_6-tagged protein of interest. The tagged protein was immunoprecipitated with anti-Myc antibody and Protein-A/Sepharose beads. Each sample was separated by SDS-PAGE and analysed by immunoblotting with anti-Myc or anti-Ret2p antibody. The total, unbound and bound fractions were examined for Myc_6-GMT (pMAV426; lanes 1-3), Myc_6-GMTΔ4 (pMAV424; lanes 4-6), Myc_6-GMTΔ12 (pMAV425; lanes 7-9), GMT-Myc_6 (pMA12; lanes 10-12), GMTΔ12-Myc_6 (pMA50; lanes 13-15) and Anp1p-Myc_6 (pSV314; lanes 16-18).

cases, the amount of Ret2p-3HA was considerably reduced but still detectable by immunoblotting (Fig. 3, lanes 4 and 5). We replaced the basic amino acids with glutamine. Both replacement of RK with QQ and of KQKK with QQQQ significantly reduced the amount of Ret2p bound to the beads (Fig. 3, lanes 6 and 7). Furthermore, replacement of one or two lysines in KQKK with glutamine similarly reduced the binding activity (Fig. 3, lanes 8 to 13). The amounts of Ret2p bound to the K-to-Q mutant peptides were small and varied among five independent experiments and it was difficult to get mean values to evaluate their binding activity. Altogether, these results indicate that the basic amino acids in the C-terminus of GMT have an important role in binding to Ret2p in vitro.

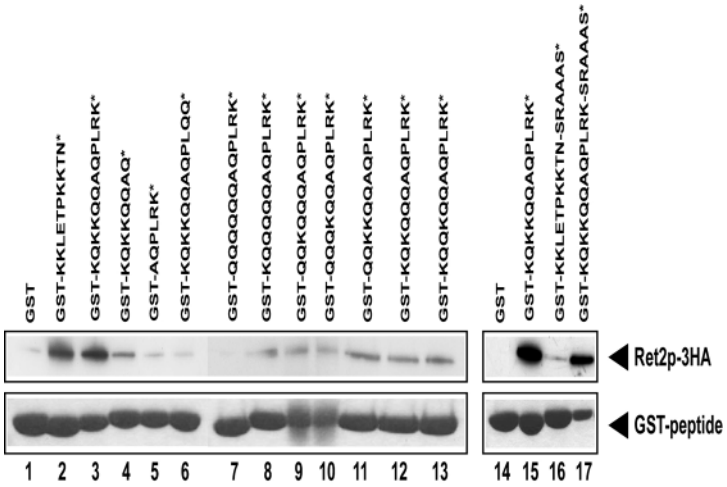

Fig. 3. The coatomer subunit Ret2p binds to the GST-fusion peptides related to the cytoplasmic tail of GMT. The GST-fusion proteins were produced in *E. coli* and affinity purified by glutathione-Sepharose beads. The yeast lysate was prepared from the cells producing the 3HA-tagged Ret2p with glass beads and 0.5% Triton X-100 and used as the labelled coatomer. The beads were mixed with the lysate, incubated for 16 hours at 4°C and washed extensively, and the bound proteins were analysed by SDS-PAGE. The bound Ret2p was detected by immunoblot analysis with 12CA5 (top). The amount of GST-fusion peptide was shown by Coomassie Brilliant Blue staining (bottom). In lanes 1 and 14, the purified GST was used as the negative control. In lanes 2 and 15, the GST-fusion protein containing the Wbp1p peptide was used as the positive control. In lane 3, the GST-fusion protein containing the wild-type GMT C-terminal peptide was used.

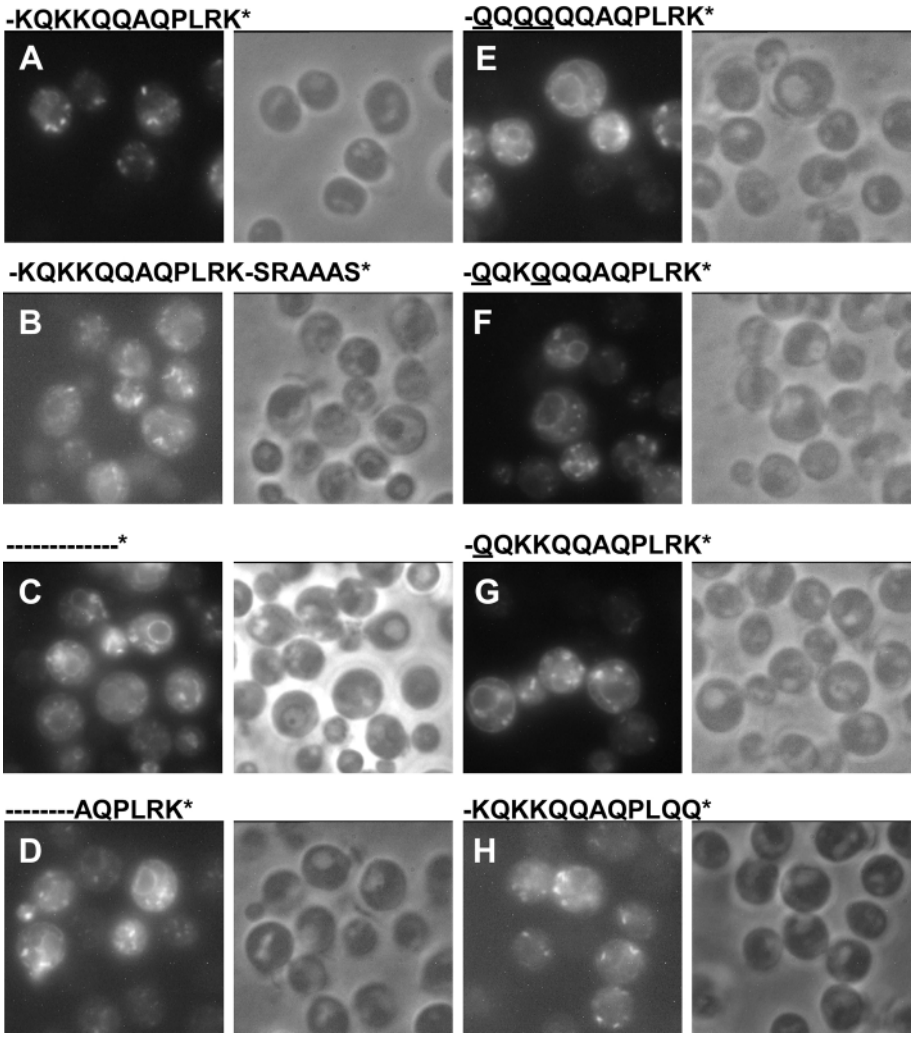

Fig. 4. Subcellular localization of the GFP-GMT tail mutants in the *S. cerevisiae* cells by indirect immunofluorescence. The GFP-tagged GMT tail mutant genes were expressed from the original *VIG4* promoter on a *CEN ARS* plasmid in the wild-type cell. The cells were fixed with formaldehyde and observed by indirect immunofluorescence using anti-GFP antibody. The C-terminal sequences are shown. For expression of GFP-tagged GMT mutant derivatives, the plasmids used were pMA89 (A), pMA86 (B), pMAV587 (C), pMAV594 (D), pMAV595 (E), pMAV597 (F), pMAV599 (G) and pMAV601 (H).

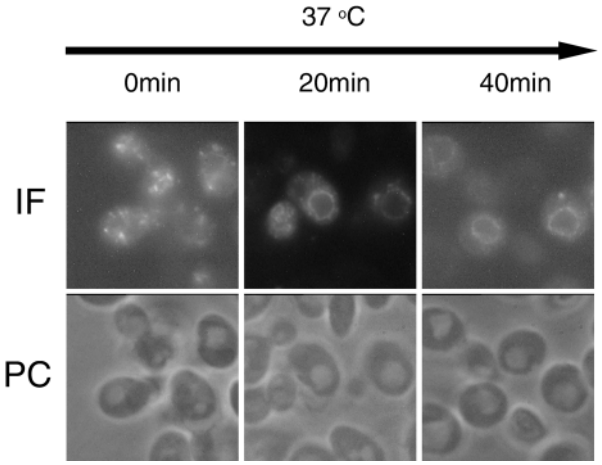

Fig. 5. The *sec21*^{ts} mutant cells expressing a GFP-tagged GMT. The *sec21*^{ts} mutant cells expressing a GFP-tagged GMT were treated with cycloheximide (0.1 mg ml^{-1}) before being shifted to 37°C. The cells were fixed with formaldehyde and observed by indirect immunofluorescence using anti-GFP antibody (IF) and by phase-contrast microscopy (PC).

C-terminal peptide has a role in steady-state Golgi localization of GMT

We next examined whether the localization of mutant GMT correlates with the in vitro binding activity of its tail peptide to Ret2p. The N-terminus of GMT derivatives was tagged with GFP, which is detectable even if the fusion proteins are delivered to the vacuole. The wild-type GFP-GMT fusion protein is functional as determined by the complementation of *vig4-1* phenotype (Fig. 6, lane 2). The localization of this protein was to the punctate pattern characteristic of the Golgi (Fig. 4A). Two GMT tail mutants with a C-terminal addition of SRAAAS and replacement of the terminal RK with QQ were also detected in the punctate structure and therefore mainly localized to the Golgi as the wild type (Fig. 4B,H). Other GMT tail mutants that have deletion of KQKK (Fig. 4C,D) or K-to-Q replacements (Fig. 4E-G) within the C-terminal peptide were found not only in the punctate structure but also in rings that represented the vacuole membrane. Among 100 randomly selected cells in the immunofluorescent images, the proportion of cells having a large circular structure was 10% in Fig. 4A, 12% in Fig. 4B and 4% in Fig. 4H, whereas it was 46% in Fig. 4C, 52% in Fig. 4D, 48% in Fig. 4E, 41% in Fig. 4F, and 41% in Fig. 4G. These results indicate the steady Golgi localization of GMT largely requires the binding of the GMT tail to coatomer and when the binding is impaired GMT is mislocalized to the vacuole membrane.

COPI-dependence of the Golgi localization of GMT

We further examined the intracellular behaviour of GMT in a coatomer mutant. The *sec21-1* gene encodes a temperature-sensitive subunit of coatomer, the yeast homologue of mammalian γ-COP. If the steady-state Golgi localization of GMT is achieved by the retrograde transport, it will be damaged at the nonpermissive temperature in the *sec21-1* mutant. To test this prediction, we produced the GFP-tagged GMT in the *sec21-1* mutant from a *CEN* plasmid. As shown in Fig. 5, the punctate pattern of the Golgi rapidly decreased after the temperature shift and the staining of the vacuole membrane increased instead. At 0 minutes, 20 minutes and 40 minutes after the temperature shift up, the ratio of cells that had GMT in the vacuole were 22%, 63% and 92%, respectively. This result further supports the importance of the COPI-dependent retrograde transport for localization of GMT in the Golgi.

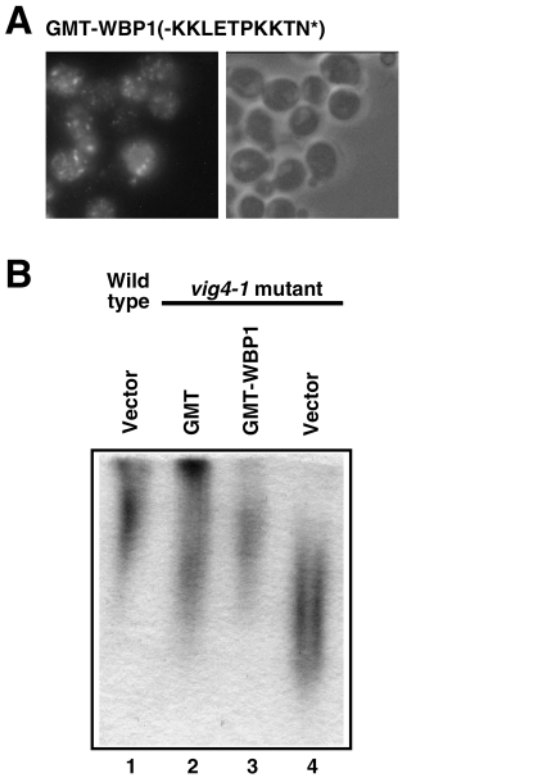

Fig. 6. Subcellular localization of the GFP-GMT-Wbp1p chimeric protein, which exchanges the C-terminal peptide of the GMT tail for that of Wbp1p. This chimeric protein is tagged with GFP and produced by *VIG4* promoter and *CEN ARS* plasmid (pMAV592) in the wild-type cell. (A) The cells were fixed with formaldehyde and observed by indirect immunofluorescence using anti-GFP antibody (left) or by phase contrast microscopy (right). (B) Activity staining of invertase in 7.5% SDS-PAGE of the periplasmic fraction of the wild-type and *vig4-1* transformant cells.

If the major role of the C-terminal peptide of GMT is to bind to COPI coatomer to engage in the retrograde transport, we predicted that the peptide could be substituted with other C-terminal peptides containing an active binding sequence, such as the dilysine motif. To test this possibility, we constructed a chimeric protein GFP-GMT-Wbp1p by exchanging the C-terminal peptide (12 amino acids) of GMT with the C-terminal peptide (14 amino acids) of Wbp1p. The chimeric protein was produced from the *VIG4* promoter on a *CEN* plasmid. GFP-GMT-Wbp1p was exclusively found in a punctate pattern characteristic of the Golgi apparatus (Fig. 6A). Next, we introduced the expression plasmid into the *vig4-1* mutant to examine whether the chimera GMT can efficiently transport GDP-mannose. The invertase in the periplasmic fraction of the *vig4-1* mutant is not fully glycosylated and therefore migrates

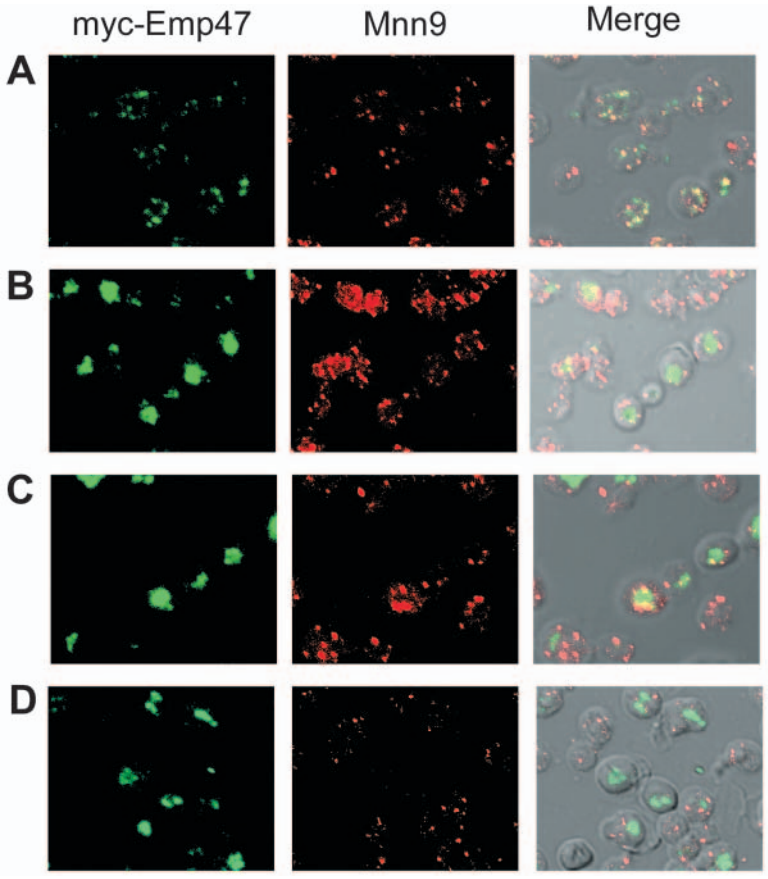

Fig. 7. Localization of the mutant Emp47p with modified C-terminus. Cells were stained by immunofluorescence using anti-Myc and anti-Mnn9 antibodies to visualize Emp47p (myc-Emp47) and the Golgi mannosyltransferase (Mnn9), respectively. Merged images for phase-contrast microscopy are shown at the right. (A) The wild-type Emp47p (-RIRQEIIKTKLL). (B) A truncated Emp47p (-RIRQEII). (C) Addition of the GMT C-terminal peptide to the truncation (-RIRQEIIGSKQKKQQAQPLRK). (D) A C-terminal replacement with the GMT C-terminal peptide (-GSKQKKQQAQPLRK).

faster in SDS-PAGE (Fig. 6B, lane 4). Slow migration of invertase was recovered by introduction of the *GFP-GMT(VIG4)* gene by a *CEN* plasmid (Fig. 6B, lane 2) but the *vig4Δ12* did not have the activity to rescue this defect (Abe et al., 1999). However, when GFP-GMT-Wbp1p was produced in the *vig4-1* mutant, invertase migrated considerably more slowly (Fig. 6B, lane 3). This result indicates that the C-terminal peptide of GMT could be functionally replaced with a dilysine motif peptide and the COPI-mediated retrograde transport is essential for the localization and function of GMT in the Golgi.

We further examined whether the GMT C-terminal peptide is able to functionally substitute the K(x)Kxx motif-containing C-terminal peptide of other Golgi membrane proteins that retrieve to the Golgi depending on the COPI-mediated retrograde transport. We selected a Myc-tagged Emp47p (Schroder et al., 1995) (a kind gift of H. Riezman, University of Geneva, Geneva, Switzerland) as the reporter protein and a *pep4*Δ host was used to avoid its degradation in the vacuole. Emp47p has the sequence RIRQEIIKTKLL at the C-terminus and removal of KTKLL to make RIRQEII resulted in localization of the truncated protein in the vacuole (Fig. 7B). Addition of the GMT C-terminal peptide to this truncated peptide to make RIRQEIIGSKQKKQQAQPLRK, where GS are encoded by the *Bam*HI recognition sequence (Fig. 7C), or a total C-terminal replacement to make GSKQKKQQAQPLRK (Fig. 7D) resulted in the same localization of the chimeric protein. This indicates that the GMT C-terminal sequence is not functional when it is attached to Emp47p.

Discussion

The yeast Golgi apparatus has several mannosyltransferase and a single GMT to conduct modification of glycoprotein as one of the Golgi's major roles. The mechanism to keep the resident components in an organelle has been a major subject in the study of vesicular traffic. The major ways to realize the steady-state composition of a dynamic organelle are considered to be the retention to prevent the residents from going out and the retrieval to collect the escaped residents. Our result using the temperature-sensitive *sec* mutants indicated the Golgi-resident GMT also has a dynamic character.

Table 2. The C-terminal peptide of NST-family proteins has multiple lysine residues

Accession	Species	Amino acid sequence*
AAG48813	*Arabidopsis thaliana*	-**K**WR**K**LQRMQ**KKKK**A
AAG53653	*Chinese hamster*	-SSRGDLITEPFLP**K**LLT**K**V**K**GS
AAK50396	*Caenorhabditis elegans*	-EA**K**SD**K**V**K**LLGRDGNAAEESV
AAK51705	*Homo sapiens*	-EM**KK**TPEEPSP**K**DSE**K**SAMGV
AAK51897	*Candida glabrata*	-**K**Q**KK**VQ**K**N
AAK74075	*Candida albicans*	-**K**Q**K**QQ**K**EQSQQLPTT**K**
BAA13390	*Homo sapiens*	-S**K**QSEANN**K**LDI**K**G**K**GAV
CAB46704	*Schizosaccharomyces pombe*	-**K**ILNNNSTA**KKK**AS
NP_005818	*Homo sapiens*	-DA**K**FG**K**GA**KK**TSH
NP_010912	*Saccharomyces cerevisiae*	-GSIHPRQND**K**GAI**KKSK**
NP_011290	*Saccharomyces cerevisiae*	-**K**Q**KK**QQAQPLR**K**
NP_015080	*Saccharomyces cerevisiae*	-EALN**KKK**ANIP**K**A**K**SA
XP_040602	*Homo sapiens*	-EEQLS**K**QSEANN**K**LDI**K**G**K**GAV

*The C-terminal hydrophilic sequences of NSTs and possible NSTs registered in the current database are listed and lysine residues are shown in bold.

In a temperature-sensitive COPII mutant *sec23-1*^{ts}, GMT moved to the ER after a shift to the restrictive temperature. This suggests that the GMT removed from the Golgi is retrieved to the ER and then rapidly transported to the Golgi in the normal condition. It seems redundant for the retrieval of GMT to be directed to the ER instead of the early Golgi compartment. However, the quality-control process of the ER might be important to check whether the returned GMT is suitable for its reuse. In contrast to GMT, Anp1p and Van1p were not found in the ER and remained in punctate structures that seem somewhat smaller than the structures at the permissive temperature. Wooding and Pelham (Wooding and Pelham, 1998) reported a similar observation that Anp1p was observed in a fine dotted appearance in a *sec12*^{ts} mutant with defect in the assembly of COPII coat on the ER. Anp1p and Van1p are subunits of two distinct Mnn9p-complex mannosyltransferases that generally colocalize with GMT. Because GMT supplies the substrate for mannosyltransferases, their colocalization seems biochemically favourable. However, the mechanism of localization seems to be different for GMT, a polytopic and homodimeric membrane protein, and the subunits of type II membrane protein complexes. A possibly conflicting result was presented by Todorow et al. (Todorow et al., 2000), who detected that the Mnn9p complexes had recycled to the ER from Golgi in *sec12*^{ts} cells. In addition to the use of different mutants, cells were treated with cycloheximide for 1 hour at 24°C and then incubated for 30 minutes at 37°C in their experiments. However, we suspect that a longer arrest of new protein synthesis might lead to a shortage of proteins and induce a complementary response of the cell. At least, there is a kinetic difference between GMT and Mnn9p-containing complexes for retrieval to the ER.

The N-terminal region mediates export of GMT from the ER (Gao and Dean, 2000) and the C-terminal region is essential for both its dimerization and export from the ER (Abe et al., 1999). Especially, the 12-amino acid C-terminal hydrophilic peptide that is exposed to the cytosol (Gao and Dean, 2000) is not necessary for GMT to dimerize and exit from the ER, but its truncation resulted in a considerably reduced activity (Abe et al., 1999). Because GMTΔ13 overproduced by a multicopy plasmid supports the growth of a mutant that does not have the wild-type GMT by glucose repression of the chromosomal *GAL1-VRG4* (Gao and Dean, 2000), correct folding and correct topology of GMT is likely to be achieved in the absence of the C-terminal 13 amino acids. Overproduction of the wild-type GMT induces a progression of the stacked Golgi, but GMTΔ12 does not have this activity (Hashimoto et al., 2002). Our previous experiments using a Myc_6 tag could not detect mislocalization of GMTΔ12, but GMTΔ12 was found in the vacuole when Myc_6 was replaced with GFP, which is more resistant to digestion in the vacuole (Fig. 4C). This suggests that the GMT removed from the Golgi is delivered to the vacuole and degraded there unless it is retrieved back to the ER.

Immunoprecipitation experiments showed that the wild-type GMT binds to the COPI coat but GMTΔ12 does not (Fig. 2). Therefore, the mislocalization of GMTΔ12 in the vacuole of the wild-type cell is likely to be caused by its failure in binding to the COPI coat. It is a complementary phenomenon that the wild-type GMT localizes in the vacuole of the *sec21-1* cell with defective COPI coats at the nonpermissive temperature. A chimera GFP-GMTΔ12-Wbp1p with the 14 amino-acid C-terminal peptide of Wbp1p (which has a COPI-binding KKxx motif) was found in the Golgi punctate pattern and had a good activity to rescue the *vig4-1* defect. These results indicated that the C-terminal tail of GMT is required for the proper localization by retrieval through binding to the COPI coat. However, this vital role seems to act only in the context of GMT molecule, because the 12-amino-acid peptide could not functionally replace the C-terminal peptide of Emp47p to localize it to the Golgi.

The C-terminal peptide of GMT has a novel sequence recognized by COPI coat. In the case of the dilysine motif (KKxx or KxKxx), its position at the C-terminus is crucial (Cosson and Letourneur, 1994). However, in the case of the C-terminal peptide of GMT, the position of lysine from the C-terminus was not important (Fig. 3). This is consistent with the fact that the addition of C-terminal tags had not impaired the function and localization of GMT. As similar cases, it has been reported that the C-terminal peptides of Erv25p (KNYFKTKHII) and Rer1p (KYRYIPLDIGKKKYSHSSN), which also recycle between the ER and Golgi, bind to coatomer (Belden and Barlowe, 2001; Sato et al., 2001). Rer1p localizes to the Golgi at the steady state (Sato et al., 2001), like GMT. There is no sequence similarity between these peptides but the presence of a cluster of lysines (at least three within seven amino acids) is significant. Tyrosine residues are also important for retrieval of Rer1p (Sato et al., 2001), but the tail of GMT does not have an aromatic residue.

The 12-amino-acid peptide fused to GST binds to COPI coat as efficiently as the C-terminal peptide of Wbp1p, a type-I membrane protein subunit of the ER-resident oligosaccharyltransferase that has a typical dilysine motif. In the search for the crucial motif to bind to coatomer, we constructed a GST fusion of mutant derivatives replacing arginine or lysine with glutamine and tested the binding with coatomer. All the mutant derivatives had a weaker binding activity than the wild type, but the defect was not attributed to certain residues. The binding to coatomer in GST pull-down assay in vitro (Fig. 3) and mislocalization of the mutant GMT to the vacuole in vivo (Fig. 4) had a good agreement. An exception was the case of the terminal RK replaced with QQ (Fig. 3, lane 6, Fig. 4I). Although GST-KQKKQQAQPLQQ had a reduced ability to bind to Ret2p in vitro, the GMT with this peptide localized to the Golgi like wild-type GMT in vivo. The reason for this is not clear, but the presence of the membrane affects the protein-protein interaction, as we have shown elsewhere (Kosodo et al., 2002). It should be realized that the three clustered lysines are present only in this derivative, as in the wild-type GMT. Therefore, a cluster of at least three lysines in the vicinity of the membrane-spanning domain might be the signal for such recycling.

Candidates of the member of NST family can be found in the increasing data of genome projects. By examining the amino acid sequences of their C-terminal peptides, the presence of at least three lysines is a common characteristic in 13 proteins including those of *Homo sapiens*, *S. cerevisiae*, *Schizosaccharomyces pombe*, *Arabidopsis thaliana*, *Candida albicans* and *Caenorhabditis elegans* (Table 2). Mostly, three-lysine clusters are found in a seven-amino-acid stretch, or else as four lysines within 14 amino acids that do not have an aromatic residue. Although further study is necessary, we here

propose a testable hypothesis that the localization of a member of NSTs in the ER-Golgi compartments depends on the coatomer and clustered lysines in the vicinity of the membrane-spanning domain.

We thank F. Letourneur for antiserum and H. Riezman and R. Schekman for yeast strains. This work was supported by a grant-in-aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan, grants for 'Bioarchitect Research' from the Institute of Physical and Chemical Research (RIKEN), a grant from the Kato Memorial Bioscience Foundation (to Y.N.), and a grant from the Nagase Science and Technology Foundation (to K.Y.).

References

Abe, M., Hashimoto, H. and Yoda, K. (1999). Molecular characterization of Vig4/Vrg4 GDP-mannose transporter of the yeast *Saccharomyces cerevisiae*. *FEBS Lett.* **458**, 309-312.

Abeijon, C., Robbins, P. W. and Hirschberg, C. B. (1996). Molecular cloning of the Golgi apparatus uridine diphosphate-*N*-acetylglucosamine transporter from *Kluyveromyces lactis*. *Proc. Natl. Acad. Sci. USA* **93**, 5963-5968.

Belden, W. J. and Barlowe, C. (2001). Distinct roles for the cytoplasmic tail sequences of Emp24p and Erv25p in transport between the endoplasmic reticulum and Golgi complex. *J. Biol. Chem.* **276**, 43040-43048.

Berninsone, P. M. and Hirschberg, C. B. (2000). Nucleotide sugar transporters of the Golgi apparatus. *Curr. Opin. Struct. Biol.* **10**, 542-547.

Bretscher, M. S. and Munro, S. (1993). Cholesterol and the Golgi apparatus. *Science* **261**, 1280-1281.

Cosson, P. and Letourneur, F. (1994). Coatomer interaction with di-lysine endoplasmic reticulum retention motifs. *Science* **263**, 1629-1631.

Eckhardt, M., Muhlenhoff, M., Bethe, A. and Gerardy-Schahn, R. (1996). Expression cloning of the Golgi CMP-sialic acid transporter. *Proc. Natl. Acad. Sci. USA* **93**, 7572-7576.

Fiedler, K., Veit, M., Stamnes, M. A. and Rothman, J. E. (1996). Bimodal interaction of coatomer with the p24 family of putative cargo receptors. *Science* **273**, 1396-1399.

Gao, X. D. and Dean, N. (2000). Distinct protein domains of the yeast Golgi GDP-mannose transporter mediate oligomer assembly and export from the endoplasmic reticulum. *J. Biol. Chem.* **275**, 17718-17727.

Goto, S., Taniguchi, M., Muraoka, M., Toyoda, H., Sado, Y., Kawakita, M. and Hayashi, S. (2001). UDP-sugar transporter implicated in glycosylation and processing of Notch. *Nat. Cell Biol.* **3**, 816-822.

Graham, T. R. and Emr, S. D. (1991). Compartmental organization of Golgi-specific protein modification and vacuolar protein sorting events defined in a yeast *sec18* (NSF) mutant. *J. Cell Biol.* **114**, 207-218.

Hashimoto, H. and Yoda, K. (1997). Novel membrane protein complexes for protein glycosylation in the yeast Golgi apparatus. *Biochem. Biophys. Res. Commun.* **241**, 682-686.

Hashimoto, H., Abe, M., Hirata, A., Noda, Y., Adachi, H. and Yoda, K. (2002). Progression of the stacked Golgi compartments in the yeast *Saccharomyces cerevisiae* by overproduction of GDP-mannose transporter. *Yeast* **19**, 1413-1424.

Hausler, A. and Robbins, P. W. (1992). Glycosylation in *Saccharomyces cerevisiae*: cloning and characterization of an α-1,2-mannosyltransferase structural gene. *Glycobiology* **2**, 77-84.

Jungmann, J. and Munro, S. (1998). Multi-protein complexes in the cis Golgi of *Saccharomyces cerevisiae* with α-1,6-mannosyltransferase activity. *EMBO J.* **17**, 423-434.

Jungmann, J., Rayner, J. C. and Munro, S. (1999). The *Saccharomyces cerevisiae* protein Mnn10p/Bed1p is a subunit of a Golgi mannosyltransferase complex. *J. Biol. Chem.* **274**, 6579-6585.

Kojima, H., Hashimoto, H. and Yoda, K. (1999). Interaction among the subunits of Golgi membrane mannosyltransferase complexes of the yeast *Saccharomyces cerevisiae*. *Biosci. Biotechnol. Biochem.* **63**, 1970-1976.

Kosodo, Y., Noda, Y., Adachi, H. and Yoda, K. (2002). Binding of Sly1 to Sed5 enhances formation of the yeast early Golgi SNARE complex. *J. Cell Sci.* **115**, 3683-3691.

Ma, D., Russell, D. G., Beverley, S. M. and Turco, S. J. (1997). Golgi GDP-mannose uptake requires *Leishmania* LPG2. A member of a eukaryotic family of putative nucleotide-sugar transporters. *J. Biol. Chem.* **272**, 3799-3805.

Miura, N., Ishida, N., Hoshino, M., Yamauchi, M., Hara, T., Ayusawa, D. and Kawakita, M. (1996). Human UDP-galactose translocator: molecular cloning of a complementary DNA that complements the genetic defect of a mutant cell line deficient in UDP-galactose translocator. *J. Biochem.* **120**, 236-241.

Munro, S. (1995). An investigation of the role of transmembrane domains in Golgi protein retention. *EMBO J.* **14**, 4695-4704.

Nakanishi-Shindo, Y., Nakayama, K. I., Tanaka, A., Toda, Y. and Jigami, Y. (1993). Structure of the *N*-linked oligosaccharides that show the complete loss of α-1,6-polymannose outer chain from *och1*, *och1 mnn1*, and *och1 mnn1 alg3* mutants of *Saccharomyces cerevisiae*. *J. Biol. Chem.* **268**, 26338-26345.

Nilsson, T., Slusarewicz, P., Hoe, M. H. and Warren, G. (1993). Kin recognition. A model for the retention of Golgi enzymes. *FEBS Lett.* **330**, 1-4.

Nilsson, T., Hoe, M. H., Slusarewicz, P., Rabouille, C., Watson, R., Hunte, F., Watzele, G., Berger, E. G. and Warren, G. (1994). Kin recognition between medial Golgi enzymes in HeLa cells. *EMBO J.* **13**, 562-574.

Poster, J. B. and Dean, N. (1996). The yeast *VRG4* gene is required for normal Golgi functions and defines a new family of related genes. *J. Biol. Chem.* **271**, 3837-3845.

Rayner, J. C. and Munro, S. (1998). Identification of the *MNN2* and *MNN5* mannosyltransferases required for forming and extending the mannose branches of the outer chain mannans of *Saccharomyces cerevisiae*. *J. Biol. Chem.* **273**, 26836-26843.

Romero, P. A., Lussier, M., Veronneau, S., Sdicu, A. M., Herscovics, A. and Bussey, H. (1999). Mnt2p and Mnt3p of *Saccharomyces cerevisiae* are members of the Mnn1p family of α-1,3-mannosyltransferases responsible for adding the terminal mannose residues of *O*-linked oligosaccharides. *Glycobiology* **9**, 1045-1051.

Sato, K., Sato, M. and Nakano, A. (2001). Rer1p, a retrieval receptor for endoplasmic reticulum membrane proteins, is dynamically localized to the Golgi apparatus by coatomer. *J. Cell Biol.* **152**, 935-944.

Schroder, S., Schimmoller, F., Singer-Kruger, B. and Riezman, H. (1995). The Golgi-localization of yeast Emp47p depends on its di-lysine motif but is not affected by the ret1-1 mutation in α-COP. *J. Cell Biol.* **131**, 895-912.

Tabuchi, M., Tanaka, N., Iwahara, S. and Takegawa, K. (1997). The *Schizosaccharomyces pombe gms1*$^{+}$ gene encodes an UDP-galactose transporter homologue required for protein galactosylation. *Biochem. Biophys. Res. Commun.* **232**, 121-125.

te Heesen, S., Janetzky, B., Lehle, L. and Aebi, M. (1992). The yeast *WBP1* is essential for oligosaccharyl transferase activity in vivo and in vitro. *EMBO J.* **11**, 2071-2075.

Todorow, Z., Spang, A., Carmack, E., Yates, J. and Schekman, R. (2000). Active recycling of yeast Golgi mannosyltransferase complexes through the endoplasmic reticulum. *Proc. Natl. Acad. Sci. USA* **97**, 13643-13648.

Wooding, S. and Pelham, H. R. (1998). The dynamics of Golgi protein traffic visualized in living yeast cells. *Mol. Biol. Cell* **9**, 2667-2680.

Yip, C. L., Welch, S. K., Klebl, F., Gilbert, T., Seidel, P., Grant, F. J., O'Hara, P. J. and MacKay, V. L. (1994). Cloning and analysis of the *Saccharomyces cerevisiae MNN9* and *MNN1* genes required for complex glycosylation of secreted proteins. *Proc. Natl. Acad. Sci. USA* **91**, 2723-2727.