Proteins in the early Golgi compartment of *Saccharomyces cerevisiae* immunoisolated by Sed5p

Jung-Hwa Cho, Yoichi Noda, Koji Yoda*

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

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Abstract The yeast tSNARE Sed5p is considered to mainly reside in the early Golgi compartment at the steady state of its intracellular cycling. To better understand this compartment, we immunoisolated a membrane subfraction having Sed5p on the surface (the Sed5 vesicles). Immunoblot studies showed that considerable portions (20–30%) of the Golgi mannosyltransferases (Mnt1p, Van1p, and Mnn9p) were simultaneously recovered while the late Golgi (Kex2p) or endoplasmic reticulum (Sec71p) proteins were almost excluded. The N-terminal sequences of the polypeptides detectable by Coomassie blue staining indicated that the prominent components of the Sed5 vesicles include Anp1p, Emp24p, Erv25p, Erp1p, Ypt52p, and a putative membrane protein of unknown function (Yml067c).

Key words: Early Golgi compartment; Immunoisolation; Sed5p; *Saccharomyces cerevisiae*

1. Introduction

In eukaryotic cells, secretory and membrane proteins are transferred from the endoplasmic reticulum (ER) to various areas of the cell via the Golgi compartments in a process that involves the formation and fusion of transport vesicles [1–3]. During the traffic, important posttranslational modifications such as glycosylation and proteolytic processing of polypeptides occur in the Golgi compartments. More importantly, sorting of the cargo molecules in specific vesicles for the delivery to their destined areas of the cell also occurs in the Golgi by mostly yet unknown mechanisms.

Maintenance of the Golgi complex is considered to be achieved by the balance of the incoming and outgoing of transport vesicles. Basically, two models have been proposed to explain the mechanism. The 'vesicular transport' model claims that the Golgi cisternae are a stable existence and vesicles carry proteins through the Golgi compartments and also back to the ER [2,4], and the 'cisternal maturation' model claims that the Golgi cisternae move progressively carrying cargo molecules through the stacks and the vesicles are involved only in the backward transport [5,6]. In *Saccharomyces cerevisiae*, the Golgi complexes are dispersed throughout the cytoplasm and immunological staining or green fluorescent protein fusion showed punctate patterns with slight differences depending on the particular Golgi proteins. Development of the Golgi subcompartments was also reported by studies of secretion mutants [7,8]. The tSNARE Sed5p that is abundant in the early Golgi compartment [9] is also in a dynamic state, recycling through the ER and early Golgi [10]. To fully understand the molecular architecture of the Golgi, it seems necessary to purify and examine the subcompartments.

Until now, most of the subcellular vesicles were purified from the crude cell lysate by time-consuming repeats of sedimentation and flotation in density gradients of an appropriate solute [11,12]. Although the procedure needs a long time of centrifugation, the purity and intactness of the vesicles is not guaranteed. An alternative method is immunoisolation [13]. Bryant and Boyd successfully applied it in isolation of the yeast late Golgi vesicles using the antibody to the cytoplasmic region of Kex2p [14]. They further planned to develop a generalized method by the use of protein A tagging of the proteins whose specific antibodies are not available. Unfortunately, in the case of Kex2p, tagging modified the targeting signal and the fusion protein localized on the vacuolar membrane [15]. Since then, few reports have appeared which use epitope tagging in immunoisolation of vesicles.

In this study, we used the myc-tagged Sed5p as the probe for adsorption and successfully immunoisolated a specific membrane vesicle fraction. The identification of the Sed5 vesicles as the early Golgi compartments was demonstrated by simultaneous enrichment of a group of proteins as detected by immunoblotting and amino acid sequencing. The coincidence of glycosyltransferases and putative cargo receptors with Sed5p is characteristic. We also found a novel protein of yet undetermined function was enriched in the fraction.

2. Materials and methods

2.1. Strains, plasmids, and media

Escherichia coli DH5α (F$^-$, ϕ80*lac*Δ*ZM15*, *supE44* Δ*lacU169 hsdR17 recA1 endA1 gyrA96 thi-1 relA1*) was used for manipulation of recombinant DNA and was grown in LB medium (1% Bacto tryptone, 0.5% yeast extract, 0.5% NaCl). The *KEX2-3HA* construct (pSN218) was a generous gift from Dr. Steven Nothwehr (University of Missouri-Columbia, MO), the *6myc* construct (pRH360) from Dr. Randy Hampton (University of California, San Diego, CA), *3HA-MNT1* from Dr. Hitoshi Hashimoto (University of Tokyo), and *3HA-SEC71* from Drs. Ken Sato and Akihiko Nakano (Riken, Wako).

S. cerevisiae KA31-1A (*MATa*, *his3 trp1 leu2 ura3*), CJY119 (*MATa*, Δ*sed5::[6myc-SED5 URA3] his3 trp1 leu2 ura3*), CJY123 (KA31-1A/pYN117 [*CEN*, *TRP1 3HA-SEC71*]), CJY124 (CJY119/pYN117), CJY125 (KA31-1A/pYN119 [*CEN*, *TRP1 HA3-MNT1*]), CJY126 (CJY119/pYN119), CJY127 (KA31-1A/pYN126 [*CEN*, *TRP1 KEX2-3HA*]), and CJY128 (CJY119/ pYN126) were used. Yeasts were grown in YEPD (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, and appropriate amino acids) medium. Solid media were made with 2% agar.

*Corresponding author. Fax: (81)-3-5841 5337.
E-mail: asdfg@mail.ecc.u-tokyo.ac.jp

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2.2. Construction of CJY119

The 5′ region of *SED5* (nucleotides −338 to −1, nucleotide 1 was defined as adenine of the initiation ATG of Sed5p), the six tandem myc-encoding sequence, and the ORF and 3′ region of *SED5* (nucleotides 1–1141) were amplified by PCR and used to generate a gene encoding myc6-Sed5p in the replacement plasmid pCJ13 (*URA3*). Transformation of KA31-1A with the linearized pCJ13 replaced the chromosomal copy of *SED5* with myc6-Sed5. Gene replacement in CJY119 was verified by Southern blotting.

2.3. Immunoisolation of the Sed5 vesicles

The yeast cells were grown at 30°C in 100 ml YEPD or SD medium to early exponential phase ($OD_{560\,nm}$ ca. 1). Cells were harvested by centrifugation and washed once with water. All steps hereafter were performed at 4°C. Cells were resuspended in a glass tube in B88-500 buffer (20 mM HEPES, pH 6.8, 500 mM potassium acetate, 5 mM magnesium acetate, 200 mM sorbitol, 1/1000 volume of a protease inhibitor cocktail (1 mg/ml each of chymostatin, aprotinin, leupeptin, pepstatin A, and antipain) and 1 mM phenylmethylsulfonyl fluoride). Glass beads were added to the mixture, which was then vortexed four times for 1 min with 1-min incubation on ice between each burst. The unbroken cells were discarded by centrifugation at $500\times g$ for 5 min. The supernatant was then centrifuged in a TLA100 rotor (Beckman) at 30 000 rpm ($50\,000\times g$) for 20 min to yield a supernatant, S50.

Pansorbin (formaldehyde-fixed *Staphylococcus aureus* cells, Calbiochem) was washed three times with B88-500 buffer, incubated with 10 mg/ml bovine serum albumin in B88-500 for 1 h at 4°C, and washed once with B88-500. The S50 was mixed with Pansorbin and rotated for 1 h at 4°C to yield the precleared lysate. The monoclonal anti-myc antibody (9E10, Berkeley Antibody) was added to the precleared lysate and incubated for 1 h at 4°C. Pansorbin was then added to the mixture and incubated for 1 h at 4°C. After this incubation, Pansorbin was collected by centrifugation at $500\times g$ for 5 min, and the supernatant (unbound fraction) was retained. The pellets were washed twice with B88-500 and twice with B88 (same as B88-500 but potassium acetate was omitted) buffers. The vesicle proteins were eluted from Pansorbin by solubilizing the membrane with 1% Triton X-100 in B88 buffer (TX-soluble fraction). Pansorbin was again pelleted and remaining materials were eluted by boiling for 2 min in 1×Laemmli sample buffer. The volume of elution was one-twentieth the volume of the starting precleared sample. These fractions were analyzed by SDS–PAGE in 10% polyacrylamide gel followed by immunoblotting with various antibodies described in the text.

2.4. Antibodies and immunoblotting

The 6myc-tagged and 3HA-tagged proteins were detected using monoclonal antibodies 9E10 and 12CA5, respectively. Anti-Sly2p/Sec22p and Ypt1p rabbit polyclonal antibodies were from Dr. Dieter Gallwitz (Max-Planck-Institute, Göttingen). Anti-Mnn9p and Van1p rabbit antisera were described previously [16]. Immunoblots were detected using enhanced chemiluminescence (ECL kit, Pierce) and AR-GUS-50/2D luminometer (Hamamatsu Photonics) or X-ray film (Hyperfilm MP, Amersham Pharmacia Biotech).

2.5. Protein sequencing

The Sed5 vesicles were obtained from 1 l culture of CJY119 by scaling up the procedure described above. Proteins were resolved on a 10% polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Immobilon, Millipore). Abundant proteins were detected with Coomassie brilliant blue, excised, and their N-terminal amino acid sequences were determined with automated protein sequencer (492 Procise Protein Sequencing system, PE Biosystems).

3. Results and discussion

3.1. Immunoisolation of the Sed5 vesicles

The SNAREs are on the surface of the transport vesicles or the target membranes specifically. Therefore, SNAREs should be a proper probe in immunoisolation of specific compartments. As Sed5p is localized on the early Golgi membrane [9,10] and the tagging with the HA epitope or green fluorescent protein at the N-terminus did not impair its activity or localization [10], we examined if it could be used in immunoisolation.

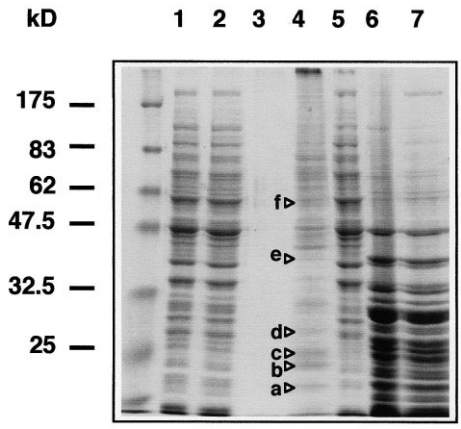

Fig. 1. Protein profiles of the Sed5 vesicles and other subcellular fractions. *S. cerevisiae* CJY119 was used except lane 3 (KA31-1A, mock). Lane 1 represents the precleared lysate and lane 2 the unbound fraction. Lanes 3 and 4 represent TX-soluble fractions of the Sed5 vesicles prepared from KA31-1A and CJY119, respectively. The Triton X-100-soluble crude membrane fraction (lane 5) and the insoluble residue (lane 6) were prepared from the pellet of a centrifugation of S50 at $100\,000\times g$ for 1 h (lane 7). Proteins were separated by SDS–PAGE on 10% gel and detected by staining with Coomassie brilliant blue. The N-terminal amino acid sequences of the proteins indicated by the arrowheads were determined as a: HNVLL (Emp24p), b: LHFDI (Erv25p), c: FYYYT (Erp1p), d: MLQFKLVL (Ypt52p), e: XXLKTFDAF (Yml067c), and f: MKYNN (Anp1p).

A yeast strain CJY119 in which the 6myc-tagged version was the sole copy of the *SED5* gene was constructed. CJY119 showed no detectable growth retardation in comparison with the parent. The Sed5 vesicles were isolated on Pansorbin by immunoadsorption using anti-myc antibody as described in Section 2. Fig. 1 shows the polypeptide composition of the Sed5 vesicles. When the parental strain that had the authentic Sed5p was used to monitor non-specific adsorption, essentially no protein band was recovered from Pansorbin (lane 3, mock). The protein profile of the TX-soluble fraction from the Sed5 vesicle (lane 4) was clearly distinct from that of the precleared lysate (lane 1), unbound fraction (lane 2) or crude membrane fraction prepared from S50 (lane 5). This result suggested a specific subcellular fraction was obtained as the Sed5 vesicles.

3.2. Relative recovery of the marker proteins in the Sed5 vesicles

Characteristic marker proteins were used to assess the Sed5 vesicles (Fig. 2 and Table 1). More than 95% of 6myc-Sed5 in the precleared lysate was removed by 9E10 and Pansorbin. By Triton X-100 extraction, only 5% of 6myc-Sed5p was recovered in the extract. So, almost all 6myc-Sed5p was still bound to Pansorbin after solubilization of the membrane.

Sec22p/Sly2p is the vSNARE that appears on the ER-derived vesicles and represents a docking partner for Sed5p on the early Golgi [17]. Approximately 60% of Sec22p in the precleared lysate was recovered in the Sed5 vesicles and extractable from Sed5p-bound Pansorbin by Triton X-100. Therefore, although more detailed analysis is needed, the ma-

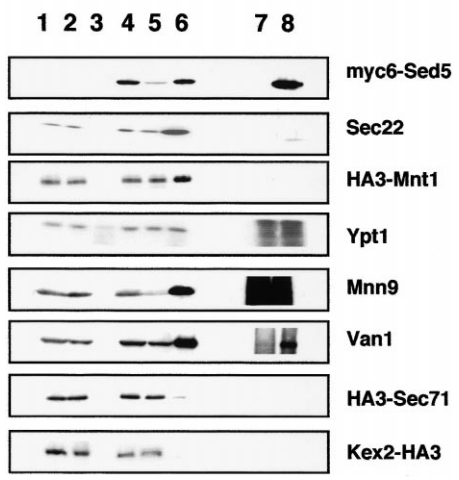

Fig. 2. Assessment of the Sed5 vesicles by the amount of marker proteins by immunoblotting. The Sed5 vesicles were isolated from KA31-1A (mock, lanes 1, 2, 3 and 7) or from CJY119 (lanes 4, 5, 6 and 8). Samples from each fraction (lanes 1 and 4, precleared lysate; lanes 2 and 5, unbound fraction; lanes 3 and 6, TX-soluble fraction; lanes 7 and 8, proteins still bound to Pansorbin after Triton X-100 extraction) were resolved by SDS–PAGE on 10% polyacrylamide gels followed by immunoblotting using various antibodies. In the case of HA3-Mnt1, HA3-Sec71, and Kex2-HA3, appropriate transformants of KA31-1A and CJY119 were used. The volume of elution was one-twentieth the volume of the starting precleared sample (see Section 2).

jority of Sec22p seems to locate on the same vesicles but physically free from Sed5p in its steady state.

Mnn9p and Van1p are the residents of the early and medial Golgi compartments as the components of α-1,6- and α-1,2-mannosyltransferase complex [12,16]. Ten to 20% of these proteins in the precleared lysate were recovered in the Sed5 vesicles. Mnt1p is the α-1,3-mannosyltransferase and a resident of the medial Golgi [18]. Twenty percent of 3HA-Mnt1p was recovered in the Sed5 vesicles.

Ypt1p, a small GTP-binding protein, is prenylated and localized partly on the Golgi membrane and in the cytoplasm. Although Ypt1p probably regulates the vesicle tethering and/or targeting to the early Golgi, only a small amount (4%) was recovered in the Sed5 vesicles.

No detectable amount of Kex2p-3HA was found on the Sed5 vesicles, in agreement with the finding that the late Golgi compartments were distinct from the earlier ones. A small fraction of 3HA-Sec71p (0.4%), an ER protein, was recovered

Table 1
Relative amounts of marker proteins in the Sed5 vesicles

Protein[a]	Unbound[b]	TX-soluble[b]
6myc-Sed5p	3 ± 0.2	5.4 ± 0.4
Sec22p	33 ± 7.1	60 ± 5.4
3HA-Mnt1p	58 ± 5.3	20 ± 7.4
Ypt1p	100 ± 11	4.1 ± 1.9
Van1p	70 ± 6.4	10 ± 6.4
Mnn9p	54 ± 12	20 ± 9.1
3HA-Sec71p	92 ± 2.8	0.4 ± 0.1

[a]Immunoreactive bands were visualized using enhanced chemiluminescence and the bands on the X-ray film were quantified using NIH image.
[b]The average percent and the standard deviation were calculated relative to the values in precleared lysate from at least three independent experiments.

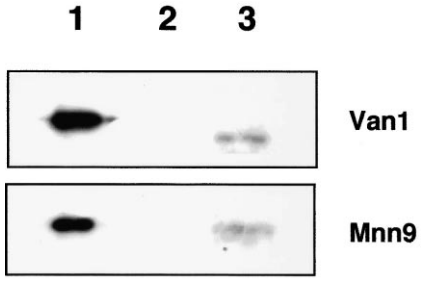

Fig. 3. Resistance of Van1p and Mnn9p to trypsin digestion in the Sed5 vesicles. Proteins in the Sed5 vesicles bound to Pansorbin (lane 1) were challenged with trypsin (10 mg/ml) in the presence (lane 2) or absence (lane 3) of 1% Triton X-100 for 10 min at room temperature. After addition of trypsin inhibitor, TX-soluble fractions were processed for SDS–PAGE and immunoblotting with anti-Van1 or anti-Mnn9 antiserum.

in the Sed5 vesicles. As Sec71p recycles between the ER and Golgi [19], this might represent the fraction that is in transit through the early Golgi compartment.

3.3. Intactness of the Sed5 vesicles

Protease protection experiments of the type II membrane proteins, Mnn9p and Van1p [12,16], were performed to see whether the Sed5 vesicles on Pansorbin are sealed or not. As shown in Fig. 3, only the short cytoplasmic domains were digested and the large lumenal parts were protected in the absence of detergent. This result suggested a considerable part of the membranes were sealed in the correct orientation.

3.4. Identification of the polypeptides enriched in the Sed5 vesicles

We determined the N-terminal amino acid sequences of several polypeptides apparently enriched in the Sed5 vesicles. Protein labeled with arrowhead f in Fig. 1 (lane 4) was identified as Anp1p. This coincides with the immunological data (Fig. 2) as Anp1p is a component of a Golgi mannosyltransferase complex including Mnn9p [10,14].

Proteins labeled with arrowheads a, b, and c were Emp24p [11], Erv25p [20], and Erp1p [21], respectively. These proteins form a complex and are assumed to participate in concentration of cargo molecules as the cargo receptors. Schimmöller et al. showed a bipartite distribution of Emp24p in sucrose density fractionation, i.e. a minor portion sedimented with the Golgi marker while the majority sedimented with the ER marker [11]. Our results clearly confirmed that the Emp24p/Erv25p/Erp1p complex and Sed5p stay exactly on the same vesicles for a while during the recycling process.

The protein labeled with arrowhead d was Ypt52p. Ypt52p was previously suggested to be required for early endosomes as its homolog Ypt51p/Vps21p [22]. As the precise localization and function have not yet been determined, there remains a possibility that Ypt52p may participate rather in the earlier traffic events. Our results showed that at least a part of Ypt52p localizes on the vesicles having Sed5p on their surface.

We also found a putative membrane protein of unknown function (Yml067c, 352 amino acids) was recovered in the TX-soluble fraction of the Sed5 vesicles (arrowhead e). Further studies to examine the main localization and function of this 40.7 kDa protein by epitope tagging are in progress. Detailed examination of the components of the Sed5 vesicles and its comparison to other Golgi subcompartments would be of

great interest because it will allow us to obtain a comprehensive understanding of the molecular architecture and function of the yeast Golgi complex.

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