Ykt6 Forms a SNARE Complex with Syntaxin 5, GS28, and Bet1 and Participates in a Late Stage in Endoplasmic Reticulum-Golgi Transport*

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The yeast SNARE Ykt6p has been implicated in several trafficking steps, including vesicular transport from the endoplasmic reticulum (ER) to the Golgi, intra-Golgi transport, and homotypic vacuole fusion. The functional role of its mammalian homologue (Ykt6) has not been established. Using antibodies specific for mammalian Ykt6, it is revealed that it is found mainly in Golgi-enriched membranes. Three SNAREs, syntaxin 5, GS28, and Bet1, are specifically associated with Ykt6 as revealed by co-immunoprecipitation, suggesting that these four SNAREs form a SNARE complex. Double labeling of Ykt6 and the Golgi marker mannosidase II or the ER-Golgi recycling marker KDEL receptor suggests that Ykt6 is primarily associated with the Golgi apparatus. Unlike the KDEL receptor, Ykt6 does not cycle back to the peripheral ER exit sites. Antibodies against Ykt6 inhibit in vitro ER-Golgi transport of vesicular stomatitis virus envelope glycoprotein (VSVG) only when they are added before the EGTA-sensitive stage. ER-Golgi transport of VSVG in vitro is also inhibited by recombinant Ykt6. In the presence of antibodies against Ykt6, VSVG accumulates in peri-Golgi vesicular structures and is prevented from entering the mannosidase II compartment, suggesting that Ykt6 functions at a late stage in ER-Golgi transport. Golgi apparatus marked by mannosidase II is fragmented into vesicular structures in cells microinjected with Ykt6 antibodies. It is concluded that Ykt6 functions in a late step of ER-Golgi transport, and this role may be important for the integrity of the Golgi complex.

The secretary pathway of eukaryotic cells contains a highly dynamic set of membrane compartments that are maintained by the continuous anterograde and retrograde flow of proteins and lipids. Transport vesicles formed from a donor compartment are specifically targeted to an acceptor compartment, where they deliver cargo through membrane fusion (1–6). Soluble NSF-ethylmaleimide-sensitive factor (NSF)1 (or its yeast counterpart, Sec18p) and soluble NSF attachment proteins (SNAPs) (or the yeast counterpart, Sec17p) have been shown to participate in many different transport events (7–10). NSF and SNAP serve to regulate the functional status of SNAP receptors (SNAREs) that play a major role in the final stage of vesicular transport. It is generally believed that the final event of docking and fusion of vesicles with the target compartment is mediated by interaction between v-SNAREs on vesicles and t-SNAREs on the target membrane (11–14). The action of NSF and SNAP is to prime the SNAREs by dissociating cis-SNARE complex so that unpaired free SNAREs on the vesicles and the target compartment can interact to form trans-SNARE complexes to mediate the final event of docking and fusion.

Six SNAREs including Bet1p, Sec22p/Sly12p, Bos1p, Ykt6p, Gos1p, and Sed5p have been shown to participate in ER-Golgi transport in yeast (14). Mammalian SNAREs known to participate in ER-Golgi transport include Bet1 (Bet1p homologue) (15–16), Sec22b (also known as ERS-24) (Sec22p homologue) (17–19), GS28 (also known as GOS-28) (homologous to yeast Gos1p) (20–22), and syntaxin 5 (Sed5p homologue) (23–24). Although GS27/membrin has also been implicated in intra-Golgi transport (25), a recent study suggests that syntaxin 5, GS27/membrin, Sec22b, and Bet1 form a SNARE complex that functions in homotypic fusion of ER-derived COP II vesicles (26), which may lead to the formation of dynamic large transport intermediates referred to as vesicular-tubular clusters (VTC) or ER-Golgi transport containers/carriers that actually undergo subsequent fusion with the Golgi apparatus (27). Consistent with this possibility, Sec22b and Bet1 are preferentially associated with the intermediate compartment (IC) and behave like constituents of intermediates involved in ER-Golgi transport (16, 19). Although preferentially associated with the Golgi apparatus, a significant portion (particularly an alternatively spliced longer form) of syntaxin 5 is associated with the IC and may be a component of the transport intermediate (24, 28). The majority of GS28 is associated with the cis-Golgi and is implicated in both ER-Golgi as well as intra-Golgi transport (20–21).

Yeast Ykt6p was originally identified by co-immunoprecipitation with Sed5p and is required for ER-Golgi transport and/or intra-Golgi transport (29–30). A recent study also showed that a significant portion of Ykt6p is associated with the vacuole and is involved in yeast vacuole homotypic fusion, suggesting that it may participate in multiple transport events (31). Mammalian proteins (Ykt6) homologous to Ykt6p have been identified, and the human gene can rescue the lethality of Ykt6p gene deletion in yeast (30, 32). Among the 35 known mammalian SNAREs (33), Ykt6 is the most conserved across phate; ERES, endoplasmic reticulum exit site; ERGIC, endoplasmic reticulum-Golgi intermediate compartment; PBS, phosphate-buffered saline; endo H, endoglycosidase H.

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1 The abbreviations used are: NSF, N-ethylmaleimide-sensitive factor; SNAP, soluble N-ethylmaleimide-sensitive factor attachment protein; SNARE, soluble N-ethylmaleimide-sensitive factor attachment protein receptor; ER, endoplasmic reticulum; PAGE, polyacrylamide gel electrophoresis; VSVG, vesicular stomatitis virus envelope glycoprotein; VTC, vesicular-tubular cluster; IC, intermediate compartment; FITC, fluorescein isothiocyanate; GST, glutathione S-transferase; GFP-β, guanosine 5′-3′-O-(thio)triphosphate; ERES, endoplasmic reticulum exit site; ERGIC, endoplasmic reticulum-Golgi intermediate compartment; PBS, phosphate-buffered saline; endo H, endoglycosidase H.
different species, suggesting an indispensable function in protein traffic. Another unique feature of Ykt6p and Ykt6 is that they contain COOH-terminal CAAX motif for prenylation. Despite these information, the exact subcellular localization, detailed functional studies, and interacting partners of Ykt6 have not been reported.

In this study, we have generated specific antibodies against mammalian Ykt6 to investigate the subcellular localization, interacting partners, and functional involvement of Ykt6 in protein traffic in mammalian cells. Our results suggest that Ykt6 forms a SNARE complex with syntaxin 5, GS28, and Bet1; participates in ER-Golgi transport; and is involved in maintaining the integrity of the Golgi apparatus.

MATERIALS AND METHODS

Expression and Purification of Recombinant GST-Ykt6—For the production of recombinant glutathione S-transferase (GST) fusion protein, the coding region of Ykt6 (residues 1–198) was retrieved by PCR with oligonucleotides 1 (5′-GGT CCT GTA GAC ATG AAG CTC AGC CTT AGT-3′) and 2 (5′-GGG CCG CTC GAG TCA CAT GAT TCA GCA AGA-3′) from a mouse kidney cDNA library and its sequence was confirmed by DNA sequencing. The polymerase chain reaction products were digested with NdeI and XhoI. The gel-purified DNA fragment was subcloned into the compatible sites of the bacterial expression vector pGEX-KG (34). The ligated DNA was transformed into DH5α cells, and ampicillin-resistant colonies expressing the GST fusion protein were screened as described (25, 35). Purification of GST-Ykt6 was performed as described previously (35).

Antibodies—For the preparation of polyclonal antibodies against Ykt6, GST-Ykt6 (400 µg) emulsified in complete Freund’s adjuvant was injected subcutaneously into New Zealand White rabbits. Booster injections containing similar amounts of the antigen emulsified in incomplete Freund’s adjuvant were performed after 2, 4, 6, 9, and 12 weeks. Rabbits were bled 10 days after the second and subsequent booster injections. Affinity purification of specific antibodies was performed using the GST-Ykt6 coupled to cyanogen bromide-activated Sepharose (3 mg/ml Sepharose bead). Affinity purification was carried out as described previously (35). Monoclonal antibodies against mammalian KDEL receptor (KDEL R), GS28 (also referred to as GOS-28), and GS27 (also referred to as membrin) have been described previously (25, 36–37). A monoclonal antibody against Golgi mannosidase II was purchased from Babco (Berkeley, CA). Rabbit polyclonal antibodies against syntaxin 5, Bet1, Sec22B, syntaxin 6, and Vti1-rp2 were described previously (16, 19, 38–40).

Immunofluorescence Microscopy—Immunofluorescence microscopy was performed as described previously (16, 19). Briefly, cells grown on coverslips were washed twice with PBSCM (PBS containing 1 mM CaCl2 and 1 mM MgCl2) and then fixed with 3% paraformaldehyde in PBSCM at 4 °C for 30 min. After washing with PBS/0.1% Triton X-100, cells were permeabilized with 100 µg/ml saponin (PBSCSM containing 0.1% saponin) for 20 min at room temperature. Incubation with primary antibodies (5–10 µg/ml) in fluorescence dilution buffer (PBS/0.1% bovine serum albumin, pH 7.6) was performed for 1 h at room temperature. After washing three times with PBSCSM, cells were incubated with rhodamine- or FITC-conjugated secondary antibodies for 1 h at room temperature. Cells were then washed five times with PBSCSM, mounted with Vectastain (Vector Laboratories), and then viewed. Confocal microscopy was performed using a Bio-Rad MRC1024 scan head connected to a Carl Zeiss Axiohot microscope equipped with epifluorescence optics. For temperature treatment of cells, NRK cells were incubated at 15 °C for 3 h and then processed for immunofluorescence microscopy. Infection of cells with the ts045 strain of VSV and the subsequent processing for immunofluorescence microscopy were performed as described previously (16, 19).

Immunoprecipitation and Immunoblot Analysis—Ykt6-specific antibodies (100 µg) bound to protein A-Sepharose beads (Amersham Pharmacia Biotech) were incubated overnight with 3 mg of Golgi extracts in incubation buffer (Hepes pH 7.2, 120 mM KCl, 1 mM dithiothreitol, 10 mM EDTA, 0.2 mM ATP with 15 mM Tris-X-100 with 4 °C). Both beads were then washed twice in incubation buffer with 0.5% Triton X-100, and then twice in incubation buffer with 0.2% Triton X-100. Immunoprecipitates were separated on SDS-PAGE and analyzed by immunoblot using antibodies against Ykt6, syntaxin 6, GS28, Bet1, Sec22B, GS27, Vti1-rp2, and syntaxin 6. Total membranes, cytosol, and fractionated membranes were derived from rat liver (37). Membranes were extracted, and the extracts were separated by SDS-PAGE and transferred to a Hyb-C 2 extra nitrocellulose filter. Immunoblot analysis was performed as described (16, 19).

In Vitro ER-Golgi Transport—The ER to Golgi transport assay using serum starvation cells was performed as described previously (16, 19, 41–42). Briefly, NRK cells grown on 10-cm Petri dishes in PBSCM were covered with a temperature-sensitive strain of the vesicular stomatitis virus, VSVts045, at 32 °C for 3 h. The cells were pulse-labeled with [35S]methionine (100 µCi/ml) at the restrictive temperature (40 °C) for 10 min and perforated on ice by hypotonic swelling and scraping. These semi-intact cells were then incubated in a complete transport buffer (40 °C) for 10 min and perforated on ice. After a 60-min incubation, the samples were digested overnight at 37 °C in the presence of 2.5 units of endoglycosidase H (endo H) and the reaction was terminated by adding 5× concentrated gel sample buffer. The samples were analyzed on 7.5% SDS-polyacrylamide gels. The transport was quantified using a Phosphorimager system (Molecular Dynamics). For antibody inhibition of the transport assay, Ykt6 antibodies were added into the complete assay mixture and incubated on ice for 60 min to allow the antibodies to diffuse into the semi-intact cells. In the case of the two-stage assay, after a stage 1 incubation for 60 min at 32 °C in a complete assay mixture supplemented with 12.5 mM EGTA but without Ca2+, membranes were spun for 20 s at full speed in an Eppendorf table-top centrifuge and subsequently resuspended in fresh assay mixture with Ca2+ by pipetting up and down 10 times with a yellow pipette tip. Additional reagents were added as indicated under “Results.” Samples were incubated for 30 min at 32 °C, and transport was terminated.

Morphological Analysis of VSVG Transport in Digitonin-permeabilized Cells—This was performed essentially as described previously (16, 19). Briefly, L2 cells were grown to confluence on 12-mm round coverslips. Cells were infected with VSV to a t045 at 32 °C for 1 h, followed by incubation at 40 °C for 2–3 h. Cells were rapidly transferred to ice and washed immediately with ice-cold washing buffer (50 mM Heps, pH 7.2, 90 mM KOAC, 2 mM MgOAc). Cells were permeabilized with digitonin (20 µg/ml in the washing buffer) on ice for 5 min. Coverslips were then transferred to 16-mm wells in washing buffer and incubated for 20 min on ice to release the cytosol. Coverslips were incubated in transport mixture supplemented with rat liver cytosol in the presence or absence of indicated antibodies on ice for 1 h, followed by an incubation at 32 °C for 2 h. Transport was terminated by washing with ice-cold washing buffer, and the cells were fixed immediately by 3% paraformaldehyde at 4 °C for 30 min.

Microinjection of L2 Cells with Antibodies—Microinjection was performed using a Zeiss Axiohot 35 microscope with a heated stage using an Eppendorf micromanipulation system as described (35). Injected cells were further incubated at 37 °C for 60 min before fixation. Microinjected cells were identified by the detection of microinjected antibodies using anti-rabbit secondary antibodies conjugated with FITC.

RESULTS

Membrane-associated Ykt6 Behaves like an Integral Membrane Protein and Is Enriched in the Golgi Membrane—The coding region of mouse Ykt6 predicts that it is very hydrophilic in nature and does not possess a transmembrane-spanning domain. However, Ykt6 contains a carboxyl-terminal CAAX motif, suggesting that this protein may be post-translationally modified by the addition of a 15- or 20-carbon isoprenoid group (30, 32). Prenylation of yeast Ykt6 is required for its membrane association, which is critical for its biological function (30). When isolated membranes from cells from NRK cells was analyzed by immunoblot using Ykt6 antibodies, a major polypeptide of about 26 kDa was detected (Fig. 1A, lane 1). Detection of this polypeptide was abolished by pre-incubating antibodies with recombinant GST-Ykt6 (Fig. 1A, lane 2) but not with GST (Fig. 1A, lane 2).

To study the cellular distribution of Ykt6, we separated soluble and membrane fraction from rat
liver homogenate. When equal fractions of rat liver cytosol (rlc) and total membranes were analyzed by immunoblot, it was found that 70–80% of total Ykt6 exists in the cytosol whereas the remaining 20–30% are membrane-associated (Fig. 1B). The detection of cytosolic pool of Ykt6 is not due to contamination of vesicles containing Ykt6 because, under the same condition, the vesicle-associated membrane protein Bet1 was not found in the cytosol (Fig. 1C). We further analyzed the distribution of Ykt6 using equal amounts of rat liver cytosol, total membranes (TM), microsomal membranes (MM), and Golgi/IC enriched membranes (GM) (16, 19, 37). Immunoblot analysis of these fractions with Ykt6 antibodies showed that Ykt6, like Golgi/IC-

**Ykt6 in ER-Golgi Transport**

**Ykt6 Interacts with Syntaxin 5, GS28, and Bet1**—To gain understanding about the biochemical and functional aspects of Ykt6, we have investigated its interaction with other SNAREs operating in the early secretory pathway. Golgi extracts were immunoprecipitated with Ykt6 antibodies or control antibodies. The immunoprecipitates of Ykt6 antibodies (IP) and control antibodies (control IP) as well as one-fifth of the immunoprecipitation supernatants (S) were analyzed by immunoblot to detect a variety of proteins as indicated.

![Fig. 1](http://www.jbc.org/content/27482/Fig1)

**Fig. 1.** A, antibodies raised against GST-Ykt6 recognize a specific 26-kDa protein. The postnuclear total membrane fraction of NRK cells was resolved by SDS-PAGE and transferred to a filter. The filter was incubated with Ykt6 antibodies (lane 1), in the presence of GST (lane 2), or GST-Ykt6 (lane 3). B, Ykt6 exists in both cytosol and membranes. Rat livers were homogenated and separated into cytosol and membranes. Equal fractions of cytosol (rlc) and total membranes (TM) were analyzed by immunoblot with Ykt6 antibodies. C, membrane-associated Ykt6 is enriched in Golgi/IC membranes. Cytosol (rlc), Golgi/IC enriched membranes (GM), microsomal membranes (MM), and total membranes (TM) derived from rat livers were analyzed by immunoblot to detect Ykt6 and IC enriched membrane protein Bet1. D, membrane-associated Ykt6 behaves like an integrated membrane protein. Golgi/IC enriched membranes were extracted with a range of different reagents as indicated and then separated by high speed centrifugation into pellet (P) and supernatant (S) fractions, which were further analyzed by immunoblot to detect Ykt6. NP40, Nonidet P-40.

![Fig. 2](http://www.jbc.org/content/27482/Fig2)

**Fig. 2.** Ykt6 exists in a novel SNARE complex with syntaxin 5, GS28, and Bet1. Golgi extracts were immunoprecipitated with Ykt6 antibodies or control antibodies. The immunoprecipitates of Ykt6 antibodies (IP) and control antibodies (control IP) as well as one-fifth of the immunoprecipitation supernatants (S) were analyzed by immunoblot to detect a variety of proteins as indicated.

enriched Bet1, is enriched in the Golgi/IC enriched membrane fraction (Fig. 1C). Unlike Bet1, which is exclusively membrane-associated, Ykt6 is also detected in the cytosol. To determine the type of association of Ykt6 with the membrane, we extracted the Golgi/IC enriched membrane fraction with PBS, 2 M KCl, 2.5 M urea, 0.15 M sodium bicarbonate (pH 11.5), 1% Triton X-100, or 1% Nonidet P-40. It was found that the membrane pool of Ykt6 was not solubilized by PBS, 2 M KCl, 2.5 M urea, or 0.15 M sodium bicarbonate (pH 11.5) (Fig. 1D, lanes 1–8) and was only partially solubilized by 1% Triton X-100 or 1% Nonidet P-40 (Fig. 1D, lanes 9–12). This suggests that the membrane pool of Ykt6 behaves as an integral membrane protein. Our results suggest that Ykt6 is enriched in the Golgi membrane, in addition to its presence in the cytosol.

Ykt6 Interacts with Syntaxin 5, GS28, and Bet1—To gain understanding about the biochemical and functional aspects of Ykt6, we have investigated its interaction with other SNAREs operating in the early secretory pathway. Golgi extracts were immunoprecipitated with immobilized Ykt6 antibodies, and the immunoprecipitate was analyzed by immunoblot (Fig. 2). As expected, Ykt6 itself was efficiently immunoprecipitated. In addition, significant amounts of syntaxin 5, GS28, and Bet1 were detected, GS28 and syntaxin 5 are two cis-Golgi SNAREs involved in ER-Golgi and intra-Golgi transport events (20–24, 44). Bet1 cycles in the early secretory pathway and participates in ER-Golgi transport (16). The coimmunoprecipitation of these SNAREs is specific because they were not detected in the immunoprecipitates of control antibodies. Under identical conditions, Sec22b/ERS-24 (17–19), GS27/membrin (17, 25), Vti1-rp2 (39), and syntaxin 6 (40, 45) were not coimmunoprecipi-
tated by Ykt6 antibodies. Endosomal SNAREs such as endobrevin, syntaxin 7, 8, and 12/13 were not co-immunoprecipitated (data not shown). Sec22b/ERGIC-53, Bet1, GS27/membrin, and Vti1-rp2 have been reported to interact with the cis-Golgi SNARE syntaxin 5. The preferential coimmunoprecipitation of a subset of syntaxin 5 interacting proteins with Ykt6 antibodies suggests that Ykt6 exists in a unique syntaxin 5 containing SNARE complex. Although Sec22b, GS27/membrin, and Bet1 are implicated in ER-Golgi transport, only Bet1 but not Sec22b or GS27/membrin was co-immunoprecipitated with Ykt6, suggesting that Ykt6 interacts with a specific subset of SNAREs involved in ER-Golgi transport. Ykt6, syntaxin 5, GS28, and Bet1 may therefore form a unique SNARE complex. Our previous studies (19) also indicated that Sec22b may act before Bet1. The co-immunoprecipitation of Bet1 but not Sec22b therefore suggests that Ykt6 may be involved in a late event in ER-Golgi transport.

Association of Ykt6 with the Golgi Apparatus—The association of Ykt6 with Golgi-enriched membranes and its unique interaction with syntaxin 5, GS28, and Bet1 suggest that Ykt6 may be localized to the Golgi apparatus. To provide more direct insight into the subcellular localization of Ykt6, we performed indirect immunofluorescence microscopy. Ykt6 antibody labeling of NRK cells revealed that Ykt6 is mainly associated with perinuclear membrane structures (Fig. 3a). Diffuse, cytosolic labeling was seen to some extents but not obvious, which might be due to lower concentrations of Ykt6 in the cytosol. The perinuclear structure positive for Ykt6 is the Golgi apparatus because it is also marked by the medial Golgi marker mannosidase II (46) (Fig. 3, a–c). The Golgi labeling of Ykt6 is specific because it was totally abolished when the antibodies were pre-incubated with GST-Ykt6 but not with GST (data not shown). Similar Golgi labeling of Ykt6 was also detected in other cells (data not shown). The KDEL receptor cycles between the Golgi, the ER exit sites (ERES), and the ER-Golgi intermediate compartment (ERGIC) and is dynamically distributed in the cis-Golgi and peripheral ERES and ERGIC (36, 47–49). When Ykt6 was double-labeled with the KDEL receptor, Ykt6 colocalized only with the Golgi-associated pool of the KDEL receptor but not with the peripheral pools (Fig. 4, a–c).

Nocodazole is known to fragment the Golgi apparatus into smaller mini-Golgi elements distributed more peripherally, although still enriched in the perinuclear region (50). When cells were treated with nocodazole, Ykt6 was seen to colocalize well with Golgi mannosidase II in the fragmented Golgi structures (Fig. 3, d–f). When Ykt6 was double-labeled with the KDEL receptor in nocodazole-treated cells, it was found that only the perinuclear structures contain both Ykt6 and the KDEL receptor, while the more peripheral structures marked by the KDEL receptor (likely representing the ERES and the ERGIC) was negative for Ykt6, suggesting again that Ykt6 colocalizes only with the Golgi pool of the KDEL receptor.

Incubation of cells at 15 °C is known to inhibit transport from the ERES/ERGIC to the Golgi and cause a shift of cycling proteins such as the KDEL receptor, ERGIC-53, Bet1, Sec22b, syntaxin 5, and members of the p24 protein family from the Golgi pool to the peripheral ERES/ERGIC (16, 19, 36, 47–49, 51–52). When cells were examined after 3 h of incubation at 15 °C, the tight Golgi structure marked by Ykt6 and mannosidase II was only slightly dispersed into several smaller patches (Fig. 3, g–i). It is interesting to note that structures marked by Ykt6 and mannosidase II are adjacent and partially overlapping with each other, but the labeling do not totally colocalize, suggesting that Ykt6 and mannosidase II may be localized to different subcompartments of the Golgi apparatus. Under the same condition, the peripheral spotty structures labeled by the KDEL receptor became more prominent with concomitant reduction in the dispersed Golgi patches (compare Fig. 4, b with h). Ykt6 was only colocalized with the KDEL receptor in the dispersed Golgi patches but not in the peripheral structures (Fig. 4, g–i). Unlike the case with mannosidase II, the colocalization of Ykt6 with the KDEL receptor in the dispersed Golgi is essentially complete. Since the KDEL receptor is enriched in the cis-Golgi while mannosidase II is confined more to the medial-Golgi (46–48), the observed distribution of Ykt6 sug-
gests that it is enriched in the cis-Golgi. Taken together, these results suggest that Ykt6 is predominantly localized to the cis-Golgi and does not recycle back to the peripheral ER-ERGIC, a property similar to GS28 but distinct from the cycling property of syntaxin 5, Bet1, and Sec22b.

Antibodies against Ykt6 and Recombinant GST-Ykt6 Inhibit in Vitro ER-Golgi Transport—The preferential localization of Ykt6 in the cis-Golgi and its existence in a unique SNARE complex with syntaxin 5, GS28, and Bet1 suggest that Ykt6 may be involved in a late step in protein transport from the ER to the Golgi in mammalian cells. To investigate this, we have examined whether in vitro ER-Golgi transport could be affected by antibodies against Ykt6. We used the well established in vitro ER-Golgi transport assay using semi-intact VSV ts045-infected NRK cells (16, 19, 41–42). Infected NRK cells were pulse-labeled with [35S]methionine at 40 °C so that the labeled G protein is restricted to the ER. The plasma membrane was then selectively perforated, and the cells were depleted of endogenous cytosol. Synchronized transport of VSVG could be reconstituted when these semi-intact cells are incubated at the permissive temperature (32 °C) supplemented with exogenous cytosol and an ATP-regenerating system. ER-Golgi transport was measured by following the extents of conversion of ER-restricted endo H-sensitive VSVG into endo H-resistant Golgi form. As shown in Fig. 5A, no transport was detected when semi-intact cells were incubated on ice (panel a, lane 1). The majority of VSVG was converted into endo H-resistant Golgi form when incubated at 32 °C (lane 2). Transport from the ER to the Golgi was, however, inhibited by antibodies against Ykt6 in a dose-dependent manner (lanes 2–7). Approximately 50% of the VSVG was not converted into the Golgi form with 0.5 μg of antibodies. Transport was almost completely inhibited when 1 μg or more of Ykt6 antibodies were added. The inhibition is specific because the same amount of heat-denatured antibodies had no effect on transport (panel b) and comparable amounts of antibodies against the KDEL receptor and several other control antibodies had no effect as well (data not shown). Importantly, VSVG transport from the ER to the Golgi was also inhibited by recombinant GST-Ykt6 in a dose-dependent manner. A clear inhibition by GST-Ykt6 could be seen when 7 μg or more of the recombinant protein was added to the transport assay (panel c).

Furthermore, inhibition exhibited by antibodies against Ykt6 could be neutralized by a non-inhibitory amount of GST-Ykt6 (panel d). G protein transport to the Golgi was completely inhibited by 2 μg of Ykt6 antibodies (lane 4). However, pre-incubation of 2 μg of Ykt6 antibodies with 2 μg of GST-Ykt6 resulted in ~80% of VSVG being converted into the Golgi form (lane 5). These results, taken together, suggest that inhibition of ER-Golgi transport by Ykt6 antibodies occurs by specific interaction of antibodies with endogenous Ykt6. Ykt6 is therefore essential for ER-Golgi transport.

In vitro ER-Golgi transport could be inhibited by EGTA at a late stage after docking of transport intermediates but before the actual fusion event (42, 53, 54). We have found that Ykt6 antibodies must be present before the EGTA-sensitive stage in order to exhibit an inhibitory effect (Fig. 5B). In this experiment, in vitro ER-Golgi transport was first performed in the presence of EGTA to arrest transport at the EGTA-sensitive stage. Semi-intact cells were then washed, resuspended in complete transport mixture, and proceeded to a second stage of incubation to continue the events between the EGTA-sensitive stage and the actual membrane fusion. VSVG remained in the endo H-sensitive ER form after the first stage of incubation (lane 4). A second stage of incubation in fresh cytosol and complete transport mixture allowed more than 50% conversion of the EGTA-arrested ER form into the endo H-resistant Golgi form (lane 5). Inclusion of Ykt6 antibodies in standard transport assay inhibited the transport to the background level (lane 6). However, when Ykt6 antibodies were included only in the second stage of incubation, transport was essentially not affected (lane 7), suggesting that Ykt6 antibodies could not inhibit the transport when present only in the second stage of

![Fig. 5](image-url)
transport assay. As shown previously, GTPγS (53) and Bet1 antibodies (16, 19) were also no longer inhibitory to ER-Golgi transport when supplemented only at the second stage of incubation (lanes 8–11). These results suggest that Ykt6 antibodies can no longer gain access to Ykt6 or that Ykt6 is no longer required after the EGTA-sensitive stage.

VSVG Transport Is Arrested at a Late Stage at the Golgi Apparatus by Ykt6 Antibodies—VSVG transport from the ER to the Golgi can also be followed morphologically in digitonin-permeabilized cells (42, 43). In this assay, ER-exported G protein was first seen in peripheral ERES mediated by COP II, and then in larger transport intermediates, referred to as VTC, ERGIC, or transport containers, which migrate to and then fuse with the Golgi apparatus. COP I is associated with larger transport intermediates and is important for their maturation (27, 49). Previous studies showed that Sec22b/ERS-24 and Bet1 antibodies block the VSVG transport in the peripheral ERES and peri-Golgi larger transport intermediates, respectively (19). To gain additional insight into the functional stage of Ykt6, we performed morphological studies to investigate the site of inhibition of VSVG G protein transport in the presence of Ykt6 antibodies. L2 cells were used because of larger sizes. When transport was performed in the absence of cytosol at 32 °C for 120 min, G protein was detected the ER (Fig. 6A, panel b). Ykt6 labeling was still concentrated in the perinuclear Golgi region (Fig. 6A, panel a), although becoming more fragmented (characteristic of the Golgi in the semi-intact cells). When transport was performed in the complete transport mixture for 120 min, VSVG was transported into large vesicular structures in the Golgi region and colocalized significantly with Ykt6 (Fig. 6A, panels d–f). At this stage, Ykt6 was colocalized with mannosidase II, suggesting that VSVG had reached the Golgi (data not shown). When the transport assay was performed in the presence of antibodies against Ykt6, VSVG was similarly accumulated in large vesicular structures in the Golgi region and colocalized significantly with Ykt6 (Fig. 6B, panels a–c), suggesting that VSVG transport was inhibited in this stage. Double labeling of Ykt6 with Golgi mannosidase II revealed that they are, although closely apposing each other in the Golgi region, essentially not colocalized (Fig. 6B, panels d–f). These results suggest that Ykt6 antibodies prohibited VSVG from entering the Golgi apparatus. This conclusion is in agreement with that derived from our in vitro biochemical transport studies. The likely explanation is that Ykt6 antibodies prevented fusion of large transport intermediates with the Golgi apparatus. Alternatively, it could be interpreted as that Ykt6 antibodies prevented formation of new Golgi cisternae from these transport intermediates, in the context of the maturation model for the secretory pathway (55–58).

Microinjection of Ykt6 Antibodies Causes Golgi Fragmentation—To sustain our in vitro results and to understand the role of Ykt6 in vivo, we microinjected Ykt6 antibodies into L2 cells to inhibit the function of endogenous Ykt6. Cells were incubated at 37 °C for 60 min after microinjection and then fixed. Indirect immunofluorescence microscopy was performed to reveal the injected antibodies and the Golgi apparatus marked by Golgi mannosidase II. As shown in Fig. 7, the Golgi apparatus marked by Golgi mannosidase II was essentially not affected in cells microinjected with control antibodies (rabbit IgG) (panels a–c). However, the Golgi apparatus marked by mannosidase II became fragmented into punctuated vesicular structures in cells microinjected with antibodies against Ykt6 (panels d–f). In cells microinjected with antibodies against Sec22b, Golgi mannosidase II was completely redistributed into the ER (panels g–i). These results not only highlight an important role of Ykt6 in the early secretory pathway in vivo but also support the interpretation that Ykt6 acts later than Sec22b.

**DISCUSSION**

Our present study suggests that Ykt6 is preferentially localized to the cis-Golgi and its associated structures. This conclusion is based on several lines of evidence. First, Ykt6 was present at high levels in Golgi-enriched membranes. Second, indirect immunofluorescence labeling with antibodies against Ykt6 revealed that Ykt6 is present in the Golgi apparatus marked by Golgi mannosidase II. When cells were incubated at 15 °C, Ykt6-positive structures only partially overlapped with that of Golgi mannosidase II, suggesting that it is localized to a subcompartment distinct from the medial-Golgi enriched for Golgi mannosidase II (46). Since Ykt6 colocalized well with the Golgi pool of the KDEL receptor at 15 °C and the KDEL receptor is enriched in the cis-Golgi (47–48), Ykt6 is likely to be enriched in the cis-Golgi. Although the Golgi structures marked by the KDEL receptor are positive for Ykt6, the peripheral structures (ER/CRCI) marked by the KDEL receptor are devoid of Ykt6 either at steady state or upon incubation at 15 °C, which is known to induce shift of the KDEL

**FIG. 6.** Ykt6 antibodies inhibit VSVG transport at the Golgi region. A, VSV ts045-infected L2 cells grown on coverslips were permeabilized with digitonin. Cells were incubated at 32 °C for 120 min in transport buffer without (panels a–c) or with (panels d–f) rat liver cytosol (rlc). Cells were fixed and double-labeled with antibodies against Ykt6 (a and d) and VSVG (b and e). Merged images are also shown (c and f). B, VSV ts045-infected L2 cells grown on coverslips were permeabilized with digitonin and incubated for 120 min at 32 °C in complete transport mixture buffer supplemented with Ykt6 antibodies. Cells were fixed and double-labeled to reveal the Ykt6 antibodies (a and d) with either VSVG (b) or mannosidase II (Man II) (c). Merged images are also shown (c and f). Bars, 10 μm.
receptor and other cycling proteins from the Golgi to the peripheral structures. This property of Ykt6 is different from that of Bet1 and Sec22b (16, 19), which are both detected in and become more concentrated in the peripheral structures upon 15 °C treatment. Third, the existence of Ykt6 in a unique SNARE complex with Bet1, syntaxin 5, and GS28 is consistent with this conclusion because syntaxin 5 and GS28 are both enriched in the cis-Golgi, whereas Bet1 and syntaxin 5 cycles between the cis-Golgi and earlier ERES/ERGIC. In conjunction with previous studies, our results suggest that the six SNAREs involved in ER-Golgi transport could be used in a combinatorial manner to form at least two distinct SNARE complexes.

The high amino acid sequence identity of Ykt6 with Ykt6p and the ability of human Ykt6 to substitute for Ykt6p in yeast suggest that Ykt6 may function as the mammalian equivalent of Ykt6p in ER-Golgi transport. The enrichment of Ykt6 in the cis-Golgi and its existence in a distinct SNARE complex further point to such a role. Several lines of direct evidence suggest that Ykt6 indeed participates in ER-Golgi transport. First, ER-Golgi transport of VSVG in semi-intact cells was inhibited by antibodies against Ykt6 in a dose-dependent manner. This inhibition is specific because the same amount of antibodies denatured by boiling did not inhibit ER-Golgi transport of VSVG. Furthermore, the inhibitory effects can be neutralized by preincubation of antibodies with a non-inhibitory amount of recombinant GST-Ykt6. Second, recombinant GST-Ykt6 also exhibited dose-dependent inhibition on ER-Golgi transport of VSVG, although GST-Ykt6 is not as potent as its antibodies. The relatively lower potency of GST-Ykt6 could be due to lack of membrane association of GST-Ykt6 (not prenylated) and/or the possibility that only a fraction of the recombinant protein being properly folded. An additional understanding of the involvement of Ykt6 in ER-Golgi transport was revealed by the demonstration that Ykt6 antibodies must be present before the EGTA-sensitive stage in order to be inhibitory. Since EGTA inhibits ER-Golgi transport at a late stage after docking of transport intermediates with the cis-Golgi but before the actual membrane fusion event (42, 53–54), our results indicate that once transport intermediates have docked onto the cis-Golgi membrane, Ykt6 antibodies are no longer inhibitory in the transport assay. This observation can be explained in two alternative ways. The first is that Ykt6 is only important for the docking process but not for the fusion event.

Alternatively, once transport intermediates have docked onto the cis-Golgi membrane, Ykt6 becomes incorporated into a large SNARE complex in such a way that Ykt6 is no longer accessible to the antibodies. In view of the recent demonstration that SNAREs are not only involved in the docking but also participate directly in the fusion event by forming a highly twisted structure of four helical bundles (59–62), we favor the latter possibility. Similarly, antibodies against Bet1, Sec22b (16, 19), or syntaxin 5 must also be added before the EGTA-sensitive stage in order to exhibit an inhibition on ER-Golgi transport.

Finally, the functional importance of Ykt6 in intact cells was established by the observation that the Golgi marked by mannosidase II is dispersed into smaller vesicular structures in cells microinjected with Ykt6 antibodies. In addition to the possibility that Ykt6 may have a direct role in maintaining the integrity of the Golgi structure by a yet to be defined mechanism, the observed effects could be also due to a direct inhibition on ER-Golgi transport at the stage of docking and fusion of large transport intermediates with the cis-Golgi. The vesicular structures could consist of the unfused transport intermediates as well as fragmented Golgi stack resulting from inadequate influx of materials required to sustain the structure of dynamic Golgi apparatus. Such an inhibition could result in a shift of steady-state Golgi distribution of Golgi mannosidase II into these vesicular structures. On the other hand, microinjection of antibodies against Sec22b resulted in a shift of Golgi mannosidase II into the ER, similar to a inhibition on ER export exhibited by mutant Sar1 (63). This suggests that Sec22b antibodies exhibited an inhibition at an earlier stage in vivo, such that its effect is indistinguishable from that resulted from inhibition on vesicular budding from the ER by mutant Sar1. These observations could thus be interpreted by proposing that ER-Golgi transport involved two distinct SNARE complexes that act sequentially. The first SNARE complex, consisting of Sec22b/ERS-24, GS27/membrin, Bet1, and syntaxin 5, is involved in the formation of large transport intermediates (VTC, ERGIC, or transport containers) from newly derived COPII vesicles (26). The second SNARE complex identified in our current study, consisting of Ykt6, GS28/GOS-28, Bet1, and syntaxin 5, may be utilized for the fusion of these large transport intermediates with the cis-Golgi. The observed effects of anti-Ykt6 antibodies in morphological transport assay as well as the microinjection experiments are consistent with this speculation. Both Sec22b (component of the first SNARE complex) and Ykt6 (of the second SNARE complex) antibodies inhibited ER-Golgi transport of VSVG before the EGTA-sensitive stage. However, there is a clear distinction between the effect of Sec22b antibodies as compared with Ykt6 in both morphological transport assay as well as in microinjection experiments. This distinct effect is supportive to the hypothesis of two dis-

2 T. Zhang and W. Hong, unpublished observation.
tinct SNARE complexes acting sequentially during ER-Golgi transport. Although several (up to seven) distinct Sed5p-containing yeast quaternary SNARE complexes could be formed by combinatorial SNARE interactions in vitro (64), there is no equivalent complex consisting of Ykt6p, Gos1p, Bet1p, and Sed5p. This observation may reflect the fact that no equivalence to large transport intermediates characteristic of VTC, ERGIC, or transport containers have ever been identified in yeast. The Ykt6-GS28-Bet1-syntaxin 5 quaternary SNARE complex may therefore be uniquely found in mammalian cells for the fusion of large transport intermediate with the cis-Golgi. More studies are needed to address this possibility.

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Ykt6 in ER-Golgi Transport

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