Ykt6p, a Prenylated SNARE Essential for Endoplasmic Reticulum-Golgi Transport*

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Vesicular transport between secretory compartments requires specific recognition molecules called SNAREs. Here we report the identification of three putative SNAREs, p14 (S01p), p28 (Gos1p), and a detailed characterization of p26 (Ykt6p). All three were originally isolated as interacting partners of the cis Golgi target membrane-associated SNARE Sed5p, when Sec18p (yeast NSF) was inactivated. YKT6 is an essential gene that codes for a novel vesicle-associated SNARE functioning at the endoplasmic reticulum-Golgi transport step in the yeast secretory pathway. Depletion of Ykt6p results in the accumulation of the p1 precursor (endoplasmic reticulum form) of the vacuolar enzyme carboxypeptidase Y and morphological abnormalities consistent with a defect in secretion. Membrane localization of Ykt6p is essential for protein function and is normally mediated by isoprenylation. However, replacement of the isoprenylation motif with a bona fide transmembrane anchor results in a functional protein confirming that membrane localization, but not isoprenylation per se, is required for function. Ykt6p and its homologues are highly conserved from yeast to human as demonstrated by the functional complementation of the loss of Ykt6p by its human counterpart. This is the first example of a human SNARE protein functionally replacing a yeast SNARE. This observation implies that the specific details of the vesicle targeting code, like the genetic code, are conserved in evolution.

The N-ethylmaleimide-sensitive fusion protein (NSF) and the soluble NSF attachment proteins (SNAPs) are required for vesicular transport at multiple steps (1–6). However, transit through the secretory pathway requires a mechanism to ensure the fidelity of transport vesicles docking to their correct target membrane. The known molecular machinery mediating this process consists of SNAP receptors (SNAREs) localized to vesicles (v-SNAREs)† that form a specific match with t-SNAREs

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† The abbreviations used are: v-SNARE, vesicle-associated SNARE; t-SNARE, target membrane-associated SNARE; CPY, carboxypeptidase Y; ER, endoplasmic reticulum; PCR, polymerase chain reaction; ORF, open reading frame; EST, expressed sequence tag; PAGE, polyacrylamide gel electrophoresis; VAMP, vesicle-associated membrane protein.

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novel proteins, p28, p14, and p26 (36).

Here we report the identification of the putative SNAREs p28, p14, and p26 and the detailed characterization of p26, a unique SNARE with close homologues throughout the eukaryotic lineage, each characterized by an isoprenylation signal.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains and Media—**Yeast strains (Table I) were routinely grown in rich media (1% yeast extract, 2% peptone, and either 2% dextrose-YPD, 8% dextrose-YPDS, or 2% raffinose and 0.5% galactose-YPRG) for wild type cells, MSY60, and JMY89. Synthetic complete media included 0.67% yeast nitrogen base (Difco) and 2% dextrose-YPD, 8% dextrose-YPD8, or 2% raffinose and 0.5% galactose-SCDG or 2% raffinose, 0.5% galactose (SCRG), and the appropriate auxotrophic supplements (Bio101).

**Glucose Depletion of HyKt6—**A 500-ml pre-culture of JMY89 was grown overnight in YPRG media. At time 0, 200 A 500 units of cells were harvested by centrifugation at 1,500 × g for 5 min. One liter of YPRG or YPDS (8% glucose) was inoculated at 0.1 A 500/ml. Aliquots were taken at 2-h intervals to measure cell density. Additionally, 20 A 500 units were harvested, washed in water, and frozen as cell pellets in liquid nitrogen. At 10 and 24 h an additional 10 A 500 units were recovered for electron microscopic analysis. Glass bead extracts were prepared from the 2-h time points, and equal amounts of the extracts were analyzed with the anti-carboxypeptidase Y monoclonal antibody 10A5-B5 (Molecular Probes) at a dilution of 1:1,000.

**Sequence Alignment—**The Multiple Sequence Alignment was performed using PILEUP from the University of Wisconsin Genetics Computer Group sequence package (40), and the shading was done using BOXSHADE. The sequences represented in Fig. 1 are: Sc Ykt6p is S. cerevisiae chromosome XI ORF YKL196c (Z28196); Hs Ykt6p represents several overlapping human ESTs (THC111483) (H23796, H0958, H18232, J76979, H23795, H18270, and H40165); Ce Ykt6p is an amino acids 519–720 of the ORF B0361.8 from the cosmid B0361 (U00031); Ec Ykt6p in Euplotes crassus ORF1 (M73925); At Ykt6p is an EST from Arabidopsis thaliana cDNA clone 15ID57T (T76779), Mm Ykt6p is an EST from Mus musculus cDNA clone 403610 (W82343); and Rn Ykt6p is EST110382 (H39392) from Rattus norvegicus.

**Preparation of Recombinant Ykt6p and Generation of Ykt6p Antisera—**Recombinant, His-tagged Ykt6p was purified from inclusion bodies in Escherichia coli (XL1-Blue) expressing pMS130 using nickel-nitrilotriacetic acid-agarose according to the manufacturer’s instructions. Two New Zealand White Rabbits (215 and 216) were immunized with the fusion protein and bleeds were obtained 21 days after immunization.

**Peptide Sequencing and Mass Spectrometry—**Proteins separated by SDS-PAGE were electroblotted onto nitrocellulose, and the visualized bands were processed for internal amino acid sequence analysis as described (41). Briefly, membrane bound proteins were digested in situ with trypsin, and the resulting peptides were separated by narrow bore reverse phase high pressure liquid chromatography. Selected peptides were then subjected to chemical microsequencing and matrix-assisted laser desorption ionization mass spectrometry, also as described previously (42, 43).

The 14-kDa band (p14) in the Sed5p immunoprecipitate from extracts of sec18–1 at the permissive temperature was identified by laser desorption ionization mass spectrometry, also as described previously (42, 43).
Identification of Sed5p Interacting Proteins—The identities of p28, p26, and p14 were determined by microsequencing and mass spectrometry. The isolated Sed5p complex was resolved by SDS-PAGE and blotted on to nitrocellulose. p26 (Ykt6p) was previously reported to be encoded by the ORF YKL196c on chromosome XI (36). Microsequencing and mass spectrometry of tryptic peptides derived from the 28-kDa band revealed that p28 is identical to the 223-amino acid protein with a predicted molecular mass of 25,394 Da encoded by the ORF YHL031c on chromosome VIII. This protein, named Gos1p (Golgi SNARE) is a SNARE protein with a carboxyl-terminal membrane anchor. 4 Similarly, peptide sequence obtained from the 14-kDa protein led to the identification of p14 as a 97-amino acid (10,960 Da) protein located on chromosome XI. p14 is identical to the product of the SFT1 gene, a gene identified independently by Pelham and colleagues as a high copy suppressor of a sed5-1 allele (44).

BLAST searches of various data bases revealed that Ykt6p has homologues in many species including man and that all possess a high degree of sequence similarity (Fig. 1A) (51). The yeast protein is 47% identical in amino acid sequence to the human protein, HaYkt6p, whereas the Caenorhabditis elegans ORF is 57% identical to HaYkt6p. Hydropathy analysis by the method of Kyte and Doolittle (52) predicts that each of these proteins is a transmembrane protein with a hydrophobic sequence at position 20, which is characteristic is peculiar for a SNARE molecule, although post-translational modifications including S-N and O-glycosylation have been reported for other SNAREs (54–56).

Sequence comparisons of Ykt6p with defined SNAREs show a significant similarity to the SEC22 gene product as well as a lower but statistically significant similarity to the late acting v-SNAREs Snc1p and Snc2p.

RESULTS

Identification of Sed5p Interacting Proteins—The identities of p28, p26, and p14 were determined by microsequencing and mass spectrometry. The isolated Sed5p complex was resolved by SDS-PAGE and blotted on to nitrocellulose. p26 (Ykt6p) was previously reported to be encoded by the ORF YKL196c on chromosome XI (36). Microsequencing and mass spectrometry of tryptic peptides derived from the 28-kDa band revealed that p28 is identical to the 223-amino acid protein with a predicted molecular mass of 25,394 Da encoded by the ORF YHL031c on chromosome VIII. This protein, named Gos1p (Golgi SNARE) is a SNARE protein with a carboxyl-terminal membrane anchor. 4 Similarly, peptide sequence obtained from the 14-kDa protein led to the identification of p14 as a 97-amino acid (10,960 Da) protein located on chromosome XI. p14 is identical to the product of the SFT1 gene, a gene identified independently by Pelham and colleagues as a high copy suppressor of a sed5-1 allele (44).

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Ykt6 Is Essential and Can Be Functionally Replaced by Human YKT6—To address the function of Ykt6p, one copy of YKT6 was replaced with the LEU2 gene in the diploid strain W303 (Fig. 1C). Sporulation of this strain (MSTY41) resulted in only two viable progeny, both of which were Leu−. This result

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demonstrates that YKT6 serves an essential function either in germination, vegetative growth, or both. The ΔYKT6 strain could be recovered by an extra-chromosomal copy of the YKT6 gene expressed from the galactose inducible GAL1–10 promoter. Additionally, an amino-terminal epitope-tagged copy of YKT6 was completely functional (Table III).

The human YKT6 gene (HsYKT6) was identified by comparisons of the yeast sequence with the data base of human ESTs. The complete sequence of the open reading frame encoding human Ykt6p was obtained from the data base by splicing together several overlapping ESTs. The human cDNA was cloned by PCR from a human pancreatic cDNA library, and its sequence was confirmed. The HsYKT6 gene was put under the control of GAL1 promoter in a multicopy vector and transformed into the YKT6/ΔYKT6 heterozygous diploid strain JMY64. All of the resulting spores from this strain were viable when grown on rich media with galactose as a carbon source. Analysis of these progeny showed that all of the Leu" spores were also Ura", confirming that viability was plasmid-dependent. The HsYKT6 complemented ΔYKT6 strain (JMY89) grew approximately 25% slower in liquid culture than wild type (Table III). This is the first documented case of a human SNARE functionally complementing its yeast counterpart.

Membrane Anchoring, but Not Isoprenylation, Is Necessary for Ykt6p Function—The unique feature distinguishing Ykt6p from other described SNAREs is the absence of a transmembrane spanning domain and the presence of a carboxyl-terminal CAA box sequence (CCIIM) specifying isoprenylation. Because isoprenylation of an otherwise hydrophilic protein seems to be the only mode to anchor Ykt6p to the membrane, we determined the functional importance of this post-translational modification. To this end, a mutant was generated that eliminates the isoprenylation signal by mutating both cysteines at residues 196 and 197 within the CCIIM sequence to serine. This construct, Ykt6p-C196S-C197S, could not rescue the YKT6 deletion (Table III). Expression of this construct was confirmed in wild type cells (Fig. 2 and data not shown). To determine if a bona fide transmembrane domain could func-
tationally substitute for isoprenylation, we replaced the CCIIM at the carboxyl terminus of Ykt6p by the membrane spanning region of Gos1p. Interestingly, this hybrid, termed Ykt6p/Gos1TM, also rescued the YKT6 deletion (Table III).

Next, we determined the membrane association of Ykt6p, Ykt6p-C196S-C197S, and Ykt6p/Gos1TM. For this purpose these proteins were expressed in a wild type background (W3031B), some with an amino-terminal myc epitope. Yeast extracts from the different strains were detergent extracted and phase partitioned. In contrast to the wild type protein, myc-Ykt6p-C196S-C197S partitioned exclusively to the aqueous phase (Fig. 3B). Importantly, soluble Ykt6p, prepared simply by breaking total yeast in the absence of detergent and removing membranes by ultracentrifugation (Fig. 2), is found in the Triton X-114 detergent phase (Fig. 3C), suggesting that the soluble pool is also modified by isoprenylation.

Finally, we wanted to determine the prenylation status of the soluble pool of Ykt6p. In yeast, in contrast to mammalian cells, it is currently not possible to test for the presence of an isoprenyl group by growing yeast in the presence of radiolabeled isoprenoid precursors (57). Therefore we chose to assay this isoprenyl group by growing yeast in presence of radiolabeled methionine and using the detergent Triton X-114. The cleared extract was then extracted with 2% Triton X-114, phase partitioned, and analyzed by SDS-PAGE, Western blots of extracts from wild type cells and MSY54 grown in glucose containing media with glucose concentrations up to 8% maintained for several weeks. This phenomena is likely attributable to small amounts of transcription of the gene in glucose, resulting in the production of sufficient Ykt6p to allow growth. Immunoblots of extracts from wild type cells and MSY54 grown in glucose show that Ykt6p is reduced at least 10-fold in MSY54 but is still present in detectable amounts (data not shown).

### Table III

| Strain   | Protein          | Carboxyl terminus | Rescue of null strain | Doubling time in YPG (min) |
|----------|------------------|-------------------|-----------------------|---------------------------|
| W3031A   |                  |                   |                       |                           |
| W3031B   |                  |                   |                       |                           |
| JMY59    | HsYkt6p          | KTARKQN5CCAIM      | ++                    | 150 (3)                   |
| MSY54    | Ykt6p            | KQAKKN55CCCIIM     | +++                   | 142 (3)                   |
| MSY56    | myc-Ykt6p        | KQAKKN55CCCIIM     | +++                   | ND                        |
|          | Ykt6p-C196S-C197S| KQAKKN55SSIIIM     | ++                    | ND                        |
| MSY60    | Ykt6p/Gos1TM     | KQAKKN55KNAFLATITTLCLIPFFTW | +++                | 152 (4)                   |

- Number of replicates; ND, not determined.
Because it was not possible to reduce Ykt6p to a level that would slow cell growth, we replaced the plasmid-borne Ykt6p with its human homologue, assuming that the homologue might work less effectively than Ykt6p itself. Indeed this strain (JMY89) ceased growing when shifted to media containing glucose as the only carbon source as shown in Fig. 4.

To determine whether depletion of HsYkt6p function influences transport along the secretory pathway, the processing of a well-characterized vacuolar protein, carboxypeptidase Y (CPY), was analyzed (Fig. 4). CPY is translocated into the ER, where it receives core oligosaccharides generating the p1 form, transits the Golgi where the core sugars are elongated yielding the p2 form and is proteolytically processed in the vacuole to the mature form. When HsYkt6p is expressed in the presence of galactose, the majority of CPY is found in its mature form, and small amounts of both p1CPY and p2CPY are detectable, consistent with the observed reduced growth rate. During HsYkt6p depletion induced by glucose, the p1 form of CPY steadily accumulates. A small amount of p2CPY also appears to persist until it is masked by the increased p1CPY signal. This may suggest that additional transport steps at the level of the Golgi are also affected by the loss of Ykt6p function. At later time points, mature CPY also appears to migrate slightly faster in SDS-PAGE. It is unclear why this occurs but might be explained by partial proteolysis or changed processing of the covalently attached sugar side chains of CPY.

Next, the HsYkt6p-depleted cells were analyzed morphologically by electron microscopy. Fig. 5 shows wild type cells grown in 8% glucose (W3031A; Fig. 5A), the ΔYKT6 strain expressing HsYkt6p in galactose (JMY89; Fig. 5B), and HsYkt6p-depleted cells grown in 8% glucose (JMY89; Fig. 5, C and D). There is a marked accumulation of 50 nm diameter transport vesicles in the HsYkt6p-depleted cells. A wild type cells (W3031A) were grown in 8% glucose to early log phase and then processed for analysis by electron microscopy as described under “Experimental Procedures.” B, the ΔYKT6 strain carrying the HsYkt6p gene (JMY89) cultured under plasmid-expressing growth conditions (2% raffinose, 0.5% galactose). C and D, the ΔYKT6 strain carrying the HsYkt6p gene cultured under plasmid-repressing growth conditions (8% glucose). Arrowheads indicate the 50 nm diameter vesicle, asterisks denote the undefined exaggerated membrane structure, and the arrows illustrate the accumulation of ER membrane. The bar equals 0.5 μm.

Finally, we asked if Ykt6p could suppress the phenotype of certain temperature-sensitive secretion mutants. Overexpression of Ykt6p suppressed the temperature-sensitive phenotype.

5 M. Craighead, T. H. Sölter, and J. E. Rothman, unpublished results.
of two SEC22 alleles, sec22–1 (44), and sec22–3 as well as a temperature-sensitive Bos1p mutant (sec22–1) (data not shown); both genes encode v-SNAREs found on ER-derived transport vesicles. It suppressed neither mutants in sec12, the GTP-exchange factor for Sar1p involved in budding of COPII-coated transport vesicles, nor mutants in sec18, the NSF homologue, which is involved in vesicle consumption at several intracellular transport steps (data not shown). This provides indirect evidence for an involvement of Ykt6p at the level of the SNARE complex in ER-Golgi transport.

**DISCUSSION**

Vesicular traffic through the secretory pathway requires specific pairwise interaction of targeting molecules on vesicles and on the destination membrane to maintain the cellular compartmentalization and directionality of transport. This recognition mechanism is provided by cognate v-SNARE and t-SNARE interactions. In this report we characterize a new v-SNARE, Ykt6p, involved in ER-Golgi transport, increasing the number of potential v-SNAREs at this transport step to four: Bet1p, Bos1p, Sec22p, and Ykt6p. Ykt6p has several hallmarks of a v-SNARE, present in a detergent extract of total yeast membranes, of the VAMP/synaptobrevin family like Sec22p. Additionally, Ykt6p, present in a detergent extract of total yeast membranes, interacts directly or as a part of a v-t-SNARE complex with recombinant GST-Sec17p (data not shown). A striking feature distinguishing Ykt6p from other SNAREs is the presence of a CAAX box providing a signal for isoprenylation and thereby mediating membrane attachment. In all other known v-SNAREs, hydrophobic peptide sequence function as membrane anchors. In this respect, all of the Ykt6p homologues, ranging from yeast to man, are unique SNARE molecules.

A chromosomal deletion of YKT6 is lethal, but it can be rescued by expression of wild type Ykt6p, amino-terminally myc-tagged Ykt6p, the human Ykt6p homologue, and a Ykt6p chimera, whose isoprenylation signal has been substituted by a proteinaceous membrane anchor. Ykt6p is present at ~0.05% of total protein, but cells are completely viable at levels at least 10-fold below this under the conditions we tested. This would indicate that wild type yeast can adapt to low Ykt6p levels. However, Ykt6p function is still clearly required for viability, because these cells could not lose the URA3-based plasmid on 5-fluoroorotic acid plates after many generation in glucose media.

Interestingly, Ykt6p was found in two cellular pools, one in the cytoplasm and another associated with membranes. Triton X-114 phase partitioning would suggest that both the membrane bound and soluble Ykt6p are prenylated, raising the question how the hydrophobic group is masked in the cytosol. Currently, we cannot answer this question, but our data clearly indicate that the membrane-associated pool of the Ykt6p represents the functional form. Mutant Ykt6p, which lacks the isoprenylation signal, cannot recover the YKT6 deletion. In addition, the chimera Ykt6p/Gos1pTM, containing the Gos1p membrane spanning region instead of the isoprenylation signal, was functionally active, and a cytoplasmic pool of this chimera was not detectable. It was not possible to localize the membrane-bound population of Ykt6p by immunofluorescence microscopy to a distinct intracellular membrane, because fluorescence due to the cytosolic pool of Ykt6p resulted in too large of a background signal.

Biochemical studies and the presence of Ykt6p in the isolated Sed5p complex indicate that Ykt6p is involved, at the very least, in ER to Golgi transport. These observations suggest a localization within these compartments. Depletion of Ykt6p function stops cell growth and manifests a transport block at the level of the endoplasmic reticulum, as shown by the accumulation of the p1 form of CPY (Fig. 4). Genetic evidence confirms an ER-Golgi function for Ykt6p. Overexpression of Ykt6p suppresses the temperature-sensitive phenotype of two ER-Golgi v-SNAREs, Sec22p (sec22–1 and sec22–3), and Bos1p (sec32–1). Additionally, a temperature-sensitive allele of Uso1p (uso1–1) can be suppressed by all four ER-Golgi SNARE molecules including Ykt6p (38). Morphological data obtained from yeast cells containing reduced levels of the human homologue of Ykt6p, which replaces the endogenous Ykt6p, gave a heterogeneous phenotype. The depleted cells accumulate 50 nm transport vesicle and exaggerated ER membrane further supporting a Ykt6p function in ER-Golgi transport. Similarly vesicle and ER accumulation has been observed in Sed5p-depleted yeast (46). It is not clear if the exaggerated ER is the primary or secondary effect of the transport block. However, it seems likely that vesicles initially accumulate, and when Ykt6p becomes limiting, production of new vesicles is reduced, leading to the ER accumulation. This would be consistent with a mechanism, which couples vesicle generation to v-SNAREs packaging and therefore ensures generation of docking and fusion competent vesicles. It would also be consistent with a selective block in anterograde transport.

We cannot exclude that Ykt6p might also act at transport steps other than ER-Golgi because a cytoplasmic pool of Ykt6p could provide an interacting partner for several SNAREs localized to multiple compartment. Further analysis of Ykt6p localization and distribution will help to determine its precise role in vesicle targeting and fusion.

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