Tlg2p, a Yeast Syntaxin Homolog That Resides on the Golgi and Endocytic Structures*

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Intracellular membrane fusion events in eukaryotic cells are thought to be mediated by protein-protein interactions between soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex proteins. We have identified and analyzed a new yeast syntaxin homolog, Tlg2p. Tlg2p is unique among known syntaxin family proteins in possessing a sizeable hydrophilic domain of 63 amino acids that is C-terminal to the membrane spanning region and nonessential for Tlg2p function. Tlg2p resides on the endosome and late Golgi by co-localization with an endocytic intermediate and co-fractionation with markers for both endosomes and late Golgi. Cells depleted for Tlg2p missort a portion of carboxypeptidase Y and are defective in endocytosis. In addition, we report that Tlg2p forms a SEC18-dependent SNARE complex with Snc2p, a vesicle SNARE known to function in Golgi to plasma membrane trafficking. Based on these findings we propose that Tlg2p is a t-SNARE that functions in transport from the endosome to the late Golgi and on the endocytic pathway.

The intracellular flux of lipids and proteins that constitutes vesicular trafficking in eukaryotic cells is a highly regulated process. The correct sorting of these components defines cellular compartmentalization and is thus vital for cellular function (1). Our understanding of the control of intracellular membrane fusion has been highlighted in recent years by the formulation of the SNARE hypothesis (2). Integral membrane proteins residing on different membrane-bound compartments (e.g. synaptic vesicles and plasma membrane) called t- and v-SNAREs are able to interact, and this interaction and its function are conserved from yeast to man (2–5). The complex formed between SNAREs is thought to span the lipid bilayers of both interacting compartments and to play a pivotal role in the docking and fusion processes. In agreement with this suggestion, a number of toxins that abrogate synaptic transmission do so by site-specific proteolysis of SNARE proteins (6–8), and antibodies to SNARE complex proteins inhibit in vitro membrane fusion assays (9). Complex formation between t-SNAREs on the target membrane and v-SNARE proteins on the vesicle membrane is thought to be modulated by a cycle of multiprotein complex assembly and disassembly that involves a number of accessory proteins (2). NSF, a multimeric ATPase, interacts with SNAREs via soluble NSF attachment proteins in an ATP-dependent manner. Hydrolysis of ATP by NSF induces conformational changes in SNAREs that lead to the disassembly of the SNARE complex. Recent data have indicated that this cycle may function at a point prior to docking, in rearranging intramembrane, inactive SNARE complexes (10). A basic implication of the SNARE hypothesis is that the fate of any patch of membrane, be it a transport vesicle or cellular compartment, is determined at least in part by the set of v- and/or t-SNAREs that are present on its surface (3). As such, an interesting question arises as to the precise fashion by which SNARE content correlates with membrane flux. It was originally suggested that t-SNAREs and v-SNAREs interact in a specific and exclusive fashion (11, 12) and that this specificity underlies the fidelity of vesicular transport. It has since been demonstrated, however, that a single v-SNARE can function at two different transport steps, with different t-SNAREs specific for each transport step (13). These findings have been accommodated within the SNARE model by suggesting that specificity arises out of additional, uncharacterized interactions that modulate the t-SNARE/v-SNARE coupling.

In the endocytic vacuolar system of Saccharomyces cerevisiae, the endosome (also known as the prevacuolar compartment) is a point of convergence for membrane traffic originating in the Golgi complex and in the plasma membrane (14, 15). It has been shown that Pep12p, a yeast protein that is homologous to the prototypical mammalian t-SNARE syntaxin, acts as a t-SNARE required for docking and/or fusion of Golgi-derived vesicles with the endosome/prevacuolar compartment (16, 17). It has also been shown that delivery of pheromone receptors to the prevacuolar compartment requires the action of the NSF ATPase, Sec18p (18). In this paper we describe a new syntaxin homologue, Tlg2p, which fractionates in a bimodal fashion with protein markers for the endosome and late Golgi compartments in density gradients and is required for efficient endocytosis. Unlike Pep12p, tlg2Δ cells do not accumulate the p2 form of the vacuolar protease carboxypeptidase Y (CPY) but missort a fraction of p2 CPY to the cell surface. We suggest that Tlg2p is a t-SNARE that is required in trafficking from the prevacuolar compartment to the late Golgi or alternatively for endocytosis-related membrane fusion events.

EXPERIMENTAL PROCEDURES

Materials—Enzymes used in DNA manipulations were purchased from New England Biolabs (Beverly, MA) or Boehringer Mannheim Biochemicals. ECL reagents, 125I-protein A, horseradish peroxidase-
labeled streptavidin, and 35S translate were from Amersham Pharmacia Biotech. Nitrocellulose type HAB5 paper was obtained from Schleicher & Schuell. Antiserum to yeast carboxypeptidase Y was a gift from Peter Novick. Zymolyase 100T (Kirin Brewery) was purchased from Seikagaku (Tokyo, Japan). Antiseras to Pep12p and to Kar2p were kindly donated to us by Scott Emr and Mark Rose, respectively. Anti-Ste3p antiserum was a gift from G. Sprague. Lucifer Yellow-CH was from Fluka (Buchs, Switzerland). Protein A-Sepharose was from Amersham Pharmacia Biotech and Sigma Immunochromics. Pronase and Biotinyl-anti-HA antibodies were from Boehringer Mannheim. Other chemicals were from Sigma, standard sources, or as indicated.

**Strains, Media, and Microbiological Techniques**—The strains used in this study are listed in Table I. Strains were constructed by standard genetic techniques and grown in rich YEPD medium (1% yeast extract, 2% peptone, 2% dextrose), galactose-based rich YEPG medium (1% galactose, 2% dextrose), and synthetic defined media (SD) with appropriate supplements as described by Sherman et al. (47). YEPD was supplemented with 1 M NaCl for high salt selection assays.

**Strain Construction**—Genomic DNA isolated from SFY562 yeast was used as template in a PCR reaction to amplify an XbaI-SalI fragment carrying the TLG2 gene. The PCR product was digested, gel purified, and inserted into pRS305 and pRS315 or pRS316 (19) at the XbaI-SalI sites to form plasmids pHAB1, pHAB2, and pHAB3, respectively. Plasmid pHAB6 was constructed by first amplifying an EcoRI-internal fragment mentioned above, and the HA-SNC2 gene cloned in frame with the GAL1 promoter and the 3′ untranslated region. The flanking regions of this construct are identically. The construct was inserted into the pEG111 vector that was obtained using the Splicing by Overlap Extension technique described in Ref. 20 (gene SOEing). Using two nested gene SOEing reactions, the TLG2 promoter region was fused to the GFP open reading frame, and the product of this reaction was in turn fused in-frame with the TLG2 coding sequence and 3′ untranslated region. The flanking regions of this construct are identical with those of the XbaI-SalI fragment mentioned above, and the linear PCR product was inserted into pRS305 as before. Plasmid pHAB15 was constructed by cloning an EcolI-BclI PCR amplified URA3 gene into the EcoI-BclI and BclI sites of the TLG2 gene, in pHB36. Plasmid pHAB16 was constructed by cloning a PCR product encoding amino acids 1–250 of Tlg2p into pET14b (Novagen). Plasmid pEGL111 is an integrating vector containing the HA-SNC2 gene cloned between the GAL1 promoter and the ADH terminator. Triple HA tagging of the SNC2 gene at the C terminus was according to the method of Schneider et al. (21). The HA-SNC2 gene was PCR amplified using a PstI primer 49 base pairs upstream of the 5′ end of the coding sequence and a HindIII primer 1351 base pairs downstream of the 3′ end of the coding sequence. The PCR product was subcloned into pRB529, a pRS426-based yeast expression vector containing GAL1 promoter and ADH terminator sequences. Site-directed mutagenesis was performed using the C-terminal hydrophilic domain of Tlg2p was according to Kunkel et al. (22), using the Muta-Genie kit (Bio-Rad). All constructs were verified by DNA sequencing according to the Sanger dyeideoxy nucleotide method. Recombinant Tlg2p Purification and Antibody Production—Plasmid pHAB16 was transformed into BL21 cells (Strategene), iso-1-thio-β-D-galactopyranoside induction of His6-tagged recombinant Tlg2p was confirmed by Coomassie staining of acrylamide gels. Recombinant His6 Tlg2p (5 mg) was purified to homogeneity from 1 liter of logarithmically growing bacterial cells using nickel-chelate chromatography, following the pET system manual (Novagen). Antiseras were raised in New Zealand White rabbits by Cocalico, Inc. (Reamstown, PA). CPY Maturation Studies—CPY maturation was followed essentially as described previously (23). Briefly, 4 OD units of cells from a culture grown to A600 of 0.5 were collected, treated for 10 min at room temperature with 100 mM Tris, pH 9.4, 10 mM dithiothreitol, washed, and resuspended in spheroplasting medium (SD plus nutrients, 1.2 M sorbitol and auxotrophic requirements, and incubated in the same medium at 25 °C for 10 min. Labeling was initiated by adding 200 µCi 35S PRO-MIX™ ([35S]methionine and [35S]cysteine at 14.4 mCi/ml; Amersham Pharmacia Biotech). After a 5-min labeling period, chase was initiated by adding cysteine and methionine to 1 and 5 mM respectively, plus 0.2% yeast extract and 4% glucose (time 0).

**Samples**—Samples of 1 A600 cell equivalent in 100 µl were taken at 0, 10, 30, and 90 min after initiation of chase, brought to 5 mM NaN3, and cooled in ice. Samples were spun to separate intracellular and extracellular fractions. The spheroplast pellets were collected, washed with SD medium supplemented with 1.2 M sorbitol and auxotrophic requirements, and incubated in SDs boiling buffer (50 mM Tris, pH 7.5, 1% SDS, 1 mM EDTA) and dialyzed for 5 min. The lysates were diluted with dilution buffer (50 mM Tris, pH 7.5, 2% Triton X-100, 0.1 mM EDTA, 150 mM NaCl) and immunoprecipitated with anti-CPY antibody. The immunoprecipitates were resolved by SDS-PAGE and 35S-labeled CPY was visualized and quantified using a Molecular Dynamics PhosphorImager™ and ImageQuant™ software. The colony lift immunoblotting technique (24) was used to assay gross CPY release from cells.

**Lucifer Yellow Uptake and Ste3p Endocytosis Assays**—Lucifer Yellow uptake was assayed as described previously (25). Briefly, 1 ml of logarithmically growing yeast cells (5 × 107 cells/ml in YEPD) were collected and resuspended in 900 µl of YEPD. 100 µl of Lucifer Yellow stock solution was added to a final concentration of 4 mg/ml, and the cells were incubated for 1 h at 25 °C with vigorous shaking. The cells were then washed three times in ice-cool buffer (50 mM succinate-NaOH, pH 5.0, 20 mM NaCl) and mounted in 0.8% low melt agarose.

The cells were observed using a Zeiss axiophot microscope equipped with fluorescence and Nomarski optics. Photographs were taken using Kodak T-Max 400 film (Eastman Kodak Co.). Ste3p endocytosis and Pronase sensitivity assays were done as described by Davis et al. (26).

**GFP Fluorescence Imaging**—For viewing GFP-tagged proteins, logarithmically growing cells were fixed by suspension in –20 °C methanol following by –20 °C acetone and finally resuspended in TE (10 mM Tris-HCl, pH 7.5, 1 mM EDTA). Cells were mounted in 0.8% low melting point agar and viewed using a Zeiss Axioplan microscope equipped with a 100× Apochromat objective. For fluorescence imaging of living cells, cells were mounted in YEPD medium containing the low melting agarose and viewed using a Zeiss Axiotax 10 microscope coupled to a Bio-Rad MRC600 confocal laser apparatus and equipped with a 63× Apochromat objective.

**SNARE Complex Immunoprecipitations**—Cell were grown to early log phase in YEPD or YEPG medium, pelleted, and resuspended in medium pre-warmed to 25 or 37 °C. After a 10-min incubation, the cells
Yeast Culture in YEPD were spun in the presence of 10 mM NaN₃ and resuspended in 100 mM Tris, pH 9.4, 10 mM dithiothreitol, and 10 mM sorbitol. Cells were incubated at room temperature for 10 min, collected and resuspended in 5 ml of ice-cold YEPD containing 0.6 M mannitol, 0.1 M KCl, 50 mM Tris, pH 7.5, 1 mM EGTA, and clarified by centrifugation at 100,000 g for 1 h. Protease protection assays (16) were conducted on the 100,000 g pellet fraction. Membrane pellets (80 ug of protein) were resuspended in lysis buffer and incubated with combinations of 0.29 mg/ml proteinase K and/or 0.4% Triton X-100 for 5 min, and then the reactions were quenched with 10% trichloroacetic acid. Samples (15 ug of protein) were resolved by SDS-PAGE and analyzed by immunoblotting for Kar2p and Tlg2p.

RESULTS

The TLG2 Gene Encodes a Syntaxin-like Protein—We scanned the yeast data base for proteins showing similarity to the conserved juxta-membrane coiled-coil domain of Sed5p, a known syntaxin-like yeast t-SNARE that is localized to the endoplasmic reticulum and Golgi complex (30). An open reading frame (YOL018c), located on chromosome XV, was found to encode a novel syntaxin-like protein. Unlike other members of the syntaxin family, the predicted transmembrane domain encoded by this sequence is not at the C terminus of the open reading frame. We therefore cloned the TLG2 gene using the PRETTYBOX™ program. Identical residues are highlighted in black, and conservative substitutions are shaded.

Fig. 1. Three-way comparison of Pep12p, Vam3p, and Tlg2p. The first 336 residues of Tlg2p (lacking the putative luminal domain) are aligned with Pep12p and Vam3p using the PRETTYBOX™ program. Identical residues are highlighted in black, and conservative substitutions are shaded.

Low speed supernatant and low speed pellet were generated by a 15-min centrifugation at 13,000 × g. The low speed supernatant fraction was centrifuged for 1 h at 100,000 × g to generate high speed supernatant and high speed pellet fractions. Equivalent volumes of each fraction were used for SDS-PAGE and immunoblot analysis. 1 ml of high speed pellet fraction (120 A₅₀₀₀ unit equivalents) in lysis buffer was loaded on a sucrose step gradient and fractionated as described (16).

Tlg2p and Pep12p were detected by standard immunoblot technique. Kex2p activity was assayed according to Fuller et al. (29) using t-butylocarbonyl-Gln-Arg-Arg 4-methylcoumarin-7-amide as substrate and following the evolution of 7-amino-4-methylcoumarin by fluorescence (385 nm excitation wavelength, 465 nm emission wavelength). One unit of Kex2p activity was defined as 1 pmol of 7-amino-4-methylcoumarin released per minute.

Protease Protection Assays—Spheroplast preparation was as described above. Spheroplasts were lysed by Dounce homogenizing in 0.3 M mannitol, 0.1 M KCl, 50 mM Tris, pH 7.5, 1 mM EGTA, and clarified extracts were centrifuged at 100,000 × g for 1 h. Protease protection assays (16) were conducted on the 100,000 × g pellet fraction. Membrane pellets (80 ug of protein) were resuspended in lysis buffer and incubated with combinations of 0.29 mg/ml proteinase K and/or 0.4% Triton X-100 for 5 min, and then the reactions were quenched with 10% trichloroacetic acid. Samples (15 ug of protein) were resolved by SDS-PAGE and analyzed by immunoblotting for Kar2p and Tlg2p.
Spheroplasts were formed, pulsed for 5 min with 35S-PRO- under-stand the origin of the extracellular CPY in (34). We performed pulse-chase analysis of CPY maturation to route in the cell. Upon translocation to the lumen of the endo- some other mechanism. The mobility of CPY in SDS gels can be extracellular CPY by the colony filter assay could be the con- sensus of sequence identity within those cellular functions. Yeast defective in vacuolar endocytic trafficking often show sensitivity to osmotic stress (33) and defects in the sorting of vacuolar proteases (23, 33). In 40 tetrads scored, the Ura haploid segregants released CPY to the extracellular medium. In addition, light microscopic analysis revealed that tlg2Δ yeast had abnormally fragmented vacuoles. Detection of extracellular CPY by the colony filter assay could be the conse- quence of cell lysis, secretion of the late Golgi (p2) form, or some other mechanism. The mobility of CPY in SDS gels can be used as a gauge of intracellular location within its biosynthetic route in the cell. Upon translocation to the lumen of the endo- plasmic reticulum, CPY is glycosylated by the addition of four N-linked core oligosaccharides, and this precursor form of the protein, called p1 CPY, migrates as 67 kDa in SDS-PAGE. As CPY transits through the later compartments of the Golgi complex, it acquires additional mannose residues to give rise to p2 CPY, which migrates at 69 kDa. Upon delivery to the vac- uole, the 90-amino acid propeptide on p2 CPY is proteolysed, giving rise to the 61-kDa mature form of the protein (mCPY) (34). We performed pulse-chase analysis of CPY maturation to understand the origin of the extracellular CPY in tlg2Δ cells. Spheroplasts were formed, pulsed for 5 min with [35S]-PRO- MIX™, and chased for various times as described under “Ex- perimental Procedures.” We found that CPY maturation in tlg2Δ cells is not blocked. Although tlg2Δ cells were slightly delayed in the appearance of the p2 form at very early time points (not shown), they processed p2 CPY to the mature vacu- olar form within the same time frame as that of wild type cells (Fig. 2c). However, approximately 20% of the total CPY syn- thesized was secreted from these cells as the Golgi-modified p2 form.

Tlg2p is unique among members of the syntaxin family in possessing a sizeable, putatively luminal, hydrophilic C-termi- nal domain. In an attempt to understand the function of this domain, we assayed the ability of a truncated form of Tlg2p lacking the C-terminal hydrophilic domain to complement the observed high salt growth defect and colony-lift CPY missort- ing assay phenotypes of the TLG2 disruption. A termination codon was introduced at position 338 of the open reading frame as described above to form a mutant version of the gene encoding the truncated protein, Tlg2pΔ338. Cells deleted for endoge- nous TLG2 but carrying the truncated form express a protein of the predicted molecular weight that reacts with anti-Tlg2p antibody (Fig. 2c). The integration vector carrying the truncated gene was introduced into the LEU2 locus of diploid TLG2/ tlg2Δ cells, and the resultant transformants were sporulated and dissected. In the 35 tetrads examined, leucine prototrophy co-segregated with suppression of the high salt sensitive growth and the CPY missorting phenotypes, as shown for a typical tetrate type tetrad in Fig. 2a. Thus, although cells lacking full-length Tlg2p are deficient in growth on high salt and se- crete p2 CPY, cells that express Tlg2pΔ338 instead of the normal form of the protein show wild type behavior. We there- fore conclude that the C-terminal hydrophilic region of Tlg2p is not

### Figure 2

**tlg2Δ cells missort p2 CPY, and the Tlg2pΔ338 truncated protein can complement this defect.**

- **a.** Diploid yeast containing one disrupted TLG2 allele (HAY30) were transformed with an integrating vector carrying a mutant version of the TLG2 gene in which amino acid 339 was replaced with a nonsense codon followed by a frameshift (tlg2Δ338). Sporulation of the diploid (HAY30) gave four genotypes that were assayed for ability to grow on high salt and for CPY release into the extracellular medium; wild type (TLG2); tlg2Δ; tlg2Δ338, TLG2; and tlg2Δ338. A typical tetratype tetrad is shown, replica plated on YPD containing 1 M NaCl, and assayed for CPY missorting by colony immunoblotting from YPD plates. Genotypes were confirmed by replica plating on selective media (not shown).
- **b.** Immunoblot analysis of yeast carrying TLG2 variants with anti-Tlg2p antibody. Lane 1, wild type; lane 2, tlg2Δ; lane 3, tlg2Δ338. Blots were performed as described under “Experimental Procedures.” c, wild type and tlg2Δ cells were pulse-labeled for 5 min with [35S]cysteine/methionine and chased with an excess of unlabeled cysteine and methionine. Aliquots (1 A600 equivalents) were removed at 0, 40, and 90 min of chase and placed on ice in the presence of 5 mM NaCl. Cells were separated from the medium and lysed, and both cell lysates and medium were subjected to immunoprecipitation with antibody to CPY as described under “Experimental Procedures.” i, intracellular fraction; o, extracellular fraction.
essential for these aspects of Tlg2p function.

**tlg2Δ Yeast Are Deficient in Lucifer Yellow Uptake and Show a Delay in Ste3p Turnover**—Because a number of vacuolar protein sorting mutants that missort CPY are also deficient in endocytosis, we examined tlg2Δ mutant cells for defects in aspects of endocytosis. Yeast cells constitutively take up soluble material from the medium and transport it through the endocytic vacuolar system. This process can be studied using Lucifer Yellow, a fluorescent dye that is an established marker for fluid phase endocytosis (25). Wild type cells incubated with Lucifer Yellow at 25 °C for 1 h show a distinct vacuolar staining. In tlg2Δ cells, however, only a small amount of dye appears to have been internalized into vacuolar structures, as defined by Nomarski optics (Fig. 3). This result suggests that Tlg2p is necessary for efficient fluid phase endocytosis.

If fluid phase uptake is impaired in tlg2Δ cells, the trafficking of membrane proteins from the plasma membrane to the vacuole may also be defective. To address this possibility, we followed the turnover of the a-factor receptor, Ste3p, which undergoes constitutive and rapid ligand-independent turnover through the endocytic pathway that delivers it to the vacuole for proteolytic degradation. The rate at which Ste3p is endocytosed can be analyzed by pulse-chase experiments (26, 35). The data from such an experiment are presented in Fig. 4b. We found that the half-life of Ste3p in wild type cells was approximately 21 min (Fig. 4), and this agreed with published data (26, 35). In tlg2Δ cells the half-life was 43 min, and Pronase sensitivity data showed that the residual Ste3p was at the cell surface, indicating that the increase in half-life was due to a defect in internalization rather than inefficient proteolysis in the vacuole (Fig. 4c). These results corroborate the Lucifer Yellow data in demonstrating an endocytic defect for tlg2Δ cells.

**Tlg2p Is a Type II Membrane Protein That Fractionates in a Bimodal Fashion with Endosomal and Late Golgi Marker Proteins**—In differential centrifugation experiments, 70% of the total cellular Tlg2p is found in the 100,000 × g pellet, whereas 15% is found in the 12,000 × g pellet, and 10% is found in the 100,000 × g supernatant (Table II). The association of Tlg2p with the 100,000 × g pellet was efficiently disrupted by 2% Triton X-100, but not by 1.5 M NaCl, 2 M urea, or 0.3 M Na2CO3, pH 11.4 (not shown). These results imply that Tlg2p is tightly associated with membranes, presumably through the putative transmembrane domain. The N-terminal domain of Tlg2p is sensitive to addition of proteinase K both in the absence and presence of 0.4% Triton X-100, whereas the lumenal marker protein, Kar2p, is protected from proteolysis in the absence but not in the presence of detergent (Fig. 5). Thus, Tlg2p appears to be an integral membrane protein oriented such that its N-terminal domain is facing the cytoplasm.

We next fractionated the P100 pellet on an equilibrium sucrose density gradient. We used conditions shown by others to separate late Golgi markers from those of the prevacuolar compartment (13, 16, 36). The P100 fraction, containing the majority of cellular Tlg2p, was fractionated by equilibrium density gradient centrifugation. As shown in Fig. 6, approximately 50% of Tlg2p co-sedimented with Pep12p, toward the top of the gradient, at a sucrose concentration of 24%. The rest of Tlg2p migrated in denser fractions; these dense fractions also contain the major peak of Kex2p activity, a late Golgi marker protein that cycles between the late Golgi and the prevacuolar compartment. The simplest explanation of this data is that Tlg2p resides on both late Golgi and endosomal membranes.

**GFP-Tlg2p Co-localizes with Endocytic Intermediates Observed during FM4-64 Uptake**—In order to directly observe Tlg2p localization in living cells, the open reading frame of GFP was fused to the TLG2 promoter as an N-terminal fusion to the Tlg2p reading frame, yielding a GFP fusion gene transcribed from the endogenous TLG2 promoter ("Experimental Proce-
dures”). GFP-Tlg2p is expressed at normal levels when compared with wild type Tlg2p and functionally complements the high salt growth defect and CPY missorting of tlg2Δ (not shown). The GFP fusion protein shows punctate cytoplasmic staining in wild type cells, with typically 2–5 bright dots/cell. In type E vacuolar mutants, the normally punctate staining that is observed with markers of the endosome/prevacuolar compartment is found as a single, abnormally large structure. We reasoned that if this staining was endosomal or late Golgi, then a class E type vacuolar mutant would show a consolidation of these punctate structures to one perivacuolar compartment (15, 23). Indeed, vps27 tlg2Δ cells containing the GFP-Tlg2p fusion show the expected pattern, with an average of 1.29 dots/cell (n = 66 cells, SD = 0.65) as compared with an average of 3.21 dots/cell in wild type (n = 64 cells, SD = 0.88). The single dot in vps27 cells is much larger than that in wild type and has a perivacuolar localization.

If Tlg2p is a t-SNARE residing on endosomes, one would expect it to co-localize with endocytic intermediates. The styryl dye, FM4-64, has been shown to be endocytosed via a route involving the prevacuolar compartment, and its excitation-emission spectrum is compatible with GFP for double-fluorescence studies (not shown). The availability of the GFP-Tlg2p fusion allowed us to test this hypothesis by comparing the fluorescence patterns of GFP-Tlg2p and FM4-64 under conditions that trap the fluorescent dye in endocytic compartments that can then be chased to the vacuole (28). The results, shown in Fig. 7, demonstrate that FM4-64 fluorescence is localized to punctate cytoplasmic structures in cells that were incubated at 15 °C during dye uptake, as has been previously described (Fig. 7). This contrasts with the pattern seen in cells that were incubated at 30 °C, in which the dye stains the vacuolar mem-

![FIG. 4. Ste3p endocytosis is delayed in tlg2Δ cells.](image)

**FIG. 4.** Ste3p endocytosis is delayed in tlg2Δ cells. a, wild type (WT) and tlg2Δ cells were pulsed for 10 min with 35S Pro-mix labeling mix as described under “Experimental Procedures.” Samples (1 A600 equivalent) taken at 2, 15, 30, 60, and 90 min of chase were incubated on ice with 10 Mm each of potassium fluoride and sodium azide. Extracts were prepared by treating the cells with zymolyase (250 mg/ml) for 30 min in digestion buffer (1.4 M sorbitol, 25 mM Tris, pH 7.5, 10 mM NaN3, 10 mMKF, 2 mM MgCl2, 0.3% β-mercaptoethanol) and precipitated with anti-Ste3p antiserum. Quantitation of the data was by densitometric analysis of PhosphorImager output. b, wild type and tlg2Δ cells were pulse-chased as above. Aliquots were taken at the indicated time points and divided; one half received Pronase treatment (380 units/ml), and the other half was mock-treated with buffer (25).

![FIG. 5. The N-terminal domain of Tlg2p is accessible to proteases.](image)

**FIG. 5.** The N-terminal domain of Tlg2p is accessible to proteases. Whole cell extracts were spun at 100,000 × g, and the resuspended membranes were treated with protease K in the presence or absence of 0.4% Triton X-100. Samples (15 μg of protein) were quenched with 10% trichloroacetic acid before and after protease treatment, resolved by SDS-PAGE, and analyzed by immunoblotting.

**TABLE II**

**Distribution of Tlg2p, Pep12p, and Kex2p activity during differential centrifugation**

Whole cells extracts were prepared as detailed under “Experimental Procedures.” Equivalent amounts of subcellular fractions were separated on 10% SDS-PAGE and immunoblotted for Tlg2p and Pep12p. Immunoblots were quantitated by densitometry. Kex2p activity was determined as described under “Experimental Procedures.” Results are shown as percentages of recovery in the particular fraction, relative to cleared whole lysate.

| Cleared lysate | 12,000 × g pellet | 100,000 × g sup | 100,000 × g pellet |
|----------------|-------------------|-----------------|-------------------|
| Kex2p          | 100               | 0               | 12               | 87               |
| Pep12          | 100               | 2               | 3                | 90               |
| Tlg2p          | 100               | 15              | 10               | 70               |
brane. The punctate structures stained by FM4-64 in cells incubated at 15 °C colocalize with GFP-Tlg2p fluorescence. This co-localization is not observed in cells incubated at 30 °C, although GFP-Tlg2p fluorescence retains its punctate nature. It has previously been demonstrated (28) that the punctate FM4-64 stained structures that accumulate at 15 °C can be chased to the vacuole during a 25-min incubation at room temperature. In agreement with these previous observations, we found that the FM4-64 uptake intermediates that co-localize with GFP-Tlg2p at 15 °C can also be chased to the vacuole and are therefore bona fide endocytic intermediates (not shown).

Fig. 7. Co-localization of GFP-Tlg2p with FM4-64 internalization intermediates. Cells expressing GFP-Tlg2p (HAY24) were labeled on ice for 30 min with 0.6 mM FM4-64, washed, and incubated at 15 °C (top panels) or 30 °C (bottom panels) for 30 min. Each column represents one cell viewed with different fluorescence filters. Cells were visualized by confocal microscopy. FM4-64 fluorescence was observed using the red fluorescence channel (top row of each panel) and GFP fluorescence with the green fluorescence channel (middle row of each panel). The bottom row of each panel shows overlays of the two images. No bleedthrough fluorescence was observed between the red and green channels under our experimental conditions (not shown).

Tlg2p Interacts with the v-SNARE, Snc2p, in a SEC18-dependent Fashion—Although sequence homology suggests that Tlg2p is a t-SNARE, we sought functional evidence for this by assaying its ability to interact with known v-SNAREs. Golgi to plasma membrane trafficking requires one of the two functionally redundant v-SNAREs, Snc1p and Snc2p (37). The Snc proteins are known to interact with the plasma membrane localized t-SNAREs Sso1p, Sso2p, and Sec9p (38, 39). We sought to determine whether the Snc proteins also interact with Tlg2p by assaying the ability of anti-Tlg2p antibodies to co-precipitate Sncp. A small amount of Sncp co-precipitated with Tlg2p in wild type cells (Fig. 8a). Sncp from a tlg2 Δ strain does not co-precipitate with anti-Tlg2p antibodies (Fig. 8b). In sec18-1 cells, the amount of Sncp co-precipitated with Tlg2p dramatically increased after shifting the cells to the restrictive temperature, 37 °C, for 10 min. This phenomenon, typical for SNARE complexes in yeast, is thought to reflect a defect in the ability of the mutant Sec18p to disassemble SNARE complexes (38, 44).2

To validate the co-precipitation assay, we sought to demonstrate that the Tlg2p-Snc interaction exists before the cells are lysed. Two sec18-1 strains were used for this experiment, a tlg2 Δ strain expressing HA-Sncp (HA34) and a TGL2 strain (NY1217) expressing untagged Sncp. The cell populations were mixed before lysis and then subjected to immunoprecipitation with anti-Tlg2p antibody. The Sncp from the TGL2 strain co-precipitated, but the HA-Sncp from the tlg2 Δ strain did not, indicating that HA-Sncp cannot form a complex with Tlg2p

Fig. 8. Tlg2p forms a SEC18-dependent SNARE complex with Snc2p in vitro. a, temperature dependence of Sncp co-precipitation with α-Tlg2p antibodies in wild type and sec18-1 cells. Tlg2p was precipitated from lysates prepared from NY1217 (sec18-1) or NY605 (wild type) cells grown overnight at 25 °C and then either shifted for 10 min to 37 °C or allowed to remain at 25 °C. Snc1 and Snc2 proteins co-precipitating with Tlg2p were detected on an immunoblot with anti-Sncp antibodies. b, specificity of Snc2p co-precipitation. Immunoprecipitations were carried out after a 37 °C shift from EGY213 (TGL2 HA-SNC2 sec18-1), HAY34 (tig23 HA-SNC2 sec18-1), and a mixture of HAY34 (tig23 HA-SNC2 sec18-1) and NY1217 (TGL2 sec18-1) prepared prior to lysis. The immunoprecipitates were blotted for HA-Sncp and total Sncp using anti-HA and anti-Sncp antibodies. After lysis (Fig. 8b). Therefore, we conclude that the Tlg2p-Sncp interaction exists in vivo.

DISCUSSION

Although the SNARE machinery of the early and late secretory pathway in yeast has been under intense study in the last few years, less is known about the corresponding SNARE complexes and auxiliary proteins that control the vacuolar endocytic system.

Recently, SNARE complexes required for vacuole-vacuole fusion and for late Golgi to endosome transport have been described, and a requirement for NSF (Sec18p) activity in early endosome to late endosome transport in yeast was reported (17, 18, 40). We now report that a third syntaxin-like protein, Tlg2p, is important for normal functioning of the vacuolar endocytic system in yeast. Tlg2p shows a stronger resemblance to Vam3p and Pep12p than to other characterized yeast syntaxin-like proteins: Sso1p, Sso2p, Sed5p, Utb1p, and Sft1p (31). Pep12p and Vam3p receive the most significant BLAST probability scores when Tlg2p is used as query against the yeast data base (P values of 7.9e-19 and 1.3e-11, respectively). Perhaps significantly, one of the regions in which the similarity of Tlg2p with Pep12p is higher is the hydrophobic transmemen-

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2 E. Grote and P. Novick, unpublished data.
brane domain, which has been shown to be important for localization of previously characterized t-SNAREs, including Pep2p (32). These three proteins thus appear to comprise a subfamily of yeast syntaxin-like t-SNAREs involved in controlling membrane flux and protein trafficking through the vacuolar/endocytic system.

Tlg2p contains an unusual hydrophilic C-terminal domain that is absent from all other known syntaxin family proteins. The presence of this domain does not alter the overall topology of the rest of the protein. Tlg2p thus appears to be a type II integral membrane protein, as are all the syntaxin family proteins. In addition, the C-terminal hydrophilic region is not required for complementation of two phenotypes that we have associated with deletion of the TLG2 gene: failure to grow on high salt medium and partial secretion of p2 CPY. This result implicates the syntaxin-like cytoplasmic and transmembrane regions as both necessary and sufficient for these functions.

Yeast carrying vacuolar biogenesis mutations known as “class E” mutants accumulate a membrane-bound compartment, the class E compartment, generally thought to be an exaggerated late endosome or prevacuolar compartment (23). The class E compartment accumulates several types of proteins: resident endosomal proteins, as well as proteins that cycle between the late Golgi and the endosome, proteins that transit between the plasma membrane and the endosome, and proteins en route to the vacuole (15). Consistent with the idea that Tlg2p functions in the vacuolar endocytic pathway, the pattern of GFP-Tlg2p fluorescence is shifted in the class E vps27 mutants to a single, perivacuolar compartment. The fact that Tlg2p co-fractionates in a bimodal fashion with both Pep12 and Kex2p in density gradient centrifugation experiments, together with the class E compartment localization in vps27 cells, suggests that Tlg2p may be cycling between the late Golgi and the endosome. As such, it may also function in transport from the endosome to a late Golgi compartment.

Several lines of evidence link Tlg2p to a membrane fusion step or steps that pertain to endocytosis. Although pep12Δ yeast, vam3Δ yeast, and tlg2Δ yeast all show aberrations in CPY processing (16, 41), tlg2Δ cells are unique in that intra-vacuolar CPY processing (16, 41), the endosome to Golgi and the endosome, proteins that transit between the plasma membrane and the endosome, and proteins en route to the vacuole (15). Consistent with the idea that Tlg2p functions in the vacuolar endocytic pathway, the pattern of GFP-Tlg2p fluorescence is shifted in the class E vps27 mutants to a single, perivacuolar compartment. The fact that Tlg2p co-fractionates in a bimodal fashion with both Pep12 and Kex2p in density gradient centrifugation experiments, together with the class E compartment localization in vps27 cells, suggests that Tlg2p may be cycling between the late Golgi and the endosome. As such, it may also function in transport from the endosome to a late Golgi compartment.

In vivo

We have demonstrated that Sncp co-precipitates with Tlg2p in a SEC18-dependent fashion. This result suggests that Sncp may form a functional SNARE complex with Tlg2 as it recycles from the plasma membrane back to the late Golgi, presumably via the endosome. Because there is no other evidence that Snep functions in endocytosis, we carried out a mixing experiment to prove that the Snep-Tlg2p interaction occurs in vivo. The data support a model whereby Snep is involved in SNARE complexes with distinct t-SNAREs, Ssoo and Tlg2p, as it cycles between the Golgi and the plasma membrane. A similar model has previously been proposed for Sec22p, a v-SNARE that cycles between the endoplasmic reticulum and cis-Golgi. Sec22p interacts both with the cis-Golgi t-SNARE Sed5p for anterograde transport and with the endoplasmic reticulum t-SNARE Ufe1p for retrograde transport (42–44). Evidence is accumulating that the mammalian homologs of Snep, the syntaxobrevins (VAMPs) and cellubrevin, also interact with multiple t-SNAREs in a similar fashion. These proteins were originally postulated to interact with syntaxin 1 during fusion of vesicles with the plasma membrane (45). However, more recently there have been reports that they interact with Golgi/endosome localized t-SNAREs including syntaxin 6 (46). A clearer understanding of the specific membrane fusion events in which Tlg2p functions (endocytosis versus endosome to Golgi traffic) and knowledge of the protein-protein interactions required for these functions will be necessary to test this hypothesis.

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Tlg2p, a Yeast Syntaxin Homolog That Resides on the Golgi and Endocytic Structures
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