The N-terminal peptide of the syntaxin Tlg2p modulates binding of its closed conformation to Vps45p

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The Sec1/Munc18 (SM) protein family regulates intracellular trafficking through interactions with individual SNARE proteins and assembled SNARE complexes. Revealing a common mechanism of this regulation has been challenging, largely because of the multiple modes of interaction observed between SM proteins and their cognate syntaxin-type SNAREs. These modes include binding of the SM to a closed conformation of syntaxin, binding to the N-terminal peptide of syntaxin, binding to assembled SM–syntaxin–SNARE complexes, and/or binding to nonsyntaxin SNAREs. The SM protein Vps45p, which regulates endosomal trafficking in yeast, binds the conserved N-terminal peptide of the syntaxin Tlg2p. We used size exclusion chromatography and a quantitative fluorescent gel mobility shift assay to reveal an additional binding site that does not require the Tlg2p N-peptide. Characterization of Tlg2p mutants and truncations indicate that this binding site corresponds to a closed conformation of Tlg2p. Furthermore, the Tlg2p N-peptide competes with the closed conformation for binding, suggesting a fundamental regulatory mechanism for SM–syntaxin interactions in SNARE assembly and membrane fusion.

Sec1/Munc18 protein | SNARE | membrane fusion

Eukaryotic cell growth and survival require membrane-bound vesicles to transport proteins and membrane between the various organelles within the cell and to the plasma membrane for secretion. These conserved vesicle trafficking mechanisms require exquisite regulation to ensure specificity (1, 2). Crucial components are the SNAREs, which form a parallel four-helix bundle called the SNARE complex to bridge the vesicle and target membranes for fusion (3, 4). The Sec1/Munc18 (SM) protein superfamily regulates SNARE complex assembly and membrane fusion through direct interactions with their cognate SNAREs (5, 6). The SM family is divided into four subfamilies: Sec1p, Vps45p, Sly1p, and Vps33p. The Sec1p family is exocytic and includes yeast Sec1p and mammalian Munc18 isoforms; Munc18a is neuronal specific, whereas Munc18c functions in multiple cell types. The Vps45p family regulates endosomal trafficking, whereas the Sly1p family regulates trafficking between the ER and the Golgi, and Vps33p functions in trafficking to the vacuole/lysosome. The different SM proteins are structurally similar, consisting of three mixed α-helical and β-sheet domains arched around a central cleft, suggesting a conserved function (7–10).

Although SM proteins are thought to perform similar roles in the regulation of vesicle fusion, a wealth of conflicting data from SM homologues in different trafficking steps and species has made the mechanism of SM action unclear (for review, see ref. 5). SM proteins appear to have both positive and negative roles in vivo, which may reflect different aspects of their function in the SNARE assembly/disassembly cycle. Consistent with these different roles, SM proteins interact not only with individual SNAREs, but also with assembled SNARE complexes. Most of the SM proteins interact directly with syntaxins at one of two distinct binding sites, which are separated by >30 Å. One site is formed by the central cleft of Munc18a and the closed conformation of syntaxin 1a (Fig. 1A, mode 1; ref. 7). In contrast, other SMs bind individual syntaxins and SNARE complexes, using a hydrophobic pocket in domain 1 of the SM protein to bind the N-terminal peptide of the syntaxin (Fig. 1A, mode 2). These include Sly1p–Sec35p (9, 11), Vps45p–Tlg2p (12, 13), and Munc18c–syntaxin 4 (10). Thus, the N-peptide interaction appears to be the most common mode of binding, and the closed conformation interaction has been hypothesized to be specific to the neuronal Munc18a (6). Additionally, the yeast exocytic Sec1p binds assembled SNARE complexes in the absence of the syntaxin N-peptide (14); this mode may also be used by other SMs (13, 15). Moreover, several SM proteins have been shown to interact with nonsyntaxin SNAREs (13, 16–18). The multiple distinct modes of interaction between SM proteins and SNARE partners suggested that the SMs may not share a common mode of action (5), although the high degree of sequence and structural similarity between them argues that SMs should have a common function in vesicular trafficking.

Recent studies are beginning to resolve this quandary. Although the closed conformation seemed to be the predominant mode of the Munc18a–syntaxin 1a interaction, the syntaxin 1a N-peptide appears to also be important for the regulation of SNARE complex assembly, the interaction between Munc18a and assembled neuronal SNARE complexes, and stimulation of membrane fusion (15, 19–22). The N-peptide alone is not sufficient for binding of syntaxin 1a to Munc18a, but these functional data suggested that Munc18a may bind the syntaxin 1a N-peptide similarly to other SM proteins. Moreover, when the original Munc18a-syntaxin 1a structure was re-examined, density corresponding to several residues of syntaxin 1a’s N-peptide bound to Munc18a was observed, confirming that syntaxin 1a can bind Munc18a through dual modes (Fig. 1A, mode 3; ref. 22).

Here, we focus on yeast Vps45p, which interacts with the N-peptide of Tlg2p (Fig. 1A, mode 2). Mutational analyses had previously suggested that only this N-peptide binding site was important for the Vps45p–Tlg2p interaction (12, 13). Additionally, NMR studies of truncated Tlg2p had suggested that, unlike syntaxin 1a and Sso1p (23, 24), Tlg2p does not form a stable closed conformation (12). The above data, along with homology to other SM–syntaxin pairings, led to the hypothesis that the N-peptide of Tlg2p is solely responsible for the interaction...
between Vps45p and Tlg2p. However, the absence of a phenotype for mutants that disrupt binding between Vps45p and Tlg2p (13), the evidence for dual binding modes for Munc18a-syntaxin 1a, and recent data indicating that residues outside of the N-peptide of syntaxin 16 stabilize the binding of syntaxin 16 to mammalian Vps45 (22) led us to re-examine the possibility of additional interaction modes between Vps45p and Tlg2p. We discovered a second binding site for Vps45p on Tlg2p, which corresponds to a Tlg2p closed conformation (Fig. 1A, mode 1).

We demonstrate that either of these Vps45p binding sites is sufficient for Tlg2p function in vivo, but abrogation of both results in a phenotype similar to tlg2/H9004 cells. Furthermore, we show that the N-peptide of Tlg2p modulates the affinity of the closed conformation binding site, indicating a role for the N-peptide in controlling accessibility of Tlg2p for SNARE complex assembly. These data suggest that a common mechanism for SM–syntaxin interactions is a dual mode, whereby the SM interacts with two distinct sites on the syntaxin, the N-peptide and the closed conformation.

Results

Vps45p Interacts with Tlg2p in the Absence of the Tlg2p N-Peptide.

Various truncations of Tlg2p were created to examine the role that individual domains play in the interaction with Vps45p and were designed using secondary structure predictions, sequence alignments with other syntaxin homologues, and domains delineated by NMR experiments (12). The full-length cytosolic Tlg2p(1-318) protein contains: the N-peptide region; the Habc domain, which acts as an autoinhibitory domain in many syntaxin-type SNAREs; a short linker region; and the SNARE motif region, which is used for binding to the other SNARE proteins (Fig. 1B). We also purified wild-type Vps45p and Vps45p–L117R, a mutant with abrogated binding to the Tlg2p N-peptide (13). Each protein is monomeric and predominantly α-helical, as determined by size exclusion chromatography (SEC) and circular dichroism (CD) (Fig. S1A and B). The exception is Tlg2p(221-318), which is predominantly unfolded, as expected for an isolated SNARE motif region (25, 26).

The interaction between the recombinant Vps45p and Tlg2p(1-318) proteins was investigated. Vps45p and Tlg2p(1-318) were incubated together, and SEC was used to separate the complex from the free proteins (Fig. S1C). The individual elution peaks of both Vps45p and Tlg2p(1-318) shift to a larger apparent molecular weight when these two proteins are mixed, indicating that Vps45p interacts with the cytosolic region of Tlg2p, corroborating previous studies (12, 13). We next demonstrated that a construct lacking the N-peptide, Tlg2p(37-318), also interacts with Vps45p (Fig. 2A). This result was surprising, because previous studies had indicated that Tlg2p lacking the
N-peptide could not bind Vps45p (12, 13). Our results clearly demonstrate that Vps45p can interact with another binding site in the C-terminal region of Tlg2p.

To determine the apparent affinity between Vps45p and this second binding site, we developed a native EMSA using Tlg2p(37-318) protein fluorescently labeled with Alexa Fluor 488 dye [Tlg2p(37-318)]. This well-established method has been widely used in the field of protein–nucleic acid interactions (27).

First, increasing concentrations of Vps45p were equilibrated with limiting labeled material (27). Tlg2p(1-318), which contains the Tlg2p N-Peptide Modulates Affinity of the C-Terminal Binding Site. Collectively, these results suggest that the two binding sites are not independent; in fact, they appear to be mutually exclusive. This apparent negative allosteroy between the two binding sites likely explains the weaker apparent affinity observed for the cytoplasmic region of Tlg2p compared with the N-peptide alone, although steric hindrance and/or interactions between the N-peptide and the 37-318 region cannot currently be ruled out.

Our conclusions are supported by several critical point mutations. First, we examined an N-peptide mutant, Tlg2p(1-318)–F9A/L10A. The dual alanine mutations abolish the interaction with the hydrophobic pocket on domain 1 of Vps45p and modulate the conformation of Vps45p such that it can no longer bind the C-terminal binding site on Tlg2p. Alternatively, the N-peptide could bind directly to Tlg2p(37-318)* and compete for Vps45p binding, or the N-peptide could bind to a different site on Vps45p that precludes binding of Tlg2p(37-318)*. To discriminate between these possibilities, we analyzed the ability of the N-peptide to compete for binding to Vps45p–L117R. We discovered that, even at concentrations as high as 1 μM, Tlg2p(1-33) was unable to compete with Tlg2p(37-318)* for binding to Vps45p–L117R. Thus, the N-peptide cannot compete for binding to Vps45p when its hydrophobic pocket binding site has been disrupted. These results suggest that the two binding sites are not independent; in fact, they appear to be mutually exclusive. This apparent negative allosteroy between the two binding sites likely explains the weaker apparent affinity observed for the cytoplasmic region of Tlg2p compared with the N-peptide alone, although steric hindrance and/or interactions between the N-peptide and the 37-318 region cannot currently be ruled out.

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CPY secretion requires the presence of at least one of the two binding sites, but not the case for Tlg2p-F9A/L10A/I285A. Thus, Tlg2p function in the overexpression observed for the Tlg2p–F9A/L10A mutant complements the CPY secretion defect (Fig. 4B-I285A, in which both binding sites are disrupted, does not rescue the CPY-invertase secretion phenotype (Fig. 4C)). The ability of the Tlg2p–I285A mutant to compete for binding to Vps45p was measured by competition EMSA and the K_c,app was calculated as in A. (C) The ability of the Tlg2p–I285A mutant to compete for binding to Vps45p was measured by competition EMSA. Results were analyzed as in A. The Tlg2p(37-1285A) and Tlg2p(1-318)-I285A data are plotted on the same graph for comparison.

**Mutation of Both Binding Sites Abrogates the Function of Tlg2p in Vivo.** Previous studies indicated that the N-peptide binding mode is not required for the function of Sly1p or Vps45p in vivo (13, 16), although recent evidence indicates a role for the syntaxin N-peptide in Caenorhabditis elegans (30, 31). To determine the functional importance of the Tlg2p C-terminal binding site, we assessed the ability of Tlg2p containing the I285A mutation to complement the CPY trafficking defect of cells lacking endogenous Tlg2p. To measure the effect of the loss of one or both Vps45p binding sites on Tlg2p function, full-length TLG2 (encoding residues 1–397) and mutant tlg2 constructs were expressed in tlg2Δ cells producing a CPY-invertase fusion protein that is used to quantitatively measure the amount of CPY secreted (Fig. 4A) (32). Fig. 4B demonstrates that wild-type cells secrete ∼5% of the CPY-invertase fusion protein, reflecting the fact that the CPY fusion protein is properly sorted to the vacuole in these cells (32). In contrast, tlg2Δ cells secrete ∼25% of the fusion protein (Fig. 4B) (33). Production of either wild-type Tlg2p or the Tlg2p–F9A/L10A mutant complements the CPY-invertase secretion phenotype of tlg2Δ cells (Fig. 4B), consistent with our previous finding that abolition of the N-peptide mode of binding by the Vps45p–L117R mutant did not affect CPY sorting (13). Interestingly, mutation of Tlg2p at I285A, which disrupts the C-terminal binding mode in vitro, also rescued the CPY-invertase secretion phenotype (Fig. 4B), indicating that neither binding site individually is essential for Tlg2p function. However, expression of the triple mutant, Tlg2p–F9A/L10A/I285A, in which both binding sites are disrupted, does not rescue the CPY secretion defect (Fig. 4B). Although it is possible that the overexpression observed for the Tlg2p–F9A/L10A mutant could result in sufficient levels of functional Tlg2p, this is clearly not the case for Tlg2p–F9A/L10A/I285A. Thus, Tlg2p function in CPY secretion requires the presence of at least one of the two Vps45p binding sites.

**Vps45p Interacts with the Closed Conformation of Tlg2p.** We hypothesized that the C-terminal binding site is comprised of the Habc domain, linker, and SNARE motif regions folded into a closed conformation akin to that observed for other syntaxins (Fig. 1A, mode 1). To test this, Tlg2p(37-192) containing the Habc domain and Tlg2p(221-318) containing most of the linker and the SNARE motif (Fig. 1B) were incubated separately with Vps45p and their binding was analyzed by SEC and EMSA. Neither region is able to directly bind to Vps45p, nor compete with Tlg2p(37-1285A) for binding to Vps45p (Fig. S2 and Table 1). Moreover, one prediction of the closed conformation model is that residues critical for the Munc18a-closed syntaxin interaction would also be important for Vps45p–Tlg2p (37-1285A). Results from the I285A mutants support this idea. The I285 residue is present in the SNARe motif region of Tlg2p, and, by analogy to the syntaxin 1a-Munc18a structure, should contact Vps45p when Tlg2p is closed. We found that the I285A mutation abrogates binding to the C-terminal binding site of Tlg2p (Fig. 3C), indicating that Tlg2p is bound to Vps45p in a conformation structurally similar to closed syntaxin 1a.

To directly probe the closed conformation of Tlg2p, we made

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**Fig. 3.** The N-peptide of Tlg2p negatively affects the binding of Tlg2p(37-318). (A) The full-length cytosolic Tlg2p(1-318) protein competes with the Tlg2p(37-318)–Vps45p interaction. Increasing concentrations of Tlg2p(1-318) were added to Vps45p–Tlg2p(37-318). The presence of unbound or bound Tlg2p(37-318) was detected as in Fig. 2B. The Tlg2p(37-318) fraction bound vs. [Tlg2p(1-318)] was graphed and the data were fit to a modified version of the Lin and Riggs equation (28) to generate the curve and K_c,app. The first lane contains no Vps45p or competitor; the second lane contains complex with no added competitor; and the other lanes contain increasing concentrations (3.1 nM to 3.1 μM) of the competitor Tlg2p(1-318) protein. Representative data are shown; values reported are the average K_c,app and standard deviation for three replicates. (B) The ability of Tlg2p(37-318) to compete for binding to Vps45p was measured by competition EMSA and the K_c,app was calculated as in A. (C) The ability of the Tlg2p–I285A mutant to compete for binding to Vps45p was measured by competition EMSA. Results were analyzed as in A. The Tlg2p(37-1285A) and Tlg2p(1-318)-I285A data are plotted on the same graph for comparison.

**Fig. 4.** Abrogation of the two individual binding modes is required to disrupt Tlg2p function. (A) The expression levels of wild-type Tlg2p or Tlg2p mutants were monitored in tlg2Δ cells. Tlg2p constructs were expressed from a plasmid and their presence was detected by immunoblot analyses using α-Tlg2p antibodies (the same filter was probed with antibodies against phosphoglycerate kinase (PGK) as a loading control). (B) Levels of secretion of a CPY-invertase fusion were measured in wild-type cells, tlg2Δ cells, and tlg2Δ cells expressing the wild-type and mutant Tlg2p proteins. Values shown are mean ± standard deviation from four experiments.
mutations in the Habc domain designed to destabilize the closed conformation (Fig. 5A). The closest structural homologues to Tlg2p were used to create a homology model for the Habc domain (see SI Text). Using this model, and our previous “open” Sso1p mutants as guides (23, 34), we designed and created putative open Tlg2p mutants. The triple alanine mutant Tlg2p(37-318)–K134A/K137A/K163A shows several characteristics consistent with an open conformation, which would be caused by the SNARE motif residues no longer packing against the Habc domain. The Tlg2p(37-318) mutant’s α-helical secondary structure is decreased ~20% and its apparent molecular weight, as monitored by SEC, is increased when compared with wild-type Tlg2p(37-318) (Fig. 5B and C). Supporting our hypothesis that Vps45p interacts with the Tlg2p closed conformation, this mutant is unable to compete for binding to Vps45p (Fig. 5D).

Discussion

Here, we have used a fluorescent EMSA to reveal a previously uncharacterized binding site for the endosomal Sec1/Munc18 protein, Vps45p, on the syntaxin Tlg2p. We show that a construct lacking the N-peptide of Tlg2p interacts tightly with Vps45p (Fig. 2), demonstrating the presence of a C-terminal binding site. The Vps45p–L117R mutant, which does not bind the N-peptide of Tlg2p, binds to Tlg2p(37-318) with a similar affinity as wild type. Moreover, mutation of Tlg2p residues F9 and L10 to alanine, which disrupts binding of the N-peptide to Vps45p, competes for binding to Vps45p similarly to the N-peptide deletion (Table 1). This Tlg2p–Vps45p interaction explains the lack of a trafficking defect when the Vps45p–L117R mutant is expressed in yeast as the sole copy of Vps45p (13). Our results indicate that the absence of Vps45p, the closed conformation of Tlg2p inhibits SNARE complex assembly. Second, we have recently demonstrated that either removal of the Tlg2p Habc domain or the addition of Vps45p increases the rate of endosomal SNARE complex assembly in vitro (36). Here, we show that binding of Vps45p to the C-terminal binding site requires the Habc linker and SNARE motif regions of Tlg2p. In addition, the Tlg2p(37-318)–I285A mutant is unable to compete for binding to Vps45p; I285 is a residue predicted from analogous mutations in other SNAREs (7, 22, 29) to interact directly with Vps45p only when Tlg2p is in a closed conformation. Furthermore, the Tlg2p(37-318)–K134A/K137A/K163A mutant, which appears to form an open conformation as analyzed by CD and SEC, no longer competes for binding to Vps45p. Finally, evidence from mammalian cells leads to the conclusion that residues in the C-terminal region of syntaxin16 contribute to the overall binding affinity for Vps45p, although no binding was detected in the absence of the syntaxin16 N-peptide (22).

In contrast, NMR evidence suggested that Tlg2p does not adopt a stable closed conformation (12). However, the Tlg2p construct used in that study (amino acids 60–283) lacked additional N-terminal residues and a key section of the SNARE motif, which may have destabilized the closed conformation. Analogous SNARE motif residues in Sso1p, although unstructured, are ordered in the crystal structure of the closed conformation and pack against the Habc domain, possibly contributing to the stability of the closed conformation (23). Similarly, SNARE motif residues of Tlg2p(37-318) that are C-terminal to residue 283 may stabilize the closed conformation. These would be predicted to pack in close proximity to the location of residues K134, K137, and K163, which we found to be important for stabilizing the closed Tlg2p conformation (Fig. 5). Consistent with its open conformation and lack of additional SNARE motif residues, the Tlg2p(60-283) truncation protein does not compete for binding to Vps45p (Table 1). It remains possible that Tlg2p is not tightly closed and is in equilibrium between closed and open states; in this case, the closed conformation would be stabilized by binding to Vps45p. Further biological and/or structural characterization will be necessary to test these models. Nonetheless, our results indicate that, by binding to the closed conformation of Tlg2p, Vps45p joins Munc18a as proteins that possess both modes 1 and 2 of binding to their cognate syntaxin proteins.

Our results also demonstrate that the Tlg2p N-peptide modulates the affinity of the Tlg2p closed conformation for Vps45p,
indicating that these two sites do not bind simultaneously, e.g., using either mode 1 binding or mode 2, but excluding mode 3. Our competition assays reveal that the N-peptide cannot competes with the Vps45p-L117R mutant, confirming that binding of the Tlg2p-N-peptide to the hydrophobic pocket on domain 1 of Vps45p is required for this competition. This result was surprising because the two distinct binding sites in both Munc18a and mammalian Vps45 appear to positively influence each other (mode 3; ref. 22). These discrepancies likely reflect real differences between the SM proteins in diverse organisms and suggest that the N-peptide may be an attractive target for regulating the accessibility of the syntaxin SNARE motif for SNARE complex assembly (switching from mode 1 to 2). Alternatively, these results may suggest that, rather than the Munc18a cleft, Vps45p may use a different surface to bind to closed Tlg2p, one that overlaps with the N-peptide binding pocket. The fact that the Vps45p-L117R mutant binds the closed Tlg2p with similar affinity to the wild type argues strongly against this possibility.

In conclusion, our use of quantitative in vitro binding analyses led to the discovery of a closed conformation binding site on Tlg2p for the yeast SM protein Vps45p. Together with previous studies, these results now reveal that Vps45p uses all of the binding modes observed for other SM proteins and their cognate syntaxins. These diverse modes of binding to SNAREs and SNARE complexes allow SM proteins to function at multiple key stages during the SNARE assembly and membrane fusion processes. The plethora of different binding modes provides ample opportunities for exquisite regulation of SM-mediated membrane fusion in diverse trafficking steps. Further quantitative in vitro studies, combined with in vivo analyses of specific SM and SNARE mutants, will tease apart the detailed regulatory mechanisms. A comprehensive molecular understanding necessitates the expansion of these studies to include the function of SMs in conjunction with tethering complexes, Rab GTPases, and specialized regulators, such as Munc13, syntaxotagmins, and complexins.

Materials and Methods

Protein Purification and Analyses. Recombinant Tlg2p and Vps45p constructs with N-terminal His6 tags were expressed in Escherichia coli and purified by affinity, ion-exchange, and SEC. The Tlg2p (1-33) peptide containing a cysteine at the C terminus was synthesized (Quality Controlled Biochemicals). For SEC experiments, protein samples were run on a Superdex 200 10/30 column (GE) and monitored using the absorbance at 280 nm. Complex formation was verified by running elution fractions on SDS/PAGE and staining with Coomassie blue. Further purification and SEC details are given in SI Text.

EMSA. Tlg2p (37-318) was labeled [Tlg2p (37-318)] at Cys-316 with maleimide-conjugated Alexa Fluor 488 dye. The apparent affinity (K_{app}) between Vps45p and Tlg2p (37-318) was determined by EMSA using a protocol adapted from ref. 37. Additional details are given in SI Text. The data were fit to a sigmoidal dose–response function to determine the half-maximal saturation point (K_{app}) and the apparent Hill coefficient (n), by using Eq. 1 in SI Text. Competition experiments were performed similarly to the direct titration experiments, but increasing concentrations of unlabeled Tlg2p constructs ("competitor") were incubated with a subconcentrated concentration of Vps45p–Tlg2p (37-318)*. The apparent equilibrium dissociation of the competitor (K_{app}) and the shape factor were determined as described in SI Text.

CPY Secretion Assays. The ability of various Tlg2p constructs to complement the CPY trafficking defects of tlg2A cells was monitored by using a CPY-invertase fusion protein as described (32).

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Supporting Information

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SI Text

Protein Expression and Purification. Tlg2p(1-318), Tlg2p(37-318), Tlg2p(60-283), Tlg2p(37-192), and Tlg2p(221-318) truncations were generated by PCR and cloned into NdeI and BamHI sites of pET15b (Novagen), creating an N-terminal His6 tag on each. Tlg2p(37-192) also contains a C-terminal cysteine that did not affect its expression or solubility. Tlg2p(1-318)–F9AL10A, Tlg2p(1-318)–I285A, Tlg2p(37-318)–I285A, and Tlg2p(37-318)–K134A/K137A/K163A constructs were generated by using PCR mutagenesis. Cloning of His6–Vps45p and His6–Vps45p–L117R has been described (1). Constructs were confirmed by sequencing. Vps45p constructs were expressed in E. coli BL21(DE3) cells containing the GroEL and GroES expressing plasmid pIK213 (a gift from J. Kahana). Cells containing Tlg2p(1-318), Tlg2p(37-318), Tlg2p(60-283), Tlg2p mutants, Vps45p, and Vps45p–L117R, were shifted to 15 °C at an OD600 of ~0.3; protein expression was induced at an OD600 of 0.6–0.8 with 0.1 mM IPTG for 12–16 h. Cells expressing Tlg2p(37-192) and Tlg2p(221-318) were induced at 37 °C for 3 h. All proteins were purified by affinity and ion-exchange chromatography. Protease inhibitor tablets (Roche) were included in the lysis and affinity chromatography buffers used for Tlg2p proteins, except Tlg2p(37-318) and mutants Tlg2p(60-283), Tlg2p(37-192), and Tlg2p(221-318). Glycerol was added to 10% in all buffers, except for those used for Tlg2p(1-318) and Tlg2p(221-318). All proteins, except Tlg2p(221-318), were further purified by Superdex 200 (GE) SEC. Kphos (10 mM potassium phosphate, 140 mM potassium chloride) plus 1 mM DTT buffer was used for Tlg2p(1-318). Kphos, 10% glycerol, plus 1 mM DTT was used for the other constructs. All proteins, except Tlg2p(221-318) were concentrated by using a Stirred Ultrafiltration Cell 10/30 column equilibrated in KPhos plus 10% glycerol and 1 mM TCEP. Tlg2p(37-318) was concentrated by using an Amicon Centrifugal Concentrator (MWCO 5,000) (Millipore). Tlg2p(221-318) was concentrated by using an Amicon Centrifugal Concentrator (MWCO 5,000) (Millipore). Protein concentrations were determined by a quantitative ninhydrin protein assay (2). Proteins were stored at ~80 °C in Kphos plus 10% glycerol and 1 mM DTT or 2 mM TCEP. The Tlg2p(1-33) peptide containing a cysteine at the C terminus was synthesized (QCB). Purity was confirmed by HPLC and mass spectrometry. The peptide was lyophilized and stored at ~80 °C. Before use in the competition experiments, the peptide was resuspended in Kphos, 10% glycerol, and 1 mM TCEP. The concentration was determined by a quantitative ninhydrin protein assay (2). Proteins were stored at ~80 °C in Kphos plus 10% glycerol and 1 mM DTT or 2 mM TCEP. The Tlg2p(1-33) peptide containing a cysteine at the C terminus was synthesized (QCB). Purity was confirmed by HPLC and mass spectrometry. The peptide was lyophilized and stored at ~80 °C. Before use in the competition experiments, the peptide was resuspended in Kphos, 10% glycerol, and 1 mM TCEP. The concentration was determined by a quantitative ninhydrin protein assay (2). Proteins were stored at ~80 °C in Kphos plus 10% glycerol and 1 mM DTT or 2 mM TCEP.

Fluorescent Labeling of Tlg2p(37-318). Aliquots of Tlg2p(37-318) were incubated in KPhos plus 10% glycerol and 2 mM TCEP (reaction buffer) for 45 min. Maleimide-conjugated Alexa Fluor 488 dye (Invitrogen) was resuspended in reaction buffer and added at a 14:1 molar ratio (dye/Tlg2p). Tlg2p(37-318) contains two cysteine residues. One cysteine, Cys-316, is close to the C-terminal end of the protein. Homology modeling of the Habc domain predicts that the other cysteine (residue 129) is completely buried in the hydrophobic core. Mass spectrometry confirmed that only a single cysteine was labeled, and EMSA experiments showed that Vps45p affinity for the unlabeled Tlg2p(37-318) was ~2-fold different (slightly weaker) than for the labeled protein. The labeling reaction was incubated for 2 h in the dark at room temperature and centrifuged, and the supernatant was purified in the dark by using a Superdex 200 10/30 column equilibrated in KPhos plus 10% glycerol and 1 mM TCEP. Fractions containing labeled Tlg2p(37-318) were pooled and concentrated by using a Stirred Ultrafiltration Cell with a YM regenerated cellulose membrane (MWCO 10,000) (Millipore). Protein was stored and concentration determined similarly to above.

EMSA. The apparent affinity (Kd,app) between Vps45p and Tlg2p(37-318)* was determined by EMSA using the protocol adapted from ref. 6. Each experiment was replicated at least three times. Increasing concentrations of Vps45p were incubated with ~15 nM Tlg2p(37-318)* in Kphos, 10% glycerol, 0.01% Nonidet P-40, and 1 mM TCEP (binding buffer) and equilibrated for 3 h at room temperature in the dark. Each sample was mixed with a 1:9 dilution of Bromocresol green dye solution (2% wt/vol in 30% glycerol) and loaded onto a 6% horizontal slab polyacrylamide native gel (29:1 acrylamide/bis-acrylamide in 8.6 mM Imidazole and 7 mM Hepes, pH 7.4) previously equilibrated in the dark at room temperature and centrifuged, and the supernatant was purified in the dark by using a Superdex 200 10/30 column equilibrated in KPhos plus 10% glycerol and 1 mM TCEP. Fractions containing labeled Tlg2p(37-318)* were pooled and concentrated by using a Stirred Ultrafiltration Cell with a YM regenerated cellulose membrane (MWCO 10,000) (Millipore). Protein was stored and concentration determined similarly to above.

SEC. For SEC, protein samples (1–10 μM each) were incubated in KPhos for 1 h at 18 °C. One hundred microliters of each sample was loaded on a Superdex 200 10/30 column (GE) preequilibrated in Kphos plus 1 mM DTT and elution of the protein from the column was monitored by using the absorbance at 280 nm. The gel filtration column was calibrated by using standards (thyroglobulin, 670 kDa; γ-globulin, 158 kDa; ovalbumin, 44 kDa; myoglobin, 17 kDa; Bio-Rad). We observed that Vps45p and the L117R mutant had slightly smaller apparent molecular mass than expected; however, mass spectrometry confirmed that the recombinant purified Vps45p is full length. Conversely, the Tlg2p constructs generally eluted with an apparent molecular mass slightly larger than calculated, which is consistent with residues 37–69 and 308–318 being unstructured (3) The monomeric state of Tlg2p(37-318) was confirmed by analytical ultracentrifugation and at concentrations as high as 100 μM Tlg2p(37-318) did not aggregate when assayed by SEC. Each CD and SEC experiment was repeated at least three times.

CD Spectroscopy. CD spectra were recorded as described (4) in KPhos, 10% glycerol, and 0.5 mM TCEP (reaction buffer) for 45 min. Maleimide-conjugated Alexa Fluor 488 dye (Invitrogen) was resuspended in reaction buffer and added at a 14:1 molar ratio (dye/Tlg2p). Tlg2p(37-318) contains two cysteine residues. One cysteine, Cys-316, is close to the C-terminal end of the protein. Homology modeling of the Habc domain predicts that the other cysteine (residue 129) is completely buried in the hydrophobic core. Mass spectrometry confirmed that only a single cysteine was labeled, and EMSA experiments showed that Vps45p affinity for the unlabeled Tlg2p(37-318) was ~2-fold different (slightly weaker) than for the labeled protein. The labeling reaction was incubated for 2 h in the dark at room temperature and centrifuged, and the supernatant was purified in the dark by using a Superdex 200 10/30 column equilibrated in KPhos plus 10% glycerol and 1 mM TCEP. Fractions containing labeled Tlg2p(37-318)* were pooled and concentrated by using a Stirred Ultrafiltration Cell with a YM regenerated cellulose membrane (MWCO 10,000) (Millipore). Protein was stored and concentration determined similarly to above.

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signal for Tlg2p(37-318)* in the bound fraction, and b is the greatest signal for Tlg2p(37-318)* in the unbound fraction.

Competition experiments were performed as above, except that increasing concentrations of unlabeled Tlg2p constructs (“competitor”) were incubated with a saturated concentration of Vps45p and Tlg2p(37-318)*. The fraction bound of Tlg2p(37-318)* vs. [competitor] was graphed, and the half maximal inhibition constant (IC50) was calculated by using Eq. 2, where [C] is the concentration of competitor protein, and s is the shape factor.

\[
\phi = b + \frac{(m - b)}{1 + \left(\frac{IC_{50}}{[C]}\right)^s}
\]  

These values were converted to the apparent equilibrium dissociation of competitor (K\text{d,app}) by applying the Lin and Riggs correction (7), where [P] is the concentration of Vps45p, [\text{T}]* is the concentration of labeled Tlg2p(37-318)*, and K\text{d,app} is the apparent dissociation constant for Vps45p–Tlg2p(37-318)*:

\[
K_{\text{d,app}} = \frac{(2K_{\text{d,app}}IC_{50})}{2[P] - [\text{T}]* - 2K_{\text{d,app}}} 
\]  

For competition experiments, the shape of the curve accounts for several complicating features of the competitions, including possible cooperativity in the competition mechanism, the presence of multiple competing half-sites per mole of competitor added, and multiple nonequivalent classes of binding complexes between the macromolecule and the labeled ligand. A parameter related to but not equivalent with the Hill coefficient termed the shape factor (s) reflects the magnitude of the deviation from ideality. For simple direct competition between a competitor and a ligand in a bimolecular complex, the shape factor is expected to be one. In our experiments, competitor proteins that contain a single functional binding site reveal a shape factor of 2.4–2.8 (Table 1), whereas the presence of both binding sites in Tlg2p(1-318) leads to an increased shape factor of 4.4. The profound increase in the shape factor in constructs that contain both binding sites is consistent with the dual binding site model proposed above. Surprisingly, the Tlg2p(1-318)–I285A mutant also shows an elevated shape factor, suggesting that the presence of the N-terminal peptide increases the local concentration of the C-terminal 37–318 region, which is sufficient to overcome the detrimental effect of the I285A mutation.

**CPY Secretion Assays.** The ability of various Tlg2p constructs to complement the CPY trafficking defects of tlg2Δ cells was monitored by using a CPY-invertase fusion protein as described (8). Wild-type [BHY10 (9)] or tlg2Δ [SGSY2, made by replacing the entire TLG2 ORF in BHY10 with the KANMX4 module as described (10)] cells expressing a CPY-invertase fusion (but lacking endogenous invertase) were transformed with plasmids harboring either wild-type TLG2 [pHA-TLG2 (10)] or versions harboring the I285A mutation (pCMDO13), the F9A/L10A mutation (pCOG066), or all three mutations (F9A/L10A/I285A; pCMDO11). The amount of invertase activity secreted by these cells was measured (8) and expressed as a percentage of the total activity.

**Tlg2p Homology Model.** To facilitate our mutagenesis experiments, we built a high-resolution homology model of the structure of yeast Tlg2p Habc domain based on structures of other syntaxin proteins. We performed a structure-based sequence alignment of Tlg2p with other syntaxin family members in the nonredundant sequence database from the National Center for Biotechnology Information using the program HHpred (http://toolkit.tuebingen.mpg.de/hhpred), which constructs a hidden Markov model and searches the selected HMM databases for homologs (11, 12). We used these alignments to derive a homology model from rat syntaxin-1a (Protein Data Bank ID code 1ez3; ref. 13), our structure of yeast Sso1p (Protein Data Bank ID code 1fio; ref. 14), squid syntaxin-12 (Protein Data Bank ID code 1s94; ref. 15), and human syntaxin-12 (Protein Data Bank ID code 2dnx; from the Riken Structural Genomics Initiative) using MODELLER (16) on the same website.

Fig. S1. CD analysis of Tlg2p (A) and Vps45p (B) constructs expressed in *E. coli* and purified by ion-exchange and SEC. (Insets) Purified proteins were analyzed by SDS/PAGE and stained with Coomassie blue. (C) The interaction between purified Vps45p and Tlg2p(1-318) was observed by SEC. Vps45p and Tlg2p(1-318) were incubated together and applied to a Superdex 200 gel filtration column. The elution profile of the complex is shown compared with the profiles of the individual proteins. Absorbance was monitored at 280 nm and the mobility of the molecular mass standards is indicated at the top.
Fig. S2. Vps45p interacts with the closed conformation of Tlg2p. (A and B) The interaction between Vps45p and Tlg2p(37-192) or Tlg2p(221-318) was analyzed by SEC. Presence of a complex was detected as in Fig. S1C. Failure of the elution peaks to shift indicates that no complex was formed. (C) The ability of Tlg2p(37-192) to compete for binding to Vps45p was measured by competition EMSA. Results were analyzed as in Fig. 3A. Failure to observe a shift in Tlg2p(37-318)* indicates an inability of the competitor to compete for binding to Vps45p. At very high concentrations of competitor, some nonspecific aggregation is observed in the wells of the gel.

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