The Sec1p/Munc18 protein Vps45p binds its cognate SNARE proteins via two distinct modes

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Sec1p/Munc18 (SM) proteins are essential for SNARE-mediated membrane trafficking. The formulation of unifying hypotheses for the function of the SM protein family has been hampered by the observation that two of its members bind their cognate syntaxins (Sxs) in strikingly different ways. The SM protein Vps45p binds its Sx Tlg2p in a manner analogous to that captured by the Sly1p–Sed5p crystal structure, whereby the NH2-terminal peptide of the Sx inserts into a hydrophobic pocket on the outer face of domain 1 of the SM protein. In this study, we report that although this mode of interaction is critical for the binding of Vps45p to Tlg2p, the SM protein also binds Tlg2p-containing SNARE complexes via a second mode that involves neither the NH2 terminus of Tlg2p nor the region of Vps45p that facilitates this interaction. Our findings point to the possibility that SM proteins interact with their cognate SNARE proteins through distinct mechanisms at different stages in the SNARE assembly/disassembly cycle.

Introduction

Intracellular compartmentalization into discrete membrane-bound organelles is a defining feature of eukaryotic cells. Communication between organelles is achieved through membrane fusion, which is a highly conserved and regulated process. Central to the membrane fusion machinery are the SNARE family of proteins, which are characterized by a helical motif preceding their COOH-terminal transmembrane domain (Jahn and Sudhof, 1999). A SNARE motif from one membrane assembles into a four-helix bundle with three SNARE motifs on another membrane, including one contributed by a syntaxin (Sx) family member (Jahn and Sudhof, 1999). The neuronal Sx, Sx1A, adopts two distinct conformations (Dulubova et al., 1999): in the closed conformation, the autonomously folded NH2-terminal Habc domain folds back onto the SNARE motif, rendering it unavailable for core complex formation; in the open conformation, the Habc domain moves away from the SNARE motif, leaving it free to participate in the core complex.

Although SNARE proteins are sufficient to drive bilayer fusion in vitro (Weber et al., 1998), other factors control membrane fusion in vivo. The Sec1p/Munc18 (SM) family of proteins are essential for SNARE-mediated traffic, but their mode of action is far from understood (Gallwitz and Jahn, 2003; Toonen and Verhage, 2003). Unlike SNARE proteins, which are common only in their shared SNARE motif, SM proteins are conserved across their entire length (600–700 residues; Jahn and Sudhof, 1999).

Munc18a was originally identified as a Sx1A-binding protein (Hata et al., 1993) whose binding to Sx1A prevents SNARE complex formation in vitro (Pevsner et al., 1994). The crystal structure of the Munc18a–Sx1A complex also supports a model in which Munc18a acts as a negative regulator of SNARE complex assembly, revealing that the SM protein is an arch-shaped molecule with three distinct domains forming a central cavity that cradles the Sx in its closed conformation (Misura et al., 2000). Consistent with this, no binding of Munc18a to Sx1A–SNARE complexes containing Sx1A in the open conformation is observed (Yang et al., 2000). In marked contrast, other SM proteins bind to assembled SNARE complexes (Gallwitz and Jahn, 2003); indeed, the yeast plasma membrane SM protein Sec1p was originally thought to bind only to the assembled SNARE complex and not to uncomplexed Sso1p (Carr et al., 1999), although this has recently been disputed (Scott et al., 2004).

Consistent with the crystal structure of the Munc18a–Sx1A complex, which shows contacts of the central cavity of the SM protein with both the SNARE and Habc domains of the Sx (Misura et al., 2000), most of the cytosolic portion of
Sx1A is required to bind the SM protein (Kee et al., 1995; Misura et al., 2000). Therefore, it was surprising that short NH$_2$-terminal sequences of Sx5 and 16 are both necessary and sufficient to capture their respective cognate SM proteins Sly1p and Vps45p (Dulubova et al., 2002, 2003). The crystal structure of Sly1p in complex with the NH$_2$-terminal 45 residues of Sed5p, the yeast homologue of Sx5, reveals that the structure of Sly1p is very similar to that of Munc18a, with three domains arranged in an arch shape, forming a central cavity of $\sim$15 Å (Bracher and Weissenhorn, 2002). As described above, the central cavity of Munc18a cradles Sx1A in its closed conformation, with contacts made between the Sx and domains I and IIIa of the SM protein (Misura et al., 2000). The interaction between Sed5p and Sly1p is strikingly different from this, with the NH$_2$-terminal peptide of the Sx buried in a hydrophobic pocket on the outer face of domain I (Bracher and Weissenhorn, 2002). This binding has been disrupted through mutations in both Sly1p and Sed5p and is not essential for membrane traffic (Peng and Gallwitz, 2004). As both proteins are essential for membrane traffic, it is therefore likely that their essential functions involve interactions other than that revealed by the crystal structure of the Sly1p–Sed5p complex (Peng and Gallwitz, 2004).

The differences between the interactions of SM proteins with their cognate Sxs have made understanding the action of this family of proteins difficult. We have previously shown that the SM protein Vps45p controls the entry of its cognate Sx Tlg2p into functional SNARE complexes (Bryant and James, 2001) and that it interacts with both uncomplexed Tlg2p and cis-SNARE complexes in vivo (Bryant and James, 2003). Tlg2p binds to Vps45p via its NH$_2$-terminal region in a manner analogous to the high-affinity interaction between Sed5p and a hydrophobic pocket on the outer face of domain I of Sly1p (Dulubova et al., 2002). This hydrophobic pocket is formed by five residues of Sly1p (L137, L140, A141, I153, and V156) that surround F10 of Sed5p (Bracher and Weissenhorn, 2002). Sequence alignment of Vps45p and Sly1p indicates that four of these residues are conserved (Fig. 1 A), with the fifth residue, I153, being replaced by a valine in Vps45p. Although this position is occupied by an isoleucine residue in Sly1p from *Saccharomyces cerevisiae*, a valine is found here in Sly1p sequences from other species, including humans and *Drosophila melanogaster* (Peng and Gallwitz, 2004).

The mutation of Sly1p L137 to an arginine residue abolishes the binding of the SM protein to Sed5p without affecting membrane transport (Peng and Gallwitz, 2004). We found that the mutation of L117 of Vps45p to arginine similarly disrupts the binding of Vps45p to Tlg2p. A protein A (PrA) fusion protein containing the cytosolic domain of Tlg2p (Tlg2p-PrA) efficiently binds tagged Vps45p (HA-Vps45p) from a cell lysate (Fig. 1 B). However, Tlg2p-PrA is unable to bind a version of HA-Vps45p harboring the L117R mutation (Fig. 1 B). Similarly, immunoprecipitation of HA-Vps45p$_{1-117}$ from a
Vps45p binds to the v-SNARE Snc2p

Peng and Gallwitz (2004) reported the novel finding that an SM protein interacts directly with non-Sx SNAREs, leading them to propose a model for Sly1p function whereby the SM protein forms a bridge between t-SNAREs and their cognate v-SNAREs. We investigated whether Vps45p physically interacts with the non-Sx SNAREs present in the Tlg2p–SNARE complex. We expressed the cytosolic domains of Snc2p and Vti1p as PrA fusion proteins. Attempts to express a similar fusion protein containing the cytosolic domain of Tlg1p were unsuccessful, so we used a GST fusion protein (Tlg1p-GST; Coe et al., 1999). Fig. 2 A demonstrates that His$_6$-Vps45p interacts directly with the cytosolic domains of both Tlg2p and the v-SNARE Snc2p (Snc2p-PrA). We found no evidence for an interaction of Vps45p with the cytosolic domains of either Tlg1p or Vti1p (Fig. 2 A). Fig. 2 B demonstrates that the binding between the cytosolic domain of Snc2p and Vps45p is specific, as Snc2p-PrA does not bind to another SM protein family member, Munc18c. Munc18c shares 50% similarity with Vps45p and binds specifically to its cognate Sx (Sx4; Fig. 2 B; Tellam et al., 1997).

Unlike Sx t-SNAREs, the v-SNARE Snc2p has a short NH$_2$-terminal region of 19 residues preceding the SNARE motif. A version of Snc2p-PrA lacking these residues (Snc2p$_{22-19}$-PrA) efficiently bound Vps45p (Fig. 2 C), demonstrating that the SNARE motif accounts for the Vps45p–Snc2p interaction. This is consistent with the binding of Sly1p to the v-SNAREs Bet1p and Sft1p, which is mediated through the SNARE motif of these v-SNAREs (Peng and Gallwitz, 2004). Snc2p does not bind Vps45p through the same mechanism as Tlg2p because HA-Vps45p$_{L117R}$, which is unable to bind Tlg2p-PrA (Fig. 1 B), binds to Snc2p-PrA (Fig. 2 D).

Figure 2. Vps45p binds directly to the v-SNARE Snc2p. Recombinant proteins purified from E. coli were immobilized on glutathione-agarose or IgG-Sepharose before incubation with either His$_6$-tagged Vps45p or Munc18c purified from E. coli. After extensive washing, bound proteins were subject to SDS-PAGE followed by Coomassie blue staining (top) or immunoblotting with Vps45p or Munc18c antibodies. (A) Proteins comprising the cytosolic domains of Tlg2p, Snc2p, Vti1p, and Tlg1p fused to either PrA or GST were tested for their ability to bind His$_6$-Vps45p. (B) The binding of Munc18c to Snc2p-PrA and GST-tagged syntaxin 4 (Sx4) was assessed. (C) The ability of Snc2p-PrA, Snc2p$_{22-19}$-PrA, and the PrA moiety alone to bind His$_6$-Vps45p was analyzed. (D) The ability of Snc2p-PrA to bind either His$_6$-tagged Vps45p (wild type) or the same harboring the L117R mutation purified from bacteria was assessed and run next to 5% of the lysate used (input).

The finding that Tlg2p and Snc2p use different mechanisms to bind to Vps45p (Fig. 2 D) supports the bridging hypothesis of Peng and Gallwitz (2004). However, we were unable to observe Tlg2p and Snc2p binding to Vps45p simultaneously (Fig. 3 A). Tlg2p-PrA immobilized on IgG-Sepharose is unable to bind to the cytosolic domain of Snc2p (Snc2p$_{cyto}$), which was produced using thrombin cleavage to remove the PrA tag from Snc2p-PrA (Fig. 3 A, lane 4). Attempts to bridge the two SNARE proteins by the addition of purified His$_6$-Vps45p proved unsuccessful (Fig. 3 A, lane 5), with only His$_6$-Vps45p and Tlg2p-PrA binding to IgG-Sepharose. Note that His$_6$-Vps45p and Snc2p$_{cyto}$ are able to bind under these conditions, as Snc2p$_{cyto}$ can be bound to Ni$^{2+}$–nitrilotriacetic acid–agarose in the presence of His$_6$-Vps45p (Fig. 3 A, lane 7).

Although our inability to observe the two SNARE proteins binding to the SM protein does not disprove the bridging hypothesis of Peng and Gallwitz (2004), as such a bridging interaction is likely to be tightly regulated and may require a transient conformation of the SM protein that is not accessed in our experimental system, it does indicate that the Tlg2p- and Snc2p-binding sites on Vps45p may overlap or influence each other. Consistent with this, we found that Tlg2p-PrA is able to displace Snc2p-PrA from His$_6$-Vps45p (Fig. 3 B). In contrast, Snc2p-PrA is not able to displace Tlg2p-PrA from the SM protein (Fig. 3 B).
A dominant-negative version of Vps45p binds SNARE complexes through a mechanism that is distinct from the Sly1p–Sed5p hydrophobic pocket interaction

With the aim of gaining mechanistic insight into Vps45p function, we sought to isolate alleles of \( \text{VPS45} \) that were dominant negative for CPY sorting (see Materials and methods). Fig. 4 A demonstrates that a version of Vps45p carrying a single amino acid substitution (W244R) exerts a dominant-negative effect on the sorting of CPY. Wild-type cells expressing Vps45pW244R from a multicopy (\( 2 \mu \) based) plasmid missort 60% of their newly synthesized CPY. The wild-type gene expressed at the same level caused no defects in CPY sorting (Fig. 4 A).

Introduction of a second multicopy plasmid carrying wild-type \( \text{VPS45} \) suppressed the CPY missorting phenotype of this strain, indicating that Vsp45pW244R acts as a competitive antagonist of Vps45p function (Fig. 3 B). This is consistent with the finding that when Vsp45pW244R was introduced into a wild-type strain on a single-copy (\( \text{CEN} \) based) plasmid, no CPY secretion was observed (unpublished data). Thus, we conclude that the mutant is a dosage-dependent inhibitor of normal Vps45p function (an antimorph). The requirement for the mutant to be in excess relative to the normal protein may indicate that it competes for interaction with a target protein or proteins with which it forms a nonproductive complex. Indeed, it is clear that Vsp45pW244R is not able to perform all of the functions of its wild-type counterpart because cells in which the mutant gene is the only source of Vps45p are unable to sort CPY correctly (Fig. 4 C).

![Figure 3. Tlg2p and Snc2p do not bind simultaneously to Vps45p.](image)

![Figure 4. Tlg2p binding is required for the efficacy of a dominant-negative version of Vps45p.](image)
between Vps45p/W244R and SNARE complexes is not mediated by Snc2p, whereas Vps45pL117R does not. Fig. 5 B demonstrates that Vps45p binds to SNARE complexes on the NH2-terminal region of Tlg2p via a mode analogous to that captured by the Sly1p–Sed5p crystal structure (Bracher and Weissenhorn, 2002) required for Vps45p to interact with Tlg2p, whereas Vps45p does not bind to the cytosolic domain of Tlg2p. This binding is facilitated through the NH2-terminal peptide of Tlg2p, as Vps45p did not bind to a version of Tlg2p lacking the first 36 residues (Tlg2pΔ2–36-PrA; Fig. 4 D). These data reveal that the binding of Vps45p to the NH2-terminal region of Tlg2p is not dependent on the NH2-terminal peptide of Tlg2p (in a manner analogous to the binding captured by the Sly1p–Sed5p interaction (through the NH2 terminus of Tlg2p)). This suggests that Vps45p blocks traffic through the endosomal system by titrating out the v-SNARE Snc2p. Therefore, we looked at the interaction of Vps45p with the cytosolic domain of Snc2p. Fig. 4 D demonstrates that Vps45p binds to Snc2p-PrA in a manner analogous to that used for its interaction with Tlg2p (Fig. 2 D).

In addition to interacting with monomeric Tlg2p, Vps45p binds to cis-SNARE complexes (Bryant and James, 2003). Given that we found no differences between the binding of Vps45p and its wild-type counterpart to either Tlg2p or Snc2p, we went on to investigate their binding to assembled SNARE complexes. Fig. 5 B demonstrates that Vps45p binds to SNARE complexes formed in vitro, whereas Vps45p does not. Fig. 5 B also shows that Vps45p binds to these complexes, as does the double mutant Vps45pL117R/W244R, indicating that the interaction between Vps45p and SNARE complexes is not mediated through the NH2 terminus of Tlg2p and a hydrophobic pocket of Vps45p. To further investigate this interaction, we prepared SNARE complexes using the version of Tlg2p lacking the first 36 residues of the Sx (Tlg2pΔ2–36-PrA; Fig. 5 B). These complexes are unable to bind either wild-type Vps45p or Vps45pL117R, but, importantly, both Vps45p and the double mutant Vps45pL117R/W244R were able to bind these SNARE complexes. These data suggest that the W244R mutation locks Vps45p in a form that interacts with the assembled SNARE complex and that this interaction does not require the NH2 terminus of Tlg2p.

The CPY sorting defect that Vps45p bestows upon wild-type cells is suppressed by the overexpression of SNC2 (Fig. 4 B). Overproduction of other SNARE proteins, including Tlg2p, did not suppress the phenotype of Vps45p (Fig. 4 B). This suggests that Vps45p blocks traffic through the endosomal system by titrating out the v-SNARE Snc2p. Therefore, we looked at the interaction of Vps45p with the cytosolic domain of Snc2p. Fig. 4 D demonstrates that Vps45p binds to Snc2p-PrA in a manner analogous to that used for its interaction with Tlg2p (Fig. 2 D).

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Figure 5. The NH2-terminal peptide of Tlg2p is not required for Vps45p to bind assembled SNARE complexes. (A) SNARE complexes containing either the entire cytosolic domain of Tlg2p (WT) or lacking residues 2–36 (Δ2–36) bound to Ni2+–agarose were resolved by SDS-PAGE on a 14% gel and were visualized by Coomassie staining. Note that the relatively weak band for Snc2p is caused by its poor Coomassie staining (McNew et al., 2000). The bands migrating between Tlg2p and Tlg1p in the wild-type lane are Tlg2p degradation products (confirmed by immunoblotting; not depicted). (B) The binding of HA-Vps45p and indicated mutants from yeast cell lysates described in Fig. 4 to the complexes represented in A was assayed as described in Materials and methods. Bound material was analyzed through immunoblotting with anti-HA antibodies and anti-Vt1p (to ensure that binding to equivalent amounts of complex was being assessed).

The interaction between Vps45p and the SNARE complex is weaker than the binding of wild-type Vps45p to the same (Fig. 5 B). This likely reflects the fact that the two versions of the SM protein are binding to the SNARE complexes via different mechanisms. Wild-type Vps45p binds to the assembled SNARE complex through the NH2-terminal peptide of Tlg2p (in a manner analogous to the binding captured by the Sly1p–Sed5p crystal structure). This is evident from the observation that wild-type Vps45p does not bind to complexes formed using Tlg2p that lacks its first 36 residues (Tlg2pΔ2–36; Fig. 5 B) and also by the observation that Vps45pL117R does not bind to SNARE complexes (containing full-length Tlg2p; Fig. 5 B). We speculate that the W244R mutation results in a protein that is locked in a conformation that binds to the assembled SNARE complex. It binds to this complex via a mode distinct from what is analogous to the Sly1p–Sed5p interaction (through the NH2 terminus of Tlg2p and the putative hydrophobic pocket in domain I of Vps45p). This is supported by the observation that the Vps45pL117R double mutant binds to Tlg2pΔ2–36-containing SNARE complexes and also by the finding that the Vps45pL117R/W244R double mutant binds to SNARE complexes (Fig. 5 B). The Vps45pL117R/W244R double mutant binds less well to SNARE complexes (containing wild-type Tlg2p) than Vps45pL117R/W244R, presumably because the latter is able to bind to these complexes both through the Sly1p–Sed5p-type interaction and also via the binding mode enabled by the W244R mutation. This conclusion is supported by the reduced...
amount of Vps45pW244R binding to SNARE complexes that were formed using Tlg2p22,36 (Fig. 5 B).

Vps45p is a peripheral membrane protein that attaches to membranes through its interaction with Tlg2p (Bryant and James, 2001). Like endogenous Vps45p (Bryant and James, 2001), HA-Vps45p is distributed between membrane and cytosol fractions obtained from wild-type cells, as is the dominant-negative mutant Vps45pW244R (Fig. 6). Consistent with its inability to bind to the cytosolic domain of Tlg2p, Vps45pL117R does not associate with membranes but instead localizes to the cytosol (Fig. 6). However, the double mutant Vps45pL117R/W244R is found attached to membranes despite its inability to bind monomeric Tlg2p (Fig. 4 D). Collectively with the data presented in Fig. 5, this indicates that Vps45pW244R binds to SNARE complexes in a way that does not require an interaction with Tlg2p akin to that captured by the Sly1p–Sec5p crystal structure. We suggest that this mode of interaction is not observed for wild-type Vps45p (or the L117R mutant; Fig. 5), as the W244R mutation is required to lock the molecule in a conformation capable of binding the SNARE complex in this way. However, the observation that the dominant-negative effect of Vps45pW244R is suppressed by an increased dose of the wild-type protein (Fig. 4 B) indicates that Vps45p does adopt this conformation during the course of its normal functional cycle. If the W244R mutation caused Vps45p to fold in such a way that it binds to the SNARE complex nonspecifically, it would be a neomorphic mutant (where the mutant protein has gained a new or significantly altered function with respect to the wild type). This is ruled out by our finding that an increased dose of the wild-type gene suppresses the dominant-negative effect of Vps45pW244R (Fig. 4 B).

Discussion

We set out to investigate the modes of interaction between Vps45p and the components of the Tlg2p–SNARE complex (Tlg2p, Tlg1p, Snc2p, and Vti1p). Building upon the findings of Dulubova et al. (2002) and Peng and Gallwitz (2004), we created a mutant version of Vps45p (L117R) in which the interaction between Vps45p and the NH2-terminal region of Tlg2p is abolished. This confirmed that Vps45p and Tlg2p bind to each other in a manner analogous to the interaction revealed by the Sly1p–Sec5p crystal structure (Bracher and Weissenhorn, 2002, Dulubova et al., 2002). We also identified a mutation in Vps45p, W244R, that creates a dominant-negative phenotype. Peng and Gallwitz (2004) proposed that the hydrophobic pocket mode of binding might serve to recruit the SM protein to sites of trans-SNARE complex formation. Our finding that the L117R mutation abolishes the membrane association of Vps45p is consistent with this (Fig. 6). We have demonstrated that as well as binding to Tlg2p in this mode, Vps45p binds to SNARE complexes via an unrelated mechanism. The SM protein likely goes through an ordered sequence of these interactions as the SNARE complex assembly/disassembly cycle proceeds with ongoing rounds of membrane fusion. These interactions are likely to be carefully orchestrated within the cell, with the SM protein switching between binding modes. Vps45pW244R could subvert this orderly progression by acting in the two modes simultaneously, and this may explain the deleterious effects of this mutant. Our studies of the Vps45pW244R mutant have revealed that as well as binding directly to the NH2-terminal segment of Tlg2p, Vps45p binds to SNARE complexes via an unrelated mechanism. The SM protein likely goes through an ordered sequence of these interactions as the SNARE complex assembly/disassembly cycle proceeds with ongoing rounds of membrane fusion. These interactions are likely to be carefully orchestrated within the cell, with the SM protein switching between binding modes. Vps45pW244R could subvert this orderly progression by acting in the two modes simultaneously, and this may explain the deleterious effects of this mutant.

Sequence alignments reveal that W244 of S. cerevisiae Vps45p is conserved in all Vps45p and Sly1p homologues; in most Sec1p homologues (including Munc18a and squid Sec1 [sSec1]), there is a leucine residue at this position. Structures of Munc18a (Misura et al., 2000), sSec1 (Bracher et al., 2000), and yeast Sly1 (Bracher and Weissenhorn, 2002) show that these residues (Sly1W278, nSec1L247, and sSec1L244) project into the protein interior to form part of a conserved hydrophobic cluster that includes F336 and L411 (in Sly1p; I296/Y358 in nSec1 and I293/Y356 in sSec1). In all three cases, this pair of residues lies at the internal ends of the two helices that form the helical hairpin of domain II; thus, the conserved hydrophobic cluster interfaces between the projecting helical hairpin and the rest of domain II. Insertion of a polar residue here may cause a conformational change that opens the arch, allowing unregulated binding to the SNARE complex. An open structure of this type is seen in sSec1, and it has been suggested that movements of the helical hairpin could affect the release of Sx1A during SNARE complex formation (Bracher et al., 2000).

The conserved hydrophobic cluster also interfaces with (1) the five-helix helical repeat of domain II, which forms part of the molecular surface at the top of the arch; and (2) the surface loop insertion in Sly1p, a motif that is well positioned to regulate access to the surface of the helical hairpin and that includes the site of the sly1-20 mutation, which renders Sly1p independent of the rab protein Ypt1 (Bracher and Weissenhorn, 2002). Thus, the W244R mutation could disrupt two different surface regions of the protein and affect binding sites for regulatory factors.

| wild-type | W244R | L117 | L117/W244 |
|-----------|-------|------|-----------|
| W | P | S | W | P | S |
| Vps45p(674D) | PGK(453D) | AP(634D) |

Figure 6. Vps45p associates with membranes through a manner distinct from the binding represented by the Sly1p–Sec5p crystal structure. Vps45p∆ (NOzY1) cells expressing HA-tagged versions of wild-type Vps45p (pNB706), W244R (pNB707), L117R (pNB708), or L117R/W244R (pNB709) from centromeric plasmids were fractionated into membrane pellet (P) and cytosol (S) fractions (W, whole cell extract). The amount of Vps45p in each of these fractions was analyzed through immunoblotting. Phosphoglycerate kinase (PGK) and AP were included in this analysis as markers for membranes and cytosol, respectively.
In conclusion, this study demonstrates that the SM protein Vps45p not only binds to its cognate Sx Tlg2p but also to the v-SNARE Snc2p, which participates in SNARE complexes with Tlg2p. We have also demonstrated that Vps45p uses two distinct binding modes to interact with Tlg2p and Tlg2p-containing SNARE complexes. This is an important finding, as one of the major hindrances to the formulation of a unifying hypothesis of SM protein function has been the observed differences in the interactions of members of this family with their cognate Sx–SNARE complexes. Our finding uncovers the possibility that SM proteins interact with their cognate SNARE proteins through distinct mechanisms at different stages in the SNARE assembly/disassembly cycle. Determining the functional significance of these two different modes of interaction between Vps45p and its cognate SNARE proteins will require a detailed analysis of the conformational state of Vps45p that is bound to the assembled SNARE complex.

Materials and methods

Reagents
Antibodies against Vps45p, Vtlp, AP, phosphoglycerate kinase, and Munc18c have been described previously (Piper et al., 1994; Tellam et al., 1997; Coe et al., 1999; Bryant and James, 2001). The monoclonal antibody for the influenza HA epitope 3F10 was obtained from Roche. Yeast strains and plasmids used in this study are listed in Tables I and II.

Construction of plasmids made during the course of the study

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Table I. Yeast strains used in this study

| Strain   | Genotype                           | Reference |
|----------|------------------------------------|-----------|
| RPY10    | MATa ura3-52 leu2-3 112 his4-519 ade6 gal2 | Piper et al., 1994 |
| SFB38-9D | MATa ura3-52 leu2-3 112 his4-519 ade6 gal2 pep4-3 | Rothman and Stevens, 1986 |
| NO2Y1    | MATa ura3-52 leu2-3 112 his4-519 ade6 gal2 pep4-3 vps45Δ::Kan’ | Bryant and James, 2001 |
| NO2Y2    | MATa ura3-52 leu2-3 112 his4-519 ade6 gal2 vps45Δ::Kan’ | Bryant and James, 2001 |

All strains are congenic to RPY10.
Table II. Plasmids used in this study

| Plasmid       | Description                                                                 |
|---------------|-----------------------------------------------------------------------------|
| pCOG070       | Yeast expression (2μ URA3) plasmid encoding wild-type HA-Vps45p              |
| pCOG071       | As pCOG070 encoding HA-Vps45p<sub>117R</sub>                                 |
| pCOG072       | As pCOG070 encoding HA-Vps45p<sub>W244R</sub>                                |
| pCOG073       | As pCOG070 encoding HA-Vps45p<sub>117R,W244R</sub>                          |
| pCOG065       | Yeast expression (2μ LEU2) plasmid encoding HA-Vps45p<sub>W244R</sub>       |
| pNB706        | Yeast expression (CEN, URA3) plasmid encoding wild-type HA-Vps45p           |
| pNB707        | As pNB706 encoding HA-Vps45p<sub>W244R</sub>                                |
| pNB708        | As pNB706 encoding HA-Vps45p<sub>117R</sub>                                 |
| pNB709        | As pNB706 encoding HA-Vps45p<sub>117R,W244R</sub>                           |
| pNB710        | E. coli expression vector encoding His<sub>6</sub>-Vps45p                   |
| pCOG067       | E. coli expression vector encoding His<sub>6</sub>-Vps45p<sub>117R</sub>     |
| pCOG022       | E. coli expression vector encoding two IgG-binding domains of S. aureus PrA |
| pCOG025       | E. coli expression vector encoding Tlg2pPrA                                 |
| pCOG026       | E. coli expression vector encoding Tlg2p<sub>12–34</sub>PrA                 |
| pCOG045       | E. coli expression vector encoding Snc2pPrA                                 |
| pCOG046       | E. coli expression vector encoding Snc2p<sub>12–19</sub>PrA                 |
| pCOG048       | E. coli expression vector encoding Vti1pPrA                                 |
| pCOG054       | Yeast expression (2μ URA3) plasmid driving the overproduction of Scn2p      |
| pCOG038       | Yeast expression (2μ URA3) plasmid driving the overproduction of Tlg1pGST   |
| pCOG032       | Yeast expression (2μ URA3) plasmid driving the overproduction of Snc2p      |
| pCOG033       | As pCOG032, but with the cystosolic domain of Tlg2p lacking residues 2–36  |
| pCOG054       | Yeast expression (2μ URA3) plasmid driving the overproduction of Scn2p      |
| pHA-TLG2      | Yeast expression (2μ URA3) plasmid driving the overproduction of Tlg2p<sup>+</sup> |

All plasmids were constructed during the course of this study unless otherwise referenced. A detailed account of plasmid construction can be found in Materials and methods.

<sup>a</sup>Coe et al., 1999.

<sup>b</sup>Seron et al., 1998.

oligonucleotide B (5′-GTTAGCTACCTGATTACC-3′; flanking primer in the vector lying outside the SphI site); oligonucleotide C (5′-CATGACAGG-ACGATAATTACC-3′; VP545 primer approximately 200 bp upstream of the Bsu361 site); and oligonucleotide D (5′-TCGACCTGAGCTGGATATCCGAATACTGGAAGCAGCGTATTTCGATACGATCCATATCC-3′; HA tag + stop codon; same as in primer A [but complementary] plus last 10 codons of VPS45 ORF [without the stop codon]). The final 719-bp PCR product (obtained using oligonucleotides B and C as primers) was cloned as a Bsu361–SphI fragment into YEpVPS45. pCOG070 thus constructed encodes a Vps45p with the following COOH-terminal sequence: [YMDSSIRSKAS-ypydvpdyayp]-ypydvpdyayp.

Site-directed mutagenesis was used to construct pCOG071, pCOG072, and pCOG074 encoding versions of HA-Vps45p harboring L117R, W244R, and both L117R and W244R, respectively. pCOG065 was constructed by subcloning the ~3.7-kb BamHI–SphI fragment containing the VP545 ORF from pCOG072 into YEpplac181 (2μ LEU2; Gietz and Sugino, 1988). pNB706, pNB707, pNB708, and pNB709 (CEN versions of pCOG070, pCOG071, pCOG072, and pCOG073) were created by subcloning the ~3.7-kb BamHI–SphI fragment containing the VP545 ORF from pCOG070, pCOG071, pCOG072, and pCOG073 into YCplac111 (CEN URA3; Gietz and Sugino, 1988). pCOG054, a multicopy (URA3) plasmid driving the overproduction of an NH<sub>ε</sub>-terminally tagged version of Scn2p, was constructed by using PCR to amplify a 1.1-kb fragment beginning 300 bp downstream of the SNC2 ATG and extending ~300 bp downstream of the stop codon from yeast genomic DNA. The resulting PCR product was subcloned into XhoI–NcoI HindIII. Approximately 50,000 Ura<sup>+</sup> transformants were screened for a Vps<sup>+</sup> phenotype using a CYP colony blot assay (Rothman and Stevens, 1986). Plasmids were rescued from six colonies that were found to strongly secrete CPY, and three of these resulted in CPY secretion upon retransformation into wild-type cells. Sequence analysis revealed that each of these plasmids carried multiple
In vitro SNARE complex formation and isolation

E. coli transformations were induced for the coexpression of His6-Snc2p, the untagged cytosolic domain of Tlg1p (both from pCCGO38), the untagged cytosolic domain of Vh1p and Tlg2p-PrA (from pCCGO32), or Tlg2p-2xPrA (from pCCGO33) as in Protein expression and purification. IgG-Sepharose was added to cleared cell lysates as in Protein expression and purification. After binding for 1 h at 4°C and extensive washing with PBS, 25 U thrombin protease (Sigma-Aldrich) in 500 μl PBS was added to cleave the cytosolic domain of Tlg2p and bound material from the PrA tag during a 4-h incubation at 25°C. Ni2+-agarose was added to the supernatant containing the thrombin-cleaved complexes, and binding through His6-Snc2p was achieved at 4°C for 1 h followed by extensive washing with PBS containing 20 mM imidazole.

In vitro binding assays

The binding of Vps45p, mutants thereof, and Munc18c to fusion proteins and to assembled SNARE complexes was assessed by incubating the immobilized proteins with either a yeast cell lysate (prepared as previously described; Bryant and James, 2001) or purified His6-tagged Vps45p or Munc18c for 16 h at 4°C. After extensive washing with PBS, bound material was subjected to SDS-PAGE and Coomassie staining or immunoblot analysis.

Immunoprecipitation of Vps45p

HA-Vps45p and bound proteins were immunoprecipitated from yeast cell lysates as described previously (Bryant and James, 2003).

Pulse-chase radiolabeling and immunoprecipitation of CPY

The fate of newly synthesized CPY was followed by immunoprecipitation of the protein from intracellular and extracellular fractions of cells that had incorporated radiolabeled methionine into proteins synthesized during a 5-min time period as previously described (Piper et al., 1994). Subcellular fractionation

Cells were fractionated using differential centrifugation after osmotic lysis of spheroplasts as previously described (Bryant and James, 2001), with the modification that the two membrane fractions were combined into one. In brief, 10-ml cultures were grown to OD600 = 1 and were incubated in 1 ml of 50 mM Tris-HCl, pH 8.0, and 1% 2-mercaptoethanol for 10 min at 30°C. Cells were converted to spheroplasts using 150 μg/ml zymolyase 1 mg/ml sorbitol, 50 mM Tris-HCl, pH 7.5, and 1 mM magnesium chloride) at 30°C for 45 min. Spheroplasts were washed with 1.2 M sorbitol before osmotic lysis in 1.2 ml of cold lysis buffer [0.2 M sorbitol, 50 mM Tris-HCl, pH 7.5, and 1 mM EDTA]. Unbroken cells were removed by centrifugation for 5 min at 500 g (this and all subsequent centrifugation steps were performed at 4°C), yielding 1.2 ml of whole cell lysate. A 1.0 ml aliquot of whole cell lysate was subjected to centrifugation at 100,000 g for 30 min to yield membrane pellet and cytosolic/supernatant fractions. Membrane pellet was resuspended in 200 μl SDS sample buffer. Proteins were precipitated from the cytosolic/supernatant and 0.2 ml of whole cell lysate using 10% TCA and were resuspended in 200 and 40 μl SDS sample buffer, respectively.

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