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A t-SNARE of the endocytic pathway must be activated for fusion

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The t-SNARE in a late Golgi compartment (Tlg2p) syntaxin is required for endocytosis and localization of cycling proteins to the late Golgi compartment in yeast. We show here that Tlg2p assembles with two light chains, Tlg1p and Vti1p, to form a functional t-SNARE that mediates fusion, specifically with the v-SNAREs Snc1p and Snc2p. In vitro, this t-SNARE is inert, locked in a non-functional state, unless it is activated for fusion. Activation can be mediated by a peptide derived from the v-SNARE, which likely bypasses additional regulatory proteins in the cell. Locking t-SNAREs creates the potential for spatial and temporal regulation of fusion by signaling processes that unleash their fusion capacity.

Introduction

Eukaryotic cells use endocytosis to recycle or degrade plasma membrane proteins and lipids, internalize nutrients, and control the transport and receptor activities in the plasma membrane (for review see Mellman, 1996). The early endosome, the first compartment in this pathway, receives proteins from both the plasma membrane and the Golgi compartment. From the early endosomes, proteins can be transported to the Golgi compartment, returned to the plasma membrane, or dispatched to late endosomes (termed prevacuolar compartments in yeast) for subsequent degradation in lysosomes (vacuoles in yeast) (for review see Geli and Riezman, 1998). A complex matrix of trafficking pathways links these compartments (Pelham, 1999).

Members of the SNARE family of proteins (Söllner et al., 1993) are required for most fusion events in vivo. These proteins are ubiquitously expressed in eukaryotic species and distinct members are localized on the surface of the various intracellular organelles (Bock et al., 2001). Most of the SNAREs are integral membrane proteins and all possess a cytoplasmic heptad-repeat region ("SNARE motif") that is likely able to assemble and form a parallel rod–like four-helix bundle (Canaves and Montal, 1998; Poirier et al., 1998; Sutton et al., 1998). Three of these four helices are contributed by the t-SNARE, generally a preassembled complex consisting of one heavy chain (a syntaxin family member) and two distinct light chains that mark the target membrane for vesicle fusion. The fourth helix is derived from a v-SNARE localized on the other membrane partner (Fukuda et al., 2000; Parlati et al., 2000). As the bundle assembles between two membranes to form a SNAREpin, the membranes are forced into close apposition and fusion results (Weber et al., 1998; Nickel et al., 1999; Parlati et al., 1999). Specificity in membrane fusion results from the precise pairing of cognate v-SNARE and t-SNAREs (McNew et al., 2000a).

Of the seven syntaxins in the yeast genome, three are directly involved in endocytosis (for review see Pelham, 1999). Pep12p is concentrated in the prevacuolar compartment and is required for fusion at this compartment (Becherer et al., 1996; Gerrard et al., 2000). Vam3p is the heavy chain of the t-SNARE that marks vacuoles for homotypic fusion (Wada et al., 1997); its light chains are Vam7p and Vti1p. This t-SNARE enables fusion by pairing with its cognate v-SNARE, Nyv1p (Nichols et al., 1997, Fukuda et al., 2000). The precise roles played in trafficking by the third syntaxin, Tlg2p, are less clear. It is localized to both early endosomes and late Golgi compartment (Abeliovich et al., 1998; Holthuis et al., 1998a). It is not required for the secretory pathway, but it is needed for efficient endocytosis and for retrieval of late Golgi complex–resident proteins lost to the endocytic pathway (Abeliovich et al., 1998; Holthuis et al., 1998a; Seron et al., 1998; Lewis et al., 2000).

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Here, we investigate the fusion potential of the endocytic syntaxin, Tlg2p. Coimmunoprecipitation revealed that this syntaxin is associated with several other SNARE proteins involved in endocytosis in binary and ternary complexes containing various combinations of Tlg1p, Vti1p, and Snc2p (Abeliovich et al., 1998; Holthuis et al., 1998a; Coe et al., 1999). However, it is not known whether these four proteins constitute a single stable quaternary complex, or whether multiple complexes may exist, including other as yet unidentified SNAREs.

Vti1p is a light chain of the vacuolar t-SNARE (Fukuda et al., 2000), but it is also found in endosomes and Golgi (Fisher von Mollard et al., 1997; Lupashin et al., 1997). Snc2p, the v-SNARE used in Golgi complex to plasma membrane fusion (Brennwald et al., 1994; Gerst, 1997; McNew et al., 2000a) is also required for endocytosis (Gurunathan et al., 2000). Tlg1p was first identified as an endosomal protein (Holthuis et al., 1998a). Although it was originally classified as a syntaxin, its homology is actually closer to that of a light chain or a v-SNARE (Weimbs et al., 1997). Thus, all of these SNARE proteins are known to be involved in endocytosis, and many participate in other trafficking steps as well.

Results

Tlg2p, Tlg1p, Vti1p, and Snc2p form a functional SNARE complex

We began by testing the simplest possibility, that Tlg2p, Tlg1p, Vti1p, and Snc2p form a single quaternary SNARE complex in which Tlg2p is the heavy chain, Tlg1p and Vti1p are the light chains of the t-SNARE and Snc2p is the cognate v-SNARE. To test this, we expressed recombinant Tlg2p, Tlg1p, Vti1p and Snc2p as his_6- or glutathione *S*-transferase (GST)*-tagged proteins in *Escherichia coli*. Tlg2p expression was improved by the truncation of the luminal domain that was previously demonstrated to be nonessential for its function (Abeliovich et al., 1998) and by a small additional NH_2-terminal truncation (amino acids 1–35). Different combinations of these purified SNARE proteins were tested in binding studies to identify the binary and ternary SNARE complexes as well as the quaternary SNARE complex. All binding experiments were performed in high salt (400 mM KCl) using transmembrane proteins in the presence of detergent.

As shown in Fig. 1, the proposed light chains Vti1p and Tlg1p interact (lane 3), but Vti1p is not sufficient to pull down either the syntaxin Tlg2p or the v-SNARE Snc2p (lanes 2 and 4, respectively). In fact, binding of either SNARE also requires Tlg1p (lanes 5 and 6, respectively). No interaction is observed among Tlg2p, Vti1p and Snc2p (lane 7). Finally, all four proteins bind together, suggesting that these four proteins likely represent a complete SNARE complex (lane 8). These interactions are specific as no binding of any SNARE to GST beads was observed (unpublished data). Two ternary complexes can be formed among the set of four

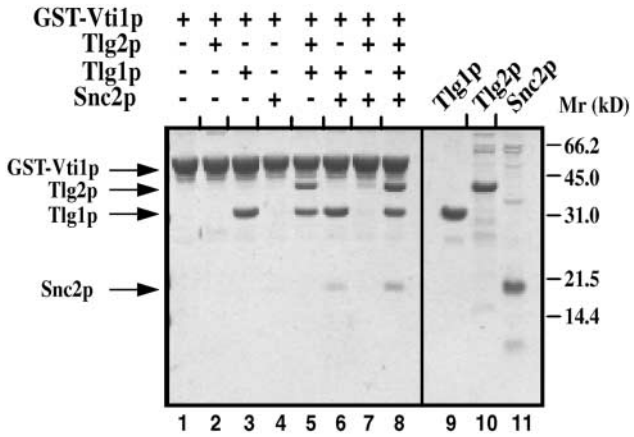

Figure 1. **Tlg2p, Tlg1p, Vti1p and Snc2p form a quaternary complex.** GST-Vti1p was immobilized on glutathione-agarose and incubated with his_6-Tlg2p, his_6-Tlg1p, and his_6-Snc2p as indicated. Bound proteins were then analyzed by SDS-PAGE and Coomassie staining (lanes 1–8). For comparison, purified his_6-Tlg1p, his_6-Tlg2p, and his_6-Snc2p were loaded on lanes 9, 10, and 11, respectively. Note that the weak band for Snc2p is due to its poor Coomassie staining (McNew et al., 2000a).

SNAREs, (a) Tlg2p/Tlg1p/Vti1p and (b) Tlg1p/Vti1p/Snc2p. Tlg2p is a syntaxin homologue and by definition should be a constituent of the t-SNARE, whereas Snc2p is a v-SNARE and should be on the vesicle membrane (Fukuda et al., 2000). Tlg2p/Tlg1p,Vti1p therefore likely represents the physiological t-SNARE complex.

This deduction was confirmed by testing the function of this complex in the liposome fusion assay (Weber et al., 1998). We reconstituted the proposed t-SNARE (Tlg2p/Tlg1p,Vti1p) into "acceptor" liposomes and the proposed v-SNARE Snc2p into fluorescent "donor" liposomes (Fig. 2 A). Donor liposomes contain NBD-PE and rhodamine-PE derivatives at trace levels. The fluorescence of NBD is quenched by the close proximity of rhodamine in the same bilayer. Fusion with the bilayer of nonfluorescent acceptor liposomes separates the two fluorophores, resulting in an increase of NBD fluorescence, which can be converted according to a calibration curve to measure the efficiency of fusion (Parlati et al., 1999).

When the acceptor t-SNARE liposomes (containing Tlg2p, Tlg1p, and Vti1p) were simply incubated with the donor v-SNARE liposomes (containing Snc2p), no fusion was observed (Fig. 2 C). One possible explanation for this surprising result could be that this t-SNARE is intrinsically unreactive, and relies on additional proteins in the cell to activate it for fusion. Indeed, some t-SNAREs are known to be subject to regulation in vivo (for review see Gonzalez and Scheller, 1999). In particular, the mammalian plasma membrane syntaxin 1 is kept in a closed conformation when its NH_2-terminal regulatory domain folds back on its coil region, stabilized by the regulatory protein n-Sec1 (Misura et al., 2000; Yang et al., 2000). The exocytic t-SNARE syntaxin 1/SNAP-25 fuses slowly because it pairs slowly with the cognate v-SNARE vesicle-associated membrane protein (VAMP)2. SNAREpin assembly is accelerated when the NH_2-terminal domain is removed, increasing the rate of fusion (Parlati et al., 1999). This exocytic t-SNARE can also be activated by binding a peptide

*Abbreviations used in this paper: GST, glutathione *S*-transferase; Tlg, t-SNARE in a late Golgi compartment; VAMP, vesicle-associated membrane protein.

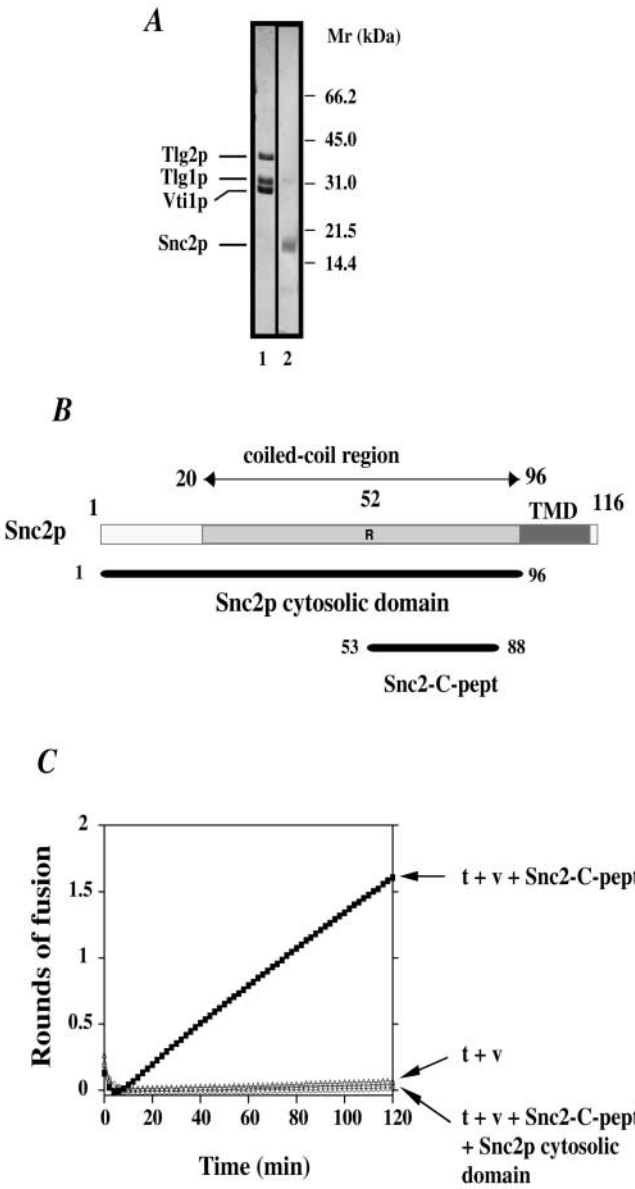

Figure 2. **Tlg2p, Tlg1p, Vti1p, and Snc2p form a functional fusogenic complex.** (A) Reconstitution of acceptor and donor liposomes. 10% (4.5 μl) of acceptor liposomes (lane 1) and 100% (5 μl) of donor liposomes (lane 2) used in a typical fusion reaction were analyzed by SDS-PAGE and Coomassie blue staining. (B) Snc2-C peptide corresponds to amino acids 53–88 of the Snc2p v-SNARE. The cytosolic domain of Snc2p corresponds to amino-acids 1 to 96 of the Snc2p v-SNARE (TMD: trans-membrane domain). (C) Membrane fusion. Donor liposomes and acceptor liposomes were mixed (5:45 μl) in a microtitre plate with either 10 μl buffer or 5 μl snc2-C-pept (3.5 nmol) plus 5 μl buffer, or with 5 μl snc2-C-pept (3.5 nmol) plus 5 μl snc2p cytosolic domain (6 nmol) as indicated. The plate was transferred to a 37°C fluorescent plate reader and NBD fluorescence was monitored. The results were converted to rounds of fusion as described (Parlati et al., 1999).

corresponding to the membrane-proximal COOH-terminal half of its cognate v-SNARE, VAMP2. The v-SNARE then displaces the peptide and fusion occurs. Peptide binding changes the conformation of the t-SNARE, switching the cognate membrane–proximal region of the COOH terminus of SNAP-25 from a protease-sensitive to a protease-resistant and presumably helical state (unpublished data).

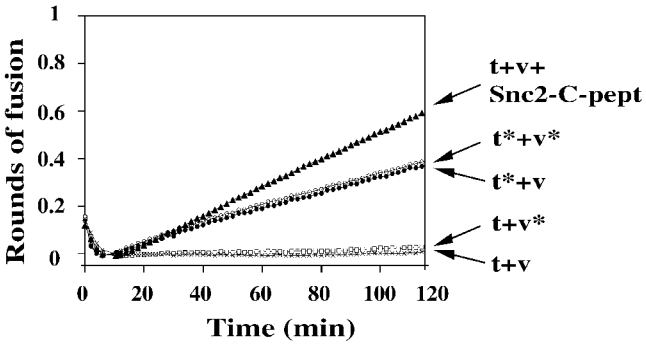

Figure 3. **The Snc2-C peptide binds and activates the t-SNARE complex.** Acceptor and donor liposomes were incubated with Snc2-C peptide (called t* and v*, respectively) or buffer (called t and v, respectively) overnight at 4°C and then refloated to remove the free peptide. Fusion reactions were performed with these liposomes: 45 μl of t* plus 5 μl of v* (t*+v*); 45 μl of t* plus 5 μl of v (t*+v); 45 μl of t plus 5 μl of v* (t+v*), 45 μl of t plus 5 μl of v (t+v); or 45 μl of t plus 5 μl of v plus 3.5 nmol of Snc2-C-pept (t+v+Snc2-C-pept). The plate was then transferred to a 37°C fluorescent plate reader and NBD fluorescence was monitored and converted to rounds of fusion. Note that the maximum signal is lower than usual (compare Fig. 2 C) likely due to some snc2-C-pept dissociation from t* during the refloatation.

Therefore, we tested whether the analogous peptide from Snc2p could similarly activate the proposed endosomal t-SNARE, Tlg2p/Tlg1p,Vti1p. When this peptide (Fig. 2 B; named snc2-C-pept) is added, the donor and acceptor liposomes now fuse efficiently, reaching 1.6 rounds of fusion in 2 h (Fig. 2 C). As expected, the addition of the entire Snc2p-cytosolic domain (outlined in Fig. 2 B) completely inhibits this fusion. This implies that peptide bound to t-SNAREs is displaced by v-SNAREs, and confirms that free t-SNAREs on the acceptor liposomes must be available to interact with v-SNAREs of donor liposomes for fusion to occur. This also shows that snc2-C peptide is not intrinsically fusogenic. Under no conditions did the v-SNARE or t-SNARE liposomes fuse with protein-free liposomes (unpublished data). In addition, the snc2-C-pept is not able to activate another functional complex, the Golgi compartment t-SNARE Sed5p/Sec22p, Bos1p (unpublished data).

These results show that Snc2p and Tlg2p/Tlg1p,Vti1p can form a functional SNAREpin. A formal possibility is that one or more of the SNAREs expressed in and purified from *E. coli* may not be correctly folded. However, when Tlg2p, Tlg1p, and Vti1p are coexpressed in bacteria, fusion still requires snc2-C peptide (unpublished data). Furthermore, Vti1p and Snc2p are functional in other contexts (Fukuda et al., 2000; McNew et al., 2000a) and are evidently capable of forming the ternary t-SNARE and the quaternary v/t-SNARE complex in solution (Fig. 1).

The snc2-C peptide directly activates the t-SNARE complex

Since the activating peptide corresponds to the COOH-terminal part of the v-SNARE core, it is expected to act by binding to the corresponding portion of the t-SNARE. If this were the case, preincubation with peptide should preactivate the t-SNARE liposomes but not the v-SNARE liposomes, and the t-SNARE liposomes should remain

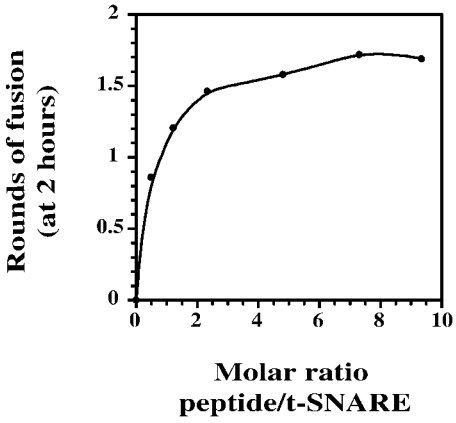

Figure 4. **The effect of the Snc2-C peptide is concentration dependent and saturable.** Donor (5 μl) and acceptor liposomes (45 μl) were mixed in a microtitre plate in the presence of increasing amounts of peptide (from 0 to 2.6 nmol). The plate was then transferred to a 37°C fluorescent plate reader and NBD fluorescence was monitored and converted to rounds of fusion. Peptide/t-SNARE molar ratio was calculated and plotted as well as the fusion rate obtained after 2 h.

activated subsequently, even in the absence of further peptide. To test this, t-SNARE or v-SNARE liposomes were separately incubated either with Snc2-C peptide (termed t* and v* liposomes, respectively), or with buffer as control. Then, the donor or acceptor liposomes were reisolated by flotation to remove free peptide and tested in the fusion assay. Indeed, the preincubated t* liposomes are activated and remain so after reisolation (Fig. 3) but v* liposomes are not fusion competent (assayed with t-liposomes). This result directly established that the peptide targets the t-SNARE and not the v-SNARE. The slightly reduced fusion efficiency of t* liposomes compared with standard fusion reactions containing excess free Snc2-C peptide (Fig. 3; t+v+snc2-C-pept) is likely due to some dissociation of the snc2-C-pept from the t* liposomes during their reisolation.

As expected for a stoichiometric binding reaction, the activation of fusion is saturable with respect to peptide concentration, and approximately one peptide per t-SNARE is needed for maximal activation of fusion (Fig. 4).

Topological restriction of fusion based on an endosomal SNARE complex

All three members of this endosomal t-SNARE contain transmembrane domains. To test whether in this case, as in others (Parlati et al., 2000), there is topological restriction of fusion, we tested all possible combinations in which any three SNAREs are reconstituted in acceptor vesicles while the fourth, remaining SNARE, is reconstituted in the donor population. Additionally, we prepared all combinations in which Tlg2p and any one other SNARE reside in the acceptor liposomes with the other two in the donor liposomes (Fig. 5). Fusion was tested both in the presence and in the absence of the snc2-C peptide.

Fusion was only observed when Tlg2p, Tlg1p, and Vti1p were reconstituted in the same bilayer with Snc2p in the opposing bilayer, and further only when the C-peptide was added (Fig. 5). This confirms the uniqueness of Tlg2p/Tlg1p,Vti1p as the functional t-SNARE and specifically assigns to Snc2p the role of v-SNARE in this quaternary complex. This was of special interest because the binding assay revealed that two ternary complexes can be formed in detergent micellar solution (Vti1p/Tlg1p/Tlg2p and Vti1p/Tlg1p/Snc2p; see Fig. 1), but topological restriction shows that the latter complex cannot function as a t-SNARE with Tlg2p as a v-SNARE.

Specificity of fusion with the t-SNARE Tlg2p/Tlg1p,Vti1p for the cognate v-SNARE

The fusion activity of all of the potential v-SNAREs encoded in the yeast genome was tested by independently re-

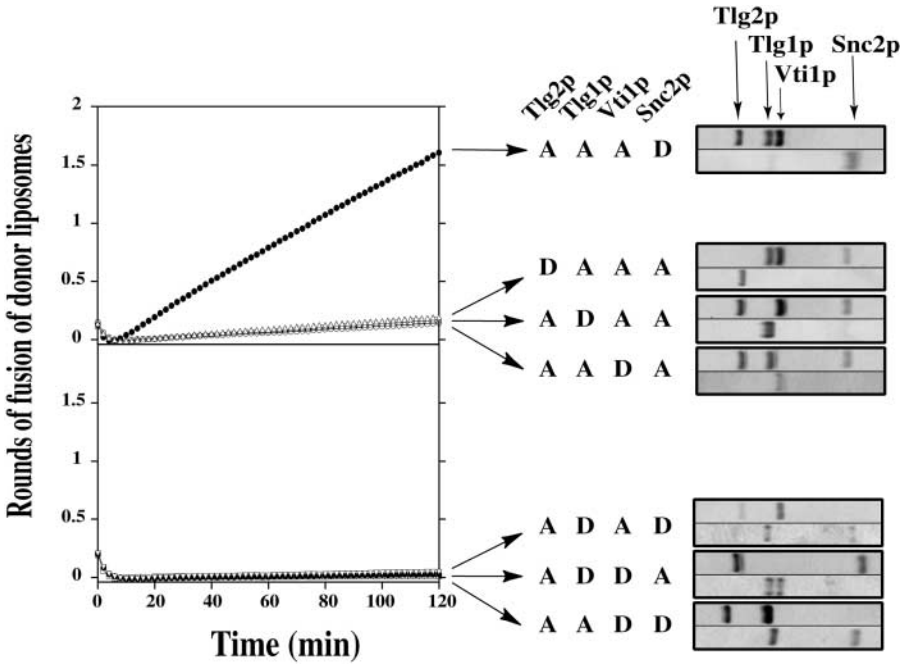

Figure 5. **Topological restriction of the endocytic SNARE complex.** Different combinations of proteins were reconstituted in acceptor liposomes (A) and in donor liposomes (D) as indicated. Reconstitution efficiency was analyzed by SDS-PAGE followed by Coomassie staining (right, −10% of acceptor liposomes and 100% of donor liposomes). Three to one combinations are represented on the top, whereas two to two combinations are on the bottom. The acceptor and donor liposomes were mixed (45:5 μl) as indicated in the presence of snc2-C-pept (3.5 nmol) and the increase of fluorescence was monitored over 120 min. Fluorescence values were converted to rounds of fusion as described (Parlati et al., 1999).

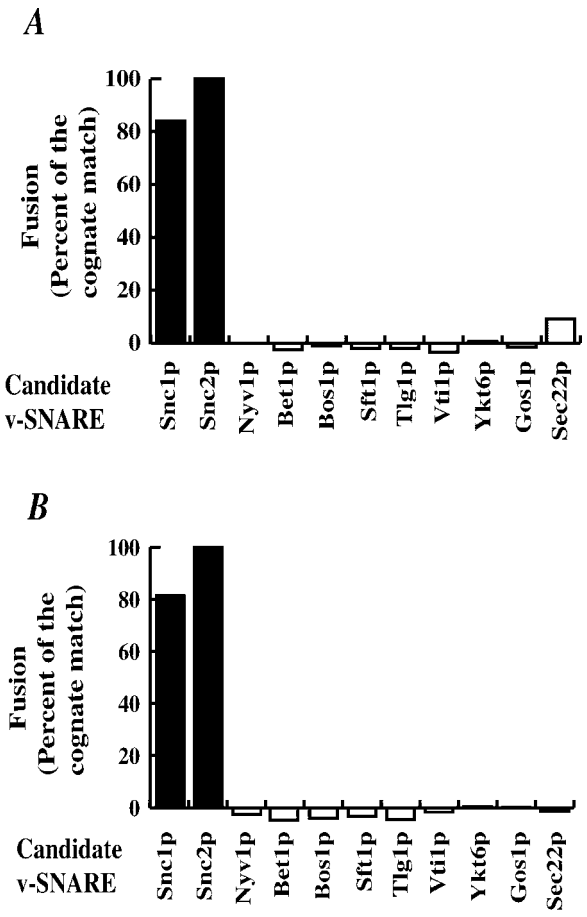

Figure 6. **Specificity of fusion between the endocytic t-SNARE and all potential v-SNAREs.** (A) Fusion between reconstituted t-SNARE liposomes and reconstituted potential v-SNARE liposomes in the presence of Snc2-C peptide. Snc2-C-pept (3.5 nmol per reaction) was added to all reactions. (B) Fusion between reconstituted t-SNARE liposomes and reconstituted potential v-SNARE liposomes in the presence of each corresponding peptide. Peptides corresponding to the potential v-SNARE (Table I; 3.5 nmol per reaction) were added to all reactions. The extent of fusion at 120 min was normalized to the amount of cognate v-SNARE fusion (Snc2p; black bars). Fusion with protein free liposomes (0.0434 rounds) was subtracted from all the fusion results. The maximal extent of fusion (100%) with Snc2p donor liposomes and snc2-C-pept was 0.977 rounds (A), and 0.934 rounds (B). Ykt6p was anchored to the membrane by the attachment of a synthetic geranylgeranyl lipid anchor as described (McNew et al., 2000b).

constituting each SNARE into donor liposomes and incubating them with Tlg2p/Tlg1p,Vti1p t-SNARE acceptor liposomes. Fusion was tested both in the presence or absence of the snc2-C peptide. No fusion activity was observed without the snc2-C peptide with any potential v-SNARE (unpublished data). In the presence of snc2-C peptide, significant fusion was seen with both Snc1p and Snc2p as v-SNAREs, but not with any other potential v-SNARE (Fig. 6 A). Snc2p is very similar to Snc1p and they are largely interchangeable functionally, since only when both genes are deleted is viability compromised (Protopopov et al., 1993). Each potential v-SNARE was also tested for fusion in the presence of a C-peptide based upon its own sequence (Table I). Even so, only the Snc2p and Snc1p liposomes were able to fuse with Tlg2p/Tlg1p,Vti1p acceptor liposomes (Fig. 6 B).

Discussion

It has been shown previously that functional yeast t-SNAREs marking the Golgi compartment, the plasma membrane, and the vacuole are each composed of a distinct heavy chain from the syntaxin family and, depending on the particular membrane, one or two nonsyntaxin light chains (Fukuda et al., 2000; McNew et al., 2000a; Parlati et al., 2000). The architecture of the endosomal t-SNARE further establishes the generality of this concept with Tlg2p as the heavy chain and Tlg1p and Vti1p as its two light chains, respectively, functioning exclusively with Snc2p as its v-SNARE. Moreover, only one topological arrangement of these four proteins between two membranes results in a fusogenic complex, establishing their roles as t-SNARE and v-SNARE, and extending the concept of topological restriction (Parlati et al., 2000).

In yeast, the 21 SNARE proteins have been grouped into four different categories defined by the sequence homology in the SNARE motif: the syntaxins (Ufe1p, Sso1p, Sso2p, Sed5p, Pep12p, Tlg2p, and Vam3p), the Bet1p group (Sec9p-C, Spo20p-C, Vam7p, Bet1p, Sft1p, and Tlg1p), the Bos1p group (Sec9p-N, Spo20p-N, Vti1p, Bos1p, Gos1p, and Sec20p), and a fourth group termed R-SNAREs (Snc1p, Snc2p, Nyv1p, Sec22p, and Ykt6p) (Pelham, 2001; note Sec9p and Spo20p, like their animal homologue SNAP-25, have two SNARE motifs, C and N). All results to date indicate that fusogenic SNARE pins must contain one subunit from each group: t-Sso1p/Sec9p and v-Snc1p or v-Snc2p at the plasma membrane (McNew et al., 2000a); t-Sed5p/Sec22p,Bos1p and v-Bet1p at the Golgi compartment (Parlati et al., 2000); and t-Vam3p/Vam7p,Vti1p and v-Nyv1p at the vacuole (Fukuda et al., 2000). The endosomal t-SNARE also fits this rule, suggesting that it has a concrete structural basis which can be used to predict additional fusogenic SNARE complexes. However, the unique v-SNARE within a particular complex (i.e., based on topological restriction) can be drawn either from the R-SNARE group (Snc1p or Snc2p, Nyv1p) or from the Bet1p group (Bet1p, Sft1p) and potentially (given the limited number of results to date) from the Bos1p group.

Certainly, Tlg2p, Tlg1p, and Snc2p function in endocytosis, but there is also some evidence showing that they are involved in the retrieval of proteins to the TGN from the cell surface or endosomes (Abeliovich et al., 1998; Holthuis et al., 1998b; Seron et al., 1998; Gurunathan et al., 2000; Lewis et al., 2000). These functions are of course related in that endosomes play an important role in maintaining the steady-state distribution of late Golgi membrane proteins (Conibear and Stevens, 1998). Therefore, it is possible that the fusogenic SNARE complex we have identified here, t-Tlg2p/Tlg1p,Vti1p and v-Snc1p or Snc2p, is involved in more than just one trafficking step. Indeed, this complex is also required in TGN homotypic fusion (Brickner et al., 2001, this issue).

Our results imply that the fusion activity of this endomal t-SNARE is intrinsically switched "off" due to auto-inhibi-

Table I. **C-peptide alignment**

Name	Sequence	Identity
		%
Snc2-C-pept	^{53}GERLTSIEDKA DNLAISAQGFKRGAN RVRKQMWWKD^{88}	100.0
Snc1-C-pept	^{54}GERLTSIEDKA DNLAVSAQGFKRGAN RVRKAMWYKD^{89}	91.6
Nyv1-C-pept	^{191}QERVSLLVDKT SQLNSSSNKFRRKAV NIKEIMWWQK^{226}	30.5
Ykt6-C-pept	^{166}GEKLDNLVDKS ESLTASSKMFYKQAK KSNSCC^{197}	28.1
Vam7-C-pept	^{285}NELLTALEDDV DNTGRRLQIANKKAR HFNNSA^{316}	28.1
Tlg1-C-pept	^{167}GQLLDNMDEGM DGVVNKLARGRRQLE WVYEKNKEKY^{202}	16.6
Sec22-C-pept	^{157}GDSLDKMSDMS SSLKETSKRYRKSAQ KINFDLLISQ^{192}	13.8
Bos1-C-pept	^{187}NKILSKVQDRM SNGLRTLGVSEQTIT SINKRVFK^{220}	11.7
Gos1-C-pept	^{170}SNVLNTANNKV LQTLQRIPGVNQLIM KINTRR^{201}	9.3
Sft1-C-pept	^{42}SSVINQMTDSL GSMFTDIKNSSSRLT RSLKAGNSIW^{77}	8.3
Vti1-C-pept	^{159}RETLENARQTL FQADSYVDKSIKTLK TMTRRLVA1^{92}	5.8
Bet1-C-pept	^{87}NQTIDQLGDTF HNTSVKLKRTFGNMM EMARRSGIS^{121}	5.7

The sequence of each peptide corresponds to the COOH-terminal half of the core domain. In bold are the amino acids that are identical to snc2-C-pept. The percentage of identity with snc2-C-pept is indicated on the right.

tion. Its fusion activity can be unleashed by binding a peptide corresponding to the COOH-terminal part of the cognate v-SNARE. The capacity to switch the endosomal t-SNARE "on" is specific for peptide derived from its cognate v-SNARE, and when activated the endosomal t-SNARE will only fuse with its cognate v-SNARE Snc1/2p and no other potential v-SNARE encoded in the genome of yeast. Most likely peptide binding conformationally switches the endosomal t-SNARE from "off" to "on" states when it binds, as it does for the neuronal exocytic t-SNARE (unpublished data).

The intrinsic inactivity of the endosomal t-SNARE is a significant finding because it implies that cells must possess mechanisms to activate it for fusion. Presumably, peptide binding throws the switch by tapping into a mechanism that is physiologically reserved for certain regulatory proteins. Indeed, a very recent study showed that Tlg2p is locked in an inactive state, unable to bind its light chains Tlg1p and Vti1p, unless Vps45p is present (Bryant and James, 2001). Interestingly, none of the other t-SNAREs tested to date show a strict requirement for peptides to be functional in the in vitro fusion assay (Fukuda et al., 2000; McNew et al., 2000a; Parlati et al., 2000), suggesting that the endosomal t-SNARE might be auto-inhibited to a greater extent.

Of course, regulatory proteins in the cell could tip the balance further toward (or against) the "off" state. Snc1/2p is the sole example to date of a multifunctional v-SNARE. Snc2p is required in the endocytic pathway (in association with t-Tlg2p/Tlg1p,Vti1p) as well as for fusion of secretory vesicles with the plasma membrane (in association with t-Sso1p/Sec9p [McNew et al., 2000a]). Thus, a single v-SNARE suffices for fusion with the plasma membrane and with the two compartments with which the cell surface interfaces for endocytosis and secretion, early endosomes and late Golgi compartment. This neatly solves the problem of how the v-SNAREs are recycled among these compartments. If the only source of specificity for vesicle targeting in these pathways were SNARE pairings, this would imply that the pattern of transport among these compartments could be relatively random. Interestingly, this pattern is extremely complex (Pelham, 1999) and since, in contrast to the genes and organelles in the secretory pathway which are essential, the entire pathway is not essential in yeast (Holthuis et al., 1998b), it is not inconceivable that even a random pattern in these pathways might suffice and would certainly cause no harm. Therefore, it is unclear whether the large number of transport links among endocytic compartments (Pelham, 1999) is due to an equal number of uniquely specific fusion steps, or whether movement could be more random than that. The latter would require a smaller genetic load, but would also result in less overall efficiency in endocytosis.

If the transport pattern were precise, how could the cell direct Snc2p-containing v-SNARE vesicles to one versus another of its potential target membranes? The tight autoregulation of the endosomal t-SNARE, so that its fusion activity is intrinsically locked-up, would be important in this connection. If there is a lock then there is presumably a key, and a simple possibility is that Snc2p-containing vesicles in the cell have additional proteins encoding their origin that act as keys to preferentially unlock one or another different t-SNARE at one or another different target membrane, adding a further level of specificity. Such a "key–lock" system could certainly include such Sec1 family proteins as Vps45p, but also rab GTPase switch proteins or cognate tether proteins (Mellman and Warren, 2000; Zerial and McBride, 2001). Each of the two different t-SNAREs so far known to be fusogenic with v-Snc2p (or Snc1p) has a distinct Sec1 family member: t-Sso1p/Sec9p functions with Sec1p (Carr et al., 1999) and t-Tlg2p/Tlg1p,Vti1p with Vps45p (Nichols et al., 1998). Additional elements, such as cytoskeletal structures, may also play a less direct role in directing Snc2p-containing v-SNARE vesicles to different target membranes. Further studies are needed to establish the extent to which the plasma membrane–endosomal compartments–TGN network operates according to stochastic or deterministic principles.

Materials and methods

Peptides

Peptides presented on Table I were synthesized by the Microchemistry Core Facility of Memorial Sloan Kettering Cancer Institute. All peptides were dissolved in 10 mM HCl and then diluted in reconstitution buffer (25 mM Hepes-KOH, pH 7.4, 100 mM KCl, 10% glycerol) to a concentration of ~3 mg/ml.

Plasmid constructs

The coding sequence of truncated Tlg2p (amino acids 36–335) was amplified by PCR from *Saccharomyces cerevisiae* genomic DNA (Novagen) with

primers FO34 (GGGCATATCATATGTTTAGAGATAGAACTAAT) and FO11 (CGGGATCCTCATTTCAACATAACAAAGAA). The PCR product was digested with NdeI and BamH1 and ligated either in pET28a vector (Novagen) resulting in FD7, or in pGEX-2T (Amersham Pharmacia Biotech) resulting in FD13. The coding sequence of full-length Tlg1p was amplified with primers FO7 (GGGAATTCCATATGAACAACAGTGAAGATCCG) and FO8 (CGCGGATCCTCAAGCAATGAATGCCAAAAC), digested by NdeI/BamH1 and ligated either in pET28a or in pGEX-2T resulting in FD1 and FD10, respectively. The coding sequences of Vti1p, Ykt6p, and Nyv1p were obtained as described (Fukuda et al., 2000). The coding sequences of Snc1p, Snc2p, and the cytosolic domain of Snc2p were obtained as described (McNew et al., 2000a). The coding sequences of Bos1p, Gos1p, Sft1p, Bet1p, and Sec22p were obtained as described (Parlati et al., 2000). All plasmids are propagated in DH5α strain (GIBCO BRL).

Protein expression and purification

Plasmids used for protein expression were transformed into the *E. coli* strain BL21 (DE3) (Invitrogen). Transformed cells were grown at 37°C to an absorbance at 600 nm of 0.7. Protein expression was induced with 1 mM IPTG (Boehringer) for 4 h at 37°C for his_6-Tlg2p and his_6-Tlg1p or with 0.2 mM IPTG for 2 h at 37°C for GST-Tlg2p. Then the cells were collected by centrifugation and lysed by several passages through an Avestin cell disrupter at >10,000 psi in buffer A (25 mM Hepes-KOH, pH 7.4, 400 mM KCl, 10% glycerol, 4% Triton X-100, 5 mM β-mercaptoethanol, 1 mM PMSF). Lysates were clarified by centrifugation at 35,000 rpm (Ti45; Beckman Coulter) for 45 min. Lysates containing his-tagged proteins were bound to Ni-NTA agarose and then washed with buffer B (25 mM Hepes-KOH, pH 7.4, 400 mM KCl, 10% glycerol, 1% n-Octyl-β-D-glucopyranoside, 5 mM β-mercaptoethanol, 1 mM PMSF) containing 50 mM imidazole. Proteins were eluted with a 50 mM to 1 M imidazole gradient (in buffer B). Lysates containing GST-tagged protein were bound to glutathione agarose beads and then washed with buffer B. Tlg2p was cleaved from GST with 0.05 U/μl thrombin in buffer B. We produced Snc2p-his_6, Snc1p-his_6, and GST-Snc2p cytosolic domain as described (McNew et al., 2000a). GST-Gos1p, GST-Ykt6p, and GST-Sft1p were produced as described (McNew et al., 1998), except that GST-Sft1p was reconstituted as a GST fusion protein and thrombin cleaved on liposomes. We produced his_6-Vti1p, GST-Vti1p, and GST-Nyv1p as described (Fukuda et al., 2000). his_6-Bos1p, his_6-Sec22p, and his_6-Bet1p were also produced as described (Parlati et al., 2000). To isolate the assembled t-SNARE complex, we cotransformed the *E. coli* strain BL21 (DE3) with plasmids expressing GST-Tlg2p, his_6-Tlg1p, and Vti1p. After induction, we first purified the complex via the his-tag (purification of his_6-Tlg1p, his_6-Tlg1p/Vtip, and his_6-Tlg1p/Vtip/GST-Tlg2p). Then we repurified this product via the GST tag (purification of his_6-Tlg1p/Vtip/GST-Tlg2p), thereby isolating only the ternary complex.

Protein binding assay

For preparation of GST-Vti1p or GST affinity matrices, we proceeded as described (Fukuda et al., 2000). Briefly, lysates prepared from cells containing pGEX-Vti1 or pGEX-4T3 were incubated at 4°C for 2 h with glutathione agarose equilibrated in binding buffer C (25 mM Hepes-KOH, pH 7.4, 400 mM KCl, 10% glycerol, 1 mM DTT, and 1% Triton X-100). After several washes, beads (0.2 μmol of protein) were incubated with a 15-fold excess of SNARE proteins in the presence of bovine serum albumin (500 μg/ml) at 4°C overnight. The beads were then washed three times with buffer C and 30 μl of binding buffer D (25 mM Hepes-KOH, pH 7.4, 100 mM KCl, 10% glycerol, 1 mM DTT, and 1% Triton X-100) was added. One eighth of each sample was mixed with Novex SDS-PAGE buffer, boiled for 5 min and resolved by SDS-PAGE.

Reconstitution

For acceptor liposomes containing t-SNARE, ~13 nmol of Tlg2p, 13 nmol of Tlg1p, and 13 nmol of Vti1p were preincubated 20 h at 4°C. 500 μl of the reaction were used for the reconstitution. For donor liposomes containing v-SNARE, 7.5 nmol of proteins in 100 μl were used. SNAREs were reconstituted as described, except that all the buffers used for reconstitution contained 400 mM KCl (Weber et al., 1998). The lipid components in the acceptor liposomes were 85% palmitoyl-oleoyl-phosphatidylcholine (POPC) and 15% 1,2-dioleoyl-phosphatidylserine (DOPS), corresponding to 15 mM total lipids in $CHCl_3$. The donor liposomes contained 82% POPC, 15% DOPS, 1.5% 7-nitrobenz-2-oxa-1,3-diazole-dipalmitoyl phosphatidyl ethanolamine (NBD-DPPE), 1.5% Rhodamine-DPPE, corresponding to 3 mM total lipids in $CHCl_3$. All lipids were obtained from Avanti Polar Lipids, Inc. The typical lipid recovery efficiency in the recovered Nycodenz fraction was ~50% for acceptor liposomes containing Tlg2p, Tlg1p, Vti1p, and ~30% for donor liposomes containing Snc2p.

Peptide binding assay

Donor and acceptor liposomes were preincubated for 16 h at 4°C in the presence of peptide (10 times in excess). Then these liposomes were refloated and harvested as described previously (Weber et al., 1998). Bound peptide was then analyzed by fusion assay.

Fusion assay

The lipids mixing assay was conducted as described (Weber et al., 1998; Parlati et al., 1999). Briefly, 45 μl of acceptor liposomes were mixed with 5 μl of donor liposomes in a 96-well FluoroNunc microtitre plate (Nunc). For some experiments, 3.5 nmol of peptide, or buffer, or 6 nmol of cytosolic domain of Snc2p were added as indicated in figure legends. Microtitre plates were then placed in a Fluoroscan II Platereader (Labsystems) equilibrated at 37°C and NBD fluorescence was measured over 2 h at 2-min intervals (excitation 460 nm, emission 538 nm). After 2 h, 10 μl of 2.5% wt/vol n-dodecyl-maltoside (Boehringer) was added to dissolve the lipids and measure the maximum NBD fluorescence. The data were converted to rounds of fusion as described (Parlati et al., 1999). Note that the small decrease observed during the first 10 min of each fusion reaction is due to the temperature equilibration of the plate reader.

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