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A Rab Protein Is Required for the Assembly of SNARE Complexes in the Docking of Transport Vesicles

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Summary

Rab proteins are generally required for transport vesicle docking. We have exploited yeast secretion mutants to demonstrate that a rab protein is required for v-SNAREs and t-SNAREs to assemble. The absence of the rab protein in the docking complex suggests that, in a broad sense, rab proteins participate in a reaction catalyzing SNARE complex assembly. In so doing, rab proteins could help impart an additional layer of specificity to vesicle docking. This mechanism likely involves the Sec1 homolog Sly1, which we identified in isolated docking complexes. We also report the identification of a novel v-SNARE (Ykt6p) component of the yeast ER-Golgi docking complex that has a CAAX box and is predicted to be lipid anchored. The surprising finding that docking complexes can contain many distinct species of SNAREs (Sed5p, Bos1p, Sec22p, Ykt6p, and likely Bet1p, p28, and p14) suggests that multimeric interactions are features of the fusion machinery, and may also improve the fidelity of vesicle targeting.

Introduction

The ATPase N-ethylmaleimide-sensitive fusion protein (NSF) and the soluble NSF attachment proteins (SNAPs) are required for the fusion of multiple species of transporting vesicles, each with its respective target membrane (Wilson et al., 1989; Clary et al., 1990; Whiteheart et al., 1993). Yet, the propagation and maintenance of subcellular compartments relies upon a mechanism of transport vesicle fusion that must be based on the pairwise matching of specific donor and acceptor compartments. The SNARE hypothesis proposes that this is accomplished by the partnering of a particular set of identifying markers carried by transport vesicles, termed v-SNAREs, with their cognate t-SNAREs, associated with the intended target membrane (Söllner et al., 1993a).

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This hypothesis was prompted by the discovery that the membrane receptor at nerve terminals for the general fusion protein SNAP (i.e., SNAP receptor, abbreviated SNARE) is a complex of three membrane proteins (Söllner et al., 1993a), one originating in synaptic vesicles (vesicle-associated membrane protein [VAMP], or alternatively, synaptobrevin) (Trimble et al., 1988; Baumert et al., 1989) and two (syntaxin and SNAP-25 [unrelated to SNAP; SNAP-25 is an abbreviation for synaptosomal-associated protein 25 kDa]) originating from the plasma membrane (Bennett et al., 1992; Oyler et al., 1989), the target for fusion. The formation of this complex in cells would thus serve to dock synaptic vesicles to the plasma membrane and enable the subsequent binding of the general fusion proteins SNAP and NSF to these sites, thereby forming a 20S particle, to initiate the fusion process (Söllner et al., 1993a, 1993b; Wilson et al., 1992). The physiological relevance of this 20S fusion particle is clear from the fact that the yeast homologs of NSF and SNAP, the products of the *SEC18* and *SEC17* genes, are required for fusion in living cells (Wilson et al., 1989; Griff et al., 1992), and because each of the three SNAP receptor subunits is a target for a particular form of botulinum or tetanus neurotoxins known to block neurotransmitter release from synapses (Schiavo et al., 1992; Blasi et al., 1993a, 1993b).

Because these three particular membrane proteins, VAMP, syntaxin, and SNAP-25, are limited to synapses and certain specialized secretory cells (Bennett and Scheller, 1994), while the action of the SNAP–NSF system is general within and among cell types (Rothman and Orci, 1992), it follows that homologs of these molecules likely exist that would be localized to all the pairs of membrane compartments connected by vesicle transport pathways to act similarly in vesicle docking and perhaps in fusion, in each case providing a framework for the assembly of NSF and SNAP. Thus, v-SNAREs would constitute a family of membrane proteins related to VAMP, while the t-SNAREs would be a family of proteins related to syntaxin and SNAP-25, most likely associated as heterooligomers. A key prediction of the SNARE hypothesis is that v- and t-SNAREs should be able to bind each other directly and specifically. This has been tested and confirmed in the synaptic case (Söllner et al., 1993b).

The SNARE hypothesis is also strongly supported by an accumulation of evidence from genetic studies in yeast that has identified likely v-SNAREs and t-SNAREs that are compartment specific both in their physical localization and in their secretory phenotypes when mutated (for review see Bennett and Scheller, 1993). The VAMP homologs Snc1p and Snc2p and the syntaxin homologs Sso1p and Sso2p are thus required for the docking and/or fusion of Golgi-derived transport vesicles to the plasma membrane in vivo (Protopopov et al., 1993; Aalto et al., 1993). Similarly, the VAMP homologs Sec22p/Sly2p and Bos1p (Dascher et al., 1991; Shim et al., 1991) are concentrated in endoplasmic reticulum (ER)-derived transport vesicles, in which they are required for docking/fusion with Golgi

both in vitro and in vivo (Newman et al., 1990; Lian and Ferro-Novick, 1993). A third protein related to VAMP, Bet1p/Sly12p, is essential for ER–Golgi transport (Dascher et al., 1991; Newman et al., 1990), but it is unclear whether it is in transport vesicles, as would be expected for a v-SNARE (Lian and Ferro-Novick, 1993; Barlowe et al., 1994). Sed5p is significantly homologous to syntaxin, required for docking/fusion of ER-derived vesicles with Golgi in vivo, and physically localized to the cis side of the Golgi stack (Hardwick and Pelham, 1992; Banfield et al., 1994). Thus, Sec22p and Bos1p are likely v-SNAREs specifying attachment of ER-derived vesicles with Golgi, forming a match with a cognate t-SNARE containing Sed5p. Similarly, Snc and Sso proteins are expected to be SNAREs involved in trans-Golgi to plasma membrane transport. Mammalian homologs of neuronal syntaxin and VAMP (Cain et al., 1992; McMahon et al., 1993; Bennett et al., 1993) localized to nonneuronal cell surface membranes and endocytic compartments have been described, including an animal cell protein homologous to Sed5p (Bennett et al., 1993). This protein, termed syn5, is localized to the entry (cis) face of the Golgi stack (Bennett et al., 1993; Banfield et al., 1994), further substantiating the proposal that Sed5p is a Golgi t-SNARE.

In addition to the SNAREs, other proteins and genes have been described that are important for vesicle docking. Members of the rab family of small GTP-binding proteins are always required in vivo and in vitro (for review see Ferro-Novick and Novick, 1993). Ypt1p and its animal equivalent rab1 are required for vesicle docking in ER to Golgi transport and are also required for vesicular transport through the Golgi stack (Segev et al., 1988; Segev, 1991; Rexach and Schekman, 1991; Plutner et al., 1991; Tisdale et al., 1992). The *SEC4* gene is required for post-Golgi vesicle docking to the plasma membrane in yeast (Salminen and Novick, 1987), and the rab3a protein is likely important for synaptic vesicle exocytosis (Fischer von Mollard et al., 1991). Because rab3a is not found in the purified synaptic SNARE complex or in the 20S fusion particle (Söllner et al., 1993b), rab proteins are not likely to contribute to core docking interactions. Despite intensive efforts, no significant insight has been gained concerning the precise role of rab proteins in docking since their discovery in this connection (Salminen and Novick, 1987). The recognition of the pivotal role of SNAREs in docking may have provided the handholds that can be used to forge a linkage between the rab and SNARE systems, a relationship that would be of fundamental importance for understanding the basis of the specificity of cellular compartments.

Any model of the docking/fusion machinery would also have to take into account an additional gene family, related to the yeast *SEC1* gene (Aalto et al., 1992; Bennett and Scheller, 1993). A *sec1* mutant accumulates post-Golgi transport vesicles (Novick et al., 1980), and is suppressed by overexpressing the putative plasma membrane t-SNAREs encoded by *SSO1* or *SSO2* (Aalto et al., 1993). Yeast homologs of *SEC1*, termed *SLY1* (Dascher et al., 1991) and *SLP1* (Wada et al., 1990), have been described (Aalto et al., 1992), genes required for transport from ER to Golgi and from Golgi to vacuole, respectively. A neuronal homolog of Sec1p (N-sec1) binds tightly to neuronal syntaxin (Hata et al., 1993; Pevsner et al., 1994a; Garcia et al., 1994) and prevents VAMP from binding (Pevsner et al., 1994b). Thus, Sec1p and other members of this family are expected to prevent v-SNAREs from docking to t-SNAREs, in effect opposing the positive action of rab proteins in promoting docking. Consistent with this is the finding that a deletion of *YPT1* (encoding the ER and Golgi rab protein) can be overcome either by mutating *SLY1* (the ER-to-Golgi *SEC1* family member) or by overexpressing *SEC22* (encoding the proposed ER v-SNARE) or *BET1* (Dascher et al., 1991). Because N-sec1 is not an obligatory component of the synaptic SNARE complex (Söllner et al., 1993a, 1993b; Pevsner et al., 1994b), neither the *SEC1* family nor the rab family are likely to contribute to the core docking interactions.

All of these observations can be encompassed in a simple model for docking and the initiation of membrane fusion, in which the core docking interaction is due to the partnering of v-SNAREs with their cognate t-SNAREs to create a scaffold on which SNAP and then NSF assemble. An additional network of proteins, including the rab and Sec1p families, would serve to control SNARE assembly to improve fidelity by proofreading, or by imposing an additional layer of specificity, or both.

We have utilized the well-developed genetics of the yeast secretory pathway (Novick et al., 1980; Pryer et al., 1992) to test the critical predictions of this model (and the SNARE hypothesis that underlies it) by accumulating ER-derived transport vesicles in various states in vivo, using rab, SNARE, SNAP, and NSF mutants, and exploring the consequences for the accumulation of complexes among the proposed SNARE proteins. The predicted ER v-SNAREs (Sec22p and Bos1p) and Golgi t-SNARE (Sed5p) components accumulate in a well-defined stoichiometric complex when fusion is blocked in an NSF mutant. In addition, this complex contains three previously uncharacterized proteins (p28, p14, and p26/Ykt6p) as well as SNAP (Sec17p), and a Sec1 family member (Sly1p) that additionally forms a stoichiometric complex with the free t-SNARE subunit (Sed5p), but does not contain the rab protein (Ypt1p) even though the complex does not accumulate in a *ypt1* mutant and thus relies upon rab function to assemble.

Results

In Vivo Accumulation of SNARE-Containing Complexes When Vesicle Fusion Is Blocked

In wild-type yeast cells in which intracellular transport proceeds rapidly, the steady-state level of transport vesicles is low (Kaiser and Schekman, 1990). Thus, in this condition, the ER-derived v-SNAREs can be expected still to reside mainly in the ER, unassociated with the Golgi-localized and mainly unbound t-SNAREs (Figure 1A). When ER-to-Golgi transport is blocked by mutation after vesicle budding, transport vesicles will accumulate, and based on studies in the cell-free system (Rexach and Schekman, 1991; Lian and Ferro-Novick, 1993), should

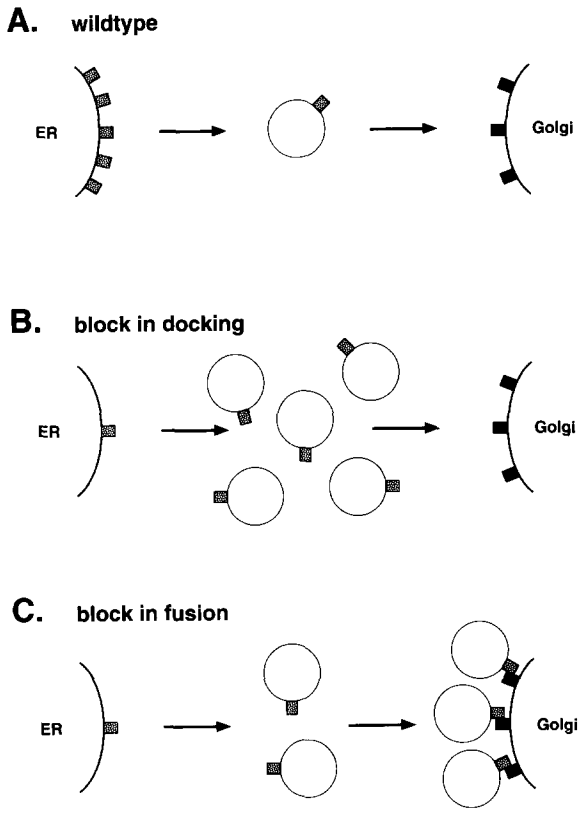

Figure 1. Accumulation of Transport Vesicles in Different States

The models show the accumulation of transport vesicles in the following: (A) a yeast wild-type strain, in which transport proceeds normally, (B) a strain blocked in docking (for example, due to a v-SNARE or rab mutation), and (C) a strain blocked in fusion (for example, due to a NSF mutation). The stippled rectangles denote v-SNAREs, and the closed ones indicate t-SNAREs.

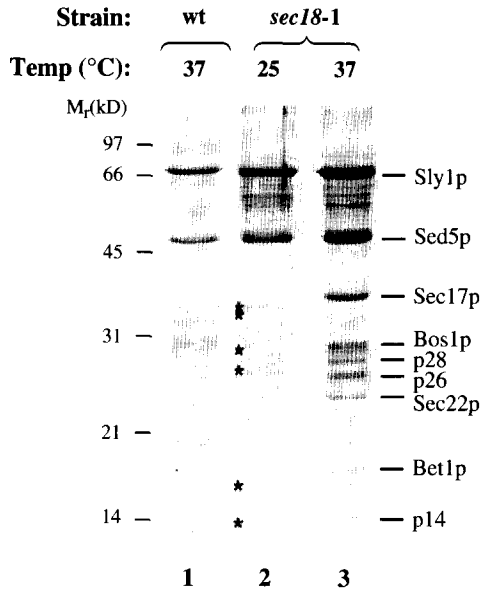

Figure 2. Formation of the ER–Golgi SNARE Complex in *sec18* Mutant Cells

Spheroplasts of yeast wild-type (RSY255, lane 1) or *sec18-1* (RSY271, lanes 2 and 3) cells were preincubated for 1 hr at the indicated temperatures, 37°C being a restrictive temperature for RSY271, and Triton X-100 extracts were prepared as described in the Experimental Procedures. Each of the different detergent extracts (20 mg of protein) was incubated with anti-Sed5p antibodies (200 μg), which had been covalently coupled to protein G–Sepharose Fast Flow beads (100 μl), and the bound proteins eluted with a glycine buffer (pH 2.6) and precipitated with trichloroacetic acid (TCA) (see Experimental Procedures). Samples were resolved by SDS–polyacrylamide gel (12%) electrophoresis (SDS–PAGE), and the polypeptides were stained with Coomassie brilliant blue. One-third of the immunoprecipitate was loaded in each lane. Bands that are labeled with an asterisk are present in minor amounts and are impurities that are not specific for the SNARE complex because they are present both at permissive temperature (lane 2) and restrictive temperature (lane 3).

now contain a large fraction of the ER-derived pool of v-SNAREs. Should the transport block be at the level of docking, these vesicles should accumulate in a free state so that their v-SNAREs would not be bound to Golgi-localized t-SNAREs in the cell (Figure 1B). On the other hand, should the transport block be in the process of fusion that follows docking, the vesicles should accumulate in the docked state in which, according to the SNARE hypothesis, a large fraction of the ER-derived v-SNAREs are bound to t-SNAREs at the Golgi (Figure 1C).

NSF is needed for vesicle fusion after docking because when NSF is inactivated uncoated transport vesicles accumulate docked to Golgi cisternae both in vitro and in vivo (Orci et al., 1989), and because in the case of the synapse NSF binds to and exerts its effects upon a preassembled SNARE complex (Söllner et al., 1993b). The *SEC18* gene encodes NSF in yeast (Wilson et al., 1989). Therefore, when NSF is inactivated by incubating temperature-sensitive *sec18* mutant yeast cells at the restrictive temperature, the predicted SNARE-containing complexes should accumulate (as in Figure 1C).

Spheroplasts of *sec18-1* mutant cells were incubated for 60 min at the restrictive temperature of 37°C before harvesting, and whole-cell extracts were prepared in non-ionic detergent. Figure 2 presents a Coomassie blue–stained SDS gel of polypeptides coimmunoprecipitated with antibodies against Sed5p (a proposed Golgi t-SNARE). The bound proteins were eluted from beads to which the affinity-purified antibodies remain covalently attached. A total of nine specific polypeptides were observed, including the antigen itself. These were identified by microsequencing, mass spectrometry, and Western blotting with affinity-purified monospecific antibodies (see the Experimental Procedures for details) as Sly1p (75 kDa), Sed5p (39 kDa), Sec17p (the yeast homolog of α-SNAP; 33 kDa), Bos1p (27 kDa), Sec22p (24 kDa), and Bet1p (16 kDa), and in addition three previously undescribed proteins of apparent molecular masses of 28 kDa (p28), 26 kDa (p26), and 14 kDa (p14). Scanning the Coomassie gels (on an Omnimedia 6cx scanner (XRS) using Bioimage software (Millipore) and correcting for molecular masses, the molar ratio of each band to Sed5p in the anti-Sed5p immunoprecipitates is as follows (average of three experiments except in the case of p14): Sly1p (1.07 ± 0.15), Sec17p (0.79 ± 0.07), Bos1p (0.56 ± 0.11), p28 (0.29 ±

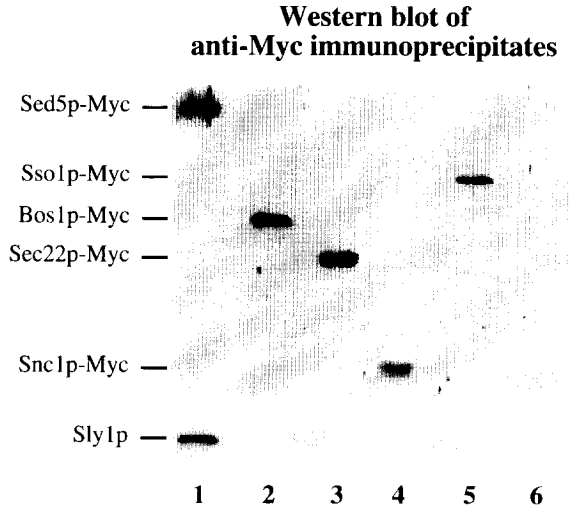

Figure 3. Sly1p Is Specifically Associated with Sed5p

Purified recombinant Sly1p-His_6 (~ 140 pmol) was mixed with the C-terminally Myc-His_6–tagged cytoplasmic domains (~ 200 pmol) of Sed5p, Bos1p, Sec22p, Snc1p, or Sso1p, respectively, in presence of anti-Myc antibodies (9E10; 200 μg) covalently coupled to protein G–Sepharose Fast Flow beads (100 μl) in 1 ml of buffer E containing 1 mg/ml ovalbumin and rotated overnight at 4°C (the recombinant proteins were purified from bacterial extracts by Ni–NTA chromatography as described in the Experimental Procedures). Experiments described in lanes 1 and 6 are identical, except that protein G beads without anti-Myc antibodies were used in the latter case. The bound proteins were eluted by low pH treatment and were TCA precipitated. Samples were analyzed after SDS–PAGE and transfer to nitrocellulose by immunodecoration. Bound antibodies were visualized by the ECL method (Amersham). The upper panel shows the different Myc-tagged cytoplasmic domains recognized by anti-Myc antibodies; the lower panel shows the coisolated Sly1p, recognized by anti-Sly1p antibodies. For detection of the Myc-tagged proteins, 1/1000 of the immunoprecipitate was loaded on the gel; for detection of Sly1p, 1/2 of the immunoprecipitate was used.

0.03), p26 (0.47 ± 0.12), Sec22p (0.32 ± 0.10), Bet1p (0.21 ± 0.04), p14 (0.19).

The ability to isolate this distinct complex of discrete subunit composition containing stoichiometric amounts of the proposed v-SNAREs Sec22p and Bos1p, the t-SNARE Sed5p, and the yeast equivalent of SNAP protein from a whole-cell extract is striking confirmation of a key prediction of the SNARE hypothesis in living cells.

That the SNARE complex we have isolated has formed in vivo, rather than in vitro after detergent extraction is clear for the following reasons. First, the appearance/lack of appearance of this complex varies with the treatment of whole cells or spheroplasts (restrictive versus permissive temperature). Second, while NSF is known to disrupt SNARE complexes in vitro (Söllner et al., 1993b), this activity requires both Mg^{2+} and ATP. We employ EDTA during all immunoprecipitations to prevent this from occuring on the basis of endogenous Sec18p. Third, it is known, in any case, that when extracts are prepared from the *sec18* mutant strain incubated at permissive temperature that Sec18p activity is lost at all temperatures (Wilson et al., 1989).

The finding of additional polypeptides in this docking complex warranted further investigation. Sly1p is a homolog of Sec1p that on the basis of analogy to the properties of N-sec1 is expected to bind to Sed5p. Indeed, when Sed5p is immunoprecipitated from wild-type cells or from *sec18-1* cells at permissive temperatures (Figure 2), Sly1p coprecipitates with Sed5p under these conditions in which t-SNAREs are predicted to be free (see Figure 1B). Scanning Coomassie-stained gels reveals that almost exactly one mole (1.03 ± 0.19; two experiments) of Sly1p is bound per mole of Sed5p and that no other major polypeptide chains appear to be present in this complex (Figure 2). The identity of Sly1p was established by microsequencing. The stoichiometric coimmunoprecipitation of Sly1p, but not other major polypeptides, with Sed5p was observed at all temperatures examined in wild-type cells (25°C, 30°C, or 37°C).

To confirm the specificity of the interaction between Sly1p and Sed5p further, recombinant Sly1p protein was incubated with recombinant, Myc-tagged cytoplasmic domains from the Sed5p, Bos1p, Sec22p, Snc1p, or Sso1p proteins and was only found to bind to the domain from the Sed5 protein (Figure 3). Efficient immunoprecipitation of the Myc-tagged proteins was confirmed by blotting of the postimmunoprecipitation supernatants (data not shown).

Bet1p, having an overall similarity to the VAMP family, is a protein required for ER–Golgi transport and has been reported to be present in ER-derived transport vesicles (Barlowe et al., 1994), although there is contradictory evidence on the latter point (Barlowe et al., 1994; Lian and Ferro-Novick, 1993). *BOS1* was isolated as a multicopy suppressor of a temperature-sensitive *bet1* mutant (Newman et al., 1990), and indeed Bet1p coimmunoprecipitates with anti-Bos1p antibody under all conditions including those in which vesicles are not accumulating (see Figure 5). Bet1p is also found in precipitates employing anti-Sed5p antibodies, but only under conditions in which NSF is inactive (Figure 4). Thus, our data are most consistent with the idea that Bet1p is an ER–Golgi v-SNARE. Further studies will be needed to show whether the coisolation of Bet1p with Sed5p is dependent or independent of the presence of Bos1p.

p26 Is a Novel, Presumably Farnesylated, VAMP Homolog (Ykt6p)

Microsequencing and mass spectrometry of four tryptic peptides derived from p26 (Figure 6; see Experimental Procedures for details) revealed that p26 is identical to the 22.7 kDa protein encoded by the open reading frame YKL196c (or *YKT6*) on chromosome XI (Dujon et al., 1994). p26/Ykt6p is predicted to be a v-SNARE because of its pattern of homologies. Searching the GenBank data base with the BLAST program revealed that p26/Ykt6p is significantly homologous to a number of proteins over the last third (amino acids 130–190) of the molecule; typically, 30% identity and 60% similarity (including conservative substitutions) were observed. Homologs include (in this order) two predicted proteins encoded by Caenorhabditis elegans (accession number U00031) and Euplotes crassus (accession number S35107) of unknown function, the predicted yeast v-SNAREs Sec22p, Snc2p, and Snc1p,

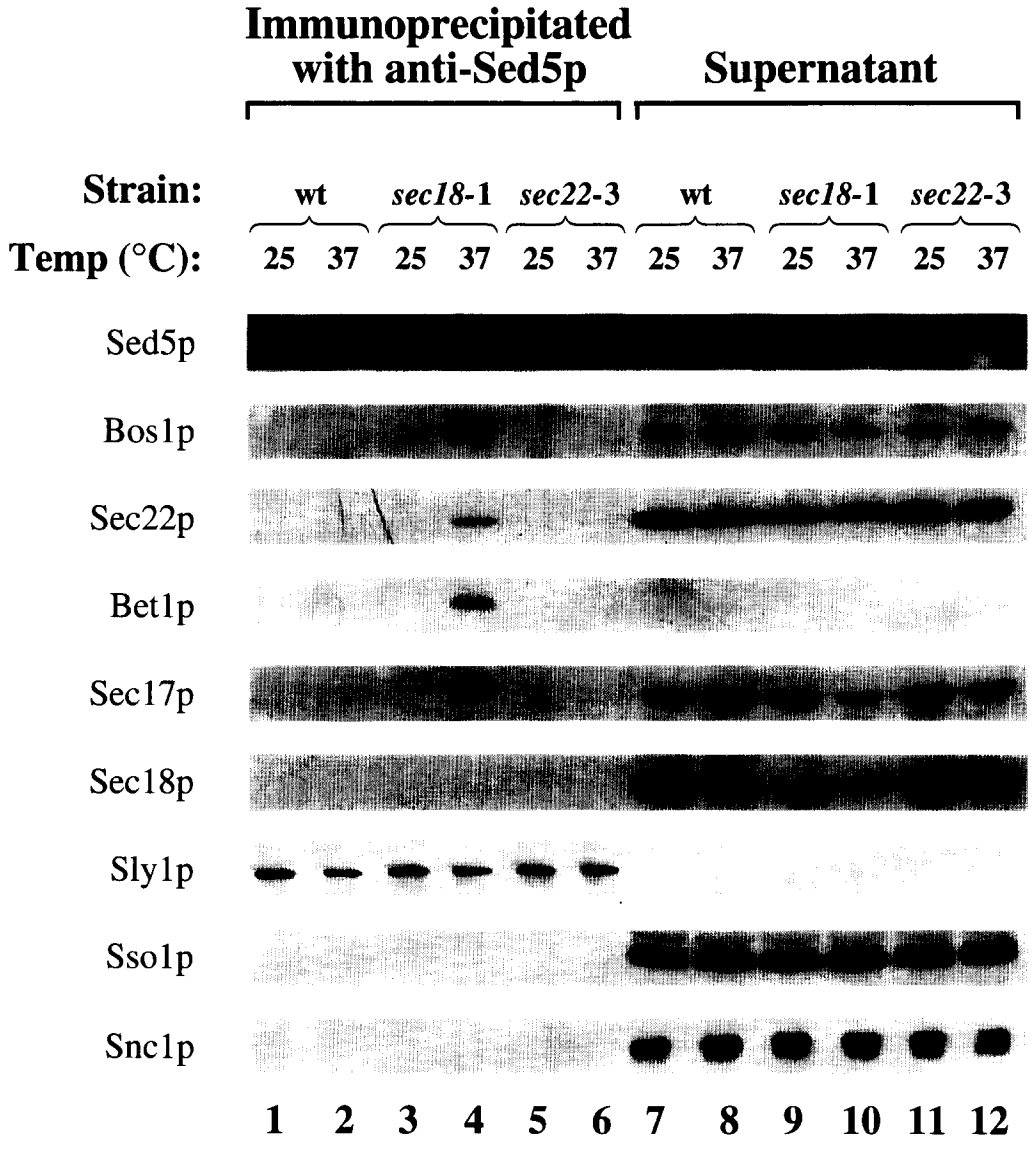

Figure 4. The Specific Assembly of the ER–Golgi SNARE Complex Does Not Occur in Presence of Active Sec18p or in Absence of the Active v-SNARE Sec22p

Wild-type (RSY255), *sec18-1* (RSY271), and *sec22-3* (RSY279) spheroplasts were incubated for 1 hr at either 25°C or 37°C prior to lysis with Triton X-100. The detergent extracts (20 mg of protein) were incubated with anti-Sed5p antibodies (~200 μg) covalently coupled to protein G–agarose beads (100 μl) as described in the Experimental Procedures. The immunoprecipitates were isolated by centrifugation, and the bound proteins eluted by low pH treatment. Both the eluates (lanes 1–6) and the supernatants of the centrifugation (lanes 7–12) were TCA precitated. Equivalent amounts (corresponding to 25 μg protein of starting material) were loaded on the SDS–polyacrylamide gel, except for Bet1p and Sly1p analyses, in which cases 40 times more immunoprecipitate than supernatant was used. The separated polypeptides were transferred to nitrocellulose and analyzed by immunodecoration with affinity-purified antibodies directed against the proteins indicated at the left side of the figure. Bound antibodies were visualized by the ECL method (Amersham).

mammalian cellubrevin, VAMP2 and VAMP1, and in addition, VAMP homologs from other sources.

Unlike other, previously identified v-SNAREs, p26/Ykt6p is not predicted to have a proteinaceous membrane anchor at its C-terminus. Instead it has a C-terminal CAAX box consensus sequence (-C-I-I-M) for farnesylation (Reiss et al., 1991), presumably anchoring Ykt6p to membranes. The C. elegans and E. crassus homologs are also predicted to have CAAX boxes (-C-N-Y-V and -C-T-L-S, respectively) indicative of isoprenylation.

Our finding of Ykt6p in the yeast ER–Golgi SNARE complex indicates that it is another ER–Golgi v-SNARE and suggests that such proteins can participate in docking/fusion without being integral membrane proteins.

SNARE-Containing Complexes Do Not Accumulate When NSF Is Active or When a v-SNARE Is Inactive

To test further the specificity of the docking complex isolated when NSF action is blocked, we wished to confirm that the complex accumulates only when fusion is blocked, to test whether post-Golgi SNAREs are excluded, and to

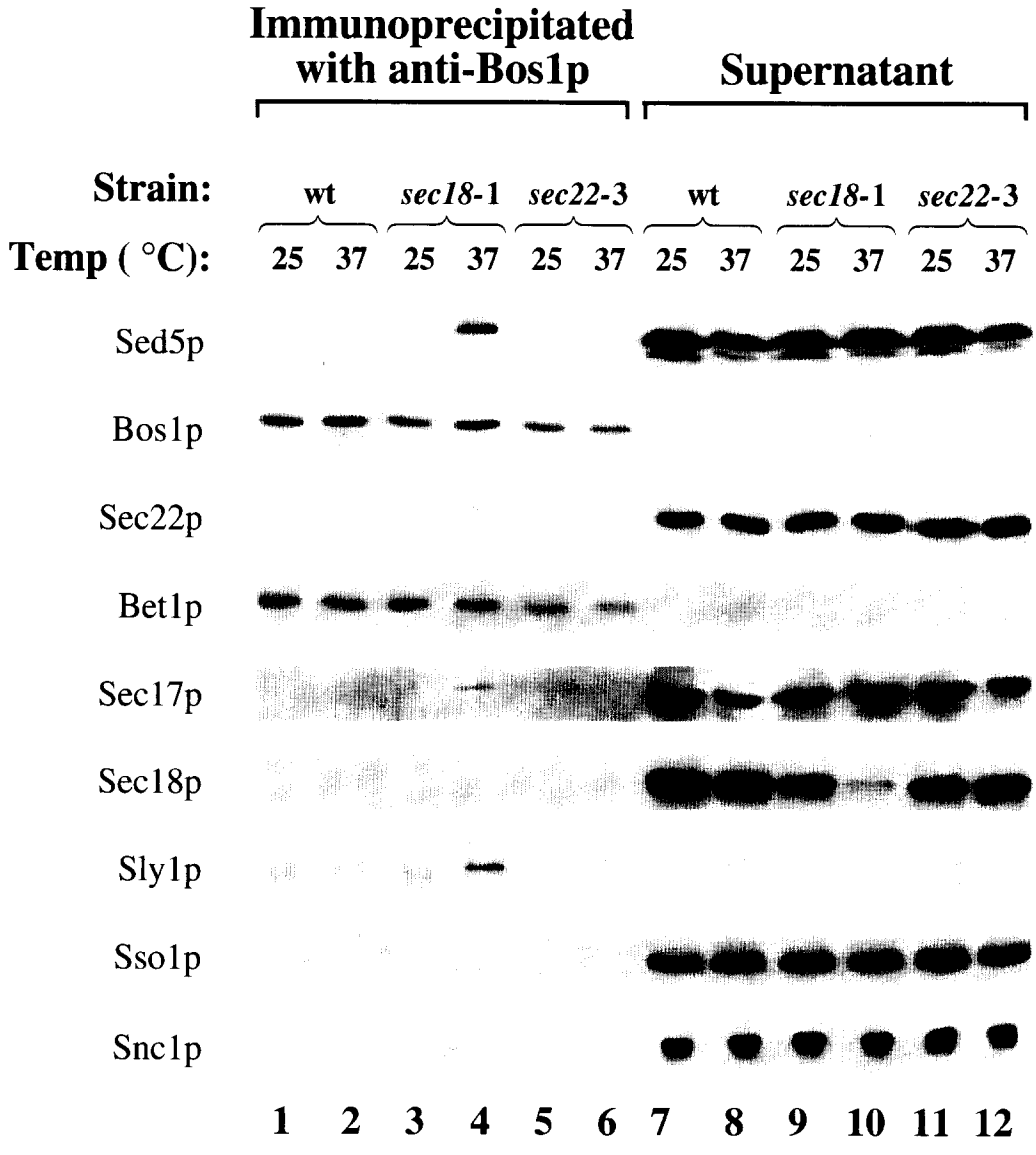

Figure 5. Bos1p and Sed5p Are Part of a Similar SNARE Complex

The composition of the ER–Golgi SNARE complex was analyzed as described in the legend to Figure 4, but anti-Bos1p antibodies were used instead of anti-Sed5p antibodies. Note that Bos1p and Bet1p form a stable complex independent of activities of Sec18p and Sec22p.

examine the requirements for formation of the complex in vivo. For this purpose, we examined the composition of the immunoprecipitates by Western blotting.

Figure 4 shows the analysis of precipitates obtained using anti-Sed5p antibody. Wild-type, *sec18-1*, and *sec22-3* temperature-sensitive mutant cells were tested after incubation at permissive (25°C) or restrictive (37°C) temperatures. In wild-type cells at either temperature, few transport vesicles should be present at steady-state (see Figure 1A), so SNARE complexes should not be present to a significant extent. When fusion is blocked in the *sec18-1* mutant at restrictive temperature (see Figure 1C), SNARE-containing complexes should accumulate. Since *SEC22* is predicted to encode a v-SNARE, vesicles should accumulate in this mutant prior to docking (see Figure 1B), so SNARE-containing complexes should not be present at any temperature.

The results were in complete accord with these predictions (Figure 4, left side). In wild-type or *sec22-3* mutant cells at either temperature, or in *sec18-1* mutant cells at permissive temperature, none of the proteins examined (Bos1p, Sec22p, Bet1p, Sec17p, Sso1p, Snc1p, or Sec18p) coprecipitated with Sed5p antibody, except for Sly1p, which remains associated under all conditions.

MRIYYIGVFR SGGEKALELS EVKDLSQFGF FERSSVGQFM TFFAETVASR TGAGQRQSIE EGNYIGHVYA
RSEGICGVLI TDKEYPVRPA YTLLNKILDE YLVAHPKEEW ADVTETNDAL KMKQLDTYIS KYQDPSQADA
IMKVQQELDE TKIVLHKTIE NVLQRGEKLD NLVDKSESLT ASSKMFYKQA KKSNSCCIIM

Figure 6. p26 Is a Putatively Farnesylated VAMP Homolog

Tryptic peptides of p26/Ykt6 that were identified by sequencing and confirmed by mass spectrometry are underlined in the sequence of ORF YKL196c of yeast chromosome XI. See Experimental Procedures for details. The CAAX box of p26 indicating farnesylation is marked in bold.

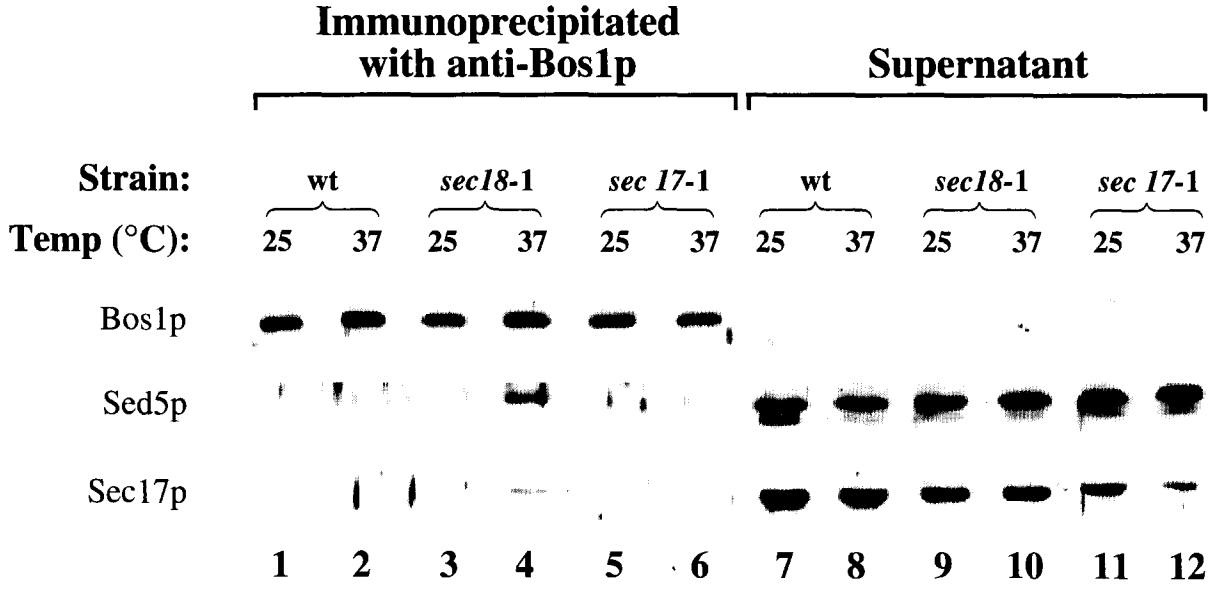

Figure 7. Sec17p Is Necessary for Formation or Stabilization of the ER–Golgi SNARE Complex

Wild-type (RSY255), *sec18-1* (RSY271), or *sec17-1* (RSY269) spheroplasts were incubated at 25°C or 37°C for 1 hr, then lysed with Triton X-100. Extracts were incubated with anti-Bos1p antibodies, and immunoprecipitates and postimmunoprecipitates prepared as described in the legend to Figure 4, except that five times more immunoprecipitate was loaded on the gel. The presence of Sed5p (as a corollary of SNARE complex formation), Bos1p, and Sec17p was analyzed in immunoprecipitates as well as in supernatants by decoration with the corresponding affinity-purified antibodies. See the Experimental Procedures for details.

Only when fusion was blocked by inactivating Sec18p/NSF at restrictive temperature did SNARE-containing complexes accumulate (Figure 4). Note that these complexes lack detectable Sso1p and Snc1p proteins and contain an appreciable fraction (up to 50%) of the total cellular pool of coprecipitating Bos1p, Sec22p, Bet1p, and Sec17p proteins (Figure 4, right side). Similar results were obtained when precipitates from these same cell extracts were made with anti-Bos1p antibody (Figure 5) with the exception noted already that Bet1p was found to be associated with Bos1p under all conditions examined. This indicates that Sed5p and Bos1p are part of the same docking complex.

The SNAP Protein Is Required for Formation or Stability of the SNARE Complex at the ER-Golgi Step in Yeast

The SNARE-containing complex does not form, or cannot be isolated if it does form in vivo, when ER-derived transport vesicles are accumulated in a *sec17* (α-SNAP) mutant (Figure 7). Because the Sec17 protein is a component of the SNAP receptor-containing complex accumulating when fusion is blocked, this implies either that the SNAP protein is required for SNARE assembly, or that SNAP binds after SNARE assembly. The latter interpretation would be more in keeping with the finding that synaptic SNARE complexes form in the absence of SNAP protein. It is noteworthy that Sec17p does not coimmunoprecipitate with anti-Bos1p (see Figure 5) or anti-Sed5p (see Figures 3 and 4) when this v-SNARE and t-SNARE are uncombined, also suggesting that SNAP associates after the SNAREs have partnered in the ER–Golgi case. Consistent with this finding, we were unable to detect coimmunoprecipitation of either radiolabeled or recombinant Sec17p with any of the recombinant cytoplasmic domains of Sed5p, Sec22p, Bos1p, Sso1p, or Snc1p (data not shown). Similar results were obtained when α-SNAP was used instead of Sec17p.

The Rab Protein Ypt1p Is Required for Assembly of SNARE Complexes

The Ypt1p protein is required for ER-to-Golgi transport in vivo (Segev et al., 1988) and is also needed for the attachment and/or fusion of ER-derived transport vesicles with Golgi in vitro (Segev, 1991; Rexach and Schekman, 1991). However, neither the function of this nor any other rab protein has been further defined than this. Since the act of docking results in, and indeed is almost certainly due to, the assembly of the SNARE-containing complex, we tested whether the Ypt1p protein is required for assembly of the SNARE-containing complex. For this purpose, we used thermolabile *ypt1* mutants, which accumulate ER-derived transport vesicles upon shift to the restrictive temperature (Barlowe et al., 1994). This is consistent with the finding that active Ypt1p has to be provided from an external source to transport vesicles devoid of functional Ypt1p, for transport to occur (Salama et al., 1993). Therefore, Ypt1p has to act at the step of vesicle docking or consumption. In both cases, one would expect the observed vesicle accumulation as outlined in Figures 1B and 1C, but only the latter would result in accumulation of the SNARE complex.

The *ypt1-3* allele seems to encode an unstable Ypt1p protein, as inferred from the absence of Ypt1p in postimmunoprecipitate supernatants from extracts of *ypt1-3* cells

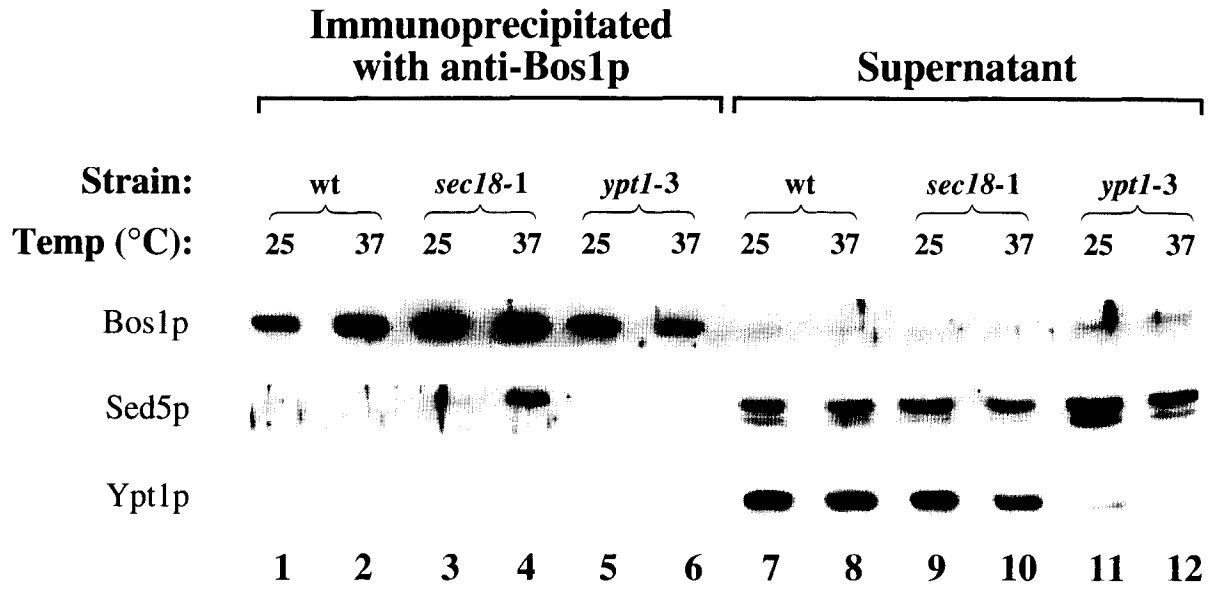

Figure 8. Ypt1p Function Is Required for SNARE Complex Assembly

Wild-type (RSY255), *sec18-1* (RSY271), or *ypt1-3* (RSY977) spheroplasts were incubated at 25°C or 37°C for 1 hr, then lysed with Triton X-100. Extracts were incubated with anti-Bos1p antibodies, and immunoprecipitates and postimmunoprecipitates prepared as described in the legend to Figure 4, except that five times more immunoprecipitate was loaded on the gel. The presence of Sed5p (as a corollary of SNARE complex formation), Bos1p, and Ypt1p was analyzed in immunoprecipitates, as well as in supernatants, by decoration with the corresponding affinity-purified antibodies. See Experimental Procedures for details.

incubated at the restrictive temperature, and from the significantly reduced amounts of Ypt1p even in extracts from cells incubated at the permissive temperature (Figure 8). Inactivation of Ypt1p at the restrictive temperature prevented the accumulation of the SNARE-containing complex (Figure 8). Similar results were obtained when the *ypt1^{ts}::LEU2* allele was used (data not shown). This clearly implies that Ypt1p function is required for assembly of the ER–Golgi SNARE complex. Whether this effect is exerted directly cannot be conclusively established by such a genetic argument, so the formal possibility that rab acts upstream but at a distance from the SNAREs cannot be rigorously excluded.

However, the fact that the complex accumulating when Sec18p is inactivated includes all major suppressors of *YPT1* deletion (so-called *SLY* genes), including both Sly1p, which we show binds specifically and with high efficiency to the t-SNARE Sed5p, and two v-SNAREs (Sec22p and Bet1p) makes it likely that Ypt1p acts directly in the SNARE complex assembly reaction. While we can not exclude the formal possibility that Ypt1p and Sec18p act in independent pathways (we have been unable to construct a *ypt1 sec18* double mutant for epistasis test), in light of the genetic interaction among v-SNAREs, Ypt1p, and Sly1p and known physical interaction between Sly1p and Sed5p, and NSF and SNAREs in the synaptic system, two independent pathways seems an untenable notion.

It is important that even though the function of Ypt1p is required for the assembly of SNAREs, the Ypt1p protein is not itself found in the SNARE-containing complex (Figure 8). This means that Ypt1p does not contribute a core interaction necessary for the stability of this docking complex.

Discussion

The finding that Ypt1p functions to enable the assembly of SNARE complexes, and thus vesicle docking, unites two lines of evidence involving two classes of proteins, each of which has been implicated in docking.

One compartment-specific class of docking proteins is the SNAREs (Söllner et al., 1993a). The core interactions in docking are almost certainly due to the partnering of v-SNAREs with t-SNAREs, because in the synapse a stable interaction requires only VAMP, syntaxin, and SNAP-25 (Söllner et al., 1993b). In the case of the ER–Golgi interface, the v-SNAREs that partner in docking vesicles include Sec22p, Bos1p, and possibly p26/Ykt6p and Bet1p proteins, and the t-SNAREs include or consist of Sed5p. Two additional unknown components are present, p14 and p28, that may prove to be additional SNARE family members, or a new class of docking component. The understanding of how these SNAREs work is still incomplete; for example, it cannot be excluded that some may play a role in retrograde transport.

The second compartment-specific class includes rab and Sec1 proteins (Ferro-Novick and Novick, 1993; Aalto et al., 1992). These proteins, possibly together with other proteins, likely regulate the docking process. In doing so, they may provide an additional layer of specificity, but they do not contribute to the core docking interaction in an obligatory way, because neither rab3a nor N-sec1 is a necessary component for stable binding of synaptic SNAREs (Söllner et al., 1993b), nor is Ypt1p a component of the yeast ER–Golgi docking complex we describe here.

The docking complex described here contains Sly1p, the ER–Golgi specific member of the Sec1 family, as a

major constituent. The fact that Sly1p is present following assembly of SNARE complexes does not preclude that Sly1p may prevent v-SNAREs from binding to Sed5p under certain conditions, as N-sec1 prevents VAMP from binding to syntaxin (Pevsner et al., 1994b); it is not known whether the Sly1p bound to the SNARE complex is bound in the same manner as Sly1p bound to Sed5p alone. It is possible that a conformational change in Sly1p while bound to Sed5p exposes v-SNARE-binding sites on Sed5p. Another fact that has to be considered is that the molar ratios of Sly1p to Sed5p in the Bos1p immunoprecipitate (approximately 1:2) is lower than in the Sed5p immunoprecipitates (approximately 1:1) (data not shown). Since Sed5p forms dimers (Banfield et al., 1994), it might be that one subunit of a Sed5 dimer binds a v-SNARE while the other subunit is still bound to Sly1p. Further investigations are needed to solve this question.

The data reported here link rab and SNARE proteins in two ways. First, by the finding that Ypt1p is required for SNARE complex assembly. In principle, this could be a direct or an indirect (downstream) effect. But, the second line of evidence strongly implies that rabs acts directly on SNAREs, because *SLY1*, *SEC22*, and *BET1* have each been found to interact genetically with *YPT1* (Dascher et al., 1991), and we now report a stable complex between Sly1p and the t-SNARE Sed5p, and the presence of Sly1p, Sec22p, Bet1p (and Bos1p) in the SNARE complex. Together with genetic interactions of *YPT1* with *SEC22* and *BET1*, this links the action of Ypt1p, and more generally the rab protein family, to SNARE complex assembly.

In that a rab protein is required for the SNARE complex to assemble, but not a part of the product of its action, the docking complex, the rab protein acts as a catalyst. In doing so, it may work, together with other proteins like Sec1 family members, to impart additional specificity according to a kinetic mechanism. This could entail the presentation of v- and t-SNAREs to each other in a more reactive state, or a proofreading mechanism to disrupt inappropriate SNARE complexes having less than a minimum stability. Whatever the detailed mechanism, only a limited degree of compartmental specificity can be involved, since a synthetic rab protein containing elements of both Ypt1p and Sec4p can simultaneously fulfill the requirements of both genes and allow transport from the ER to the cell surface (Brennwald and Novick, 1993; Dunn et al., 1993), and because cells can survive a deletion of the *YPT1* gene by mutating *SLY1* (Dascher et al., 1991).

The ER–Golgi docking complex contains at least three species of v-SNAREs, Sec22p, Bos1p, and Ykt6p, which coimmunopreciptate with antibodies to both Bos1p and Sed5p. This means that docking complexes are multivalent with respect to v-SNAREs, and thus oligomeric structures. The use of a combinatorial code of v- and t-SNAREs would of course be a means to improve the fidelity of targeting. The true molecular mass and the number of subunits in the synaptic SNARE complex and in the NSF and SNAP-containing fusion particle cannot be accurately determined owing to the presence of bound detergent. However, all models for protein-dependent bilayer fusion postulate an assembly of proteins at the fusogenic interface between apposed bilayers that walls off an environment within which the bilayer is destabilized and fusion is initiated (White, 1992). It is conceivable that the SNAREs assemble into such a structure by themselves, or when SNAP and NSF are additionally bound.

The behavior of the yeast SNARE proteins in their assembly properties in living cells under the control of various *SEC* and related genes is in complete accord with the predictions of the SNARE hypothesis, and provides the first direct evidence that the protein complexes proposed on the basis of binding experiments to be involved in docking (Söllner et al., 1993b; Calakos et al., 1994) exist in living cells and are indeed required for vesicle docking. Together with the discovery of a direct and specific interaction between synaptic v-SNAREs and t-SNAREs, and with the identification of these same membrane proteins as the targets for neurotoxins blocking exocytosis (Schiavo et al., 1992; Blasi et al., 1993a, 1993b), the essential tenets of the SNARE hypothesis now seem to be established.

Experimental Procedures

Expression and Purification of Recombinant Protein in Escherichia coli

SED5 (Hardwick and Pelham, 1992), *BOS1* (Shim et al., 1991), *SLY2/SEC22* (Dascher et al., 1991), *SSO1* (Aalto et al., 1993), *SNC1* (Protopopov et al., 1993), *SLY1* (Dascher et al., 1991), *YPT1* (Gallwitz et al., 1983), and *SLY12/BET1* (Dascher et al., 1991) genes were cloned by PCR using published sequence information for primer design and yeast genomic DNA (Clonetech) as a template. The sequence of cloned, PCR-amplified DNA was verified by the dideoxynucleotide method using Sequenase (United States Biochemical) according to the instructions of the manufacturer.

Two derivatives of the E. coli expression vector pET3a (Novagen) were prepared by replacing the NdeI–BamHI fragment of pET3a with phosphorylated, synthetic linkers. These vectors had the following DNA inserted between the NdeI and BamHI sites of pET3a: in pMS/His, NdeI-CAT ATG GGA TCC CAC CAT CAC CAT CAC CAT TAA GAG CTC AGA TCC-(BamHI); in pMS/MycHis, NdeI-CAT ATG GGA TCC GAA CAA AAA CTT ATT TCT GAA GAA GAC TTG CAC CAT CAC CAT CAC CAT TAA GAG CTC AGA TCC-(BamHI). PCR products encoding the proteins of interest and containing a 5′ NdeI site comprising the initiation ATG and a 3′ BamHI site added after the last codon were digested with NdeI and BamHI and inserted into pMS/His or pMS/MycHis. In this way, vectors encoding fusion proteins with C-terminal Gly-Ser-His_6 or Gly-Ser-Myc-His_6 tags were constructed. Gly-Ser is derived from the BamHI recognition sequence, His_6 was added to facilitate purification on Ni^{2+}–NTA agarose, and the Myc tag to allow immunodetection or immunoprecipitation experiments with the monoclonal antibody 9E10 (Evan et al., 1985) directed against the c-*myc* protooncogene. Cytoplasmic domains of putative SNAREs (i.e., amino acids 1–324 of Sed5p, amino acids 1–222 of Bos1p, amino acids 1–194 of Sec22p, amino acids 1–267 of Sso1p, or amino acids 1–115 of Snc1p) were expressed with the appropriate C-terminal tag in BL21(DE3) E. coli cells (Novagen). Plasmids directing the synthesis of Sly1p (amino acids 1–513)-Gly-Ser-His_6, Sly1p-Gly-Ser-His_6, and Ypt1p (1–206)-Gly-Ser-His_6 fusion proteins were prepared in a similar way. The cytoplasmic domain of Bet1p (amino acids 1–124) was expressed as a C-terminal fusion to GST in the vector pGEX2T (Pharmacia), and purified on glutathione–agarose (Pharmacia) according to the instructions of the manufacturer. GST–Bet1p was expressed in XL1-blue cells (Stratagene).

BL21(DE3) and XL1-blue cells harboring the appropriate plasmids were cultured at 37°C in 1 liter of LB medium with ampicillin, expression induced by addition of IPTG to 1 mM at a OD_{600} of 0.6, and the cells incubated for a further 2 hr before harvesting. Cell paste (20 ml in 50 mM Na-phosphate [pH 8.0], 300 mM NaCl, 10 mM β-mercaptoethanol, 1 mM PMSF) was passed through a French Press cell (Aminco) three times at 500 lb/in^2. The lysate was cleared by centrifuga-

tion at 100,000 × g for 30–60 min. The supernatant was applied to a 10 ml Ni^{2+}–NTA agarose column (QIAgen), washed with 50 mM Na-phosphate (pH 8.0), 300 mM NaCl, 10% glycerol (buffer A) until a stable baseline was obtained and then with 150 ml of 10 mM imidazole (pH 7.0), 100 mM NaCl, 10% glycerol (buffer B) and eluted with a 100 ml of 0–250 mM linear gradient of imidazole gradient in buffer B. Alternatively, Bos1p fusion proteins were purified from inclusion bodies. The pellet obtained after French Press lysis was solubilized in 6 M GuHCl, 100 mM Na-phosphate, 10 mM Tris (pH 8.0) (buffer C) for 2 hr at room temperature. The extract was cleared by centrifugation (100,000 × g, 30 min), applied on a Ni^{2+}–NTA column (10 ml) equilibrated with buffer C, washed extensively with buffer C and then with buffer A, and eluted with imidazole in buffer B as described above. More than 100 mg of soluble protein was obtained from a 1 liter cell culture.

Antibody Production and Purification

Gly-Ser-His_6-tagged recombinant proteins (Sly1p [amino acids 1–513]-Gly-Ser-His_6 in the case of Sly1p) and the GST–Bet1p fusion protein were used for preparation of polyclonal antibodies in rabbits. Recombinant His_6–Sec18p was provided by Dr. W. Whiteheart. Either the soluble protein or DMSO-dissolved nitrocellulose strips (Knudsen, 1985) containing the protein of interest were used for immunization. Antibodies were affinity-purified on nitrocellulose strips containing the corresponding recombinant protein, except for Sed5p, Bos1p, and Bet1p antibodies, which were isolated using an antigen–Sepharose 4B column (Harlow and Lane, 1988). Coupling of the antigen to CNBr-activated Sepharose 4B (Pharmacia) was performed according to the instructions of the manufacturer. Antibodies were produced by East Acres Biologicals or by the Memorial Sloan–Kettering Cancer Center animal facility.

Yeast Spheroplast Detergent Extracts

S. cerevisae SEY2102 (wild type) (Griff et al., 1992), RSY255 (wild type), RSY269 (*sec17-1*), RSY271 (*sec18-1*), RSY279 (*sec22-3*) (Kaiser and Schekman, 1990), RSY950 (*ypt1^{ts}::LEU2*), and RSY977 (*ypt1-3*) (M. Rexach and R. Schekman, unpublished data) were grown at 25°C, and spheroplasts were prepared essentially as described (Waters and Blobel, 1986). In brief, cells (6–18 liters) were resuspended in YPD, 1 M sorbitol to 0.3 g/ml, DTT was added to 10 mM and NaOH to a pH of 7. Zymolyase 100T (Seikagaku or ICN) was added to 0.125 mg/ml, and the suspension was gently stirred until the A_{600} of a 1:200 dilution in water had decreased >90%. The spheroplasts were collected at 1,000 × g, washed with YPD–sorbitol, resuspended in YPD–sorbitol, and incubated with gentle shaking for 1 hr at different temperatures. Half the spheroplasts were incubated at 25°C, the other half at 37°C before harvesting. Alternatively, SEY2102 was cultured and spheroplasts incubated at 30°C. The isolated spheroplasts were subsequently "dounced" 20 times on ice in buffer D (20 mM HEPES, KOH [pH 7.0], 500 mM KCl, 1 mM DTT, 1 mM $MgCl_2$, 0.5 mM ATP, 1 mM PMSF, 0.5 mM phenantroline, 2 μM pepstatin A, 0.5 μg/ml leupeptin, 2 μg/ml aprotinin, 2% [w/v] Triton X-100), and debris was removed by centrifugation (100,000 × g, 60 min). The extracts were collected, avoiding the lipid layer at the top of the tube, and frozen in aliquots in liquid nitrogen.

Immunoprecipitations

Affinity-purified anti-Sed5p or anti-Bos1p antibodies as well as the 9E10 monoclonal anti-Myc antibody were bound to protein G–Sepharose Fast Flow (Pharmacia) at a concentration of 2 A_{280} units of antibody per milliliter of beads and cross-linked with dimethylsuberimidate (Harlow and Lane, 1988).

Spheroplast detergent extracts were diluted to a final protein concentration of ~2 mg/ml protein with buffer E (20 mM HEPES, KOH [pH 7.0], 100 mM KCl, 1 mM DTT, 2 mM EDTA, 0.5 mM ATP, 1 mM PMSF, 0.5 % [w/v] Triton X-100) and rotated overnight at 4°C with antibodies coupled to protein G beads. For experiments described in Figures 2, 4, and 5, antibody-coupled protein G–Sepharose Fast Flow beads were incubated with extract (20 mg of protein), washed three times with 15 ml of buffer E, transferred to a disposable polystyrene column (Pierce), washed with five consecutive 4 ml washes (20–40 column volumes) of buffer E adjusted to final concentrations of 0.2 M, 0.35 M, 0.5 M, 0.75 M, and 1 M KCl, respectively, followed by elution with 1 ml of 0.1 M glycine (pH 2.6), 0.25% Triton X-100, and then 1 ml of 0.1 M glycine (pH 2.6). For experiments in Figures 7 and 8, anti-Bos1p protein G–Sepharose Fast Flow beads (~25 μl) were incubated with extract (2 mg of protein), washed four times with 15 ml of buffer E, eluted twice with 0.5 ml of 0.1 M glycine (pH 2.6), 0.25% Triton X-100 and once with 1 ml of 0.1 M glycine (pH 2.6). Postimmunoprecipitation supernatants (25 μl, ~50 μg of protein) were precipitated with 10% (w/v) TCA as was glycine eluates, in which case Na-deoxycholate was added to 0.01% w/v (Harlow and Lane, 1988) and resuspended in sample buffer. Immunodecoration of Western blots were performed using affinity-purified antibodies followed by horseradish peroxidase coupled goat anti-rabbit IgG secondary antibodies (Bio-Rad) at a 1:3000 dilution in PBST, 5% milk. The bound antibodies were visualized by enhanced chemiluminescence, ECL (Amersham), and exposed to Kodak X-OMAT AR film.

Peptide Sequencing and Mass Spectrometry

Polypeptides separated by SDS–PAGE were electroblotted onto nitrocellulose, and the stained protein bands were processed for internal amino acid sequence analysis, essentially as described (Tempst et al., 1990). In brief, membrane bound proteins were in situ digested with trypsin, and the resulting peptides were separated by microbore RP-HPLC; the modular LC system used in this study has been described elsewhere (Elicone et al., 1994). Selected peptides were then subjected to chemical microsequencing and MALDI mass spectrometry also as described previously (Söllner et al., 1993a; Geromanos et al., 1994).

The 75 kDa band in the Sed5p immunoprecipitate from a spheroplast extract of the wild-type strain SEY2102 (Griff et al., 1992) incubated at 30°C was identified by combined chemical sequencing/MALDI-mass spectrometry: VLILDIKSTATISSVLR, SDKYSEFYINFTSSLPR, QVYDQYLDFIVTEPELFSLEISN, and SLDTFFEVEQDPGSTK peptides are perfect matches to the published Sly1p sequence (Dascher et al., 1991). Furthermore, three unsequenced peptides had m/z values within 0.022% of the calculated $[MH^+]$ for predicted Sly1p tryptic peptides.

The structure of a similarly sized 75 kDa protein in the Bos1p immunoprecipitate from the RSY271 (*sec18-1*) spheroplast extract (cells incubated at 37°C) was analyzed by mass spectrometry; at least ten peptides, purified from the tryptic digest mix, had m/z values matching the expected $[MH^+]$ values for the predicted Sly1p tryptic peptides within 0.03%, unequivocally establishing the expected identity.

The 24 kDa band in the Sed5p immunoprecipitate from the same extract was identified by chemical sequencing: NLAFSYLNDIAQEFEHxxAN, LTPQSATEATLE, and STLIYREDGLPL sequences revealed a perfect match to the N-terminal portions of three predicted Sec22p tryptic peptides (Dascher et al., 1991).

Finally, four tryptic peptides from the 26 kDa band in the Sed5p immunoprecipitate from the RSY271 spheroplast extract (cells incubated at 37°C), IYYIGVFR, QSIEEGNYIGHVYAR, SEGICGVLITDKE, and ILDEYLVAHPKEExAxVTETN have sequences encoded by the yeast chromosome XI open reading frame YKL196c (Dujon et al., 1994). The first two peptides were sequenced all the way to the C-terminus; the others were not. However, mass analysis on those (amino acids 72–96 and amino acids 97–121) gave m/z values that were in good agreement (within 0.031%) with the calculated $[MH^+]$ for the predicted ORF YKL196c tryptic peptides, so confirming identity of the peptides.

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