SNARE proteins represent a family of related proteins that are thought to have a central role in vesicle targeting and fusion in all eukaryotic cells. The binding properties of the neuronal proteins synaptobrevin 1 (VAMP1), syntaxin 1, SNAP-25, and soluble N-ethylmaleimide-sensitive factor attachment protein (α-SNAP), have been extensively studied. We report here the first biochemical characterization of a nonneuronal SNARE complex using recombinant forms of the yeast exocytic SNARE proteins Snc1, Sso1, and Sec9 and the yeast α-SNAP homolog, Sec17. Despite the low level of sequence identity, the association properties of the yeast and neuronal complexes are remarkably similar. The most striking difference we have found between the yeast and neuronal proteins is that individually neither of the target membrane SNAREs (t-SNAREs), Sso1 nor Sec9, show any detectable binding to the synaptobrevin homolog, Snc1. However, as a hetero-oligomeric complex, Sec9 and Sso1 show strong binding to Snc1. The clear dependence on the Sso1-Sec9 complex for t-SNARE binding suggests that regulating the formation of this complex may be a key step in determining the site of vesicle fusion.

In addition, we have used this in vitro assay to examine the biochemical effects of several mutations in Sec9 that result in pronounced growth defects in vivo. As expected, a temperature-sensitive mutation in the region most highly conserved between Sec9 and SNAP-25 is severely diminished in its ability to bind Sso1 and Snc1 in vitro. In contrast, a temperature-sensitive mutation near the C terminus of Sec9 shows no defect in SNARE binding in vitro. Similarly, a deletion of the C-terminal 17 residues, which is lethal in vivo, also binds Sso1 and Snc1 normally in vitro. Interestingly, we find that these same two C-terminal mutants, but not mutants that show SNARE assembly defects in vitro, act as potent dominant negative alleles when expressed behind a strong regulated promoter. Taken together these results suggest that the C-terminal domain of Sec9 is specifically required for a novel interaction that is required at a step following SNARE assembly.

In recent years, studies on neuronal exocytosis, biochemical analysis of in vitro transport systems, and yeast genetic analysis have converged on a set of structurally related proteins known as SNARE proteins, as critical for the process of vesicle targeting and fusion in eukaryotic cells (1). This has led to the SNARE hypothesis, which suggests that SNARE proteins on the surface of vesicles (v-SNAREs)1 can interact specifically with SNARE proteins on the target membrane (t-SNAREs) to form a complex that recruits factors required for fusion of the two membrane bilayers (2). In neurons the vesicular protein, synaptobrevin, associates with the two plasma membrane proteins syntaxin and soluble SNAP-25 to form a complex that acts as a receptor for α-SNAP and NSF. Hydrolysis of ATP by NSF leads to SNARE complex disassembly and is thought to be linked to membrane fusion, although precisely how NSF is involved in the membrane fusion event is still unclear (3).

Binding studies with recombinant neuronal proteins have shown that although syntaxin and SNAP-25 can bind to one another with high affinity, they can also individually bind to the vesicle protein, synaptobrevin, with lower affinities (4). The presence of both t-SNAREs, however, potentiates the interaction of syntaxin with synaptobrevin about 10-fold and that of SNAP-25 with synaptobrevin approximately 2-fold. This suggests that the formation of a highly stable ternary complex drives the interaction between these proteins and consequently aids in determining the overall specificity of synaptic vesicle docking (4).

The regions of each neuronal SNARE protein that mediate these binding activities have been extensively characterized. Within the cytoplasmic domain of syntaxin 1, for example, a small 73-residue juxtamembrane region, predicted to form coiled-coils, can mediate both binding to synaptobrevin (5, 6) and SNAP-25 (5, 7, 8). The amino-terminal region of syntaxin is also capable of binding the carboxy-terminal domain of the protein, and this interaction appears to be somewhat inhibitory to synaptobrevin binding (5). The amino-terminal half of SNAP-25, which is also predicted to form a coiled-coil structure, is required for binding to syntaxin, while the interaction between SNAP-25 and synaptobrevin requires the entire protein (7, 8). Truncation of the carboxy-terminal 9 residues of SNAP-25 by botulinum neurotoxin A, was shown in vitro to diminish the interaction of SNAP-25 with synaptobrevin (7) and inhibit the ability of SNAP-25 to form an SDS-resistant complex with syntaxin and synaptobrevin (8). Recent studies, however, suggest that toxin poisoning of synaptosomes does not

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1 The abbreviations used are: v-SNARE, vesicle SNARE; t-SNARE, target membrane SNARE; NSF, N-ethylmaleimide-sensitive factor; SNAP, soluble NSF attachment protein; GST, glutathione S-transferase; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction.

6 This work was supported by grants from the March of Dimes, the Council for Tobacco Research, and the Pew Scholars in Biomedical Sciences program (to P. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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affect SNARE complex assembly or disassembly but rather acts at a later stage (9, 10). Also, studies in neuroendocrine cells have shown that a C-terminal 20-mer corresponding to the BoTx A cleavage of SNAP-25 can inhibit Ca^{2+}-dependant exocytosis presumably via interactions with other components of the fusion complex (11). Binding of synaptobrevin to syntaxin and SNAP-25 requires the presence of the entire conserved domain of the protein (8), and mapping of the interacting sequences revealed that complex formation correlated with the presence of coiled-coil domains.

The t-SNAREs syntaxin and SNAP-25 can each bind specifically to α-SNAP, and the interaction between α-SNAP and syntaxin was mapped to the carboxy-terminal fragment of the protein (6, 12, 13). Although the v-SNARE synaptobrevin does not directly bind α-SNAP, it greatly potentiates α-SNAP binding to syntaxin (13) by formation of a third binding site in the SNARE complex (8).

In yeast, SNC1 and SNC2 represent a duplicated gene family that encodes homologs of synaptobrevin that are required for post-Golgi transport and have been localized to post-Golgi vesicles (14). Likewise, SSO1 and SSO2 represent a duplicated gene family whose protein products are homologous to syntaxin. Like their neuronal counterparts, the Sec9, Sso1-(1–265), and Snc1-(1–200) proteins can be co-precipitated as a complex in yeast (15). Like their mammalian counterparts in their primary sequences, their binding properties are extremely similar and form a complex with Sec17 that appears to associate with identical stoichiometry. This suggests that Sec9 and Sec17 represent a duplicated gene family whose protein products are homologous to synaptobrevin that are required for post-Golgi transport and have been localized to post-Golgi vesicles (15). Their neuronal counterparts, the Sec9, Sso1-(1–265), and Snc1-(1–200) proteins can be co-precipitated as a complex from detergent extracts of yeast, and this complex is disassociated in the presence of ATP and magnesium (15).

In this paper we examine the binding properties of reconstituted forms of the yeast exocytic SNARE proteins Snc1, Sso1, and Sec9 and the α-SNAP homolog Sec17. We have found that although the yeast proteins show limited conservation with their mammalian counterparts in their primary sequences, their binding properties are extremely similar and form a complex with Sec17 that appears to associate with identical stoichiometry to those found in the neuronal SNARE-α-SNAP complex. We have used this system to begin to assess the biochemical defects associated with four distinct mutant alleles of Sec9 with clear in vivo defects in function. We show that in two of these alleles the in vivo defect correlates precisely with a clear biochemical defect in SNARE interactions, while two alleles containing mutations in the extreme C terminus of Sec9 show no detectable defect in SNARE interactions and act as dominant negative mutants when expressed behind a strong regulated promoter. Taken together, these mutants appear to identify a functional requirement for the C terminus of Sec9, which appears to play a role following the assembly of Sec9 into SNARE complexes.

**Experimental procedure**

**Plasmid Constructions—**ss–sec9 was subcloned into the pGEX4T1 expression vector (Pharmacia Biotec Inc.) using fragments generated by PCR with EcoRI and SalI sites introduced in the PCR primers. Sec17(1–292) was subcloned as a XhoI–BamHI fragment into the same vector. The details of the Sec9-(402–851) GST fusion construct will be described elsewhere. All constructs were verified by sequence analysis to ensure that no mutations had been introduced during their construction.

**Purification of the Recombinant Proteins—**Plasmids expressing GST-Sec9 and GST-Sec17 were transformed in BL21 cells. The remaining GST-fused proteins were expressed in DH5α. Cells were grown to an A_{600} = 0.3 and induced with 0.1 mM isopropyl-1-thio-β-D-galactopyranoside at 30 °C for 2 h. Cells were then pelleted, resuspended in 3.5 ml of ice-cold PBS with phenylmethylsulfonyl fluoride (1 mM) and 4,2-ami-noethylbenzenesulfonyl fluoride hydrochloride (2 mM) and frozen in Eppendorf tubes on dry ice. The cell pellets were then thawed on ice and sonicated for 30 s with a probe sonicator. PBS with 1% Triton X-100 was then added at a 1:1 ratio to the cell lysate, and after 15 min on ice, the lysate was spun in a microcentrifuge for 10 min, and the supernatant containing the soluble recombinant protein was pooled and incubated with 3 ml of a 5% slurry of glutathione-Sepharose beads on ice with gentle mixing. The beads were subsequently washed twice with 1 × PBS, three times with 1 × PBS plus 1% Triton X-100, and then stored at 4 °C in 1 × PBS with 10 mM NaCl. Soluble recombinant proteins were obtained from the fusion proteins bound to beads by thrombin cleavage in buffer containing 20 mM Tris, pH 7.5, and 150 mM NaCl using 2.5 units of thrombin (Pharmacia) per 20 ml of beads for 2 h at room temperature. In the case of the GST-Sec9 and GST-Sec17 fusion proteins, however, 4 and 1.25 units of thrombin were added for 4 and 1 h, respectively. The supernatant was then separated from the beads, and the beads were washed twice in cleavage buffer. The supernatants containing the soluble proteins were then pooled and treated with benzamidine-Sepharose beads (Pharmacia) to remove any thrombin activity by raising the NaCl concentration to 0.5 M and then adding approximately 0.2 ml of 1:1 slurry of benzamidine-Sepharose beads. 0.1 M of original glutathione-Sepharose bead bed volume. After 30 min on ice, the supernatant was separated from the beads and dialyzed against 20 mM Tris, pH 7.5, 150 mM NaCl and then concentrated in a Centricon concentrator (Amicon). Protein concentrations were then quantitated by BCA protein assay (Pierce) and by comparison to purified standards following SDS-PAGE and Coomassie staining.

**Antibodies—**Affinity-purified antibodies against the C-terminal 70 residues of Sec9 were described previously (15). Rabbit antisera were raised against recombinant GST-Sec17 and GST-Sso1 (Cocalico Biologicals) and affinity-purified as described previously (15).

**In Vitro Transcription-Translation—**Wild-type or mutant Sec9 sequences were placed under control of a T7 promoter by PCR amplification with the following primer pair: the S9/T7 primer, TAATAAC-GACT-CAC-TAT ATAAG CGTCATGCAC TTTATCCC AAAACAGACA GAAGTGCC AAAAGAGGGGCTCGCCA, and the S9–DS primer, GCGTA CAGCT-TGGGATCC AAAGGTAT CTCCATACC.

**Binding Assays—**For the binding of radiolabeled Sec9 to the glutathione-Sepharose-bound fusion proteins, 4 µl of the [35S]methionine-labeled in vitro transcription-translation reaction mixture was added to a 100-µl reaction containing a final concentration of 2 µM of immobilized fusion protein in 10 mM Hepes/KOH, pH 7.4, 140 mM KCl, 2 mM MgCl₂, and 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, and 2 mM 4,2-aminoethylbenzenesulfonyl fluoride hydrochloride. The reaction was incubated overnight at 4 °C, and the supernatants were separated from the pellets by centrifugation. The supernatants were boiled in 2 × sample buffer, while the pellets were washed three times with binding buffer and then boiled in 200 µl of sample buffer. Equal volumes of the samples were then subjected to SDS-PAGE, stained, destained, and reacted with sodium salicylate (1 M) dried, and exposed to a PhosphorImager screen. The binding reactions with recombinant Sec9 and Sso1 proteins were performed identically except that SDS-PAGE gels were transferred to nitrocellulose, probed with the appropriate affinity-purified antisera, detected with [125I]-Protein A, and quantitated on a PhosphorImager (Molecular Dynamics).

For the Sec9 binding experiment the SNARE complex was preassembled onto GST-Snc1 beads by incubating the beads overnight in binding buffer. The beads were then washed in binding buffer and used for the incubation with Sec17 (3 µM) at 4 °C for 2 h. The samples were processed by SDS-PAGE and immunoblot analysis using 125I-Protein A to detect bound antibodies. For the saturation experiments, the soluble protein was incubated with the glutathione beads in a final volume of 50 µl. The concentration of protein bound to the glutathione beads was kept constant at approximately 0.4 µM, while the concentration of the soluble protein was varied between 0.05 and 30 µM. The binding reaction was carried out at 4 °C for 2 h. The reactions were then treated as above and analyzed by SDS-PAGE followed by immunoblot analysis. The binding was quantitated by use of a PhosphorImager, and the EC_{50} values were obtained using a Prism program.

**Mapping and Cloning the Mutations—**The pet1 site in pBS316 (CEN, URA3) was removed by filling in with Klenow and religating. A BamHI/SalI fragment of SEC9 was subcloned into this vector and then digested with PstI and XbaI to create a gap over most of the SNAP-25 domain. This gapped DNA was used to transform a yeast strain, BY70 (a, sec9–7, ura3–52). Transformants, which arise from repair of this gapped plasmid with sequences present at the chromosomal sec9–7.
locus, were scored for temperature sensitivity. Approximately half of the transformants were temperature-sensitive for growth at 37 °C, and half showed normal growth at 37 °C. This indicated that the site of temperature sensitivity was not within the gapped region but just outside the boundary of the gap. We showed that the mutation was present in the C-terminal side of this gap by subcloning the XbaI-SacI region from a plasmid recovered from a temperature-sensitive transformant. The resulting subclone gave rise to 100% temperature-sensitive transformants. Sequencing of the SNAP-25 domain of the temperature-sensitive Sec9 clone showed a single base change in the expected region causing a leucine to histidine substitution at residue 627.

Effects when these mutants were expressed behind their own promoter were apparent from the normal growth seen in the his haploid progeny were analyzed by replica plating for the presence of the deleted allele. Approximately half of the viable spores were his', demonstrating that both the sec9-Δ17 and sec9-338 mutations are lethal to Sec9 function. The lack of dominant effects when these mutants were expressed behind their own promoter was apparent from the normal growth seen in the his', leu' segregants, which have both the wild type and mutant copies of Sec9.

To look for possible dominant negative phenotypes associated with moderate overexpression of these alleles, DNA fragments were PCR-amplified from the cloned SEC9, sec9-4, sec9-7, sec9-Δ17, and sec9-338 alleles and subcloned behind the GALI promoter in a LEU2 integration vector (pRS316). Two independent subclones of each allele were linearized with PstI to target integration at the LEU2 locus and transformed into a GAL+ strain (strain BY-TN-201, a diploid yeast strain heterozygous for a chromosomal deletion of SEC9). BY-TN-201 was transformed with pRS316-sec9-4, pRS316-sec9-7, pRS316-sec9-Δ17, and pRS316-sec9-338. The plasmids containing the mutations were cleaved within the LEU2 gene to target chromosomal integration at the LEU2 locus. The transformants were colony-purified and sporulated, and tetrads were dissected with a micromanipulator on YPD plates. The plates were grown at 25 °C, and the haploid progeny were analyzed by replica plating for the presence of the integrated mutants (scored as leu') and for the presence of a deleted copy of SEC9 (scored as his'). Analysis of 24 tetrads for each of the two deletion mutants showed 2:2 segregation for viability, and all of the viable spores were his', demonstrating that both the sec9-Δ17 and sec9-338 mutations are lethal to Sec9 function. The lack of dominant effects when these mutants were expressed behind their own promoter was apparent from the normal growth seen in the his', leu' segregants, which have both the wild type and mutant copies of Sec9.

RESULTS

Preparation of Recombinant Sso1, Snc1, and Sec9—We have previously described a SNARE-like complex among Sec9, Sso1, and Snc1 in co-purification experiments from detergent lysates of yeast (15). To study more directly the specific interactions seen between the yeast t-SNAREs Sec9p and Sso1p and the v-SNARE Snc1 and compare these interactions to those observed with the neuronal SNARE proteins, we prepared GST fusion constructs containing the cytoplasmic domains of Sec9 (residues 1–93), Sso1 (residues 1–265), and the essential, SNAP-25-like domain of Sec9 (residues 402–651). Since the yeast syntaxin homolog, SSO1 and SSO2, and the yeast synaptobrevin homologs SNC1 and SNC2 are encoded by duplicated gene families with redundant functions, we chose one member of each gene family for our analysis. While the Snc1 and Sso1 constructs readily expressed fusion protein (see Fig. 1), attempts to express recombinant Sec9 were unsuccessful in a variety of Escherichia coli expression systems. The most likely explanation for the lack of expression was the poor codon usage in this domain, since the SNAP-25 domain of Sec9 contains 26 arginine AGG and AGA codons, which are the two rarest codons in Gram-negative bacteria. To overcome this problem, we prepared a synthetic version of this domain of Sec9 whose codon usage was optimal for E. coli. As predicted, GST-Sec9 constructs containing the synthetic coding sequence expressed large amounts of fusion protein of the expected size.
Sec9 \textsuperscript{p} binding was in the presence of excess (3 \textmu{}M) as high as 10 \textmu{}M failed to see detectable binding even at concentrations of Sec9 recombinant Sec9-(402–651) at a final concentration of 1 \textmu{}M. In B, Sec9\textsuperscript{p} binding was in the presence of excess (3 \textmu{}M) recombinant full-length or truncated Sso1. The binding reaction was incubated overnight at 4 \textdegree{}C, and the supernatants were separated from the pellets by centrifugation. The supernatants were boiled 2 \times sample buffer, while the pellets were washed 3 \times with binding buffer and then boiled in a proportional volume of sample buffer. The samples were then electrophoresed, transferred to nitrocellulose, and blotted with antibodies to Sec9. Binding was quantitated by use of the PhosphorImager and expressed as a percentage of the total protein. Results show that recombinant soluble Sso1 could bind to GST-Snc1 beads in conditions in which it does not bind to GST-Snc1 and that soluble Sso1p promotes the binding of Sec9\textsuperscript{p} to GST-Snc1 beads.

failed to see detectable binding even at concentrations of Sec9 as high as 10 \textmu{}M (data not shown). Since the affinity of the interaction of synaptobrevin with syntaxin has been shown to be dramatically increased by the presence of SNAP-25, we examined whether the presence of soluble Sec9 protein could promote the interaction of Sec9 with Snc1 fused to GST. The results, shown in Fig. 2B, demonstrate that full-length Sso1 (present at 3 \textmu{}M) is able to promote significant binding of the SNAP-25 domain of Sec9 to GST-Snc1 (12\% of Sec9 is bound), while the presence of the same concentration of soluble truncated Sso1 results in nearly all (98\%) of the Sec9 being associated with the GST-Snc1 beads. This is not due to a nonspecific aggregation of Sec9 protein and Sso1, since no binding was observed in identical samples incubated with GST protein alone immobilized on the beads.

Sso1 \textsuperscript{p} Binds Directly to the SNAP-25 Domain of Sec9 but Only Interacts with Snc1 in the Presence of Sec9 \textsuperscript{p}—We next examined the interaction between soluble recombinant Sso1 and GST-Sec9 or GST-Snc1 beads (Fig. 3). Results show that while the full-length Sso1 protein (Fig. 3A) can bind to GST-Sec9 beads, it cannot bind to GST-Snc1 beads under the same binding conditions. Binding was found to be insignificant up to 10 \textmu{}M of soluble Sso1 protein (data not shown); however, Sso1 protein could bind to GST-Sec9 beads in the presence of recombinant Sec9. Taken together with the result seen in Fig. 2, this suggests that in order for Sec9 and Sso1 to bind to Snc1 they must first bind to one another to form an active t-SNARE.

We also examined the ability of the truncated Sso1 protein (Fig. 3B) to bind to GST-Sec9 and GST-Snc1 beads. We found that the truncated protein bound to the GST-Sec9 beads but was unable to bind to GST-Snc1 beads in the absence of soluble Sec9p. In the presence of Sec9p, however, the truncated form of Sso1 could bind to GST-Snc1 beads, and the extent of the binding (96\%) confirmed the fact that in the same binding conditions this fragment can form more t-SNARE complex with Sec9 than the full-length protein (40\% binding).

**Fig. 3. Sso1 protein binds directly to Sec9\textsuperscript{p} but only binds to GST-Snc1 protein in the presence of soluble Sec9\textsuperscript{p}**. A, binding of full-length cytoplasmic domain of Sso1 (1 \textmu{}M) to different GST fusion proteins was analyzed in the presence or absence of soluble excess Sec9\textsuperscript{p} (3 \textmu{}M). B, binding of \textsuperscript{35}S-labeled, truncated Sso1 (1 \textmu{}M) to different GST fusion proteins was analyzed in the presence or absence of soluble excess Sec9\textsuperscript{p} (3 \textmu{}M) as in A. Binding reactions were carried out as for Fig. 2A. Results show that while Sso1p can bind to GST-Sec9, it can only bind to GST-Snc1 beads in the presence of recombinant Sec9p. Radiolabeled Sso1-(193–265) was prepared by thrombin cleavage of in vivo \textsuperscript{35}S-labeled GST-Sso1 fusion protein. The radiolabeled protein was incubated at 1 \textmu{}M final concentration in the absence or presence of excess (3 \textmu{}M) recombinant Sec9p with the different GST fusion protein beads as described above. Results show that, like the full-length protein, the truncated Sso1p can bind to GST-Sec9, but it only binds to GST-Snc1 in the presence of soluble Sec9p protein.
duration binding assays with recombinant Sec17. Binding assays were transferred to nitrocellulose, and blotted with antibodies to Sec17p.

The stoichiometry of the yeast SNARE complex was assessed by preassembling complexes of Sec9, Sso1(1–265), and GST-Snc1 and subsequently incubating in the presence or absence of saturating amounts of Sec17 (30 μM). After washing to remove unbound Sec17, the complexes were boiled in sample buffer with 1% SDS and subjected to SDS-PAGE, and the gel was stained with SYPRO-Red (Molecular Probes) and visualized on STORM 860 (Molecular Dynamics) according to the manufacturers directions. Quantitation of four such experiments gave the molar ratio of GST-Snc1/Sso1/Sec17 as 1.0:1.3:1.2:7, consistent with the 1:1:1:3 stoichiometry observed with the neuronal SNARE complex (17).

**The Binding of the in Vitro Translated SNAP-25 Domain of Sec9 to Sso1 and Snc1 Is Similar to That of Recombinant Sec9 and Shows the Importance of the N-terminal Region in the Interaction with Sso1**—To readily examine the binding of mutant alleles of Sec9 in the assay described above, we designed a set of PCR primers to amplify the SNAP-25 domain of Sec9 such that the PCR-amplified product could be directly transcribed and translated in a rabbit reticulocyte transcription/translation extract. When amplified material is added to the lysate, two translation products of roughly equal intensity appear, one of about 32 kDa and a second of about 25 kDa (Fig. 7A). Both of these proteins are quantitatively immunoprecipitated by polyclonal antibodies (15) raised to the C-terminal 70 residues of Sec9 (data not shown). In *vitro* translation of C-terminal truncation mutants along with the reactivity with the C-terminal antibody show that the lower band is most likely to be due to the use of a downstream methionine for initiation of translation (see Fig. 6). Binding of the full-length SNAP-25 domain translation product appeared to be indistinguishable from that of recombinant Sec9 described above. It binds partially to GST-Sso1 and nearly completely to the truncated Sso1 and interacts with GST-Snc1 only if soluble Sso1 protein is present in the binding reaction.

Since the lower band corresponds to an N-terminally truncated form of Sec9, which results in removal of a region that, in the homologous region of SNAP-25, is required for the interaction with both syntaxin and synaptobrevin, we were also able to determine the association of this mutant form of Sec9. Again, as predicted by the results of similar truncations in its neuronal homolog SNAP-25 (7, 8), this domain appears to be important for the interactions of Sec9 with Sso1, since this protein was unable to bind with either Sso1 or Snc1 in any of the assays (Fig. 7, A and B). However, since the interaction with Snc1 is dependent on the ability of Sec9 to interact with Sso1, it is not possible to determine the effect of a mutation or deletion in Sec9 on the interaction with Snc1 unless it is able to interact with Sso1.

To further delineate the importance of the N-terminal domain of Sec9 for the interaction with Sso1, we examined the binding properties of a mutant Sec9 protein containing a single amino acid substitution in a highly conserved residue in this region. The sec9–4 allele contains a Gly to Asp change at...
residue 458 (see Fig. 6), which confers its temperature sensitivity (15). This glycine is conserved among all known homologs of SNAP-25 and Sec9 and resides between two predicted coiled-coil domains. To determine whether this mutation results in a defect in SNARE interaction, we examined the ability of in vitro translated Sec9–4 protein to bind to Sso1 and Snc1 in vitro. The results, shown in Fig. 8, demonstrate that this mutation has a profound effect on the ability to bind to Sso1. While one would expect this would also eliminate the ability of this protein to form ternary interactions with Snc1 in the presence of Sso1, we found 21% binding in the ternary complex assay, suggesting that the presence of Snc1 can stabilize the interaction of the Sec9–4 mutant protein with Sso1 protein.

Effects of Deletions of the C Terminus of Sec9 That Approximate the Cleavage of SNAP-25 by Botulinum Toxin A—SNAP-25 is inactivated in vivo by proteolytic cleavage of the C-terminal 9 residues by botulinum toxin A (19, 20). Analysis of the binding properties of this C-terminally truncated protein indicated that cleavage primarily effects the interaction of SNAP-25 with synaptobrevin and has little or no effect on the interaction with syntaxin (7, 8). To determine if the C terminus of Sec9 also had a selective role in interacting with Snc1, we prepared two C-terminal deletion alleles of Sec9 by site-directed mutagenesis. One of these alleles, sec9–D38, approximates the botulinum toxin A cleavage of SNAP-25 by the sequence alignment shown in Fig. 6 and results in removal of
the terminal 38 residues of Sec9. The second deletion allele, sec9-Δ17, approximates the cleavage by botulinum toxin A by the distance from the end of the third putative coiled-coil domain. To investigate the biochemical effect of these deletions on the interactions with Sso1 and Snc1, both mutants were in vitro translated and assayed for their ability to interact in a binary assay with GST-Sso1 and for the ternary interaction with GST-Snc1 in the presence of soluble Sso1. The results, shown in Fig. 8, demonstrate that the deletion of the C-terminal 38 residues in the Sec9-Δ38 mutant resembles the effect of botulinum toxin A cleavage; while it has a relatively mild effect on the interaction with Sso1 (55% mutant binding compared with 99% binding for wild type) (7, 8), it completely blocks any detectable ternary interaction with Snc1 (Fig. 8B) (7, 8). In contrast, the binding observed with Sec9-Δ17 mutant protein was virtually indistinguishable from wild type in both the binary and ternary assays (Figs. 8, A and B).

To characterize the in vivo phenotype of these mutants, we introduced them in a diploid yeast strain containing a disrupted copy of one of the SEC9 genes. We then assessed the ability of the mutant alleles to rescue the lethality of the disrupted copy of Sec9 by tetrad analysis of the sporulated diploids (see “Experimental Procedures”). The results demonstrate that both of these alleles are incapable of complementing the disrupted copy and that both the sec9-Δ38 and sec9-Δ17 alleles result in a recessive lethal phenotype when expressed behind the endogenous SEC9 promoter (Table I). Since the sec9-Δ17 mutation had no observable effect on SNARE binding, this strongly suggests that the C-terminal domain is required for a function of Sec9 that is distinct from assembly of the SNARE complex.

The Temperature Sensitivity of the sec9-7ts− Allele Is Due to a Single Leu to His Mutation at the C Terminus of Sec9—In addition to the C-terminal deletions described above we have mapped the site of one of the original temperature-sensitive alleles of Sec9, sec9-7, to the C terminus of Sec9. The temperature-sensitive sec9-7 allele was cloned by the gap repair method and the mutation was found to map to the C-terminal end of the SEC9 coding sequence (see “Experimental Procedures”). The mutation was identified as a T to A transversion within codon 627 changing a leucine codon to a histidine codon (see Fig. 6).

We next examined the effect of this mutation on the ability of the mutant protein to assemble with Sso1 and Snc1 in the binding assay described above. The results shown in Fig. 8 demonstrate that the interaction of Sec9-7 protein with Sso1 and Snc1 in the binary and ternary assays is indistinguishable from that of wild-type Sec9 and thus has no detectable defect in SNARE assembly. Therefore, like the nearby sec9-Δ17 mutation, the in vivo defect associated with this mutation is likely to reside in an interaction that is important for Sec9 function but is not involved in the ability of the protein to assemble into SNARE complexes.

Mutations That Block Sec9 Function but Allow SNARE Assembly Act as Dominant Negative Alleles When Expressed behind the GAL1 Promoter—If the ability of the Sec9-Δ17 and Sec9-7 mutant proteins to assemble into SNARE complexes in vitro correlates with a similar ability in vivo, incorporation of defective components in such a complex would result in a dominant negative phenotype. While the phenotypes of both of these mutants are recessive when these genes are present at single copy, in many cases dominant negative phenotypes require higher than normal levels of expression of the offending protein to become apparent. We therefore expressed the SEC9, sec9-4, sec9-7, sec9-Δ17, and sec9-Δ38 alleles behind the regulated GAL1 promoter, which is strongly repressed in glucose-containing media and activated in galactose-containing media. The constructs were introduced at single copy at the LEU2 locus by integrative transformation and tested for growth on different carbon sources at different temperatures. As expected, the expression of wild type and two Sec9 mutants that block SNARE assembly, sec9-4 and sec9-Δ38, have no effect on growth under any condition tested. In contrast, yeast strains containing the GAL1::sec9-Δ17 constructs show normal growth on glucose media but are unable to grow on galactose-containing media (Fig. 9A), demonstrating a clear dominant negative phenotype. Interestingly, transformants containing the GAL-sec9-7 construct gave normal growth on galactose media at 25 °C but not at 37 °C, showing an unusual temperature-sensitive dominantly negative phenotype. This strongly suggests that the Sec9-7 protein is truly temperature-sensitive for function, rather than the more common situation of a temperature-sensitive mutant resulting from a constitutive defect that, at higher temperature, is unable to keep up with the increased rate of function required. Immunoblot analysis of these strains with Sec9 antisera following 4 h of induction show that all of the constructs express similar amounts of Sec9 (data not shown), indicating that the lack of dominant negative phenotypes seen in GAL-SEC9, GAL-sec9-4, and GAL-sec9-Δ38 is not due to a lack of overexpression.

### TABLE I

<table>
<thead>
<tr>
<th>SEC9 allele</th>
<th>Description</th>
<th>Phenotype as sole copy of SEC9</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>sec9-4</td>
<td>Residue 458 Gly to Asp change</td>
<td>Recessive temperature-sensitive</td>
<td>15, 22</td>
</tr>
<tr>
<td>sec9-7</td>
<td>Residue 627 Leu to His change</td>
<td>Recessive temperature-sensitive</td>
<td>15, this study</td>
</tr>
<tr>
<td>sec9-38</td>
<td>Deletes residues 614–651</td>
<td>Recessive lethal</td>
<td>This study</td>
</tr>
<tr>
<td>sec9-Δ17</td>
<td>Deletes residues 634–651</td>
<td>Recessive lethal</td>
<td>This study</td>
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**FIG. 9.** Mutations in the C terminus of SEC9 that block Sec9 function but allow SNARE assembly act as dominant negative alleles when expressed behind the GAL1 promoter. Each of the alleles shown was subcloned into a GAL1 expression vector (pNB527) and introduced at single copy into a wild type yeast strain by integrative transformation. Transformants were replica-plated onto media containing either 2% glucose (YPD), which represses expression, or 3% galactose (YP-GAL), which induces expression, and plates were incubated at the indicated temperature for 2–3 days. Results show that the sec9-7 and sec9-Δ17 mutants manifest a dominant negative effect on cell growth when overexpressed in the wild type strain.

### DISCUSSION

We report here the first analysis of the biochemical interactions that make up the core of the yeast exocytic SNARE complex. The overall levels of sequence identity observed between Sec9 and synaptobrevin (29%), Sso1 and syntaxin (28%), and Sec9 and SNAP-25 (18.5%) are relatively low, yet the biochemical interactions among the cytosolic domains of these proteins appear to be remarkably well conserved. In addition to the similarity of the interactions among the three “core” SNARE components, the complex of Sso1-Sec9-Snc1 acts as a receptor for the α-SNAP homolog, Sec17, with an affinity similar to that of α-SNAP for the neuronal SNARE complex. Moreover, the stoichiometry of the assembled components of the
yeast SNARE complex appears to be identical to that of the neuronal complex, binding as a 1:1:1:3 complex of Sec9, Sso1, Snc1, and Sec17, respectively. The major difference we see with the yeast SNARE proteins is a strict requirement for a heterooligomeric complex between Sec9 and Sso1, to make an active t-SNARE capable of binding to the v-SNARE, Snc1. While synaptobrevin 1 (VAMP1) can bind individually both to recombinant syntaxin and SNAP-25, it is also clear that it binds with higher affinity and stability when both t-SNARE proteins are present (4, 8). The lack of a detectable one-to-one interaction between Snc1 and the Sec9 and Sso1 proteins may reflect an increased requirement in yeast to keep the t-SNARE activity regulated \textit{in vivo}. Membrane-spanning SNARE proteins, such as Sso1, are inserted into the endoplasmic reticulum immediately following their synthesis and then must follow the secretory pathway to reach their functional destination; for Sso1, this is the plasma membrane (15, 21). Therefore, the Sso1/2 proteins must be present on "inappropriate" membranes during their delivery to the plasma membrane. One mechanism to ensure that the activity of a plasma membrane t-SNARE is restricted to the plasma membrane is to assemble the active complex directly at the site of action. Sec9 is a soluble protein that is peripherally associated with the plasma membrane and is capable of binding to it directly. One prediction of this model is that localization of Sec9 to the plasma membrane should be independent of Sso1. Consistent with this prediction, we have recently found that the membrane localization of Sec9 is unaffected by either \textit{in vivo} depletion of Sso1/2 protein or by mutations (sec9–4 for example) that block the interaction with Sso1 \textit{in vitro}. Therefore, the localization of Sec9 to the plasma membrane is, in fact, likely to be independent of the localization of Sso1/2 to this membrane. This suggests a mechanism by which targeting of Golgi-derived vesicles to the correct membrane would result from the overlap of two independent t-SNARE targeting mechanisms. While each SNARE localization mechanism by itself is likely to be imperfect, the requirement for a dimeric t-SNARE complex between two independently localized t-SNAREs would be expected to guarantee a substantial improvement in the specificity of the targeting reaction. With this in mind, we can understand why the direct interaction among v-SNARES and individual t-SNAREs would be selected against in yeast. The steady state of metabolically active yeast cells is quite different from that of mature neurons, since in yeast it involves constant growth and high level production of new plasma membrane with its complement of new t-SNARE proteins. Therefore, the lack of one-to-one interaction among the v- and t-SNAREs in yeast may be the result of selective pressure to prevent mistargeting of secretory vesicles via interaction with integral membrane t-SNAREs (such as Sso1/2) that are enroute to the plasma membrane. In neurons, where there is a much lower rate of \textit{de novo} synthesis of plasma membrane and SNAREs, it is unlikely that the amount of syntaxin on inappropriate membranes would be sufficient to result in mistargeting.

This system allows us to pursue a combination of biochemical and genetic approaches to understanding the function of SNARE proteins in vesicle transport. For example, we can assess the effect of particular changes in the primary sequence of SNARE proteins on their function \textit{in vivo} as we have done for the two C-terminal deletion mutants, sec9–Δ58 and sec9–Δ17, and we can screen random mutants in SNARE proteins to determine the precise biochemical defects associated with the mutants’ alleles, as we have done with the two temperature-sensitive alleles, sec9–4 and sec9–7. As an example of the utility of such an approach, we have examined \textit{in vitro} the assembly of a number of mutant alleles of Sec9 that have conditional lethal or lethal phenotypes \textit{in vivo}. This analysis has identified a pair of mutations in the C-terminal 17 residues of Sec9, whose binary interactions with Sso1 and ternary interactions with Snc1 are normal \textit{in vitro}, yet are lethal or conditional lethal mutants as the only source of Sec9 \textit{in vivo}. This suggests that the \textit{in vivo} defects observed with these two mutations are likely to result from a defect in the ability of Sec9 to interact with components of the transport machinery other than the SNARE proteins themselves. Consistent with the notion that the mutant proteins are able to assemble into nonproductive SNARE complexes \textit{in vivo}, we find that these same two mutants exhibit a strong dominant negative phenotype when overexpressed behind a regulated promoter. However, the expression of mutants that show clear defects in SNARE assembly \textit{in vitro} shows no detectable effect on growth. We are in the process of identifying extragenic suppressors of the sec9–7 mutant, to isolate candidate gene product(s) that are likely to interact with this domain. Such a factor(s) may play an important role in the function of SNARE-mediated vesicle fusion.

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\textbf{REFERENCES}

Analysis of a Yeast SNARE Complex Reveals Remarkable Similarity to the Neuronal SNARE Complex and a Novel Function for the C Terminus of the SNAP-25 Homolog, Sec9

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