Yeast Snc Proteins Complex with Sec9

FUNCTIONAL INTERACTIONS BETWEEN PUTATIVE SNARE PROTEINS*

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Yeast possess two homologs of the synaptobrevin family of vesicle-associated proteins that are proposed to be involved in membrane recognition and to act as receptors for components of the fusion machinery in neurons. We have previously described the yeast homologs, Snc1 and Snc2, and demonstrated that they localize to secretory vesicles and are required for normal secretion. Yeast lacking Snc protein expression accumulate post-Golgi transport vesicles that contain secretory proteins. Therefore, Snc proteins are essential for the fusion of carrier vesicles with the plasma membrane, and this property appears to have been conserved in evolution.

We have now examined whether Snc proteins interact with other components of the late secretory pathway in yeast. Here we show that Snc proteins form a tight genetic and physical interaction with a plasma membrane protein, Sec9. Sec9 is the yeast equivalent of SNAP-25, a second receptor protein from neurons that has been shown to interact with synaptobrevin. We suggest, then, that recognition of the plasma membrane by secretory vesicles may involve the formation of a Snc-Sec9 complex and that this interaction has evolved as a fundamental step in secretory processes.

The identification of membrane-localized receptors for components of the fusion machinery has significantly altered our perception of the mechanisms that control the trafficking and fusion of carrier vesicles with their target membranes. These receptors, known as SNAREs (soluble NSF attachment protein (SNAP) receptors), are present on both the vesicle (v-SNAREs) and target membrane (t-SNAREs) and are thought to physically associate as cognate pairs (reviewed in Refs. 1 and 2). This finding has allowed others to suggest a simple model for the recognition of target membranes by transport vesicles. Moreover, evidence from both yeast and mammalian systems implies that this mechanism for membrane recognition has been well conserved in evolution.

We have previously identified two yeast homologs of the synaptobrevin family of vesicle-associated membrane proteins that are proposed to act as v-SNAREs in the process of synaptic vesicle docking and fusion with the presynaptic membrane in neurons (3, 4). Snc proteins, Snc1 (5) and Snc2 (6), localize to post-Golgi transport vesicles and are required for the normal secretion competence of yeast cells (6). Yeast lacking Snc protein expression accumulate large numbers of transport vesicles and show a variety of conditional-lethal phenotypes that result from the blockage of vesicle fusion. This finding lends credence to the idea that the Snc/synaptobrevin family of proteins provides compartmental specificity to the trafficking of post-Golgi vesicles and is the principal SNARE on these vesicles.

Numerous other proteins of the late secretory pathway in yeast have already been identified. They include other putative t-SNAREs, Sso1 and Sso2 (7), which are homologs of syntaxins A and B from neurons (8, 9), and Sec9 (10), which is the yeast equivalent of SNAP-25 (11). Both syntaxins and SNAP-25 were previously identified as major proteins of synaptic vesicles and, more importantly, were reisolated as membrane receptors for components of the cell fusion machinery (e.g. SNAPs, NSF, etc.) (3, 4). Thus, one might predict that the yeast homologs of neural t-SNAREs are likely to foster similar interactions with proteins carried on secretory vesicles.

Other yeast genes that are essential for post-Golgi vesicle transport and fusion include those identified in the original sec mutant analysis of Novick et al. (12): Sec1, -2, -3, -4, -5, -6, -8, -10, and -15 (12). Many of these genes have been cloned and aspects of their functions investigated. For example, the role of Sec1 in exocytosis is not yet known; however, both SSO genes were first identified as multicyclop suppressors of the sec1 temperature-sensitive defect (7). Recent studies have now identified Sec1 homologs from Caenorhabditis elegans and mammals, and they have been shown to complex directly with the syntaxins (13, 14). A second gene proven critical for the vectorial transport of carrier vesicles is SEC4 (12). SEC4 encodes a novel Ras-like GTPase that is specific to the late secretory pathway (15). Moreover, cycling of the Sec4 protein between its GTP-bound and GDP-bound states is critical for exocytosis to occur (16). Thus, the regulation of nucleotide binding, hydrolysis, and release must also play a major role in secretion (17, 18). Also, several large multisubunit complexes that contain Sec proteins of unknown function are thought to be required for the vectorial transport of vesicles. A 19.5 S particle, peripherally associated with the plasma membrane, consists of the Sec8 and Sec15 proteins (19), while Sec6 is associated with a large soluble complex having a sedimentation coefficient of 14 S (20). How these complexes interact to bring about vesicle transport, docking, and fusion is not yet known.

In this initial work we have looked for both genetic and physical interactions between the principal v-SNAREs from late secretory pathway of the yeast, Snc1 and Snc2, and putative t-SNAREs from the plasma membrane. Using both genetic and biochemical approaches we have now identified Sec9 as a likely candidate for forming interactions with Snc proteins.

EXPERIMENTAL PROCEDURES

DNA Manipulations—Molecular cloning techniques were performed as described by Sambrook et al. (21). DNA sequencing was performed
Yeast Sacn Proteins Interact with Sec9

Yeast strains created for this study included JG4-9 (Mat a leu2 trpl sncl::URA3 sec-4 pTGAL-SNC1), and JG8-9 (Mat a sncl::URA3 sec2::LEU2 sec-4 pTGAL-SNC1). These strains were created by first taking JG4 cells and crossing them to NY782 cells and dissecting tetrad from the resultant crosses. Segregation of each of the following genes: sec-4, URA3, and trpl was 2:2. All tetrad were leu2 and were unable to grow in the absence of leucine. Next, JG4-9 cells were transformed sequentially with plasmid pTGAL-SNC1 (see above) and a linear DNA fragment containing the LEU2 disruption of SNC2 to yield JG9-9 cells. All disruptions were verified by Southern blot analysis.

RESULTS

Sacn Proteins Suppress Temperature-sensitive Defects in Certain Late-acting Genes on the Secretory Pathway—In order to determine whetherSacn proteins have in common certain aspects of the yeast secretory pathway we first examined if their overexpression could suppress temperature-sensitive (ts) mutations in other known Sec genes. sec mutants can be divided into at least three broad groups defining defects in vesicle budding from the endoplasmic reticulum, endoplasmic reticulum to Golgi, and post-Golgi vesicle transport. It has been shown that the overexpression of genes that encode proteins having overlapping or cognate functions can suppress the temperature-sensitive defects in proteins functioning at a similar level of the secretory pathway. Thus, we tested whether overexpression of SNC1 from a multicopy plasmid could suppress temperature-sensitive mutations in late-acting components of the pathway (summarized in Table I). The levels of SNC1 gene expression were on the order of 10-fold over wild-type levels in these experiments. At temperatures not permissive for the growth of sec mutants (e.g. 34.5–39 °C), we found that the overexpression of SNC1 could weakly suppress the ts defects of sec1 cells. Overexpression of SNC1 could also suppress the ts defect of sec9 cells over a wide temperature range and could confer robust growth at 34.5 °C. Cells transformed with empty vector as a control did not show this suppression pattern (data not shown). In contrast, SNC1 overexpression could not suppress the ts defects seen in sec2, -4, -5, -6, -8, -10, and -15 cells. Neither could SNC1 overexpression suppress a ts defect in a type V myosin (myo2) that is essential for vesicle transport.

Table I: Effect of overexpression of yeast Sacn proteins on mutational hotspots of late-acting components of the secretory pathway.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Mutation</th>
<th>Suppression of Defects</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>34.5 °C</td>
</tr>
<tr>
<td>NY768</td>
<td>sec-1</td>
<td>+</td>
</tr>
<tr>
<td>NY770</td>
<td>sec-21</td>
<td>-</td>
</tr>
<tr>
<td>NY405</td>
<td>sec-4-8</td>
<td>ND*</td>
</tr>
<tr>
<td>NY776</td>
<td>sec-5-24</td>
<td>NT</td>
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<tr>
<td>NY778</td>
<td>sec-6-4</td>
<td>ND</td>
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<tr>
<td>NY780</td>
<td>sec-8-9</td>
<td>NT</td>
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<tr>
<td>NY782</td>
<td>sec-9</td>
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</tr>
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</tr>
<tr>
<td>NY1002</td>
<td>myo2-66</td>
<td>ND</td>
</tr>
<tr>
<td>JG8</td>
<td>sncl1 sec2</td>
<td>ND</td>
</tr>
<tr>
<td>H458</td>
<td>sos1 sos2</td>
<td>ND</td>
</tr>
</tbody>
</table>

* ND, not determined.
NT, not temperature-sensitive.

2 P. Novick, personal communication.

Media and Genetic Manipulations—Yeast were grown in medium containing 2% glucose or 4% galactose as a carbon source. Standard medium (YPD): yeast extract (10 g/L), Dextrose (20 g/L), synthetic minimal medium (SC), and SC drop-out minimal medium, lacking an essential amino acid or nucleotide base, were prepared according to Rose et al. (24). Standard methods were used for the introduction of DNA into the various yeast strains, the preparation of genomic DNA, and for tetrad dissection (24).

Phenotypic Assays and Selections—Assays for cell growth on amino acid-rich medium (YPD) and temperature-sensitive growth on synthetic medium at 37 °C were performed as described (23).

Immunoprecipitation and Immunoblot Analysis—Cells lysates for use in immunoprecipitation experiments and immunoblots were prepared by using glass beads to break intact cells. Between 25 and 50 mg of cells in log phase growth were harvested, washed, and resuspended in a volume of 250–300 μL of lysis buffer consisting of a phosphate-buffered saline solution with 1% Triton X-100, leupeptin (10 μg/mL), aprotonin (10 μg/mL), soybean trypsin inhibitor (10 μg/mL), and 100 μg phenylmethylsulfonyl fluoride. Cell extracts were prepared by adding an equal volume of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and (3 min) prior to electrophoresis on 10–12% acrylamide gels.

For immunoblotting, gels were electroblotted onto GeneScreen Plus nylon membranes (DuPont NEN). Blots were blocked and probed as described previously (23), except that TWEEN 20 was added to a concentration of 0.1%. Protein expression was detected using the ECL chemiluminescence system (Amersham Corp.).

Affinity-purified anti-Sec9 antibody was generously provided by Drs. P. Brennwald and P. Novick. A polyclonal antiserum was raised in rabbits against the last 70 amino acids of the carboxyl terminus of Sec9 fused to the maltose-binding protein. Affinity purification was performed by passing the crude serum over a column containing the Sec9 antigen coupled with CNBr.

Plasmids—Plasmids used in this study included the following previously described plasmids: pADH-SNC1, which contains genomic SNC1 under the control of the ADH1 promoter (5); pTGAL-SNC1 (pGAL-TSNC1), which is a vector pSE358 bearing an ECORI/SacI fragment, containing the GAL10 promoter fused to SNC1, that is cloned into the SmaI/SacI sites (6); and pADH-HASN1C (pADH-LSN1C), which contains a SalI/SacI fragment of SNC1 subcloned into the SalI/SacI sites of vector pAH54 (6). This subcloning results in the addition of a peptide epitope from the influenza virus hemagglutinin antigen (HA) upstream and in-frame to SNC1 (13). Vectors included pSE58, a CEN4-based plasmid bearing the TRPI selectable marker.

Plasmids created for this study included: pGAL-HASN1C, which contains an EcoRI fragment, containing the GAL10 promoter fused to HA-SNC1, cloned into the EcoRI site of pSE585; and pSN2CL, a disruption construct for SNC2 that was created by cloning the BamHI fragment of LEU2 into the BglII site of the SNC2 genomic locus carried in the pUC-based plasmid, p2H (2).
nor could it suppress the lethality of a sso1 sso2 mutation (7). Thus, SNC1 overexpression can ameliorate defects only in specific components of the late secretory pathway. We have also tested whether SNC1 overexpression could suppress ts defects in earlier mutations in the secretory pathway. In particular, we tested whether Snc proteins could suppress defects in components of the cell fusion machinery. Yeast genes SEC17 and SEC18 encode homologs of α-SNAP (26) and NSF (27), respectively. These proteins were first identified as effectors of vesicle fusion in in vitro reconstitution assays for intra-Golgi vesicle transport. Moreover, they appear to be general factors that can mediate bilayer fusion at many different levels of the secretory pathway (1). Overexpression of SNC1 was unable, however, to suppress ts defects in these and other early acting alleles (data not shown).

Synergy between Mutations in SNC Genes and SEC9—Since we have established a genetic link between Snc and Sec9 function, we decided to test whether cells bearing dual sets of mutations (e.g. snc null mutations and the sec9 mutation) show synergy in their growth defects. Such an interaction between mutant alleles could lead to enhanced temperature sensitivity, or even inviability, which would be indicative of a functional association between these proteins. To test this we created yeast strains bearing gene disruptions in both SNC1 and SNC2 and carrying a sec9 temperature-sensitive allele (see “Experimental Procedures”). snc1 sncl sec9 cells (JG9–9) bear a galactose-inducible SNC1 gene and were maintained continually on galactose-containing medium at 25 °C to ensure the viability of the mutants during the selection process.

Yeast sncl null cells, in addition to their inability to secrete normally and their accumulation of secretory vesicles, also show a variety of conditional lethal phenotypes (6). Such cells are temperature-sensitive for growth at 37 °C and are unable to grow on amino acid-rich medium (e.g. YPD) (6). In contrast, sec9 cells are only temperature-sensitive, which implies that the mutant Sec9 protein is partially functional. By definition then, snc1 sncl sec9 mutant strains (JG9–9 cells) must be unable to grow on YPD as well as being temperature-sensitive at 37 °C. If, however, Sec9 and Snc proteins cooperate to bring about the same cellular function, cells bearing both types of mutations would suffer from a more severe phenotype. To determine whether these mutations are synergistic (i.e. yielding synthetic lethality), we examined JG9–9 cells for temperature-sensitive growth at 37, 30, and 25 °C, in the absence of SNC gene expression (Fig. 1). Under the conditions tested, we were unable to culture snc1 sncl2 sec9 cells on glucose-containing medium at any temperature. Such cells were completely inviable in the absence of Snc protein expression. However, these same cells could grow normally, on galactose-containing medium at temperatures ≤30 °C. Thus, the combined mutations result in a lethal phenotype that can be rescued by SNC gene expression. This implies that Snc proteins and Sec9 interact either physically, or in concert, to yield the same cellular event.

In other experiments, we found earlier that snc1 sncl2 sec6 triple mutants did not show synthetic lethality and were viable in the absence of SNC gene expression (6) (data not shown). Thus, the synergy seen between the snc null alleles and the sec9 temperature-sensitive allele is specific and is not a general feature seen with all components of the late secretory pathway.

Sec9 Protein Co-immunoprecipitates with Snc1—Since Snc proteins and Sec9 demonstrate a tight genetic relationship in vivo, we next determined whether they are capable of forming a complex in yeast, as has been described for their neuronal counterparts (3, 4). To do this, we performed immunoprecipitation experiments using an antibody directed against an epitope from the HA. We have previously demonstrated that an HA-tagged form of Snc protein is fully functional and can be used for the immunolocalization of Snc proteins in yeast (6). Immunoprecipitated HA-Snc1 protein complexes, prepared from cells bysed in a non-denaturing detergent, were electrophoresed on SDS-PAGE gels, blotted, and probed separately with antibodies able to detect the HA-Snc1 and Sec9 (see “Experimental Procedures”). The results of a representative experiment are shown in Fig. 2.

HA-tagged Snc1 protein was readily detectable as a single band of 23 kDa in cell lysates and immunoprecipitates from yeast expressing the protein from a galactose-inducible promoter (Fig. 2). In contrast, this band was eliminated from immunoprecipitates formed in the presence of excess HA peptide. Thus, the monoclonal antibody specifically recognizes and precipitates HA-Snc1. In identical immunoblots, performed in parallel, we were able to specifically detect the presence of Sec9, using an affinity-purified anti-Sec9 polyclonal antiserum. Sec9 was detected as a single band of low abundance, corresponding to a molecular weight of approximately 100 kDa, in both total cell lysates and immunoprecipitates (Fig. 2). Moreover, the amount of Sec9 that could be co-immunoprecipitated with HA-Snc1 was roughly comparable with that detected in 50 μg of total cell lysate. It would appear, then, that a significant fraction of Sec9, perhaps as much as 5% of that present in the immunoprecipitation reaction, could be co-immunoprecipitated with Snc protein. In contrast, this 100-kDa band was not observed in immunoprecipitates formed in the presence of excess

![Fig. 2. Sec9 co-immunoprecipitates with overexpressed Snc1 protein.](image-url)
In yeast, secretory carrier vesicles interact with the plasma membrane via two principal receptor moieties: one located on the surface of vesicles (e.g., Sec1 and Sec2) and one located on the plasma membrane (e.g., Sec9). Interaction between these receptors may further involve complexing with other putative receptors of the plasma membrane (e.g., Sec1 and Sec2). Complex formation is likely to be the initial event leading to vesicle docking and formation of the prefusion complex. This may require other components specific to the late secretory pathway (e.g., Sec1-6, -8, -10, and -15). After the vesicle associates with the plasma membrane, interaction of the general fusion machinery (e.g., Sec1-17 and -18) may proceed and is, presumably, the rate-limiting step in bilayer fusion. After fusion, soluble components are released into the cytosol, while membrane components are thought to be recycled via an endocytic-type mechanism.

HA peptide. Thus, the Sec9 protein appears to co-immunoprecipitate with Secn in a specific fashion.

**DISCUSSION**

We have begun to examine the protein requirements for vesicle docking and fusion in constitutive exocytosis. In this preliminary work, we have added to earlier findings that demonstrated complex formation in vitro between proteins that act as SNAP receptors (SNAREs) (3, 4). Previously, it was shown that the SNARE proteins, SNAP-25 and synaptobrevin/VAMP, along with syntaxins A and B, assemble into the prefusion particle that forms between synaptic vesicles and the presynaptic membrane. The assembly of this complex appears to be a prerequisite for the fusion of these membrane-bound compartments.

Here we show that the yeast homologs of SNAP-25 and synaptobrevin interact genetically and also form a tight physical association. The yeast SNAP-25 homolog, Sec9, is a low abundance protein that is localized to the plasma membrane and is a candidate for having possible t-SNARE functions (10). We have shown that overexpression of Secn protein partially suppresses the temperature sensitivity of the sec9 mutant and that combined sec null and sec9 mutations result in cellular inviability. To our knowledge this is the first demonstration of these proteins interacting in vivo, and these data support earlier conclusions made about SNAP-25 and synaptobrevin function (3, 4). Finally, we have shown that Sec9 protein can co-immunoprecipitate with Sec1. A similar finding has been also observed by others (10, 11), and together, these results suggest that Secn proteins and Sec9 participate in the same cellular function. Since both sec null and sec9 mutant cells accumulate secretory vesicles it is likely that these proteins cooperate in bringing about the initial events that lead to membrane fusion, e.g., membrane recognition and vesicle docking.

Based on these observations, as well as those by others, we offer a simple model for recognition of plasma membrane by secretory vesicles in yeast (Fig. 3). In this model receptors on vesicles, Sec1 and Sec2, are capable of interacting directly with a receptor on the plasma membrane, Sec9, to promote vesicle docking. Once brought into close proximity with the membrane, other factors that could aid in vesicle identification and membrane association, such as Sec1, Sec9, and Sec2, may complete the formation of the primary docking event and allow general components of the fusion machinery (e.g., Sec17 and Sec18) to form the fusion complex. Alternatively, the Sec proteins may complex directly with Secn proteins. The generation of a fusion pore between membranes, along with the regulation of ATP binding and hydrolysis by Sec18, probably occurs in a sequential order, as has been proposed for the fusion of synaptic vesicles (4). Since secretion in yeast is constitutive these events must take place in rapid succession over a very short time period. It is unclear whether some form of negative regulation can restrict the fusion of carrier vesicles in yeast, as has been demonstrated for synaptic vesicles.

What is clear, however, is that both the Secn proteins and Sec9 are essential for vesicle docking and fusion to occur. Thus, the roles of these proteins in constitutive secretion in yeast parallel those described for synaptobrevin and SNAP-25 in regulated secretion in neurons. It is likely, then, that this interaction arose early in eukaryote evolution. Moreover, it implies that SNARE complex formation is a fundamental and necessary step in the events leading to the fusion of cellular compartments.

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**REFERENCES**