The yeast lgl family member Sro7p is an effector of the secretory Rab GTPase Sec4p

Bianka L. Grosshans,1 Anna Andreeva,2 Akanksha Gangar,2 Sherry Niessen,3,4,5 John R. Yates III,3,4,5 Patrick Brennwald,2 and Peter Novick1

1Department of Cell Biology, Yale University School of Medicine, New Haven, CT 06520
2Department of Cell and Developmental Biology, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599
3Department of Cell Biology, 4Department of Chemistry, and 5The Skaggs Institute for Chemical Biology, The Scripps Research Institute, La Jolla, CA 92037

Rab guanosine triphosphatases regulate intracellular membrane traffic by binding specific effector proteins. The yeast Rab Sec4p plays multiple roles in the polarized transport of post-Golgi vesicles to, and their subsequent fusion with, the plasma membrane, suggesting the involvement of several effectors. Yet, only one Sec4p effector has been documented to date: the exocyst protein Sec15p. The exocyst is an octameric protein complex required for tethering secretory vesicles, which is a prerequisite for membrane fusion. In this study, we describe the identification of a second Sec4p effector, Sro7p, which is a member of the lethal giant larvae tumor suppressor family. Sec4-GTP binds to Sro7p in cell extracts as well as to purified Sro7p, and the two proteins can be coimmunoprecipitated. Furthermore, we demonstrate the formation of a ternary complex of Sec4-GTP, Sro7p, and the t-SNARE Sec9p. Genetic data support our conclusion that Sro7p functions downstream of Sec4p and further imply that Sro7p and the exocyst share partially overlapping functions, possibly in SNARE regulation.

Introduction

Cell polarization is important for processes as diverse as cell movement, axonal outgrowth, secretion of hormones, and cell differentiation. Polarization requires the vectorial delivery of secretory vesicles to, and their subsequent fusion with, the plasma membrane. The yeast Saccharomyces cerevisiae is an excellent model organism to study mechanisms of polarization because it propagates through the polarized outgrowth of a bud.

In S. cerevisiae, the polarized transport of secretory vesicles to the bud tip or, later in the cell cycle, to the mother-bud neck, depends on the actin cytoskeleton and the actin-based myosin motor protein Myo2p (Pruyne et al., 1998; Karpova et al., 2000). An important factor in this transport event is Sec4p, the founding member of the Rab branch of the Ras superfamily of small GTPases (Salminen and Novick, 1987; Goud et al., 1988). Rab proteins are so-called molecular switches, which cycle between an “on” (GTP bound) and “off” (guanosine 5′-diphosphate [GDP] bound) state. This GTPase switch is influenced by guanine nucleotide exchange factors (GEFs), which trigger the binding of GTP, thus activating the Rabs, and GTPase-degrading activating proteins, which accelerate hydrolysis of the bound GTP to GDP, inactivating the GTPase (for review see Pfeffer, 2001; Segev, 2001). Rabs are thought to accomplish their function by binding specific effector proteins in their GTP-bound state (for review see Pfeffer, 2001; Zerial and McBride, 2001).

Sec4p was found in a screen for mutants that block exocytosis and accumulate secretory vesicles at a restrictive temperature (Novick et al., 1980). Mutants of SEC2, the Sec4p GEF, show an accumulation of vesicles randomly distributed throughout the cell, implying that the activation of Sec4p by Sec2p directs the polarized delivery of secretory vesicles (Walch-Solimena et al., 1997). Supporting the view that activated GTP-Sec4p promotes Myo2p-dependent movement of secretory vesicles along actin cables, Sec4p was found to coimmunoprecipitate with Myo2p (Wagner et al., 2002). Although mutations in SEC4 tightly block secretion (Novick et al., 1980), mutations in actin (ACT1) and MYO2 depolarize secretion and cell surface growth but do not block secretion (Govindan et al., 1995; Karpova et al., 2000). These results imply that Sec4p has at least one function in addition to its role in polarized vesicle delivery. A clue toward one such function came from the identification of Sec15p as a Sec4p effector (Guo et al., 1999b). Sec15p is a subunit of the exocyst, an octameric complex required for tethering secretory vesicles to the plasma membrane in preparation for fusion (TerBush et
larized membrane traffic. Data are accumulating that lgl family members function in polarized function of Sec4p. However, are caused by defects in the actin cytoskeleton (Strand et al., 2003). Combined with the evidence that Sec4p regulates both the transport of secretory vesicles to and fusion with the plasma membrane, it seems likely that there are still more Sec4p effectors to be found. The identification of additional effectors would provide important new insights into the molecular function of Sec4p.

Lethal giant larvae (lgl) was first identified as a tumor suppressor gene in the fly Drosophila melanogaster (for review see Wodarz 2000; Bilder 2004). lgl mutant flies were found to develop malignant tumors in the larval brain and imaginal discs that appear to result from a loss of cell polarity. Subsequently, homologues of lgl have been identified in many organisms, ranging from yeast to humans (for review see Bilder 2004). Because lgl family members are often found to be associated with the actin cytoskeleton, it has been argued that the observed polarity defects in cells bearing lgl mutations are caused by defects in the actin cytoskeleton (Strand et al., 1994; Peng et al., 2000; for review see Baek, 2004). However, data are accumulating that lgl family members function in polarized membrane traffic. D. melanogaster lgl is required for the targeting of proteins to the basolateral membrane (Peng et al., 2000), and the yeast homologues of lgl, Sro7p, and Sro77p (also known as Sop1p and Sop2p, respectively) are redundantly required for exocytosis (Lehman et al., 1999). Sro7p and Sro77p are 55% identical (Kagami et al., 1998; Lehman et al., 1999). Both proteins were originally identified as high-copy suppressors of rho3Δ (Kagami et al., 1998), a Rho GTPase required for actin cytoskeleton polarity and polarized exocytosis (Matsui and Toh-e, 1992b; Imai et al., 1996; Adamo et al., 1999).

In this study, we describe the identification of Sro7p as an effector of Sec4p. Sro7p from yeast extracts as well as purified Sro7p interact specifically with the GTP-bound form of Sec4p. Sro7p was found to coimmunoprecipitate with Sec4p, demonstrating that the interaction we observed in vitro also occurs in vivo. Moreover, we found that Sro7p, Sec4p, and the t-SNARE Sec9p can form a ternary complex, suggesting that Sec4p regulates SNARE function through Sro7p. In agreement with this, genetic analysis shows that Sro7p shares a function with the exocyst downstream of Sec4p.

**Results**

**Identification of Sec4p effectors**

We used an affinity purification approach to identify potential Sec4p effectors. GST-tagged Sec4p was purified from bacteria (Fig. 1, A and B). GST-Sec4p bound to glutathione beads was loaded with either GTP7S or GDP and, subsequently, incubated with wild-type yeast extract. As a control, GST bound to beads was also incubated with extract. The beads were washed several times, and bound proteins were eluted with high salt (see Sec4p affinity chromatography for details). Despite the presence of background proteins (Fig. 1 C), GST lane, differences could be readily detected between the protein bands in the GST-Sec4p-GTP7S and GST-Sec4p-GDP affinities chromatography samples (Fig. 1 C). The protein mixture of each sample was TCA precipitated and subjected to mass spectrometry analysis. Common background proteins were identified by their abundance in both samples and by a survey of published literature (Ho et al., 2002). By definition, an effector binds more strongly to the GTP-bound form of a GTPase than to the GDP-bound form. One measure of the relative abundance of a protein in a protein mixture examined by mass spectrometry is the percentage of residues in the protein sequence that are represented by at least one peptide. We considered all proteins with an at least threefold higher coverage in the sample retrieved from the GTP7S-bound versus the GDP-bound form of Sec4p to be potential Sec4p effectors. Among the identified proteins meeting these criteria was Sro7p, a yeast member of the lgl family of proteins.

**Sro7p binds preferably to the activated, GTP-bound form of Sec4p**

As shown in Table 1, Sro7p displayed fivefold higher mass spectrometry coverage when Sec4p was GTP7S bound compared with its GDP-bound form. To validate these findings, we repeated the affinity chromatography with an extract from yeast cells expressing an integrated HA3-tagged allele of Sro7p. The tag did not interfere with the functionality of the protein, as demonstrated by its ability to suppress the cold sensitivity of an sro7Δ sro77Δ double mutant and the salt sensitivity of an sro7Δ...
mutant strain (Fig. 2 A and not depicted). Western blot analysis confirmed that Sro7-HA₃p migrates at its expected molecular weight (Fig. 2 B). An extract of this strain was incubated with GST-Sec4p in its different nucleotide-bound states or in the nucleotide-free state, GST-Ypt1p and GST. The latter two were used as specificity controls. Ypt1p is another member of the Rab GTPase family that is required for ER-to-Golgi transport in S. cerevisiae (Jedd et al., 1995). To test whether this method indeed distinguishes the different nucleotide-bound states and the nucleotide-free state of Sec4p, we used antibodies to probe for Sec2p and Sec15p. As mentioned above, Sec15p is the only previously documented effector of Sec4p, which was shown by two-hybrid analysis to bind preferentially to a hydrolysis-deficient (GTP locked) allele of Sec4p (Guo et al., 1999b). Sec2p is the exchange factor of Sec4p, which preferentially binds to the nucleotide-free state of Sec4p and, with lesser affinity, to the GDP-bound form of Sec4p (Walch-Solimena et al., 1997; Ortiz et al., 2002). As shown in Fig. 2 C, Sec15p binds specifically to the GTPₙ/S-bound form of GST-Sec4p (α-Sec2), whereas Sec2p binds with the highest affinity to the nucleotide-free state of GST-Sec4p (α-Sec2), thus confirming the nucleotide specificity of the method. To detect Sro7-HA₃p, these samples were analyzed by Western blotting with an α-HA antibody. Confocal microscopy of the Western blot is shown as a loading control. (C) Sro7-HA₃p binds preferentially to GTPₙ/S-Sec4p. GST-Sec4 (Sec4), GST-Ypt1 (Ypt1), or GST immobilized on glutathione beads were incubated with an extract of an SRO7-HA₃ strain, and copurifying proteins were subjected to Western blot analysis using the indicated antibodies. GST-Sec4p and GST-Ypt1p were either stripped of nucleotide (NF) or loaded with GTPₙ/S (GTP) or GDP. Ponceau staining of the Western blot is shown as a loading control. The input lanes represent 0.2 and 0.3%, respectively.

Table I. Mass spectrometry results for Sro7p

| Recovery of Sro7p | GTPₙ/S-Sec4p | GDP-Sec4p |
|-------------------|-------------|-----------|
| Percentage of protein sequence | 9.9 | 1.9 |
| Number of identified peptides | 7 | 1 |
| Identified peptides | R.TVFETENVFPQDYNIR.D | R.SSDDNNANHPHEQYTKPTRK.G |

Figure 2. Sro7-HA₃p from yeast extracts binds preferentially to GTPₙ/S-Sec4p. [A] Expression of SRO7-HA₃ suppresses the cold sensitivity of an sro7Δ sro77Δ strain. Wild-type (WT), sro7Δ sro77Δ, and SRO7-HA₃ sro77Δ yeast strains were spotted in 10-fold dilutions onto YPD media plates and grown at the indicated temperatures. [B] Extracts of a wild-type or SRO7-HA₃ strain [HA] were subjected to Western blot analysis using an α-HA antibody. Ponceau staining is shown as a loading control. [C] Sro7-HA₃p binds preferentially to GTPₙ/S-Sec4p. GST-Sec4p [Sec4], GST-Ypt1p [Ypt1], or GST immobilized on glutathione beads were incubated with an extract of an SRO7-HA₃ strain, and copurifying proteins were subjected to Western blot analysis using the indicated antibodies. GST-Sec4p and GST-Ypt1p were either stripped of nucleotide (NF) or loaded with GTPₙ/S (GTP) or GDP. Ponceau staining of the Western blot is shown as a loading control. The input lanes represent 0.2 and 0.3%, respectively.
Sro7p binds directly to Sec4p

We next determined whether purified Sro7p and Sec4p bind to each other in the absence of other proteins. Because Sro7p cannot be purified from bacteria (unpublished data), it was purified from yeast using a multistep purification protocol (see Purification of full-length Sec9 and Sro7 for details). This purification resulted in an Sro7p preparation that appears homogeneous by SDS-PAGE and Coomassie staining (Fig. 3 A) and dissociated from its binding protein, Sec9p (Lehman et al., 1999; unpublished data). As shown in Fig. 3 B, purified Sro7p binds to GST-Sec4p preferentially in the presence of GTPγS. The amount of Sro7p bound to GST-Sec4p ranges from ~5 to 20% of total Sro7p bound to GTPγS-Sec4p, and 1/20–1/10 of this amount bound to the GDP-bound or nucleotide-free Sec4p (Fig. 3 B). The observed binding is specific because Sro7p does not bind to any of the nucleotide-bound and -free forms of GST-Ypt1 or GST alone (Fig. 3 B and not depicted). Thus, purified Sro7p binds to Sec4p in its activated state. Given the strength of the observed interaction (Fig. 3 B) and the purity of Sro7p (Fig. 3 A), we conclude that this interaction is very likely to be direct because any copurifying factor would be substoichiometric.

Sro7p and Sec4p interact in vivo

To test whether this interaction takes place in vivo, coimmunoprecipitation experiments were performed. Because of the low abundance of Sro7p (Ghaemmaghami et al., 2003; Huh et al., 2003), Sro7p and HA-tagged Sec4p or, as a control, HA-tagged Ypt1p, were cooverexpressed in yeast. The HA-tagged Rab proteins were immunoprecipitated using an α-HA antibody, and coimmunoprecipitating Sro7p was detected by Western blotting with an α-Sro7p antibody. As shown in Fig. 4, Sro7p coimmunoprecipitates with HA-Sec4p. This interaction is specific because (1) only a background amount of Sro7p is found to interact with Ypt1p; (2) the signal requires the cooverexpression of both Sec4p and Sro7p; and (3) no signal can be detected when the antibody is omitted from the precipitation reaction (Fig. 4). These data further support the conclusion that Sro7p binds directly to Sec4p because no third protein was overexpressed. Quantification of the interaction revealed that ~2% of total Sro7p bound to GST-Sec4p (immunoprecipitated amount set to 100%). This relatively low amount might reflect the predominantly inactivated state of Sec4p in a yeast lysate. Together, our in vivo and in vitro data establish Sro7p as an effector of Sec4p.

Sec4p, Sro7p, and Sec9p form a trimeric complex

Sro7p and its parologue, Sro77p, belong to the lgl tumor suppressor family (Kagami et al., 1998; Larsson et al., 1998; Lehman et al., 1999). Both proteins have been found to localize to the plasma membrane and to be required for exocytosis in yeast (Kagami et al., 1998; Larsson et al., 1998; Lehman et al., 1999). Moreover, it has been shown that Sro7p binds to the plasma membrane t-SNARE Sec9p and that this interaction is required for Sro7p’s function (Lehman et al., 1999; Gangar et al., 2005). Although no direct, GTP-specific interaction of Sec4p with SNAREs was previously found (Grote and Novick, 1999), our data suggested the possibility that GTP-Sec4p directly signals to SNAREs via Sro7p. To explore this possibility, we performed in vitro binding assays using recombinant GST-Sec4p, Sec9p, and purified Sro7p. As shown in Fig. 5, Sec9p interacts with Sec4p, but only in the presence of GTPγS and Sro7p. Under the conditions used in this assay, ~5–10% of the input Sro7p binds to Sec4p. Only background binding of Sec9p to GTPγS-Sec4p was observed when Sro7p was omitted.
from the binding assay, suggesting that Sec9p binds to Sec4p through Sro7p and implying that Sec4p and Sec9p use different binding sites on Sro7p (Fig. 5). The specificity of this interaction was demonstrated by the fact that no significant binding of Sro7p or Sec9-His6p to GDP-Sec4p or GST was detected (Fig. 5, GST). Therefore, our data imply that GTP-Sec4p, Sro7p, and Sec9p are able to form a ternary complex.

**Sro7p interacts genetically with Sec3p, a subunit of the exocyst complex**

Our data suggest that Sec4p might play a role in SNARE regulation via its effector, Sro7p. The other known effector of Sec4p is Sec15p, a member of the exocyst complex (Guo et al., 1999b). The exocyst is required for the tethering of secretory vesicles to the plasma membrane (TerBush et al., 1996; Guo et al., 1999a), a step that precedes the final SNARE-mediated fusion of those vesicles with the plasma membrane. If Sro7p and the exocyst act in converging pathways, each downstream of Sec4p, the overexpression of Sro7p might be expected to compensate for the loss of exocyst function, and, conversely, the loss of Sro7p would exacerbate the phenotype of exocyst mutants. As shown in Fig. 6 A, overexpression of SRO7 on a \( 2\mu \) plasmid partially rescues the temperature sensitivity of a sec3\( \Delta \) mutant. In agreement with this, it has recently been published that the overexpression of SRO7 rescues the exocytosis defect of the sec3\( \Delta \) mutant (Zhang et al., 2005). Interestingly, however, the level of suppression achieved by overexpression of SRO7 is not as strong as that achieved by 2\( \mu \) SEC4 (Fig. 6 A, compare sec3\( \Delta \) 2\( \mu \) SRO7 with sec3\( \Delta \) 2\( \mu \) SEC4). We found that the deletion of SEC3 increases the doubling time of a yeast strain about twofold compared with wild-type yeast at 30\( ^\circ \)C in synthetic complete (SC) minimal medium (225 \( \pm \) 21 min vs. 125 \( \pm \) 7 min; Fig. 6 B). Overexpression of SEC4 rescues this growth defect to nearly wild-type levels (145 \( \pm \) 7 min), but sec3\( \Delta \) cells overexpressing SRO7 still display a strong growth defect (190 \( \pm \) 14 min; Fig. 6 B). Similarly, 2\( \mu \) SRO7 does not suppress the lethality of sec3\( \Delta \) on yeast peptone dextrose (YPD) media (Fig. 6 C; Wiederkehr et al., 2003). One interpretation of these data is that Sec4p signals to other effectors in addition to Sro7p and the exocyst to achieve vesicle fusion.

In support of our suggestion of converging functions for Sro7p and the exocyst, deletion of SRO7 in a sec3\( \Delta \) strain further aggravates its growth defect (Fig. 7 A). We observed that the doubling time of a sec3\( \Delta \) sro7\( \Delta \) strain (360 \( \pm \) 28 min) is almost twice that of a sec3\( \Delta \) single mutant strain (225 \( \pm \) 21 min; Fig. 7 B). This growth phenotype is accompanied by a significant drop in exocytosis in the sec3\( \Delta \) sro7\( \Delta \) mutant of \( \sim \)10% compared with the sec3\( \Delta \) mutant (73 \( \pm \) 7\% vs. 60 \( \pm \) 5\% of secreted invertase; Fig. 7 C). This further implicates Sro7p and the exocyst in interrelated functions on the exocytic pathway.

Because we observed a synthetic growth defect in the sec3\( \Delta \) sro7\( \Delta \) mutant strain, we decided to test whether the deletion of SRO7 would have a similar influence on the growth of other exocyst mutant strains. For that purpose, we used a collection of temperature-sensitive late secretory mutant strains (sec1-1, sec2-41, sec3-2, sec4-8, sec5-24, sec6-4, sec8-9, sec9-4, sec10-2,
and sec15-1). We observed that sec2-41, sec3-2, sec4-8, sec9-4, and sec15-1 in combination with sro7Δ displayed slower growth compared with the single mutant strains at the permissive temperature of 24°C (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200510016/DC1). Interestingly, the most striking feature we observed is that combining a deletion of SRO7 with almost all of the heat-sensitive mutants leads to a synthetic growth defect at 14°C (Table II and Fig. S1). Double deletion of SRO7 and its parologue SRO7Δ has been found to lead to cold sensitivity of the resulting double mutant strain (Kagami et al., 1998; Lehman et al., 1999), which demonstrated that their gene products share a common function. We found that the double mutants of the Rab GTPase Sec4p (sec4-8) or its GEF Sec2p (sec2-41) with sro7Δ display synthetic lethality at 14°C (Table II and Fig. S1). This result further indicates a common function of Sro7p and Sec4p. The fact that sro7Δ causes sec1-1, a heat-sensitive mutant of the SNARE-interacting protein Sec1p, and sec9-4, a mutant of the t-SNARE Sec9p, to become cold sensitive (Table II and Fig. S1) adds genetic evidence for a role of Sro7p in SNARE function in yeast. Interestingly, we also found that the deletion of SRO7 leads to cold sensitivity in only a subset of the temperature-sensitive exocyst mutant strains (Table II and Fig. S1). These data might either reflect the relative strength of these mutant alleles at low temperature or indicate that only those subunits share a function with Sro7p and, therefore, add further evidence for a functional specialization of exoyct subunits within the complex (Wiederkehr et al., 2004). Altogether, our genetic data support a possible role for Sro7p in transferring the signal of the Rab GTPase downstream of Sec4p.

### Genetic evidence for a role of Sro7p downstream of Sec4p

Although Sec3p is the only nonessential exocyst protein (Wiederkehr et al., 2003), a previous study revealed that SEC5 and EXO70 also could be deleted if either Sec4p or Sec1p were overproduced (Wiederkehr et al., 2004). Given our data that overproduction of Sro7p rescues the growth defect of a sec3Δ mutant strain, we hypothesized that Sro7p might also be able to overcome the lethality of exo70Δ and sec5Δ mutant strains. As shown in Fig. 8, overexpression of SRO7 on a 2μ plasmid indeed rescues the lethality of both exo70Δ (Fig. 8 A) and sec5Δ (Fig. 8 B) mutant strains. Because we found that Sro7p is an effector of Sec4p with an exocyst-related function, it appeared likely that Sro7p would be required for the Sec4p-mediated rescue of exo70Δ and sec5Δ mutant strains (Wiederkehr et al., 2004). Indeed, the deletion of SRO7 reduces the growth of both exo70Δ and sec5Δ strains rescued by the overexpression of SEC4 from a 2μ plasmid (Fig. 9, A and B; compare exo70Δ 2μ SEC4 or sec5Δ 2μ SEC4 with exo70Δ sro7Δ 2μ SEC4 or sec5Δ sro7Δ 2μ SEC4). We found that the doubling times of the exo70Δ sro7Δ 2μ SEC4 (240 ± 21 min) or sec5Δ sro7Δ 2μ SEC4 (242 ± 31 min) yeast strains are significantly increased compared with the yeast strains without additional deletion of SRO7 (190 ± 14 min and 185 ± 21 min, respectively; Fig. 9, C and D). In striking contrast, deletion of SRO7 was found to only have minor influences on the growth of both exo70Δ and sec5Δ mutant strains rescued by overexpression of SEC1 from a 2μ plasmid on either solid or liquid media (Fig. 9, compare exo70Δ 2μ SEC1 or sec5Δ 2μ SEC1 with exo70Δ sro7Δ 2μ SEC1 or sec5Δ sro7Δ 2μ SEC1). Therefore, these data provide genetic evidence that Sro7p functions downstream of Sec4p in an exocyst-related function. They further imply that although Sec4p signaling is upstream

### Table II. Synthetic growth defects of temperature-sensitive secretory mutant strains when combined with sro7Δ

| Mutant            | Growth at 14°C |
|-------------------|----------------|
| sec1-1 sro7Δ      | Slow           |
| sec9-4 sro7Δ      | Slow           |
| sec2-41 sro7Δ     | None           |
| sec4-8 sro7Δ      | None           |
| sec3-2 sro7Δ      | None           |
| sec5-24 sro7Δ     | Wild-type like |
| sec6-4 sro7Δ      | Wild-type like |
| sec8-9 sro7Δ      | None           |
| sec10-2 sro7Δ     | Slow*          |
| sec15-1 sro7Δ     | None           |

*Single temperature-sensitive mutant strains grow comparably with wild-type yeast at 14°C. Double temperature-sensitive sro7Δ mutant strains display the indicated synthetic growth phenotypes at this temperature after growth for 7 d. *Only slight growth defects were observed.
of Sro7p and the exocyst, Sec1p functions downstream of both (summarized in the model in Fig. 10). Our results are also consistent with previous data that showed that Sec4p and Sec1p use different mechanisms for suppression of exocyst deletion mutations (Wiederkehr et al., 2004).

**Sro7p does not bypass all exocyst functions**

Given the fact that both Sro7p and the exocyst are Sec4p effectors and that the overexpression of SRO7 suppresses the phenotypes of three different exocyst deletions, it appeared possible that Sro7p would be able to completely bypass the exocyst, which would imply that Sro7p and the exocyst have identical functions. We assessed this possibility by investigating whether the overexpression of SRO7 (or, if necessary, SRO7 in combination with SEC4) would rescue the lethality of a sec15Δ strain. Sec15p is the subunit of the exocyst that directly interacts with Sec4p (Guo et al., 1999b). It has been previously shown that the overexpression of SEC4 and SRO7 was able to suppress the temperature sensitivity of a sec15-1 mutant strain.
Figure 10. Model for the Sec4p signaling pathways. Secretory vesicles (V) carry the Rab GTPase Sec4p and its GEF Sec2p, which keeps Sec4p in its activated, GTP-bound state. Sec15p, a member of the exocyst complex, is one effector for Sec4p, and the interaction of these two proteins is required for the assembly of this complex and its tethering function in exocytosis (Guo et al., 1999b). Sec1p interacts with the exocyst (Wiederkehr et al., 2004) and binds to assembled SNARE complexes, possibly stabilizing them (Carr et al., 1999). Another effector of Sec4p, Sro7p (this study), interacts with the exocyst subunit Sec9p (Lehman et al., 1999), and genetic data indicate that this interaction is required for Sec4p's role in exocytosis (Brennwald et al., 1994; Lehman et al., 1999; and this study). A recent study showed that the exocyst and the yeast Igl family members interact (Zhang et al., 2005), allowing an integrated response. Arrows indicate physical interactions.

Discussion

Sro7p is an effector of the secretory Rab GTPase Sec4p

Sec4p is a member of the Rab GTPase family that plays important roles in the yeast secretory pathway (Salminen and Novick, 1987; Goud et al., 1999b; Lehman et al., 1999). However, under all tested conditions (different media and temperatures), neither 2μ SRO7 nor 2μ SRO7 in combination with 2μ SEC4 were able to suppress the lethality of a sec15Δ strain (Fig. S2 B and not depicted). These genetic data indicate that Sro7p shares some, but not all, functions with the exocyst in yeast.

Several previously published reports support our findings. Sro7p and its parologue Sro77p were originally identified as high-copy suppressors of Rho3p (Matsui and Toh-e, 1992a; Kagami et al., 1998), which is a Rho GTPase required for actin cytoskeleton polarity and polarized exocytosis in yeast (Matsui and Toh-e, 1992b; Imai et al., 1996; Adamo et al., 1999). Another protein found in this screen (Sro6p) was subsequently identified as Sec4p. These genetic data suggest that the identified proteins positively influence cell polarity and/or polarized exocytosis, as had been shown for Sec4p (Salminen and Novick, 1987; Goud et al., 1988). Subsequently, Sro7/77p have also been found to be required for exocytosis in yeast (Lehman et al., 1999). Additionally, the overexpression of Sro7p was found to suppress the cold sensitivity of a sec4-P48 mutant (Brennwald et al., 1994), which suggested that Sro7p might act downstream of Sec4p function.

Sro7p genetically interacts with the exocyst downstream of Sec4p

Genetic data presented in this study indicate that the function of Sro7p partially overlaps with that of the exocyst (Figs. 6–9), an eight-subunit vesicle-tethering complex required for tethering secretory vesicles to the plasma membrane (TerBush et al., 1996; Guo et al., 1999a). We found that the overexpression of SRO7 suppresses the growth defects of three different exocyst deletion mutants (sec3Δ, sec5Δ, and exo70Δ; Figs. 6 and 8), extending recently published data (Zhang et al., 2005). We also showed that the deletion of SRO7 impairs the growth and secretory function of a sec3Δ strain (Fig. 7), which further implies that Sro7p and the exocyst function in concert.

We recently established that the overexpression of either Sec4p or Sec1p can bypass the inviability of sec5Δ or exo70Δ strains (Wiederkehr et al., 2004). Interestingly, we now demonstrate that the deletion of SRO7 in exo70Δ or sec5Δ strains reduces growth only when SEC4 is overexpressed (Fig. 9). If SEC1 is overexpressed, no significant difference in growth can be detected upon SRO7 deletion (Fig. 9). These data provide genetic evidence that Sro7p acts downstream of Sec4p in its capacity as a suppressor and demonstrate that Sro7p is involved in a Sec4p- and exocyst-related function (Fig. 10). Moreover, they further strengthen the findings by Wiederkehr et al. (2004) that indicate that Sec4p and Sec1p use different mechanisms in their suppression of the two exocyst mutants.

Although SRO7, SEC1, and SEC4 can each act as a high-copy suppressor of sec3Δ, sec5Δ, and exo70Δ, there is no simple hierarchy in the efficiency of suppression. Thus, SEC4 suppresses sec3Δ and sec5Δ better than does SRO7 (Figs. 6 and 8), but SRO7 is somewhat better at suppressing exo70Δ than is SEC4 (Fig. 8). Furthermore, SEC1 suppresses exo70Δ much better than does SEC4, but SEC4 suppresses sec5Δ much better than does SEC1 (Fig. 8). Although this pattern is presently difficult to interpret, it does support the notion that different subunits of the exocyst fulfill distinct functions (Wiederkehr et al., 2004), possibly in tethering and regulation of SNARE function.

As mentioned above, the Sec15p subunit of the yeast exocyst is also a Sec4p effector (Guo et al., 1999b). If Sro7p and the exocyst have purely redundant functions downstream of Sec4p, the overexpression of one effector should overcome the loss of the other. Contrary to this prediction, we found that...
overexpression of SRO7, either alone or in combination with SEC4, did not suppress the lethality of a sec15Δ mutant under all conditions tested (Fig. S2 B and not depicted). Thus, these data indicate that Sro7p and the exocyst have interrelated but not identical functions. In agreement with this, the overexpression of individual exocyst subunits did not suppress the salt sensitivity of the sro7Δ mutant (Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200510016/DC1) or the cold sensitivity of an sro7Δ sro77Δ mutant (Zhang et al., 2005).

Although it might simply be that more than one subunit (i.e., a subcomplex) is required for suppression, another explanation for this finding is that Sro7p and Sro77p have important cellular functions beyond their role in exocytosis. In agreement with this, overexpression of either of the t-SNAREs, SEC9 and SSO1, or the SNARE regulator SEC1 failed to suppress the sro7Δ sro77Δ phenotype (Zhang et al., 2005). Sro77p has been shown to interact biochemically with the yeast type II myosin Myo1p, an interaction that appears to play a role in the remodeling of the actin cytoskeleton (Kagami et al., 1998), which is consistent with data concerning lgl family members from other organisms (see Introduction). Another explanation for the failure of overexpression of single exocyst subunits to suppress the salt or cold sensitivity of Sro77p mutants could be that Sro7p and Sro77p act downstream of some, but not all, aspects of exocyst function (Wiederkehr et al., 2004; Zhang et al., 2005).

Sro7p links a Rab (Sec4p) to a t-SNARE (Sec9p)

Rab GTPase activity has previously been implicated in the regulation of SNARE complex assembly (Lian et al., 1994; Sogaard et al., 1994). Furthermore, genetic evidence suggested that plasma membrane SNAREs act in response to Rabs because the overexpression of SEC9 has been found to suppress the growth defect of a sec4-p48 mutant strain (Brenwald et al., 1994). Nonetheless, a direct interaction of activated Sec4p and the exocytic SNAREs could not be detected (Grote and Novick, 1999). We provide evidence in this study that GTP-bound Sec4p interacts with the plasma membrane SNAREs via Sro7p. The Sec4p effector Sro7p was found to interact with the t-SNARE Sec9p (Lehman et al., 1999), and recent data indicate that this interaction is important for Sro7p’s function in secretion (Gangar et al., 2005). We have demonstrated that Sec9p can associate with Sec4p, but only in the presence of GTPγS and Sro7p (Fig. 5). This interaction provides a link between Rab signaling and SNARE function in yeast exocytosis.

Together, two convergent signaling pathways from the Rab GTPase Sec4p appear to lead to vesicle fusion in yeast (Fig. 10). Secretory vesicles carrying activated Sec4p transmit a signal to Sec15p, which leads to exocyst assembly and vesicle tethering (Guo et al., 1999b). A recently documented association of Sec1p with the exocyst (Wiederkehr et al., 2004) potentially links this pathway to SNARE function. In a second branch of the pathway, Sec4p transmits a signal through the lgl family member Sro7p. Because Sec4p, Sro7p, and the t-SNARE Sec9p can assemble into a ternary complex, we propose that Sro7p conveys the signal from the Rab GTPase to SNARE function in yeast. These interactions may normally occur after vesicle tethering by the exocyst. There appears to be crosstalk between these two pathways because Sro7p has recently been shown to associate with the exocyst subunit Exo84p (Zhang et al., 2005). Further studies will be necessary to explore these proposals.
GST-Ypt1p were incubated with a 200-fold excess of either GTPγS or GDP in NB for 2 h at 30–37°C. To obtain the nucleotide-free state, the proteins were incubated in NB buffer supplemented with 10 mM EDTA.

Purification of full-length Sec9 and Sro7

Full-length Sec9p tagged with COOH-terminal His6 tag was purified from E. coli as described previously (Gangar et al., 2005). Sro7p with an NH2-terminal protein A/tobacco etch virus (TEV) tag was isolated from lysates prepared from overexpressing yeast strains using affinity chromatography with IgG Sepharose beads. It was then eluted by cleavage of the protein A tag with TEV protease and subsequently purified by ion exchange chromatography to apparent homogeneity based on SDS-PAGE analysis.

Sec4p affinity chromatography

The protocol was adapted from Christoforidis and Zerial (2000). In brief, 100 μl GTPγS- or GDP-bound GST-Sec4p or GST-containing beads (−750 μg of protein) were incubated with 25 ml of wild-type yeast extract (40 mg/ml) for 2 h at 4°C. After several washings, bound proteins were eluted with elution buffer (20 mM Hepes, pH 7.2, 1.5 M NaCl, 20 mM EDTA, and 1 mM DTT) supplemented with 5 mM of the opposing nucleotide. The proteins were TCA precipitated, dried, and analyzed by mass spectrometry.

For the experiment in Fig. 2, 2.5 μl of loaded beads (∼15 μg) were incubated with 1 ml of a 20-μg/ml yeast extract (lysed using a French press; Sim-Amico Spectronic Instruments). Bound proteins were analyzed by Western blotting.
Mass spectrometry

Samples were suspended in 8 M urea and 100 mM Tris, pH 8.5, reduced with 100 mM TCEP, and cysteines were alkylated with 55 mM iodoacetamide. Lys-C was used to digest the proteins for 4 h at 37°C at a concentration of 1 µg/100 µl. CaCl2 was added to ensure tryptic specificity at 1 mM, and trypsin was used to digest the samples further at 1 µg/100 µl. The digests were then analyzed by µLC/µLC-MS/MS using an ion trap mass spectrometer (LCQ Deca; ThermoElectron). Multidimensional chromatography was performed online according to MacCoss et al. (2002) using the following salt steps of 500 mM ammonium acetate: 10, 25, 35, 50, 65, 80, and 100%. Tandem mass spectra were collected in a data-dependent fashion by collecting one full MS scan (m/z range = 400–1,600) followed by the acquisition of MS/MS spectra of the three most abundant precursor ions. The collection of resulting spectra was then searched against a database of yeast ORFs obtained from the Saccharomyces genome database (release date 08/27/04) using the SEQUEST algorithm (Eng et al., 1994). Peptide identifications were organized and filtered using the DTASelect program (Tabb et al., 2002). Filtering criteria for positive protein identifications in the Sm3p purification were the identification of two unique, fully tryptic peptides with Xcor values >2.0 for +1 spectra, 2.2 for +2 spectra, and 3.75 for +3 spectra.

In vitro binding assays

Glutathione beads carrying 6 µM GST-Sec4p or GST were washed with 20 mM Tris, pH 7.5, 100 mM NaCl, and 1 mM DTT and were incubated in 20 mM Tris, pH 7.5, 100 mM NaCl, 5 mM EDTA, and 1 mM DTT in the presence of either 100 µM GTPγS, 100 µM GDP, or no nucleotide for 15 min at 25°C. Then, MgCl2 was added to a final concentration of 25 mM and incubated 45 min at 25°C. Binding assays were performed in binding buffer (20 mM Tris-HCl, pH 7.4, 140 mM NaCl, 5 mM MgCl2, 1 mM DTT, and 0.5% Triton X-100). The final concentrations of GST/GST-Sec4p in the binding reactions were 4 µM, whereas concentrations of Sro7p or Sec9p were 1 µM. Samples were incubated at 4°C for 1 h. The beads were washed four times with binding buffer, and bound proteins were subjected to Western blot analysis. Sro7p and Sec9p were detected with rabbit a-Sro7p or a-Sec9 antibodies and a-rabbit IgG conjugated to AlexaFluor680 and were analyzed on an Odyssey Infrared Imaging System (LI-COR). For the experiment in Fig. 5, Sro7p-Sec9p mixtures (or Sro7p or Sec9p alone) were preincubated on ice for 15 min at 25°C. Beads were pelleted, 7.5 µl rat a-HA antibody was added to the supernatants, and the reactions were incubated overnight at 4°C. 6 µl of 50% protein G-Sepharose beads were added and incubated for 90 min at 4°C. Beads were pelleted, 7.5 µl rat a-HA antibody was added to the supernatants, and the reactions were incubated overnight at 4°C. 6 µl of 50% protein G-Sepharose beads were added and incubated for another 30 min. The beads were pelleted, washed several times with immunoprecipitation buffer, and bound proteins were subjected to Western blot analysis.

Invertase secretion

The monitoring of invertase secretion was performed as described previously (Wiedekerke et al., 2003).

Image analysis

Data were digitalized using a scanner (HP Scanjet 4570c; Hewlett Packard).

Online supplemental material

Fig. S1 shows the growth of exocytic temperature-sensitive mutants compared with the deletion of SRO7 compared with the single mutants and wild-type yeast at 24 and 14°C (data are summarized in Table II). Fig. S2 shows that the overexpression of SEC4 and SRO7 suppresses the growth phenotype of a sec13-1 mutant strain [A] but not the deletion of SEC15 [B]. Fig. S3 shows that the overexpression of single exocyst subunits does not suppress the salt sensitivity of an sro7Δ strain.

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