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The exocyst is an effector for Sec4p, targeting secretory vesicles to sites of exocytosis

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Polarized secretion requires proper targeting of secretory vesicles to specific sites on the plasma membrane. Here we report that the exocyst complex plays a key role in vesicle targeting. Sec15p, an exocyst component, can associate with secretory vesicles and interact specifically with the rab GTPase, Sec4p, in its GTP-bound form. A chain of protein–protein interactions leads from Sec4p and Sec15p on the vesicle, through various subunits of the exocyst, to Sec3p, which marks the sites of exocytosis on the plasma membrane. Sec4p may control the assembly of the exocyst. The exocyst may therefore function as a rab effector system for targeted secretion.

Keywords: exocyst/membrane traffic/rab/vesicle targeting

Introduction

Intracellular membrane traffic in eukaryotic cells requires an efficient mechanism to direct different vesicular carriers to their appropriate target compartments. Our studies on the exocyst complex indicate that it may be the central component of the machinery responsible for targeting secretory vesicles to the appropriate exocytic sites on the yeast plasma membrane. The exocyst consists of one copy each of Sec3p, Sec5p, Sec6p, Sec8p, Sec10p, Sec15p and Exo70p (TerBush *et al*., 1996). Components of the exocyst are concentrated in subdomains of the plasma membrane that represent sites of active vesicle fusion (TerBush and Novick, 1995; Mondesert *et al*., 1997; Finger *et al*., 1998). These sites relocate with the yeast cell cycle. As cells enter the cycle, exocyst components are found in a patch at the pre-bud site and, at bud emergence, they are found at the tip of the bud. In large budded cells, when the growth pattern switches from apical to isotropic, the patch disperses around the membrane of the bud. At the time of cytokinesis, exocyst components re-concentrate first as one ring, and then as two rings at the neck separating the mother cell and bud. The localization of Sec3p to these sites is independent of ongoing secretion and of mutations in the other components of the exocyst. These observations led to the proposal that Sec3p represents a spatial landmark for exocytosis and that it may be the component of the exocyst complex most proximal to the target membrane (Finger *et al*., 1998).

The exocyst complex is evolutionarily conserved. A complex was isolated from rat brain consisting of subunits that share significant sequence homologies with the yeast exocyst components (Ting *et al*., 1995; Hsu *et al*., 1996; Guo *et al*., 1997; Hazuka *et al*., 1997; Kee *et al*., 1997). Recently, it was shown that the mammalian Sec8p is essential for the biogenesis of epithelial cell polarity (Grindstaff *et al*., 1998). It is anticipated that related complexes function to direct vesicles to specialized exocytic sites in all eukaryotic cells.

If, as proposed, the exocyst functions in vesicle targeting, it must not only associate with the target membrane, but must also be capable of interacting directly or indirectly with the vesicles. A clue to the nature of a possible connection emerged from studies on Sec15p overproduction (Salminen and Novick, 1989). Expression of Sec15p from the strong *GAL1* promoter inhibits cell growth and leads to the accumulation of a cluster of secretory vesicles. While the normal level of Sec15p is not detectable by immunofluorescence, the overexpressed protein is visible as a distinct patch present in the cytoplasm. This patch of Sec15p co-localizes with Sec4p, the rab protein associated with secretory vesicles (Roth *et al*., 1998). Overexpression of Sec15p in various secretory mutants revealed that the ability of Sec15p to form a patch is dependent upon both Sec4p and Sec2p function, but not on the other components of the exocytic machinery (Salminen and Novick, 1989). Since we now know that Sec2p is a nucleotide exchange factor for Sec4p (Walch-Solimena *et al*., 1997), these results imply that Sec15p responds, either directly or indirectly, to activated Sec4p. In the context of Sec15p overexpression, the result of the response is the clustering of vesicles and the formation of a Sec4p/Sec15p patch. At normal levels of Sec15p expression, the response might be the docking of vesicles carrying activated Sec4p to a site on the plasma membrane marked by Sec3p.

In this study, we demonstrate for the first time that Sec15p associates with vesicles and binds to activated Sec4p. Furthermore, we define a network of protein–protein interactions that connects Sec4p on the vesicle surface, through various components of the exocyst, to Sec3p on the target membrane. We also provide evidence that Sec4p may control the assembly of the exocyst complex. These protein–protein interactions may form the basis for targeting secretory vesicles to specific domains of the plasma membrane.

Results

Sec15p associates with secretory vesicles

To test the hypothesis that Sec15p can associate with secretory vesicles, we have examined the localization of

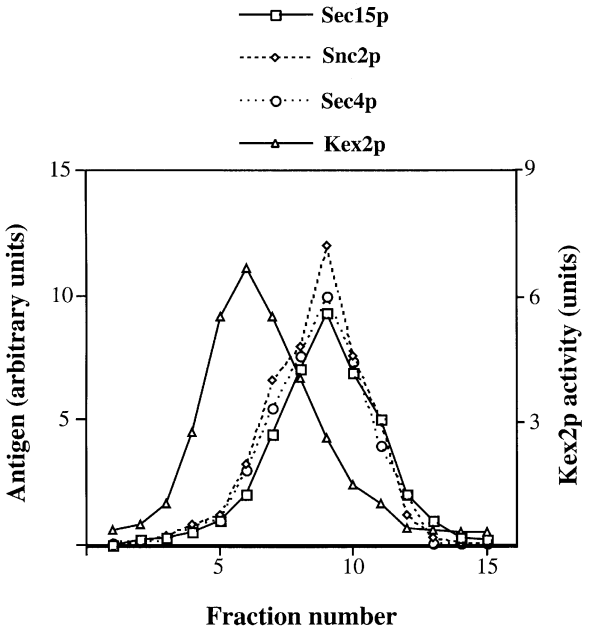

Fig. 1. Sec15p co-migrates with secretory vesicles on sucrose density gradients. Microsomal membranes of NY724 cells overexpressing Sec15p were fractionated on 15–65% sucrose density gradients. The fractions were collected from the top of the gradient and analyzed for Kex2p activities and by Western blotting using antibodies against Sec15p, Sec4p and Snc2p. Sec15p co-migrates with Sec4p and Snc2p, markers of secretory vesicles.

Sec15p by subcellular fractionation. The microsomal pellet (P3) of a lysate from cells expressing Sec15p from the *GAL1* promoter (NY724) was loaded onto sucrose gradients and centrifuged to equilibrium. Fractions were collected and analyzed by Western blotting. As shown in Figure 1, Sec15p co-fractionates with Sec4p and Sncp, the rab protein and v-SNARE associated with secretory vesicles (Goud *et al*., 1988; Protopopov *et al*., 1993). As a control, Sec15p did not co-migrate with Kex2p endoprotease, a *trans*-Golgi marker (Redding *et al*., 1991). This result suggests that the overexpressed Sec15p, like Sec4p and Sncp, associates with secretory vesicles.

To visualize Sec15p directly *in situ*, we examined its localization in these cells using immunoelectron microscopy. Immunogold labeling using affinity-purified anti-Sec15p antibody was found associated with secretory vesicles and the cell wall (Figure 2A and B). The cell wall staining represents a non-specific antigen recognized by anti-Sec15p antibody because an equivalent level of cell wall staining was observed in cells that were not overexpressing Sec15p (Figure 2C). In contrast, the colloidal gold labeling within the cells appears to be specific since it was ~5-fold reduced in non-overexpressing cells (Figure 2C). The labeling is enriched in the vesicles that are close to mother–daughter necks or bud tips. On average, 75 ± 17 gold particles were found to associate with secretory vesicles in each cell section (n = 9), whereas only 20 ± 5 gold particles were found in the remaining cytoplasmic areas. Sec15p labeling was compared with Sec4p labeling in these cells. As shown in Figure 2D, Sec4p is also associated with the accumulated vesicles. This observation is consistent with the immunofluorescence data showing co-localization of Sec15p and Sec4p (Roth *et al*., 1998) as well as with the subcellular fractionation data (Figure 1). No cell wall staining was seen by immunofluorescence labeling with Sec15p antibody (Roth *et al*., 1998; Salminen and Novick, 1989), because the cell walls were enzymatically digested before incubation with antibody in the immunofluorescence labeling procedure.

Sec15p interacts with Sec4p

The results described above indicate that both Sec4p and Sec15p associate with secretory vesicles. We have therefore examined whether Sec4p physically interacts with Sec15p. Immunoprecipitation experiments were performed in non-ionic detergent using anti-Sec15p polyclonal antibody and chemically cross-linked lysates from cells expressing Sec15p from the *GAL1* promoter (NY724). Due to the close proximity of Sec4p and Ypt1p to immunoglobulin light chain on SDS gels, we could not detect these proteins by Western blot. Instead, we used a [^{32}P]GTP overlay assay. As shown in Figure 3A, anti-Sec15p antibody, but not pre-immune serum, immunoprecipitates Sec15p and a small GTP-binding protein. To confirm that this protein is Sec4p rather than another GTP-binding protein, the immunoprecipitate was subjected to a secondary immunoprecipitation with anti-Sec4p or anti-Ypt1p antibody. As shown in Figure 3B, Sec4p antibody efficiently re-precipitated the GTP-binding protein, while the Ypt1p antibody did not re-precipitate any GTP-binding protein from the primary precipitate. These results indicate that Sec4p, but not Ypt1p, can be cross-linked specifically and co-precipitated with Sec15p. Anti-Sec15p antibody was not able to co-immunoprecipitate Sncp, another vesicle protein (not shown). Sec4p can also be co-precipitated with Sec15p without prior cross-linking, although with somewhat reduced efficiency (not shown).

To probe further the interaction between Sec15p and Sec4p, we used the yeast two-hybrid system. Full-length Sec15p was fused with the *GAL4* activation domain in pACTII vector (Durfee *et al*., 1993). Sec4 was fused with the *GAL4* DNA-binding domain in pAS-CYH2. The constructs were introduced into Y190 yeast cells by transformation. Western blotting with anti-hemagglutinin (HA) monoclonal antibody (12CA5) confirmed the expression of the proteins fused with the HA epitope. A panel of *SEC4* constructs was examined to explore the interaction with Sec15p (Table I). Among them were the wild-type Sec4 (Sec4wt), Sec4 with C-terminal residues deleted (Sec4ΔC), and Sec4 Gln79 to Leu mutant (Sec4L79), Sec4 Asn133 to Ile mutant (Sec4I133) and Sec4 Ser29 to Val mutant (Sec4V29). Using the two-hybrid assay, we found that Sec4wt, Sec4ΔC and Sec4L79 interact with Sec15p, whereas Sec4V29 and Sec4I133 do not. Deletion of the C-terminal cysteines abolishes the geranylgeranylation of Sec4p that is necessary for the membrane attachment of this protein (Walworth *et al*., 1989; Jiang *et al*., 1993). The interaction of Sec4ΔC with Sec15p suggests that the C-terminal lipid modification of Sec4p is not required for its interaction with Sec15p. The Sec4L79 mutant has a lower intrinsic GTP hydrolysis rate, and is thought to be predominantly in its GTP-bound form (Walworth *et al*., 1992). This allele yielded somewhat greater β-galactosidase activity than wild-type Sec4p (Table I). Sec4I133 mutant contains an Asn to Ile substitu-

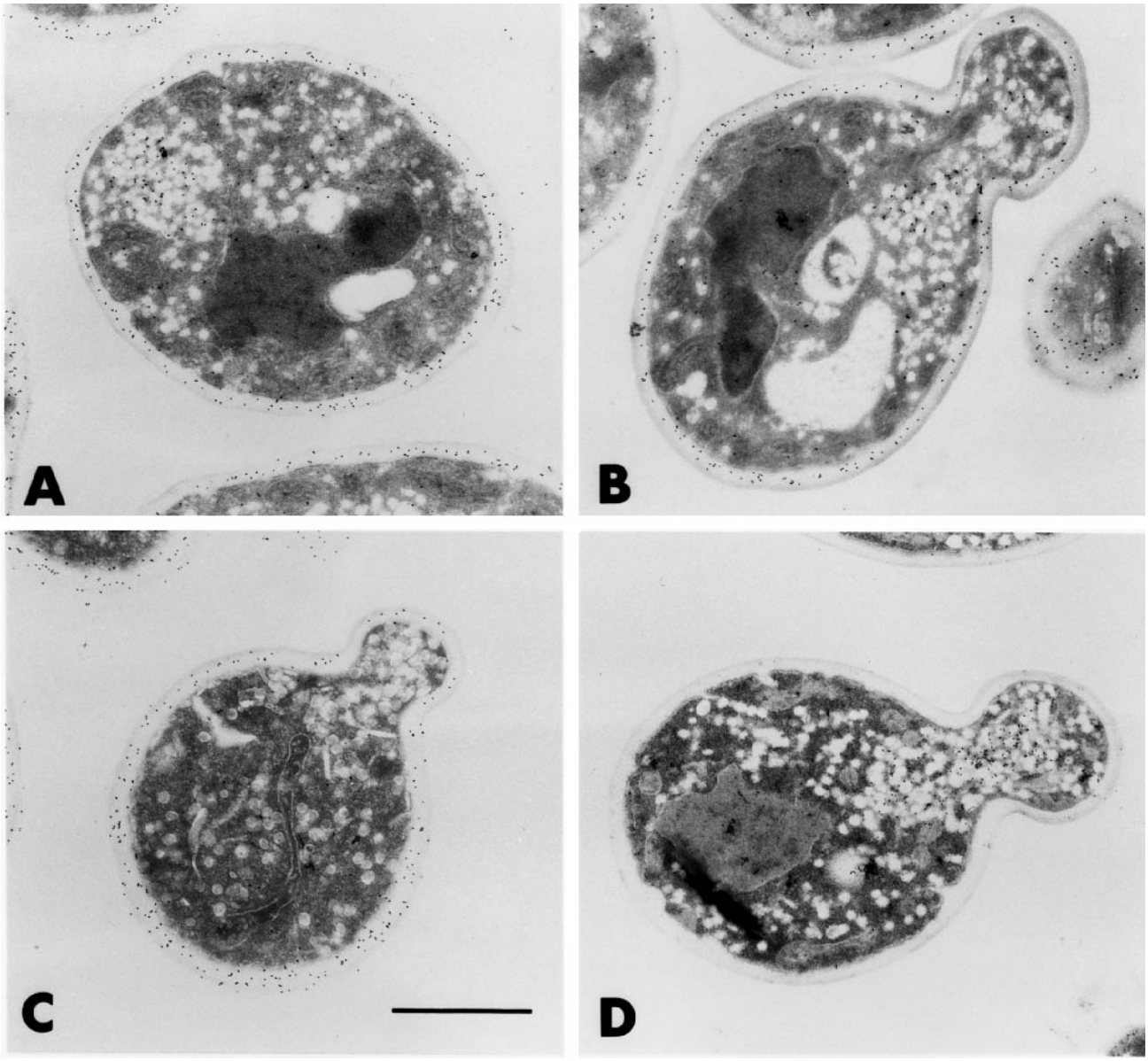

Fig. 2. Immunoelectron microscopic labeling of Sec15p and Sec4p. The cells overexpressing Sec15p were processed and stained using affinity-purified anti-Sec15p antibody (**A** and **B**) and anti-Sec4 antibody (**D**). (**C**) Demonstrates the labeling of Sec15p in *sec6-4* mutant cells as a control for cell wall staining. Protein A–immunogold (10 nm) was used for secondary labeling. (Bar: 1 μm).

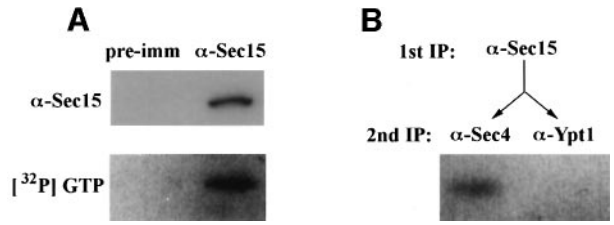

Fig. 3. Sec4p co-immunoprecipitates with Sec15p. Immunoprecipitation experiments were performed using anti-Sec15p antibody with pre-immune serum as a control. The immunoprecipitates were analyzed by Western blotting using anti-Sec15p antibody or [^{32}P]GTP overlay. For double immunoprecipitations (right panel), the anti-Sec15p antibody immunoprecipitates were boiled in a buffer containing 50 mM Tris, pH 8.0, 25 mM EDTA, 100 mM DTT and 1% SDS. The samples were then diluted 1:25 in immunoprecipitation buffer and used in secondary immunoprecipitation with anti-Sec4p or anti-Ypt1p polyclonal antibodies. The immunoprecipitated Sec4p and Ypt1p were detected by [^{32}P]GTP overlay.

tion that abolishes the ability of the protein to bind guanine nucleotides (Walworth *et al*., 1989). This nucleotide-free form of Sec4p does not interact with Sec15p, although it interacts strongly with the nucleotide release factor Dss4p and the nucleotide exchange factor Sec2p (Collins *et al*., 1997; Walch-Solimena *et al*., 1997). In the Sec4V29 mutant, a valine residue substitutes a non-conserved serine. Although the biochemical characteristics of this mutant are not well understood, it is thought to assume a GDP-bound conformation as this mutant binds to GDP dissociation inhibitor (GDI) and a nucleotide exchange protein for Sec4p, Sec2p (Collins *et al*., 1997; Walch-Solimena *et al*., 1997). This mutant fails to bind Sec15p. This pattern of interaction suggests that Sec15p binds specifically to the GTP-bound form of Sec4p. Ypt1p and Ypt51p, two other yeast rab proteins we have tested, failed to interact with Sec15p in the two-hybrid assay, indicating specificity.

Using the two-hybrid assay, we further found that the

Table I. Summary of Sec15p yeast two-hybrid interactions

pAS1-CYH2 constructs	Description	Activity (U)
Sec4 (wt)	wild-type Sec4	39.67 ± 2.70
Ypt1 (wt)	wild-type Ypt1	0.28 ± 0.06
Ypt5 (wt)	wild-type Ypt5	0.21 ± 0.04
Sec4ΔC	non-prenylated	42.24 ± 2.29
Sec4V29	probably GDP-bound conformation	1.69 ± 0.89
Sec4L79	GTP hydrolysis-deficient	75.75 ± 9.12
Sec4I133	nucleotide-free	1.61 ± 0.89
Sec4-EF^{Ypt1}	Sec4 effector domain replaced with the corresponding region of Ypt1	0.53 ± 0.49
Ypt1-EF^{Sec4}	Ypt1 effector domain replaced with the corresponding region of Sec4	0.43 ± 0.18
Sec4-(L7, HV)^{YP}	Sec4 loop 7 and C-terminal hypervariable region replaced with those of Ypt1	8.90 ± 0.55

β-Galactosidase activities (units) are presented as mean ± SEM obtained from three independent transformations.

Sec4p-specific effector domain is crucial for the binding to Sec15p. A comparison of the effector domain of Sec4p (corresponding to loop 2 in the ras structure) with its closest yeast homolog, Ypt1p, reveals only four amino acids that are not conserved (residues 46–49) in this region (Brennwald and Novick, 1993). We have replaced these four amino acids in Sec4p with the corresponding sequence of Ypt1p (Sec4-EF^{ypt1}) and tested its ability to interact with Sec15p. The four amino acid substitutions abolished the binding of Sec4p to Sec15p. This result indicates that the Sec4p-specific effector domain is crucial for the binding to Sec15p. Conversely, we have also replaced the corresponding region of Ypt1p with that of Sec4p. The resulting Ypt1p mutant (Ypt1-EF^{Sec4}) was not able to interact with Sec15p. Thus, the Sec4p effector domain is necessary but not sufficient for the binding specificity to Sec15p.

Previously, we had identified a single Sec4/Ypt1 chimera that can function simultaneously as Ypt1p or Sec4p in yeast cells (Brennwald and Novick, 1993). This chimera, Sec4-(L7, HV)^{YP}, consists of the Sec4p sequence with its loop 7 and C-terminal hypervariable domains replaced with those of Ypt1p. We subcloned this chimera into pAS-CYH2 vector and tested its ability to interact with Sec15p. Positive interaction was found using this mutant. However, this interaction was ~4-fold less than the interaction between Sec15p and the wild-type Sec4p as determined by β-galactosidase activity assay (Table I). As this chimera is able to complement the deletion of *SEC4* in yeast cells, the two-hybrid interaction result is fully consistent with the genetic data.

The assembly of the exocyst complex

The exocyst complex has been suggested to restrict post-Golgi secretion to specific domains of the plasma membrane (TerBush and Novick, 1995; Finger *et al*., 1998). It was proposed that Sec3p is a spatial landmark for polarized secretion on the plasma membrane (TerBush and Novick, 1995; Finger *et al*., 1998). However, to direct secretory vesicles to this landmark effectively, there must be a trail of protein–protein and/or protein–lipid interactions that connects secretory vesicles to Sec3p. The results described above indicate that Sec15p is able to associate with secretory vesicles. It is possible that the assembly of the exocyst complex may provide the link between Sec15p on the vesicle surface and Sec3p at specific domains of the plasma membrane. We therefore investigated the subunit interactions of the exocyst complex. Since the genes for all the components have been identified, we have examined the direct protein–protein interactions *in vitro*. The cDNAs for individual components were subcloned into the pcDNA3 expression vector and the proteins were *in vitro* translated in rabbit reticulocyte lysates. To identify the interactions between the subunits, co-immunoprecipitation experiments were carried out on all pair-wise combinations of co-synthesized proteins. We tagged the individual components with specific epitope sequences, including HA, c-Myc and FLAG. The tagged proteins were then immunoprecipitated using commercially available monoclonal antibodies against the epitope sequences and the precipitates were analyzed for the presence of the non-tagged subunit. The pair-wise interactions identified are shown in Figure 4. For each panel, the left lane shows the precipitation of the protein tagged by the epitope specific to the antibody used. The center lane, as a negative control, shows that the antibody does not precipitate the untagged partner when synthesized alone. The right lane demonstrates the co-precipitation of the untagged protein in combination with the tagged subunit. For example, the direct interaction between Sec6p and Sec8p was identified. As shown in Figure 4, the anti-FLAG antibody was able to precipitate FLAG-Sec6p from the *in vitro* translation mixture. When Sec8p and FLAG-Sec6p were co-synthesized, the antibody was able to precipitate Sec8p in addition to FLAG-Sec6p. In contrast, Sec8p was not precipitated by anti-FLAG antibody in the absence of FLAG-Sec6p. This experiment indicates that Sec8p specifically binds Sec6p. The interaction was also observed in the converse experiment in which 9E10 anti-myc monoclonal antibody only precipitated Sec6 in the presence of Myc-tagged Sec8p (not shown).

We have also utilized the yeast two-hybrid assay to probe the pair-wise interactions between the exocyst components. We have found positive interactions between Sec3p and Sec5p, Sec10p and Sec5p, and Sec10p and Sec15p. These results are consistent with those obtained using *in vitro* translated proteins. All possible pair-wise interactions have been examined using these two strategies. A map summarizing the identified interactions is shown in Figure 5A. They include Sec3p–Sec5p, Sec5p–Sec6p, Sec5p–Exo70p, Sec10p–Sec15p, Sec5p–Sec10p and Sec6p–Sec8p.

These results are fully consistent with previous studies

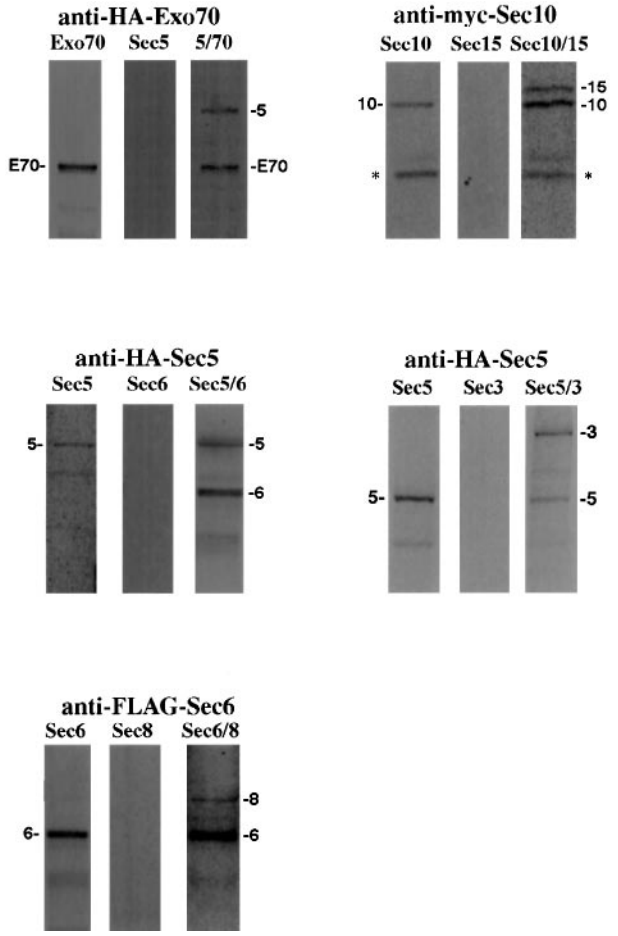

Fig. 4. Protein–protein interactions of *in vitro* synthesized exocyst components. Individual panels show the interactions of the *in vitro* synthesized exocyst proteins by immunoprecipitation using antibodies against the epitopes tagged on the exocyst proteins. Only the identified positive interactions are shown in this figure. For each panel, the left lane shows the immunoprecipitation of the protein tagged by the epitope that can be recognized by the immunoprecipitating antibody. As a negative control, the center lane, shows that the antibody does not immunoprecipitate the untagged binding partner. The right lane demonstrates the co-precipitation of the co-synthesized binding protein. The asterisks indicate a Myc-tagged fragment of Sec10p generated by *in vitro* translation.

in which partially assembled exocyst structures were immunoprecipitated from lysates of various mutant strains. For example, in *sec10-2* mutant cells, the mutant Sec10p is absent from the complex immunopurified using 9E10 antibody against the c-Myc epitope fused to Sec8p (TerBush and Novick, 1995). In addition to Sec10p, Sec15p is also absent from the complex although Sec15p is expressed normally. This result suggests that Sec15p requires Sec10p to associate with the exocyst complex. Our finding that Sec15p physically interacts with Sec10p is consistent with these data (Figure 5B). In *sec5-24* mutant cells, Sec10p, Sec15p and Exo70p are all absent, as is the mutant Sec5p. This is consistent with our finding that Sec5p binds to Sec10p, Exo70p and Sec6p, and, through its binding to Sec6p, Sec5p is connected to Sec8p (Figure 5B).

Except for the positive interactions shown in the map (Figure 5), all the other pair-wise combinations were found to be negative by these methods. The identified interactions link all the individual components of the exocyst. However, we speculate that additional interactions may exist that were not detected by the two strategies mentioned above. For example, Sec3p may bind Sec6p and/or Sec8p since, in a *sec5* mutant, only Sec3p, Sec6p and Sec8p are in the immunoisolated complex, although the amount of Sec3p decreases.

Sec10p and Sec15p interact with each other in a subcomplex

The isolated exocyst complex consists of seven subunits, each at single copy (TerBush *et al*., 1996). However, it is unclear whether all the components exist only within this 19.5S particle. To determine whether all the components were exclusively in the complex, yeast lysates were centrifuged at 100 000 r.p.m. in a TL-100 rotor for 20 min. The supernatant and pellet fractions were collected and the proteins were separated by SDS–PAGE. The distribution of the exocyst components in the supernatant and the pellet was examined by Western blotting. As shown in Figure 6, Sec3p, Sec5p, Sec6p, Sec8p and Exo70p were almost completely pelleted by this centrifugation, whereas equivalent amounts of Sec10p and Sec15p were found in both the supernatant and pellet fraction. This result suggests that Sec10p and Sec15p not only bind

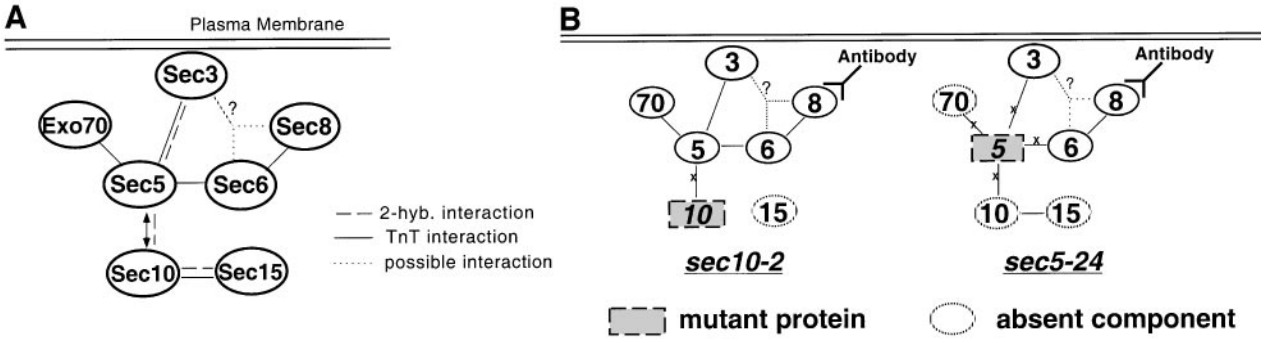

Fig. 5. Schematic representation of the protein–protein interactions of the exocyst complex in wild-type (**A**) and *sec10* -2 and *sec5-24* mutant strains (**B**). The mutant protein and proteins absent from the exocyst complex are indicated. 9E10 antibody against the c-Myc epitope tagged to Sec8p was used in immunoisolation of the complex (TerBush and Novick, 1995; TerBush *et al*., 1996). Interactions identified by TnT immunoprecipitation are indicated by solid lines. The interactions identified by yeast two-hybrid assay are indicated by dashed lines. Possible interactions between Sec3p and Sec8p and/or Sec6p that are not identified using the above two methods are indicated with a question mark.

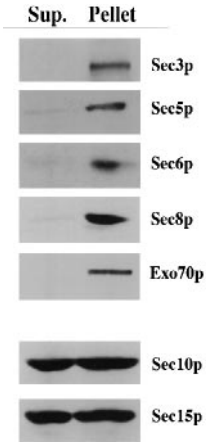

Fig. 6. Sec10p and Sec15p exist both in the exocyst complex and in a free pool. The exocyst pelleting assay was performed as described in Materials and methods. The pellet and supernatant of the centrifugation were separated by SDS–PAGE. The distribution of each exocyst protein in the two fractions was detected by Western blot analysis.

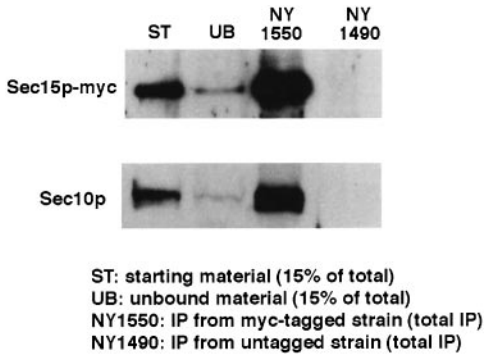

Fig. 7. Sec10p co-immunoprecipitates with Sec15p from the high-speed spin supernatant fraction of the yeast lysates. Sec15p was epitope tagged with the triple c-Myc sequence (strain NY1550). The high-speed supernatants of the lysate from NY1550 cells were used as the starting material for immunoprecipitation using 9E10 monoclonal antibody. Anti-Sec15p (upper panel) and anti-Sec10p antibodies (lower panel) were used for Western blotting. ST, starting material (15% of total); UB, unbound material (15% of total); NY1550, the immunoprecipitates (total amount) from the NY1550 strain (the tagged strain); NY1490, the immunoprecipitates (total amount) from the untagged parental strain.

the other components in the 19.5S particle, but are also present outside the complex.

Sec10p and Sec15p both have free pools in the cell and, as shown above (Figure 4), the *in vitro* synthesized Sec15p co-immunoprecipitates with epitope-tagged Sec10p. To study the interaction of Sec10p with Sec15p in the free pool, we have performed immunoprecipitation experiments using the high-speed supernatant. The lysate of the yeast strain containing triple c-Myc-tagged Sec15p (NY1550) was centrifuged to clear the cytosol of complete exocyst particles and the supernatant was used as the starting material for immunoprecipitation with 9E10 monoclonal antibody. The proteins were separated by SDS–PAGE gel and immunoblotted for Sec10p and Sec15p. As shown in Figure 7, Sec15p was precipitated from the high-speed supernatant and Sec10p co-immunoprecipitated, indicating that these two proteins can interact *in vivo*. Sec10p was co-precipitated with Sec15p only in the tagged strain. This result indicates that Sec10p and

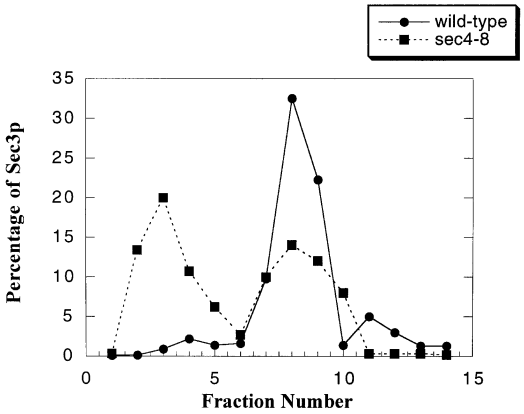

Fig. 8. The sedimentation profiles of Sec3p-HA in wild-type (●) and *sec4-8* mutant cells (■) in 10–35% glycerol velocity gradients.

Sec15p form a subcomplex in addition to their presence in the exocyst complex.

Sec4p affects the assembly of the exocyst proteins with Sec3p

The exocyst complex may assemble during the process of vesicle docking, and a defect in Sec4p should affect the assembly of the exocyst complex. A previous study in our laboratory indicates that the composition of the exocyst is not affected in *sec4* mutant cells because the complex immuno-isolated using an antibody against the epitope-tagged Sec8p contains all the exocyst components (TerBush *et al*., 1996). However, this result does not reveal whether all the exocyst proteins are fully assembled in the cell. It is possible that a mutation in Sec4p would affect the assembly of the exocyst subunits with Sec3p on the target membrane. To test this, complex formation was examined by velocity gradient fractionation and Western blot using the antibody against the HA epitope-tagged to the C-terminus of Sec3p. As shown in Figure 8, in wild-type cells, Sec3p migrates almost exclusively in the exocyst complex whereas, in *sec4-8* mutant cells, a significant amount of Sec3p sediments more slowly than the fully assembled exocyst complex, suggesting that a significant amount of the exocyst fails to assemble into a complex. This result indicates that efficient recruitment of the exocyst proteins to Sec3p requires functional Sec4p. Consistent with this result, recent studies have shown that the localization of Sec3p to sites of exocytosis is independent of ongoing membrane traffic, while other components (Ayscough *et al*., 1997; Finger *et al*., 1998; unpublished data) of the exocyst require functional Sec4p for their polarized localization.

Discussion

Prior studies have demonstrated that the exocyst is localized specifically to sites of active secretion, suggesting that this protein complex plays an important role in the spatial regulation of exocytosis (TerBush and Novick, 1995; Mondesert *et al*., 1997; Finger *et al*., 1998). Here we have defined the molecular interactions of the exocyst that provide a network connecting the secretory vesicles to the sites of vesicle docking on the plasma membrane.

The first of these interactions we will discuss involves

Sec15p. We provide two lines of evidence demonstrating the association of Sec15p with secretory vesicles: (i) subcellular fractionation demonstrates co-fractionation of Sec15p with Sec4p and Sncp, supporting the association of Sec15p with secretory vesicles; and (ii) immunoelectron microscopy confirms that both Sec4p and Sec15p are associated with clusters of secretory vesicles that accumulate in response to Sec15p overexpression. While in each case overproduction of Sec15p was necessary to observe its association with vesicles (Figure 2C), we believe that the association reflects a normal affinity of Sec15p for vesicles rather than non-specific binding of an overproduced protein. The Sec15p overexpression mutant allows us to observe the interaction, which might be too transient to observe in wild-type cells.

Sec15p interacts with Sec4p, but not other closely related yeast rab proteins such as Ypt1p and Ypt51p. Interactions are observed with wild-type Sec4p, Sec4ΔC (non-prenylated) and Sec4L79 (GTP-bound), but not Sec4I133 (nucleotide-free state) or Sec4V29 (probable GDP-bound state), suggesting that it is the GTP-bound form which is preferred and that prenylation is not required. In contrast, Gdi1p prefers the prenylated, GDP-bound form (Sasaki *et al*., 1990; Garrett *et al*., 1994), while the nucleotide release factor Dss4p and the exchange protein Sec2p prefer the nucleotide-free form (Collins *et al*., 1997; Walch-Solimena *et al*., 1997). The interaction with Sec15p requires the effector domain of Sec4p, since replacement of this domain with the corresponding region of Ypt1p abolishes the interaction. In total, these results define Sec15p as an effector molecule for Sec4p. This conclusion is also supported by the previous observation that the ability of Sec15p to form a patch upon overproduction requires activated Sec4p, and by the genetic observation that overexpression of Sec4p suppresses partial but not complete loss of Sec15p function (Salminen and Novick, 1989). Sec4p is probably not responsible for recruiting Sec15p to the secretory vesicles because Sec15p co-migrates with Snc2p, a vesicle marker, even in *sec4-8* mutant cells (data not shown). We hypothesize that Sec15p associates with vesicles through its interaction with an as yet unidentified receptor or possibly lipid.

Sec15p shares no obvious sequence similarities to other proposed rab effectors such as rabphilin (Shirataki *et al*., 1993), rabaptin-5 (Stenmark *et al*., 1995), EEA1 (Simonsen *et al*., 1998), rabkinesin-6 (Echard *et al*., 1998), rab8ip (Ren *et al*., 1996), rim (Wang *et al*., 1997) and a rab9 effector (Diaz *et al*., 1997). This should not be suprising as these effectors share, at best, limited homology with each other. Different rabs may employ different effectors. Indirect evidence suggests that Sec15p may not be the only effector for Sec4p. Defects in the Sec4p exchange protein Sec2p result in the failure of vesicles to concentrate at sites of exocytosis, implying that activation of Sec4p is required for polarized vesicle transport (Walch-Solimena *et al*., 1997). In contrast, defects in Sec15p, or in any of the other exocyst subunits, block exocytosis, but vesicles are still found concentrated at the appropriate pole of the cell (Govindan *et al*., 1995; Walch-Solimena *et al*., 1997). In addition to working through Sec15p to stimulate vesicle docking, Sec4p may also promote vesicle transport by activating a cytoskeletal motor, analogous to the proposed interaction of rab6 with rabkinesin-6 (Echard *et al*., 1998).

Using co-immunoprecipitation and the two-hybrid assay, we have defined a map of protein–protein interactions within the exocyst. We have found that Sec15p directly binds to Sec10p, and a Sec10p/Sec15p subcomplex is normally present at levels comparable to that of the complete exocyst complex. Sec10p is needed to link Sec15p to the remainder of the complex. Indeed, Sec15p was absent from the partial complex isolated from a *sec10-2* mutant strain (Figure 5B) (TerBush and Novick, 1995). The binding of the Sec10p/Sec15p subcomplex to the rest of the exocyst is mediated by the interaction of Sec10p with Sec5p. Sec5p also makes key links to Exo70p, Sec6p and Sec3p. Thus, Sec5p appears to be at the core of the complex. In support of this model, loss of Sec5p function leads to the failure of Sec10p, Sec15p, Exo70p and the mutant Sec5 protein to co-precipitate with Sec8p (Figure 5B) (TerBush and Novick, 1995; TerBush *et al*., 1996). The co-precipitation of Sec3p was reduced, suggesting that Sec5p also plays an important role linking this protein to Sec8p, presumably through the association of Sec5p with Sec6p and Sec6p with Sec8p. Sec3p may also bind directly to Sec8p or Sec6p, since some residual Sec3p was found in the complex isolated from the *sec5-24* strain.

In the light of the map of interactions described above, we can now re-evaluate the molecular mechanisms underlying several genetic observations. Since Sec15p must interact with both Sec4p and Sec10p, the lethality resulting from Sec15p overproduction may reflect the saturation of Sec4p with Sec15p lacking its partner, Sec10p. Consistent with this proposal, overexpression of either Sec4p or Sec10p was found to suppress the growth defect resulting from Sec15p overexpression (data not shown). It was shown previously (Roth *et al*., 1998) that when a truncated form of Sec10p (Sec10p with its C-terminal 282 amino acids deleted, termed Sec10ΔC) was co-overexpressed with Sec15p, this Sec10p fragment localized to the Sec15p–Sec4p 'patch'. Since Sec10ΔC was found to interact physically with Sec15p, the specific localization of Sec10ΔC is probably due to its association with Sec15p on the secretory vesicles that accumulate upon Sec15p overproduction. This study suggests that Sec10p may be able to bind to Sec15p on the vesicle containing Sec4p and thereby link the vesicles to the remainder of the exocyst complex. Sec10ΔC may fail to complete these interactions that would allow the vesicles to dock at exocytic sites.

The results presented in this study provide a network of molecular interactions that could function to link secretory vesicles to specific sites on the plasma membrane (Figure 9). The localization of Sec3p to specific domains of the plasma membrane and the interaction of Sec15p with the GTP-bound Sec4p suggest that the exocyst complex plays an important role in rab/GTPase-directed membrane docking and fusion. Sec4p may play an activating role in the assembly of the exocyst components onto Sec3p, which marks the site of secretion. Consistent with this hypothesis, we have shown that a pool of Sec3p fails to assemble into a complete complex in *sec4-8* cells (Figure 8). This is also consistent with previous immunofluorescence data showing that some exocyst components

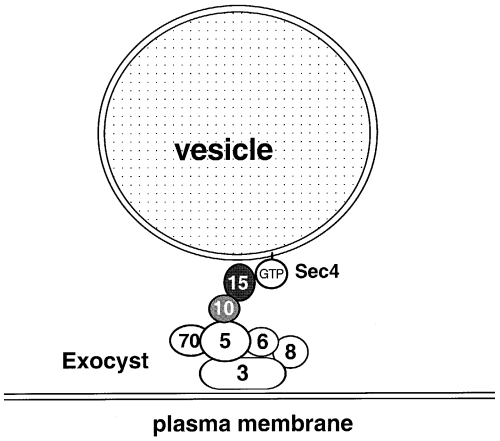

Fig. 9. Schematic presentation of a network of molecular interactions that links secretory vesicles to specific sites of the plasma membrane. Sec15p can interact with the GTP-bound form of Sec4p on the secretory vesicles; Sec3p marks specific sites of exocytosis. Through protein–protein interactions of the exocyst complex, secretory vesicles can be targeted to specific exocytic sites on the plasma membrane. Although the sequence of events leading to exocyst assembly has not yet been defined, Sec4p may play a role in activating this process.

lose their polarized pattern of localization in *sec4-8* mutant cells while the localization of Sec3p is unaffected (Finger *et al*., 1998; unpublished observation). Although the exocyst proteins can still assemble (TerBush and Novick, 1995), the amount of fully assembled exocyst complex significantly decreases in the *sec4-8* mutant in comparison with that in the wild-type cells (Figure 8). It is possible that the fully assembled pool in *sec4-8* cells represents complex assembled prior to the temperature shift. Disassembly following docking may be a slow event since, in wild-type cells, most exocyst subunits are found predominantly in the assembled pool.

A prior genetic study suggested that Sec4p may function upstream of the SNARE complex. Overexpression of Sec9p, a SNAP25 homolog, was shown to suppress a mutation in the effector domain of Sec4p as well as several exocyst mutants (Brennwald *et al*., 1994). The regulation of SNARE function by Sec4p might be mediated by the exocyst. This could serve to couple SNARE complex formation to vesicle docking. In this light, it will be interesting to explore further possible molecular interactions between the exocyst and SNAREs.

Why are there so many proteins in the complex? We believe that vesicle targeting and docking is a highly regulated process. The exocyst may communicate with the cell cycle machinery, the cytoskeleton, the SNAREs and other proteins in the cell. In this study, we found that Sec15p may play an important role in its communication with the rab protein and secretory vesicles. Overexpression of the C-terminal portion of Sec10p leads to an elongated cell shape without affecting the kinetics of protein secretion, suggesting that Sec10p interacts with the morphogenetic machinery (Roth *et al*., 1998). The other components may also interact with proteins outside of the complex. The assembly of the exocyst complex may integrate various sources of cellular information to ensure correct vesicle targeting and docking. The map of protein–protein interactions depicted in this study will be important for future studies concerning the functional regulation of vesicle docking.

A rapidly growing number of proteins have been identified recently that may play key roles in membrane targeting (Pfeffer, 1996). These include: a complex of p115, GM130 (Nakamura *et al*., 1997) and giantin (Sönnichsen *et al*., 1998) that tethers Golgi vesicles; Uso1p (Sapperstein *et al*., 1996; Cao *et al*., 1998) and a large complex termed TRAPP (Sacher *et al*., 1998) implicated in targeting endoplasmic reticulum–Golgi vesicles; rabaptin 5 and EEA1, rab5-interacting proteins that function in the homotypic interaction of endosomes (Stenmark *et al*., 1995; Simonsen *et al*., 1998); DOC involved in synaptic vesicle delivery (Verhage *et al*., 1997); and a RING finger protein complex in yeast vacuole targeting (Rieder and Emr, 1997). The sequences of these components are often conserved among diverse eukaryotic species, indicating that their functions developed early in evolution. However, proteins such as these that function at different stages of transport appear to share no detectable sequence similarity, suggesting that their functions are unique for each target compartment. The unique sequence and distinctive localization of these proteins make them good candidates for the targeting machinery that directs the docking of different classes of vesicles to the appropriate membrane.

Materials and methods

Yeast strains and media for growth

The genotypes of the *Saccharomyces cerevisiae* strains used in this study are listed in Table II. The triple c-Myc-tagged Sec15p strain (NY1550) was constructed using the method of Schneider *et al*. (1995) with NY1490 as the parental strain. Cells were grown in YP medium (1% Bacto-yeast extract, 2% Bacto-peptone) supplemented with either 2% dextrose (YPD) or other carbohydrate sources as illustrated in the text. To induce protein overexpression under the control of the *GAL* promoter, cells were grown in YP medium containing 2% glycerol (YP-glycerol) until early log phase. Galactose (2%) was then added to the medium and the cells were allowed to grow further for 6–9 h.

Immunoelectron microscopy

Sec15p overproduction was performed as described above. The cells were collected by vacuum filtration using a 0.45 μm nitrocellulose membrane and fixed for 2 h at room temperature in a buffer containing 3% formaldehyde, 40 mM potassium phosphate, pH 6.7, 0.8 M sorbitol, 1 mM $MgCl_2$ and 1 mM $CaCl_2$. The cells were processed further as described by Mulholland *et al*. (1994). After dehydration and embedding in LR White resin (Polysciences, Inc.), thin sections were cut and transferred onto 400-mesh uncoated nickel grids (Electron Microscopy Sciences). Immunolabeling was performed as described previously (Walch-Solimena *et al*., 1997). Affinity-purified anti-Sec4p and anti-Sec15p polyclonal antibodies were used in the primary staining, and 10 nm colloidal gold-conjugated protein A was used for secondary labeling.

Cell membrane fractionation

Sec15p overproduction was performed as described above. The cells were washed once with 10 mM NaN_3 in ddH_2O and resuspended in spheroplast medium containing 50 mM KPi, pH 7.5, 5 mM NaN_3, 1.4 M sorbitol, 40 mM β-mercaptoethanol and 0.15 mg/ml zymolase-100T. After spheroplasting, the microsomal pellets (P3) were prepared as described previously (Goud *et al*., 1988). The pellets were resuspended and loaded onto 15–65% (w/v) sucrose gradients, and spun at 170 000 *g* for 16 h in a SW28 rotor (Beckman) at 4°C. Fifteen fractions were collected from the top of the gradient and analyzed by 12% SDS–PAGE. The proteins were transferred to nitrocellulose, and antibodies against Sec15p, Sec4p and Sncp were used for Western blotting. Kex2p endoprotease assay was performed as described by Cunningham and Wickner (1989).

Immunoprecipitation of Sec15p and Sec4p

NY724 cells were grown, induced with galactose and spheroplasted as described above. After spheroplasting, the cells were lysed by triturating

Table II. Relevant yeast strains

Strain	Genotype
NY17	*Mat* **a**, *ura3-52, sec6-4*
NY604	*Mat* α, *ura3-52, leu2-3,112*
NY724	*Mat* **a**, *ura3-52::(GAL-SEC15, URA3)*
NY1060	*Mat* **a**, *ura3-52/ura3-52, leu2-3,112/leu2-3/112*
NY1245	*Mat* α, *ura3-52, leu2-3,112::(LEU2, SEC8-c-myc-6xhis), sec5-24*
NY1248	*Mat* α, *ura3-52, leu2-3,112::(LEU2, SEC8-c-myc-6xhis), sec10-2*
NY1468	*Mat* α, *ura3-52::(URA3, EXO70-HA), leu2-3,112::(LEU2, SEC8-3xc-myc), sec8-9, L-A-*o
NY1490	*Mat* **a**, *leu2-3,112, ura3-52, trp1, his3Δ200, L-A-o*
NY1550	*Mat* **a**, *leu2-3,112, ura3-52, trp1, his3Δ200, L-A-o, SEC15-3xc-myc*
NY1562	*Mat* **a**/α *ura3-52::(URA3, Gal-SEC15)/ura3-52, leu2-3,112/leu2-3,112*
NY1577	*Mat* **a**/α *ura3-52::(GAL-SEC15, URA3)/ura3-52, leu2-3,112/leu2-3,112 SEC10::(GAL-SEC10, LEU2)*
NY1588	*Mat* **a**, *ura3-52::(SEC5-HA3, URA3), his4-619*
NY2130	*Mat* **a**, *ura3-52, leu2-3,112, sec3::(SEC3-4XHA, URA3)*

in ice-cold lysis buffer containing phosphate-buffered saline (PBS), 1 mM EDTA, 4 mM $MgCl_2$ and protease inhibitors [1 mM phenylmethylsulfonyl fluoride (PMSF) and 5 μg/ml each of leupeptin, chymostatin, pepstatin, aprotinin and antipain]. The cell lysates were cross-linked using 1 mM 3,3′-dithiobis(sulfosuccinimidyl)propionate (DSP; Pierce Chemical Co., Rockford, IL) as described previously (Brennwald *et al*., 1994). Immunoprecipitation was carried out in a PBS buffer supplemented with 1% Triton X-100 and 0.1% bovine serum albumin (BSA) with anti-Sec15p polyclonal antibody. The immunoprecipitates were washed with PBS buffer containing 0.2% SDS, boiled in sample buffer containing 100 mM dithiothreitol (DTT) for 10 min and then subjected to SDS–PAGE. Anti-Sec15p antibody was used to detect Sec15p in Western blots of the immunoprecipitated material. For Sec4p, detection by Western blotting was difficult because its molecular weight is very similar to that of the antibody light chain. Therefore, a [^{32}P]GTP overlay assay was performed (Goud *et al*., 1988). The secondary immunoprecipitation was performed basically as described previously (Collins *et al*., 1997). Briefly, the Sec15p antibody immunoprecipitates were boiled in a buffer containing 50 mM Tris, pH 8.0, 25 mM EDTA, 100 mM DTT and 1% SDS to release the bound proteins, inactivate the anti-Sec15p antibody and cleave the DSP-induced cross-links. The samples were then diluted 1:25 in immunoprecipitation buffer containing 20 mM Tris, pH 8.0, 0.5% Tween-20 and 100 mM NaCl. The protein A–Sepharose was spun down and discarded. The samples were then subjected to secondary immunoprecipitation using anti-Sec4p or anti-Ypt1p polyclonal antibodies. Sec4p and Ypt1p were detected by [^{32}P]GTP overlay.

Yeast two-hybrid assay

The yeast two-hybrid assay (Chien *et al*., 1991) was performed as described previously (Durfee *et al*., 1993; Walch-Solimena *et al*., 1997). The yeast strain Y190 was used as the host. The interactions were quantified using a β-galactosidase assay.

Identification of protein–protein interactions in vitro

Epitope-tagged open reading frames were constructed in the pcDNA3 expression vector (Invitrogen) for *in vitro* translation of the exocyst components using the TnT system (Promega). To examine the interactions of the exocyst proteins, pairs of the exocyst components were synthesized in reticulocyte lysates and then diluted 1:100 in the binding buffer (20 mM PIPES, pH 6.8, 0.5% Tween-20, 150 mM NaCl, 1 mM EDTA, 1 mM DTT). Immunoprecipitations were carried out using antisera (BabCO) against the tagged epitopes (including HA, c-Mycand FLAG) of the proteins. Interactions were detected by coimmunoprecipitation of the untagged protein with the tagged proteins. As a control, we evaluated the precipitation of the untagged protein by the antibody in the absence of the tagged binding partner.

Pelleting assay of the exocyst proteins

Yeast cells were grown in YPD to mid-log phase, and 50 A_{600} units of cells were harvested. Cells were washed twice in pelleting buffer (20 mM PIPES pH 6.8, 150 mM NaCl, 1 mM DTT, 1 mM EDTA), resuspended in a final volume of 1 ml and kept on ice for 10 min. Just prior to lysis, the protease inhibitor cocktail (see above) was added, and cells were lysed by vortexing with glass beads. The lysates were then extracted from the glass beads by addition of 1 ml of pelleting buffer containing fresh protease inhibitor cocktail. Cell debris was pelleted by centrifugation at 10 000 *g* for 10 min at 4°C in a microcentrifuge. The resulting supernatant was centrifuged at 100 000 r.p.m. for 15 min in a TL-100 rotor (Beckman). Supernatant and pellet fractions were collected. The pellet fraction was resuspended in the same amount of pelleting buffer as the supernatant, and equal volumes of these two fractions were loaded onto SDS–PAGE. The individual exocyst components present in the two fractions were analyzed by Western blotting using polyclonal antisera for Sec6p, Sec8p, Sec10p and Sec15p. To identify Sec3p, Sec5p and Exo70p, strains expressing these proteins as HA-tagged fusions as their sole genomic copy were used in the experiment and Western blot analysis was carried out with a monoclonal antibody (12CA5) directed against the HA epitope.

Immunoprecipitation of Sec15p and Sec10p

High-speed supernatants of yeast lysates were prepared as described above in buffer A (20 mM PIPES pH 6.8, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.5% Tween-20) containing protease inhibitors. To study the interaction of the free pool of Sec15p and Sec10p, 2 mg of high-speed supernatant lysate (in 1 ml) was used in each immunoprecipitation experiment. The supernatant was first pre-cleared using 4 mg of protein A–Sepharose for 45 min and then incubated with 9E10 monoclonal antibody against the c-Myc epitope of Sec15p for 1 h. After further incubation with 4 mg of protein A–Sepharose for 45 min, the immunoprecipitate was washed three times with buffer A. All the immunoprecipitation experiments were performed at 4°C. The immunoprecipitated proteins were boiled in protein sample buffer for 5 min and separated by 10% SDS–PAGE. The proteins were transferred to nitrocellulose and probed by Western blot analysis with 9E10 antibody and anti-Sec10p antibodies (YU58) at a dilution of 1:2000 (Roth *et al*., 1998).

Glycerol gradient analysis of the exocyst complex

Wild-type and *sec4-8* strains expressing Sec3p-4×HA as the sole copy were spheroplasted and lysed in a buffer containing 20 mM HEPES, pH 7.4, 120 mM NaCl, 1 mM DTT, 1 mM EDTA, 1% Triton X-100 and protease inhibitors. After spinning at 16 000 *g* in a microfuge, the supernatants were loaded onto 10–35% (v/v) glycerol gradients and centrifuged at 50 000 r.p.m. for 5 h in an SW50.1 rotor (Beckman) at 4°C. Fractions were collected from the top of the gradient and analyzed by Western blotting using anti-HA antibody.

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