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The Exocyst is a multiprotein complex required for exocytosis in *Saccharomyces cerevisiae*

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In the yeast *Saccharomyces cerevisiae*, the products of at least 15 genes are involved specifically in vesicular transport from the Golgi apparatus to the plasma membrane. Previously, we have shown that three of these genes, *SEC6*, *SEC8* and *SEC15*, encode components of a multisubunit complex which localizes to the tip of the bud, the predominant site of exocytosis in *S.cerevisiae*. Mutations in three more of these genes, *SEC3*, *SEC5* and *SEC10*, were found to disrupt the subunit integrity of the Sec6–Sec8–Sec15 complex, indicating that these genes may encode some of the remaining components of this complex. To examine this possibility, we cloned and sequenced the *SEC5* and *SEC10* genes, disrupted them, and either epitope tagged them (Sec5p) or prepared polyclonal antisera (Sec10p) to them for co-immunoprecipitation studies. Concurrently, we biochemically purified the remaining unidentified polypeptides of the Sec6–Sec8–Sec15 complex for peptide microsequencing. The genes encoding these components were identified by comparison of predicted amino acid sequences with those obtained from peptide microsequencing of the purified complex components. In addition to Sec6p, Sec8p and Sec15p, the complex contains the proteins encoded by *SEC3*, *SEC5*, *SEC10* and a novel gene, *EXO70*. Since these seven proteins function together in a complex required for exocytosis, and not other intracellular trafficking steps, we have named it the Exocyst.

Keywords: Exocyst/exocytosis/*Saccharomyces cerevisiae*/ *SEC* genes/secretory pathway

Introduction

The basic elements of the machinery mediating vesicular transport have been conserved, not only among eukaryotes as diverse as yeast and man, but also between the different stages of transport that exist in any given cell (Bennett and Scheller, 1993; Ferro-Novick and Jahn, 1994; Rothman, 1994; Rothman and Warren, 1994). This conservation of mechanism is evident in the amino acid sequences and topology of the proteins implicated in different transport steps. Every transport event is thought to require at least one member of a number of different protein families. Synaptobrevin/VAMP is an integral membrane protein found on the surface of synaptic vesicles, and distinct synaptobrevin homologs are found on vesicular carriers responsible for transport from the endoplasmic reticulum (ER) to Golgi (Newman *et al.*, 1990; Hay *et al.*, 1996), intra Golgi (Banfield *et al.*, 1995; Nagahama *et al.*, 1996) and Golgi to cell surface (Trimble *et al.*, 1988; Baumert *et al.*, 1989; Gerst *et al.*, 1992; Protopopov *et al.*, 1993). Similarly, syntaxin is an integral membrane protein found predominantly on the presynaptic membrane of neurons, and unique syntaxin homologs are found on different target membranes (Hardwick and Pelham, 1992; Aalto *et al.*, 1993; Bennett *et al.*, 1993; Dascher *et al.*, 1994; Søgaard *et al.*, 1994; Becherer *et al.*, 1996). At each stage of transport, the cytoplasmic domain of the relevant synaptobrevin family member is believed to bind to the cytoplasmic domain of the corresponding syntaxin family member, and the specificity of this protein–protein interaction contributes to the specificity of the overall transport reaction (Söllner *et al.*, 1993).

Members of additional protein families may be responsible for the regulation of the interaction of each pair of synaptobrevin and syntaxin homologs. SNAP25 is found on the presynaptic membrane (Oyler *et al.*, 1989) in association with syntaxin and acts to increase its affinity for synaptobrevin (Pevsner *et al.*, 1994a). Sec1p, a hydrophilic yeast protein identified through its role in exocytosis, has family members involved in ER to Golgi transport (Sly1p) (Dascher *et al.*, 1991), Golgi to endosome transport (Vps45p) (Cowles *et al.*, 1994; Piper *et al.*, 1994) and in synaptic transmission (nSec1/Unc-18/rop) (Gengyo-Ando *et al.*, 1993; Salzberg *et al.*, 1993; Garcia *et al.*, 1994; Pevsner *et al.*, 1994b). Studies using purified recombinant proteins indicate that nSec1 can bind syntaxin and thereby block its interaction with synaptobrevin (Garcia *et al.*, 1994; Pevsner *et al.*, 1994b). The Sec1-related proteins may function in response to signals from members of another family of proteins typified by the Sec4 protein of yeast. Sec4p is a small GTPase of the Ras superfamily found on Golgi-derived secretory vesicles (Salminen and Novick, 1987; Goud *et al.*, 1988). Different homologs of Sec4p, known as Rab proteins in animal cells and Ypt proteins in yeast, function on different portions of the exocytic or endocytic pathways. Genetic evidence indicates that these GTPases act upstream of their corresponding Sec1p, synaptobrevin and syntaxin homologs (Dascher *et al.*, 1991; Novick and Brennwald, 1993; Lian *et al.*, 1994; Søgaard *et al.*, 1994). However, the molecular mechanism of this regulation has not yet been elucidated.

Genetic analysis has defined 13 genes, in addition to *SEC1* and *SEC4* described above, whose products are required for exocytosis in yeast (Gerst *et al.*, 1992; Schekman, 1992; Aalto *et al.*, 1993; Nelson *et al.*, 1996).

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Many of them encode members of one of these protein families. Snc1p and Snc2p are members of the synaptobrevin family and are found on secretory vesicles (Gerst *et al.*, 1992; Protopopov *et al.*, 1993). Sso1p and Sso2p are members of the syntaxin family and are found predominantly on the plasma membrane (Aalto *et al.*, 1993). Sec9p is a member of the SNAP25 family (Brennwald *et al.*, 1994). It is found on the plasma membrane and can associate with Sso and Snc proteins (Brennwald *et al.*, 1994).

The functions of the remaining gene products, Sec2p, Sec3p, Sec5p, Sec6p, Sec8p, Sec10p, Sec15p and Scd5p are largely unknown. Previous analysis has demonstrated that Sec6p, Sec8p and Sec15p are components of a 1×10^6–2×10^6 Da complex that contains a total of eight polypeptides ranging in size from 70 to 144 kDa (TerBush and Novick, 1995). This complex is found both in the cytosol and peripherally associated with the plasma membrane (Bowser and Novick, 1991; Bowser *et al.*, 1992). Immunofluorescence microscopy has demonstrated high concentrations of Sec8p at the tip of small buds, which represents a very active site of exocytosis (TerBush and Novick, 1995). Isolation of the Sec6–Sec8–Sec15 complex from all of the late-acting *sec* mutants has shown that its structure is altered in *sec3-2, sec5-24* and *sec10-2* strains, but not in *sec1-1, sec2-41, sec4-8* or *sec9-4* strains (TerBush and Novick, 1995). To probe the structure of this complex further, we have taken two independent approaches. We have cloned and sequenced *SEC5* and *SEC10*, and we have isolated the complex in amounts that have allowed amino acid sequence determination of the unknown polypeptides. Together, these experiments have shown that the complex consists of one copy of Sec3p, Sec5p, Sec6p, Sec8p, Sec10p, Sec15p and one protein encoded by a novel gene, *EXO70*. This multiprotein complex which is required for exocytosis, and not other intracellular trafficking steps, we now term the Exocyst.

Results

Cloning of SEC5 and SEC10

The *SEC5* and *SEC10* genes were isolated from a genomic yeast library based on the shuttle vector, YCp50, by the ability to complement the *sec5-24* and *sec10-2* temperature-sensitive growth defects. The smallest region of the clone capable of complementing *sec5-24* was a 4.1 kb fragment defined by *Kpn*I sites (Figure 1). By a similar analysis, the smallest region of the clone capable of complementing *sec10-2* consists of a 4.4 kb *Cla*I–*Sac*I fragment (Figure 1). Marker integration experiments verified that these cloned fragments could direct integration by homologous recombination to the appropriate loci (data not shown). Taken together, these data indicate that these fragments contain the *SEC5* and *SEC10* genes, respectively.

Sequencing of SEC5 and SEC10

The complementing regions of pNB498 (*SEC5* complementing library clone) and pNB497 (*SEC10* complementing library clone) were sequenced. For the *SEC5* complementing region, an open reading frame (ORF) of 2916 bases within the *Kpn*I–*Kpn*I fragment was identified. Comparison with recent releases of the GenBank DNA

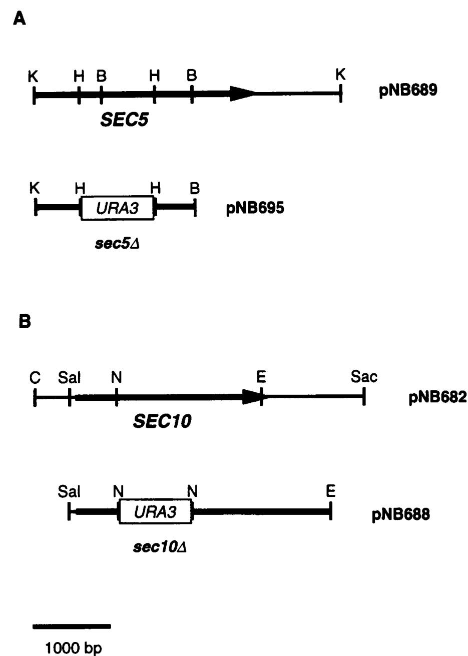

Fig. 1. *SEC5* and *SEC10* constructs. (**A**) The smallest complementing fragment of *SEC5* is defined as a 4.1 kb *Kpn*I–*Kpn*I fragment. The *sec5*Δ construct was made by inserting a 1.1 kb *URA3* gene into the *Kpn*I–*Bgl*II fragment of *SEC5* which replaces an internal *Hind*III fragment. (**B**) The smallest complementing fragment of *SEC10* is defined by a 4.4 kb *Cla*I–*Sac*I fragment. The *sec10*Δ construct was designed by inserting a 1.1 kb *URA3* gene at an internal *Nco*I site of a *Sal*I–*Eco*RV fragment. B, *Bgl*II; C, *Cla*I; E, *Eco*RV; H, *Hind*III; K, *Kpn*I; N, *Nco*I; Sac, *Sac*I; Sal, *Sal*I.

database indicated that the 5′ 1238 bases of this ORF are coded for by the incomplete sequence YD9489.01c (GenBank accession no. Z47813) and the 3′ 2676 bases are coded for by the incomplete sequence YD8358.20c (GenBank accession no. Z50046) with a 998 base overlap. Another putative ORF of 1335 bases was identified by sequence comparison with the GenBank sequences which is 3′ of the 2916 bp ORF (but it is missing its initial 272 bases) in the *Kpn*I–*Kpn*I complementing fragment. To ensure that this partial ORF does not encode *SEC5*, an *Xba*I–*Sna*BI fragment which completely lacks any of the putative ORF was subcloned into a *CEN* plasmid. This fragment fully complements the *sec5-24* temperature-sensitive growth defect (data not shown). Thus, the 2916 bp ORF encodes *SEC5* and it is located on chromosome IV.

SEC5 encodes an essential gene. *SEC5* was disrupted by inserting *URA3* at an internal *Hind*III site (Figure 1), and a fragment containing the disrupted gene was transformed into a diploid yeast strain. *URA*$^+$ transformants were selected, sporulated and tetrads dissected. All tetrads yielded two viable and two dead spores, indicating that *SEC5* is an essential gene.

For the *SEC10* complementing region, we sequenced the first 826 bases of a putative ORF within the *Cla*I–*Sac*I fragment. Our partial sequence exactly matched the 5′ end of a 2616 bp ORF sequenced by the yeast genome project (gene L9362.12; GenBank accession no. U51921). This fragment fully complements the *sec10-2* temperature-sensitive growth defect on a *CEN* plasmid (data not shown). Thus, the 2616 bp ORF encodes *SEC10* and it is located on chromosome XII.

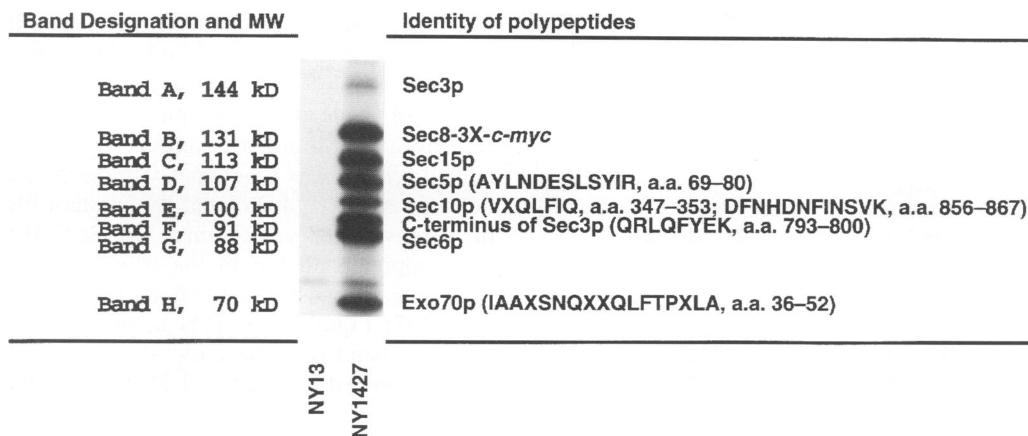

Fig. 2. The immunopurified Exocyst complex contains Sec3p, Sec5p, Sec10p and Exo70p. Radiolabeled yeast lysates from NY13 and NY1427 were prepared and the polypeptides which specifically co-immunoprecipitate with Sec8-3X-c-*myc* are shown. Exocyst components which have been identified previously (Sec6p, Sec8p and Sec15p) are shown together with the polypeptides identified in this study to be Sec3p (144 and 91 kDa), Sec5p (107 kDa), Sec10p (100 kDa) and Exo70p (70 kDa). The peptides which were sequenced are shown next to the corresponding protein. Single amino acid code, (X) indicates an uncertain amino acid from the sequencing of the peptide.

SEC10 encodes an essential gene. *SEC10* was disrupted by inserting *URA3* at an internal *Nco*I site (Figure 1), and a fragment containing the disrupted gene was transformed into a diploid yeast strain. URA^+ transformants were selected, sporulated and tetrads dissected. Twenty three of 24 tetrads yielded two viable and two dead spores, indicating that *SEC10* is an essential gene.

The Exocyst purification

Concurrent with the molecular analysis of *SEC5* and *SEC10*, the protein components of the Exocyst complex were purified for amino acid sequence analysis. The Exocyst complex proteins were purified by co-immunoprecipitation via a c-*myc* epitope tag on Sec8p and sequential SDS–PAGE separating (Laemmli, 1970) and funnel tube concentrating gel electrophoresis (D.R. TerBush and P.Novick, in preparation). In order to purify sufficient protein for peptide microsequencing of all of the subunits, two purifications were done. The first purification was from 6.4 g of starting yeast lysate (NY1008 yeast strain) containing a single c-*myc* epitope-tagged Sec8 protein as the sole copy of Sec8p. This yielded sufficient amounts of protein for tryptic digestion, HPLC separation and peptide microsequencing of bands D, E and H (see Figure 2). An unusually large amount of protein immunoprecipitated in the 90 kDa range in this experiment, and subsequent peptide microsequencing identified it as the yeast L-A virus coat protein, gag (data not shown). Further experiments confirmed that the virus was present in the NY1008 strain and that the virus coat protein had affinity for the 9E10 antibody fixed to protein A beads. Therefore, gag was clearly a contaminant and not an integral Exocyst component (data not shown).

Before the purification was repeated, a new strain (NY1427) which lacked the L-A virus and which contained a triple c-*myc* epitope-tagged Sec8p as the sole copy of Sec8p was constructed and characterized. Western blot analysis of co-immunoprecipitation experiments using the NY1008 and NY1427 strains indicated that 4-fold greater amounts of Sec15p and triple c-*myc* Sec8p co-immunoprecipitate compared with the amounts of Sec15p and single c-*myc* Sec8p at comparable expression levels of the respective proteins (data not shown). An autoradiogram of the immunoprecipitated proteins from a triple c-*myc* Sec8p radiolabeled lysate is shown in Figure 2. The pattern is similar to that which we have observed before using the single c-*myc* Sec8p strains as the source material (see Figure 4, TerBush and Novick, 1995).

The second purification was from 4.8 g of starting yeast lysate (NY1427 yeast strain) containing a triple c-*myc* epitope-tagged Sec8 protein as the sole copy of Sec8p. This yielded sufficient amounts of protein for lys-C digestion, mass spectrometry and peptide microsequencing of the HPLC-separated peptides of bands A and F. A full description of the Exocyst purification method using funnel tube concentrating gel electrophoresis will be published elsewhere (D.R.TerBush and P.Novick, in preparation).

The Exocyst complex contains Sec3p, Sec5p, Sec10p and a novel protein encoded by EXO70

Components of the purified Exocyst complex were identified by a combination of peptide microsequencing and mass matching. From the first purification, we were able to identify tryptic peptides from bands D, E and H (Figure 2). Band D was identified as Sec5p. We obtained one peptide sequence from band D: AYLNDESLSYIR. This peptide exactly matches amino acids 69–80, is preceded by an arginine residue and has an observed M_r of 1446.1 Da, which is in good agreement with the predicted M_r of 1444.6 Da.

Band E was identified as Sec10p. We obtained two peptides from band E: VXQLFIQ and DFNHDNFINSVK. These peptides correspond to amino acids 347–353 and 856–867 of Sec10p. Both are partial sequences of predicted tryptic peptides with M_rs of 1326.6 and 1579.7 Da, respectively. These are in good agreement with the observed M_rs of 1329.8 and 1576.87 Da for the full peptides, respectively.

Band H is encoded by an ORF originally sequenced due to its proximity to the tRNA ligase 1 gene [ORF2, GenBank accession no. Y00473 (Komatsoulis *et al.*, 1987; Westaway *et al.*, 1988)] We obtained one peptide from band H: IAAXSNQXXQLFTPXLA. This peptide corresponds to amino acids 36–52 of a partially sequenced

Table I. Mass pattern matching of peptides derived from the 144 kDa (band A) polypeptide

Observed mass (Da)[a]	Predicted mass (Da)	Predicted Sec3p peptide (aa)
1068.3	1068.2	1–9
2162.3	2164.48	195–211
1967.3	1967.15	310–327
2861.9	2862.10	328–349
1229.7	1229.41	361–369
1372.0	1371.50	429–439[b]
1517.7	1516.7	429–440
1729.2	1727.06	756–769
1112.3	1112.26	793–800
1199.6	1199.3	1092–1101
1394.0	1393.58	1313–1323

[a]All masses shown were determined with reflectron and are within the predicted accuracy of 0.15%. Six masses, not shown above, clearly do not match predicted Sec3p peptides. Two additional peptides with masses which closely match predicted Sec3p-derived peptides were not included since they were just outside the predicted accuracy.
[b]Assumes an N-terminal pyroglutamine modification.

tryptic peptide. The peptide had an observed M_r of 1920.3 Da, which is in good agreement with the predicted M_r of 1919.2 Da for the full peptide. We have renamed this gene *EXO70*, for Exocyst component of 70 kDa.

From the second purification, we were able to identify the protein which encodes both band A and its proteolytic breakdown product band F. Band A is full-length Sec3p and band F corresponds to the C-terminus of Sec3p. *SEC3* is identical to *PSL1* (profilin synthetic lethal 1) accession no. L22204 (Haarer *et al.*, 1996; F.Finger and P.Novick, in preparation). We sequenced one peptide from band F: QRLQFYEK. This peptide is an exact match to Sec3p amino acids 793–800 and is preceded by a lysine residue as predicted. The peptide has an observed M_r of 1112.1 Da, which agrees well with the predicted M_r of 1112.27 Da. Thus, band F is clearly derived from Sec3p. Sec3p is a 1336 amino acid protein with a predicted M_r of 154.7 kDa; therefore, it was unlikely that band F was the full-length Sec3 protein. The HPLC profiles of the lys-C digests of band F closely mirrored those of band A, which suggested that band F was a proteolytic breakdown product of band A (data not shown). Mass spectrometry was performed on some of the corresponding peak fractions for mass pattern comparisons. Band A clearly contains peptides derived from the full-length Sec3 protein (amino acids 1–1323; Table I). Band F contains peptides from the C-terminus of Sec3p (amino acids 770–1323, data not shown). There are a few peptides in the fractions we massed from the band A digest which are clearly not derived from Sec3p (six of 19 peptides, Table I). These peptides may be derived from a very minor protein species of 142 kDa which co-immunoprecipitates with the Exocyst but is not readily apparent at the exposure shown in Figure 2. Peptide sequencing of one of these peptides resulted in such low signal levels that the resulting sequence is suspect and does not match any yeast protein sequence.

The co-purification and peptide sequencing data indicate that Sec3p, Sec5p, Sec10p and Exo70p are integral components of the Exocyst complex. To verify this, we either epitope tagged or prepared polyclonal antibodies to several of these proteins for co-immunoprecipitation experiments. The *SEC5* gene was modified at its 3′ end to include the sequence encoding the 12CA5 HA (influenza hemagglutinin) epitope (Wilson *et al.*, 1984). The resulting *SEC5-HA* gene fully complemented the temperature-sensitive *sec5-24* allele at single copy, indicating that the tagged protein was functional. The Sec5-HA protein was detected as a 107 kDa band by Western blotting lysates of a transformed yeast strain. The Sec5-HA protein co-immunoprecipitated with the triple c-*myc* Sec8 protein from a strain transformed with both epitope-tagged alleles (NY1457; Figure 3A). This confirmed the presence of Sec5p as band D of the Exocyst.

We prepared a polyclonal antibody to the N-terminus (amino acids 2–296) of Sec10p, which recognizes a protein of 100 kDa that is overexpressed when *SEC10* is present as a second copy on a *CEN* plasmid. A smaller (35 kDa) proteolytic breakdown fragment is also overexpressed (data not shown). Double immunoprecipitation experiments were performed with [^{35}S]cysteine/methionine-labeled lysates from strains NY13 (wild-type Sec8p) and NY1427 (triple c-*myc*-tagged Sec8p) with and without prior cross-linking with dithiobis(succinimidylpropionate) (DSP). When c-*myc* immunoprecipitates from NY1427 are re-immunoprecipitated with polyclonal antibody to Sec10p, a 100 kDa band is brought down specifically (Figure 3B). This band fails to immunoprecipitate from NY13. If we perform the same experiment, but first cross-link NY1427 lysates with DSP, then the entire Exocyst complex co-immunoprecipitates as expected (Figure 3B). This confirmed the presence of Sec10p as band E of the Exocyst. Additional support for the presence of Sec3p, Sec5p and Sec10p in the Exocyst is provided by the observed physical disruption of the complex isolated from strains containing temperature-sensitive mutations in these genes (TerBush and Novick, 1995) and the synthetic lethal interactions between the *sec8-9* allele and the *sec6-4*, *sec15-1*, *sec3-2*, *sec5-24* and *sec10-2* alleles (Bowser *et al.*, 1992; Potenza *et al.*, 1992). Further support for identification of Sec3p as bands A and F will be provided elsewhere (F.Finger and P.Novick, in preparation).

Since a non-specific protein of 70 kDa weakly immunoprecipitates from non-c-*myc*-tagged Sec8p strains (Figure 3B and see Figure 6 in TerBush and Novick, 1995), we wanted to establish unequivocally that the protein identified as Exo70p by peptide sequencing was the true 70 kDa Exocyst component. The *EXO70* gene was modified at its 3′ end to include the sequence encoding the 12CA5 HA epitope (Wilson *et al.*, 1984). The resulting *EXO70-HA* gene was transformed into a diploid yeast strain disrupted for one copy of *EXO70*. Analysis of 11 dissected tetrads established that the tagged Exo70 protein fully complemented the disrupted allele for growth at temperatures ranging from 14 to 37°C (data not shown). We transformed NY1393 (*SEC8-3X-c-myc*) with an *EXO70-HA* integrating construct (strain NY1468) and then analyzed immunoprecipitates for the presence or absence of the respective tagged proteins by Western blot (Figure 3C). We were able to co-immunoprecipitate the tagged proteins from NY1468 whether we used the 12CA5 or the 9E10 monoclonal antibodies for the immunoprecipitation (Figure 3C). Exo70-HA did not immunoprecipitate in the absence of added antibody, indicating that it is not the minor back-

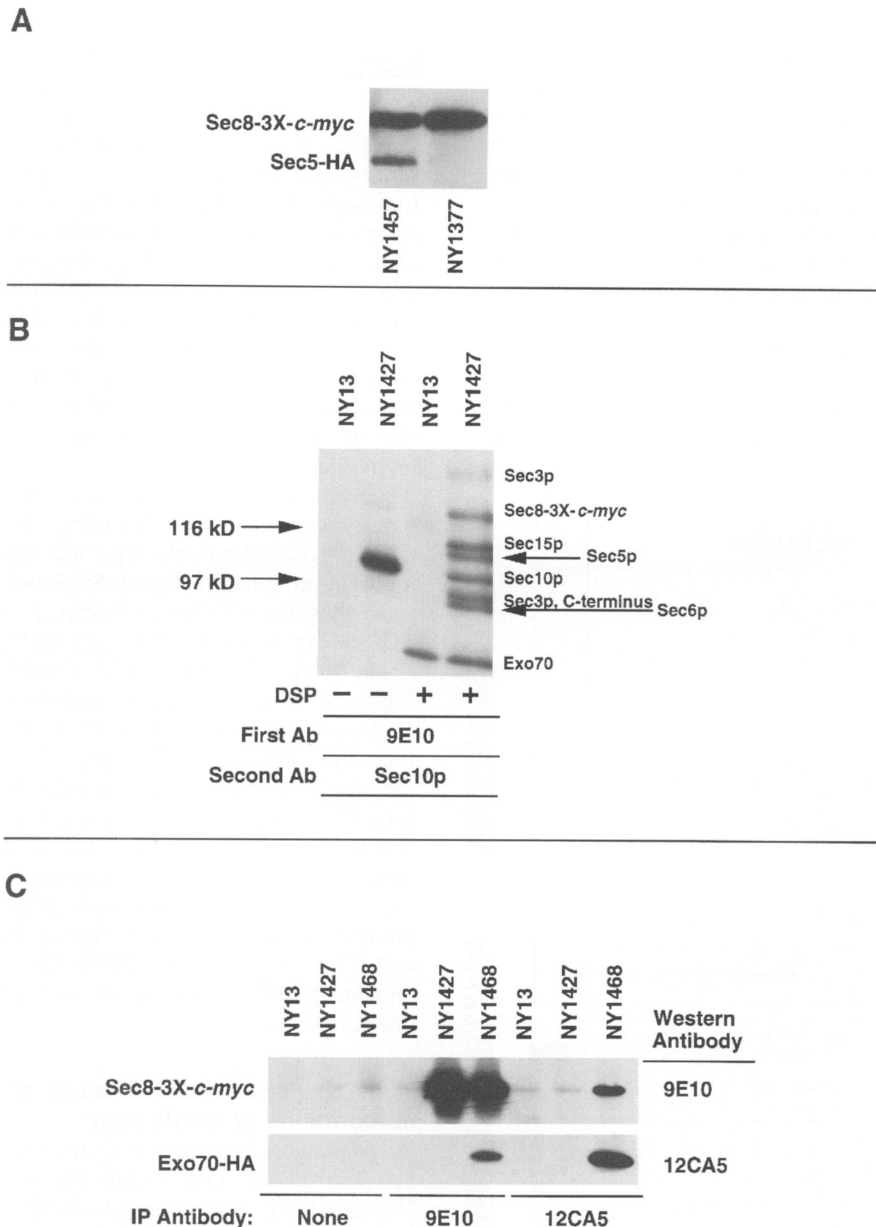

Fig. 3. Sec5-HA, Sec10p and Exo70-HA co-immunoprecipitate with triple c-*myc* epitope-tagged Sec8p. (**A**) Anti-c-*myc* (9E10) immunoprecipitates were prepared from yeast strains NY1377 (Sec8-3X-c-*myc*) or NY1457 (Sec8-3X-c-*myc*, Sec5-HA). The immunoprecipitates were separated by SDS–PAGE (7.5% gel) and transferred to nitrocellulose. The blots were first probed by ECL-based Western blotting with anti-c-*myc* which only detected the Sec8-3X-c-*myc* protein (data not shown). Then, without stripping the blot, it was reprobed by ECL with anti-HA (12CA5) to detect Sec5-HA. Shown is the reprobed blot which also detects Sec8-3X-c-*myc* due to residual anti-HRP bound to the first antibody. Sec5-HA co-immunoprecipitates with Sec8-3X-c-*myc*. (**B**) Radiolabeled lysates (1×10^8 c.p.m./IP) were prepared from NY13 (Sec8p) or NY1427 (Sec8-3X-c-*myc*) and treated with or without 1 mM DSP as described in Materials and methods. Double immunoprecipitations were then performed as detailed in Materials and methods. The second immunoprecipitates were collected, boiled in sample buffer containing 100 mM DTT to break the chemical cross-links and separated on a 7% gel. The immunoprecipitates were then imaged by autoradiography. Anti-Sec10p antibody recognizes the 100 kDa component of the Exocyst. (**C**) Anti-c-*myc* or anti-HA immunoprecipitates were prepared from yeast strains NY13 (Sec8p), NY1427 (Sec8-3X-c-*myc*) or NY1468 (Sec8-3X-c-*myc*, Exo70-HA). The immunoprecipitates were separated by SDS–PAGE (7% gel) and transferred to nitrocellulose. The blots were then cut in half and the bottom was probed for Exo70-HA with the 12CA5 antibody and the top was probed for Sec8-3X-c-*myc* with the 9E10 antibody by ECL. Either first antibody co-immunoprecipitated Sec8-3X-c-*myc* and Exo70-HA from NY1468. In the absence of antibody, there was no detectable Exo70-HA protein, indicating that it is associated specifically with the Exocyst complex. All experiments were performed at least twice.

ground protein. This confirmed that Exo70p is band H of the Exocyst.

The new Exocyst complex proteins

We have identified four proteins as new components of the Exocyst complex. The proteins encoded by *SEC5* and *SEC10* have not been described previously and the protein encoded by the *EXO70* gene was only mentioned previously in a sequencing report (Komatsoulis *et al.*, 1987). Sec5p is 971 amino acids long with a predicted M_r of 112.1 kDa and an apparent M_r of 107 kDa (see above). It is hydrophilic, lacks any predicted transmembrane domain, has a predicted pI of 5.67 and a net charge at neutral pH of −11.6. Sec10p is 871 amino acids long with a predicted

and apparent M_r of 100.4 kDa (see above). It is hydrophilic, lacks any transmembrane domains, has a predicted pI of 6.09 and a net charge at neutral pH of –9.3. The sequences of Sec5p and Sec10p are shown in Figure 4. Exo70p is 623 amino acids long with a predicted M_r of 71.3 kDa and an observed M_r of 70 kDa (see above). It is hydrophilic, lacks any transmembrane domains, has a predicted pI of 7.39 and a net charge of 1.32 at pH 7. Sec3p is described elsewhere (Haarer *et al.*, 1996), and a summary of the physical properties of all Exocyst proteins is given in Table II.

A search of these four proteins against the non-redundant GenBank protein database, against the dbEST database and against the now completed yeast genome database by BLAST indicates that none of these proteins have homologs except Sec10p. Sec10p is highly homologous to a putative *Caenorhabditis elegans* protein encoded by gene C33H5.9 (GenBank accession no. U41007) and a human expressed sequence tag (EST; GenBank accession no. H96462). The *C.elegans* protein has a BLAST probability score of 8.5×0^{-14} and aligns with the full length of Sec10p (although the alignment contains three significant gaps since the *C.elegans* ORF encodes a protein of 659 amino acids versus 871 for Sec10p), indicating a high likelihood that they are derived from a common ancestor. BESTFIT analysis indicates that they share 21% identity and 47% overall similarity. The alignment score resulting from a comparison of randomized fragments of the two sequences decreases by 18.7 SD (standard deviations), indicating that the homology between the two proteins is not due to overall similarity in amino acid composition, but is dependent on the order of the amino acids within the protein sequence. The homology between Sec10p and C33H5.9 is comparable with that observed between the rat brain proteins rSec6 and rSec8 and their corresponding yeast proteins (rSec6p to Sec6p, $P < 3.7 \times 10^{-10}$, SD = 9.0; rSec8p to Sec8p, $P < 1.4 \times 10^{-14}$, SD = 13.0) (Ting *et al.*, 1995). The human EST H96462 encodes a protein fragment which is highly homologous to Sec10p and *C.elegans* (C33H5.9). The EST encodes an 84 amino acid fragment (bases 2–253) which is homologous to the extreme C-termini of Sec10p and C33H5.9. As predicted for a C-terminal protein fragment, the region of homology is followed by a stop codon (two stop codons in the next three codons). A gap-free alignment of the 76 amino acid region of homology is shown in Figure 5. In this region, the EST has 25% identity to Sec10 (19 of 76 amino acids) and 37% identity to C33H5.9 (28 of 76 amino acids), indicating that it may encode part of the human Sec10 protein homolog.

A SEC5 Protein Sequence

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1     MDRFQIGDEQLLRFYQLKTINPTHSWAQDSSKLNNEEATSNELGVETSFD    50
51    ILKDFKYGNQISIDKESRAYLNDESLSYIRDPLNGQEMSKELQHLPNDSM   100
101   RLNYLVNSKQFNVKAFLRDMHKQDSFNDLNNSLDRLDSDIQDQSIHLKQL   150
151   VGKNFTKYVKIKNKLDQIYKEFDEKTNEKNQCDSPKENQINVESLNKKVD   200
201   EVIRTTTFKLKPLMDNYQKILNYQATKKFIELNKFYFNLPKSLKRCLTNN   250
251   DFNEFIIEYSKGLTLRRRFNQSSDASQSLVIKRIWTQIENLLVTYKDLIW   300
301   NSLINSNFNIDQPQETILSLFSKLLNLENFINNNQRESESGNKNTTSSSN   350
351   ENPILRWMSIKMNGFQNELNELSGHMISKIIHSQRLILQNNTNQDKSQGC   400
401   VELSYYLKINQLFQIISDTGKDSEGLKSTVEPNKVNTISGTSYLNLNCQP   450
451   SSQGLTDSPTIIEMWLLILKYINDLWKICDQFIEFWEHIEKFLDGTYQNS   500
501   IINEKRKENILIGDSNIIESYQKSLILKEEQINEVRLKGEEFITSVSQNL   550
551   ISFFTSSQSSLPSSLKDSTGDITRSNKDSGSPLDYGFIPPNCNGLSCLRY   600
601   LPKIVEPILKFSTELAQLNITTNGITICRNTLSTIINRCVGAISSTKLRD   650
651   ISNFYQLENWQVYETVTFSSKSQDSSKNLTFEYGVTQFPEIVTSFQEVSI   700
701   KTTRDLLFAYEKLPIINGISVVSYPSKQLLTGIEIQQIISMEAVLEAILK   750
751   NAAKDKDNPRNSHTILTLTNLQYFRECAFPNILQYFDDAFEWNLASKNLE   800
801   LFSLLSKMESSIFGNYLSDLKINLRDTLEEKFHEINWPMYTSNSFRVGDY   850
851   IIEALMILIVVHSECFRIGPQLIHKILIETQIFIARYLFEAFKPYVGNLS   900
901   NDGSLQIIVDLEFFQKVMGPLLEKDTEATLRACLQNCFQNDTNRLQKCIN   950
951   EINPIVSANLKRTAIQFAAFS                                971
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B Sec10 Protein Sequence

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1     MNSLYELDPKWKKLLKTDNFLGGLTVNEFVQELSKDHRNDVLIDANTKNL    50
51    PTNEKDQDAIREAIWKQLDPKPYIRTFESTLKELKNLNEETLNKRQYFSE   100
101   QVATQEVIHSENVIKLSKDLHTTLLTFDKLDDRLTNVTQVVSPLGDKLET   150
151   AIKKKQNYIQSVELIRRYNDFYSMGKSDIVEQLRLSKNWKLNLKSVKLMK   200
201   NLLILSSKLETSSIPKTINTKLVIEKYSEMMENELLENFNSAYRENNFTK   250
251   LNEIAIILNNFNGGVNVIQSFINQHDYFIDTKQIDLENEFENVFIKNVKF   300
301   KEQLIDFENHSVIIETSMQNLINDVETVIKNESKIVKRVFEEKATHVIQL   350
351   FIQRVFAQKIEPRFEVLLRNSLSISNLAYVRILHGLFTLFGKFTKSLIDY   400
401   FQLLEIDDSNQILSTTLEQCFADLFSHYLYDRSKYFGIEKRSLEAILVDM   450
451   TSKFTVNYDKEINKRVLLDKYKEKLSTNVDAFMHSPRGNTHSRQDSTSRS   500
501   KLSQFNSFLKTHLDKDHLSLNRTNTLSDSFNNSSSSTQYDVANNSSSLVN   550
551   SSFTASDIDNSPNSPANYSLNDVDSMLKCVVESTARVMELIPNKAHLYIL   600
601   EILKIMFLGIVDSYMEIALEVAYWKICKVDINKTAGVVNLNFLKFISMST   650
651   EILDLLSISIKSIFLPLLNNSPEIKAQIIEMTNSQIQKMEILINIILQET   700
701   ITVISTKFSAILCKQKKKDFVPKSQELLDQDTLPAIEIVNILNLIFEQSS   750
751   KFLKGKNLQTFLTLIGEELYGLLLSHYSHFQVNSIGGVVVTKDIIGYQTA   800
801   IEDWGVASLIDKFATLRELANLFTVQPELLESLTKEGHLADIGRDIIQSY   850
851   ISNREDFNHDNFINSVKLNFR                                871
```

Fig. 4. (**A**) Sec5p and (**B**) Sec10p protein sequences. Regions with a $P > 0.5$ of forming a coiled-coil are double underlined. The sequenced regions are boxed in black. Amino acids are represented by the single letter code.

The Exocyst complex contains all identified components at single copy

Previous work has suggested that the Exocyst complex is between 1×10^6 and 2×10^6 Da in mass (Bowser and Novick, 1991; Bowser *et al.*, 1992). The apparent mass of the seven proteins of the Exocyst is 743 kDa if all the components are present at single copy, but higher copy numbers would be equally consistent with the previously determined size. Since we know the amino acid sequences of all Exocyst proteins, we can establish their relative

Table II. A comparison of the Exocyst proteins

Protein	Length a.a.	Apparent mol. wt	Predicted pI	Charge pH 7.0	Chromosome	Motifs
Sec3p	1336	144	5.33	–38.42	V	G, C2, K, Y
Sec8p	1065	121	6.56	–5.20	XVI	G, C2, K, Z(50–71)
Sec15p	910	113	4.68	–39.67	VII	G, C2, K, Y
Sec5p	971	107	5.67	–11.59	IV	C2, K, Y
Sec10p	871	100	6.09	–9.29	XII	C2, K, Y
Sec6p	733	88	4.89	–31.05	IX	C2, K, Y
Exo70p	623	70	7.39	1.32	X	G, C2, K, Y

G, potential cAMP- and cGMP-dependent protein kinase phosphorylation site(s); C2, potential casein kinase II phosphorylation site(s); K, potential protein kinase C phosphorylation site(s); Y, potential tyrosine phosphorylation site(s); Z, potential leucine zipper, the amino acids which may form a leucine zipper are in parentheses.
Potential glycosylation sites, amidation sites and internal myristylation sites have not been included due to their unlikely biological significance.
Some of the above information has been published elsewhere (Komatsoulis *et al.*, 1987; Salminen and Novick, 1989; Bowser *et al.*, 1992; Potenza *et al.*, 1992; Haarer *et al.*, 1996).

```
Sec10       784- SIGGVVVTKDIIGYQTAIEDWGVASLIDKFATLRELANLFTVQPELLESLTKEGHLADIGRDIIQSYISNREDFNH -859 aa
C. elegans  578- STGAVLLLCDVGELRTLVSKWRVQNALTQWESLQALTNLLAVLPDQVNETAHSSLLENVDRQLIHDFVRLRTDFRS -653 aa
Human         2- CMGGMLAICDVAEYRKCAKDFKIPMMLHLFDTLHALQNLLVVAPDNLKQVCSGEQLANLDKNILHSFVQLRADYRS -229 bases
```

Fig. 5. Sequence alignments between the C-terminus of Sec10p, the C-terminus of the putative *C.elegans* Sec10 homolog (cosmid with GenBank accession no. U41007, gene C33H5.9) and a translation of a human EST (GenBank accession no. H96462) encoding a sequence homologous to both the yeast and *C.elegans* C-terminal sequences. The sequences in this region align without gaps. Identical residues between any two sequences are boxed in black. For the human EST sequence, the numbers refer to the bases in the sequenced clone. For Sec10p and the protein encoded by C33H5.9, the numbers refer to amino acid positions.

stoichiometry by cutting and counting the [^{35}S]cysteine/methionine-labeled bands which specifically co-immunoprecipitate with triple c-*myc*-tagged Sec8p. If we then determine the copy number of one of the Exocyst components, the copy number of all the components could be defined based on their stoichiometry relative to this component.

To facilitate this analysis, we constructed a yeast strain (NY1393) which contains both a wild-type copy of Sec8p (121 kDa) and a triple c-*myc* epitope-tagged copy of Sec8p (131 kDa). The tagged and untagged proteins are expressed at equal levels in this strain, are easily resolved by SDS–PAGE (Figure 6B) and are equally functional by all parameters tested (growth on rich and minimal medium at temperatures ranging from 14 to 37°C, data not shown). Therefore, the Exocyst complexes in this strain should not favor incorporation of the wild-type protein over the tagged protein. If, for example, the Exocyst complex contains two copies of Sec8p per complex, then one half of the complexes should contain both wild-type Sec8p and triple c-*myc*-tagged Sec8p, one quarter should contain only the tagged Sec8p and one quarter should contain only wild-type Sec8p (Figure 6A). c-*myc* immunoprecipitates from NY1393 were analyzed by Western blotting with a polyclonal antibody to Sec8p (Figure 6B). No wild-type Sec8p was co-immunoprecipitated with the triple c-*myc*-tagged Sec8p, indicating that Sec8p is normally present at single copy in the Exocyst complex. Next, we determined the ratio of all the Exocyst proteins to each other by cutting and counting the [^{35}S]cysteine/methionine-labeled bands which specifically co-immunoprecipitate with triple c-*myc*-tagged Sec8p (Table III). The ratios vary between 0.73 (Sec3p) and 1.03 (Sec6p) to 1 (triple c-*myc* Sec8p), indicating that all the complex components are present at single copy similarly to Sec8p.

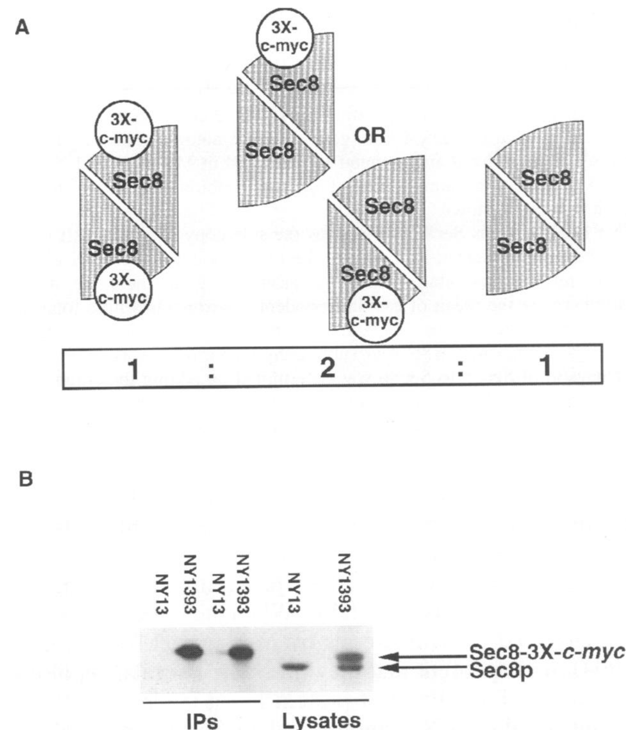

Fig. 6. The immunopurified Exocyst complex contains Sec8p at single copy. (**A**) If the Exocyst complex contains two copies of Sec8p, then there are four possible combinations of pairs of Sec8p with itself and/or with the Sec8-3X-c-*myc* protein when both are equally expressed in the same yeast cell. (**B**) Western blot analysis of lysates and Sec8-3X-c-*myc* immunoprecipitates for Sec8p. Lysates (210 μg protein) and immunoprecipitates (from 4 mg of lysate) from NY13 (contains only Sec8p) and from NY1393 (contains both Sec8p and Sec8-3X-c-*myc*) were Western blotted for total Sec8p using the Sec8p polyclonal antibody and [^{125}I]protein A for detection. Sec8p is 121 kDa and Sec8-3X-c-*myc* is 131 kDa. Only Sec8-3X-c-*myc* is present in the NY1393 immunoprecipitates. In the NY1393 lysate, both Sec8p (1517 c.p.m.) and Sec8-3X-c-*myc* (1520 c.p.m.) are present in equal amounts. In this experiment, the immunoprecipitations were done in duplicate. The data shown is from one of two experiments.

Discussion

The subunit composition of the Exocyst complex has now been defined. The structure, as isolated, contains one copy of seven different proteins. Six of these subunits are encoded by genes previously defined through the isolation of late-acting *SEC* mutants (Novick and Schekman, 1979; Novick *et al.*, 1980). The newly identified gene, *EXO70*, is an essential gene (Westaway *et al.*, 1988) and, as with the other six genes, we expect it to be required for secretion. We cannot rule out the possibility that there are additional subunits which are difficult to detect due to a weak association with the complex, instability or small size. Furthermore, we cannot exclude the possibility that one or more of these subunits has additional functions outside the confines of this complex, either alone or in an alternative complex. Defects in the genes encoding the Exocyst subunits, by and large, affect only exocytosis. Overall, it is striking that the products of six of the 10 *SEC* genes required for exocytosis form a stable structure that is found on the plasma membrane, concentrated at a site of active exocytosis (TerBush and Novick, 1995).

The amino acid sequences of the seven subunits offer few direct clues to their biochemical functions or to that of the complex as a whole. However, all the Exocyst complex proteins share several common properties (Table II). All of the proteins overall are hydrophilic and lack any obvious domain capable of spanning a membrane. All of the proteins are predicted to be acidic and negatively charged at neutral pH, except Exo70p which has a predicted pI and charge at neutral pH of 7.39 and 1.32, respectively. They all contain a number of putative phosphorylation sites and, with the exception of a possible leucine zipper

Table III. All Exocyst proteins are present at single copy in the immunoisolated complex

Exocyst component[a]	Cys + Met residues	Ratio of immunoprecipitated proteins to Sec8p NY1427[b]
Sec3p[c]	37	0.73
Sec8p	30	1.0
Sec15p	31	0.76
Sec5p	28	0.94
Sec10p	19	0.75
Sec6p	31	1.03
Exo70p	19	0.96

[a]Radiolabeled bands corresponding to identified components were excised from dried SDS–PAGE gels using the autoradiogram as a template, solubilized and counted as described in Materials and methods. The total number of cysteine and methionine residues in each protein is shown.
[b]NY1427 contains Sec8-3X-c-*myc* as the sole copy of Sec8p. All numbers are presented as a ratio to the total amount of Sec8-3X-c-*myc* in the immunoprecipitates which was arbitrarily given the value of 1.0. Numbers are the mean of two independent experiments with a total of five immunoprecipitates. The standard error was ≤0.11 for each mean. Average c.p.m. for Sec8p after subtracting background was 1329.
[c]The ratio of Sec3p to Sec8p was determined by adding the c.p.m. from the full-length protein (band A) to the c.p.m. for the C-terminus of Sec3p (band F) and using this sum for all calculations.

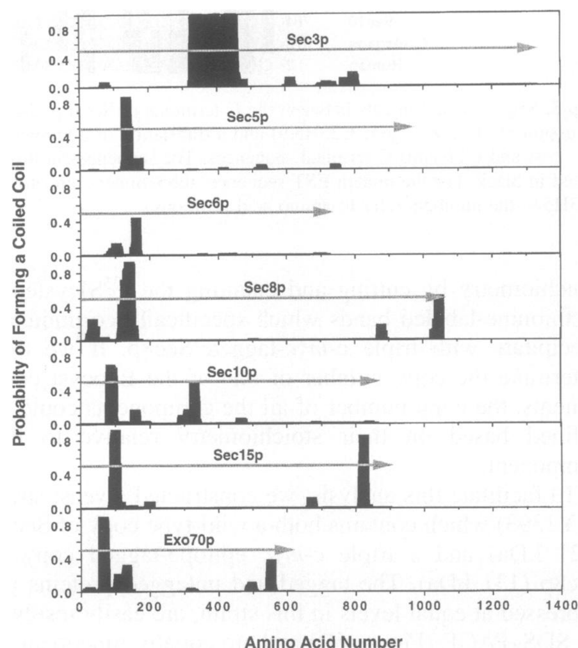

Fig. 7. Coiled-coil analysis of all seven Exocyst proteins. Shown is the probability of each Exocyst protein forming a coiled-coil domain calculated by MacStripe 1.3.4 (Knight, 1994), which is based on the Lupas algorithm (Lupas *et al.*, 1991; available at http://www.wi.mit.edu/matsudaira/coilcoil.html). The height of the arrow marks the 0.5 probability level for forming a coiled-coil and its length illustrates the size of the proteins to scale. All Exocyst proteins have some probability of forming a coiled-coil near their N-termini. Coils 2.1 (Lupas, 1996; http://ulrec3.unil.ch/software/COILS_form.html), also based on the Lupas algorithm but with a different scoring matrix (MTIDK), yielded qualitatively similar results. Paircoil (Berger *et al.*, 1995; http://ostrich.lcs.mit.edu/cgi-bin/score), which uses much stricter criteria than the Lupas algorithm, predicts that only Sec3p has a >0.5 probability of forming a coiled-coil.

motif in Sec8p (Bowser *et al.*, 1992), they lack any other, biologically significant, protein domain identifiable by MOTIFS (GCG, Madison, WI) and PRINTS (Attwood *et al.*, 1996). However, these programs fail to predict coiled-coil domains in proteins. When the Exocyst proteins are analyzed for coiled-coils by Macstripe 1.3.4 (Knight, 1994), it is apparent that they all share a similar topology (Figure 7). Each Exocyst protein has a tendency to form a coiled-coil in its N-terminus, and Sec3p has a particularly high likelihood of forming a long coiled-coil structure near its N-terminus (Haarer *et al.*, 1996). These coiled-coil domains may contribute significantly to the subunit interactions stabilizing the complex.

None of the Exocyst components show significant sequence homology to any well characterized protein. However, an indirect clue to the function of the Exocyst can be inferred from the absence of any homologs to its subunits in the recently completed yeast database. Many of the other components of the secretory machinery are the products of gene families. Each stage of vesicular transport appears to require at least one member of each of the Rab, synaptobrevin, syntaxin and Sec1p families, reflecting the common underlying molecular mechanisms of all transport events (Bennett and Scheller, 1993; Ferro-Novick and Jahn, 1994; Rothman, 1994; Rothman and Warren, 1994). In contrast, there are evidently no Exocyst-related structures needed for the earlier stages of the secretory pathway, for the endocytic pathway or for Golgi to vacuole transport. This complex appears to fulfill a requirement that is unique for exocytosis.

Homologs of several of the subunits have been identified in higher eukaryotes (Ting *et al.*, 1995). Prior studies have identified mammalian genes encoding proteins with weak, but statistically significant, similarity to Sec6p and Sec8p, and preliminary evidence suggests that these, like their yeast counterparts, are members of a large complex partially associated with the plasma membrane (Ting *et al.*, 1995). We have shown here that there are proteins with clear similarity to Sec10p in both the *C.elegans* and human databases. All of these proteins, similarly to their yeast counterparts, have a significant probability of forming an N-terminal coiled-coil domain (Ting *et al.*, 1995; data not shown). Although there is no directly comparable complex needed for other transport events in yeast, a similar complex is probably needed in all eukaryotes to permit exocytosis.

To gain some insight into the nature of the Exocyst function, we can consider the aspects of exocytosis that distinguish it from other membrane fusion events. One distinguishing feature is the division of the exocytic target membrane into different specialized domains. Fusion of any given class of exocytic vesicle is usually restricted to a limited region of the plasma membrane (Simons, 1995). This allows for cellular polarization with regards to the composition of the plasma membrane and cell surface growth (Drubin and Nelson, 1996). In most other membrane fusion events in the cell, there is no obvious need for such subspecialization of the target membrane. In the case of exocytosis in yeast, vesicle fusion is most active at the tips of small buds. Since this is also the site of highest Exocyst concentration (TerBush and Novick, 1995), we propose that the complex serves to direct vesicles or restrict their fusion to this site. While this is an appealing model, we must accommodate the observation that 75% of Sec8p and Sec15p (and presumably the other subunits as well) are found in the cytoplasm upon subcellular

fractionation. This soluble pool would interfere with vesicle targeting unless it could be maintained in an inactive form prior to recruitment to the plasma membrane. Given the complexity of the Exocyst, such regulation is readily conceivable.

Another distinguishing feature of exocytosis is the need for the vesicles to traverse a dense cytoskeletal layer to reach the target membrane (reviewed in Trifaró and Vitale, 1993). In regulated secretory cells and neurons, the cortical actin network restricts access of a pool of secretory vesicles to the sites of exocytosis. Upon the arrival of a secretory stimulus, this pool of vesicles is released from cytoskeletal constraints and diffuses to the site of exocytosis (Trifaró and Vitale, 1993; Pieribone *et al.*, 1995). Yeast also contain dense cortical actin patches near their sites of exocytosis (Adams and Pringle, 1984; Mulholland *et al.*, 1994). The Exocyst could be involved in rearranging cortical actin to provide access to the site of fusion during exocytosis in yeast. While there is, to date, no direct evidence for interaction between actin and the components of the Exocyst at a biochemical level, the identification of *SEC3* as *PSL1* (a profilin synthetic lethal gene) (Haarer *et al.*, 1996; F.Finger and P.Novick, in preparation) does provide a genetic link to the actin-based cytoskeleton.

A third distinguishing feature of exocytosis is that the target membrane faces the extracellular space. The environment of the extracellular space can vary greatly with respect to pH, osmotic strength and ionic conditions. This variation can be particularly severe in the case of a free-living microorganism like yeast. Cells may have evolved a mechanism, provided by the Exocyst complex, that ensures fusion of an exocytic vesicle to the plasma membrane, even when the plasma membrane is exposed to a hostile environment. In contrast, the fusion of other transport vesicles to their intracellular acceptor membranes does not face such extremes and may not require this unknown function.

In total, the molecular description of the Exocyst, together with the completion of the yeast genome sequence, have emphasized the unique function of the complex in membrane traffic. The role most consistent with the data at present is in restricting vesicle fusion, and hence addition of new membrane, to the appropriate region of the cell surface. The molecular mechanisms by which this is achieved await further analysis.

Materials and methods

Materials

Oligonucleotides for mutagenesis and primers for sequencing and primer extension were prepared by DNA Lab, Group #241, Yale University (New Haven, CT). Restriction enzymes, T4 DNA ligase and polymerase were from New England Biolabs (Beverly, MA). Pwo polymerase was from Boehringer Mannheim Corp. (Indianapolis, IN). Bacto-peptone, bacto-tryptone, bacto-agar, yeast nitrogen base without amino acids and bacto-yeast extract were from Difco Laboratories (Detroit, MI). Zymolyase 100T was from Seikagaku Corp. (Tokyo, Japan). The 9E10 ascites was prepared by the Pocono Rabbit Farm & Laboratory, Inc. (Canadensis, PA). The Nutridoma-grown, 9E10 antibody was prepared at the Department of Cell Biology Core Facility, Yale University (New Haven, CT). The 12CA5 ascites was a gift from Peter Kim (Whitehead Institute, MIT, Cambridge, MA). Monoclonal antibodies to the yeast L-A virus coat protein, gag, were provided by David Toft (Mayo Clinic, Rochester, MN) and polyclonal antibodies to gag were from Reed Wickner (NIH, Bethesda, MD). All other chemicals were purchased from Sigma, J.T.Baker or from American Bioanalytical, except as noted in the text.

Sec10 antibody production

An N-terminal Sec10 six histidine (His_6-Sec10) fusion protein was constructed for the preparation of polyclonal antibodies. Primer extension was performed using pNB682 as the template and the primers were constructed to place a *Bam*HI site at the 5′ end and a *Sal*I site at the 3′ end of an 888 bp segment of *SEC10* (bp 4–891, which correspond to amino acids 2–296). The primer extension reaction yielded a single 0.9 kb fragment of DNA which was gel purified, cut with *Bam*HI–*Sal*I, and ligated into a *Bam*HI–*Sal*I-digested pQE30 (Qiagen Inc., Chatsworth, CA) to yield plasmid pNB709. XL1Blue cells were transformed with pNB709 and the His_6-Sec10 was expressed in response to addition of isopropyl-β-D-thiogalactopyranoside (IPTG). Induced fusion protein was isolated on Ni^{2+}-NTA resin (Qiagen, Chatsworth, CA) by standard methods. Polyclonal antisera to the N-terminal Sec10 fusion protein was prepared in rabbits by Cocalico Biologicals Inc. (Reamstown, PA).

Cloning, sequencing and disrupting SEC5 and SEC10

Yeast strains containing the *sec5-24* and *sec10-2* temperature-sensitive alleles were transformed with a library of wild-type yeast genomic inserts in YCp50. The plasmids which fully complemented the temperature-sensitive phenotype of these alleles at 37°C were pNB498 and pNB497, respectively. The smallest region complementing *sec5-24* was defined by a 4.1 kb *Kpn*I–*Kpn*I fragment which was subcloned into the *Kpn*I site of pNB402 (pRS316; Sikorski and Hieter, 1989) to yield pNB689. The smallest fragment complementing *sec10-2* was a 4.4 kb *Cla*I–*Sac*I fragment which was subcloned into the *Cla*I–*Sac*I sites of pNB402 to yield pNB682. The nucleotide sequence was determined by the dideoxy chain termination method (Sanger *et al.*, 1977) using the Sequenase 2.0 kit (United States Biochemical Corp., Cleveland, OH) for *SEC5* and for the 5′ end of *SEC10*. DNA primers were either the universal M13 sequencing primers or synthetic primers complementary to parts of the *SEC5* or *SEC10* DNA sequences. The full *SEC10* DNA sequence was determined by searching our partial sequence against the preliminary, unpublished chromosome XII DNA sequence which matched a 2616 bp ORF (search performed courtesy of Mark Johnston, Washington University, St Louis, MO).

Constructs to disrupt the yeast genomic *SEC5* and *SEC10* genes were designed. For *SEC5*, the *Kpn*I–*Kpn*I complementing fragment in pNB689 was digested with *Hin*dIII to remove a 991 bp fragment within the *SEC5* structural gene and religated. A 1.2 kbp *Bgl*II–*Kpn*I fragment from a partial digest of the *SEC5* gene was then cloned into a *Bam*HI–*Kpn*I-cut vector pNB451 (pRS306; Sikorski and Hieter, 1989). The resulting vector was cut at *Hin*dIII, and a 1.1 kb *Hin*dIII fragment containing *URA3* was inserted to yield pNB695. For *SEC10*, a 2.6 kb *Sal*I–*Eco*RV fragment containing 0.1 kb of DNA sequence 5′ of the *SEC10* start codon and 2.5 kb of the coding sequence was subcloned into pUC119 at *Eco*RV–*Sal*I. The *SEC10* gene was then cut at an internal *Nco*I site and blunt ended. Into this site a blunt-ended 1.1 kb *URA3* fragment was ligated to yield pNB688. See Figure 1 for an overview of the *SEC5* and *SEC10* constructs.

Oligonucleotide-directed mutagenesis

12CA5 epitope-tagged *SEC5* (*SEC5-HA*) and *EXO70* (*EXO70-HA*) genes and a triple 9E10 epitope-tagged *SEC8* gene (*SEC8-3X-c-myc*) were constructed by oligonucleotide-directed mutagenesis of single-stranded DNA as described previously (Kunkel *et al.*, 1987; TerBush and Novick, 1995). The 3′ end of the *SEC5* gene was altered so that the encoded protein contained the sequence 971-YPYDVPDYA*-980 at its extreme C-terminus. The 3′-end of the *EXO70* gene was altered to encode a protein with the sequence 624-AKYPYDVPDYAA*-635 at its C-terminus. The 3′ end of the clone encoding the single c-*myc SEC8* epitope-tagged protein was altered to encode a triple c-*myc SEC8* epitope-tagged protein with the sequence 1053-KEQKLISEEDLVEQKLISEEDLVEQKLISEE-DLHTANEK*-1091 at its C-terminus. The corresponding epitope tags are underlined for each protein and the asterisk indicates the location of the stop codon. Full-length Sec5p, Exo70p and Sec8p are 971, 623 and 1065 amino acids long, respectively. The *SEC5-HA* gene was subcloned into the *URA3* integrating plasmid pRS306 (Sikorski and Hieter, 1989) as an *Xba*I–*Sna*BI fragment into *Xba*I–*Sma*I sites (pNB723). The *EXO70-HA* gene was subcloned from pBluescript KS+ into the *URA3* integrating plasmid pRS306 as a *Kpn*I–*Bam*HI fragment into *Kpn*I–*Bam*HI sites (pNB754). The *SEC8-3X-c-myc* gene was subcloned into pNB499, a

Table IV. Yeast strains

Strain	Genotype
NY13	*MATa, ura3-52*
NY179	*MATa, leu2-3, 112, ura3-52*
NY648	*MATa/α, leu2-3, 112/leu2-3, 112, ura3-52/ura3-52*
NY776	*MATα, leu2-3, 112, ura3-52, sec5-24*
NY780	*MATα, leu2-3, 112, ura3-52, sec8-9*
NY813	*MATa/α, leu2-3, 112/leu2-3, 112, ura3-52/ura3-52, SEC8/sec8::pNB338URA$^+$, L-A+* (*SEC8* disruption)
NY1008	*MATa, ura3-52, sec8Δ::URA3, leu2-3, 112::(LEU2, SEC8-c-myc), L-A+* (sole functional copy of *SEC8* is c-*myc*-tagged)
NY1060	*MATa/α, leu2-3, 112/leu2-3, 112, ura3-52/ura3-52, GAL$^+$*
NY1314	*MATa/α, leu2-3, 112/leu2-3, 112, ura3-52/ura3-52, SEC10/sec10::pNB688URA$^+$, GAL$^+$* (*SEC10* disrupt)
NY1319	*MATa/α, leu2-3, 112/leu2-3, 112, ura3-52/ura3-52, SEC5/sec5::pNB695URA$^+$, GAL$^+$* (*SEC5* disrupt)
NY1370	*MATα, ura3-52, leu2-3, 112::(LEU2, SEC8-3X-c-myc), sec5-24*
NY1377	*MATα, ura3-52, leu2-3,112::(LEU2, SEC8-3X-c-myc), sec8-9, L-A-o*
NY1393	*MATa, ura3-52, leu2-3, 112::(LEU2, SEC8-3X-c-myc)*
NY1427	*MATα, ura3-52, sec8Δ::URA3, leu2-3, 112::(LEU2, SEC8-3X-c-myc), L-A-o* (sole functional copy of SEC8 is triple c-tagged in a strain background lacking the L-A virus)
NY1457	*MATα, ura3-52::(URA3, SEC5-HA), leu2-3, 112::(LEU2, SEC8-3X-c-myc), sec5-24*
NY1459	*MATa/α, leu2-3, 112/leu2-3, 112, ura3-52/ura3-52, SEC8/sec8::pNB338URA$^+$, L-A-o* (*SEC8* disruption in L-A virus-free strain)
NY1468	*MATα, ura3-52::(URA3, EXO70-HA), leu2-3,112::(LEU2, SEC8-3X-c-myc), sec8-9, L-A-o*

LEU2 integrating plasmid (TerBush and Novick, 1995), as a *Sma*I–*Sal*I fragment into *Sma*I–*Sal*I sites to yield pNB711.

Construction of yeast strains

A triple c-*myc*-tagged *SEC8* strain lacking the yeast double-stranded RNA virus, L-A, was constructed. First, NY813 (*MATa/α, leu2-3, 112/leu2-3, 112, ura3-52/ura3-52, SEC8/sec8:: pNB338URA3, L-A+*) was grown on YPD plates at 40°C. Individual colonies were each restreaked at 40°C and then analyzed for the loss of the L-A virus gag coat protein by Western blot. An isolate was selected which lacked detectable L-A virus gag (NY1459). Competent NY1459, NY179 or NY780 cells were prepared by the alkali cation method (Ito *et al.*, 1983) and transformed with *Bst*XI-digested pNB711. Transformants from NY1459 were plated on selective medium at 25°C. Tetrads from positive transformants were dissected and the haploid progeny were analyzed for the presence of the *LEU* and *URA* markers. The presence of the *SEC8-3X-c-myc* allele was indicated by the presence of the *LEU2* gene, and the lack of a wild-type *SEC8* was indicated by the presence of the *URA3* gene. All isolates containing *SEC8-3X-c-myc* as the sole copy of *SEC8* grew like wild-type *SEC8* strains at temperatures ranging from 14 to 37°C on YPD (1% bacto-yeast extract, 2% bacto-peptone, 2% dextrose) and SD (synthetic minimal medium containing 2% dextrose). Transformants from NY179 which contain two functional copies of *SEC8* (*SEC8-3X-c-myc* and *SEC8*) grew like wild-type, untransformed cells. Transformants from NY776 which are *sec5-24* and contain two functional copies of *SEC8* (*SEC8-3X-c-myc* and *SEC8*) remained similarly temperature-sensitive and did not grow at 34°C. Transformants from NY780 which contain both *sec8-9* and *SEC8-3X-c-myc* grew like wild-type at all temperatures tested. The yeast strain containing *SEC8-3X-c-myc* as the sole copy of *SEC8* was designated NY1427. The NY179, NY776 and NY780 strains containing *SEC8-3X-c-myc* were designated NY1393, NY1370 and NY1377, respectively. NY1370 was then transformed with *Stu*I digested pNB723 and plated on selective medium. A *SEC5-HA*-containing transformant (*URA$^+$*) was analyzed for complementation of the *sec5-24* allele at 37°C. The epitope-tagged *SEC5* allele conferred normal growth at the restrictive temperature, indicating that the tagged allele retains wild-type function. The NY1370 strain containing the *SEC5-HA* allele was designated NY1457.

Diploid yeast strains containing a *SEC5* or *SEC10* disrupt were constructed as follows. For *sec5Δ*, a 2.3 kb *Bgl*II–*Kpn*I fragment of pNB695 containing the *SEC5* disrupted with *URA3* was isolated and used to transform the diploid yeast strains NY648 and NY1060. For *sec10Δ*, a 2.2 kb *Eco*RI–*Eco*RI fragment of *SEC10* disrupted with *URA3* from pNB688 was isolated and used to transform NY648 and NY1060. *URA$^+$* colonies were selected for each disruption, induced to sporulate, and tetrads were dissected. Diploids containing one copy of *SEC5* or *SEC10* disrupted in the NY1060 strain were designated NY1319 and NY1314, respectively. The genotypes of the yeast strains used in this study are given in Table IV.

Preparation of yeast lysates, immunoprecipitations and Western blotting

Yeast strains were grown, harvested and spheroplasted as previously described (TerBush and Novick, 1995). Spheroplasts were pelleted and lysed in Buffer A [20 mM PIPES pH 6.8, 100 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol (DTT), 0.5% Tween-20] containing protease inhibitors [1 mM phenylmethylsulfonyl fluoride (PMSF), 5 μg/ml pepstatin A, 2 μg/ml chymostatin, 2 μg/ml leupeptin, 2 μg/ml antipain, 2 μg/ml aprotinin] and incubated at 4°C for 30 min on a nutator. Insoluble material was pelleted at 10 000 *g* for 20 min at 4°C. The supernatant was used as source material for the immunoprecipitations, and the protein concentration was determined by the method of Bradford (1976) with bovine IgG as a standard. Typically, 1 ml of lysate (4 mg of protein) was used per individual immunoprecipitation. The immunoprecipitations were performed as previously published (TerBush and Novick, 1995) except for using Buffer A for all wash steps. Proteins were liberated from the protein A–Sepharose beads by boiling the samples for 5 min, and the immunoprecipitated proteins were separated by SDS–PAGE (Laemmli, 1970).

Proteins separated by SDS–PAGE were transferred overnight to nitrocellulose membranes. The nitrocellulose membranes were pre-incubated in phosphate-buffered saline (PBS) and non-specific binding sites were blocked by incubation in 3% milk in PBS for 1 h or with Tris-buffered saline (TBS) containing 5 mg/ml bovine serum albumin, 10 mg/ml bovine gelatin and 0.1% Tween-20. Incubation with the primary antibody was for 2 h at room temperature. Both 9E10 and 12CA5 antibodies were used at a 1/1000 dilution of the ascites fluid. After washing, the membranes were probed for 20–30 min with anti-mouse IgG horseradish peroxidase conjugates (Sigma, St Louis, MO) at 1/5000 dilution in PBMT (PBS containing 3% milk and 0.5% Tween-20). The blots were washed once for 15 min in PBMT, washed further for 3×5 min in PBMT and rinsed briefly in PBS. Antibody detection was by ECL (Amersham, Arlington Heights, IL).

c-myc immunoprecipitation from [^{35}S]cysteine/methionine-labeled yeast and cross-linking experiments

[^{35}S]Cysteine/methionine-labeled yeast were prepared and spheroplasted as previously described (TerBush and Novick, 1995). The spheroplasts were lysed with 1 ml of Buffer A for every multiple of 0.75 A_{599} units of starting yeast, and the number of trichloroacetic acid-precipitable counts was determined by scintillation counting. To each microfuge tube, 1 ml of the radioactive lysate, 4 mg of hydrated protein A and 4 mg of non-radiolabeled protein (P30 fraction made from NY13) were added. The immunoprecipitations, SDS–PAGE separation and quantitation of radiolabeled proteins were then performed as previously described (TerBush and Novick, 1995). For quantitation, the background radioactivity which precipitates from a wild-type Sec8p strain was subtracted from the corresponding molecular weight Exocyst protein band (Table III).

In cross-linking experiments, radiolabeled yeast spheroplasts were prepared as above but given one additional wash in 1.4 M sorbitol to remove residual traces of Tris prior to lysis and cross-linking. Each 0.75 OD_{599} units of starting cells was lysed in 200 μl of ice-cold PBS containing protease inhibitors and containing either 1 mM DSP or an equal amount of vehicle (dimethylsulfoxide). The lysates were incubated on ice for 30 min and then ammonium acetate was added to a final

concentration of 200 mM for 10 min to quench the cross-linker. Each 200 μl aliquot was then diluted with 0.8 ml of Buffer A containing protease inhibitors but lacking DTT and immunoprecipitated and washed as above. The immunoprecipitates were then boiled for 5 min in buffer (75 μl) containing 10 mM Tris (pH 8.0), 1% SDS, 5 mM EDTA with or without the addition of 100 mM DTT to break the cross-links. The beads were pelleted and the supernatants were transferred to fresh tubes and 1.2 ml of Buffer A containing protease inhibitors but lacking DTT was added to dilute out the SDS. Second antibody was added and the immunoprecipitates were cleared and washed as above. The immunoprecipitates were then liberated from the beads by boiling in 75 μl of sample buffer containing 100 mM DTT to break the protein cross-links, and the samples were separated by SDS–PAGE and visualized as above.

The Exocyst purification

The Exocyst was purified by immunoprecipitation and sequential slab separating gel/funnel tube concentrating gel SDS–PAGE electrophoresis (D.R.TerBush and P.Novick, in preparation). Briefly, a 3 l culture of yeast (strain NY1008 containing c-*myc* Sec8 as the sole copy of Sec8p) which was grown to an undiluted OD_{599} of 1.5 was used as the source of protein. The yeast were spheroplasted, lysed in Buffer A, and an S30 (30 000 *g*, 30 min) fraction was prepared. Protein was quantitated by the method of Bradford (1976) and the lysate was diluted to 4 mg/ml protein concentration (6.4 g of total protein in S30 lysate). Individual proteins in the Exocyst complex were then purified by immunoprecipitation via the c-*myc* epitope on Sec8 and sequential SDS–PAGE slab gel separation and funnel tube gel concentration as described (D.R.TerBush and P.Novick, in preparation). This material was the source of protein for peptide microsequencing of bands D (107 kDa protein), E (100 kDa protein) and H (70 kDa protein). The purification was repeated using 4.8 g of protein (S30 fraction) from the NY1427 strain as the source material for bands A and F (see Figure 2). The gel slices containing the unknown 70, 91, 100, 107 and 144 kDa peptides were then provided to the W.M.Keck Sequencing Facility (Yale University, New Haven, CT) for tryptic digests (bands D, E and H) or lys-C digests (bands A and F), amino acid composition, mass spectrometry and peptide microsequencing. The peptides were compared with the non-redundant protein sequence database at the NCBI using a BLASTP program (Altschul *et al.*, 1990) to search for yeast proteins containing identical peptides.

Protein sequence analysis

The Exocyst complex protein and DNA sequences were compared with the non-redundant protein sequence and nucleotide sequence databases at the NCBI using the TBLASTN and BLASTP programs (Altschul *et al.*, 1990). In addition, sequences were compared with dbEST using XREFdb to search for homologous ESTs (Bassett *et al.*, 1995) and with the completed yeast genomic sequences at the *S.cerevisiae* genome database at Stanford. The highest scoring protein homologs were analyzed by BESTFIT with RAN (GCG Corp., Madison, WI). All proteins were analyzed further for biologically significant protein domains by MOTIFS (GCG Corp., Madison, WI) and PRINTS (Attwood *et al.*, 1996). Finally, all Exocyst proteins were analyzed for α-helical coiled-coil domains using MacStripe 1.3.4 (Lupas *et al.*, 1991; Knight, 1994), COILS 2.1 (Lupas, 1996) and PAIRCOIL (Berger *et al.*, 1995).

Accession numbers

The EMBL accession numbers for Sec5, Sec10 and Exo70 are Y08788, Y08789 and Y08787, respectively.

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