Sec15 Protein, an Essential Component of the Exocytotic Apparatus, Is Associated with the Plasma Membrane and with a Soluble 19.5*S* Particle

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Abstract. *SEC15* encodes a 116-kD protein that is essential for vesicular traffic from the Golgi apparatus to the cell surface in yeast. Although the sequence predicts a largely hydrophilic protein, a portion (23%) of Sec15p is found in association with the plasma membrane. The remainder is not associated with a membrane but is found in a 19.5*S* particle which is not dissociated by 0.5 M NaCl. Sec15p may attach directly to the plasma membrane since it is not found on the Golgi apparatus nor on the secretory vesicle precursors to the plasma membrane. Loss of function of most of the late-acting *sec* gene products does not alter the distribution of Sec15p. However, the *sec8-9* mutation and to a lesser extent the *sec10-2* mutation result in a shift of Sec15p to the plasma membrane, suggesting a role for these gene products in the regulation of the Sec15p membrane attachment/detachment processes. Depletion of Sec15p by repression of synthesis indicates that the plasma membrane bound pool is the most stable. During the course of these studies we have found that two activities associated with the yeast Golgi apparatus, Kex2 endopeptidase and GDPase, are in separable subcompartments.

EUKARYOTIC cells possess a number of vesicle mediated transport pathways. These include initial endocytic events, transport from the endoplasmic reticulum to the Golgi apparatus, intra-Golgi traffic, and transport to both the cell surface and the lysosome/vacuole. Each transport event must be properly regulated to maintain the specificity of the system. Several proteins have been identified that function at specific steps of the vesicular transport pathway (Block et al., 1988; Clary et al., 1990; Newman and Ferro-Novick, 1987; Novick and Schekman, 1979; Payne and Schekman, 1989; Pfanner et al., 1990; Segev et al., 1988; Wattenberg et al., 1990; Weidman et al., 1989). One of these proteins, NEM sensitive factor (NSF),[1] has been found to function in multiple vesicular fusion events, including ER to Golgi transport in mammalian cells (Beckers et al., 1989) and early endocytic events (Diaz et al., 1989), and has been shown to have strong homology to the yeast gene *SEC18*, a gene whose product was known to be involved in ER to Golgi transport. These results suggest that some components of the fusion apparatus may be common to different transport pathways.

In the yeast *Saccharomyces cerevisiae* 10 *SEC* genes have been identified whose products regulate vesicular traffic from the Golgi apparatus to the plasma membrane (Novick and Schekman, 1979). One of these late-acting *SEC* genes encodes the GTP-binding protein Sec4p, that associates with both the cytoplasmic surface of the plasma membrane and secretory vesicles (Goud et al., 1988; Salminen and Novick, 1987). Sec4p appears to cycle between the plasma membrane and secretory vesicles, and this cycle of localization is coupled to a cycle of GTP binding and hydrolysis. Sec4p serves to regulate secretory vesicle traffic between the Golgi apparatus and the cell surface (Walworth et al., 1989). Other GTP-binding proteins, such as Ypt1 (Segev et al., 1988) and members of the ras superfamily of proteins (Gallwitz et al., 1989; Stearns et al., 1990), regulate vesicular traffic within different pathways. Such findings suggest that while the general mechanisms controlling vesicular traffic and fusion may be applicable to many or all such transport events, each class of vesicles may be regulated by distinct sets of GTP-binding proteins. In its GTP-bound state, each GTP-binding protein may interact with its effector to specifically regulate fusion with the proper acceptor membrane.

Candidates for components of an effector pathway have been identified by studies which demonstrated very strong genetic interactions between *SEC4* and several other *SEC* genes that function at the final stage of the secretory pathway (Salminen and Novick, 1987). Increased expression of Sec4p by a simple duplication of the *SEC4* gene was found to partially suppress both the growth and secretion defects resulting from mutations in *sec2* and *sec15* (Nair et al., 1990; Salminen and Novick, 1989). However, increased expression of *SEC4* could not suppress deletions of either *SEC2* or *SEC15*. Furthermore, any combination of temperature-sensitive mutations in *sec2, sec4, sec8,* or *sec15* were found to result in lethality even at temperatures that are permissive for any of the single mutants. These results support a model portraying Sec4p as a regulator of a molecular apparatus that

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[1] *Abbreviation used in this paper*: NSF, NEM sensitive factor.

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The Journal of Cell Biology, Volume 112, Number 6, March 1991 1117–1131

includes the Sec2, Sec8, and Sec15 proteins. Increased expression of Sec4p can compensate for a partial defect in one of the components of the apparatus, while defects in any two components is synergistically deleterious.

The *SEC15* gene was cloned and sequenced and found to encode a 116-kD protein that associates with a microsomal fraction in a pH-dependent manner (Salminen and Novick, 1989). Immunolocalization was not possible due to the low signal strength. However overproduction of Sec15p by expression from the *GAL1* promoter gave a very striking immunofluorescence signal (Salminen and Novick, 1989). A bright patch of fluorescence was seen in overproducing cells. The formation of this patch of fluorescence was found to be dependent on the function of the Sec2 and Sec4 proteins. Furthermore, it was found that overproduction of Sec15p led to an impairment of vesicular traffic and the formation of a cluster of secretory vesicles in the cytoplasm. One explanation for these results, and for the genetic interactions described above, is that Sec15p has the ability to dock vesicles, but only if those vesicles carry functional Sec4p and only if Sec2p, which appears to be cytoplasmic (Nair et al., 1990), is available. Given this hypothesis, it is essential to know the normal localization of Sec15p.

In this article we show by sucrose gradient fractionation and gel filtration that Sec15p is associated with both the plasma membrane and a soluble, high molecular weight species, but is not on isolated secretory vesicles. The association of Sec15p with the plasma membrane was found to be influenced by the *SEC8* gene product, suggesting that Sec8p may interact with Sec15p and regulate its level on the plasma membrane.

Materials and Methods

Yeast Strains, Media, and Reagents

The *Saccharomyces cerevisiae* strains used in this study are listed in Table I. The cells were grown in YP medium containing 1% Bacto-yeast extract and 2% Bacto-peptone (Difco Laboratories Inc., Detroit, MI), supplemented with either 2% glucose (rich medium, YPD) or 0.2% glucose (low glucose media). To induce overproduction of Sec15p from the GAL promoter cells were grown in YP medium containing 2% lactose until early log phase (A_{599} = 1). Galactose was then added to 1% and the cells incubated for 6 h. To deplete cellular levels of Sec15p, NY799 cells were first grown 16 h in the presence of 0.5% galactose in YP medium containing 2% raffinose. The cells were washed free of galactose using YP, and resuspended in YPD medium and incubated for various lengths of time.

Chemicals for SDS gel electrophoresis were obtained from Bio-Rad Laboratories (Richmond, CA). Zymolyase 100-T was obtained from ICN ImmunoBiologicals (Costa Mesa, CA). Sephacryl S-500 and S-1000 was obtained from Pharmacia Fine Chemicals (Piscataway, NJ). Nucleotide phosphates were purchased from Boehringer Mannheim Diagnostics, Inc. (Indianapolis, IN). Cytochrome *c* type III, NADPH, *o*-dianisidine, peroxidase, PMSF, sorbitol, EDTA, 4-aminodiphenylamine hydrochloride, and Triton X-100 were obtained from Sigma Chemical Co. (St. Louis, MO). Boc-Gln-3-Arg-MCA (Kex2 substrate) was purchased from Peninsula Laboratories Inc. (Belmont, CA). Production of rabbit anti-Sec15p antibodies was previously described (Salminen and Novick, 1989).

Electrophoresis

For SDS-PAGE, samples were heated for 5 min at 100°C in sample buffer containing 2% SDS and run on 8% slab gels according to Laemmli (1970). After transfer to nitrocellulose overnight at 4°C, Sec15p was probed with polyclonal αSec15$^{1-241}$ antisera at 1:1,000 dilution as previously described (Salminen and Novick, 1989).

Table I. Yeast Strains

Strain	Genotype
NY3	*MATa ura3-52, sec1-1*
NY11	*MATa his4-619*
NY17	*MATa ura3-52, sec6-4*
NY57	*MATa ura3-52, sec9-4*
NY61	*MATa ura3-52, sec10-2*
NY67	*MATa his4-619, sec15-1*
NY130	*MATa ura3-52, sec2-41*
NY400	*MATa his4-619, sec5-24*
NY405	*MATa ura3-52, sec4-8*
NY410	*MATa ura3-52, sec8-9*
NY440	*MATa ura3-52, his4-619*, pNB148 (2 μm, *SEC15, URA3*)
NY648	*MATa/α leu2-3, 112/leu2-3, 112, ura3-52/ura3-52*
NY724	*MATa ura3-52*, Gal+, *SEC15*::pNB304 (*GAL1-SEC15, URA3*)
NY799	*MATa leu2-3, 112, sec15::LEU2, ura3-52::pNB304* (URA3, GAL-SEC15) (*GAL1-SEC15, URA3*)
NY821	*MATa* NY410 transformed with pNB330 (*ura3-52::URA3, SEC8*)

Sucrose Gradient Fractionation

Cells (200–250 A_{599} U) grown at 25°C in YP medium containing 2% glucose, were pelleted and transferred to YP supplemented with 0.2% glucose and incubated with shaking for 1 h at 37°C. After washing once with 10 mM NaN_3, the cells were resuspended in spheroplast media (50 mM Tris pH 7.5, 10 mM NaN_3, 1.4 M sorbitol, 40 mM β-mercaptoethanol, 0.125 mg/ml Zymolyase-100T), and incubated at 37°C for 45 min. Spheroplasts were pelleted, cooled on ice, and resuspended in 20 ml ice cold lysis buffer (0.8 M sorbitol in 20 mM triethanolamine, 1 mM EDTA pH 7.2) containing 1 mM PMSF and 10 μl/10 ml protease inhibitor cocktail: leupeptin, chymostatin, pepstatin, aprotinin, and antiparin (all at 1 mg/ml). The following steps were performed at 4°C. The suspension was homogenized 20 times in a 40-ml Wheaton tissue grinder (Wheaton Scientific, Millville, NJ), pestle A, and centrifuged at 450 *g* for 3 min. The pellet (P1) was resuspended in the same volume lysis buffer, homogenized and centrifuged as above, and the supernatants (S1) pooled. 2 ml of 1 M MES, 2-(*N*-morpholino) ethane sulfonic acid, pH 6.5 was added to S1 and the supernatant was spun at 10,000 *g* for 10 min. The supernatant (S2) was spun at 100,000 *g* for 1 h to produce a P3 pellet and S3 supernatant. The P2 and P3 pellets were resuspended in 2 ml of 55% sucrose (wt/wt) containing 10 mM MES pH 6.5 and homogenized by four strokes in a 2-ml Wheaton tissue grinder (Wheaton Scientific). The P2 or P3 homogenate was placed at the bottom of a SW41 (Beckman Instruments, Inc., Palo Alto, CA) tube and overlaid with the following sucrose solutions: 1 ml 50%, 1 ml 47.5%, 1.5 ml 45%, 1.5 ml 42%, 1.5 ml 40%, 1 ml 37.5%, 1 ml 35%, 1 ml 30%, all containing 10 mM MES pH 6.5. The gradients were spun at 170,000 *g* in a SW41 rotor (Beckman Instruments, Inc.) for 16 h. Fractions were collected from the bottom and any pellet resuspended in an identical volume (as other fractions) of 55% sucrose and labeled as fraction 1.

For velocity gradient analysis an S2 from NY11 cells was isolated as above, except that the cells were lysed in 8 ml total volume of lysis buffer. The protein concentration was determined and 3.4 mg of protein (in ~450 μl) was layered on top of a 10–30% (wt/wt) continuous sucrose gradient containing 10 mM MES, pH 6.5, and +/− 0.1% Triton X-100. The gradient was centrifuged at 49,000 rpm for 2–8 h in a SW50.1 rotor (Beckman Instruments, Inc.). Fractions were collected from the bottom of the tube and any pellet was resuspended in an identical volume and labelled as fraction 1. BSA (4.5*S*), catalase (11.5*S*), horse spleen ferritin (16.5*S*), and thyroglobulin (19.3*S*) were used as standards.

Gel Filtration

Cells (usually 200–300 A_{599} U for S-500 columns or 500–800 A_{599} U for S-1000 columns) grown at 25°C in YP medium containing 2% glucose, were pelleted, resuspended in YP medium containing 0.2% glucose at 37°C, and incubated at 37°C with shaking for 1 h. After washing once in

10 mM NaN_3 the cells were resuspended in 20 ml of spheroplast medium and incubated at 37°C for 45 min. The spheroplasts were pelleted, cooled on ice, and resuspended in 4 ml (or 20 ml for S-1000 columns) of ice cold lysis buffer (see above). The cell suspension was homogenized by 20 strokes in a 7-ml (or 20 ml) Wheaton tissue grinder using pestle A (Wheaton Scientific). After a 3-min spin at 750 *g* (4°C), the pellet was resuspended in 4-ml lysis buffer and the homogenization repeated. The 750 *g* supernatants were combined to form the S1. For S-500 gel filtration the pH of the lysate was maintained at pH 7.5. For S-1000 gel filtration 1 M MES pH 6.5 was added to 10 mM. The S1 was centrifuged at 10,000 *g* for 10 min to form the S2 and P2. The protein concentration of the S2 was determined by Bradford analysis (Bio-Rad Laboratories). 20–25 mg of protein, in 1–1.5 ml of lysis buffer, was layered on the top of the S-500 column and fractionation performed at 4°C using 20 mM Hepes, 50 mM NaCl, 1 mM $MgCl_2$, 1 mM DTT, pH 7.5 as column buffer. 1.1 ml fractions were collected, analyzed by SDS gel electrophoresis, and the elution profile compared to that of molecular mass standards.

For Sephacryl S-1000 column fractionation for secretory vesicle isolation, the P3 was resuspended into 1 ml lysis buffer, pH 6.5 and layered on the top of the S-1000 column. 0.8 M sorbitol, 10 mM triethanolamine, 1 mM EDTA, 10 mM MES, pH 6.5 was used as the column buffer. 4.1 ml fractions were collected and analyzed by SDS gel electrophoresis and Western blot analysis.

Enzyme Assays

The enzyme assays for the plasma membrane ATPase, endoplasmic reticulum, and mitochondria were performed as previously described (Walworth et al., 1989). The protein concentration was determined by Bradford analysis. The GDPase assay was performed basically as described by Abeijon et al. (1989) and Bradan and Fleischer (1982). After incubation of each reaction tube containing 30 μl of the fraction for 30 min at 37°C the reaction was stopped by addition of 20 μl of 5% SDS. Liberated phosphate was measured using the Fiske-Subbarow reducing kit (Sigma Chemical Co.) The activity was expressed as nmoles of phosphate released per fraction per min. Kex2 activity was measured using the method of Julius et al. (1984) and Cunningham and Wickner (1989). The reaction tubes containing 50 μl of each fraction were incubated at 30°C for 1 h in the presence or absence of Triton X-100 and stopped by boiling for 5 min. The liberated product was determined by measuring the emission in a fluorometer at ex380$_{nm}$ and em460$_{nm}$. Latent Kex2 activity was determined by subtracting the values of +/− Triton X-100 and the result expressed as the amount of latent Kex2 activity per fraction.

Nucleic Acid Techniques

To generate *SEC8*+ transformants of *sec8-9* cells, the following was performed. The *SEC8* gene was cloned in a 2-μm library (results of cloning and sequencing the *SEC8* gene to be published elsewhere), and the Sma I-Sal I fragment inserted into a YIp5 vector. This integrating plasmid, pNB330, containing the entire *SEC8* coding sequence, was linearized with Stu I and integrated into NY410 cells (a *ura3-53, sec8-9*) by transformation (Ito et al., 1983). Transformants were selected for Ura+ on SD medium at 25°C.

NY799 cells were produced as follow. Integrating plasmid pNB291 (YIp5, *sec15::LEU2* gene disruption; 3-kb Bgl II–Bgl II fragment from YIp13 (*LEU2*) replacing the Bgl II–Bgl II internal fragment of *SEC15* in pNB192 [Salminen and Novick, 1989]) was used to transform NY648 cells to Leu+. These diploids were then transformed with pNB304 (YIp5, *GAL1-SEC15*) and dissected. URA+, LEU+ spores were isolated that grow on YP + 1% galactose + 2% raffinose but not YPD medium. These cells contain their sole copy of the *SEC15* gene under control of the *GAL1* promoter.

Electron Microscopy

Electron microscopy was performed on NY799 cells, after overnight incubation in YP + 0.2% galactose medium and subsequent incubation in YPD medium for 0–24 h, as previously described (Salminen and Novick, 1987).

Results

Localization of Sec15p to the Plasma Membrane

In the preliminary fractionation study (Salminen and Novick, 1989), it was shown by differential centrifugation that the *SEC15* gene product is in association with both the 100,000-*g* supernatant and 100,000-*g* pellet of a wild-type (NY451) yeast lysate. Association with the pellet fraction was found to be both pH sensitive and ionic in nature. Sec15p was not detected in a low speed (10,000 *g*) pellet. However, in that study, detection was limited by the low level of protein used on Western blots. In this study we have done more extensive cell fractionation and we consider in detail the various forms of Sec15p found in a yeast lysate.

Table II. Percent of Sec15p Localized in Subcellular Fractions of Late-acting SEC Mutants

Cell type	Percent in S2	Percent in P2	Percent in S3	Percent in P3
Wild type	77	23	31	45
sec1-1	79	22		
sec2-41	75	26		
sec4-8	75	26		
sec5-24	71	28		
sec8-9	52	45	27	38
sec9-4	74	21		
sec10-2	65	32	19	32
NY821 (*sec8-9*::SEC8+)	78	19		

Differential centrifugation analysis of Sec15p distribution in late-acting *SEC* mutants. 75 A_{599} U of each strain were harvested, lysed in a 6-ml volume, and centrifuged to form S2 and P2 fractions. A S3 and P3 fraction was also isolated from *sec8-9* and *sec10-2* cells. Sec15p was quantitated by Western blot analysis in fractions from each strain. The results are expressed as the percent of Sec15p located in P2 or P3, in relation to the total cellular Sec15p (levels of Sec15p in the initial total lysate). Each value represents the average of two to five experiments.

Analysis by differential centrifugation of a wild-type (NY11) cell lysate has revealed that ∼23% of the total cellular Sec15p is located in the 10,000-*g* pellet, whereas 40–45% resides in the 100,000-*g* pellet, and the remaining 31% is in the 100,000-*g* supernatant (Table II). To identify membrane components with which Sec15p associates, sucrose gradient fractionation was performed on both the 10,000-*g* and 100,000-*g* pellets of wild-type yeast cells (see Materials and Methods). NY11 cells were grown in rich media (YPD) at 25°C, harvested, and converted to spheroplasts. After osmotic lysis, the lysate was centrifuged at 750 *g* to remove unlysed cells. This lysate (S1) was spun at 10,000 *g* to form a supernatant (S2) and pellet (P2). The S2 was further centrifuged for 1 h at 100,000 *g* to form a high speed supernatant (S3) and pellet (P3). Both the P2 and P3 were resuspended in 55% sucrose, pH 6.5, and overlaid with various sucrose solutions. After centrifugation to equilibrium at 170,000 *g*, the gradient was fractionated and each fraction subjected to SDS gel electrophoresis and transferred to nitrocellulose. The Sec15p present in each fraction was determined using anti-Sec15p antibody and ^{125}I-protein A. Individual lanes were quantitated and the results expressed as the level of radiolabelled Sec15p per 50 μl of fraction.

The results of subfractionation of the P2 pellet of wild-type cells are shown in Fig. 1. Sec15p cofractionates with the plasma membrane enzyme marker, vanadate sensitive Mg^{2+}-ATPase activity (Bowman and Slayman, 1979). The plasma membrane marker enzyme is 18-fold enriched relative to the total lysate in the fractions containing the peak of Sec15p (Fig. 1, *Fractions 6–9*). Sec15p clearly does not

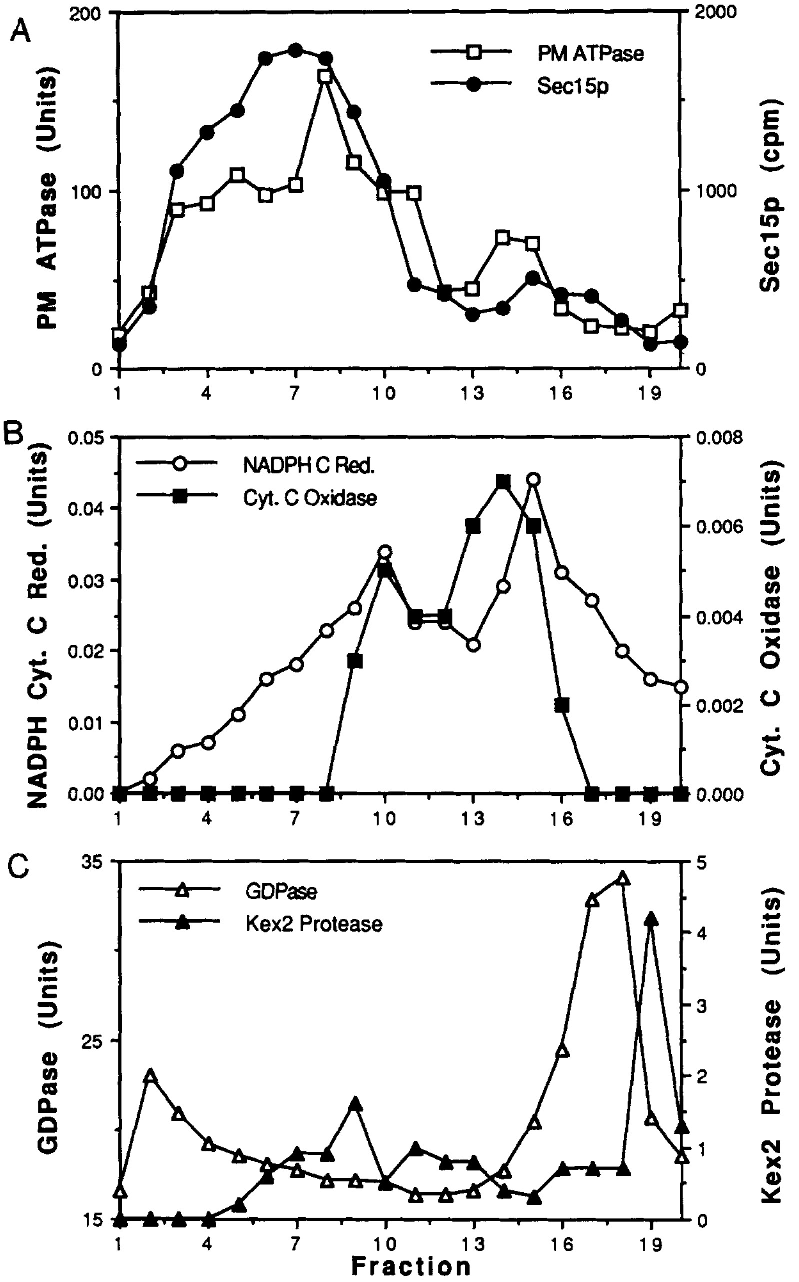


Figure 1. Localization of Sec15p and organelle enzyme marker activities within sucrose gradient fractions of a 10,000-*g* pellet from NY11 cells. A P2 was generated by differential centrifugation and resuspended in 2 ml 55% sucrose, 10 mM MES pH 6.5 and loaded on the bottom of a 30–55% gradient. The gradient was spun until equilibrium, fractionated, and aliquots of each fraction were assayed for Sec15p, plasma membrane ATPase, cytochrome *c* reductase, cytochrome *c* oxidase, GDPase, Kex2, protein concentration, and sucrose density. (*A*) Sec15p and plasma membrane ATPase activity cofractionate within the gradient. Sec15p was quantitated from Western blots by quantitative determination of ^{125}I-labeled protein A secondary antibody on cut out strips of nitrocellulose, and expressed as the number of ^{125}I counts per 50 μl of each fraction (●). The recovery of Sec15p from the gradient was 75% of the loaded P2 pellet fraction. Plasma membrane ATPase activity was determined by measuring the release of inorganic phosphate for 10 min at 37°C and the result expressed as the nmoles of liberated phosphate per fraction per min (□). (*B*) The gradient profile for cytochrome *c* reductase (○) and cytochrome *c* oxidase (■) activities. Both are expressed as the rate of increase in the A_{550nm} of the reaction using 20 μl of sample. (*C*) Localization of GDPase (△) and Kex2 (▲) activities within the gradient. The GDPase activity is expressed as the nmoles of liberated phosphate per fraction per minute using 30 μl of each fraction in the reaction. The Kex2 activity is expressed as the units of latent Kex2 activity per 50 μl of each fraction. Fraction 1 is the gradient pellet and fraction 20 is the top of the gradient.

cofractionate either the NADPH-cytochrome *c* reductase or cytochrome *c* oxidase enzyme activities. These enzymes are markers for the endoplasmic reticulum (Kreibich et al., 1973) and mitochondria (Mason et al., 1973), respectively. Sec15p also fails to localize with two marker enzymes for the yeast Golgi apparatus, Kex2 and GDPase. Kex2 is an endoprotease involved in prohormone maturation and believed to localize to a very late Golgi compartment (Cunningham and Wickner, 1989; Julius et al., 1984). GDPase converts the GDP that is liberated after transfer of mannose from GDP-mannose to glycoprotein acceptors to GMP + P_i and cofractionates with a presumed Golgi enzyme, α-1,2 mannosyltransferase (Abeijon et al., 1989). It is interesting to note that in wild-type cells the Kex2 and GDPase containing compartments pelletable at 10,000 *g* are separable from one another and from other organelles (see below). From these results we conclude that Sec15p associates with the plasma membrane in a P2 from wild-type yeast cells.

Soluble Sec15p Is Found in a High Molecular Mass Particle

We next determined if Sec15p localizes to other membrane components. Differential centrifugation analysis revealed that ~40–45% of the total Sec15p of a NY11 lysate will pellet at 100,000 *g*, while the remainder either sediments at 10,000 *g* or is soluble, Table II. Sec15p could be in association with secretory vesicles, or other membrane components, localized to this high speed pellet. Alternatively, Sec15p could be in a nonmembraneous, particulate form.

To address these possibilities we performed sucrose gradient fractionation of a P3 from NY11 cells. Since the secre-

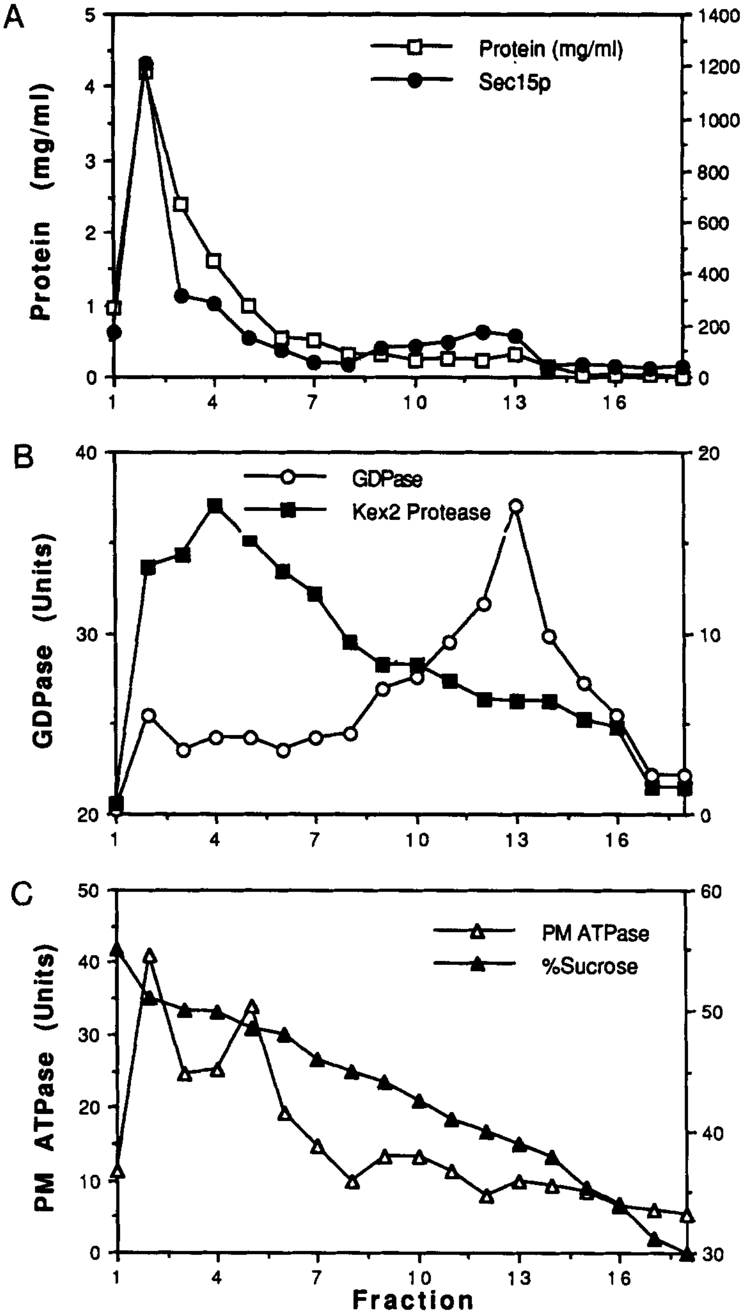


Figure 2. Localization of Sec15p and organelle enzyme marker activities within sucrose gradient fractions of a 100,000-*g* pellet from NY11 cells. A P3 was generated by differential centrifugation and resuspended in 2 ml of 55% sucrose, 10 mM MES pH 6.5, and placed at the bottom of a 30–55% gradient. After centrifugation until equilibrium, aliquots of each fraction were assayed for Sec15p by Western blot analysis and for each of the marker enzyme activities as in Fig. 1. The recovery of Sec15p was 108% of the loaded P3 fraction (average of three independent gradients). (*A*) Sec15p (●) and protein concentrations of each fraction (□). (*B*) GDPase (○) and Kex2 (■) activities. (*C*) Plasma membrane ATPase activity (△) and percent sucrose (▲).

tory pathway of wild-type yeast cells is extremely rapid (Novick et al., 1981), few secretory vesicles are localized in the high speed pellet. However sucrose gradient analysis of wild-type cells will indicate if Sec15p associates with other membrane components contained in P3 that enter the gradient. Upon fractionation, however, we find that Sec15p remains where it was loaded, at the bottom of the gradient, cofractionating with a peak of protein (Fig. 2). Sec15p does not localize with a peak of plasma membrane ATPase that enters the gradient. It also fails to localize with either of the two Golgi markers, Kex2 or GDPase. These results suggest that Sec15p is not associated with any membrane component of the high speed pellet, but may rather be in a nonmembraneous protein complex or aggregate.

The Kex2 and GDPase activities pelletable at 100,000 *g* are easily resolved by sucrose gradient fractionation (Fig. 2). Kex2 activity fractionates near the bottom of the gradient, whereas GDPase activity remains near the top of the gradient. This data provides evidence that Kex2 and GDPase containing compartments are distinct from one another (see below).

To further characterize Sec15p that is contained in both the high speed pellet and supernatant fractions, we performed Sephacryl S-500 gel filtration of a 10,000-*g* supernatant from NY11 cells. We chose to analyze a 10,000-*g* supernatant (S2) since it contains all Sec15p not associated with the plasma membrane. By Western blot analysis of the resulting fractions we observe that Sec15p elutes in a single peak with

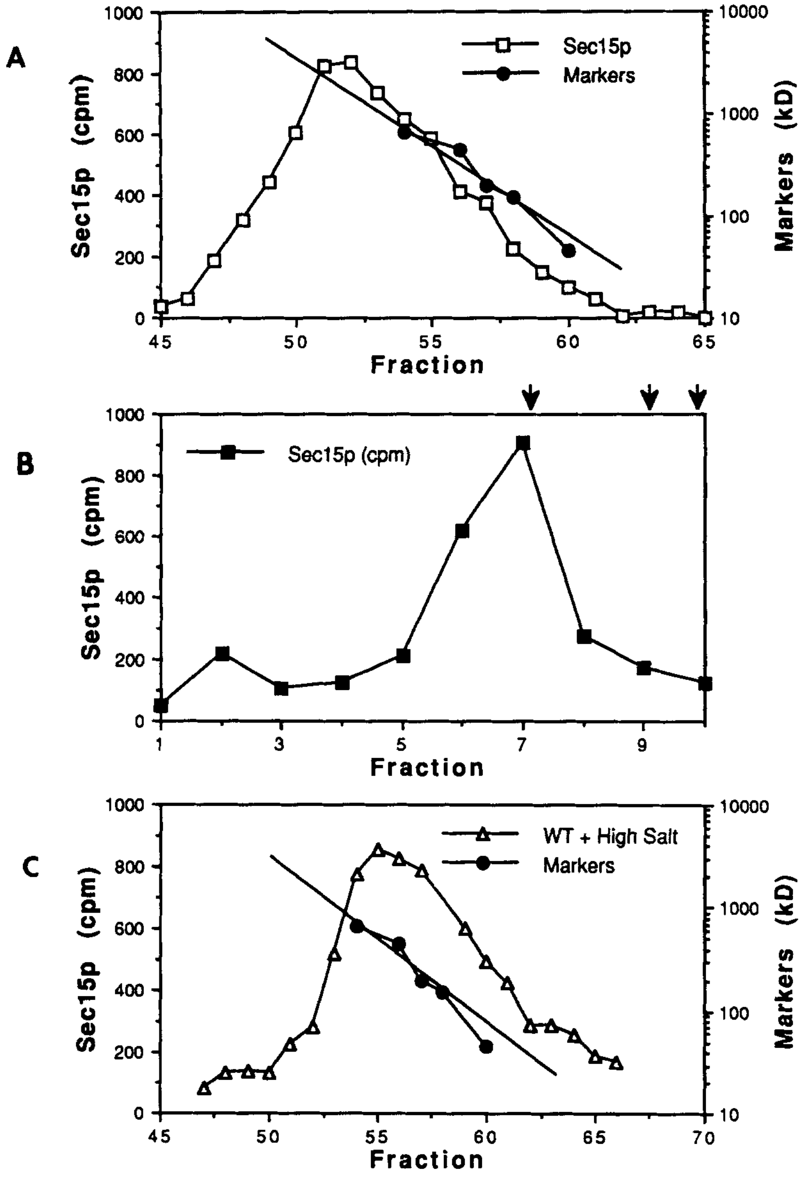


Figure 3. Soluble Sec15p is associated in a high molecular mass particle of 19.5*S*, identified by (*A*) Sephacryl S-500 gel filtration and (*B*) sucrose velocity gradients. (*A*) 1.5 ml of a S2 (20 mg of protein) from NY11 cells was layered on a S-500 column and the elution profile of Sec15p was determined by quantitative Western blot analysis (□). The column elution profile of markers was also determined by Western blot analysis. Markers used were thyroglobulin (669 kD), apoferritin (443 kD), β-amylase (200 kD), alcohol dehydrogenase (150 kD), and ovalbumin (45 kD). (*B*) Identification of a 19.5*S* particle containing Sec15p by velocity sucrose gradient centrifugation of a S2 from NY11 cells. Sec15p was localized in each fraction by Western blot analysis and the peak of Sec15p was found to correspond to a value of 19*S*. The recovery of Sec15p was 91% of the loaded S2 fraction. Vertical arrows, from left to right, mark the positions of thyroglobulin (19.5*S*), catalase (11.3*S*), and BSA (4.5*S*). (*C*) Dissociation of Sec15p from the plasma membrane of NY11 cells by high salt (500 mM NaCl) and pH treatment (pH 8.0) for 30 min on ice, and subsequent analysis of the released Sec15p by Sephacryl S-500 gel filtration.

a molecular mass of 1,000–2,000 kD (Fig. 3 *A*). No soluble, monomer form of Sec15p (115 kD) is present. Gel filtration of a 100,000-*g* supernatant (S3) gave identical results (not shown). Therefore soluble Sec15p localizes to a large particulate aggregate or complex of 1,000–2,000-kD apparent molecular mass. Only a single elution peak was observed, suggesting that the S3 and P3 pools of Sec15p are identical. Centrifugation of S2 for 1 h at 100,000-*g* results in partial clearance of this high molecular mass species of Sec15p. We were unable to dissociate Sec15p from the high molecular mass species by high salt treatment. An S2 from NY11 cells was column fractionated in the presence of 500 mM NaCl. We observed that Sec15p remains in the high molecular mass species, with no detectable monomer form (data not shown). Thus the high molecular mass species of Sec15p is stable in 500 mM NaCl and behaves as a tightly associated aggregate or complex.

Analysis of soluble Sec15p by sucrose velocity gradients revealed that Sec15p has a sedimentation coefficient of 19.5*S* (Fig. 3 *B*) similar to that of thyroglobulin. However by gel filtration Sec15p has an apparent molecular mass two to three times greater than that of thyroglobulin. The standards used, including thyroglobulin, are essentially globular proteins. Proteins with a more elongated shape will sediment more slowly than expected from their molecular weight (Doms, 1991). Therefore a sedimentation coefficient of 19.5*S* for the 1,000–2,000-kD species of Sec15p suggests that the shape of this high molecular mass form may be an extended or elongated oligomer.

In an attempt to determine if this high molecular mass species is a homoaggregate or a complex with other proteins, Sec15p was overexpressed from a 2μ high copy number plasmid or from a plasmid containing the inducible *GAL1* promoter. If Sec15p normally forms a complex with other proteins which are limiting in abundance it may be possible, by overproducing Sec15p, to induce monomer formation. If, however, Sec15p forms a homoaggregate, overexpression may result in increased levels of the high molecular mass

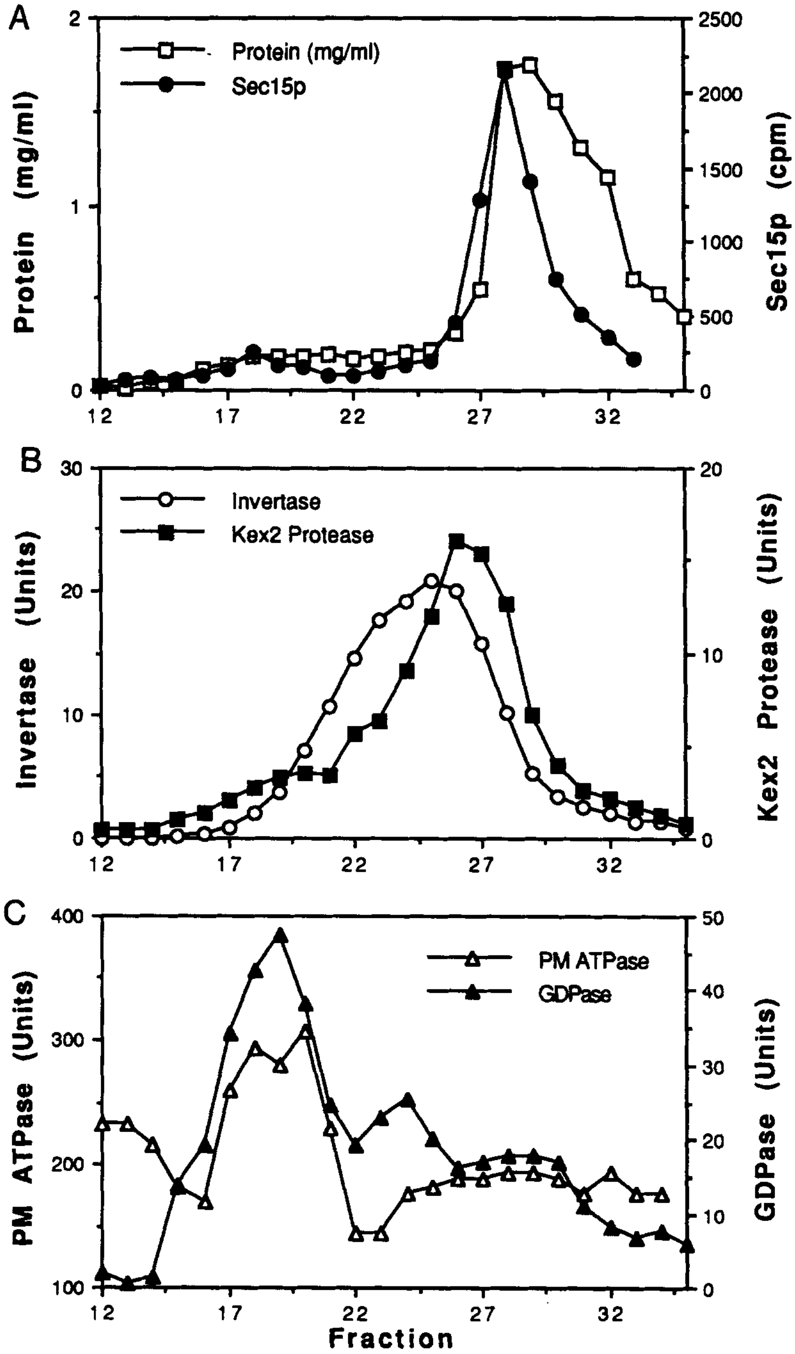


Figure 4. Secl5p is not associated with secretory vesicles isolated from a 100,000-*g* pellet of NY17 cells. A P3 from NY17 cells was resuspended in 1 ml of lysis buffer containing 10 mM MES at pH 6.5 and analyzed by Sephacryl S-1000 column fractionation. (*A*) Secl5p was localized by quantitative Western blot analysis (●). The recovery of Secl5p was 93% of the loaded P3 fraction. The protein concentration of each fraction was determined by Bradford analysis (□). (*B*) Markers for secretory vesicles (○) and Kex2 protease (■). (*C*) Elution profiles of plasma membrane ATPase (△) and GDPase (▲) activities.

form with no production of the monomer form. Upon overexpression we find that the level of Secl5p in the high molecular mass form increases, with no detectable peak of monomer form (data not shown). In principle, this is consistent with the idea that Secl5p associates in a large, soluble homoaggregate. However even by galactose induced overexpression the amount of Secl5p found in the peak of the high molecular mass soluble species increases only threefold. This level of overproduction is not high enough to assure saturation of other components of a complex, therefore this experiment is inconclusive.

We next determined if Secl5p dissociation from the plasma membrane results in release of monomer forms or if larger forms dissociate from the membrane. A P2 from NY11 cells was resuspended in 0.8 M sorbitol/TEA buffer, pH 8.0, containing 500 mM NaCl and incubated on ice for 30 min. These conditions result in solubilization of Secl5p from the membrane (Salminen and Novick, 1989). After this treatment, the membranes are pelleted and the supernatant analyzed by Sephacryl S-500 gel filtration. The results, Fig. 3 *C*, demonstrate that released Secl5p elutes with a molecular mass of ~600–700 kD. While this apparent molecular mass

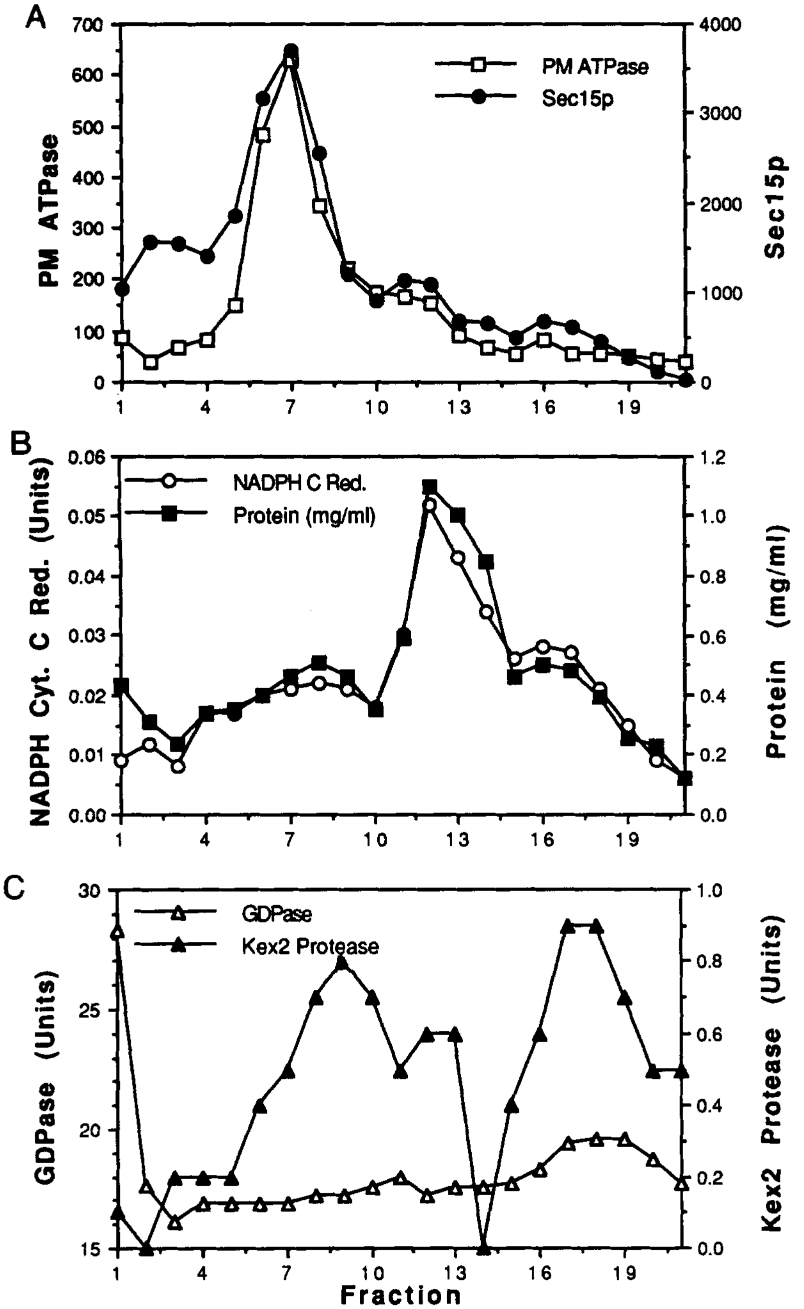


Figure 5. Sec15p localizes to the plasma membrane in the 10,000-*g* pellet of late acting *SEC* mutants. A P2 from NY17 cells was isolated and analyzed by sucrose gradient fractionation. Sec15p and marker enzyme activities were quantitated in each fraction as in Fig. 1. (*A*) Sec15p (●) and the plasma membrane ATPase activity (□) co-fractionate within the gradient. The recovery of Sec15p was 130% of the loaded P2 fraction. (*B*) Localization of NADPH cytochrome *c* reductase activity (○) and protein concentration (■) of each fraction. (*C*) GDPase (△) and Kex2 protease (▲) activities are located within distinct fractions of the gradient.

is somewhat lower than the 1,000–2,000-kD form seen upon gel filtration of the S2 fraction (Fig. 3 *A*), no monomer form of Sec15p is detectable upon dissociation from the plasma membrane. Therefore, Sec15p may be released from the plasma membrane in a large aggregate or complex, although it is possible that monomer Sec15p dissociates from the plasma membrane and quickly associates with itself or other proteins. From the above data it appears that Sec15p forms a high molecular mass particle when soluble. It is possible that Sec15p associates directly with the plasma membrane in this high molecular mass species, or indirectly through another protein(s). Further experiments are required to address these possibilities.

Sec15p Remains Associated with the Plasma Membrane in Vesicle-accumulating Mutants

Sec15p could reach the plasma membrane by direct association from a soluble pool or by prior association with the vesicular precursors to the plasma membrane. To determine if Sec15p is associated with secretory vesicles, vesicles were purified from *sec6-4* cells by Sephacryl S-1000 gel filtration (Walworth and Novick, 1987). NY17 cells were incubated for 1 h at restrictive temperature (37°C) to impose a block in the secretory pathway, allowing the accumulation of secretory vesicles, and simultaneously shifted to low glucose containing media to derepress invertase biosynthesis. Thus invertase can be used as a lumenal marker of the accumulated secretory vesicles (Walworth and Novick, 1987). Secretory vesicles were pelleted at 100,000-*g* from osmotically lysed cells, resuspended, and chromatographed on a S-1000 column (see Materials and Methods). Each fraction eluted from the column was assayed for marker enzymes and for the abundance of Sec15p by Western blot analysis. The results demonstrate that Sec15p coelutes with the leading edge of a

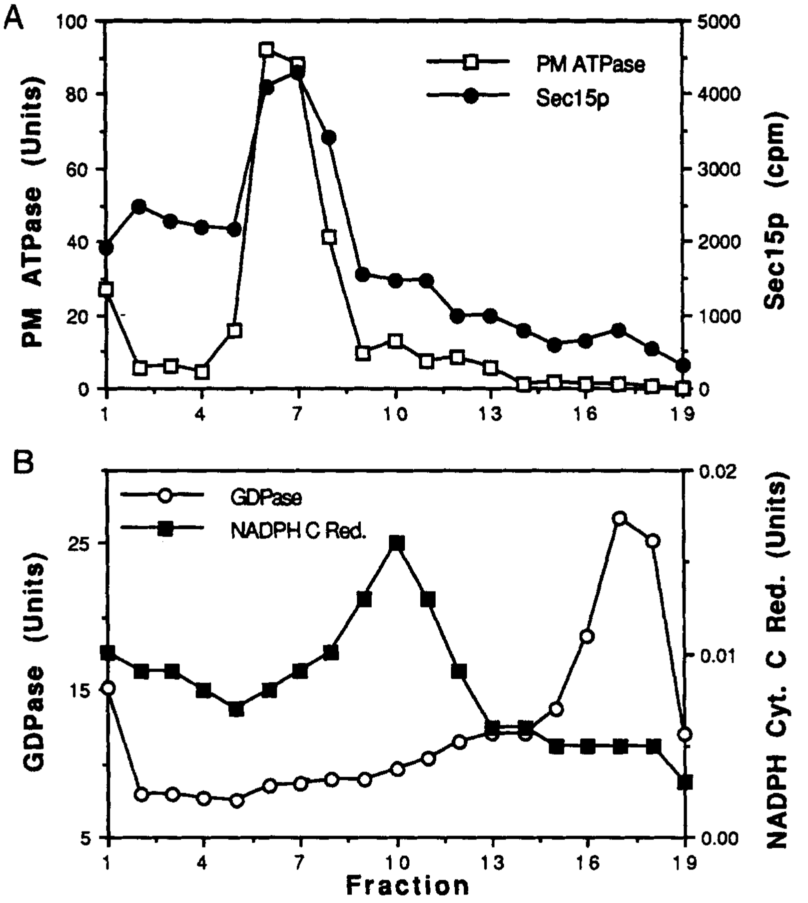


Figure 6. Localization of Sec15p on the plasma membrane of a 10,000-*g* pellet from NY410 (*sec8-9*) cells. A P2 from NY410 was resuspended in 2 ml of 55% sucrose, 10 mM MES pH6.5 and analyzed by sucrose gradient fractionation as in Fig. 1. (*A*) Sec15p (●) and the plasma membrane ATPase activity (□) cofractionate within the gradient. The recovery of Sec15p was 110% of the loaded P2 fraction. (*B*) Sec15p is not located on either the ER (■) or the GDPase containing compartment (○) in this mutant.

major protein peak (Fig. 4). The peak of protein is not associated with any identified organelle. Sec15p fails to elute with the secretory marker enzyme invertase, or with the Kex2 containing compartment (Fig. 4). Therefore Sec15p does not associate with secretory vesicles en route to the plasma membrane. Note that by this purification scheme the Kex2 containing compartment is at least partially separable from secretory vesicles. The plasma membrane ATPase activity pelletable at 100,000 *g* and the GDPase containing compartment also elute from the column before Sec15p. From this data we conclude that Sec15p contained in a high speed pellet from *sec6-4* vesicle accumulating cells fails to localize to any identified membrane component, but is found in a nonmembraneous complex or aggregate.

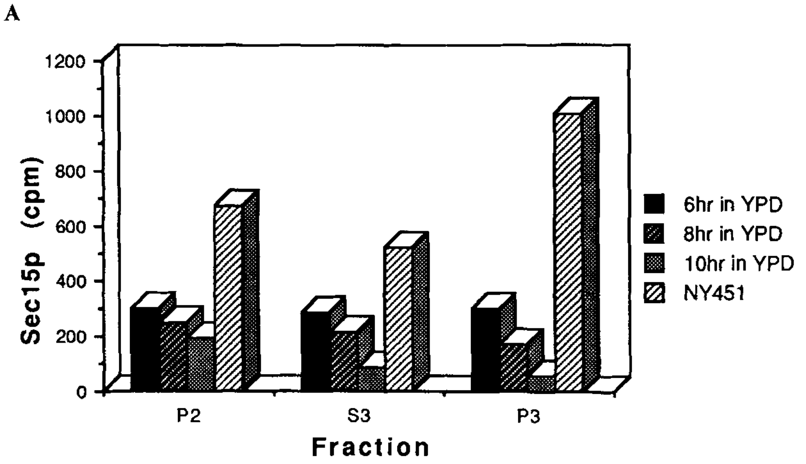
We next determined if the plasma membrane association of Sec15p was effected by the loss of function of any of the late acting *SEC* gene products. It is possible, for example, that vesicle accumulation may result in reduced levels of Sec15p on the plasma membrane and a corresponding increase in the high molecular mass soluble form. We therefore analyzed Sec15p localization in the P2 fraction of various vesicle accumulating mutants. After a 1-h incubation at the restrictive temperature, a 10,000-*g* pellet was isolated from each mutant and analyzed by sucrose gradient fractionation. The results for *sec6-4* mutant cells are shown in Fig. 5. Sec15p remains associated with the plasma membrane. Accumulation of secretory vesicles does not lead to Sec15p association with other membrane compartments localized in P2 or decrease the level of Sec15p found on the plasma membrane (Fig. 5). Therefore Sec15p localization to the plasma membrane is not dependent upon vesicular traffic or Sec6p function.

Vesicle accumulation does result in a density shift of both the Kex2 and GDPase containing compartments (Fig. 5), as the GDPase containing compartment now localizes with fraction 1 (containing the membrane pellet of the gradient) and the Kex2 containing compartment equilibrates near the middle of the gradient. This result is also apparent in *sec4-8* vesicle accumulating mutant cells.

From previous immunofluorescence studies in cells overexpressing Sec15p it was proposed that Sec15p functions downstream from Sec4p (Salminen and Novick, 1989). Therefore the localization of Sec15p may require proper Sec4p function. We performed sucrose gradient fractionation of a P2 isolated from *sec4-8* cells incubated at the restrictive temperature for 1 h to determine if Sec4-8 mutant protein effects the localization of Sec15p. The results demonstrate that Sec15p remains associated with the plasma membrane upon Sec4-8p inactivation, consistent with the results in *sec6-4* cells (data not shown). Therefore loss of Sec4p function does not result in a mislocalization of Sec15p or a decrease in the amount of Sec15p on the surface, as differential centrifugation of both wild-type and *sec4-8* mutant cells result in similar levels of Sec15p localized in P2.

We also performed sucrose gradient fractionation of a P2 from *sec15-1* mutant cells. The mutant Sec15-1p still localizes to the plasma membrane (data not shown). Therefore the mutant phenotype of these cells is not the result of a mis-

A



B Quantitation of Sec15p in subcellular fractions of the 6-10 h YPD samples expressed as the percent of Sec15p found in corresponding fractions of NY451 cells

NY799 Sample (hrs in YPD)	P2[a]	S3[b]	P3[c]
6 h	45%	55%	30%
8 h	37	40	17
10 h	29	15	5

a = NY799 P2 Sec15p (cpm)/ NY451 P2 Sec15p (cpm)

b = NY799 S3 Sec15p (cpm)/ NY451 S3 Sec15p (cpm)

c = NY799 P3 Sec15p (cpm)/ NY451 P3 Sec15p (cpm)

Figure 7. Sec15p association with the plasma membrane is stable over time after repression of Sec15p synthesis. Sec15p gene expression was controlled in NY799 cells (containing a GALp-SEC15 plasmid) by growth in either galactose or glucose containing medium. After inhibition of expression by incubation in YPD medium for 0–10 h, cells were harvested and Sec15p localized by quantitative Western blot analysis of differential centrifugation fractions. (*A*) Differential centrifugation analysis of Sec15p localization in NY799 cells incubated in YPD medium for 6, 8, or 10 h and NY451 cells (GAL+) after 10 h growth in YPD medium. P2, S3, and P3 were isolated from equal numbers of cells of each sample. (*B*) The amount of Sec15p in each subcellular fraction (P2, S3, or P3) expressed as the percent of Sec15p in the corresponding subcellular fraction from NY451 cells. After a 6-h incubation in YPD, the percent of Sec15p in each fraction from NY799 cells, relative to NY451 cells, is reduced 45–70%. After a 10-h incubation in YPD, the level of Sec15p found in both the S3 and P3 fractions has greatly decreased while the amount of Sec15p within P2 has only diminished 16%, as compared to the level in NY451 cells, and now containing the majority of the cellular Sec15p.

localization of the protein. Further analysis is required to determine the biochemical defect of the Sec15-1 mutant protein.

Analysis of Sec15p localization in other late-acting *sec* mutants displayed similar results, in that Sec15p remains localized in the 10,000-*g* pellet by differential centrifugation (Table II). However growth of *sec8-9* cells under either permissive or nonpermissive conditions results in an increase in the level of Sec15p in P2, and a corresponding decrease in S3 and P3 levels (Table II). We find that P2 of *sec8-9* cells contains between 43 and 46% of the total cellular Sec15p, under both permissive (23°C) and nonpermissive (37°C) temperatures. In all other strains, except *sec10-2*, the amount of Sec15p localized in P2 is between 22 and 28%. This data indicates that Sec8p may be involved in regulating the amount of Sec15p on the inner surface of the plasma membrane. To verify that this shift in distribution is dependent upon the function of the Sec8 protein, we transformed the *sec8-9* cell to Sec8+ with the integrating plasmid pNB330 (Materials and Methods). Upon fractionation of this transformant we find that Sec15p localization appears wild type, with only 19% of Sec15p located in P2 (Table II). Therefore the increased level of Sec15p in P2 of *sec8-9* cells is due to the loss of Sec8p function.

Sucrose gradient fractionation of *sec8-9* cells was performed to determine if the additional Sec15p accumulated in P2 resides on the plasma membrane. After a 1-h incubation of *sec8-9* cells at 37°C, Sec15p localizes exclusively to the plasma membrane upon subfractionation of the P2 pellet (Fig. 6). Therefore loss of Sec8p function results in an increased amount of Sec15p on the plasma membrane. Sucrose gradient fractionation of a P3 pellet demonstrates that Sec15p fails to associate with any membrane component of the high speed pellet, and cofractionates with a protein peak that does not enter the gradient (data not shown). Therefore loss of Sec8p function does not result in altered localization of Sec15p in the P3 pellet.

Previous evidence indicated that the *SEC15* and *SEC8* genes interact genetically (Salminen and Novick, 1987). The data described above indicates that the amount of Sec15p found in P2, on the plasma membrane, is dependent on Sec8p function. A temperature-sensitive mutation in Sec8p dramatically increases the level of Sec15p in P2. Further biochemical studies are required to determine if Sec15p directly interacts with Sec8p, and if this interaction occurs on the plasma membrane or possibly in the soluble pool.

We also observed an increase in the amount of Sec15p associated with P2 in the *sec10-2* mutant (Table II). This increase was intermediate between wild-type and *sec8-9* mutant cells, and was consistently observed. The *SEC15* gene also interacts with the *SEC10* gene (Salminen and Novick, 1987). Therefore the *SEC10* gene product may also interact directly with Sec15p, though further experiments are required to characterize this putative interaction.

The Most Stable Pool of Sec15p Is Associated with the Plasma Membrane

The different pools of Sec15p could have differing stabilities. To address this point, we constructed a strain that contains the only copy of the *SEC15* gene under control of the *GAL1* promoter. Growth of this strain, NY799, in galactose containing media is required for *SEC15* gene expression. By removal of the galactose and subsequent incubation in glucose containing media, repression of transcription from the *SEC15* gene occurs. Sec15p is an essential gene product, thus growth and division of these cells will continue in glucose containing media until Sec15p becomes the limited factor due to dilution by cell division and proteolysis. Depletion of Sec15p in NY799 cells should allow identification of the most stable pool of Sec15p.

NY799 cells were incubated in the presence of 0.5% galactose overnight to induce synthesis of Sec15p. Upon shift into glucose containing media (YPD), growth curves were first performed to determine the rate of division after repression of *SEC15* gene expression. We found that cell division in YPD occurs in a linear manner for 10–12 h, after which time division ceases (data not shown). We therefore localized Sec15p by differential centrifugation of NY799 cells after 0–10-h incubation in YPD media (see Materials and Methods).

After overnight incubation in 0.5% galactose containing media, 50 A_{600} U of NY799 cells were harvested after 0-, 6-, 8-, and 10-h incubation in YPD media. Differential centrifugation was performed on all samples and the level of Sec15p in equal aliquots of each supernatant and pellet determined by Western blot analysis. The total cellular level of Sec15p drops substantially between 0 and 6 h incubation in YPD medium, since the cells are still rapidly dividing but not synthesizing Sec15p and thus diluting the total amount of Sec15p contained within each cell. Only 25–30% of the total cellular Sec15p present in cells grown in galactose containing medium remains after a 6 hour incubation in YPD medium (data not shown).

The results for the 6–10-h YPD samples are shown in Fig. 7. We first compared the amount of Sec15p localized in each subcellular fraction to the corresponding fraction from NY451 (Gal+) cells grown in YPD medium for 10 h. The results show that the amount of Sec15p localized in P2 decreases from 45% of NY451 in the 6-h YPD sample to 29% in the 10-h sample. However the amount of Sec15p localized in either S3 of P3 decreases more considerably. The level of Sec15p found in S3 drops from 55% of NY451 in the 6-h sample to only 15% in the 10-h sample. In P3 the level of Sec15p decreases from 30% of NY451 in the 6-h sample to only 5% after 10-h incubation in YPD medium. Though no pool is completely stable over time we find that Sec15p localized in P2, residing on the plasma membrane, remains more constant after repression of *SEC15* gene expression.

NY799 cells incubated in YPD for 0, 6, 10, or 24 h were also analyzed by electron microscopy. If the level of Sec15p on the plasma membrane becomes limiting for continued expansion of the membrane surface, then secretory vesicles should accumulate in the cytoplasm. In wild-type yeast cells few secretory vesicles are apparent in the cells by electron microscopy (Novick et al., 1981). NY799 cells grown overnight in 0.5% galactose appear as wild-type (Fig. 8 *A*), as few secretory vesicles are observed. Very few vesicles are also apparent after 6-h incubation in YPD medium (Fig. 8 *B*). Since the level of Sec15p in NY799 cells incubated in YPD for 6 h is greatly diminished (Fig. 7) and further synthesis is inhibited by glucose repression, this indicates that a small pool of Sec15p is sufficient for proper function and that this pool may be reused in multiple rounds of vesicle fusion. Incubation in YPD medium for 10 h results in the accumulation of secretory vesicles in 25–30% of the cells. As the level of Sec15p continues to decrease in these cells we observe an asynchronous, but polarized, accumulation of vesicles (Fig. 8 *C*). In budded cells vesicles first accumulate specifically in the bud. Therefore vesicles are being delivered to the proper location but fail to fuse with the plasma membrane. This is consistent with Sec15p function occurring on the plasma membrane or at another very late stage of the secretory pathway, not in the delivery of secretory vesicles to the bud. Incubation of NY799 cells in YPD for 24 h results in vesicle accumulation throughout all cells (data not shown).

Kex2 and GDPase Activities Reside in Distinct Compartments

Previous studies have shown that yeast GDPase and α-1,2mannosyltransferase activities cofractionate in a sucrose velocity gradient using a 100,000-*g* pellet of wild-type cells (Abeijon et al., 1989). Cunningham and Wickner (1989) provided evidence that the Kex2 endoprotease and α-1,2mannosyltransferase activities localize in separate compartments. We demonstrate here that Kex2 and GDPase activities reside in distinct compartments (Fig. 1, 2, and 4).

Differential centrifugation of wild-type cells result in partial separation of the two yeast Golgi markers, Kex2 and GDPase (Table III). The majority of the Kex2 activity is located in P3, whereas the GDPase activity partitions much more equally between P2 and P3. GDPase activity found in S3 may be associated with membrane fragments or as soluble activity due to lysis of compartments. Sucrose gradient fractionation of both P2 and P3 further purify the Kex2 and GDPase compartments (Figs. 1 and 2). These Golgi markers are separable not only from other organelles but also from each other. Sucrose gradient fractionation of a P2 from wild-type cells results in equilibration of both Kex2 and GDPase containing compartments near the top of the gradient (Fig. 1). However the markers appear to be separable by one fraction, though in some gradients the sharp peak of Kex2 activity is more broad. The GDPase containing compartment in fractions 17–18 of Fig. 1 is 10-fold enriched over total lysate. The Kex2 containing compartment in fraction 19 of P2 is nine-fold enriched relative to the cell lysate. But only ~10% of the total Kex2 activity of the cell is located in this light compartment that pellets at 10,000 *g*.

Sucrose gradient fractionation of P3 from wild-type cells results in more extensive Kex2 and GDPase separation (Fig. 2). Kex2 activity localized in P3 is much more dense than

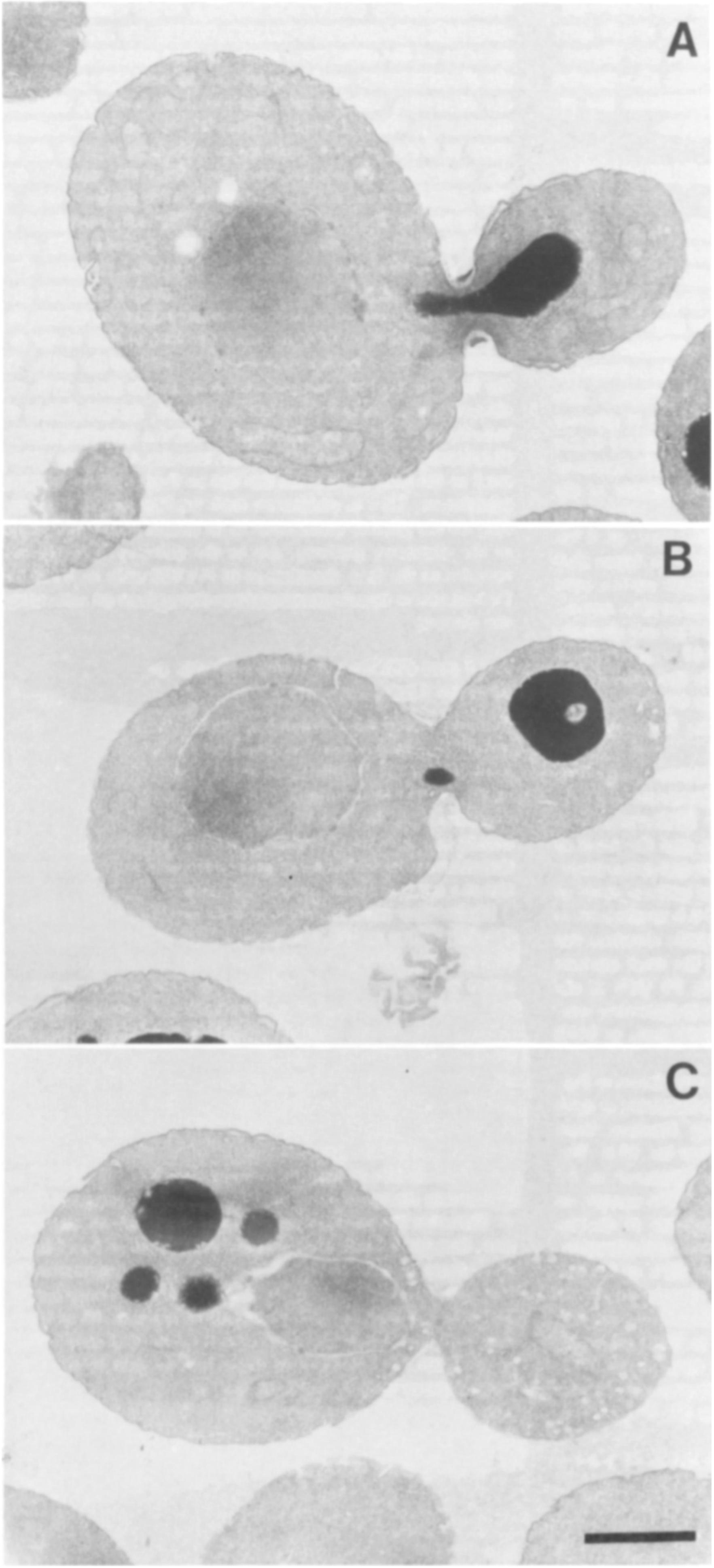


Figure 8. Electron microscopic analysis of NY799 cells after depletion of Sec15p by repression of synthesis. Cells were grown overnight in YP + 0.5% galactose to induce synthesis of Sec15p. The next day cells were harvested and incubated in YPD medium for 0, 6, or 10 h and processed for microscopy. (*A*) Cells were grown in 0.5% galactose overnight. Few secretory vesicles are apparent, as in wild-type cells. Cells grown in YPD medium for (*B*) 6 or (*C*) 10 h. After a 6-h incubation in YPD, cells accumulate few secretory vesicles and appear wild type. By 10 (*C*) secretory vesicles are apparent in 25–30% of the cells. The micrograph depicts a budded cell to demonstrate a bud-specific accumulation of secretory vesicles. Bar, 1 μm.

Table III.

Fraction	Protein	Total activity	Specific activity	Fold purification
	mg	*U*		
KEX2 distribution during differential centrifugation of NY11 cells				
Total lysate	196.8	7.6	0.039	–
P1	11.4	0.2	0.018	–
S1	176	5.3	0.030	–
P2	25.0	1.0	0.040	–
S3	134	0.0	0.0	–
P3	17.9	7.1	0.40	10.2
GDPase distribution during differential centrifugation of NY11 cells				
Total lysate	196.8	2,151	10.9	–
P1	11.4	711	62	5.6
S1	176.0	1,400	8.0	–
P2	25.0	1,031	41.6	3.8
S3	134.0	893	7.3	–
P3	17.9	1,073	48	4.4

Kex2 and GDPase activities are located in separate subcompartments. The subcellular distribution of Kex2- and GDPase-containing compartments was determined by differential centrifugation of NY11 cells. The Kex2 and GDPase activities were assayed as in Fig. 1 and the distribution and fold purification in each subcellular fraction shown.

GDPase activity, and also more dense than Kex2 activity found in P2. This dense peak of Kex2 activity may correspond to a dense peak of Kex2 previously identified in a 1000-g supernatant of wild-type cells (Cunningham and Wickner, 1989). We further characterize this peak of activity as residing in P3 and accounting for a majority of the cellular Kex2 activity (Table III). By Sephacryl S-1000 column fractionation partial separation of this dense Kex2-containing compartment from secretory vesicles occurs (Fig. 4). The peak of Kex2 activity located within the P3 sucrose gradient is highly enriched over Kex2 activity in the total lysate (Table III). Other membrane components, however, fractionate in this region of the gradient and further purification steps would be required to completely purify the Kex2 containing compartment located in P3. The GDPase activity in P3 fractionates near the top of the gradient, as in P2, and is 18-fold enriched over the total cell lysate. The density of GDPase containing compartments in both P2 and P3 is very similar, fractionating in 35–38% sucrose, indicating that both GDPase pools may be similar or identical, though further experiments are required to demonstrate this.

Discussion

We have presented evidence that the *SEC15* gene product resides and may function on the plasma membrane of the yeast *Saccharomyces cerevisiae* to regulate vesicle fusion with the plasma membrane. Sec15p is also found in a soluble 19.5*S* particle that may be cytoplasmic in origin. Sec15p is not found on the Golgi apparatus or on the accumulated secretory vesicles isolated from *sec6-4* cells. Therefore delivery of Sec15p to the plasma membrane is not dependent on prior association with elements of the secretory pathway, but may be the result of direct attachment from the soluble pool. The *Sec15-1* mutation permits plasma membrane attachment of the mutant Sec15-1p under both permissive and nonpermissive temperatures. Further biochemical analyses are required to determine the temperature sensitive characteristics of the Sec15-1 mutant protein. The association of Sec15p with the plasma membrane could be mediated by additional proteins, such as those encoded by the *SEC* genes. However, defects in the known *sec* gene products required for vesicular transport from the Golgi apparatus do not lead to a failure in Sec15p membrane attachment. Rather, in the case of *sec8-9* and possibly *sec10-2*, significant enhancement of plasma membrane association is seen. Therefore Sec8p may normally function to regulate the release of Sec15p from the plasma membrane after the completion of Sec15p function. Alternatively, loss of Sec8p function could lead to a build-up of Sec15p on the surface by a feedback mechanism. These results are in general support of the previous findings (Salminen and Novick, 1987) that demonstrated strong genetic interaction between a set of genes including *SEC8, SEC10,* and *SEC15.* Further experiments are in progress to determine if Sec8p directly associates with Sec15p.

Preliminary studies (Salminen and Novick, 1989) had shown that a large fraction of Sec15p is found in a fraction that does not pellet at 10,000 *g* but does pellet at 100,000 *g*. It is now clear that this pool of Sec15p, like that found in the 100,000-*g* supernatant, is not membrane bound, but is associated with a 19.5*S* particle. The large size of this particle leads to its partial clearance at 100,000 *g*. The subunit composition of this particle is presently unclear. Overexpression of Sec15p fails to induce monomer formation, consistent with the formation of a Sec15p homopolymer, but also fails to induce a large increase in the level of the high molecular mass species of Sec15p. Immunoprecipitation studies aimed at identifying any interacting proteins have been unsuccessful to date. It is possible that this 19.5*S* particle associates with the plasma membrane unchanged or there may be a gain or loss of some components upon attachment. However, release of Sec15p from the plasma membrane by high salt yields a particle of comparable size.

At least two other proteins involved in the fusion process, NSF and Sec23p, are known to form oligomers. Block et al. (1988) proposed a homo-tetrameric structure for NSF, which is attached to Golgi membranes by a family of NSF attachment proteins referred to as SNAPs (Clary et al., 1990). Hicke and Schekman (1990) have characterized the *SEC23* gene product, which functions in ER to Golgi transport, and have demonstrated that Sec23p associates with both the cytoplasmic surface of a membrane structure and a soluble oligomer or complex of 400 kD. The solubility properties of Sec23p are similar to Sec15p, and it is possible that the two proteins perform similar functions in different parts of the secretory pathway, yet the two proteins share no significant sequence similarity. Recently an abundant 97-kD polypeptide has been identified in a wide range of cells that forms a high molecular mass homo-oligomeric ring-shaped ATPase particle (Peters et al., 1990). This particle localizes to a 100,000-*g* supernatant and the sequence of the 97 kD polypeptide is related to both the NSF and *SEC18* genes. However p97 has not been shown to function in a fusion event.

What is the function of Sec15p on the plasma membrane? In a previous study it was proposed that the *SEC15* gene product may interact with and aggregate vesicles to one another or to the plasma membrane (Salminen and Novick, 1989). A number of mammalian proteins are known to bind to secretory vesicles and cause aggregation in vitro (Burgoyne, 1990), including members of the annexin family of calcium binding proteins (Burgoyne and Geisow, 1989). Synexin was initially characterized as a protein that causes chromaffin granules to aggregate (Creutz et al., 1978). Calpactin causes aggregation of chromaffin granules at a calcium concentration closer to physiological levels than the other annexins (Drust and Creutz, 1988), and has been shown to reside on the plasma membrane (Drust, D. S., and C. E. Creutz. 1988. *J. Cell Biol.* 107[No. 5, Pt. 2]:339a [Abstr.]). While the sequence of Sec15p is not homologous to the members of the annexin family, it does cause vesicle aggregation upon overexpression and is located, at normal levels of expression, on the plasma membrane. Overexpression of Sec15p may lead to an altered localization of Sec15p onto other membrane components, such as secretory vesicles, and cause an aggregation of vesicles to one another. In support of this hypothesis, sucrose gradient fractionation of cells overexpressing Sec15p leads to a majority of membrane components aggregating and cofractionating near the middle of the gradient along with the Sec15p (data not shown). Therefore Sec15p may normally function on the plasma membrane to dock secretory vesicles bearing Sec4p to the surface before fusion. Cytoplasmic Sec2p may be recruited to this site to assist in the docking reaction. After fusion of the secretory vesicle to the plasma membrane, the docking and fusion machinery would disassociate, possibly releasing Sec15p from the surface in a large soluble particle that is unable to associate with secretory vesicles. The release of Sec15p from the surface may require proper Sec8p function. Subsequent exocytic fusion events may require the recycling of Sec15p back to the surface. However it is possible that the large particulate form of Sec15p is not in a functional cycle but in a state of equilibrium between the cytoplasm and the plasma membrane, and this equilibrium is under control of Sec8p function.

We have also presented evidence that the GDPase and Kex2 containing compartments are distinct and separable (Table III). This data supports the notion of Golgi subcompartmentalization in *Saccharomyces cerevisiae* (Cunningham and Wickner, 1989). Payne and Schekman (1989) postulated that Kex2 recycles to the Golgi apparatus from post-Golgi secretory vesicles in a clathrin dependent manner. We have performed column fractionation of *sec6-4* cells after the accumulation of secretory vesicles and find that Kex2 is at least partially separable from secretory vesicles. This result supports the idea that a large portion of the cellular Kex2 activity localizes to a compartment distinct from both secretory vesicles and GDPase containing compartments and one can speculate that it represents a recycling vesicle intermediate.

Genetic studies have led to the identification of a number of genes that are required for the final stage of the secretory pathway in yeast and have demonstrated strong genetic interactions among a subset of these genes. Through our studies of these genes and their protein products an understanding of the physical basis for the genetic requirements and interactions is beginning to emerge. Further biochemical and genetic studies will allow us to better define the role of Sec15p in vesicle fusion and should help elucidate the general mechanisms of membrane fusion events.

We thank Hans Stukenbrok for assistance in thin section electron microscopy and Antti Salminen for preparation of the anti-Sec15 antiserum.

This work was supported by National Institutes of Health grant GM35370 to P. Novick.

Received for publication 21 September 1990 and in revised form 6 December 1990.

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