The Sec15 Protein Responds to the Function of the GTP Binding Protein, Sec4, to Control Vesicular Traffic in Yeast

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Abstract. *SEC15* function is required at a late stage of the yeast secretory pathway. Duplication of the gene encoding the *ras*-like, GTP-binding protein, Sec4, can suppress the partial loss of function resulting from the *sec15-1* mutation, but cannot suppress disruption of *sec15*. Analysis of the *SEC15* gene predicts a hydrophilic protein product of 105 kD. Anti-Sec15 antibody recognizes a protein of 116-kD apparent molecular mass which is associated with a microsomal fraction of yeast in a strongly pH dependent fashion. Overproduction of Sec15 protein interferes with the secretory pathway, resulting in the formation of a cluster of secretory vesicles, and a patch of Sec15 protein revealed by immunofluorescence. The *sec4-8* and *sec2-41* mutations, but not mutations in other *SEC* genes, prevent formation of the Sec15 protein patch. We propose that Sec15 protein responds to the function of the Sec4 protein to control vesicular traffic.

THE analysis of protein secretion in eukaryotic cells has established a well-defined intracellular transport pathway for secreted proteins. In this pathway, newly synthesized proteins pass from the ER through the Golgi complex to the plasma membrane. These transport events are mediated by interorganelle vesicular carriers that bud from the donor compartment and fuse with the acceptor compartment, releasing the transported proteins into the target organelle (Palade, 1975). Biochemical analysis has led to the identification of several factors required for vesicular transport through the Golgi apparatus (Wattenberg and Rothman, 1986; Block et al., 1988). Genetic analysis of the yeast secretory pathway has revealed 26 genes whose products are needed for protein transport from the ER to the plasma membrane (Novick and Schekman, 1979; Novick et al., 1980; Newman and Ferro-Novick, 1987; Segev et al., 1988). Studies of temperature-sensitive alleles have shown that 10 of these genes govern vesicular traffic from the Golgi apparatus to the plasma membrane.

In our earlier reports we established that one of these 10 late acting genes, *SEC4*, encodes a GTP-binding protein that is associated with the cytoplasmic face of secretory vesicles and the plasma membrane (Salminen and Novick, 1987; Goud et al., 1988). The evidence provided by these studies suggested that the Sec4 protein (Sec4p) cycles between the plasma membrane and the secretory vesicles. This cycle of Sec4p localization may be obligatorily coupled to a cycle of GTP binding and hydrolysis (Walworth et al., 1989; Bourne, 1988) that serves to control vesicular traffic. Mutational analysis of *SEC4* supports such a mechanism (Walworth et al., 1989). A related GTP-binding protein the *YPT1* gene product, has been shown to participate in yeast secretion at an earlier stage of the pathway (Segev et al., 1988). In addition, an as yet unidentified GTP-binding protein has been implicated in controlling traffic within the Golgi apparatus in a mammalian system (Melancon et al., 1987). These findings suggest that this type of mechanism may be generally applicable to all vesicular transport events, but each class of vesicles may require a different, structurally related, GTP-binding protein.

GTP binding proteins, in general, fulfill their cellular function by regulating an effector protein. In its GTP-bound state, the GTP binding protein interacts with the effector to modulate its activity. Hydrolysis of the nucleotide curtails the interaction. One of the key questions raised by our findings concerns the nature of the Sec4 effector. Possible candidates for the effector have been identified through studies on the genetic interactions between *SEC4* and other *SEC* genes required at the late stage of the secretory pathway (Salminen and Novick, 1987). Duplication of *SEC4* either on an episome or on the chromosome was found to suppress, to varying extents, the defects imposed by mutations in a subset of the other late-acting *sec* genes. This same set of mutations, when combined with the *sec4-8* mutation in a haploid cell, caused lethality at the permissive temperature. The strongest interaction was seen between the *SEC4* and the *SEC15* genes, where the duplication of *SEC4* suppressed the growth defect, the secretion defect, and the accumulation of vesicles in a *sec15-1* strain. In this article we report the analysis of the *SEC15* gene. The aim of the work is to extend our understanding of the interaction between these two gene products. We present evidence suggesting that the Sec15 protein may be the target or a component of the target of Sec4 control.

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Materials and Methods

Yeast Genetic Techniques

Yeast strains used in this study are listed in Table I. Plasmids are listed in Table II. Yeast cultures were grown in rich medium (YPD), containing 1% Bacto yeast extract, 2% Bacto peptone (Difco Laboratories Inc., Detroit, MI) and 2% glucose, or in minimal medium (SD), containing 0.7% yeast nitrogen base without amino acids (Difco Laboratories Inc.), 2% glucose, and supplemented for auxotrophic requirements as described by Sherman et al. (1974) when necessary. To induce the overproduction of the Sec15p from the GAL1 promoter, the cells were first grown to early log (A_{600} ~1.0) in YP containing 1% lactate. Galactose was added directly to the culture to 1% concentration. To obtain longer induction times the cells were inoculated in YP lactate + galactose for overnight growth (~15 h).

Yeast transformation was done by the method of alkali cation treatment (Ito et al., 1983). Transformants were selected on SD medium at 25°C. The complementation and supression assays have been described earlier (Salminen and Novick, 1987). The crosses, sporulation of diploids, and dissection of tetrads were done as described by Sherman et al. (1974).

Nucleic Acid Techniques

Bacteria and plasmid constructions were done as described earlier (Salminen and Novick, 1987; Goud et al., 1988). Plasmid pNB90 was isolated from a plasmid library of wild-type yeast inserts in YCp50, a centromere based shuttle vector containing yeast CEN4, URA3$^+$; amp^r, tet^r. This yeast genomic library was previously described (Rose et al., 1987). Plasmid pNB140 was constructed by inserting the 6.1-kb Bam HI fragment from pNB90 into the Bam HI site of YCp50. Plasmid pNB143 was generated by deletion of the 2.4-kb Hind III fragment of pNB140. To construct the multicopy plasmid, pNB148, the 3.7-kb Hind III–Bam HI fragment from pNB143 was inserted into the Hind III–Bam HI sites of the 2-μm circle based vector, pRB307 (from collection of D. Botstein, Genentech, South San Francisco, CA).

Construction of pNB186, which contains the 3.35-kb complementing fragment, involved deletion of the 0.4-kb Bam HI–Xba I fragment of pNB143. The recessed 3′ ends of the plasmid were filled using *Escherichia coli* pol I Klenow fragment (Boeringer Mannheim Biochemicals, Indianapolis, IN) and blunt-end ligated. This ligation recreates the Bam HI site but removes the Xba I site. Plasmids pNB187 (Cen) and pNB191(YIp) have been described earlier (Goud et al., 1988). Integrating plasmid pNB192 was constructed by cloning the Hind III–Bam HI fragment from pNB186 in the Hind III–Bam HI sites of pNB191. Integrating plasmid pNB193, which contains the internal 1.25-kb Eco RI–Cla I fragment, was constructed by inserting this fragment in the Eco RI–Cla I sites of pNB191. Plasmid pNB291, in which the *SEC15* gene is disrupted by the *LEU2* gene, was constructed by cloning the ~3-kb Bgl2–Bgl2 fragment carrying *LEU2* from YIp13 into the Bgl2 sites of the *SEC15* gene in pNB192. To generate the linear fragment carrying the disrupted copy of *SEC15*, pNB291 was digested with Hind III and Bam HI.

Cloning *SEC15* behind the inducible *GAL1* promoter was as follows: pNB192 was cut with Hind III and blunt ended as above. The linear vector was further digested with Kpn I and the 440-bp Hind III–Kpn I fragment and the vector fragment were purified. The isolated Hind III–Kpn I fragment was digested with Alu I (Alu I cuts 9 bp upstream from the ATG), and the resulting two fragments 200 and 240 bp were separated in 10% acrylamide gel. The 200-bp Alu I–Kpn I fragment was isolated and ligated with the Hind III (blunt)–Kpn I digested vector in the presence of kinased Bam HI linker (dGGGATCCC; Boeringer Mannheim Biochemicals). This ligation generated plasmid pNB299, carrying the *SEC15* gene on a Bam HI–Bam HI fragment. Plasmid pNB300 which contains the *SEC15* gene under *GAL1* control was constructed by cloning the Bam HI–Bam HI fragment from pNB299 into the Bam HI site of pNB187. Integrating plasmid pNB304, containing the *SEC15* gene under *GAL1* control, was constructed by cloning the 4.5-kb Pvu I–Sal I fragment from pNB300 in Pvu I–Sal I sites of pNB191.

The use of the pATH protein fusion system has been described earlier (Goud et al., 1988). Plasmid pNB164, which contains *TrpE* fused to an internal fragment of the *SEC15* gene (amino acids 257–676), was constructed by inserting the 1.25-kb Eco RI–Cla I fragment from pNB186, in frame into the Eco RI–Cla I sites in the polylinker region of pATH11. Plasmid pNB301 which contains the *TrpE* fused to the amino terminal third of the Sec15 protein (amino acids 1–241) was constructed as follows: pNB299 and pATH2 were digested with Eco RI and Hind III, respectively, and the recessed ends were blunted as described above. Both vectors were then cut with Bam HI and electrophoresed into an agarose gel. The 0.7-kb Bam HI–Eco RI (blunt) fragment from pNB299, and the pATH2 vector were purified from the agarose gel. The purified fragments were religated and used to transform *E. coli* as described before (Goud et al., 1988).

Table I. Yeast Strains Used

Strain	Genotype
NY13	*MATa, ura3-52*
NY15	*MATα, ura3-52, his4-619*
NY64	*MATa, ura3-52, sec15-1*
NY179	*MATa, leu2-3, 112, ura3-52*
NY180	*MATα, leu2-3, 112, ura3-52*
NY363	*MATa/α, leu2-3, 112/+, ura3-52/ura3-52, his4-619/+*
NY376	*MATa, ura3-52, sec15-1, SEC4*::pNB141 *(SEC4, URA3)*
NY440	*MATα, ura3-52, his4-619,* pNB148 (2 μm, *SEC15, URA3)*
NY451	*MATa, ura3-52, Gal$^+$*
NY456	*MATa, ura3-52, sec4-8, Gal$^+$*
NY467, 468	*MATα, ura3-52, his4-619, SEC15*::pNB192, *(SEC15, URA3)*
NY483	*MATa/α, leu2-3, 112/+, ura3-52/ura3-52, his4-619/+, sec15*::pNB193 (*sec15,* internal fragment, *URA3)*
NY503	*MATα, ura3-52, his4-619, sec6-4, Gal$^+$*
NY648	*MATa/α, leu2-3, 112/leu2-3, 112, ura3-52/ura3-52*
NY662	*MATa/α, leu2-3, 112/leu2-3, 112, ura3-52/ura3-52, SEC15/sec15::LEU2*
NY724	*MATa, ura3-52, Gal$^+$, SEC15*::pNB304 *(GAL1-SEC15, URA3)*
NY725	*MATa, ura3-52, Gal$^+$, sec4-8, SEC15*::pNB304 *(GAL1-SEC15, URA3)*
NY742	*MATα, ura3-52, Gal$^+$, his4-619, sec6-4, SEC15*::pNB304 *(GAL1-SEC15, URA3)*
NY748	*MATα, ura3-52, Gal$^+$, his4-619, sec1-1 SEC15*::pNB304 *(GAL1-SEC15, URA3)*
NY749	*MATα, ura3-52, Gal$^+$, his4-619, sec5-24, SEC15*::pNB304 *(GAL1-SEC15, URA3)*
NY750	*MATα, ura3-52, Gal$^+$, his4-619, sec8-9, SEC15*::pNB304 *(GAL1-SEC15, URA3)*
NY751	*MATα, ura3-52, Gal$^+$, his4-619, sec3-2, SEC15*::pNB304 *(GAL1-SEC15, URA3)*
NY752	*MATα, ura3-52, Gal$^+$, his4-619, sec9-4, SEC15*::pNB304 *(GAL1-SEC15, URA3)*
NY753	*MATa, ura3-52, Gal$^+$, his4-619, sec10-2, SEC15*::pNB304 *(GAL1-SEC15, URA3)*
NY754	*MATa, ura3-52, Gal$^+$, his4-619, SEC15*::pNB304 *(GAL1-sec15-1, URA3)*
NY755	*MATα, ura3-52, Gal$^+$, his4-619, sec2-41, SEC15*::pNB304 *(GAL1-SEC15, URA3)*

Table II. Plasmids Used (see Materials and Methods for constructions)

Plasmid	Derivation
pNB90	YCp50, *SEC15*; 16.5 kb genomic insert in the Bam HI site
pNB140	YCp50, *SEC15*; 6.1 kb Bam HI–Bam HI fragment from pNB90, in Bam HI site
pNB143	YCp50, *SEC15*; 3.7 kb Hind III–Bam HI fragment, Hind III Δ of pNB140
pNB148	2 μm, *SEC15*; 3.7 kb Hind III–Bam HI fragment from pNB143, in Hind III–Bam HI sites of vector, pRB307 (from collection of D. Botstein)
pNB164	pATH11, *TrpE-SEC15*$^{257-676}$ fusion; 1.25 kb Eco RI–Cla I fragment from pNB186 into Eco RI–Cla I sites of pATH11
pNB186	YCp50, *SEC15*; 3.35 kb Hind III–Bam HI fragment, Bam HI–Xba I Δ of pNB143
pNB187	YCp50 with *GAL1* promoter; 0.82 kb Eco RI–Bam HI fragment in Eco RI–Bam HI sites, expression under *GAL1* control by cloning into the Bam HI site
pNB191	YIp5 with unique Pvu II site removed by Bal31 digestion
pNB192	YIp5, *SEC15*; 3.35 kb Hind III–Bam HI fragment from pNB186, into Hind III–Bam HI sites of pNB191
pNB193	YIp5, *SEC15*, internal 1.25 kb Eco RI–Cla I fragment, into Eco RI–Cla I sites of pNB191
pNB291	YIp5, *sec15*::*LEU2* gene disruption; 3 kb Bgl2–Bgl2 fragment from YIp13 (*LEU2*) replacing the Bgl2–Bgl2 internal fragment of *SEC15* in pNB192
pNB299	YIp5, *SEC15*; 3.1 kb Bam HI–Bam HI fragment, Hind III–Alu I Δ of pNB192, *SEC15* leader sequence removed
pNB300	YCp50, *GAL1-SEC15*; 3.1 kb Bam HI–Bam HI fragment from pNB299 into Bam HI site of pNB187
pNB301	pATH2, *TrpE-SEC15*$^{1-241}$ fusion; 0.7 kb Bam HI–Eco RI(blunt) fragment from pNB299 into Bam HI and Hind III(blunt) sites of pATH2
pNB304	YIp5, *GAL1-SEC15*; 4.5 kb Pvu I–Sal I fragment from pNB300 into Pvu I and Sal I sites of pNB191

DNA Sequencing and Protein Homology Analyses

Nucleotide sequencing was carried out by the dideoxy chain termination method (Sanger et al., 1977) in the presence of [α-^{35}S]dATP (650 Ci/mmol; Amersham Corp., Arlington Heights, IL) (Williams et al., 1986). The template DNA was obtained by subcloning restriction fragments from pNB143 into M13 phage derivatives mp18 or mp19. The predicted protein sequence was compared with the National Biomedical Research Foundation Library by the FASTP program in the ktup-2 mode (Lipman and Pearson, 1985).

Southern Blot Hybridization

Total yeast DNA was extracted from cells by the method of Holm et al. (1986). DNA (1 μg) was digested with Bam HI, fractionated by electrophoresis in a 0.5% agarose gel, and transferred to nitrocellulose filters (Southern et al., 1975). The prehybridization, hybridization, and washing of the blots and the preparation of the probe were essentially as described elsewhere (Emanuel et al., 1986). In each hybridization experiment the isolated Eco RI–Cla I fragment from pNB186 was used as a probe.

Preparation of the TrpE-SEC15 Fusion Proteins and Rabbit Immunization

The fusion proteins were produced in *E. coli* strains, NRB164 and NRB301 (DH1 transformants containing the plasmid pNB164 or pNB301, respectively) essentially as described earlier (Goud et al., 1988). Cells from 200 ml culture were washed with 25 mM Tris-HCl, pH 7. The pellet was resuspended in 5 ml of cracking buffer (10 mM NaPi, pH 7.2, 1% β-mercaptoethanol, 1% SDS, 6 M urea [Dieckman and Tzagoloff, 1985]) by vortexing in a 50Ti centrifuge tube and incubated at 37°C for 30 min. To remove DNA the lysate was spun 30 min at 25,000 rpm in a 50Ti rotor (Beckman Instruments, Inc., Palo Alto, CA) and the pellet was discarded. Bromophenol blue was added to the lysate at 0.01% final concentration. Fusion protein was isolated from the total cell lysate, in cracking buffer, by preparative SDS gel electrophoresis. Usually 1.0–1.5 ml of the lysate was used for one 1.5-mm gel by applying the sample directly on the stacker gel without wells. After electrophoresis the gel was stained with Coomassie blue and destained. Identified fusion protein was cut out of the gel and the gel slice soaked in water for 2 h. The gel strip was cut into small cubes and the fusion protein was electroeluted at 100 V from gel cubes in dialysis buffer and collected on dialysis membrane (Hunkapiller et al., 1983). Proteins in dialysis buffer were acetone precipitated, resuspended in PBS and kept frozen at −20°C. For immunization, fusion proteins were diluted to 0.5 ml with PBS and emulsified with an equal volume of Freund's complete adjuvant. In the case of fusion protein isolated from NRB164 (TrpE-Sec15$^{257-676}$), 40 μg was used in initial subcutaneous injections into rabbits. Subsequent boosts were given subcutaneously at 4-wk intervals, mixing the fusion protein/PBS solution (20 μg) with Freund's incomplete adjuvant. After a titre was detected boosts were given by injecting the fusion protein/PBS solution (10 μg) into hind leg muscles. Immunization with the fusion protein isolated from NRB301 (TrpE-Sec15$^{1-241}$) was done essentially as described earlier (Goud et al., 1988; Louvard et al., 1982).

The affinity purification of the antibodies were carried out as described previously (Goud et al., 1988). The crude serum was first circulated through a column containing the TrpE-protein, for 3 hrs. The flow-through was circulated 3 h through column containing the TrpE-SEC15 fusion-protein. The antibodies were eluted from the immunoabsorbent according to Guesdon and Avrameas (1976).

Electrophoresis and Immunoblotting

For SDS-PAGE, samples were heated for 5 min at 100°C in sample buffer containing 2% SDS and run on 8 or 10% slab gels according to Laemmli (1970). After transfer onto nitrocellulose (BA 83, 0.22 μm; Schleicher & Schuell, Inc., Keene, NH) overnight at 4°C, SEC15 was probed with affinity-purified antisera (αSec15$^{1-241}$; 250 μg/ml, 1/2,000 dilution; or αSec15$^{257-676}$; 130 μg/ml, 1/500 dilution) and radioiodinated staphylococcal protein A (0.5 μCi/ml; 30 mCi/mg; Amersham Corp.) as described elsewhere (Burnette, 1981; Goud et al., 1988) with following modifications. Filters were blocked for 1 h in wash buffer TBS TWEEN (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% TWEEN 20), containing 1% gelatin and 0.5% BSA. Filters were then incubated in the presence of anti-Sec15 antibodies, in blocking buffer, for 3 h. Filters were washed 4 × 10 min with wash buffer. After washing, ^{125}I-protein A was added in blocking buffer for 3 h. All incubations were done at room temperature. After washing, filters were dried and autoradiographed 12–24 h.

Cell Fractionation and Extraction Experiments

Cells were grown at 25°C to an A_{600} of 2.0 as described above. Usually 200–400 A_{600} units were pelleted and washed once with buffer, 10 mM Tris, pH 7, 10 mM NaN_3. Cells were resuspended in spheroplast medium (50 mM Tris, pH 7.5, 10 mM NaN_3, 1.4 M sorbitol, 40 mM β-mercaptoethanol, and 0.125 mg/ml of Zymolyase-100T; ICN Radiochemicals, Irvine, CA) at a density of 10 A_{600} units/ml, and converted to spheroplasts during a 45-min incubation at 37°C. The spheroplasts were pelleted and resuspended in 2 ml of ice-cold lysis buffer (0.8 M sorbitol, 20 mM triethanolamine, 1 mM EDTA, at pH 7.2 or 8.0, containing 1 mM PMSF, and 10 μl/10 ml of protease inhibitor cocktail: leupeptin, chymostatin, pepstatin, antiparin, aprotinin, 1 mg/ml). The lysate was homogenized with 20 strokes in 2 ml tissue grinder (Ten Broeck; Fisher Scientific Co., Pittsburgh, PA) and centrifuged at 450 *g* for 3 min. The homogenization protocol was repeated to the pellet (P1), and the supernatants were pooled. The total pro-

tein concentration of the lysate was adjusted to 2.8 mg/ml with lysis buffer (S1). The pH of the lysate was adjusted to 6.5, when necessary, by adding 1 M MES-buffer pH 6.5, to 50 mM into S1. In differential centrifugation experiments the S1 supernatant was spun at 12,000 rpm (10,000 g_{max}) in a rotor (model 50Ti; Beckman Instruments Inc., Palo Alto, CA) in 1.5 ml volume for 10 min at 4°C. The pellet (P2) was resuspended in 1.5 ml lysis buffer. The S2 supernatant was further centrifuged at 40,000 rpm (100,000 g_{max}) in the same rotor in 1 ml volume for 1 h at 4°C and the pellet was resuspended in 1 ml lysis buffer. In crude membrane separation centrifugations the S1 supernatant was directly spun at 40,000 rpm (100,000 g_{max}). For the extraction experiments the low speed (450 *g*) supernatant was adjusted to 5 mg/ml. 0.5 ml of this concentrated lysate was mixed with 0.5 ml of lysis buffer, containing an individual extracting agent at 2× concentration (i.e., 10 M urea, 2% Triton X-100, or 2 M NaCl). The lysate was incubated on ice for 30 min, and spun 100,000 *g* as above. The resulting pellets were resuspended in appropriate extraction buffer. Samples were prepared for electrophoresis as above.

Immunofluorescence

For immunofluorescence experiments the cells were grown in YPD medium, or YP lactate-galactose medium for 15 h at 25°C, and fixed. When indicated, the induced cultures were shifted to 37°C for 2 h before fixation. 5 A_{600} units of cells were washed twice with 20 mM Tris-HCl, pH 7, 10 mM NaN_3, and fixed at a concentration of 1 A_{600} unit/ml in 3.3% formaldehyde in the wash buffer for 4 h at room temperature and stored overnight at 4°C in the presence of the fixative. Fixed cells were washed as above and 2 A_{600} units were resuspended in 1 ml of spheroplast medium (20 mM Tris-HCl, pH 7.5, 1.2 M sorbitol, 25 mM β-mercaptoethanol and 0.1 mg Zymolyase/ml) and the cell wall was digested during 40 min incubation at 37°C. Fixed and digested cells were centrifuged 450 *g*, and washed once with spheroplast buffer without β-mercaptoethanol or Zymolyase. For permeabilization, the cells were resuspended in 2 ml TBS TWEEN (for immunofluorescence; 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% TWEEN 20) containing 0.25% Triton X-100, for 10 min on ice. 15 μl of this suspension was directly applied in wells on slides (model No. 100806; Carlson Scientific, Inc., Peotone, IL) coated with polylysine (400,000 mol wt, 1 mg/ml). After 10 min at room temperature, the cell suspension was removed by aspiration. Blocking buffer (TBS TWEEN) containing 2 mg/ml BSA, was applied on the wells and incubated for 30 min. Affinity-purified anti-Sec15p antibodies ($\alpha Sec15^{1-241}$, 1/200 dilution; 1.25 μg/ml) or affinity-purified anti-Sec4p antibodies were applied on the cells in blocking buffer and incubated at room temperature for 3 h in a moist chamber. Antibody/blocking buffer was aspirated off, and the slides washed 2 × 2 min in a 50-ml vol of TBS TWEEN. Excess buffer was aspirated from the wells and second antibody (goat anti-rabbit IgG (H&L)-rhodamine, No. 6300; Tago Inc., Burlingame, CA) was applied on cells in blocking buffer at 1/500 dilution (2 μg/ml) for 3 h at room temperature. Slides were washed as above and allowed to dry. Coverslips were mounted on slides in mounting solution (90% glycerol, 10 mM K_2-HPO_4, 1 mg/ml *p*-phenylenediamine), and sealed with nailpolish.

Electron Microscopy

Cells were grown for electron microscopy as described above for immunofluorescence. Cells were prepared for electron microscopy as described before (Salminen and Novick, 1987).

Results

Cloning SEC15

In a previous study (Salminen and Novick, 1987) we identified six plasmids from a genomic plasmid bank (Rose et al., 1987) based on the centromere-containing shuttle vector, YCp50, which could complement the temperature-sensitive growth defect of *sec15-1*. Surprisingly, it was found in that study that three of these plasmids did not carry the *SEC15* gene, but rather carried the *SEC4* gene. Southern blot and restriction site analysis of the remaining three plasmids demonstrated that they did not carry the *SEC4* gene, yet were related to each other. In this study we demonstrate that these plasmids, in fact, carry the *SEC15* gene. The smallest of the three plasmids, pNB90, was selected for further study. By subcloning portions of the 6.1-kb insert, the complementing region was localized to a 3.35-kb fragment defined by Hind III and Bam HI restriction sites (Fig. 1).

To establish the identity of the complementing gene, we determined if the cloned insert could direct integration of a linked marker, *URA3*, into the *SEC15* locus. The 3.35-kb fragment was subcloned into the integrating yeast vector YIp5. The new plasmid, pNB192, was cleaved at the unique Pvu II site which is internal to the complementing region, and was used to transform a wild-type yeast strain, NY15 (*ura3-52, his4-619*) to Ura$^+$. Integration at the locus homologous to the insert would result in duplication of the cloned sequence, with the plasmid marker *URA3* between the two copies (Fig. 2 *A*) (Orr-Weaver et al., 1981). This was verified by Southern blot analysis of two transformants, NY467 and NY468 (Fig. 2 *C*). The Ura$^+$ transformant, NY467, was crossed to NY64 (*sec15-1, ura3-52*) and tetrads were analyzed. Complete linkage of the marker to the *SEC15* locus was seen; in each of 24 tetrads, temperature-sensitivity and Ura$^+$ segregated 2:2 and all temperature-sensitive spores were Ura$^-$. These data establish that the cloned sequence contains the *SEC15* gene.

SEC15 Is an Essential Locus

Although the isolation of a recessive conditional lethal mutation strongly suggests that the *SEC15* gene product is essential, this was verified by constructing a gene disruption mutation by the method described by Shortle et al. (1982). Plasmid pNB193, which carries the internal Eco RI–Cla I fragment from pNB186 (Fig. 1) was cleaved at the unique Pvu II site. This linearized plasmid was used to transform NY363, a diploid strain homozygous for the *ura3-52* mutation, thereby replacing one chromosomal copy of *SEC15* with a duplication in which both copies are truncated (Fig. 2 *B*), effectively disrupting the *SEC15* gene. Southern blot analysis of NY483, a diploid Ura$^+$ transformant of NY363, verified the integration-disruption event (Fig. 2 *C*). The diploid transformant was shifted to sporulation media, and 12 tetrads were dissected. Two spores from each tetrad were inviable, and all viable spores were Ura$^-$. The nongrowing colonies were arrested at the two-cell stage. This construction allows the synthesis of a trucated gene product, but since the expected, lethal result was obtained, this truncated product appears to be nonfunctional. This result establishes this putative null allele as a recessive lethal mutation, and the *SEC15* gene as an essential locus.

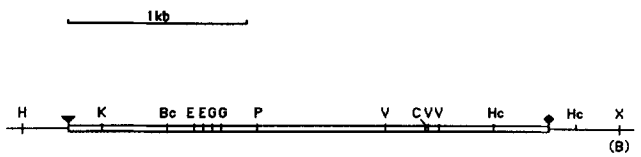

Figure 1. Restriction map of the 3.35-kb complementing region containing the SEC15 gene in plasmid pNB186. The open bar shows the coding region. The start of the gene is depicted by the solid triangle. *H*, Hind III; *K*, Kpn I; *Bc*, Bc II; *E*, Eco RI; *G*, Bgl I; *P*, Pvu II; *V*, Eco RV; *C*, Cla I; *Hc*, Hind II; *X*, Xba I; *B*, Bam HI. Note that the genomic Xba I site has been converted to Bam HI site on the plasmid.

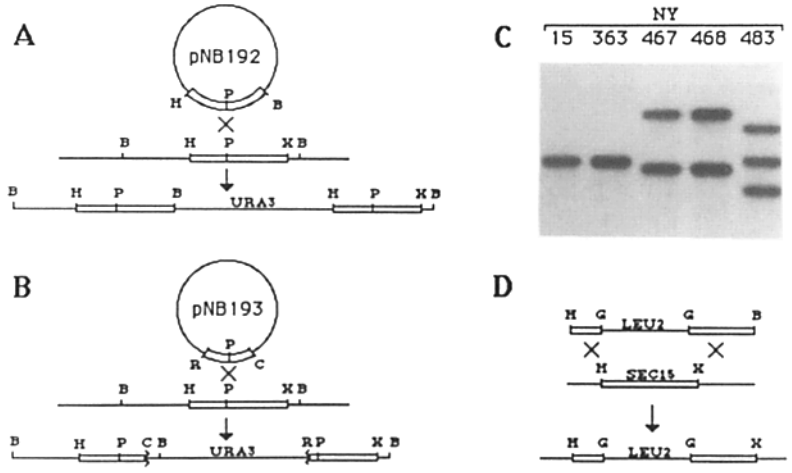

Figure 2. Cloned sequence integrates into the SEC15 locus. (*A*) Plasmid pNB192 was cleaved at the unique Pvu II site. Wild-type strain (NY15) was transformed with the linear plasmid DNA. Upon transformation the linear plasmid integrates into the genome and generates a duplication of the gene with the URA3 gene between the two copies. (*B*) Plasmid pNB193, which contains the internal Eco RI–Cla I fragment of the SEC15 gene, was cleaved with Pvu II and the linearized plasmid DNA was used to transform a diploid strain (NY363). Integration of this plasmid into the diploid genome replaces one chromosomal copy with a duplication in which both copies of the gene are truncated. (*C*) Southern blot analysis verifies the integration and disruption events. Total DNA from parental strains and from transformants derived from NY15 (NY467, 468) and from NY363 (NY-483) were digested with Bam HI and probed with the Eco RI–Cla I fragment from pNB186. Both parental strains show a single 6.1-kb band, which is replaced by 9.3- and 5.7-kb bands in the transformants carrying pNB192. In the diploid transformant, carrying pNB193, one chromosomal copy is replaced with a truncated duplication, as represented by the 7.9- and 5.2-kb bands. (*D*) SEC15 disruption with the LEU2 gene. A diploid strain (NY648) was transformed with a linear Hind III–Bam HI fragment from plasmid pNB291. This fragment contains the SEC15 gene disrupted with the LEU2 gene, and upon transformation replaces one chromosomal copy with the disrupted one.

SEC4 Duplication Cannot Suppress a Null Allele of sec15

In our earlier study we had established that duplication of the *SEC4* gene could partially (~70%) suppress the growth defect, the secretion defect, and the accumulation of vesicles in a strain carrying a temperature-sensitive *sec15* defect. We have tested the ability of *SEC4* duplication to suppress a null allele of *sec15*. A diploid strain (NY648) homozygous for the *ura3-52* and the *leu2-3, 112* mutations, was transformed to Leu$^+$ with the Hind III–Bam HI fragment from plasmid pNB291. This fragment contains the *SEC15* gene, disrupted with the *LEU2* gene at the Bg12 sites internal to *SEC15* (see Materials and Methods). Upon transformation, this linear fragment replaces one chromosomal copy of *SEC15* with the disrupted allele (Fig. 2 *D*). 12 tetrads dissected from this strain each showed two inviable spores, and the viable spores were Leu$^-$. This result confirmed our earlier finding that disruption of the *SEC15* gene is a lethal event.

The Leu$^+$, Ura$^-$, diploid strain (NY662) was then transformed to Ura$^+$ with centromere-based plasmids carrying either *SEC4* (pNB170) or *SEC15* (pNB186). Transformants were shifted to sporulation media, and tetrads were dissected. If the plasmid can complement or suppress the lethality resulting from the chromosomal disruption of *sec15*, tetrads with three and four viable spores should be observed, and these tetrads should contain one or two Leu$^+$ spores. We observed 2:2 distribution of viability among all 24 tetrads dissected from the strain transformed with pNB170 (*SEC4*), and all viable spores were Leu$^-$. Of the viable spores 12 were Ura$^+$, indicating that the *URA3*, *SEC4*, CEN plasmid, (pNB170) was segregating as an unlinked marker. Since we would expect that some of the Leu$^+$ spores would have also received the plasmid, these data indicate that duplication of the *SEC4* gene is not sufficient to suppress the total loss of *SEC15* function. Because duplication of *SEC4* can suppress the *sec15-1* defect (Salminen and Novick, 1987), these data suggest that the *sec15-1* allele must have partial function even at 37°C. As expected, many tetrads from the strain transformed with pNB186 (*SEC15, URA3*, CEN) contained three or four viable spores. Viable Leu$^+$ spores were obtained in this cross and as expected, they were all Ura$^+$ as well.

SEC15 Sequence

After localizing the *SEC15* gene to the 3.35-kb fragment we determined the nucleotide sequence of this region. The sequence contains one long open reading frame starting from nucleotide 1 and ending at nucleotide 2,733 (Fig. 3). This predicts a protein product of 105 kD in molecular mass. The predicted amino acid sequence is hydrophilic (54.5% polar, 45.5% nonpolar), and does not contain a hydrophobic domain long enough to span the lipid bilayer. 26% of the amino acids are charged, giving the protein a net negative charge (excess of 26 negative charges; pI 5.95) at neutral pH. We screened the PIR protein database for identical sequences, but found no sequence with substantial homology to the *SEC15* sequence. The upstream region of the sequence from the presumed ATG start codon contains another, in frame, start codon at nucleotide position −158–156, this reading frame is terminated by a UGA stop codon 27 nucleotides upstream from the predicted start. Since UGA is not a strong stop codon in yeast, we determined if the upstream region carried essential information or could be replaced by another promoter. The *SEC15* gene was cloned behind the inducible *GAL1* promoter, so that only nine nucleotides were left from the upstream region (see Materials and Methods). NY64 (*sec15-1, ura3-52*) was then transformed to Ura$^+$ with pNB300, a centromere based plasmid containing this construction. Transformants, selected on minimal glucose plates at 25°C, were streaked to single colonies on minimal glucose or minimal galactose plates which were incubated at 25°C for 5 h and shifted to 37°C. Growth was detected at 37°C on plates containing galactose, but not on glucose plates. Thus galactose induced the synthesis of functional *SEC15* gene product capable of complementing the *sec15-1* defect. This suggested that the wild-type *SEC15* gene is translated from

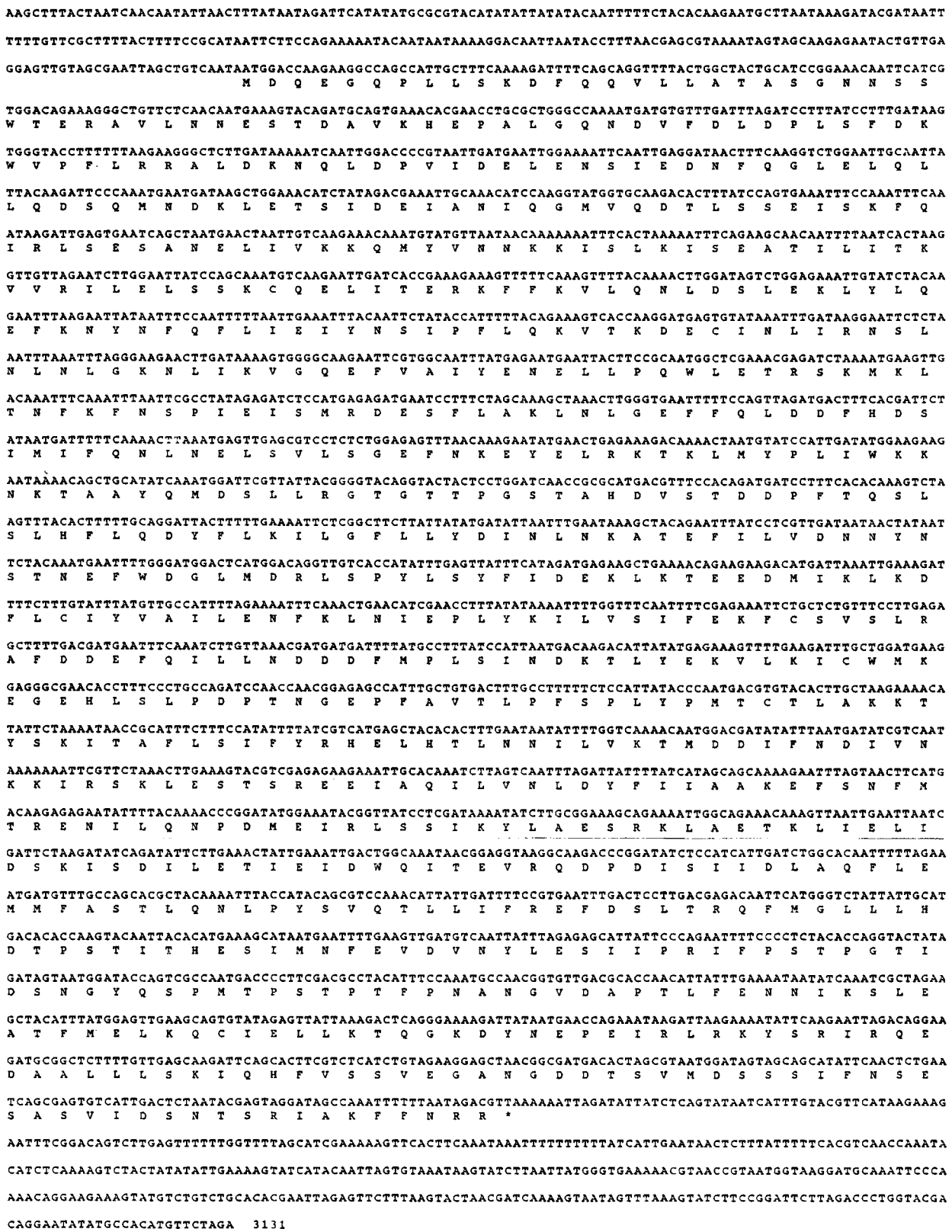

Figure 3. Nucleotide sequence of the 3.35-kb complementing region. *SEC15* gene starts at the nucleotide position 1 and ends at the position 2733. Predicted amino acid sequence of the Sec15p is shown as single letter code under the DNA sequence.

the start codon at nucleotide position 1–3. This conclusion was supported by the observation that the gene product encoded by the *GAL1-SEC15* construction had the identical mobility, in an SDS gel, to the *SEC15* gene product transcribed by its own promoter (not shown).

Generation of Antisera against the Sec15p

To address the biochemical properties of the Sec15p we have raised polyclonal rabbit antibodies against two nonoverlapping portions of the Sec15 sequence (amino acids 1–241 and 257–676), each fused to the bacterial TrpE protein (see Materials and Methods). As shown above, the nucleotide sequence of *SEC15* predicts a protein product of 105 kD. However, by Western blot analysis, these antibodies recognized a protein in a wild-type lysate that migrated at 116 kD apparent molecular mass (Fig. 4). To confirm that this band represented the *SEC15* gene product we cloned the *SEC15* gene into a yeast multicopy plasmid. A wild-type strain was transformed with this plasmid (pNB148) and transformants were selected and maintained in minimal medium (SD). Immunoblot analysis of lysates from wild-type (NY15) and plasmid containing cells (NY440) showed a 12-fold increase of the 116-kD immunoreactive band in the plasmid containing cells (NY440). This proved that the antibody recognized the Sec15p. Some additional bands were also seen. Of these the 100- and 85-kD bands were considered degradation products, since they are amplified in lysates from cells overproducing *SEC15*. The band around 50 kD comigrates with a major protein band in the lysate and probably represents nonspecific binding of the antibody. In fractionation experiments this band shows a distribution independent of Sec15p (not shown). Analysis of a *sec15-1* strain (NY64) showed that the mutant protein was shifted to a higher mobility and was present at a reduced level relative to the wild-type protein, supporting our identification of the 116-kD band as Sec15p. The nature of the *sec15-1* mutation is not known at this time. It may result in either premature truncation of the protein or cause the mutant translation product to be subject to proteolysis and therefore unstable.

Duplication of SEC4 Does Not Alter Expression or Stability of Sec15-1 Protein

To address the mechanism by which duplication of *SEC4* suppresses *sec15-1* we performed Western blot analysis using anti-Sec15p antibody on *sec15-1* strains containing one or two copies of *SEC4* (NY64 and NY376, respectively). At 25°C the two strains have approximately equal levels of Sec15-1 protein, although it is reduced in abundance and increased in its mobility with respect to the Sec15 protein in wild-type cells (NY15) (Fig. 4). When NY64 and NY376 cells are shifted to the restrictive temperature, 37°C, the amount of the Sec15-1 protein drops to 20% of the wild-type level at 25°C, however it remains approximately equal in both mutant strains (Fig. 4). Wild-type cells (NY15) exhibit a decrease of lesser magnitude in the level of Sec15 protein after growth at 37°C. The apparent loss of Sec15 and Sec15-1 protein upon a shift to 37°C is seen with antibodies directed against either the amino terminus or the internal region of the protein. The Sec15-1 protein in NY64 and NY376 shows slight, but reproducible shifts to lower mobility upon a shift to the restrictive temperature. The nature of these shifts is unknown. In total, these results indicate that duplication of *SEC4* does not increase either the expression or the stability of the Sec15-1 mutant protein.

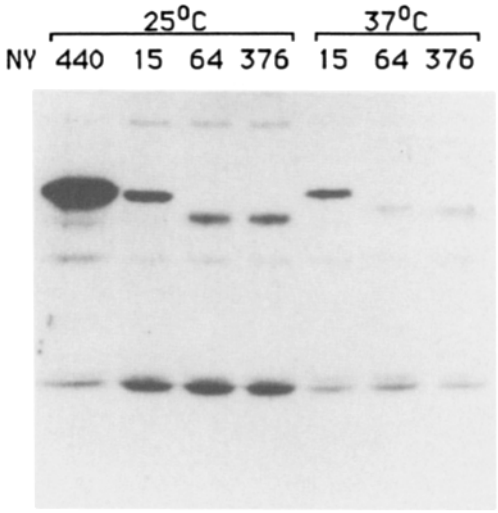

Figure 4. Antibodies generated against the *TrpE-Sec15*$^{257-676}$ fusion protein recognize the Sec15p on a western blot. NY15 (*SEC*$^{+}$), NY64 (*sec15-1*), and NY376 (*sec15-1, SEC4::SEC4*) cells were grown in YPD at 25°C to an A_{600} of 1.0. Aliquots were shifted to grow at 37°C for 2 h. NY440 cells, which contain the *SEC15* gene on a 2-μm circle based multicopy plasmid, were grown in SD minimal medium containing histidine at 25°C. 30 A_{600} units of cells were broken by agitation with glass beads at +4°C, and the lysates were suspended in 1.5 ml of ice-cold lysis buffer at pH 7.2 (see Materials and Methods). Lysates were removed from the glass beads to separate tubes, and centrifuged 450 *g* to remove unbroken cells. Aliquots were prepared for gel electrophoresis by boiling in sample buffer. 175 μg of samples per lane were electrophoresed on a 10%, 1.5 mm gel, and transferred to nitrocellulose. The filter was probed with αSec15$^{257-676}$ antibodies and iodinated protein A. The immunoreactive band at 116 kD is amplified in the lysate derived from cells carrying the *SEC15* gene on the multicopy plasmid and is shifted in the lysate derived from the mutant cells verifying the fact that this antibody recognizes the Sec15p. Other bands, seen at 100, 85, and 50 kD are discussed in Results.

Solubility of the Sec15p Is pH Dependent

The sequence of the *SEC15* gene predicts a hydrophilic protein. We have investigated the solubility properties of the Sec15p in cell fractionation experiments (see Materials and Methods). NY467 cells were grown in rich medium (YPD) at 25°C, collected by centrifugation and converted to spheroplasts. Spheroplasts were lysed osmotically and the crude lysate spun at 450 *g* to remove unlysed cells. This supernatant (S1) was routinely diluted to 2.8 mg/ml and then centrifuged at 100,000 *g* to separate soluble (S2) and membrane fractions (P2). Aliquots of the different fractions (S1, S2, P2) generated during the centrifugation were subjected to SDS gel electrophoresis and transferred to nitrocellulose. The Sec15p was visualized using the anti-Sec15p antibody and ^{125}I-protein A. We observed that in our lysis conditions (pH 7.2), both soluble and insoluble pools of Sec15p exist (Fig. 5, *top*). When the supernatant (S1) was centrifuged immediately after lysis, a substantial amount of the Sec15p was associated with the membrane fraction. Association with the membrane fraction was dependent on the pH of the lysis buffer. If the

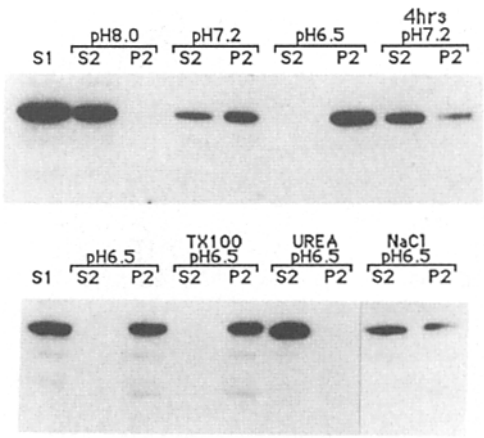

Figure 5. Analysis of the solubility properties of the Sec15 protein. The top section of the figure shows the effect of pH on the solubility of the Sec15p. The bottom section shows the solubility of the Sec15p extracted from the pH 6.5 pellet (*P2*) by various reagents (see Results). Lysates were prepared from strain NY467, which carries a duplication of the *SEC15* gene integrated into the chromosome (also see Fig. 2). Cells were grown in YPD at 25°C to A_{600} of 2.0. Lysates were prepared as described in Materials and Methods and centrifuged at 100,000 *g* to generate supernatant (*S2*) and pellet (*P2*) fractions. Aliquots of all samples were boiled in sample buffer. Equal volumes of samples (200 μg of total protein of S1) were electrophoresed in the gel and transferred to nitrocellulose. The filter was probed with $\alpha Sec15^{257-676}$ antibodies and iodinated protein A.

lysate was prepared at pH 8.0, and then centrifuged as above, all of the Sec15p was found in the supernatant. The opposite was observed when the pH of the lysate was adjusted to 6.5 by adding 2-(*N*-morpholino)ethane sulfonic acid (MES) buffer directly to the pH 7.2 lysate; all of the Sec15p was found in the membrane fraction (Fig. 5, *top*). The solubility of the Sec15p was also affected by other parameters. When the lysate, prepared at pH 7.2, was incubated on ice for 4 h before centrifugation, we observed a shift of the Sec15p from the membrane fraction to the soluble fraction (Fig. 5). A similar phenomenon was also observed when the pH 7.2 lysate was diluted twofold with lysis buffer to 1.4 mg/ml and spun immediately (not shown).

The nature of the interaction between the Sec15p and the membrane fraction was tested by using various extraction procedures. In these experiments we have extracted the Sec15p from both the S1 supernatant, adjusted to pH 6.5, and from the pH 6.5, P2 pellet and obtained essentially the same results in either case. Samples were treated with various reagents, spun at 100,000 *g* and the Sec15p was visualized by Western blot analysis. As shown in Fig. 5, *bottom*, the Sec15p is associated with the membrane fraction at pH 6.5. It is not extracted from this fraction by 1% Triton X-100. 5 M urea readily solubilizes Sec15p from the membrane fraction. Partial solubilization was observed when the lysate was treated with 1 M NaCl. These findings suggest an ionic interaction rather than a hydrophobic interaction of Sec15p with a pelletable structure.

Sec15p Associates with the Microsomal Fraction

We have studied the distribution of the Sec15p upon subcellular fractionation by differential centrifugation. In these experiments the S1 (450 *g*) supernatants derived from wild-type cells (NY 451) and cells that overproduce the Sec15p from the *GAL1* promoter (NY724) were used. NY724 was constructed by transforming the NY451 wild-type strain with the *GAL1-SEC15* integrating plasmid pNB304 (see Materials and Methods). Supernatants were centrifuged at 10,000 *g* to yield S2 and P2 and the S2 fractions were spun at 100,000 *g* to yield S3 and P3. Aliquots of the different supernatants (S1, S2, S3) and pellets (P1, P2, P3) generated during the differential centrifugation were analyzed by Western blot as above. Antibodies generated against the TrpE–Sec15 fusion protein containing the aminoterminal portion of the Sec15, $\alpha Sec15^{1-241}$, were used in this and the following experiments. These antibodies allowed improved detection of the Sec15p and also confirmed our earlier results.

Since we had observed a pH effect on the solubility of the Sec15p we did these spins at both pH 7.2 and 6.5, to study the possible difference in distribution. At pH 7.2 very little Sec15p is seen in the P2 pellet, whereas S3 and P3 contain the Sec15 protein distributed equally between these fractions (Fig. 6). This distribution is consistent with our solubility studies described above (Fig. 5, *top*). When the lysate is first adjusted to pH 6.5 and then spun, Sec15p is found primarily in the P3 fraction with no apparent soluble pool. A small pool of Sec15p is seen in the P2 pellet. In similar centrifugation experiments we have found most of the endoplasmic reticulum, plasma membrane, vacuolar, and mitochondrial markers in the P2 fraction and the secretory vesicle marker, invertase, in the P3 pellet fraction (Walworth and Novick, 1987; Goud et al., 1988). If Sec15p is associated with an organelle, possible candidates include secretory vesicles and the Golgi apparatus.

When the Sec15p is overproduced from the *GAL1* promoter by a 5 h induction with galactose, a 12-fold increase in the amount of the Sec15p is observed. Most of this overproduced Sec15p (~80%) is found in the insoluble fraction, in a 100,000-*g* spin, when lysates are prepared at pH 7.2 (not shown). Differential centrifugation experiments at pH 7.2 show that a substantial portion of this insoluble Sec15p is

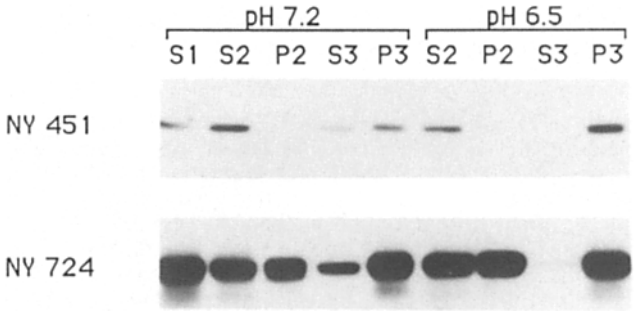

Figure 6. Differential centrifugation of lysates derived from wild-type (NY451) and Sec15p overproducing cells (NY724). Cells were grown in YP lactate overnight and then induced by addition of galactose. After 5 h of induction, cells were harvested and lysates (*S1*) were prepared as in Materials and Methods. S1 supernatants were centrifuged successively 10,000 *g* to generate supernatant S2 and pellet P2. S2 supernatants were centrifuged at 100,000 *g* to generate supernatant S3 and pellet P3. Samples were prepared for electrophoresis as in Fig. 5. Equal volumes of samples were loaded in each lane (100 μg of total protein of S1), electrophoresed in the gel, and transferred to nitrocellulose. The filter was probed with $\alpha Sec15^{1-241}$ antibodies (see Results) and iodinated protein A.

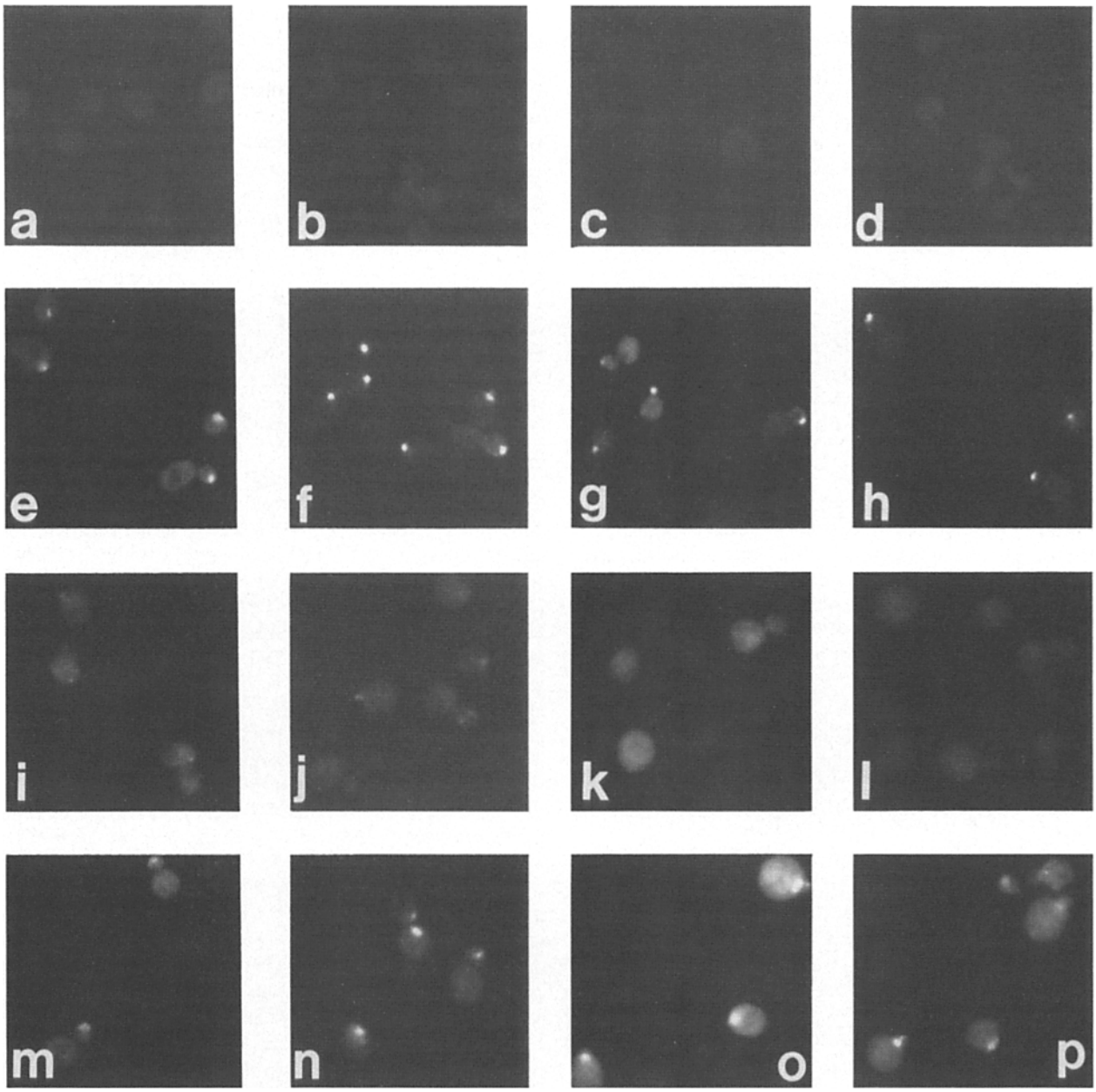

Figure 7. Immunofluorescence localization of the Sec15 and Sec4 proteins. (*a*) NY451, *SEC*$^+$, grown in galactose and labeled with αSec15$^{1-241}$ antibody; (*b*) NY724, *SEC*$^+$, *GAL-SEC15*, grown in glucose and labeled with αSEC15$^{1-241}$ antibody; (*c*) NY724, *SEC*$^+$, *GAL-SEC15* grown in galactose and processed without primary antibody; (*d*) NY15, *SEC*$^+$ grown in glucose and labeled with αSec15$^{1-241}$ antibody. Excluding the top row of the figure, cells were grown in YP lactate–galactose medium for 15 h at 25°C, and fixed for immunofluorescense (*e*, *f*, *i*, *j*, *m*, and *n*) or were shifted to 37°C for 2 h before fixation (*g*, *h*, *k*, *l*, *o*, and *p*). (*e*) NY724, *SEC*$^+$, *GAL-SEC15*, 25°C, labeled with αSec15$^{1-241}$ antibody; (*f*) NY724, *SEC*$^+$, *GAL-SEC15*, 25°C, labeled with αSec4 antibody; (*g*) NY724, *SEC*$^+$, *GAL-SEC15*, 37°C, labeled with αSec15$^{1-241}$ antibody; (*h*) NY724, *SEC*$^+$, *GAL-SEC15*, 37°C, labeled with αSec4 antibody; (*i*) NY725, *sec4-8, GAL-SEC15*, 25°C, labeled with αSec15$^{1-241}$ antibody; (*j*) NY725, *sec4-8, GAL-SEC15*, 25°C, labeled with αSec4 antibody; (*k*) NY725, *sec4-8, GAL-SEC15*, 37°C, labeled with αSec15$^{1-241}$ antibody; (*l*) NY725, *sec4-8, GAL-SEC15*, 37°C, labeled with αSec4 antibody; (*m*) NY742, *sec6-4, GAL-SEC15*, 25°C, labeled with αSec15$^{1-241}$ antibody; (*n*) NY742, *sec6-4, GAL-SEC15*, 25°C, labeled with αSec4 antibody; (*o*) NY742, *sec6-4, GAL-SEC15*, 37°C, labeled with αSec15$^{1-241}$ antibody; (*p*) NY742, *sec6-4, GAL-SEC15*, 37°C, labeled with αSec4 antibody.

present in the 10,000-*g* pellet (P2) fraction (Fig. 6, *bottom*). At pH 6.5 there is no detectable soluble pool, as in the wild-type situation, but a substantial pool of Sec15p is associated with the P2 fraction (Fig. 6, *bottom*). Thus, overproduction of Sec15p either causes a portion of the protein to associate with a larger structure than in the normal situation or causes the structure with which it is normally associated to pellet at a lower speed.

Overproduction of Sec15p Results in the Formation of a Patch of Sec15p, a Patch of Sec4p, and a Cluster of Vesicles

Immunolocalization of Sec15p in wild-type cells was problematic due to the low level of Sec15p under normal conditions. Wild-type cells (NY15) were grown in YPD at 25°C. Cells were fixed and prepared for immunofluorescence. Af-

finity-purified anti-Sec15p antibody was applied (see Materials and Methods) and the slides were visualized. Only a faint diffuse staining was observed in this situation (Fig. 7 *d*). This staining was somewhat brighter than was observed when no first antibody was used (Fig. 7 *c*).

To determine if overproduction of Sec15p would allow us to localize the protein, we analyzed cells which express Sec15 protein from the *GAL1* promoter. NY724 cells were grown in YP lactate-galactose medium at 25°C for 15 h and fixed. The Sec15p signal was greatly enhanced in these cells and the most striking observation was the presence of a brightly staining concentrated patch. This structure was located either in the bud, or adjacent to an emerging bud (Fig. 7 *e*). Observation in a confocal microscope established that the staining was intracellular rather than cortical (not shown). This signal was not detected in wild-type Gal$^+$ cells, grown in YP lactate-galactose (NY451; Fig. 7 *a*). Neither was it detected in NY724 cells grown on YP glucose (Fig. 7 *b*), nor in galactose induced NY724 cells without the first antibody (Fig. 7 *c*), indicating that the patch corresponds to Sec15p overproduced by expression from the *GAL1* promoter.

We pursued the nature of the patch structure by thin section electron microscopy. Wild-type Gal$^+$ (NY451) and Sec15p overproducing (NY724) cells were grown in YP lactate-galactose medium as described above. Cells were fixed and prepared for electron microscopy as described earlier (Salminen and Novick, 1987). These EM results demonstrate an accumulation of 100-nm vesicles in a concentrated array (Fig. 8, *b* and *d*). This vesicle cluster is often located towards the bud end of the cell. Wild-type cells grown in the same conditions did not accumulate vesicles (Fig. 8 *a*). It was not evident that this patch was held together by any apparent structure (Fig. 8 *c*).

In a previous study we have shown that Sec4p is associated with secretory vesicles (Goud et al., 1988). The observation of a cluster of vesicles in Sec15p overproducing cells therefore predicts the formation of a patch of Sec4p in these cells. As shown in Fig. 7 *f*, a patch of similar size and position to that seen with anti-Sec15p antibody was observed with affinity purified anti-Sec4p antibody, confirming that prediction. Furthermore, the result suggests that both Sec4p and Sec15p may be associated with the same vesicular structure. Double label immunofluorescence experiments are precluded by the fact that both antisera were produced in rabbits, yet immunoelectron microscopic analysis is underway to test the association of Sec15p with the cluster of vesicles.

These data suggest that the increased amount of the Sec15p, overproduced from the strong *GAL1* promoter, in some fashion interferes with the transport of the secretory vesicles at a stage between the Golgi apparatus and the plasma membrane, causing the vesicles to aggregate. Interference with the secretory pathway is generally associated with a growth defect (Novick et al., 1980). Cells induced to overproduce the Sec15p (NY724) grow at wild-type rate for 6 h, after which the growth rate slows by a factor of two. This slower growth rate is, nevertheless, maintained for up to 25 h. The slower growth rate may be an adjustment to the effect of accumulating vesicles, since we have observed by immunofluorescence with anti-Sec15p antibody and by thin section microscopy that the cluster of vesicles forms between 2 and 5 h of induction (not shown). Overproduction of the Sec15-1 mutant protein does not cause a slowing of the growth rate and does not lead to a patch by staining with anti-Sec15p antibody (not shown).

Sec15p Patch Formation Requires Function of Sec2 and Sec4

The strong genetic interactions seen between *SEC4* and *SEC15*, as well as several other *SEC* genes, suggests that their gene products may functionally interact on a dependent pathway (Salminen and Novick, 1987). These findings led us to test the effects of mutations in the various late-acting *sec* genes on the ability of overproduced Sec15 protein to form a patch. We transformed Gal$^+$ derivatives of each of the 10 vesicle accumulating mutants with the integrating plasmid pNB304 to overproduce the Sec15p in these mutant cells. Resulting transformants were grown at steady state in YP lactate-galactose at 25°C, aliquots were shifted to grow at the restrictive temperature 37°C for 2 h, and cells were fixed for immunofluorescence.

Initial studies were done with *sec4-8* and *sec6-4* strains. The analysis of NY725 (*sec4-8*) cells with anti-Sec15p antibody showed that the patch-like Sec15p signal was much reduced in the *sec4-8* mutant background. Even at 25°C, only a very small dot was seen at the tip of the cells reminiscent of the patch structure (Fig. 7 *i*). The staining of the NY725 cells grown at 25°C with anti-Sec4p antibody showed the presence of a weakly staining patch (Fig. 7 *j*). The lower intensity of the Sec4p signal can be explained by the fivefold reduction in the amount of the Sec4p present in the *sec4-8* mutant cells (Goud et al., 1988). When the NY725 cells were shifted to the restrictive temperature and analyzed with both antibodies we found that the Sec15p and the Sec4p signals were more diffuse (Fig. 7, *k* and *l*). This suggested that the loss of Sec4 function at 37°C causes a dissociation of the residual patch structure. In *SEC*$^+$ cells (NY724) shifted to 37°C the elevated temperature did not by itself affect the formation of the patches, as was seen with anti-Sec15p and anti-Sec4p antibodies (Fig. 7, *g* and *h*). In the *sec6-4* mutant background at 25°C patches were seen with both anti-Sec15p and anti-Sec4p antibodies (Fig. 7, *m* and *n*). At the higher temperature the patches were still the predominant stained structures in these cells (Fig. 7, *o* and *p*) although there was some increased staining of the cytoplasm as well and a higher frequency of cells with multiple patches. These results suggest that the *sec 6-4* mutation had a lesser effect on the formation of the patch resulting from the overproduction of Sec15p than did the *sec4-8* mutation.

We have extended these studies to include mutants in all of the late acting genes. The mutants were grown at 25°C in noninducing media, 2% lactate, induced for 4 h at 25°C by the addition of 1% galactose, and then shifted to 37°C for 1 h, before fixation in formaldehyde. In each of the mutants, with the exception of *sec2-41* and *sec4-8*, overproduction of Sec15 protein led to the formation of a patch or patches, as revealed by anti-Sec15p antibody (Fig. 9). In the case of *sec2-41* (Fig. 9 *c*), general staining of the cell was seen with only a slight tendency towards local bright spots. Consistent with our previous findings, *sec4-8* cells showed a nearly complete lack of patch formation (Fig. 9 *e*). A construction was made which overexpressed the *sec15-1* product from the *GAL* promoter in addition to the wild-type gene product from the *SEC15* promoter (NY755) (Fig. 10, lane *10*). Overexpression of the mutant protein did not lead to formation of a patch (Fig.

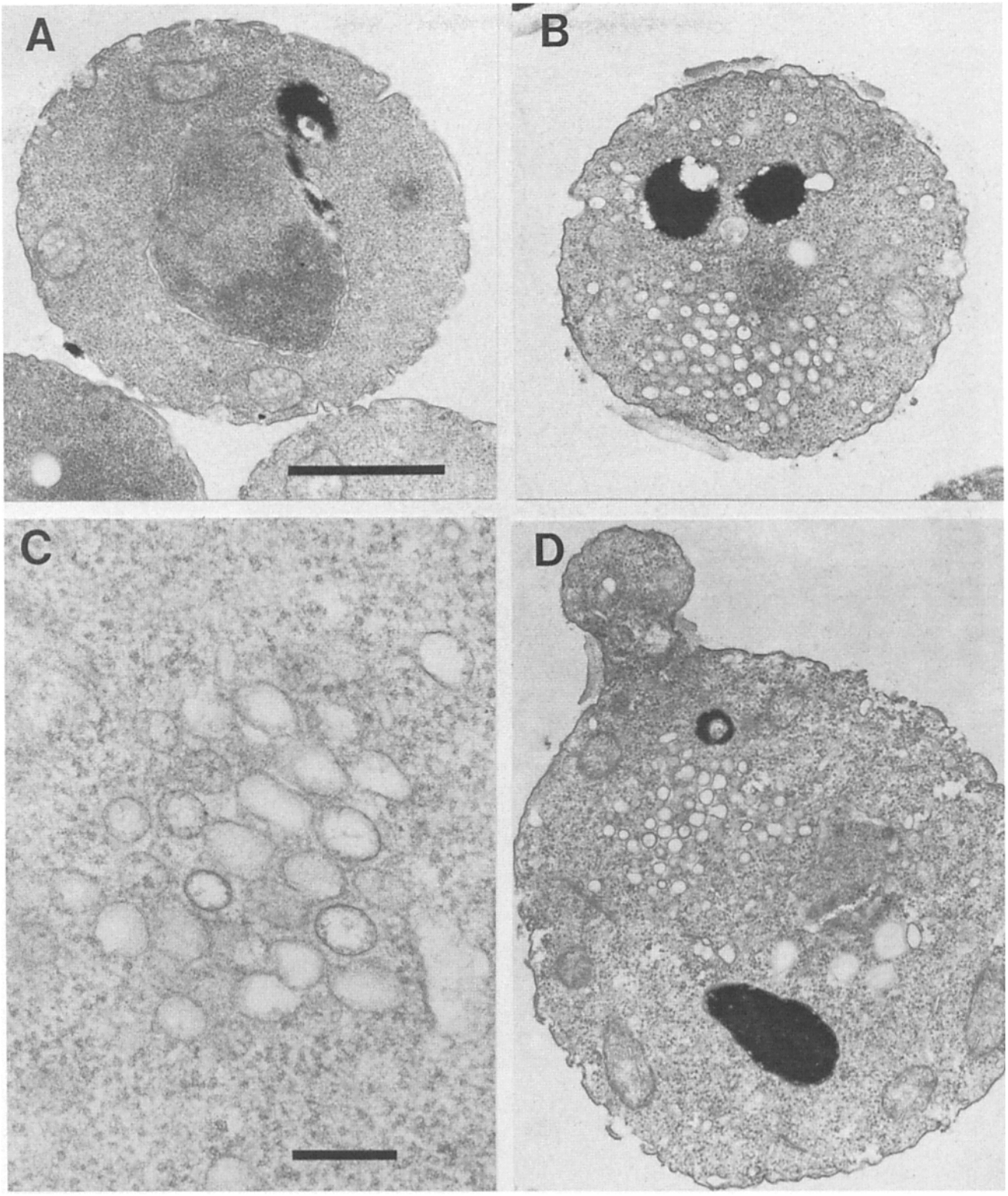

Figure 8. Electron microscopic analysis of NY724 cells overproducing the Sec15 protein from the *GAL1* promoter. Cells were grown in YP lactate–galactose medium for 15 h before processing for microscopy. (*A*) Wild-type, NY451. (*B*, *C*, and *D*) NY724 cells show a distinct patch of aggregated secretory vesicles. Bars: (*A*, *B*, and *D*) 1 μm; (*C*) 2 μm.

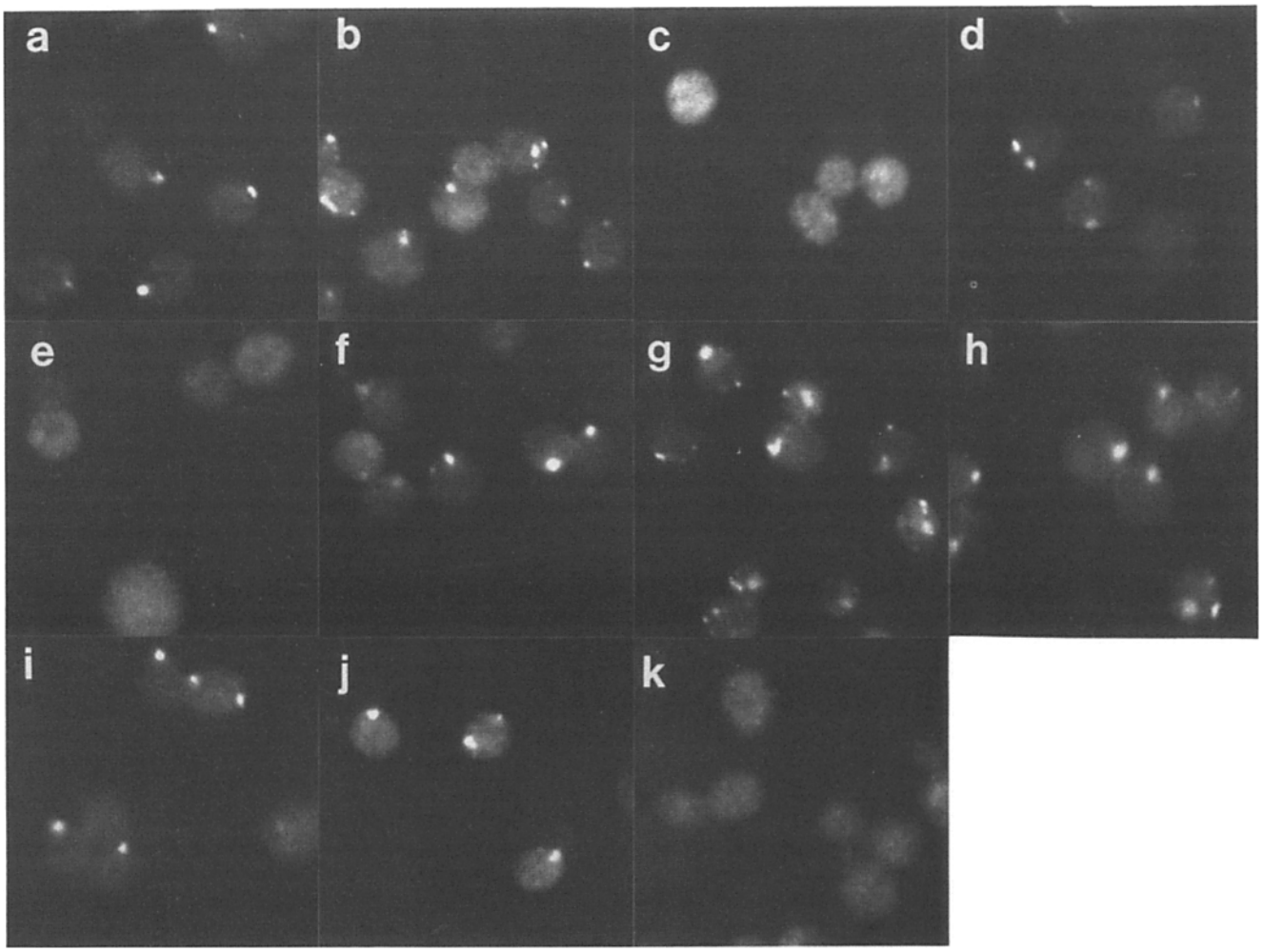

Figure 9. Immunofluorescence localization of Sec15 protein in *sec* mutants overproducing the Sec15 protein from the *GAL1* promoter. Cells were grown in YP lactate medium at 25°C then induced by addition of galactose. After 4 h of induction the cultures were shifted to 37°C for one additional hour then fixed and labeled with αSec15$^{1-241}$ antibody. (*A*) NY724, *SEC*$^+$, *GAL-SEC15*; (*B*) NY748, *sec1-1 GAL-SEC15*; (*C*) NY755, *sec2-41, GAL-SEC15*; (*D*) NY751, *sec3-2, GAL-SEC15*; (*E*) NY725, *sec4-8, GAL-SEC15*; (*F*) NY749, *sec5-24, GAL-SEC15*; (*G*) NY742, *sec6-4, GAL-SEC15*; (*H*) NY750, *sec8-9, GAL-SEC15*; (*I*) NY752, *sec9-4, GAL-SEC15*; (*J*) NY753, *sec10-2, GAL-SEC15*; (*K*) NY754, *SEC*$^+$, *GAL-sec15-1*.

9 *k*). In all cases, production of Sec15 protein was equally high as determined by Western blot analysis (Fig. 10).

Discussion

We have presented a characterization of *SEC15* and its protein product. Our specific goal in this analysis has been to understand the physical basis underlying the strong genetic interaction seen between *SEC15* and *SEC4*. Both of these genes are essential for growth. This suggests that both gene products play distinct and separate roles in the vesicular transport process. In support of this, we have extended the genetic analysis of the interaction between these genes by showing that duplication of the *SEC4* gene can suppress the *sec15-1* mutation but cannot suppress the deletion of the *SEC15* gene from the genome. Thus partial, but indispensable, function is provided by the temperature-sensitive mutant protein, Sec15-1p, even at the restrictive temperature. The fact that the *sec15-1* mutation was originally identified as incompletely restrictive with respect to invertase secretion (Novick et al., 1980) may reflect this partially functional mutant phenotype. We have also shown that the presence of a second copy of the *SEC4* gene does not significantly alter the amount of the Sec15-1p in the cells, ruling out increased synthesis or stability of the mutant protein as the mechanism of supression. We favor a model in which Sec4p acts as an upstream activator of Sec15p function. An increase in the level of Sec4p may stimulate the residual Sec15-1p to provide sufficient function. Our studies on the effects of the *sec4-8* mutation on localization of Sec15 protein are consistent with this model.

The nucleotide sequence of *SEC15* predicts a hydrophilic protein product of 105 kD, containing no hydrophobic stretches capable of spanning the lipid bilayer. Nevertheless, Sec15p is associated with the microsomal fraction by ionic interactions. This association presumably reflects an interaction with a protein component of a small organelle, possibly secretory vesicles. Efforts to pursue the fractionation studies further through the use of sucrose density gradients and gel filtration have been hampered by the sensitivity of Sec15p attachment to high molarities of sucrose, prolonged incubation, and high dilution. However, studies presented here

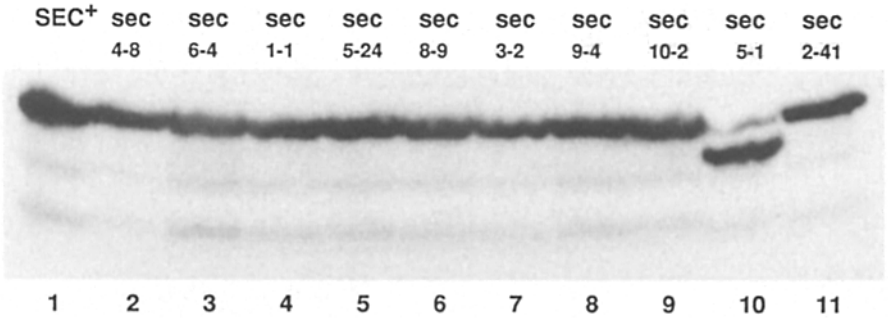

Figure 10. Expression level of Sec15 protein in *sec* mutants overproducing Sec15 protein from the *GAL1* promoter. Cells were grown in YP lactate medium then induced by addition of galactose. After 4 h of induction the cultures were shifted to 37°C for one additional hour and lysates were prepared. Samples derived from equal amounts of yeast were electrophoresed on acrylamide gels, transferred to nitrocellulose and probed with αSec15$^{1-241}$ antibody. Lane *1*, NY724, *SEC*$^+$, *GAL-SEC15*; lane *2*, NY725, *sec4-8, GAL-SEC15*; lane *3*, NY742, *sec6-4, GAL-SEC15*; lane *4*, NY748, *sec1-1, GAL-SEC15*; lane *5*, NY749, *sec5-24, GAL-SEC15*; lane *6*, NY750, *sec8-9, GAL-SEC15*; lane *7*, NY751, *sec3-2, GAL-SEC15*; lane *8*, NY752, *sec9-4, GAL-SEC15*; lane *9*, NY753, *sec10-2, GAL-SEC15*; lane *10*, NY754, *SEC*$^+$, *GAL-sec15-1*; lane *11*, NY755, *sec2-41, GAL-SEC15.*

are consistent with an interaction of Sec15p with either secretory vesicles or the Golgi apparatus.

We initially cloned the *SEC15* gene behind the strong *GAL1* promoter to overproduce the Sec15p, and thus enhance the signal in immunofluorescence. Upon induction of synthesis, a concentrated patch of Sec15p became apparent. Thin section analysis indicated that a cluster of vesicles forms in response to Sec15p overproduction suggesting a physical association of Sec15p with secretory vesicles. Immunoelectron microscopy will be necessary to prove this point, however results from cell fractionation experiments are consistent with an association of Sec15p with vesicles in wild-type cells, and with the vesicle cluster in a Sec15p overproducer. In differential centrifugation experiments with wild-type cells the Sec15p was primarily found in the 100,000-*g* pellet, while in the Sec15 overproducer a substantial pool was also found to pellet at 10,000 *g*. This shift in distribution could reflect the formation of vesicular aggregates which pellet at the lower speed.

Sec15p is apparently not associated with the plasma membrane in wild-type cells, since Sec15p is not found in the 10,000-*g* pellet, yet most of the plasma membrane marker enzyme does pellet at this speed (Walworth et al., 1987; Goud et al., 1988). If Sec15p is associated with secretory vesicles, but not the plasma membrane, then it must either dissociate before vesicle fusion or very soon after fusion. Such a transient association fits well with a model of vesicular transport in which the components of the transport machinery are recycled, and their attachment to the carrier vesicle regulated by a cyclical assembly and disassembly process.

The phenotype of the Sec15p overproducer has revealed a new, albeit aberrant function of the Sec15 protein, the ability to form a cluster of vesicles and a patch of Sec15p. We have used this property to ask if Sec15p function is dependent upon the function of any other *SEC* gene product. The mutations fall into two clear groups: *sec4-8* and *sec2-41* prevent formation of the Sec15p patch, while the other mutations do not. If we postulate that the secretion pathway consists of a linear series of dependent events, then we can conclude that Sec2p and Sec4p must function upstream of Sec15p. The other gene products may function downstream from Sec4p, or on an independent pathway. Given the somewhat artificial nature of the experiment, these conclusions must remain tentative at this time. Nonetheless, this result is consistent with the observation, discussed above, that duplication of *SEC4* can suppress a partial loss of Sec15p function, but not total loss, and supports our model of Sec4p as an upstream activator of Sec15p function. Such a model is also consistent with the known functions of other GTP binding proteins. In general, these proteins do not, by themselves, catalyze enzymatic reactions other than the very slow hydrolysis of GTP, but through their interaction with downstream effectors they serve to control a broad range of cellular functions. Sec15p could be the immediate downstream effector of Sec4p, or there may be one or more protein intermediaries between Sec4p and Sec15p function. In fact, our finding that the *sec2-41* mutation prevents patch formation by overproduction of Sec15p implicates Sec2p as a possible intermediary in such a chain of protein function. However, we cannot distinguish this from an alternative model in which Sec2p acts upstream of Sec4p.

While it may be premature to propose a specific model for Sec15 function, we can attempt to extrapolate from the vesicle aggregating phenotype of the overproducer to the normal function of the protein. Sec15 protein may, at normal concentration, serve to attach vesicles bearing Sec4 protein onto the appropriate target, the plasma membrane of the bud. At excess concentration, such a vesicle docking protein could lead to vesicle aggregation. Since a close structural homolog of Sec4, the Ypt I protein, appears to play a critical role in an earlier stage of the yeast secretory pathway (Segev et al., 1988) and an as yet unidentified GTP binding protein may function in transport through the mammalian Golgi (Melancon et al., 1987), we can speculate that there may be analogs of Sec15p. They may function, in response to their respective GTP binding protein, to attach the appropriate vesicle to the appropriate target membrane and thereby maintain the specificity of the vesicular transport mechanism.

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