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 encoding the ms-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 transport 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). 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% Bacto yeast extract, 2% Bacto peptone (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, the cells were first grown to early log (A600 ~1.0) in YP lactate + galactose for overnight growth (~15 h). To obtain longer induction times the cells were inoculated in YP containing 1% lactate. Galactose was added directly to the culture to induce the overproduction of the Sec15p. Transformants were selected on SD medium at 25°C. 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 (Salmi nen and Novick, 1987). 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*, tet*. This yeast genomic library was previously described (Rose et al., 1987). Plasmid pNB40 was constructed by inserting the 6.1-kb Bam HI fragment from pNB90 into the Bam HI site of YCp50. Plasmid pNB40 was generated by deletion of the 2.4-kb Hind III fragment of pNB40. pNB40 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 0.4-kb Barn HI Xba I fragment of pNB143. The pNB143 fragment, involved deletion of the 0.4-kb Bam HI-Xba I fragment of pNB43. The recessed 3’ ends of the plasmids 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 pNB90 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 YIp3 into the Bgl2 sites of the SEC15 gene in pNB92. 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 blunted as described 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 (dGCGATCCC; Boerigger 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 blunt-ended 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    | MATa, ura3-52, his4-619                     |
| NY64    | MATa, ura3-52, sec15-1                      |
| NY179   | leu2-3, 112, ura3-52                       |
| NY180   | leu2-3, 112, ura3-52                       |
| NY363   | MATa/a, leu2-3, 112/+ ura3-52/ura3-52, his4-619/+ |
| NY376   | MATa, ura3-52, sec15-1, SEC4::pNB141 (SEC4, URA3) |
| NY440   | MATa, ura3-52, his4-619, pNB148 (2 μm, SEC15, URA3) |
| NY451   | MATa, ura3-52, Gal*                        |
| NY456   | MATa, ura3-52, sec4-8, Gal*                |
| NY467, 468 | MATa, ura3-52, his4-619, SEC15::pNB192 (SEC15, URA3) |
| NY483   | MATa/a, leu2-3, 112/+ ura3-52/ura3-52, his4-619/+ , sec15::pNB193 (sec15, internal fragment, URA3) |
| NY503   | MATa, ura3-52, his4-619, sec6-4, Gal*       |
| NY648   | MATa/a, leu2-3, 112/leu2-3, 112, ura3-52/ura3-52 |
| NY662   | MATa/a, leu2-3, 112/leu2-3, 112, ura3-52/ura3-52, 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   | MATa, ura3-52, Gal*, his4-619, sec6-4, SEC15::pNB304 (GAL1-SEC15, URA3) |
| NY748   | MATa, ura3-52, Gal*, his4-619, sec1-1 SEC15::pNB304 (GAL1-SEC15, URA3) |
| NY749   | MATa, ura3-52, Gal*, his4-619, sec5-24, SEC15::pNB304 (GAL1-SEC15, URA3) |
| NY750   | MATa, ura3-52, Gal*, his4-619, sec8-9, SEC15::pNB304 (GAL1-SEC15, URA3) |
| NY751   | MATa, ura3-52, Gal*, his4-619, sec9-4, SEC15::pNB304 (GAL1-SEC15, URA3) |
| NY752   | MATa, ura3-52, Gal*, his4-619, sec10-2, SEC15::pNB304 (GAL1-SEC15, URA3) |
| NY753   | MATa, ura3-52, Gal*, his4-619, sec2-41, SEC15::pNB304 (GAL1-SEC15, URA3) |
| NY754   | MATa, ura3-52, Gal*, his4-619, sec1-1, SEC15::pNB304 (GAL1-SEC15, URA3) |
| NY755   | MATa, ura3-52, Gal*, his4-619, sec2-41, SEC15::pNB304 (GAL1-SEC15, URA3) |
Table II. Plasmids Used (see Materials and Methods for constructions)

| Plasmid | Derivation |
|---------|------------|
| pNB140  | YCP50, SEC15; 16.5 kb genomic insert in the Bam HI site |
| pNB143  | YCP50, SEC15; 6.1 kb Bam HI—Bam HI fragment from pNB140, in Bam HI site |
| pNB148  | YCP50, SEC15; 3.7 kb Hind III—Bam HI fragment, Hind III Δ of pNB140 |
| pNB164  | 2 μm, SEC15; 3.7 kb Hind III—Bam HI fragment from pNB143, in Hind III—Bam HI sites of vector, pBR322 (from collection of D. Botstein) |
| pNB166  | pATH11, TrpE-SEC15 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 GALI promoter; 0.82 kb Eco RI—Bam HI fragment in Eco RI—Bam HI sites, expression under GALI control by cloning into the Bam HI site |
| pNB191  | Ylp5 with unique Pvu II site removed by Bal31 digestion |
| pNB192  | Ylp5, SEC15; 3.35 kb Hind III—Bam HI fragment from pNB186, into Hind III—Bam HI sites of pNB191 |
| pNB193  | Ylp5, SEC15, internal 1.25 kb Eco RI—Cla I fragment, into Eco RI—Cla I sites of pNB191 |
| pNB291  | Ylp5, sec15::LEU2 gene disruption; 3 kb Bgl2—Bgl2 fragment from Ylp13 (LEU2) replacing the Bgl2—Bgl2 internal fragment of SEC15 in pNB192 |
| pNB299  | Ylp5, SEC15; 3.1 kb Bam HI—Bam HI fragment, Hind III—Alu I Δ of pNB192, SEC15 leader sequence removed |
| pNB300  | YCP50, GALI—SEC15; 3.1 kb Bam HI—Bam HI fragment from pNB299 into Bam HI site of pNB187 |
| pNB301  | pATH2, TrpE-SEC15 fusion; 0.7 kb Bam HI—Eco RI(blunt) fragment from pNB299 into Bam HI and Hind III(blunt) sites of pATH2 |
| pNB304  | Ylp5, GALI—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-35S]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 mp8 or mp19. The predicted protein sequence was compared with the National Biomedical Research Foundation Library by the FASTP program in the ktp2-2 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). Tissue hybridization, hybridization, and washing of the filters 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, NRBl64 and NRBl301 (DHI 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 mCi Tris-EDCI, pH 7. The pellet was resuspended in 5 ml of cold buffer (10 mCi NaI, pH 7.2, 1% β-mercaptoethanol, 1% SDS, 6 M urea) by vortexing in a 50 ml 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 50 Ti 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 10–15 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 NRBl64 (TrpE-SEC15 fusion), 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 NRBl301 (TrpE-SEC15 fusion) 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 times 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 antiserum (αSec15 results; 250 μg/ml, 1/2,000 dilution) and radiiodinated 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 (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 hrs. Filters were washed 4 × 10 min with wash buffer. After washing, 125I-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 A600 of 2.0 as described above. Usually 200–400 A600 units were pelleted and washed once with buffer, 10 mM Tris, pH 7, 10 mM NaCl. Cells were resuspended in spheroplast medium (50 mM Tris, pH 7.5, 10 mM NaCl, 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 A600 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, antipain, 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 (SI). The pH of the lysate was adjusted to 6.5, for necessary, by adding 1 M MES-buffer pH 6.5, to 50 mM into SI. In differential centrifugation experiments, the SI supernatant was spun at 12,000 rpm (10,000 g 

Results

Cubated on ice for 30 min, and spun 100,000 g as above. The resulting pellets were resuspended in 1 ml lysis buffer. In crude membrane separation centrifugation (100,000 g~), in the same rotor in 1 ml volume for 1 h at 4°C and the pellet was resuspended in 1 ml lysate buffer. In crude membrane separation centrifugation the SI supernatant was further centrifuged at 40,000 rpm (100,000 g 

For the extraction experiments the low speed (450 g) supernatant was added, the induced cultures were shifted to 37°C for 2 h before fixation. 5 A units of cells were washed twice with 20 mM Tris-HCl, pH 7.5, 1.2 M sorbitol, 25 mM B-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 B-mercaptoethanol or Zymolyase. For permeabilization, the cells were resuspended in 2 ml PBS 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 (PBS TWEEN containing 2 mg/ml BSA, was applied on the wells and incubated for 30 min. Affinity-purified anti-Sec5p antibodies (eSec5p 

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, Be II; E, Eco RI; G, Bgl I; P, Pvu II; V, Eco RV; C, Clu 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.
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. 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.

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 the presumed ATG start codon contains another, in frame, stop codon in yeast, we determined if the upstream region

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.
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). Immuno-blot 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 over-producing 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 Sec15p 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 Sec15p protein, although it is reduced in abundance and increased in its mobility with respect to the Sec15p protein in wild-type cells (NY15) (Fig. 4). When NY64 and NY376 cells were 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.

**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 125I-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
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 SI 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, top, 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 SI (450 g) supernatants derived from wild-type cells (NY 451) and cells that overproduce the Sec15p from the GALI promoter (NY724) were used. NY724 was constructed by transforming the NY451 wild-type strain with the GALI-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 (SI, 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, αSec15P1-24, 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 GALI 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

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**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 A600 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 SI) were electrophoresed in the gel and transferred to nitrocellulose. The filter was probed with αSec15P1-24 antibodies and iodinated protein A.

**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 (SI) were prepared as in Materials and Methods. SI 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 SI), electrophoresed in the gel, and transferred to nitrocellulose. The filter was probed with αSec15P1-154 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[1-24] antibody; (b) NY724, SEC+, GAL-SEC15, grown in glucose and labeled with αSEC15[1-24] antibody; (c) NY724, SEC+, GAL-SEC15 grown in galactose and processed without primary antibody; (d) NY15, SEC+ grown in glucose and labeled with αSec15[1-24] 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 immunofluorescence (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[1-24] antibody; (f) NY724, SEC+, GAL-SEC15, 25°C, labeled with αSec4 antibody; (g) NY724, SEC+, GAL-SEC15, 37°C, labeled with αSec15[1-24] antibody; (h) NY724, SEC+, GAL-SEC15, 37°C, labeled with αSec4 antibody; (i) NY725, sec4-8, GAL-SEC15, 25°C, labeled with αSec15[1-24] antibody; (j) NY725, sec4-8, GAL-SEC15, 25°C, labeled with αSec4 antibody; (k) NY725, sec4-8, GAL-SEC15, 37°C, labeled with αSec15[1-24] antibody; (l) NY725, sec4-8, GAL-SEC15, 37°C, labeled with αSec4 antibody; (m) NY742, sec6-4, GAL-SEC15, 25°C, labeled with αSec15[1-24] antibody; (n) NY742, sec6-4, GAL-SEC15, 25°C, labeled with αSec4 antibody; (o) NY742, sec6-4, GAL-SEC15, 37°C, labeled with αSec15[1-24] antibody; (p) NY742, sec6-4, GAL-SEC15, 37°C, labeled with αSec4 antibody.

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 16 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 sec2-4l 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 sec2-4l (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 SECl5 promoter (NY755) (Fig. 10, lane io). 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 GALJ 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 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 suppression. 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
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. Sec15p 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 Ypt1 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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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 αSec15-241 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-secl5-1; lane 11, NY755, sec2-41, GAL-SEC15.
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