SEC31 Encodes an Essential Component of the COPII Coat Required for Transport Vesicle Budding from the Endoplasmic Reticulum

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The COPII vesicle coat protein promotes the formation of endoplasmic reticulum- (ER) derived transport vesicles that carry secretory proteins to the Golgi complex in *Saccharomyces cerevisiae*. This coat protein consists of Sar1p, the Sec23p protein complex containing Sec23p and Sec24p, and the Sec13p protein complex containing Sec13p and a novel 150-kDa protein, p150. Here, we report the cloning and characterization of the *p150* gene. p150 is encoded by an essential gene. Depletion of this protein in vivo blocks the exit of secretory proteins from the ER and causes an elaboration of ER membranes, indicating that p150 is encoded by a *SEC* gene. Additionally, overproduction of the p150 gene product compromises the growth of two ER to Golgi *sec* mutants: *sec16–2* and *sec23–1*. p150 is encoded by *SEC31*, a gene isolated in a genetic screen for mutations that accumulate unprocessed forms of the secretory protein α-factor. The *sec31–1* mutation was mapped by gap repair, and sequence analysis revealed an alanine to valine change at position 1239, near the carboxyl terminus. Sec31p is a phosphoprotein and treatment of the Sec31p-containing fraction with alkaline phosphatase results in a 50–75% inhibition of transport vesicle formation activity in an ER membrane budding assay.

INTRODUCTION

The maturation of secretory proteins requires transit between the organelles of the secretory pathway. Secretory proteins start their journey in the endoplasmic reticulum (ER) where they receive asparagine-linked core oligosaccharide modifications. They then travel through the Golgi complex where elaboration of the N-linked carbohydrate moiety and additional modification such as sulfonation, O-linked glycosylation, or phosphorylation can occur. Finally, they are delivered either to the plasma membrane in secretory vesicles or to the lysosome (called the vacuole in yeast) cycling through endosomes. Resident proteins of various secretory pathway organelles reach their final destination either by retention in the appropriate organelle or retrieval from later compartments. Thus, travel

through the secretory pathway is bidirectional. Morphological analysis of pulse-radiolabeled secretory proteins in vivo established that transport between compartments of the secretory pathway utilizes vesicle carriers (Palade, 1975). A considerable amount of attention has focused on vesicle formation at the first transport step in the secretory pathway, between the ER and the Golgi. Two proteinaceous coats function to drive vesicle formation in this part of the pathway: COPI and COPII (Orci *et al.*, 1993; Barlowe *et al.*, 1994, Bednarak *et al.*, 1995).

COPI, originally isolated as the coat protein of transport vesicles carrying secretory proteins between the *cis* and medial compartments of the Golgi complex, contains seven polypeptides (α-ζ) and the small GTP binding protein Arf (Malhotra *et al.*, 1989; Serafini *et al.*, 1991a,b). Large scale purification of coatomer, the cytosolic assembly unit of COPI, has allowed the molecular analysis of most of the COPI subunits (Waters *et al.*, 1991). COPI functions are not restricted to intra-Golgi transport. Yeast mutants in several COPI sub-

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units do not properly retain ER resident transmembrane proteins bearing a di-lysine retrieval signal (Letourneur et al., 1994). Additionally, both mammalian and yeast COPI directly bind a di-lysine moiety fused to glutathione S-transferase (Cosson and Letourneur, 1994). Thus, COPI may direct the sorting of proteins bearing the di-lysine retention signal into retrograde transport vesicles traveling from the Golgi to the ER. Antibodies directed against the β subunit of COPI cause accumulation of secretory proteins in the ER in vivo and in vitro, pointing to an additional requirement for COPI function in anterograde transport between the ER and Golgi (Pepperkok et al., 1993; Peter et al., 1993).

In the yeast Saccharomyces cerevisiae, an approach combining genetic analysis of mutants defective for secretion in vivo and the biochemical analysis of a reaction reconstituting protein transport between the ER and the Golgi in vitro has revealed a set of proteins required for vesicle formation distinct from COPI. These proteins, in addition to being biochemically required for vesicle formation from the ER, actually coat the transport vesicles formed (Barlowe et al., 1994), thus the term COPII. COPII consists of four subunits (Sec23p, Sec24p, Sec13p, and p150) and the small GTP-binding protein Sar1p (Barlowe et al., 1994).

COPII does not form a cytosolic complex like coatomer, but instead only forms in the context of vesicle formation. Sar1p is recruited to the membrane first by the Sec12 protein (d'Enfert et al., 1991). SEC12 encodes an ER resident transmembrane glycoprotein required for transport vesicle formation in vivo and in vitro (Nakano et al., 1988; Rexach and Schekman, 1991). The cytosolic domain of Sec12p stimulates the exchange of GDP for GTP on Sar1p (Barlowe and Schekman, 1993). This activated form of Sar1p initiates recruitment of two cytosolic complexes: the Sec23p/Sec24p complex and the Sec13p/p150 complex. SEC23 encodes an 85-kDa protein that stimulates Sar1p-GTP hydrolysis to Sar1p-GDP (Yoshihisa et al., 1993). In the presence of a nonhydrolyzable analogue, GMP-PNP, as a source of guanine nucleotide, vesicles form but cannot deliver their contents to the Golgi complex (Barlowe et al., 1994). Under these conditions, COPII appears to be more tightly associated with the vesicles than in reactions performed with GTP. This indicates that Sar1p GTP hydrolysis precedes disassembly of the coat after vesicle release and uncoating precedes fusion with the Golgi membrane (Barlowe et al., 1994). SEC24 encodes a 105-kDa protein with no known homologies or biochemical activities (Yoshihisa et al., unpublished data). Sec24p does not affect Sec23p GTPase-activating protein activity in solution, but is absolutely required for vesicle formation in vivo and in vitro (Hicke et al., 1992); excess monomeric Sec23p cannot replace the Sec23p/Sec24p complex requirement for vesicle formation in vitro, and depletion of

Sec24p in vivo causes a block in ER to Golgi transport (Yoshihisa et al., unpublished data). In the context of the ER membrane, Sec24p may prevent Sec23p from interacting with Sar1p before vesicle formation has finished, thus impeding Sar1p-GTP hydrolysis and premature uncoating of the nascent vesicle. Finally, the Sec13p complex must be recruited to the membrane to complete COPII assembly. SEC13 encodes a 33-kDa hydrophilic protein containing six WD repeat motifs that make up the bulk of the coding sequence. The WD repeat, first identified in the β subunit of heterotrimeric G proteins, has been located in a number of proteins with diverse functions, including transcriptional activation, RNA splicing, and spindle pole body assembly (Neer et al., 1994). One common feature of these proteins is their participation in multiprotein complexes. Biochemical characterization of Sec13p revealed that its ability to promote vesicle formation from the ER requires an associated 150-kDa protein, p150 (Salama et al., 1993).

Here, we report the cloning and biochemical characterization of p150, now called Sec31p. Like the genes encoding the other COPII subunits, SEC31 encodes an essential protein required for ER-derived transport vesicle formation in vivo and in vitro. Although the sequence of SEC31 does not give clues to a specific biochemical activity in vesicle formation, it has characteristics of a regulator of budding.

MATERIALS AND METHODS

Strains, Materials, and General Methods

Yeast strains used in this study were RSY255 (leu2–3,112, ura 3–52, MATα; D. Botstein), RSY256 (his4–619, leu2–3,112, MATa; D. Botstein), RSY267 (sec16–2, ura3–52, his4–619), RSY281 (sec23–1, ura3–52, his4–619), RSY368 (his3–11,15, leu2–3,112, ura3–1, ade2–1, trp1–1, MATα), RSY612 (his3–11,15/his3–11,15, leu2–3,112/leu2–3,112, ura3–1/ura3–1, ade2–1/ade2–1, trp1–1/trp1–1, MATα/MATa), RSY1002 (sec31::TRP1/SEC31, his3–11,15/his3–11,15, leu2–3,112/leu2–3,112, ura3–1/ura3–1, ade2–1/ade2–1, trp1–1/trp1–1, MATα/MATa), RSY1003 (sec31::LEU2/SEC31, his3–11,15/his3–11,15, leu2–3,112/leu2–3,112, ura3–1/ura3–1, ade2–1/ade2–1, trp1–1/trp1–1, MATα/MATa), RSY1004 (sec31–1, ura3–52, leu2–3,112, MATα), and RSY1076 (pHZ134 (GAL1: p150, LEU2, ARS1, CEN4) sec31::TRP1, his3–11,15, leu2–3,112, ura3–1, ade2–1, trp1–1, MATα). Plasmid pNS3141 was made by subcloning the SEC31 BamHI-EcoRI fragment from pNS3132 described below into pCK1313 (SEC13, URA3, 2μ).

Yeast extract peptone growth medium contained 2% bacto-peptone (Difco, Detroit, MI), 1% yeast extract (Difco), and 2% glucose (YPD) or 2% galactose (YPG). Minimal medium was composed of 1.67 g/l yeast nitrogen base without amino acids and ammonium sulfate (Difco), 2% glucose, or 2% galactose, and supplemented with 100 μM $(NH_4)_2SO_4$ and amino acids where appropriate. Acetate sporulation medium contained 1% potassium acetate and 2% agar. Liquid cultures were grown with vigorous agitation at 30°C or in a 200-l New Brunswick fermenter. Yeast genetic manipulations and molecular techniques were as described (Sambrook et al., 1989; Guthrie and Fink, 1991).

Concanavalin A-Sepharose, protein A-Sepharose CL-4B, bulk Superose 6, a prepackaged Superose 6 column, and standards for the calibration of gel filtration columns were all purchased from Pharmacia (Piscataway, NJ). Molecular biology enzymes including Taq

DNA polymerase, Klenow DNA polymerase, T4 DNA ligase, polynucleotide kinase, and restriction endonucleases were purchased from Boehringer Mannheim (Indianapolis, IN). All other reagents were purchased from Sigma (St. Louis, MO) except where a different vendor is indicated.

Protein assays were performed with 1–20 μl samples in a microtiter plate using protein assay reagent (Bio-Rad, Richmond, CA). Bovine serum albumin (BSA) was used as a protein standard. Gel electrophoresis was performed according to the Laemmli (1970) SDS-PAGE method using 10% or 7.5% acrylamide. Electrophoresed proteins were visualized by silver staining (Hicke et al., 1992). For immunoblotting, electrophoresed proteins were transferred to nitrocellulose filters (Schleicher & Schuell, Keene, NH) as previously described (Towbin et al., 1979). Filters were blocked and antibody incubations were performed with 2% nonfat dry milk in 20 mM Tris-HCl (pH 7.4) and 150 mM NaCl (TBS) with 0.05% Tween 20 (Bio-Rad). Detection of filter-bound antibodies was performed using the enhanced chemiluminescence detection system (Amersham, Arlington Heights, IL) or ^{125}I-labeled protein A (ICN, Irvine, CA; Harlow and Lane, 1988). Affinity-purified anti-Sec13p antibodies and anti-Sec31p antisera were used at a 1:1000 dilution (Salama et al., 1993).

Cloning of p150

To clone the gene encoding the 150-kDa subunit of the purified Sec13-dhfrp/p150 complex, the Sec13p complex was dissociated in 2 M urea. Briefly, 1 ml (0.7 mg) of the purified Sec13-dhfrp/p150 complex (Salama et al., 1993) was diluted 1:1 in 2× urea buffer (4 M urea, 0.4 M NaCl, 0.25 M Tris-HCl, pH 7.5). This material was loaded onto a 104-ml Superose 6 column equilibrated in urea buffer using a Pharmacia FPLC system. Fractions (2 ml) were collected and analyzed by SDS-PAGE. An aliquot (1 ml) of the peak fraction containing p150 (fraction 31) was adjusted to 5 mM dithiothreitol and heated to 65°C for 5 min. An aliquot (100 μl) of this material was removed for N-terminal sequence analysis and fractionated on 10% SDS-polyacrylamide gels followed by transfer to Immobilin-P polyvinylidene difluoride membranes (Millipore, Bedford, MA). The remainder was brought to 10 mM iodoacetimide and incubated for 20 min at 25°C. Dithiothreitol was added to 10 mM and the fraction was proteolyzed with 100 ng of LysC or LysC and trypsin (Boehringer Mannheim, Mannheim, Germany) in 100 mM NH₄(CO₃)₂ and purified by reverse-phase high-pressure liquid chromatography on a 300A C8 column (Vydac, Hesperia, CA). Peptides were sequenced by standard chemistry using fast cycles on a protein peptide 477A sequencer (2.1 × 150 mm, Applied Biosystems, Foster City, CA). A portion of the gene was amplified from genomic yeast DNA (a generous gift from David Feldheim, this laboratory) by polymerase chain reaction (PCR) in a Perkin Elmer-Cetus thermocycler using degenerate oligonucleotides N4 (5'-GT-NAARYTNGCNGARTTYTC-3') and 149C (5'-TCNGCNSWYTC-NGGRTTCCA-3') corresponding to the N-terminal peptide VKLAEFSRTA and the internal peptide DNTVLLWNPESAE-QLSQFP, respectively. The PCR product was gel-purified using Geneclean (BIO 101, Vista, CA), then end-labeled by reaction with polynucleotide kinase and [γ-^{32}P]ATP (3000 Ci/mmol, New England Nuclear, Boston, MA). The gene was cloned by screening a YEp24-based library (10-kb average insert) with the labeled PCR product using standard techniques (Ausubel et al., 1987). A single clone was identified that hybridized strongly to the probe in isolated plasmid DNA. The DNA sequence of the insert was determined using standard methods with Sequenase (United States Biochemical, Cleveland, OH) according to the manufacturer's instructions.

Disruption of p150

Two null alleles of p150 were generated in vitro by digestion of pNS3101 and pNS3102 with HindIII, giving a fragment with ap-

proximately 100 bp SEC31 sequences 5' and 600 bp 3' of the TRP1 gene (pNS3101) or the LEU2 gene (pNS3102). The fragments were gel purified using Geneclean (BIO 101) according to the manufacturer's instructions. The wild-type diploid yeast strain RSY612 was transformed with the HindIII fragment from pNS3101 and a sec31–1/SEC31 heterozygous diploid (RSY1004 × RSY256) was transformed with the HindIII fragment from pNS3102 using the lithium acetate method (Ausubel et al., 1987). Transformants were selected on minimal medium lacking tryptophan, yielding RSY1002, or lacking leucine, yielding JCY218. Transformants were induced to sporulate on acetate sporulation medium, and meiotic products were separated for phenotypic analysis by micromanipulation. To generate pNS3101 and pNS3102, pNS3132 was digested with KpnI and SalI which cut uniquely at bp 78 and 3522, respectively, in p150. The intervening coding sequence was replaced with either a KpnI-SalI TRP1 fragment from pJJ248 or a SmaI-SalI LEU2 fragment from pJJ250 (Jones and Prakash, 1990). pNS3132 was generated by subcloning a 5-kb HindIII fragment from the genomic clone containing p150 into the HindIII site of YEp352k. To construct YEp352k, the KpnI site in the polylinker of YEp352 (URA3, 2 μ; Hill et al., 1986) was inactivated by digesting it with KpnI, filling in the resulting 5' overhang using Klenow DNA polymerase and deoxyribonucleoside triphosphates and religating the newly generated blunt ends.

Pulse-Chase Analysis of Carboxypeptidase Y (CPY)

To monitor the secretion phenotype of RSY1076 (pHZ134 (ARS4, CEN1, GAL1:p150 LEU2) p150::TRP1), cells were grown to 0.2 OD$_{600}$ units/ml in minimal medium with 100 μM (NH₄)₂SO₄ and 2% galactose, and then collected and resuspended in minimal medium with 100 μM (NH₄)₂SO₄ and 2% glucose. To initiate labeling, we collected and resuspended cells in minimal medium with 2% glucose lacking sulfate 10 min before adding 30 μCi/OD$_{600}$ unit cells of ^{35}S express label mix (New England Nuclear). Cells were labeled for 5 min at 30°C, and then a 0.01 volume chase cocktail (0.3% cysteine, 0.4% methionine, 100 mM (NH₄)₂SO₄) was added. Labeling was terminated by addition of an equal volume of 20 mM NaN₃ and incubation for 5 min on ice. Cells were washed once with 0.5 ml of 20 mM NaN₃ and then resuspended in 200 μl of TBS with 0.1% Triton X-100 and 0.5 mM phenylmethylsulfonyl fluoride (PMSF). This material was transferred to an Eppendorf tube, SDS was added to 1% final concentration, and acid-washed glass beads (0.5 mm in diameter) were added to the meniscus. The cells were lysed by vortexing for 90 s at maximal speed, and the sample was heated to 95°C for 5 min. This material was transferred to a fresh Eppendorf tube and 800 μl of TBS and 0.5 mM PMSF were added. The resulting fraction contained 5 OD$_{600}$ units cell equivalent/ml and 1 × 10^8 cpm/ml. Nonspecific protein aggregates were removed by adding 25 μl of 10% IgGsorb (The Enzyme Center, Malden, MA) in 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄·7H₂O and 1.4 mM KH₂PO₄ (phosphate-buffered saline), mixing, and centrifuging for 2 min at 13,000 × g in a microcentrifuge (Tomy, Seiko, Tokyo, Japan). Approximately 1 × 10^7 cpm (100 μl) were mixed with 2 μl of anti-CPY antiserum (Stevens et al., 1982) and 25 μl of 10% protein A-Sepharose CL-4B in 1 ml of immunoprecipitation buffer (150 mM NaCl, 1% Triton X-100, 0.1% SDS, 15 mM Tris-HCl, pH 7.5, 2 mM NaN₃) for 8 h with rotation at 4°C. Immune complexes were collected by centrifugation for 30 s at 13,000 × g, washed twice with 1 ml of immunoprecipitation buffer, once with 1 ml of 2 M urea, 200 mM NaCl, 1% Triton X-100, 100 mM Tris-HCl (pH 7.5), and 2 mM NaN₃, once with 1 ml of 500 mM NaCl, 1% Triton X-100, 20 mM Tris-HCl (pH 7.5), and 2 mM NaN₃, and once with 1 ml of 50 mM NaCl, 10 mM Tris-HCl (pH 7.5), and 2 mM NaN₃. The washed precipitates were resuspended in 15 μl of 2× SDS-polyacrylamide gel sample buffer, heated for 5 min at 95°C, analyzed by SDS-PAGE, and visualized with a PhosphorImager (Molecular Dynamics, Menlo Park, CA).

Preparation of Temperature-shifted Cytosolic Extracts

RSY255 (*SEC31*) and RSY1004 (*sec31–1*) were grown to 1 OD_{600} unit of cells/ml with vigorous agitation. The cultures were transferred to 38°C for 1 h and then harvested by centrifugation for 10 min at 4000 × *g* in a GSA rotor (Sorvall, DuPont, Wilmington, DE). The cell pellet was washed once with B88 (20 mM N-2-hydroxyethylpipera-zine-N'-2-ethanesulfonic acid-KOH, pH 6.8, 0.15 M KOAc, 250 mM sorbitol, 5 mM MgOAc) and then resuspended at 1000 OD_{600} units cells/ml B88 with 0.5 mM PMSF and lysed by the addition of an equal volume of acid-washed glass beads (0.5 mm in diameter) and vortexing twice for 90 s at maximal speed. The resulting lysate was clarified by centrifugation for 5 min at 5000 rpm in a Tomy refrigerated microcentrifuge (Tomy, Seiko) and then for 30 min at 100,000 × *g* in a TLA100.3 rotor (Beckman Instruments, Palo Alto, CA).

Sequencing of sec31–1

Gap repair was used to map the location of the *sec31–1* mutation. pNS3132 (*SEC31, URA3*, 2 μ) was digested with *Kpn*I and *Spe*I, *Sal*I and *Spe*I, or *Xho*I to remove the *SEC31* sequences indicated in Figure 2. The linearized gapped plasmids were gel purified and transformed into RSY1004 (*sec31–1*) using the method of Elble (1992). Transformants selected on minimal medium lacking uracil were plated at 25°C and 38°C and checked for growth. Transformants missing sequences between the two *Xho*I sites were unable to grow at 38°C, whereas cells transformed with the other two gapped plasmids grew well at 38°C. This indicated that the mutation lay within a 297-bp region between the *Sal*I site and the carboxyl-terminal coding sequence. To sequence the mutation, genomic sequences were amplified by PCR from this region in both wild-type cells (RSY255) and *sec31–1* cells (RSY1004) using primers generated during the original sequencing of *SEC31*. Genomic DNA was prepared by growing 10 ml of yeast in minimal medium to saturation (approximately 15 OD_{600} units cells/ml). The cells were centrifuged for 5 min at maximum speed in a table-top centrifuge and resuspended in 0.5 ml of H_2O. The cells were lysed by vortexing at maximum speed for 4 min in 0.2 ml of 2% Triton X-100, 1% SDS, 0.1 M NaCl, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.2 ml phenol-chloroform (1:1), and 0.3 g of glass beads (0.5 mm in diameter). After the addition of 0.2 ml of 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA (TE), the aqueous phase was removed and precipitated with 1 ml of 100% ethanol. The precipitate was collected by centrifuging for 2 min at 10,000 × *g* in a refrigerated microcentrifuge. After decanting the ethanol solution, the pellet was dried and resuspended in 0.4 ml of TE and incubated for 5 min at 37°C with 30 μg of RNase to remove RNA. DNA was precipitated by the addition of 5.4 μl of 7.5 M $(NH_4)_2OAc$ and 1 ml of 100% ethanol and then centrifuged at 10,000 × *g* for 2 min. The resulting pellet was dried and resuspended in 50 μl of TE, and 1 μl was used for each PCR amplification. Amplification reactions also contained 0.2 mM deoxyribo-nucleoside triphosphate, 1 μM primers, 1 U *Taq* DNA polymerase, and 1× incubation buffer (Boehringer Mannheim). Primer A (5'-GTCCGTATACTAATAA-3') and primer 150-Z (5'-AGCATGGTTT-GTAGCGATA-3') were used to amplify a 499-bp fragment from position 3254–3753. Amplification products were sequenced with the *fmol*™ DNA sequencing system (Promega, Madison, WI) according to the manufacturer's instructions using primer 150-Z end-labeled with [γ-^{32}P]ATP (3000 Ci/mmol, New England Nuclear).

Phosphate Labeling of Sec31p In Vivo

RSY255 (1.25 OD_{600} units) cells were inoculated in 5 ml of low phosphate and sulfate medium (Reneke *et al.*, 1988) from a fresh overnight culture and grown to 1 OD_{600} unit cells/ml at 30°C. Cells were harvested in a clinical centrifuge for 5 min at maximum speed and resuspended in 2.5 ml of low phosphate and sulfate medium. After equilibration for 10 min at 30°C, 0.5 mCi of [^{32}P]inorganic

phosphate (8500–9120 Ci/mmol, New England Nuclear) was added. Labeling was terminated after 60 min by the addition of an equal volume (3 ml) of 20 mM NaN_3 and incubation for 5 min on ice. Cells were washed once with 5 ml of 20 mM NaN_3 and then resuspended in 200 μl of TBS with 0.1% Triton X-100 and 0.5 mM PMSF. This material was transferred to an Eppendorf tube, SDS was added to 1% final concentration, and acid-washed glass beads (0.5 mm in diameter) were added to the meniscus. The samples were lysed by vortexing for 90 s at maximum speed in a multivortexer (Tomy Seiko) and heated to 95°C for 5 min. This material was transferred to a fresh Eppendorf tube and 800 μl of TBS and 0.5 mM PMSF were added, resulting in a fraction containing 5 OD_{600} units cell equivalent/ml and 3 × 10^7 cpm/ml. Nonspecific protein aggregates were removed by adding 25 μl 10% of protein A-Sepharose CL-4B in B88 with 2% BSA, mixing, and centrifuging for 2 min at 13,000 × *g* in a microcentrifuge. Approximately 3 × 10^7 cpm (1 ml) were mixed with 2 μl of anti-Sec31p antiserum (Salama *et al.*, 1993), or 2 μl of preimmune serum, and 25 μl of 10% protein A-Sepharose CL-4B in B88 with 2% BSA followed by incubation for 8 h with rotation at 4°C. Immune complexes were collected as before.

Phosphoamino Acid Analysis of Sec31p

Phosphoamino acid analysis was performed on immunoprecipitated Sec31p. Sec31p was immunoprecipitated from RSY255 cells labeled with [^{32}P]inorganic phosphate as described above. Immune complexes were resolved by 7.5% SDS-polyacrylamide gels. A PhosphorImager cassette (Molecular Dynamics) was exposed for 2 h to the gel wrapped in Saran Wrap (Dow, Pittsburgh, CA) from which the top plate had been removed. Radiolabeled Sec31p was visualized with a PhosphorImager and cut out of the gel. The gel slice was hydrolyzed in 750 μl of 6 N HCl for 1 h at 110°C and removed to a fresh tube and lyophilized. Hydrolyzed amino acids were eluted from the tube with three washes of 100 μl of deionized H_2O (dH_2O) and combined with 3 μl of unlabeled phosphoamino acid standards (1 mg/ml each of phosphoserine, phosphothreonine, and phosphotyrosine). Amino acids were separated from residual acrylamide by binding to 1 ml of 1:2 (wt/vol) AG1x8 resin (Bio-Rad) in 3.5 ml of dH_2O adjusted to pH 7.5 with 200 mM NH_4OH. Binding was carried out overnight at room temperature with rotation. The resin was washed with 3× 0.5 ml of dH_2O, and the amino acids were eluted with 1.2 ml of 0.1 N HCl. The eluate was lyophilized and then resuspended in 3 μl of dH_2O. To resolve the phosphoamino acids, 2-dimensional thin layer electrophoresis on 20 cm × 20-cm cellulose plates was performed. The first dimension was run for 2 h at 1000 V in pH 1.9 buffer (7.8% acetic acid, 2.5% formic acid). After the cellulose plate dried completely, the second dimension was run for 45 min at 1000 V in pH 3.5 buffer (5% acetic acid, 0.5% pyridine). Phosphoamino acid standards were visualized by spraying the dried plate with 0.1% ninhydrin in 95% ethanol. The radiolabeled phosphoamino acids were visualized using a PhosphorImager.

Phosphatase Treatment

Sar1p (0.6 μg), Sec23p/Sec24p (1 μg), or Sec13p/Sec31p (1.4 μg) were treated with 1 μg of grade I alkaline phosphatase from calf intestine (Boehringer Mannheim) in 0.05 M Tris (pH 8.5) and 0.1 mM EDTA for 30 min at 37°C. The phosphatase reactions were stopped by the addition of 0.01 volume of 200 mM ethylenebis(oxy-ethylenenitrilo)tetraacetic acid (EGTA) and incubating for 5 min on ice. Budding reactions (25 μl) were performed using 5 μl of the phosphatase-treated fraction as described (Salama *et al.*, 1993), except 100 mM GMP-PMP replaced the ATP-regenerating system and GTP and each reaction contained 20 mM EGTA. To determine whether the inhibition caused by the addition of phosphatase could be rescued with untreated protein fractions, parallel reactions were supplemented with Sec13p/Sec31p (0.35 μg), Sar1p (0.15 μg), or Sec23p/Sec24p (0.5 μg).

RESULTS

Cloning of p150

We previously demonstrated that the functional form of Sec13p purifies as a heterodimer with the p150 protein (Salama *et al.*, 1993). To clone the gene encoding p150, we took advantage of the observation that the Sec13p/p150 complex can be dissociated with 2 M urea and the subunits resolved by size exclusion chromatography (Figure 1). Interestingly, under these conditions, p150 fractionates as a very large protein migrating ahead of thryoglobulin (669 kDa), at the same position of the native Sec13p complex (Salama *et al.*, 1993). When analyzed by sedimentation through glycerol gradients, the Sec13p complex fractionates with an S value of 7.8 corresponding to a 166-kDa protein as would be expected for a heterodimer containing one copy of each subunit. Thus, the aberrant migration of the Sec13p complex during gel filtration probably results from the p150 subunit having a highly elongated structure.

The ability to fully resolve p150 from Sec13p allowed peptide sequencing of the purified protein. Neither amino terminal nor internal peptides displayed homology to proteins known at the time. Consequently, degenerate oligonucleotide primers were designed based on the amino acid sequence of the amino terminal peptide and one of the internal peptides. PCR amplification of yeast genomic DNA using these primers yielded a single product encoding the amino terminus of p150. A yeast genomic library was screened with a probe made from this PCR product to isolate a full-length clone. Sequencing initiated from the flanking vector sequences revealed that the amino terminus of p150 fell within 100 bp of the vector insert junction. Sequencing in the 3' direction revealed an open read-
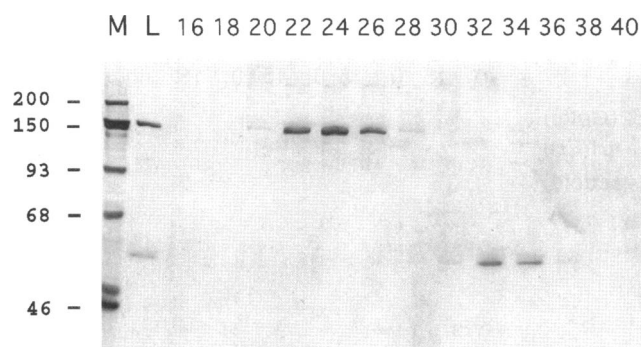
ing frame with 1273 codons encoding a protein with a predicted molecular weight of 139 kDa and a pI of 5.53. This open reading frame contains the nine peptides sequenced from the purified protein. Additionally, sequences in the 3' untranslated region show an exact match to the 5' untranslated region of the *SNF3* gene. *SNF3* and thus the gene for p150 map to the left arm of chromosome IV (Marshall-Carlson *et al.*, 1990). The sequence reveals no potential transmembrane segments or signal sequence. A homology search using the PROSITE dictionary of protein sites and patterns (release 10.3) of the GCG sequence analysis package (version 7.3, Genetics Computer Group, Inc., Madison, WI) revealed seven WD repeats near the amino terminus of the protein (Figure 2). The carboxyl-terminal half of the protein is rich in serine, threonine, and proline residues but displays no significant homologies. During the characterization of this clone, we discovered that the gene encoding p150 is identical to *WEB1*, a locus defined by a single mutation *web1–1* producing cells that require the viral protein E1A for growth in rich medium (Zieler *et al.*, 1995). The DNA sequence is available from GenBank accession number U15219 (Zieler *et al.*, 1995).

p150 Is Encoded by an Essential Gene Required for Secretion

To determine whether yeast requires p150 for viability, a null allele was constructed in vitro by replacing the majority of the coding region (from codons 26–1174) with the prototrophic marker *TRP1* or *LEU2* (Figure 2C). These alleles were integrated into one of the chromosomes in a diploid wild-type cell which was then sporulated. Dissection of four independent
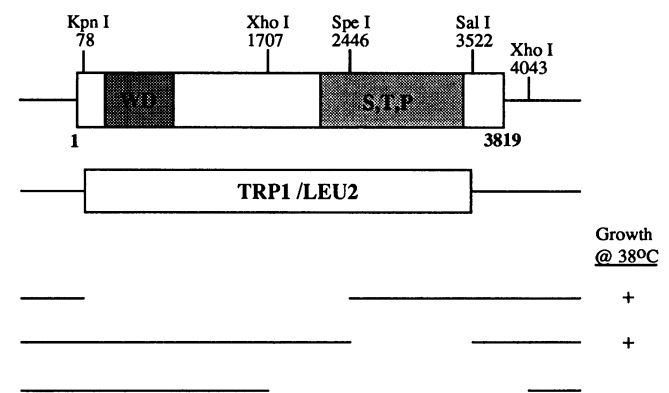

Figure 1. The Sec13p complex is sensitive to 2 M urea. Purified Sec13-dhfrp/p150 complex (0.7 mg, 1 ml) was diluted 1:1 in 2× urea buffer (4 M urea, 0.4 M NaCl, 0.25 M Tris-HCl, pH 7.5) to dissociate the Sec13p complex and loaded onto a 104-ml Superose 6 column equilibrated in 1× urea buffer. Fractions (2 ml) were collected and analyzed by SDS-PAGE followed by silver staining to visualize the proteins. The position of molecular weight markers (M) in kDa; the load (L) and fraction numbers are indicated.

Figure 2. Cloning of *p150*. Schematic representation of the *p150/SEC31* locus in pNS3132 and the growth phenotype of derivative plasmids at 38°C. The position of restriction sites used for the insertion of *TRP1* and *LEU2* sequences in the construction of a null allele and for mapping the *sec31–1* mutation are indicated. The WD-motif and the S,T,P-rich region are indicated (see text for details).

integrants yielded tetrads (12 each) displaying two living spores auxotrophic for leucine or tryptophan and two dead spores in all cases, showing p150 is required for cell viability.

To address the role of p150 in secretion, we placed the gene under control of the *GAL1* promoter. This promoter drives transcription when galactose is used as a carbon source and represses transcription when cells are grown on glucose-containing medium. The *GAL1:p150* gene on a plasmid containing the *LEU2* gene was introduced into diploid cells heterozygous for the *p150::TRP1* null allele. Transformants were sporulated and Leu⁺ Trp⁺ spores selected on galactose-containing minimal media. The cells were grown in galactose and then shifted to glucose-containing medium. Figure 3A shows that cells shifted to glucose stop growing after 8–10 h. Transport of the vacuolar protease CPY was monitored at 0, 2, 6, and 8 h after shifting to glucose. CPY is synthesized as a precursor that becomes core glycosylated upon entry into the ER, yielding the p1 form. Entry into the Golgi results in elaboration of the carbohydrate structure, causing a shift in electrophoretic mobility to the p2 form. When transported to the vacuole, pro-sequence cleavage occurs, yielding the mature (m) active protease. As shown in Figure 3B, at the 0- and 2-h time points pulse-labeled CPY matured at a normal rate. By 6 h after the shift to glucose, however, a secretion block developed and CPY accumulated in the ER as indicated by the persistence of the p1 form throughout the chase. This block to secretion preceded the cessation of cell division by 2–4 h. Ultrastructural analysis showed that the block to secretion caused by p150 depletion also led to an elaboration of ER membranes (Figure

3C). Exaggerated ER structures are characteristic of the class I ER to Golgi *sec* mutants that are defective for vesicle formation (Kaiser and Schekman, 1990).

p150 Is Allelic with SEC31

The *SEC31* gene was identified in a screen for new temperature-sensitive (ts) mutants defective in the processing of the yeast mating pheromone, α-factor (Wuestehube *et al.*, 1996). At the restrictive temperature of 38°C, *sec31–1* cells accumulate the ER precursor forms of several secretory proteins, extended ER membranes, and few if any small transport vesicles. These phenotypes suggest that the *SEC31* gene product functions during vesicle formation from the ER. A limited screen for low copy suppressors of the *sec31–1* ts growth defect led to the isolation of a single plasmid (named 32H1) that completely restored growth at 38°C. At the same time, we tested individually the ability of several known *SEC* genes, contained on low copy (CEN) plasmids, to suppress the *sec31–1* growth defect. Whereas *SEC24* showed weak suppression of the ts growth phenotype, a plasmid containing the gene encoding p150 suppressed *sec31–1* completely. Subsequent restriction analysis and DNA blot hybridization revealed that the *p150* gene and 32H1 overlap in sequence. Thus, at low doses, the *p150* gene suppressed the Ts⁻ growth and secretion phenotypes of *sec31–1*.

To test the identity of the *p150* gene and *SEC31*, the *p150::LEU2* null allele was integrated into a *sec31–1/SEC31* heterozygous diploid. Six Leu⁺ integrants had the expected genomic structure as determined by DNA blot hybridization analysis. The six integrants
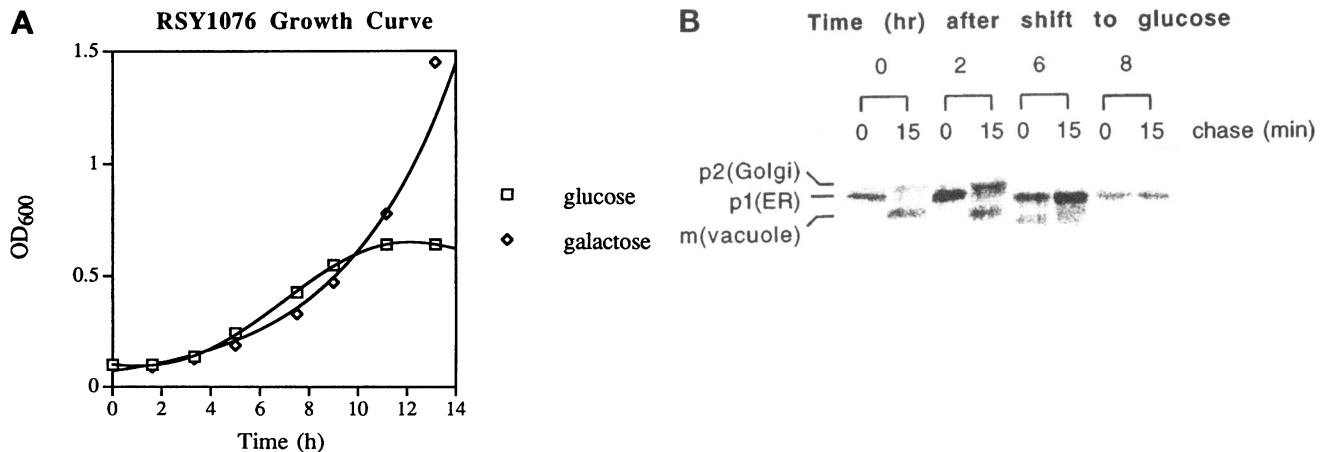

Figure 3. p150 depletion in vivo. (A) Growth curve of RSY1076 (pHZ134 (*GAL1:p150, LEU2, ARS1, CEN4*) *p150::TRP1*) grown in glucose- or galactose-containing media. (B) Secretion phenotype at 0, 2, 6, and 8 h after shift to glucose. Cells were pulse radiolabeled for 5 min followed by a 0- or 15-min chase and immunoprecipitated with anti-CPY antiserum. After pulse labeling, the ER form of CPY (p1) predominates. After a 15-min chase at the beginning of Sec31p depletion (0 h), some CPY is in the Golgi modified form (p2) while most has arrived to the vacuole where it is cleaved to the mature form (m). Cells begin to display a transport defect 6 h after shift to glucose. (C) Electron micrographs of *GAL1:p150* cells (RSY1076) at 0 and 6 h after shift to glucose. Bar, 1 μm.
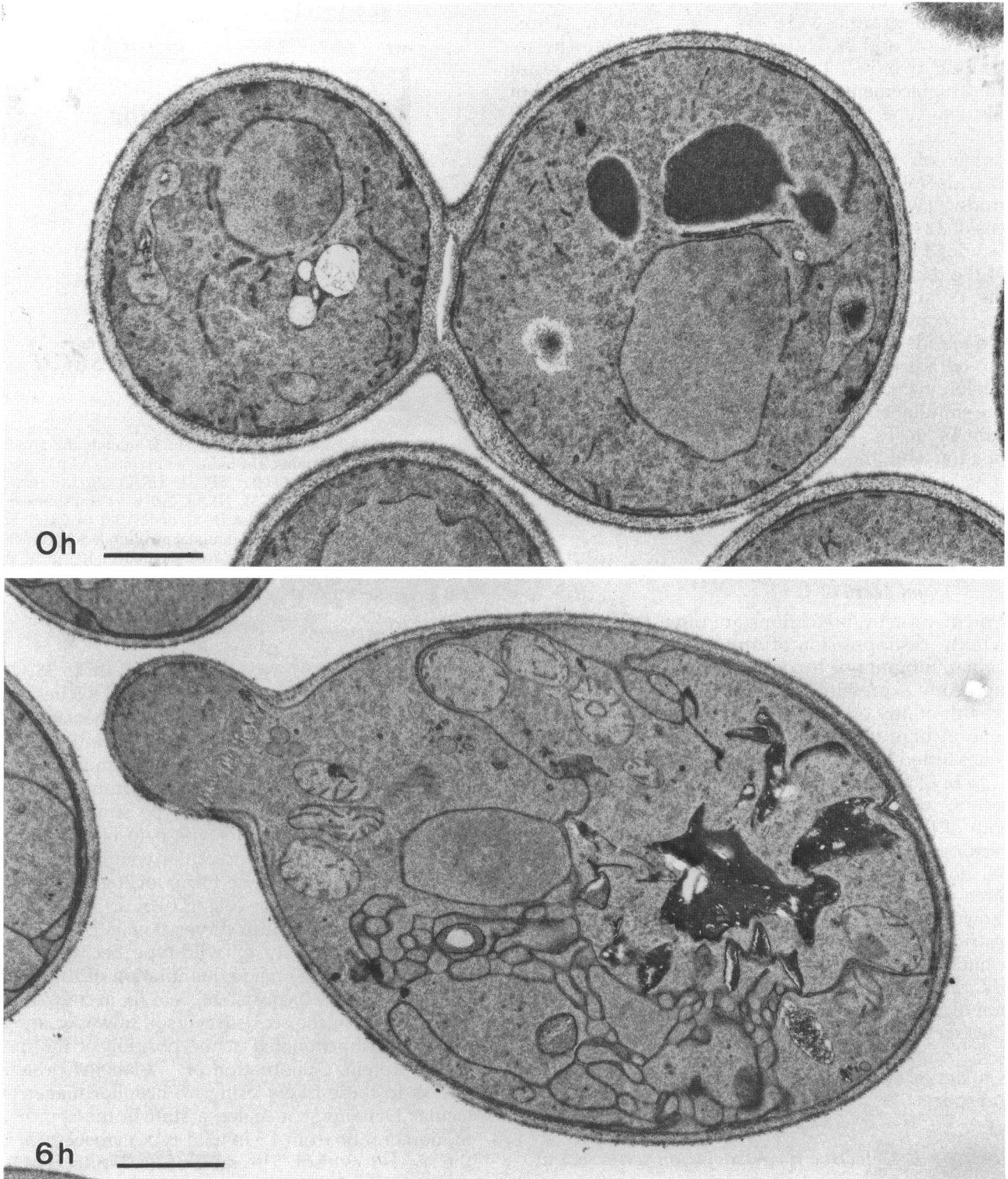
C

Oh

6h

Figure 3 (cont).

were tested for their ability to grow at 38°C. Four of these integrants were Ts⁻ for growth. Thus, p150::LEU2 and sec31–1 fail to complement. The remaining two Ts⁺ integrants are presumed to result from replacement of the sec31–1 sequence (instead of the wild-type SEC31 sequence) with the p150::LEU2 sequence.

One of the Ts⁻ diploids (relevant genotype, sec31–1/p150::LEU2) was sporulated, and the meiotic products were separated by micromanipulation. Analysis of 32 tetrads revealed that 29 segregated two Ts⁻: two dead spores and all of the Ts⁻ spores were Leu⁻. Of the remaining three tetrads, one tetrad segregated one Ts⁻:one Ts⁺: two dead; both viable spores from this recombinant tetrad were Leu⁻. The remaining two tetrads segregated three Ts⁻: one dead. These last two tetrads are presumed to be products of gene conversion events, since all three Ts⁻ spores were Leu⁻. The simultaneous conversions from Leu⁺ to Leu⁻ and from Ts⁺ to Ts⁻, in addition to the low level of recombination and the lack of complementation between p150::LEU2 and sec31–1, strongly suggest that the two mutations are allelic.

SEC31 Overexpression Is Synthetically Lethal with sec23–1 and sec16–2

Protein–protein interactions are often reflected in the pattern of suppression of mutations by plasmids encoding subunits of a complex. We checked whether high copy expression of SEC31 would suppress the ts growth of any other ER to Golgi mutants. The SEC31 gene in high copy did not suppress any of the SEC genes tested, but did lower the restrictive temperature of sec16–2 and sec23–1. The data in Figure 4 show that at 30°C, which is below the restrictive temperature of both of these mutations, cells containing the plasmid vector grew but cells containing the SEC31 gene insert did not. This toxicity is specific to these alleles since three other sec23 and two other sec16 alleles did not show growth inhibition at any temperature below the restrictive temperature. Interestingly, cells containing a high copy plasmid encoding both the SEC13 and SEC31 genes grew at this temperature. Overproduction of SEC13 alone was not toxic to these Ts⁻ strains. These results indicate that monomeric Sec31p, but not Sec31p complexed with Sec13p or monomeric Sec13p, is toxic to cells already compromised for ER to Golgi transport.

Sec31–1p Is Defective for Association with Sec13p

Sec13p functions in vesicle formation only in the form of a heterooligomeric complex with Sec31p (Pryer *et al.*, 1993; Salama *et al.*, 1993). This prompted us to determine whether Sec31–1p could engage Sec13p in such a complex. To test this, we prepared cytosol from
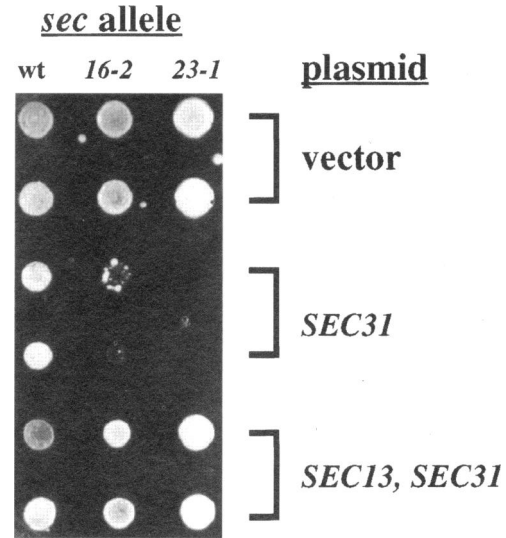

Figure 4. High copy expression of *SEC31* inhibits the growth of *sec16–2* and *sec23–1* strains. The indicated plasmids, YEp352 (vector: URA3, 2µ), pNS3132 (SEC31: SEC31, URA3, 2µ), or pNS3113 (SEC13, SEC31: SEC13, SEC31, URA3, 2µ) were transformed into RSY255 (wild type), RSY267 (*sec16–2*), or RSY281 (*sec23–1*). Single transformants were selected on minimal medium lacking uracil and restreaked. Single colonies were resuspended in 100 µl of sterile H₂O, replica plated onto a minimal medium plate lacking uracil, and grown for 3 d at 30°C.

wild-type and sec31–1 cells shifted for 1 h to 38°C, the nonpermissive temperature for sec31–1 (Wuestehube *et al.*, 1996). Complex formation was then measured using size exclusion chromatography. Figure 5 shows the profile of Sec13p and Sec31p immunoreactivity across a Superose 6 gel filtration column loaded with cytosol prepared from a wild-type strain (A) or the sec31–1 strain (B). In wild-type cytosol, Sec13p fractionated both in a high molecular weight form and in an apparently monomeric form, analogous to results previously reported for cells grown at 30°C, the normal growth temperature (Pryer *et al.*, 1993). Unfortunately, the instability of wild-type Sec31p in vitro (Salama *et al.*, 1993) made quantitation of this protein in these fractions inconsistent. Sec13p, in contrast, was quite stable. In the sec31–1 cytosol, however, most of the Sec13p fractionated at the position of the monomeric protein. Quantitation of ¹²⁵I-labeled protein A bound to these filters using a PhosphorImager (Molecular Dynamics) revealed a shift in the complex to monomer ratio from 4.5 in wild-type cytosol to 0.25 for the sec31–1 cytosol. The ratio of Sec31p to Sec13p in each of the load fractions was similar. Although we cannot exclude the possibility that Sec31–1p may be somewhat less stable than the wild-type protein, it appears that the major effect is a weaker interaction between the mutant protein and Sec13p.
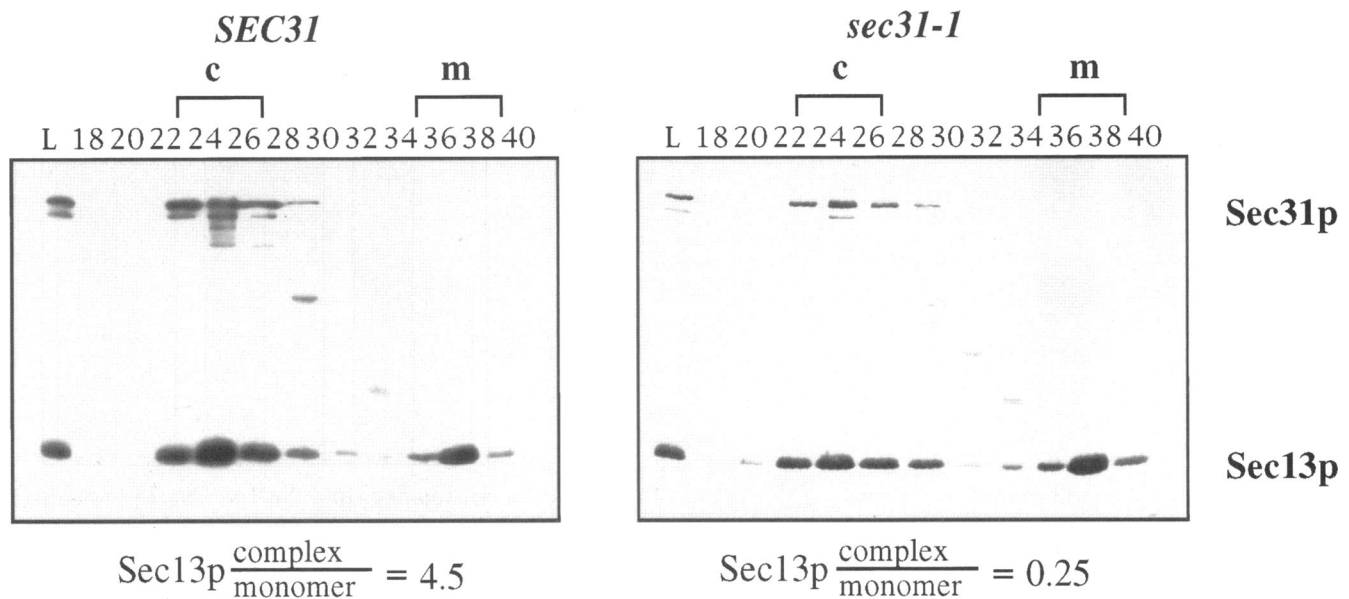
Figure 5. Sec31–1p is defective for association with Sec13p. An aliquot (200 μl) of wild-type (*SEC31*, 28 mg of protein/ml) or *sec31–1* (14 mg of protein/ml) temperature-shifted cytosol was loaded onto a 24-ml Superose 6 column equilibrated in B88 with 0.5 mM PMSF using a Pharmacia FPLC system. Fractions were collected (0.5 ml) and aliquots (10 μl) analyzed by SDS-PAGE followed by transfer to nitrocellulose. Immunoblots were probed with affinity-purified anti-Sec13p antibodies (1:1000) and developed with the enhanced chemiluminescence detection system (Amersham). The fraction numbers are indicated. c, position of the Sec13p complex; L, load fraction; m, position of Sec13p monomer. To determine the ratio of the Sec13p complex to monomer, the immunoblots were incubated with ^{125}I-labeled protein A and exposed to a PhosphorImager cassette (Molecular Dynamics). The amount of radioactivity present in arbitrary units in the complex fractions (22–26) was divided by that present in the monomer fractions (34–38).

We mapped the *sec31–1* mutation using gap repair (Orr-Weaver *et al.*, 1983). This technique relies on the ability of yeast to repair gapped plasmids with chromosomal sequences during transformation. If the gapped region does not include the mutation, repair will result in a plasmid containing a wild-type copy of the gene that can complement the ts growth phenotype. If the missing sequence includes the mutation, the repaired plasmid will also include the mutation and the resulting strain will not grow at the nonpermissive temperature. As shown in Figure 2, the *sec31–1* mutation maps to the 3′ coding region between the *Sal*I site and the end of the gene. Using PCR, we amplified this portion of the gene from genomic DNA prepared from *sec31–1* and wild-type haploid cells. Direct sequencing of the PCR products revealed a missense mutation from C to T at position 3716, changing valine 1239 to alanine. From these results we suggest that the C-terminus of Sec31p may be involved in contact with Sec13p to form the complex.

Sec31p Is a Phosphoprotein

When analyzing the protein sequence of Sec31p, we noted that the carboxyl-terminal half contains an unusually high occurrence of serine, threonine, and proline residues. These residues make up a PEST motif

found in a number of unstable proteins, and phosphorylation of these proteins may play a role in their degradation (Rogers *et al.*, 1986). We thus checked the stability of Sec31p by pulse chase analysis, but found it to be quite stable over the course of a 60-min chase. We then examined the phosphorylation of Sec31p. Cells were labeled with [32P]inorganic phosphate followed by immunoprecipitation with anti-Sec31p antiserum. The data in Figure 6 show that Sec31p was labeled with 32P; phosphoaminoacid analysis of the immunoprecipitated protein revealed that the phosphate occurs on serine residues. Phosphoaminoacid analysis of Sec31p immunoprecipitated from *sec31–1* or *sec13–1* cells yielded an identical pattern.

Phosphorylation of Sec31p Is Important for Vesicle Formation Activity

A biochemical reaction reconstituting vesicle formation from the ER in vitro requires three cytosolic proteins: Sar1p, Sec23/24p complex, and Sec13/31p complex (Salama *et al.*, 1993). We developed a means to test whether phosphorylation of Sec31p was important for vesicle formation. We treated each of the three fractions required to promote vesicle formation with phosphatase and then measured vesicle formation activity using mixtures containing one treated and two untreated proteins. As shown in Figure 7, neither
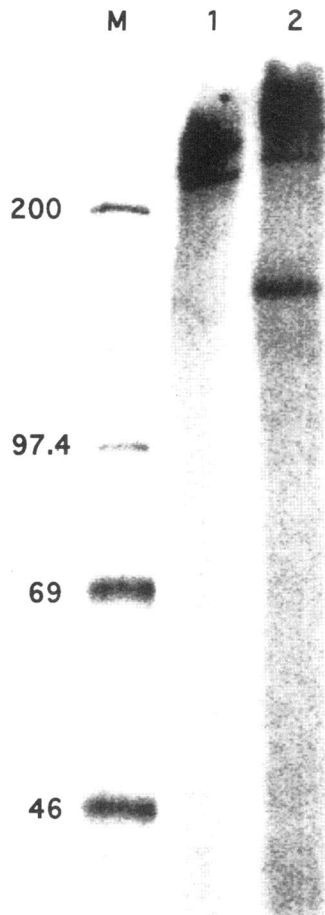
Figure 6. Sec31p is a phosphoprotein. Immunoprecipitations using anti-Sec31p antiserum (lane 2) or preimmune serum (lane 1) of cell lysates radiolabeled in vivo with [^{32}P]inorganic phosphate were analyzed by SDS-PAGE and visualized using a PhosphorImager (Molecular Dynamics). The position of molecular weight markers (lane M) in kDa is indicated.

Figure 7. Phosphatase treatment inhibits the ability of the Sec31p-containing fraction to promote vesicle formation. Sar1p (0.6 μg), Sec23p/Sec24p (1 μg), or Sec13p/Sec31p (1.4 μg) was treated with 1 μg of grade I alkaline phosphatase (Boehringer Mannheim) in 0.05 M Tris (pH 8.5) and 0.1 mM EDTA and incubated for 30 min at 37°C. The phosphatase reactions were stopped by the addition of 0.01 volume 200 mM EGTA and incubated for 5 min on ice. Budding reactions (25 μl) were performed using 5 μl of the phosphatase-treated fraction as described above, except 100 μM GMP-PNP replaced the ATP-regenerating system and GTP and each reaction contained 20 mM EGTA (hatched bars). To determine whether the inhibition caused by the addition of phosphatase could be overcome with untreated protein fractions, parallel reactions were supplemented with Sec13p/Sec31p (0.35 μg), Sar1p (0.15 μg), or Sec23p/Sec24p (0.5 μg; filled bars).

Sar1p nor the Sec23/24p complex was sensitive to phosphatase treatment. In contrast, phosphatase treatment of the Sec13/31p complex inhibited vesicle formation by 50%. The addition of untreated Sec13/31p complex overcame this inhibition. Phosphatase treatment of immunoprecipitated protein from radiolabeled yeast cells under these conditions resulted in removal of more than 90% of the phosphate from Sec31p and SDS-PAGE analysis of the Sec13/31p fraction after phosphatase treatment showed that neither protein was degraded. Because the Sec13/31p complex is essential for vesicle formation activity, we considered the possibility that phosphatase treatment disrupted the Sec13/31p complex. However, size exclusion chromatography showed that the Sec13/31p complex remained intact after phosphatase treatment. A direct comparison of radiolabeled and immunoprecipitated Sec13p and Sec31p showed phosphorus label only on Sec31p, thus we conclude that phosphorylation of this one subunit of the COPII coat is required for full activity in transport vesicle formation.
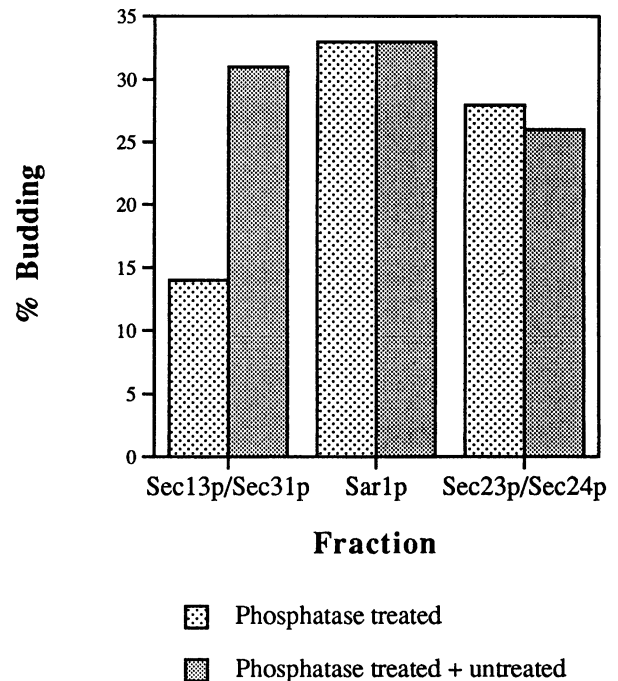
DISCUSSION

The COPII vesicle coat protein contains five protein subunits: Sar1p, Sec23p, Sec24p, Sec13p, and p150 (Barlowe *et al.*, 1994). Here, we report that the p150 protein is encoded by the *SEC31* gene which is essential for yeast cell viability. Sec31p/p150 depletion in vivo results in a secretion block between the ER and the Golgi and the elaboration of ER membranes. This phenotype is characteristic of class I ER to Golgi *sec* mutants (Kaiser and Schekman, 1990) which include *SEC13* and *SEC23*, two other subunits of COPII. *SEC31* was identified in a genetic screen for mutants accumulating unprocessed forms of the yeast secretory protein α-factor (Wuestehube *et al.*, 1996). Both the *sec31–1* mutant at the nonpermissive temperature and depletion of the wild-type protein cause disruption of ER to Golgi transport. In contrast to the depleted strain, Sec31–1 mutant protein persists in cells incu-

bated at the restrictive temperature. However, the mutant protein is defective in the recruitment of Sec13p in a heterooligomeric complex. Since monomeric Sec13p is unable to promote vesicle formation, the mutant defect may relate to the lack of Sec13/31p complex or the monomeric Sec31p may be toxic to cells. Additional insight into the phenotype of the *sec31–1* strain may come from the observation that overproduction of wild-type Sec31p, but not of the Sec13/31p complex or of Sec13p, lowers the restrictive temperature for two different ER to Golgi mutants, *sec23-1* and *sec16–2*. In these strains there appears to be a specific toxicity resulting from overexpression of monomeric Sec31p. Alternatively, these two proteins may be physically associated with Sec31p in vivo, and overexpression of Sec31p in mutant cells may alter the effective concentration of these crippled proteins. *SEC23* encodes another subunit of COPII, and Sec16p is found associated with ER-derived transport vesicles (Espenshrade *et al.*, 1995). *SEC16* encodes a 240-kDa cytosolic protein that is associated with Sec23p and is required for vesicle formation in vivo. Much of the Sec16p is tightly bound to the ER membrane, and it has not yet been possible to develop an assay to detect a requirement for this protein in the in vitro budding reaction. The sequence of Sec16p contains a large region predicted to form coiled coils consistent with a structural or scaffolding function, perhaps helping to organize the COPII coat.

The predicted amino acid sequence of Sec31p gives no obvious clues to its biochemical activity in vesicle formation. Sec31p contains seven WD repeats, a repeat motif also found in Sec13p. This sequence, originally characterized in the β subunit of heterotrimeric G proteins, does not have any known function. Proteins bearing WD repeats participate in a variety of cellular processes, including transcriptional activation, RNA splicing, and spindle pole body formation. Interestingly, COPI, the other coat protein that operates between the ER and the Golgi, also contains two subunits with WD repeats: α and β'. The α subunit (160 kDa) and the β' subunit (102 kDa) both encode large proteins with the WD repeats confined to the amino terminus as is the case for Sec31p. However, an amino-terminal deletion that removes the WD repeats of the α subunit partially complements the growth phenotype of a ts mutation in this gene (Letourneur *et al.*, 1994), thus this motif may serve a redundant function.

The WD repeats may play a role in the lateral organization of coat protomers in COPI and COPII vesicles. Interestingly, all three ts alleles of *SEC13* map to conserved residues of the WD repeats (Pryer, *et al.* 1993), but none of these strains accumulate monomeric Sec13p (Yoshihisa, unpublished results). The *sec31–1* mutation maps outside of these repeats, thus it seems likely that the WD repeats are not involved in protomer (coatomer or Sec13p/Sec31p) structure.

Coat subunits may bind directly to proteins designated for transport. Both the α and β' subunit, in combination with the ϵ subunit appear to interact directly with the di-lysine retention motif found in some ER resident transmembrane proteins (Cosson and Letourneur, 1994; Letourneur *et al.*, 1994). This has led to a model whereby COPI recruits escaped di-lysine bearing proteins into retrograde transport vesicles from the Golgi for delivery back to the ER. At this time it is not known how cargo molecules are recruited into COPII vesicles. Morphological analysis of secretory proteins in vivo has demonstrated that secretory cargo is concentrated in transport vesicles exiting the ER (Mizuno and Singer, 1993; Balch *et al.*, 1994). ER-derived transport vesicles formed using COPII in vitro exclude ER resident proteins and include vesicle resident proteins (Salama *et al.*, 1993; Barlowe *et al.*, 1994; Rexach *et al.*, 1994). The Sec13p/Sec31p complex may couple recruitment of cargo with vesicle formation, or it may serve a purely structural role in vesicle morphogenesis.

SEC31 was independently defined as a gene, *WEB1*, a mutant allele of which confers a dependence on the adenovirus transcription factor E1A for yeast cell growth (Zeiler *et al.*, 1995) The single mutant allele, *web1–1*, changes D189, a conserved residue in one of the WD repeats, to N. Cells harboring this mutation do not display a secretory defect when incubated at the nonpermissive temperature and the mutant protein associates with Sec13p normally. The connection between the E1A phenotype and vesicle budding from the ER is obscure.

We have shown that phosphorylation of serine residues on Sec31p promotes transport vesicle formation in vitro. Phosphorylation/dephosphorylation of Sec31p could provide a mechanism for regulation of membrane traffic. Unfortunately, no physiological or cell cycle control of protein transport from the ER is known in yeast. Alternatively, a kinase/phosphatase cycle could promote the assembly/disassembly of the coat structure during each round of vesicle budding. Although phosphorylation is not necessary to maintain the Sec13/31p complex, lateral association in a COPII coat could require a phosphorylated form of Sec31p. Conversely, a phosphatase located on the vesicle surface could be activated on completion of the budding cycle to dephosphorylate Sec31p and loosen the coat, allowing individual complexes to be shed from the membrane. This model predicts a role for nucleotide (most likely ATP) hydrolysis mediated by a kinase in vesicle budding; however, no such requirement is seen in the budding reaction reconstituted with purified proteins. Indeed, the only absolute nucleotide requirement is for GTP, and a nonhydrolyzable analogue (GMPPNP) suffices. If the putative kinase acts on cytosolic Sec31p, the pool of Sec13/31p isolated from a yeast lysate and used in a reconstituted

budding reaction may already be in the active phosphorylated state. In this circumstance, ATP and the kinase may not be necessary for a single round of vesicle budding. This requirement and the relevant kinase may be detected using unphosphorylated Sec13/31p complex.

ACKNOWLEDGMENTS

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