SED4 Encodes a Yeast Endoplasmic Reticulum Protein that Binds Sec16p and Participates in Vesicle Formation

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Abstract. SEC16 is required for transport vesicle budding from the ER in Saccharomyces cerevisiae, and encodes a large hydrophilic protein found on the ER membrane and as part of the coat of transport vesicles. In a screen to find functionally related genes, we isolated SED4 as a dosage-dependent suppressor of temperature-sensitive sec16 mutations. Sed4p is an integral ER membrane protein whose cytosolic domain binds to the COOH-terminal domain of Secl6p as shown by two-hybrid assay and coprecipitation. The interaction between Sed4p and Secl6p probably occurs before budding is complete, because Sed4p is not found in budded vesicles. Deletion of SED4 decreases the rate of ER to Golgi transport, and exacerbates mutations defective in vesicle formation, but not those that affect later steps in the secretory pathway. Thus, Sed4p is important, but not necessary, for vesicle formation at the ER.

Sec12p, a close homologue of Sed4p, also acts early in the assembly of transport vesicles. However, SEC12 performs a different function than SED4 since Sec12p does not bind Sec16p, and genetic tests show that SEC12 and SED4 are not functionally interchangeable.

The importance of Sed4p for vesicle formation is underlined by the isolation of a phenotypically silent mutation, sarl-5, that produces a strong ER to Golgi transport defect when combined with sed4 mutations. Extensive genetic interactions between SAR1, SED4, and SEC16 show close functional links between these proteins and imply that they might function together as a multisubunit complex on the ER membrane.

The transport of proteins between successive organelles of the secretory pathway is mediated by vesicle carriers that bud from the membrane of the donor compartment and then fuse with the membrane of the acceptor compartment (Palade, 1975). A general feature of vesicle formation is the recruitment of proteins from the cytoplasm to the membrane for assembly of a coat on the budding vesicle (Pearse and Robinson, 1990; Rothman and Orci, 1992). In Saccharomyces cerevisiae, seven proteins have been identified by genetic and biochemical methods that are required for vesicle budding from the ER (Novick et al., 1980; Nakano and Muramatsu, 1989; Kaiser and Schekman, 1990; Hicke et al., 1992; Salama et al., 1993). Five of these proteins (Sec13p, Sec31p, Sec23p, Sec24p, and Sarlp) when added in soluble form to ER membranes will drive vesicle budding (Salama et al., 1993; Barlowe et al., 1994). Under the appropriate conditions, the vesicles that form have a coat that contains all five proteins (Barlowe et al., 1994). We recently found that a sixth protein, Sec16p, is also a vesicle coat protein (Espenshade et al., 1995). However, Sec16p is unlikely to be recruited to the vesicle from the cytoplasm since there is no soluble cytoplasmic pool of Sec16p. Instead, Sec16p adheres tightly to the ER membrane and may form a peripheral membrane scaffold onto which cytosolic coat proteins assemble (Espenshade et al., 1995).

An important mechanistic problem is how coat assembly on the ER membrane is regulated so that vesicle formation occurs at the proper time and place. Sec12p is a potential early regulator of vesicle assembly because Sec12p resides in the ER membrane and is required for vesicle formation, but is not incorporated into the finished vesicle structure (Nakano et al., 1988; Rexach and Schekman, 1991; Barlowe et al., 1994). The cytosolic, NH2-terminal domain of Sec12p catalyzes exchange of GTP for GDP on the 21-kD GTPase Sarlp (Barlowe and Schekman, 1993). Overexpression of Sec12p increases the amount of Sarlp that can be bound to ER membranes in vitro, suggesting that Sec12p can recruit Sarlp to the ER membrane (d'Enfert et al., 1991b). Sarlp-GTP at the ER membrane is thought to then initiate assembly of coat proteins on the forming vesicle (Barlowe et al., 1994; Oka and Nakano, 1994).

S. cerevisiae has a second gene, SED4, that is closely related to SEC12. The NH2-terminal, cytosolic domain of Sec12p shares 45% amino acid identity with Sed4p, but the luminal domains of these proteins appear unrelated (Hard-
Molecular Biological Techniques were transformed with YEp24 library DNA. A total of 35,000 (CKY50) cation. Two temperature-sensitive (Ts) mutants. The YEp24 library (Carlson and Botstein, 1982) contains genomic inserts of sec16. Directed mutagenesis was performed using the protocol of Kunkel et al., 1987. PCR was carried out using Taq polymerase (Perkin-Elmer Cetus, Norwalk, CT). For their ability to confer temperature resistance to CKY50.

Plasmids were summarized in Table II. psi007 is a YEp24 library plasmid containing SED4. pRH26 is the 7.4-kb ApaI-Sall fragment of psi007 in pRS306-2a. pRH46 is the 5.2-kb Sall-Sall fragment of pRH26 in pRS306-2a. pRH107, pRH67, and pE187 are the 5.2-kb Sall-Sall fragment of pRH46 in pRS316, pRS306, and pRS305-2a, respectively. pRH121 and pRH120 are the 5.2-kb Sall-Sall fragments of pRH117 (see below) in pRS316 and pRS306-2a. Plasmids carrying truncations of SED4 were obtained as follows: pRH26 was cut with SpeI and the 11.6-kb fragment was self-ligated to make pRH47. pRH46 was cut with ApaI, the 5' overhang was filled in, and a 3.5-kb fragment was purified and cut with Sall-Apal to produce a 1.8-kb fragment that was ligated into Sall-SalI-cut pRS316-2a to make pRH54. pRH117 contains the 1.2-kb XhoI-Sall fragment of pRH46 ligated into XhoI-cut pRS306-2a. pRH78 contains the 1.2-kb XhoI-Sall fragment of pRH77 ligated into XhoI-Sall-cut pRH26. pRH46 was cut with HindIII and the 11-kb fragment was self-ligated to make pRH128. pRH62 was constructed using two PCR steps (Horton et al., 1989, Yan and Fried, 1989). PCR primers used were 5'-TTG TAA ATA AAG CCG TGC ACA TTG GTC TGG TTA TAG GAG AAC TGT AA-3' (nucleotides [nt] 1-124-1101, SED4, and nt 1-125-1104, SEC12, underlined), 5'-GGG ATT ACT TCT ATG GAT G-3' (nt 82-260, SEC12) and 5'-GAT GAA GAT GAA GAC GAC GCC-3' (nt 1924-1949, SED4), templates used were pSEC1230 (Nakano et al., 1988) and pRH46. The PCR product was cut with Sall-EcoRI and inserted into Sall-EcoRI cut pHRS0 (see below) to make pRH56. pRH62 is the 2.5-kb EcoRI fragment of pRH46 ligated into EcoRI-cut pRS316. pRH141 is the 2.6-kb EcoRI fragment of pRH117 (see below) ligated into EcoRI-cut pRH62. pRH148 is the 5.2-kb XhoI-Sall fragment of pRH141 in pRS316. pRH50 and pRH213 are the 3.5-kb XhoI-HindIII fragments of pSEC1230 in pRS306-2a and pRS316, respectively.

Epitope Tagging SED4 and SEC12 SED4 was tagged with the hemagglutinin (HA) epitope (Kolodziej and Young, 1991) as follows: a single copy of the HA epitope was inserted before the COOH-terminal HDEL sequence of SED4 using site-directed mutagenesis, resulting in SED4-HA1. The mutagenic oligonucleotides consisted of 27 nt encoding the HA epitope (underlined) flanked by 20 and 27 nt complementary to the SED4 sequence on the 5' and 3' end respectively (5'-CCG TAA ACT ACG CTC GGC TTC ACC CAT ACG ACC TCG CAG ACT ACG CTC ATG ACG ACC ATG TAT GAA TAT ACG GCC CAT GAC GAC TGG TGA ATA AGC-3'). A cassette containing three HA epitopes (Tyers et al., 1993) was then inserted into the newly created NotI site, creating pRH117. By DNA sequencing, pRH117 encodes SED4 containing seven tandem repeats of the HA epitope (SED4-HA).

Plasmid Constructions Plasmids are summarized in Table II. psi007 is a YEp24 library plasmid containing SED4. pRH26 is the 7.4-kb ApaI-Sall fragment of psi007 in pRS306-2a. pRH46 is the 5.2-kb Sall-Sall fragment of pRH26 in pRS306-2a. pRH107, pRH67, and pE187 are the 5.2-kb Sall-Sall fragment of pRH46 in pRS316, pRS306, and pRS305-2a, respectively. pRH121 and pRH120 are the 5.2-kb Sall-Sall fragments of pRH117 (see below) in pRS316 and pRS306-2a. Plasmids carrying truncations of SED4 were obtained as follows: pRH26 was cut with SpeI and the 11.6-kb fragment was self-ligated to make pRH47. pRH46 was cut with ApaI, the 5' overhang was filled in, and a 3.5-kb fragment was purified and cut with Sall-Apal to produce a 1.8-kb fragment that was ligated into Sall-SalI-cut pRS316-2a to make pRH54. pRH117 contains the 1.2-kb XhoI-Sall fragment of pRH46 ligated into XhoI-cut pRS306-2a. pRH78 contains the 1.2-kb XhoI-Sall fragment of pRH77 ligated into XhoI-Sall-cut pRH26. pRH46 was cut with HindIII and the 11-kb fragment was self-ligated to make pRH128. pRH62 was constructed using two PCR steps (Horton et al., 1989, Yan and Fried, 1989). PCR primers used were 5'-TTG TAA ATA AAG CCG TGC ACA TTG GTC TGG TTA TAG GAG AAC TGT AA-3' (nucleotides [nt] 1-124-1101, SED4, and nt 1-125-1104, SEC12, underlined), 5'-GGG ATT ACT TCT ATG GAT G-3' (nt 82-260, SEC12) and 5'-GAT GAA GAT GAA GAC GAC GCC-3' (nt 1924-1949, SED4), templates used were pSEC1230 (Nakano et al., 1988) and pRH46. The PCR product was cut with Sall-EcoRI and inserted into Sall-EcoRI cut pHRS0 (see below) to make pRH56. pRH62 is the 2.5-kb EcoRI fragment of pRH46 ligated into EcoRI-cut pRS316. pRH141 is the 2.6-kb EcoRI fragment of pRH117 (see below) ligated into EcoRI-cut pRH62. pRH148 is the 5.2-kb XhoI-Sall fragment of pRH141 in pRS316. pRH50 and pRH213 are the 3.5-kb XhoI-HindIII fragments of pSEC1230 in pRS306-2a and pRS316, respectively.

Materials and Methods Strains, Media, and Microbiological Techniques S. cerevisiae strains are listed in Table I. Yeast media (rich medium [YPD], minimal medium, and synthetic complete medium [SC]) were prepared, and yeast genetic and molecular biological techniques were performed using standard methods (Kaiser et al., 1994). Yeast transformations were carried out using the lithium acetate method (Gietz and Schiestl, 1991). Transformants were selected on SC medium lacking the appropriate auxotrophic supplement. All experiments on plasmid-bearing strains were performed on at least two independent transformants. To assay loss of URA3-marked plasmids, 10⁴ cells were plated on SC medium containing 0.1% 5-fluoroorotic acid (Boeke et al., 1984).

Molecular Biological Techniques DNA manipulations, subcloning, and Southern blotting were carried out using standard methods (Sambrook et al., 1989). DNA hybridizations were performed using the ECL nucleic acids detection system (Amersham Corp., Arlington Heights, IL). DNA sequencing was performed using the Sequenase kit (United States Biochemical Corp., Cleveland, OH). Site-directed mutagenesis was performed using the protocol ofunkel et al., 1987). PCR was carried out using Taq polymerase (Perkin-Elmer Cetus, Norwalk, CT).

Isolation and Analysis of Multicopy Suppressors of sec16 The YEpl4 library (Carlson and Botstein, 1982) contains genomic inserts in a vector carrying the S. cerevisiae URA3 gene and the 2μ origin of replication. Two temperature-sensitive (Ts) sec16 strains, CYK50 and CYK52, were transformed with YEpl4 library DNA. A total of 35,000 (CYK50) and 13,000 (CYK52) transformants at a density of 1.5 x 10⁴ to 1 x 10⁵ colonies per plate were replica plated and incubated at restrictive temperatures of 30, 33, and 36°C (CYK50) or 33 and 36°C (CYK52). Plasmids isolated from temperature-resistant colonies were tested for the ability to confer the temperature-resistant phenotype. A group of overlapping plasmids conferred growth up to 33°C (CYK50) and 36°C (CYK52) and represented the strongest suppressor locus. The corresponding gene was mapped to the right arm of chromosome III near the SED4 open reading frame by hybridizing an internal restriction fragment to a Southern blot of S. cerevisiae chromosomes (Clontech, Palo Alto, CA) and to a set of ordered yeast genomic clones (Riles et al., 1993). The suppressing gene was shown to be SED4 by subcloning fragments into pRS306-2a and testing for their ability to confer temperature resistance to CYK50.

1. Abbreviations used in this paper: aa, amino acid; CPY, carboxypeptidase Y; GST, glutathione-S-transferase; HA, hemagglutinin; HSP, high speed pellet; nt, nucleotide; SC, synthetic complete medium; Ts, temperature-sensitive; YPD, rich yeast medium.
amino acids at the COOH terminus. The NH2-terminal domain of SEC12 was similarly placed under control of the GALI0 promoter and tagged with the myc epitope. A 1-kb fragment encoding aa 1–354 of SEC12 plus a NotI site was amplified by PCR using pSEC1230 as a template and the following primers: 5'-AGT GAA TTC ACT ATG AAG TTC GTG ACA
-3' to +16, SEC12) and 5'-TGC GCT CGA GCT AGC GGC
-3' (nt -3 to +16, SEC12). The amplified fragment was cut with EcoRI-KpnI and ligated
into pRS313, creating pRH261. By DNA sequencing, pRH261
was similarly placed under control of the GAL10 promoter and tagged with the myc epitope. A 1-kb fragment encoding aa 1–354 of
SEC12 was cut with EcoRI-KpnI and ligated into pCD43, producing pRH186. The 3×myc cassette was ligated into
NotI-cut pRH186, the resulting product was cut with PvuI and inserted into PvuI-cut pRS313, creating pRH261. By DNA sequencing, pRH261 encodes a protein with three tandem repeats of the myc epitope plus 14 additional amino acids at the COOH terminus.

Construction of a SED4 Deletion Allele

A deletion of the entire SED4 open reading frame (sed4-D1) was constructed by site-directed mutagenesis. The mutagenic oligomer (5'-CTT

Table 1. Saccharomyces cerevisiae Strains

| Strain      | Genotype                                      | Source or Reference            |
|-------------|-----------------------------------------------|--------------------------------|
| CKY8        | MATa ura3-52 leu2-3, 112                      | Kaiser lab collection         |
| CKY10       | MATa ura3-52 leu2-3, 112                      | Kaiser lab collection         |
| CKY93       | MATa ura3-52 leu2 pep4::URA3                  | Kaiser lab collection         |
| CKY289      | MATa ura3-52 leu2 his3Δ200 trp1Δ63 lys-801 Gal+| Kaiser lab collection         |
| CKY249      | MATa/MATa SED4/sed4-D1::URA3 ura3-52/ura3-52  | This study                    |
| CKY250      | MATa/MATa SED4/sed4-D1 ura3-52/ura3-52        | This study                    |
| CKY251      | MATa sed4-D1 ura3-52 leu2-3, 112              | This study                    |
| CKY252      | MATa ura3-52 leu2-3, 112                      | This study                    |
| CKY255      | MATa sed4-D1::URA3 ura3-52 leu2-3, 112        | This study                    |
| CKY258      | MATa sed4-D1::URA3 ura3-52 leu2-3, 112        | This study                    |
| CKY291      | MATa ura3-52 leu2-3, 112                      | This study                    |
| CKY292      | MATa sed4-D1::URA3 ura3-52 leu2-3, 112        | This study                    |
| CKY293      | MATa sed4-D1::URA3 sar3-5 ura3-52 leu2-3, 112 | This study                    |
| CKY294      | MATa sar3-5 ura3-52 leu2-3, 112               | This study                    |
| CKY295      | MATa sed4-D1 SARI::URA3 ura3-52 leu2-3,112    | This study                    |
| CKY296      | MATa sed4-D1 SARI::URA3 ura3-52 leu2-3,112    | This study                    |
| CKY39       | MATa sec12-4 ura3-52 his4-619                 | Kaiser lab collection         |
| CKY45       | MATa sect1-1 ura3-52 his4-619                 | Kaiser lab collection         |
| CKY50       | MATa sect16-2 ura3-52 his4-619                | Kaiser lab collection         |
| CKY52       | MATa sect16-1 ura3-52 leu2-3, 112             | Kaiser lab collection         |
| CKY54       | MATa sect17-1 ura3-52 his4-619                | Kaiser lab collection         |
| CKY58       | MATa sect18-1 ura3-52 his4-619                | Kaiser lab collection         |
| CKY62       | MATa sect19-1 ura3-52 his4-619                | Kaiser lab collection         |
| CKY64       | MATa sect20-1 ura3-52 his4-619                | Kaiser lab collection         |
| CKY69       | MATa sect21-1 ura3-52 his4-619                | Kaiser lab collection         |
| CKY70       | MATa sec22-3 ura3-52 his4-619                 | Kaiser lab collection         |
| CKY78       | MATa sect23-1 ura3-52 his 4-619               | Kaiser lab collection         |
| CKY105      | MATa sect16-3 ura3-52 leu2-3, 112             | Kaiser lab collection         |
| CKY230      | MATa sect16-4 ura3-52 leu2-3, 112 ade2 ade3 sec13-1 | Kaiser lab collection   |
| NY768       | MATa sect-1 ura3-52 leu2-3, 112               | P. Novick (Yale University)   |
| NY770       | MATa sectc-41 ura3-52 leu2-3, 112             | P. Novick (Yale University)   |
| NY772       | MATa sectc-2 ura3-52 leu2-3, 112              | P. Novick (Yale University)   |
| NY774       | MATa sectc-8 ura3-52 leu2-3, 112              | P. Novick (Yale University)   |
| NY776       | MATa sectc-5-24 ura3-52 leu2-3, 112           | P. Novick (Yale University)   |
| NY778       | MATa sectc-5-4 ura3-52 leu2-3, 112            | Kaiser lab collection         |
| NY780       | MATa sectc-5-9 ura3-52 leu2-3, 112            | Kaiser lab collection         |
| NY782       | MATa sectc-9-4 ura3-52 leu2-3, 112            | Kaiser lab collection         |
| NY784       | MATa sectc-10-2 ura3-52 leu2-3, 112           | Kaiser lab collection         |
| NY786       | MATa sectc-15-1 ura3-52 leu2-3, 112           | Kaiser lab collection         |
| AFY72       | MATa sectc-7-1 ura3-1 his3-11 trp1-1           | R. Schekman (U.C. Berkeley)   |
| ANY123      | MATa bet1-1 ura3-52 his4-619                  | S. Ferro-Novick (Yale University) |
| ANY125      | MATa bet1-2 ura3-52 his4-619                  | S. Ferro-Novick (Yale University) |
| RSY533      | MATa sectc61-2 ura3-52 leu2-3, 112 ade2 pep4-3| R. Schekman (U.C. Berkeley)   |
| RSY530      | MATa sectc62 ura3-52 leu2-3, 112              | R. Schekman (U.C. Berkeley)   |
| RSY153      | MATa sectc63-1 ura3-52 leu2-3, 112            | R. Schekman (U.C. Berkeley)   |
| CKY234      | MATa sectc16-D1::TRP1 lys2-801 ade 2-101 trp1Δ63
|            | his3-Δ200 ura3-52 leu2-Δ1 (pPE5)              | d'Enfert et al., 1991a        |
| RSY656      | MATa/MATa SED4/sec12Δ::LEU2 ura3-3/ura3-3     | Kaiser lab collection         |
|            | leu2-3/leu2-3 trp1-1/trp1-1 ade2-1/ade2-1     | Kaiser lab collection         |
|            | his3-11/his3-11 can1-100/can1-100             | Kaiser lab collection         |
| EGY40       | MATa ura3-52 leu2 his3 trp1                 | Golemis and Brent, 1992       |
| PRY303      | MATa dpml::LEU2 leu2-3, 112 lys2-801 trp1Δ1 ura3-52 (pdpml-6) | Orlean, 1990                  |
Table II. Plasmids

| Plasmid     | Description                                                                 | Source or Reference |
|-------------|------------------------------------------------------------------------------|---------------------|
| pRS306      | integrating vector marked with URA3                                            | Sikorski and Hieter, 1989 |
| pRS316      | centromere vector marked with URA3                                             | Sikorski and Hieter, 1989 |
| pRS313      | centromere vector marked with HIS3                                             | Sikorski and Hieter, 1989 |
| pRS315      | centromere vector marked with LEU2                                             | Sikorski and Hieter, 1989 |
| pRS306-2μ   | 2μ vector marked with URA3 (pRS306 derivative)                                 | Miller and Fink, unpublished data |
| pRS305-2μ   | 2μ vector marked with LEU2 (pRS305 derivative)                                 | Miller and Fink, unpublished data |
| pRH46       | SED4 in pRS306-2μ                                                            | This study           |
| pRH107      | SED4 in pRS316                                                                 | This study           |
| pPE87       | SED4 in pRS305-2μ                                                            | This study           |
| pRH120      | SED4-HA in pRS306-2μ                                                         | This study           |
| pRH121      | SED4-HA in pRS316                                                            | This study           |
| pRH47       | SED4 (1-841) in pRH306-2μ                                                    | This study           |
| pRH54       | SED4 (1-369) in pRS306-2μ                                                    | This study           |
| pRH78       | SED4 (1-343) in pRS306-2μ                                                    | This study           |
| pRH128      | SED4 (1-294/331-1061) in pRS 306-2μ                                          | This study           |
| pRH62       | SEC12 (1-374) fused to SED4 (368-1065) in pRS306-2μ                          | This study           |
| pRH141      | SEC12 (1-374) fused to SED4-HA (368-1065) in pRS306-2μ                       | This study           |
| pRH148      | SEC12 (1-374) fused to SED4-HA (368-1065) in pRS316                          | This study           |
| pRH50       | SEC12 in pRS306-2μ                                                           | This study           |
| pRH213      | SEC12 in pRS316                                                                | This study           |
| pPE5        | SEC16 in YCP50                                                                 | Espenshade et al., 1995 |
| pPE8        | SEC16 in pRS315                                                                | Espenshade et al., 1995 |
| pKR1        | SEC12 in pRS316                                                                | Roberg and Kaiser, unpublished data |
| pCK1313     | SEC12 in YEp352                                                                | Pryer et al., 1993 |
| YCP1142     | SEC23 in YCP50                                                                 | Hicke and Schekman, 1989 |
| pRH259      | SARI in pRS316                                                                 | This study           |
| pRH262      | sarl-5 in pRS316                                                               | This study           |
| pRH279      | sarl-5 in pRS306-2μ                                                          | This study           |
| pRH280      | SARI in pRS306-2μ                                                             | This study           |
| pEG202      | lexA DNA binding domain in a 2μ vector marked with HIS3                       | Gyuris et al., 1993 |
| pG4-5       | acidic activation domain in a 2μ vector marked with TRP1                      | Gyuris et al., 1993 |
| pH318-34    | hisc gene under control of eight lexA DNA binding sites in a 2μ vector marked with URA3 | Gyuris et al., 1993 |
| pPE58       | SEC16 (1645-2194) in pEG202                                                   | Espenshade et al., 1995 |
| pPE59       | SEC16 (1-824) in pEG202                                                       | Espenshade et al., 1995 |
| pPE74       | SEC16 (447-1737) in pEG202                                                    | Espenshade et al., 1995 |
| pRH151      | SED4 (1-347) in pG4-5                                                        | This study           |
| pRH152      | SEC12 (1-354) in pG4-5                                                       | This study           |
| pRH260      | GAL10-promoted SED4-MYC (1-347) in pRH313                                      | This study           |
| pRH261      | GAL10-promoted SEC12-MYC (1-354) in pRS313                                    | This study           |
| pRD56       | GAL1-promoted GST in pRS316                                                   | R. Deshaies (California Institute of Technology) |
| pPE122      | GAL1-promoted GST-SEC16 (1638-2194) in pRS316                                 | This study           |

Numbers in parentheses indicate the amino acid numbers of the preceding gene’s product.

TTA AAC TTA GAA AAA CTA GCA TAA TAA TGG ATC CAA
GCT TGA ATA ACG AAA TAA TAT ATA TTA ATT TAA ATG
ATG-3') consisted of 32 nucleotides complementary to the 3' untranslated region of SED4. 10 nucleotides creating a HindIII and a BamHI site (underlined), and 36 nucleotides complementary to the 5' untranslated region of SED4. Mutagenesis of pRH67 deleted the entire SED4 reading frame as shown by restriction mapping. A 5-kb marker cassette (hisG-
URA3-Kan-hisG) (modification of Alani et al., 1987; kindly provided by S. Elledge, Baylor College of Medicine, Houston, TX) was inserted into the newly created BamHI site to make pRH37 (sed4-Δ1::URA3).

A diploid (CKY8 × CKY10) was transformed with a 6.9-kb, purified SacI-Sall fragment of pRH73. Tetrads analysis of Ura+ transformants gave 2:2 segregation of the URA3 marker by recombination of the hisG repeats was selected on SC medium containing 0.1% 5-fluoroorotic acid to produce sed4-Δ1.

Protein Extracts, Western Blotting, and Cell Fractionation

Yeast protein extracts were prepared from 2–6 × 107 exponentially growing cells as described (Rothblatt and Schekman, 1989). Cells were lysed in 30 μl ESB (60 mM Tris HCl, pH 6.8, 100 mM DTT, 2% SDS, 10% glycerol, 0.001% bromphenol blue) by vigorous agitation with 0.5-mm glass beads (Sigma Chemical Co., St. Louis, MO). Extracts were diluted with 70 μl of ESB and 10–20 μl were resolved by SDS-PAGE (Laemmli, 1970). Western blotting was performed using standard methods (Harlow and Lane, 1988). The following antibodies were used: anti-HA antibody (12CA5 ascitic fluid; BAbCO, Richmond, CA) at 1:10,000 dilution, anti-mycale antibody (9E10 ascitic fluid; K. Morrison, Harvard University, Boston, MA) at 1:1,000 dilution, rabbit anti-carboxy peptide Y (CPY) antibody (gift of R. Schekman) at 1:5,000 dilution, HRP-coupled sheep antibo–rabbit Ig (Amersham Corp.) at 1:10,000 dilution and HRP-coupled sheep anti-
mouse Ig (both Amersham Corp.) at 1:10,000 dilution. Blots were developed using the ECL system (Amersham Corp.). Cell fractionation was performed as described (Espenshade et al., 1995) using CKY295.

**Radiolabeling and Immunoprecipitations**

Cells were grown in selective SC medium supplemented with 2% glucose and then shifted to the indicated temperatures 2 h before labeling. Temperature, antibiotic treatment and temperature-shift experiments of the *dpr1-6* mutant were performed as described (Orlean et al., 1991). 2.6 × 10^9 exponentially growing cells (1-3 OD600 U) were radiolabeled in supplemented SD medium by incubating with 30 μCi [35S]methionine per OD600 U (Express protein labeling mix; New England Nuclear, Boston, MA), sp act 1,200 Ci/mmol. Samples were chased by the addition of 1:100 vol of a solution containing 3% (w/v) glucose, 0.1 M ammonium sulfate, 0.3% (w/v) cysteine, 0.4% (w/v) cysteine, and 0.1% (w/v) Scher's cells (Sigma Chemical Co.) and cleared by centrifugation at 12,000 g for 5 min. 0.5 μl anti-Cy5 or anti-HA antibody was added and extracts were rotated for 1 h at 25°C. Immune complexes were collected by adding 30 μl 50% protein A-Sepharose (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ) per sample and incubating for 1 h at 25°C. Protein A-Sepharose beads were washed twice with IP buffer and once with detergent-free IP buffer (50 mM Tris HCl, pH 7.4, 150 mM NaCl). Protein was released into 30 μl ESB by heating to 100°C for 2 min. 10 μl supernatant was separated by SDS-PAGE, visualized by fluorography (Harlow and Lane, 1988), and imaged on a phosphorImager (Molecular Dynamics, Sunnyvale, CA).

**Two-Hybrid Protein–Protein Interaction Assay**

Sec14p and Sec12p were tested for binding to Sec16p in vivo as described by Gryusz et al., 1993. The NH2-terminal domain of Sec4p or Sec12p was fused to the acidic activation domain in pG4-3 as follows. A fragment encoding aa 1-347 of Sec4p was amplified by PCR using pRHI66 as a template and the following primers: 5′-AGT GAA TTC ATG AAG TTC GTG ACA GCT AG-3′ (nt 1-20, SEC4) and 5′-TGC GCT CGA GCT ATT TAG AGA TTT T'T' GTT TCA 'TTG AAG-G′ (nt 1062-1037, SEC12). Segments were cut with EcoRI–Salt (SED4) or EcoRI–Xhol (SEC12) and ligated into EcoRI–Xhol-cut pG4-5 to make pRHI54 and pRHI55, respectively. pPE85, 59, and 74 are plasmids encoding aa 1645-2194, 1a-824, and aa 447-1737 of Sed4p or Sec12p, respectively. 5′ and 3′ of CTA TTT CCA AAT ATT TCG TAA AAT TGA TG-3′ (nt 1187-1165, SEC33) and 5′-AGT GAA TTC ATA ATG AGT GGC AAC TCT GC-3′ (nt -3 to +17, SED33) were amplified similarly using pSEC1230 as a template and the following primers: 5′-AGT GAA T'EC ATG AGT GGC AAC TCT GC-3′ (nt 1038-1016, SED33) and 5′-TG CTA GCT GCA GTA ATT TAG AGA TTT GT TGT TCA 'TTG AAG-G′ (nt 1002-977, SEC33).

**Immunofluorescence**

Indirect immunofluorescence was performed essentially as described by Pringle et al., 1991. Cells were fixed by adding formaldehyde (final concentration of 3.7%) to the medium and incubating for 2 h at 25°C. Fixed cells suspended in 0.1 M potassium phosphate (pH 7.2) were spheroplasted with 50 U lyticase for 30 min at 37°C. Incubations in primary or secondary antibody were for 1 h and were performed in a humid chamber at 25°C. The antibodies and concentrations used were: 12CA5 at a 1:5,000 dilution, anti-BIP polyclonal antisera (kind gift of M. Rose, Princeton University, NJ) at 1:1,000 dilution, FITC-coupled goat anti–rabbit IgG and rhodamine-coupled goat anti–mouse IgG (both Boehringer Mannheim Biochemicals) at 1:300 dilution. Cells were mounted in medium containing 4′,6-diamidino-2-phenylindole and p-phenylenediamine IMAGES were recorded on an axiostar (Carl Zeiss, Thornwood, NY) using film (T-Max 400; Eastman Kodak Co., Rochester, NY) developed according to the manufacturer’s specifications.

**Electron Microscopy**

Electron microscopy was performed as described in Kaiser and Schekman, 1990. Wild-type (CKY291), sed4-1 slr1-5 (CKY293), and sec17-1 (CKY54) cells were grown to exponential phase in YPD at 24°C and shifted to 38°C for 2 h before fixation. Cells were fixed for electron microscopy with potassium permanganate. To count vesicles, random well-stained sections were selected, photographed at a magnification of 13,000, and vesicles seen on the negative were counted. Vesicle counts were normalized to cell volume by measuring the area of the cell section and assuming a section thickness of 90 nm. 29 and 33 cell sections were counted for the sed4-1 slr1-5 and the sec17-1 strain, respectively. Data are expressed as mean ± SEM.

**In Vitro Vesicle Synthesis**

Membranes and cytosol used in the vesicle synthesis reaction were prepared as previously described (Wuestehube and Schekman, 1992) using CKY293 as a source of membranes and CKY93 as a source of cytosol. Standard vesicle synthesis reactions of 500 μl contained 100 μg of membranes, 1.2 mg of cytosol prepared in the absence of added guanine nucleotide, 1 mM GDP-mannose, 0.1 mM guanine nucleotide, and an ATP regeneration system in reaction buffer (20 mM Hepes-KOH pH 6.8, 130 mM KOAc, 5 mM MgOAc, 250 mM sorbitol) with protease inhibitors (1 mM PMSE, 0.5 μg/μl leupeptin, 1 μM pepstatin). The reaction with apyrase added contained 10 U/ml of apyrase in the place of the ATP regeneration system. Reactions were incubated at 20°C for 2 h. Donor membranes were removed by centrifugation at 32,000 rpm for 10 min at 4°C in a rotor (TLA100.3; Beckman Instruments, Inc., Fullerton, CA). Vesicles were pelleted from this medium speed supernatant by centrifugation at 60,000 rpm for 30 min at 4°C in a TLA100.3 rotor. Vesicle pellets solubilized in 30 μl ESB were proteins were analyzed by Western blotting.

Vesicles formed in vitro were freed of cytosol by gel filtration on a 14 ml (18 cm) Sephacryl S-1000 column (Sigma Chemical Co.) equilibrated in reaction buffer as described (Barlowe et al., 1994). A 0.7-ml sample of medium speed supernatant from two 0.5-ml reactions was applied to the column, eluted in reaction buffer, and 0.75-ml fractions were collected. Vesicles in each fraction were concentrated by centrifugation at 60,000 rpm for 30 min at 4°C in a TLA100.3 rotor. Proteins solubilized in 30 μl ESB were analyzed by Western blotting.
Cloning and Sequence Determination of sar1-5

sar1-5 was cloned by gap repair as follows: pRH259 contains the SARI gene on a 1.3-kb HindIII-Sall fragment of pSEC1210 (Nakano and Mura- matsu, 1989) inserted into pRS316 with a deletion from the EcoRI to the NotI sites of the polylinker. A sarl-5 sed4-ΔI double mutant was transformed with plasmid pRH259 cut with EcoRI and BamHI to produce a gap covering the SARI coding sequence. Gap-repaired plasmids carrying the mutation (pRH262) were identified by their inability to suppress the temperature sensitivity of secl6-2 and sec23-1 strains. The mutational change in a plasmid carrying sarl-5 was identified by sequencing with synthetic oligonucleotide primers. SARI and sar1-5 were placed on a 2µ plasmid by inserting the 1-kb EcoRI-HindIII fragment of pRH259 and pRH262 into pRS06-2µ, creating pRH280 and pRH279.

Results

SED4 Is a Multicopy Suppressor of sec16 Mutations

sec16-2 mutants fail to grow at temperatures above 30°C (Fig. 1, column 1). To identify genes that interact with SEC16, we screened a S. cerevisiae genomic DNA library in a multicopy (2µ) vector for plasmids that permit Ts sec16 mutants to grow at restrictive temperatures. One set of overlapping plasmids was recovered that suppressed the growth defect of sec16-2 cells up to 36°C (Fig. 1, column 2).

Cloning and sequencing identified the suppressing loci as SED4.

SED4 overexpression partially suppressed the Ts growth defect of all four sec16 alleles (not shown). However, SED4 overexpression could not bypass a sec16 null allele (sec16-ΔI::TRP1) as demonstrated in the following plasmid shuffling experiment. CKY234 carries a chromosomal sec16-ΔI::TRP1 allele and a URA3-marked plasmid containing SEC16. CKY234 transformed with SED4 on a 2µ, LEU2-marked plasmid (pPE87) could not grow without the URA3-marked SEC16 plasmid, whereas CKY234 transformed with SEC16 on a LEU2-marked plasmid (pPE87) could grow without the URA3-marked plasmid.

We examined the ability of SED4 overexpression to suppress Ts mutations in other secretion genes. The sec and bet mutants listed in Table I were transformed with either SED4 on a multicopy plasmid (pRH46) or the 2µ vector alone, and tested for growth at 30, 33, 36, and 38°C. Overexpression of SED4 partially suppressed the growth defect of sec4-1 at 33°C, but had no effect on the growth defect of any of the other mutants tested. Importantly, overexpression of SED4 did not suppress the growth defect of a strain carrying a mutation in SEC12, the gene most like SED4.

The ability of SED4 overexpression to suppress the secretion defect of sec16-2 was examined by following the maturation of the vacuolar enzyme CPY. Covalent modifications of CPY in the ER, the Golgi complex, and the vacuole mark the early events in the secretory pathway (Stevens et al., 1982). Strains were grown at 32°C for 2 h, labeled for 5 min, and then chased. In a sec16-2 strain, none of the ER form (p1) of CPY was converted into the mature, vacuolar form (m) even after 30 min (Fig. 2, lanes 1–5), whereas the same strain containing SED4 on a 2µ plasmid allowed maturation of CPY (Fig. 2, lanes 6–10).

Thus, the suppression of the secretion defect of sec16-2 by SED4 parallels the suppression of the growth defect.

Conserved NH2-terminal and Transmembrane Domains of Sed4p Confer Suppression of the Growth Defect of sec16-2 Strains

To identify the portion of SED4 required for suppression of sec16-2 truncations of SED4 were tested. In comparison to SEC12, SED4 is comprised of an NH2-terminal cytosolic domain, a transmembrane domain, and a COOH-terminal luminal domain. SED4 alleles with either a partial (sed4-T1) or a complete (sed4-T2) deletion of the COOH-terminal domain suppressed sec16-2 almost as well as wild-type SED4, demonstrating that this domain is not necessary for suppression (Fig. 3). The NH2-terminal and transmembrane domains of Sed4p were required for suppression, since deletion of a 39-amino acid segment of the NH2-terminal domain (sed4-T4) or deletion of the transmembrane domain (sed4-T3) completely abolished the ability to suppress sec16-2 (Fig. 3). To demonstrate that sed4-T4 is expressed at levels similar to SED4, we constructed an epitope-tagged allele, sed4-T4-HA, that contains the HA epitope at the same position as SED4-HA (see below). By Western blotting, the levels of Sed4p-T4-HA were identical to Sed4p-HA (not shown).

The function of the COOH-terminal luminal domain of Sed4p was further examined by fusing it to the NH2-terminal and transmembrane domains of Sec12p and testing this chimera for suppression of either sec16-2 or sec12-4. The chimera behaved like wild-type SEC12: SEC12-SED4 on either a low or a high copy plasmid did not suppress sec16-2, but complemented both sec12-4 and a chromosomal deletion of sec12 (Fig. 3, and data not shown). These results

Figure 1. SED4 overexpression suppresses the temperature sensitivity of sec16-2. Lanes 1 and 2: A sec16-2 strain (CKY50) containing vector (pRS06-2µ) or SED4 on a 2µ plasmid (pRH46). Lanes 3 and 4: A wild-type strain (CKY8) containing vector (pRS06-2µ) or SED4 on a 2µ plasmid (pRH46). Cells were spotted on selective medium and incubated at the indicated temperatures for 40 h.

Figure 2. SED4 overexpression suppresses the temperature-sensitive ER to Golgi transport defect of sec16-2. Lanes 1–5: A sec16-2 strain (CKY50) carrying vector (pRS06-2µ). Lanes 6–10: A sec16-2 strain carrying SED4 on a 2µ plasmid (pRH46). Cells were grown in selective medium at 25°C, shifted to 32°C for 2 h, and pulse-labeled with [35S]methionine for 5 min. The label was chased for the times indicated. CPY was immunoprecipitated from extracts, resolved by SDS-PAGE, and imaged on a phosphorImager.

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Figure 3. The NH$_2$-terminal domain of $SED4$ is necessary and sufficient for suppression of sec16-2. The indicated 2µ plasmids were transformed into CKY50 (sec16-2), CKY251 (sed4-Δ1), CKY39 (sec12-4), and RSY66 (SEC12/sec12Δ). Growth of single colonies assayed on selective medium at 33°C (sec16-2, sec12-4) or on rich medium at 41°C (sed4-Δ1) is shown. + + + indicates growth comparable to wild type, – indicates no growth. Complementation/suppression of sec12-4 and sec16-2 strains was also tested at 30° and 36°C with similar results. Suppression of sec12Δ was assayed by sporulating heterozygous sec12Δ diploids carrying test plasmids, dissecting 10–20 tetrads, and scoring Leu$^+$ (sec12Δ) spores. + + + indicates that Leu$^+$ spores were readily obtained, – indicates that no Leu$^+$ spores were obtained.

Figure 4. (A) Immunodetection of Sed4p-HA and comparison of the protein levels of Sed4p-HA and Sec12-Sed4p-HA fusion proteins. The SEC12-SED4 fusion construct is described in Fig. 3. Wild-type cells (CKY10) carrying the indicated plasmids (pRH121, pRH120, pRH148, pRH141, and pRH46; lanes 1–5, respectively) were grown in selective medium. Extracts of 0.2 ODr00 U of cells were resolved by SDS-PAGE on 6% gels and HA-tagged proteins were detected by Western blotting. (B) Sed4p-HA is an O-glycosylated protein. Sed4p-HA was immunoprecipitated from extracts from either wild-type (CKY10) (lanes 1, 2, and 5) or $dpm1-6$ (PRY303) (lanes 3 and 4) strains carrying $SED4$-HA on a 2µ plasmid (pRH120). Cells were grown in minimal medium, shifted to the indicated temperatures for 15 min, and radiolabeled with [35S]methionine for 15 min. Tunicamycin was added to 10 µg/ml 5 min before labeling (lane 5). HA-tagged proteins were immunoprecipitated from extracts from 1 OD$_{600}$ U of cells and labeled proteins were visualized by fluorography after SDS-PAGE on a 6% gel.

demonstrate that the function of $SED4$ is specified by its conserved NH$_2$-terminal and transmembrane domains.

Sed4p is an O-glycosylated ER Membrane Protein

Sed4p was epitope tagged by inserting seven copies of the HA epitope before the COOH-terminal HDEL sequence (see Materials and Methods). Epitope-tagged $SED4$ appeared to be functional since $SED4$-HA suppressed sec16-2 to the same extent as untagged $SED4$ (not shown). Immunoblots probed with anti-HA antibodies detected a protein that migrated more slowly than a 190-kD molecular mass standard (Fig. 4 A, lane 1). As expected for Sed4p-HA, this band was more abundant in a strain overexpressing Sed4p-HA (Fig. 4 A, lane 2) and was not present in a strain expressing untagged $SED4$ (Fig. 4 A, lane 5).

An epitope-tagged Sec12-Sed4p fusion protein (Fig. 3) was useful for estimating the abundance of Sec12p relative to Sed4p. Since both Sed4p-HA and the chimera were tagged at the same position and migrated similarly on SDS-PAGE, they were likely to be detected with equal efficiency by Western blotting. Sed4p-HA expressed from either a high or a low copy vector was 5–10 times more abundant than Sec12-Sed4p-HA, expressed from the same vector (Fig. 4 A, compare lanes 1 and 3 and lanes 2 and 4).

The difference between the observed molecular mass of Sed4p-HA (>190 kD) and the molecular mass predicted from the amino acid sequence (117 kD) prompted us to examine possible modifications of Sed4p. The COOH-terminal, luminal domain of Sed4p contains three potential N-linked glycosylation sites and is rich in serine and threonine residues that could accept O-linked glycosylation. Unglycosylated Sed4p-HA was produced in PRY303, a strain that carries a Ts mutation in dolichol phosphomannose synthase, an enzyme required for both N- and O-linked glycosylation (Orlean, 1990). Sed4p-HA, immunoprecipitated from PRY303 cells labeled at the restrictive temperature, migrated more rapidly than Sed4p-HA expressed in wild-type cells (Fig. 4 B, lane 4), indicating that Sed4p-HA is a glycoprotein. Treatment of cells with tunicamycin, an inhibitor of N-linked glycosylation, or treatment of extracts with Endo H to remove N-linked carbohydrate chains had no effect on the electrophoretic mobility of Sed4p-HA (Fig. 4 B, lane 5; and data not shown), indicating that Sed4p-HA is modified primarily by O-glycosylation. The discrepancy between the migration of Sed4p-HA without carbohydrate modifications (190 kD) with that predicted from the amino acid sequence (117 kD) is probably due to anomalous migration on SDS-PAGE since Sed4p-HA expressed in bacterial cells also migrated at 190 kD (not shown).
Sed4p-HA behaved as an ER membrane protein on cell fractionation. A large fraction of Sed4p-HA in a cell lysate pelleted at 500 g and the remainder pelleted at 10,000 g (not shown). As expected for an integral membrane protein, Sed4p-HA was partially solubilized from the 10,000-g pellet by treatment with 1% Triton-X 100, but was not released by treatment with 2.5 M urea, 0.5 M NaCl, or sodium carbonate (pH 11) (not shown).

The intracellular location of Sed4p-HA was examined further by indirect immunofluorescence. Fig. 5 (top) shows diploid cells expressing SED4-HA from a high copy plasmid stained with anti-HA antibody. The staining was chiefly at the nuclear periphery with extensions into the cytoplasm and around the periphery of the cell body. This pattern is typical for proteins located in the ER (Rose et al., 1989). The anti-HA staining pattern was identical to the anti-BiP staining observed in a double-labeling experiment (Fig. 5, bottom), indicating that Sed4p-HA is distributed throughout the ER. A similar, though weaker, staining was seen for Sed4p-HA expressed from a low copy plasmid, while no staining was apparent in a control strain transformed with untagged SED4 (not shown).

**Sed4p Is Excluded from ER Vesicles Produced In Vitro**

To investigate whether Sed4p is present on vesicles that have budded from the ER, we followed the fate of Sed4p-HA in a cell-free ER budding reaction (Wuestehube and Schekman, 1992). ER membranes were isolated from a sed4Δ deletion strain expressing Sed4p-HA from a low copy plasmid. Vesicles were produced by incubating these membranes with a guanine nucleotide and cytosol. Vesicles that had formed in vitro were isolated by first removing the donor membranes by centrifugation at medium speed and then pelleting the vesicles at high speed. Sec22p was used as a vesicle marker protein and ~10% of Sec22p was recovered in the high speed pellet (HSP) after incubation of donor membranes at 30°C with GTP and cytosol (Fig. 6). Incubation at 4°C, in the presence of apyrase, or in the absence of cytosol decreased the amount of Sec22p in the HSP by 10-fold, while incubation in the presence of GMP-PNP, a nonhydrolyzable GTP analogue, reduced vesicle formation by about twofold. The conditions that promote vesicle formation in these reactions, and the efficiency of vesicle formation are consistent with those found previously (Rexach et al., 1994). Sed4p-HA was detectable in both ER membrane and vesicle fractions, but only 0.1–0.5% of membrane-bound Sed4p-HA was released into the vesicle fraction at 20°C as compared with 10% of the input Sec22p. Even less (0.01%) was released on incubation at 4°C, without GTP or with GMP-PNP. To determine whether the small amount of Sed4p-HA released in a GMP-PNP reaction was present on ER to Golgi transport vesicles or was associated with another type of membrane, the vesicle fraction from a GMP-PNP reaction was fractionated further by gel-filtration chromatography (Barlowe et al., 1994). Most Sed4p-HA eluted before Sec22p on a Sephacryl S-1000 column (data not shown). Thus, the small amount of Sed4p that is released from the ER is not in transport vesicles and Sed4p, like Sec12p, appears to be largely excluded from budded vesicles.

![Sed4p-HA and BiP Immunostaining](image)

**NH2-terminal Domain of Sed4p Binds to the COOH-terminal Domain of Sec16p**

The genetic interaction between SED4 and SEC16 suggested that their products might physically associate. As an initial test of this possibility we used the two-hybrid sys-

![Sed4p-HA in HSP](image)

![Sed4p-HA and BiP Immunostaining](image)
system (Fields and Song, 1989; Gyuris et al., 1993). The NH2-terminal domain of SED4 was fused to an acidic transactivation domain and tested for interaction with each of three overlapping parts of SEC16 fused to the lacZ DNA-binding domain. Interaction was scored by the ability of the lacZ DNA-binding domain and the acidic activation domain to be brought together to drive transcription of a lacZ reporter gene. A strong interaction was detected for the combination of the NH2-terminal domain of SED4 and the COOH-terminal domain of SEC16 (Table III). This interaction was specific for SED4 since a parallel test of the NH2-terminal domain of SEC12 gave no interaction (Table III). The possibility that SEC12 failed to interact because of poor expression was tested by evaluating protein levels by Western blotting with antibodies against the HA tag present in the acidic activation domain. Both Sec12p and the Sed4p fusion proteins were present at comparable levels, indicating that the results of the two-hybrid test do reflect the inability of Sec12p to interact with Sed4p.

Sed4p and Sec16p were also tested for binding in cell extracts. Since Sec16p (Espenshade et al., 1995) and Sed4p are both insoluble, we tested association of only the putative interacting regions expressed in soluble form. The NH2-terminal domains of Sed4p and Sec12p were tagged with the myc epitope and expressed from the GAL1 promoter (SEC12N-MYC and SED4N-MYC). These epitope-tagged constructs were first tested for functionality as follows. We found that overexpression of either the Sec12p NH2-terminal domain or the Sed4p NH2-terminal domain has a dominant negative effect and exacerbates the temperature sensitivity of sec12-4 and other mutants defective in vesicle formation (d’Enfert et al., 1991; Gimeno, R. E., and C. A. Kaiser, unpublished observations). SEC12N-MYC and SED4N-MYC both inhibited the growth of sec12-4 to the same extent as untagged controls, indicating that addition of the epitope did not interfere with function. These tagged domains were tested for binding to the COOH-terminal domain of Sec16p fused to GST and expressed from the GAL1 promoter (GST-SEC16C). GST-Sec16Cp and associated proteins were isolated by affinity to glutathione beads from extracts prepared from yeast cells expressing GST-SEC16C and either SED4N-MYC or SEC12N-MYC. Sed4p-Myc, but not Sec12p-Myc, associated with GST-Sec16Cp bound to glutathione beads as detected by Western blotting using the anti-myc antibody (Fig. 7, lanes 1 and 2). The binding of Sed4p-Myc was dependent on the presence of Sec16p since none associated with GST alone (Fig. 7, lanes 3 and 4). Thus, the binding experiments gave the same result as the two-hybrid tests: Sed4p can bind to the COOH-terminal domain Sec16p and a parallel interaction is not seen for Sec12p.

Deletion of SED4 Slows Transport of CPY from the ER to the Golgi Complex

The genetic and physical interactions between Sed4p and Sec16p prompted us to examine more carefully the phenotypes of a chromosomal deletion of SED4 (sed4-D1). Previously, no growth or secretion defect was found in a SED4 disruption strain (Hardwick et al., 1992). Consistent with these data, sed4-D1 cells grew as well as isogenic wild-type cells at a range of different temperatures (15, 25, 38, or 40°C) and showed no accumulation of the ER form of CPY by Western blotting (not shown). However, sed4-D1 strains did not grow at 41°C, although wild-type strains grew slowly at this temperature. This growth defect of sed4-D1 strains was complemented by SED4 and could be suppressed by SAR1, SEC16, or SEC23 on a low copy vector, but not by SEC13 or SEC12 (not shown). Implementation of sed4-D1 at 41°C provided another test of SED4 function and was also used to establish the importance of the NH2-terminal domain (Fig. 3).

We examined the kinetics of secretion of CPY in sed4-D1 cells at 38°C (Fig. 8). In wild-type, 50% of the ER (p1) form of CPY was converted to the Golgi (p2) form after 4 min of chase, and CPY was completely converted to the mature form (pl) form after 8 to 10 min of chase (Fig. 8 A, lanes 7–12). In sed4-D1, p1 CPY persisted beyond 10 min of chase indicating slowed transport from the ER (Fig. 8 A, lanes 1–6). Quantitation of the rate of conversion of neutral p1 CPY to mature form (Fig. 8 B) gave a half-life of p1 CPY of 7.1 min in a sed4-D1 strain compared with 4.4 min in wild-type cells. This transport defect in sed4-D1, although subtle, was highly reproducible, and a 1.6- to 2-fold lower transport rate from the ER to the Golgi of sed4-D1 cells was found in four independent experiments.

Deletion of SED4 Exacerbates Vesicle Formation Mutations

Synthetic lethal interactions between genes that affect the

| Table III. NH2-terminal Domain of Sed4p and the COOH-terminal Domain of Sec16p Interact in the Two-Hybrid Assay |
|--------------------------------------------------|
| lexA DNA-binding domain | Activation domain | β-galactosidase activity |
|-------------------------|-------------------|------------------------|
|                        | SED4N | SEC12N | No fusion |
| SEC16C                  | 681.6 ± 77.2 | 21.6 ± 0.3 | 18.9 ± 0.1 |
| SEC16N                  | 18.6 ± 0.9 | 15.7 ± 2.8 | 16.1 ± 2.1 |
| SEC16CEN                | 18.2 ± 2.0 | 17.2 ± 0.0 | 22.5 ± 1.1 |
| No fusion               | 100.3 ± 4.5 | 146.3 ± 65.4 | 74.1 ± 7.2 |

Interactions were assayed for two independent transformants as described in Materials and Methods. The values given are means ± SD. Plasmids used were pPE68 (SEC16C), pPE59 (SEC16N), pPE74 (SEC16CEN), pRH151 (SED4N), and pRH152 (SEC12N).
secretory pathway have been found among genes required for protein translocation across the ER membrane (Rothblatt et al., 1989), genes required for vesicle formation at the ER (Kaiser and Schekman, 1990), genes required for vesicle fusion with the Golgi complex (Kaiser and Schekman, 1990; Newman et al., 1987), and genes required for fusion of secretory vesicles with the plasma membrane (Salminen and Novick, 1987). Because such interactions have only been detected between genes that affect the same step of the pathway, systematic tests for synthetic lethality can often define the step where a gene product acts. To test the interactions of sed4-Δ1, a URA3-marked sed4-Δ1 strain was crossed to a panel of Ts secretion mutants. The temperature sensitivity of mutations in each of four genes required for vesicle formation at the ER (sec12-4, sec13-1, sec16-2, sec23-1) was significantly increased when combined with sed4-Δ1::URA3 (Table IV). Importantly, these effects were specific for vesicle formation functions since sed4-Δ1 did not increase the temperature sensitivity of the mutants required for vesicle fusion (sec17-1, sec18-1, sec22-3, Table IV, and not shown) or any other secretion mutations (sec20-1, sec21-1, sec24-1, sec4-8, sec7-1, sec8-9, not shown). This pattern of synthetic lethal interactions shows that only defects in vesicle formation were made more severe by the absence of Sed4p, and therefore points to a role for SED4 in vesicle formation at the ER.

Isolation of sed4 as an Early Secretory Pathway Mutant

Perhaps the most convincing demonstration that SED4 is important for ER to Golgi transport came from the isolation of a sed4 mutant in a general screen for new secretion mutants. We examined a collection of 1,800 random Ts mutants for accumulation of the ER forms of CPY and invertase by Western blotting (Holzmacher, E., and C. A. Kaiser, unpublished data). After backcrossing and complementation testing, Ts mutations in ~15 new genes required for ER to Golgi transport have been identified. Segregation analysis of one of these mutants, designated EH874, revealed that its growth and secretion defect was caused by mutations in two unlinked genes. Analysis of crosses of EH874 to wild type showed that the double-mutant segregants were Ts at 38°C, one of the single mutants was Ts at 41°C, and the other single mutant showed no growth defect. The mutation that caused temperature sensitivity at 41°C was shown to be an allele of SED4 because it failed to complement the growth defect of sed4-Δ1 at 41°C and was completely linked to sed4-Δ1 in tetrad analysis. This allele was designated sed4-1 and in all the phenotypic tests we performed behaved the same as sed4-Δ1. The other mutation in EH874 was phenotypically silent on its own, but was needed to confer temperature sensitivity on sed4-1. Because SAR1 on a low copy plasmid complemented the temperature sensitivity of EH874, we suspected that the second mutation might be an allele of SAR1. Linkage to SAR1 was tested by crossing a sed4-Δ1 strain in which the SAR1 locus was marked with URA3 (CKY296) to EH874. Tetrads analysis of the resulting diploids demonstrated that the mutation that caused temperature sensitivity was tightly linked to SAR1. The effect of this allele, designated sar1-5, on growth and secretion is shown in Fig. 9. sar1-5 alone had no growth or secretion defect, whereas sar1-5 combined with sed4-Δ1::URA3 showed a severe growth defect and a complete block in transport of CPY to the Golgi complex at 38°C. The simplest explanation for these results is that Sed4p is needed for efficient use of Sar1p, and that in the absence of Sed4p the subtle defect caused by the sar1-5 mutation produces a strong secretion defect. The sar1-5 allele was recovered from the chromosome
Figure 9. Deletion of SED4 in combination with a mutation in SARI causes a Ts growth and secretion defect. (A) Wild-type (CKY291), sed4-Δ1::URA3 (CKY292), sarl-5 sed4-Δ1::URA3 (CKY293), and sarl-5 (CKY294) cells were spotted on rich medium and incubated for 40 h at 24 or 38°C. (B) CPY transport in the strains shown in A. Cells were grown in YPD at 30°C, shifted to 38°C for 2 h, and pulse labeled with [35S]methionine for 5 min. The label was chased for the times indicated and the different forms of CPY were immunoprecipitated from extracts, resolved by SDS-PAGE, and imaged on a PhosphorImager.

by gap repair of a SARI plasmid. The DNA sequence of sarl-5 revealed a change from G to T at nucleotide 533, replacing methionine 41 with isoleucine. Methionine 41 occurs in Sar1 proteins from all organisms examined so far and is located in a highly conserved region immediately following the G1 guanine nucleotide–binding domain and preceding the putative effector-binding domain (Kuge et al., 1994). Mutations in this region have not been previously characterized in either Sar1p or its closest homologue Arf1p.

sed4-Δ1 sarl-5 Double Mutant Accumulates ER Membranes but Not Vesicles

The finding that deletion of SED4 in a sarl-5 background causes a Ts ER to Golgi transport defect allowed us to examine in more detail the step at which Sed4p functions. Mutants that block ER to Golgi complex transport fall into two morphological classes: mutants defective in fusion of ER-derived vesicles with the Golgi complex accumulate ER membranes and a large number of 50-nm vesicles, whereas mutants defective in vesicle formation accumulate only ER membranes (Kaiser and Schekman, 1990). We examined the morphology of the sed4-Δ1::URA3 sarl-5 mutant after growth at 38°C for 2 h to impose a complete block in ER to Golgi transport (see Fig. 9). Cells were fixed with potassium permanganate to highlight membranes and were viewed by electron microscopy. sed4-Δ1::URA3 sarl-5 double-mutant cells accumulated excess ER membranes, visible as extra layers of membrane throughout the cell (Fig. 10). To determine whether sed4-Δ1::URA3 sarl-5 cells also accumulated 50-nm vesicles, we counted vesicles in random cell sections. The average number of vesicles per cubic micrometers cell volume in sed4-Δ1::URA3 sarl-5 cells was 7.4 ± 1.1. This value is similar to that previously reported for other mutants defective in vesicle formation (Kaiser and Schekman, 1990). To establish our ability to detect vesicles in this experiment, we counted vesicles in a mutant defective in vesicle fusion (sec17-1) that was grown at the restrictive temperature and was fixed for microscopy in parallel. As expected, the sec17-1 mutant accumulated vesicles (19.8 ± 2.2 vesicles/μm² cell volume). This result implies that the sed4-Δ1::URA3 sarl-5 double-mutation blocks vesicle formation at the ER, and is consistent with the genetic interactions between SED4 and vesicle formation genes and with the localization of Sed4p to the ER membrane, but not to vesicles.

sarl-5 Mutation Disrupts Interaction of SARI with SEC16 but Not SEC12

An important test for SARI function is the ability to suppress mutations in other SEC genes. SARI was first isolated because overexpression of SARI suppresses sec12 mutations (Nakano and Muramatsu, 1989). Overexpression of SARI also suppresses sec16 and sec23 mutations, although the mechanistic relationship to sec12 suppression is not known (Nakano and Muramatsu, 1989; Oka and Nakano, 1994). To explore the nature of the sarl-5 mutation, we tested sarl-5 expressed from either a low centromere or a high (2μ) copy plasmid for the ability to suppress different sec mutations. The sarl-5 mutation disrupted the interaction of SARI with SEC16 and SEC23, since sarl-5 on either low or high copy plasmids did not suppress sec16-2 or sec23-1 mutations (Table V). In contrast, sarl-5 suppressed the temperature-sensitivity of sec12-4 to the same degree as wild-type SARI (Table V). Thus, the sarl-5 allele allowed the function of SARI needed to suppress sec12 mutations to be distinguished from the function(s) needed to suppress sec16 and sec23 mutations.

In tests of sarl-5 for synthetic lethal interactions, sarl-5 exacerbated the temperature sensitivity of sec16-2, sec13-1,
Table V. Genetic Interactions of sarl-5 with Vesicle Formation Mutants

| Genotype         | Incubation temperature |
|------------------|------------------------|
|                  | 24°  | 27°  | 30°  | 33°  | 38°  |
| sec12-4          | +++  | +++  | +    | −    | −    |
| sec12-4 sarl-5   | +++  | +++  | +    | −    | −    |
| sec12-4 (psAR1)  | +++  | +++  | +++  | +++  | +++  |
| sec12-4 (psar1-5)| +++  | +++  | +++  | +++  | +++  |
| sec13-1          | +++  | +++  | +    | −    | −    |
| sec13-1 sarl-5   | +++  | ±    | −    | −    | −    |
| sec16-2 sarl-5   | +++  | ±    | −    | −    | −    |
| sec16-2 (psAR1)  | +++  | +++  | +    | −    | −    |
| sec16-2 (psar1-5)| +++  | +++  | +    | −    | −    |
| sec23-1 sarl-5   | +++  | +++  | +    | −    | −    |
| sec23-1 (psAR1)  | +++  | +++  | +    | −    | −    |
| sec23-1 (psar1-5)| +++  | +++  | −    | −    | −    |

psAR1 is plH259 or plH280, psarl-5 is plH262 or plH279. Growth of single colonies on YES after 24–48 h. ++++, growth comparable to wild type; −−, no growth.

Discussion

The major conclusion of this study is that SED4 encodes an important, but not essential, component of the machinery that assembles transport vesicles at the ER membrane. This conclusion rests on five findings. (1) Strains with a chromosomal deletion of SED4 exhibit a twofold reduction in the rate of transport of the marker protein CPY from the ER to the Golgi complex. (2) The cytosolic domain of Sed4p binds to the COOH-terminal domain of Sec16p, an ER and vesicle protein that is required for transport vesicle budding in vivo. (3) Sed4p is located in the ER membrane but not in vesicles, and therefore binding to Sec16p must take place on the ER membrane. (4) Increased dosage of SED4 suppresses sec16 mutations. (5) Deletion of SED4 exacerbates mutations in genes known to participate in vesicle budding (SEC16, SEC12, SEC13, SEC23, and SAR1), but not mutations that affect later steps in the secretory pathway. The interaction with SAR1 is particularly striking since the sarl-5 mutation alone is phenotypically silent, but when combined with sed4-Δ1 shows a strong secretion block.

An important clue to the mechanism of SED4 function is the binding of the cytosolic domain of Sed4p to the COOH-terminal domain of Sec16p. This interaction was detected both by two-hybrid assay and by binding experiments in cell extracts where the two interacting domains were expressed as soluble proteins. An internal control for the specificity of the interaction between Sed4p and Sec16p is provided by comparing binding of Sec16p to the cytosolic domains of Sed4p and Sec12p. The binding that we observe is specific to Sed4p because the cytosolic domain of Sec12p, which must have a similar structure to the cytosolic domain of Sed4p, does not interact with Sec16p by either two-hybrid or solution-binding assays. Furthermore, two-hybrid tests between Sed4p and regions of Sec16p other than the COOH-terminal domain gave no interaction and a deletion that removed 250 amino acids from the COOH terminus of Sec16p disrupted the ability to interact with Sed4p (not shown). These results show a specific association between the cytosolic domain of Sed4p and the COOH-terminal domain of Sec16p. Since both proteins are located at the ER membrane, this is presumably where they interact.

Genetic tests provide strong evidence that SED4 is important for the proper function of SEC16. When vesicle formation is impaired by sec16 mutation, increased dosage of SED4 restores function, whereas deletion of SED4 increases the severity of the defect. Since the activity of SEC16 varies according to both increased and decreased dosage of SED4, and since Sed4p binds to Sec16p, we conclude that SED4 is almost certainly needed for proper function of SEC16 in vesicle formation. SAR1 shows genetic interactions with SEC16 that are similar to the ones observed between SED4 and SEC16. Increased dosage of SAR1 suppresses sec16 mutations (Nakano and Mura-matsu, 1989), and sec16-2 is lethal at 27°C when combined with sarl-5. SAR1 also interacts genetically with SED4. We show that increased dosage of SAR1 suppresses the temperature sensitivity caused by sed4 deletion, while combination of sarl-5 and sed4-1 causes a strong transport block. These multiple genetic interactions argue that the functions of Sec16p, Sed4p, and Sarl1p are closely linked.

How the interactions of these proteins are coupled to vesicle morphogenesis can be inferred from what we know of their location with respect to the forming vesicle. Three classes of proteins that participate in vesicle budding are defined by the dissection of the membrane and cytosolic requirements for the reconstituted budding reaction and by localization experiments based on cell fractionation and immunofluorescence. The first class is associated with the ER membrane, but is not incorporated into vesicles, and therefore probably functions in the ER membrane before completion of the vesicle. Representatives of this class are Sec12p (Rexach and Schekman, 1991; Barlowe et al., 1994) and Sed4p, as shown here. The second class, represented by the COP II proteins Sec13p/Sec31p, Sec23p/Sec24p, and Sarl1p (Barlowe et al., 1994) can be recruited from the cytosol to form a coat on the budded vesicles. In the accompanying paper, we show that Sec16p represents a third class of vesicle-forming proteins that is tightly associated with the ER and is also incorporated into the vesicle coat.

From these localization studies, and from the genetic interactions and binding studies, we have developed a model for the function of Sed4p, Sec16p, Sec23p, and Sarl1p in the early steps of vesicle assembly (Fig. 11). Because Sec16p is on both the ER and on vesicles it may serve as a scaffold for incorporation of soluble coat proteins into the vesicle. In the accompanying paper we show that the COP II protein, Sec23p, binds to the COOH-terminal domain of Sec16p. The genetic interactions between SEC16 and
SAR1 are consistent with Sec16p also being a binding site for Sar1p. This proposed association of Sar1p with a complex of Sec16p and Sec23p is further supported by the observation that Sec23p stimulates Sar1p GTPase activity (Yoshishita et al., 1993). The function of Sed4p may be to promote the assembly or increase the stability of a nascent vesicle coat complex that includes Sec16p, Sec23p, and Sar1p. This would explain why deletion of SED4 exacerbates the transport defect of sar1-5, sec16, and sec23 mutations, and is consistent with SED4 being a nonessential gene.

A specific function for SED4, suggested by sequence similarity to SEC12, would be to stimulate guanine–nucleotide exchange on Sar1p. The NH2-terminal domain of Sec12p has been shown to have such activity (Barlowe and Schekman, 1993), but parallel experiments using the partially purified NH2-terminal domain of Sed4p did not show Sar1p-specific nucleotide exchange activity (Barlowe, C., personal communication). Although Sed4p does not have guanine–nucleotide exchange activity by itself, the complex between Sed4p and Sec16p may have this activity. To explore this possibility, we tested the soluble complex between the NH2-terminal domain of Sed4p and COOH-terminal domain of Sec16p for the ability to stimulate exchange of GTP for GDP by Sar1p. The complex was not active, but the truncations of Sed4p and Sec16p used to produce a soluble complex could have disrupted the capacity to associate with Sar1p. A more direct biochemical test of the interaction of Sar1p with Sec16p and Sed4p will depend on our ability to extract from membranes an active complex of these proteins.

Our data, together with the homology between Sed4p and Sec12p, suggest a role for Sed4p in the recruitment of Sar1p to a vesicle formation complex. Sec12p has been proposed to act similarly in the initial phases of vesicle formation by recruiting Sar1p to the membrane (d'Enfert et al., 1991b). However, our tests for functional overlap between SED4 and SEC12 show that these genes perform different functions. Increased dosage of SED4 does not suppress sec12 mutations and increased dosage of SEC12 does not suppress the temperature sensitivity of sed4 deletions. Moreover, increased dosage of SED4 suppresses sec16 mutations, but parallel tests show no effect of increased dosage of SEC12 on sec16. The biochemical properties of the NH2-terminal domains of Sec12p and Sed4p are also different: the NH2-terminal domain of Sed4p binds to the COOH-terminal domain of Sec16p, while no binding was detected using the corresponding domain of Sec12p.

One way to reconcile the apparently contradictory aspects of the relationship between SED4 and SEC12 would be to postulate that SARI becomes engaged in vesicle formation through two functionally independent pathways, one mediated by SEC12 and the other mediated by SED4 and SEC16. A genetic test of this idea would be to identify mutations in SARI that affect one pathway but not the other. The sar1-5 mutation appears to have this property as shown by tests for dosage-dependent suppression of sec12 and sec16 mutations. Increased dosage of sar1-5 does not suppress sec16-2, indicating that the mutation diminishes the effectiveness of Sar1p to function with Sec16p. However, increased dosage of sar1-5 does suppress sec12 mutations as effectively as wild-type SARI, showing no negative effect of sar1-5 on the interaction of Sar1p with Sec12p. Moreover, sar1-5 exacerbates sec16 mutations, but has no effect on sec12 mutations, further supporting the idea that SARI engages in two independent processes, and that sar1-5 selectively disrupts the processes that involve SEC16. The two ways that SARI functions in vesicle formation as distinguished by the sar1-5 mutation are outlined in Fig. 11.

The view suggested by our work is that Sec16p and Sed4p together may constitute a docking site needed to recruit Sar1p and coat proteins such as Sec23p to a nascent vesicle. Models for the formation of other coated vesicles have a similar outline. The binding of coatomer in formation of intra-Golgi transport vesicles and of AP-1 in formation of clathrin-coated vesicles have both been shown to depend on the action of the small GTP-binding protein, ADP-ribosylation factor (Donaldson et al., 1992; Helms et al., 1993; Stamnes and Rothman, 1993; Traub et al., 1993). These same studies showed that assembly of both types of vesicle also requires Golgi membrane factors which presumably act as docking proteins for both ADP-ribosylation factor and coat subunits. The putative docking proteins for the Golgi complex have not yet been identified. We propose that Sed4p and Sec16p carry out this function at the ER membrane.

Knowledge of the interactions between SEC16, SED4, SARI, and SEC23 offers a way to study the subunit associations in the early steps of ER vesicle assembly free from the inherent biochemical complexity of the membrane. If soluble Sec16p can be obtained in an active form either as a recombinant protein or by extraction from membranes, it should be possible to develop assays in solution for the subunit assembly steps delineated here.

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