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 Sec16p as shown by two-hybrid assay and coprecipitation. The interaction between Sed4p and Sec16p 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, *sar1-5*, 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 Sar1p) 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, NH_2-terminal domain of Sec12p catalyzes exchange of GTP for GDP on the 21-kD GTPase Sar1p (Barlowe and Schekman, 1993). Overexpression of Sec12p increases the amount of Sar1p that can be bound to ER membranes in vitro, suggesting that Sec12p can recruit Sar1p to the ER membrane (d'Enfert et al., 1991*b*). Sar1p-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 NH_2-terminal, cytosolic domain of Sec12p shares 45% amino acid identity with Sed4p, but the luminal domains of these proteins appear unrelated (Hard-

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wick et al., 1992). This similarity in sequence implies that *SED4* is involved in vesicular transport, although the step in the secretory pathway where *SED4* functions has been difficult to establish. Deletion of *SED4* does not cause a pronounced growth or secretion defect, and although *SED4* was isolated as a multicopy suppressor of a deletion of *ERD2*, the gene encoding the HDEL receptor in yeast, the mechanism of this suppression is not understood (Hardwick et al., 1992).

We became interested in the action of *SED4* in vesicular transport when we isolated *SED4* as a multicopy suppressor of *sec16* mutations. In this report, we show that *SED4* is involved in ER to Golgi transport and probably functions in conjunction with Sec16p and Sar1p in an early step in vesicle formation. Furthermore, we found that although Sed4p and Sec12p are similar in structure and location, these proteins appear to engage in functionally distinct processes.

Materials and Methods

Strains, Media, and Microbiological Techniques

S. cerevisiae strains are listed in Table I. Yeast media (rich medium [YPD][1], 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, 10^5 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 of Kunkel (Kunkel et al., 1987). PCR was carried out using Taq polymerase (Perkin-Elmer Cetus, Norwalk, CT).

Isolation and Analysis of Multicopy Suppressors of sec16

The YEp24 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, CKY50 and CKY52, were transformed with YEp24 library DNA. A total of 35,000 (CKY50) and 13,000 (CKY52) transformants at a density of 1.5×10^3 to 1×10^4 colonies per plate were replica plated and incubated at restrictive temperatures of 30, 33, and 36°C (CKY50) or 33 and 36°C (CKY52). 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 (CKY50) and 36°C (CKY52) 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 pRS306-2μ and testing for their ability to confer temperature resistance to CKY50.

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.

Plasmid Constructions

Plasmids are summarized in Table II. p5007 is a YEp24 library plasmid containing *SED4*. pRH26 is the 7.4-kb ApaI–SalI fragment of p5007 in pRS306-2μ. pRH46 is the 5.2-kb SacI–SalI fragment of pRH26 in pRS306-2μ. pRH107, pRH67, and pPE87 are the 5.2-kb SacI–SalI fragment of pRH46 in pRS316, pRS306, and pRS305-2μ, respectively. pRH121 and pRH120 are the 5.2-kb SacI–SalI fragments of pRH117 (see below) in pRS316 and pRS306-2μ, respectively.

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 ApaLI, the 5′ overhang was filled in, a 3.5-kb fragment was purified and cut with SacI–ApaI to produce a 1.8-kb fragment that was ligated into SacI–SmaI-cut pRS316-2μ to make pRH54. pRH77 contains the 1.2-kb XhoI–SspI fragment of pRH46 ligated into XhoI–SmaI-cut pRS306-2μ. pRH78 contains the 1.2-kb XhoI–SpeI fragment of pRH77 ligated into XhoI–SpeI-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; Yon and Fried, 1989). PCR primers used were 5′-TTG TAA ATA AAG CCG TGC ACA TTG TGC TTA TAG GAG AAC TGT AA-3′ (nucleotides [nt] 1124–1101, *SED4*; and nt 1125–1104, *SEC12*, underlined), 5′-GGG ATT ACT TCT ATG GAT G-3′ (nt 802–820, *SEC12*) and 5′-GAT GAA GAT GAA GAC GGC-3′ (nt 1932–1949, *SED4*), templates used were pSEC1230 (Nakano et al., 1988) and pRH46. The PCR product was cut with SalI–EcoRI and inserted into SalI–EcoRI cut pRH50 (see below) to make pRH56. pRH62 is the 2.5-kb EcoRI fragment of pRH46 ligated into EcoRI-cut pRH56. pRH141 is the 2.6-kb EcoRI fragment of pRH117 (see below) ligated into EcoRI-cut pRH62. pRH148 is the 5.2-kb XhoI–SacI fragment of pRH141 in pRS316.

pRH50 and pRH213 are the 3.5-kb XhoI–HindIII fragments of pSEC1230 in pRS306-2μ 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 oligomer 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 CTG GCC TTT ACC CAT ACG ACG TCC CAG ACT ACG CTC ATG ACG AAT TGT GAA TAA CGA AAT AA-3′). Tandem repeats of the HA epitope were inserted by introducing a NotI site between the last nucleotide of the HA tag and the COOH-terminal HDEL sequence by site-directed mutagenesis. The mutagenic oligomer consisted of a NotI site (underlined) flanked by sequences complementary to *SED4-HA1* (5′-GAC GTC CCA GAC TAC GCT AGC GGC CGC CAT GAC GAA TTG TGA ATA ACG-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*).

The NH_2-terminal domain of *SED4* was placed under control of the *GAL10* promoter and tagged at its 3′ end with a c-*myc* epitope (myc) (Munro and Pelham, 1987) as follows: pCD43 is pRS316 with a 0.6-kb EcoRI–BamHI fragment containing the *GAL1/GAL10* promoter region inserted into the polylinker. A 1-kb fragment encoding amino acids (aa) 1–346 of *SED4* plus a NotI site was amplified by PCR using pRH46 as a template and the following primers: 5′-AGT GAA TTC ATA ATG AGT GGC AAC TCT GC-3′ (nt −3 to +17, *SED4*) and 5′-ATG GGT ACC GTC GAC CTA GCG GCC GCT TTT CCA AAT ATT TCG TAA AAT TGA TG-3′ (nt 1214–1239, *SED4*). The amplified fragment was cut with EcoRI–KpnI and ligated into pCD43, producing pRH183. A cassette encoding three copies of the myc epitope flanked by NotI sites was constructed using overlapping oligonucleotides (kindly provided by B. Futcher, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). The oligonucleotides were annealed, filled in, and ligated into NotI-cut Bluescript vector (pKS^+) to make pRH177/23. The nucleotide sequence of the 3×myc cassette in pRH177/23 is 5′-GCG GCC GCT CTG AGC AAA AGC TCA TTT CTG AAG AGG ACT TGA ATG GAG AAC AGA AAT TGA TCA GTG AGG AAG ACC TCA ACG GTG AGC AGA AGT TAA TAT CCG AGG AGG ATC TTA ATA GTG CGG CCG C-3′. The 3×myc cassette of pRH177/23 was ligated into NotI-cut pRH183, the resulting product was cut with PvuI and inserted into PvuI-cut pRS313, creating pRH260. By DNA sequencing, pRH260 encodes a protein with three tandem repeats of the myc epitope plus 14 additional

Table I. Saccharomyces cerevisiae Strains

Strain	Genotype	Source or Reference
CKY8	*MATα ura3-52 leu2-3, 112*	Kaiser lab collection
CKY10	*MATa ura3-52 leu2-3, 112*	Kaiser lab collection
CKY93	*MATα ura3-52 leu2 pep4:URA3*	Kaiser lab collection
CKY289	*MATa ura3-52 leu2 his3Δ200 trp 1Δ63 lys-801 Gal*$^+$	Kaiser lab collection
CKY249	*MATa/MATα SED4/sed4-Δ1::URA3 ura3-52/ura3-52 leu2-3, 112/leu2-3, 112*	This study
CKY250	*MATa/MATα SED4/sed4-Δ1 ura3-52/ura3-52 leu2-3, 112/leu2-3, 112*	This study
CKY251	*MATα sed4-Δ1 ura3-52 leu2-3, 112*	This study
CKY252	*MATα ura3-52 leu2-3, 112*	This study
CKY255	*MATα sed4-Δ1::URA3 ura3-52 leu2-3, 112*	This study
CKY258	*MATa sed4-Δ1::URA3 ura3-52 leu2-3, 112*	This study
CKY291	*MATα ura3-52 leu2-3, 112*	This study
CKY292	*MATα sed4-Δ1::URA3 ura3-52 leu2-3, 112*	This study
CKY293	*MATa sed4-Δ1::URA3 sar1-5 ura3-52 leu2-3, 112*	This study
CKY294	*MATa sar1-5 ura3-52 leu2-3, 112*	This study
CKY295	*MATα sed4-Δ1 ura3-52 leu2-3, 112 pep4::LEU2* (pRH121)	This study
CKY296	*MATα sed4-Δ1 SAR1::URA3 ura3-52 leu2-3,112*	This study
CKY39	*MATα sec12-4 ura3-52 his4-619*	Kaiser lab collection
CKY45	*MATα sec13-1 ura3-52 his4-619*	Kaiser lab collection
CKY50	*MATα sec16-2 ura3-52 his4-619*	Kaiser lab collection
CKY52	*MATa sec16-1 ura3-52 leu2-3, 112*	Kaiser lab collection
CKY54	*MATα sec17-1 ura3-52 his4-619*	Kaiser lab collection
CKY58	*MATα sec18-1 ura3-52 his4-619*	Kaiser lab collection
CKY62	*MATα sec19-1 ura3-52 his4-619*	Kaiser lab collection
CKY64	*MATα sec20-1 ura3-52 his4-619*	Kaiser lab collection
CKY69	*MATα sec21-1 ura3-52 his4-619*	Kaiser lab collection
CKY70	*MATα sec22-3 ura3-52 his4-619*	Kaiser lab collection
CKY78	*MATα sec23-1 ura3-52 his 4-619*	Kaiser lab collection
CKY105	*MATa sec16-3 ura3-52 leu2-3, 112*	Kaiser lab collection
CKY230	*MATα sec16-4 ura3-52 leu2-3, 112 ade2 ade3 sec13-1 (pCEN-ADE3-SEC13)*	Kaiser lab collection
NY768	*MATα sec1-1 ura3-52 leu2-3, 112*	P. Novick (Yale University)
NY770	*MATα sec2-41 ura3-52 leu2-3, 112*	P. Novick (Yale University)
NY772	*MATa sec3-2 ura3-52 leu2-3, 112*	P. Novick (Yale University)
NY774	*MATα sec4-8 ura3-52 leu2-3, 112*	P. Novick (Yale University)
NY776	*MATα sec5-24 ura3-52 leu2-3, 112*	P. Novick (Yale University)
NY778	*MATα sec6-4 ura3-52 leu2-3, 112*	P. Novick (Yale University)
NY780	*MATα sec8-9 ura3-52 leu2-3, 112*	P. Novick (Yale University)
NY782	*MATa sec 9-4 ura3-52 leu2-3, 112*	P. Novick (Yale University)
NY784	*MATa sec10-2 ura3-52 leu2-3, 112*	P. Novick (Yale University)
NY786	*MATa sec15-1 ura3-52 leu2-3, 112*	P. Novick (Yale University)
AFY72	*MATa sec7-1 ura3-1 his3-11 trp1-1*	R. Schekman (U.C. Berkeley)
ANY123	*MATα bet1-1 ura3-52 his4-619*	S. Ferro-Novick (Yale University)
ANY125	*MATα bet2-1 ura3-52 his4-619*	S. Ferro-Novick (Yale University)
RSY533	*MATα sec61-2 ura3-52 leu2-3, 112 ade2 pep4-3*	R. Schekman (U.C. Berkeley)
RSY530	*MATα sec62 ura3-52 leu2-3, 112*	R. Schekman (U.C. Berkeley)
RSY153	*MATα sec63-1 ura3-52 leu2-3, 112*	R. Schekman (U.C. Berkeley)
CKY234	*MATα sec16-Δ1::TRP1 lys2-801 ade 2-101 trp1-Δ63 his3-Δ200 ura3-52 leu2-Δ1* (pPE5)	Espenshade et al., 1995
RSY656	*MATa/MATα SEC12/sec12Δ::LEU2 ura3-1/ura3-1 leu2-3/leu2-3 trp1-1/trp1-1 ade2-1/ade2-1 his3-11/his3-11 can1-100/can1-100*	d'Enfert et al., 1991*a*
EGY40	*MATα ura3-52 leu2 his3 trp1*	Golemis and Brent, 1992
PRY303	*MATα dpm1::LEU2 leu2-3, 112 lys2-801 trp1Δ1 ura3-52* (p*dpm1-6*)	Orlean, 1990

amino acids at the COOH terminus. The NH_2-terminal domain of *SEC12* 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* 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 G-3′ (nt −3 to +16, *SEC12*) and 5′-TGC GCT CGA GCT AGC GGC CGC TTT TAG AGA TTT TTT GTT TCA TTG AGG-3′ (nt 1037–1062, *SEC12*). The amplified fragment 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-Δ1*) was constructed by site-directed mutagenesis. The mutagenic oligomer (5′-CTT

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	*SEC13* in pRS316	Roberg and Kaiser, unpublished data
pCK1313	*SEC13* in YEp352	Pryer et al., 1993
YCP1142	*SEC23* in YCp50	Hicke and Schekman, 1989
pRH259	*SAR1* in pRS316	This study
pRH262	*sar1-5* in pRS316	This study
pRH279	*sar1-5* in pRS306-2μ	This study
pRH280	*SAR1* in pRS306-2μ	This study
pEG202	*lexA* DNA binding domain in a 2μ vector marked with *HIS3*	Gyuris et al., 1993
pJG4-5	acidic activation domain in a 2μ vector marked with *TRP1*	Gyuris et al., 1993
pSH18-34	*lacz* 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 pJG4-5	This study
pRH152	*SEC12* (1-354) in pJG4-5	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 ATG TTA AAT 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^r-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 pRH73 (*sed4-Δ1::URA3*).

A diploid (CKY8 × CKY10) was transformed with a 6.9-kb, purified SacI–SalI fragment of pRH73. Tetrad analysis of Ura$^+$ transformants gave 2:2 segregation of *URA3*. By Southern blot analysis, the genomic *SED4* locus was deleted in four Ura$^+$ spores examined. Loss 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 × 10^7 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 1:1,000 dilution, anti-myc antibody (9E10 ascitic fluid; K. Morrison, Harvard University, Boston, MA) at 1:1,000 dilution, rabbit anti-carboxy peptidase Y (CPY) antibody (gift of R. Schekman) at 1:5,000 dilution, HRP-coupled sheep anti–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. Tunicamycin treatment and temperature-shift experiments of the *dpm1-6* mutant were performed as described (Orlean et al., 1991). 2–6 × 10^7 exponentially growing cells (1–3 OD_{600} U) were radiolabeled in supplemented SD medium by incubating with 30 μCi [^{35}S]methionine per OD_{600} 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 0.1 M ammonium sulfate, 0.3% cysteine, 0.4% methionine. Labeled samples of 1 OD_{600} U of cells were collected into chilled tubes containing an equal volume of 40 mM sodium azide. Protein extracts were prepared in 30 μl ESB by vigorous agitation with glass beads. Extracts were diluted with 1 ml IP buffer (50 mM Tris HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.2% SDS), absorbed for 20 min with 50 μl 10% *Staphylococcus aureus* cells (Sigma Chemical Co.) and cleared by centrifugation at 12,000 *g* for 5 min. 0.5 μl anti-CPY 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

Sed4p and Sec12p were tested for binding to Sec16p in vivo as described by Gyuris et al., 1993. The NH_2-terminal domain of Sed4p or Sec12p was fused to the acidic activation domain in pJG4-5 as follows. A fragment encoding aa 1–347 of *SED4* was amplified by PCR using pRH46 as a template and the following primers: 5′-AGT GAA TTC ATA ATG AGT GGC AAC TCT GC-3′ (nt −3 to +17, *SED4*) and 5′-CTA GTC GAC CTA TTT CCA AAT ATT TCG TAA AAT TGA TG-3′ (nt 1038–1016, *SED4*). The corresponding fragment encoding aa 1–354 of *SEC12* was amplified similarly using pSEC1230 as a template and the following primers: 5′-AGT GAA TTC ATG AAG TTC GTG ACA GCT AG-3′ (nt 1–20, *SEC12*) and 5′-TGC GCT CGA GCT ATT TAG AGA TTT TTT GTT TCA TTG AGG-3′ (nt 1062–1037, *SEC12*). Fragments were cut with EcoRI–SalI (*SED4*) or EcoRI–XhoI (*SEC12*) and ligated into EcoRI–XhoI-cut pJG4-5 to make pRH154 and pRH155, respectively. pPE58, 59, and 74 are plasmids encoding aa 1645–2194, aa 1–824, and aa 447–1737 of *SEC16* fused to the *lexA* DNA-binding domain in pEG202 (Espenshade et al., 1995).

Two-hybrid interactions were tested in EGY40 (Golemis and Brent, 1992) transformed with the appropriate plasmids. *LacZ* expression was tested by patching four to eight transformants on SC medium (pH 7.0) lacking the appropriate amino acids and supplemented with 2% galactose and 40 mg/liter X-gal. For β-galactosidase assay, cells were grown to exponential phase in selective medium containing 2% raffinose, then galactose was added to 2% and growth was continued for 10 h. Extracts were prepared and assayed as described (Kaiser et al., 1994). Protein concentrations in the extracts were determined using the Bradford assay (Bio-Rad Laboratories, Melville, NY). Units of β-galactosidase are expressed as: [OD_{420} × vol of assay]/[0.0045 × protein concentration in extract × vol of extract assayed × time].

Binding to GST Fusions

The COOH terminus of Sec16p (amino acids [aa] 1638–2194) was fused to the glutathione-*S*-transferase gene (GST) expressed from the *GAL1* promoter (pRD56, a kind gift of Dr. Ray Deshaies, California Institute of Technology, Pasadena, CA) to create pPE122. The NH_2-terminal domains of Sed4p and Sec12p were expressed from the *GAL10* promoter and tagged with the myc epitope as described above (pRH260 and pRH261). pPE122 and either pRH260 or pRH261 were transformed into CKY289. For controls, CKY289 carrying pRD56 and either pRH260 or pRH261 were used.

Cells were grown to exponential phase in selective medium containing 2% raffinose, galactose was added to 2%, and extracts were prepared 4 h later. 4 × 10^7 cells were suspended in 40 μl CoIP buffer (20 mM Hepes, pH 6.8, 80 mM potassium acetate, 5 mM magnesium acetate, 0.1% Triton X-100) containing 2.5 × 10^{-4} U/ml α2-macroglobulin, 1 mM PMSF, 0.5 μg/ml leupeptin, 10 μg/ml E64, and 0.4 μg/ml aprotinin (all Boehringer Mannheim Biochemicals, Indianapolis, IN) and were lysed by vigorous agitation with 0.5 mm glass beads 4 × 20 s with 1-min intervals on ice. Extracts were diluted to 1 ml with CoIP buffer with protease inhibitors, and the lysate was cleared by centrifugation at 13,000 *g* for 5 min. Glutathione Sepharose 4B beads (Pharmacia LKB Biotechnology, Inc.) were added and samples were incubated for 1 h at 25°C. The beads were washed three times with CoIP buffer and once with detergent-free CoIP buffer. Proteins were released from the beads by boiling in 30 μl ESB. Total protein extracts were prepared from 2 × 10^7 cells lysed in ESB by agitation with glass beads.

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 antiserum (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 axioscope (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-Δ1 sar1-5* (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-Δ1 sar1-5* 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 CKY295 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, 150 mM KOAc, 5 mM MgOAc, 250 mM sorbitol) with protease inhibitors (1 mM PMSF, 0.5 ng/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 were solubilized in 30 μl ESB and proteins were analyzed by Western blotting.

Vesicles formed in vitro were fractionated 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 *SAR1* gene on a 1.3-kb HindIII–SalI fragment of pSEC1210 (Nakano and Muramatsu, 1989) inserted into pRS316 with a deletion from the EcoRI to the NotI sites of the polylinker. A *sar1-5 sed4-Δ1* double mutant was transformed with plasmid pRH259 cut with EcoRI and BamHI to produce a gap covering the *SAR1* coding sequence. Gap-repaired plasmids carrying the mutation (pRH262) were identified by their inability to suppress the temperature sensitivity of *sec16-2* and *sec23-1* strains. The mutational change in a plasmid carrying *sar1-5* was identified by sequencing with synthetic oligonucleotide primers. *SAR1* and *sar1-5* were placed on a 2μ plasmid by inserting the 1-kb EcoRI–HindIII fragment of pRH259 and pRH262 into pRS306-2μ, 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*). Subcloning and sequencing identified the suppressing locus 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-Δ1::TRP1*) as demonstrated in the following plasmid shuffling experiment. CKY234 carries a chromosomal *sec16-Δ1::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 (pPE8) 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 *sec2-41* 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 NH_2-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*, truncations of *SED4* were tested. In comparison to *SEC12*, *SED4* is comprised of an NH_2-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 NH_2-terminal and transmembrane domains of Sed4p were required for suppression, since deletion of a 39–amino acid segment of the NH_2-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 Sed4-T4-HAp were identical to Sed4-HAp (not shown).

The function of the COOH-terminal luminal domain of Sed4p was further examined by fusing it to the NH_2-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

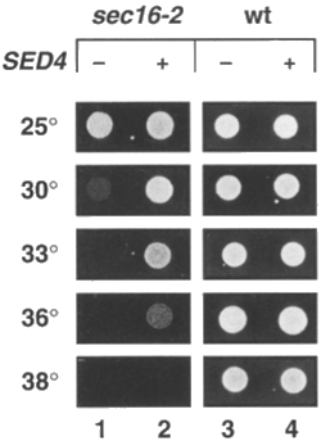

Figure 1. SED4 overexpression suppresses the temperature sensitivity of *sec16-2*. Lanes *1* and *2*: A *sec16-2* strain (CKY50) containing vector (pRS306-2μ) or *SED4* on a 2μ plasmid (pRH46). Lanes *3* and *4*: a wild-type strain (CKY8) containing vector (pRS306-2μ) or *SED4* on a 2μ plasmid (pRH46). Cells were spotted on selective medium and incubated at the indicated temperatures for 40 h.

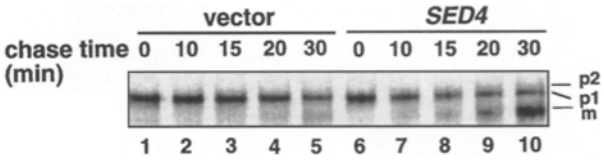

Figure 2. SED4 overexpression suppresses the temperature-sensitive ER to Golgi transport defect of *sec16-2*. Lanes *1–5*: a *sec16-2* strain (CKY50) carrying vector (pRS306-2μ). Lanes *6–10*: a *sec16-2* strain carrying *SED4* on a 2μ plasmid (pRH46). Cells were grown in selective medium at 25°C, shifted to 32°C for 2 h, and pulse-labeled with [^{35}S]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.

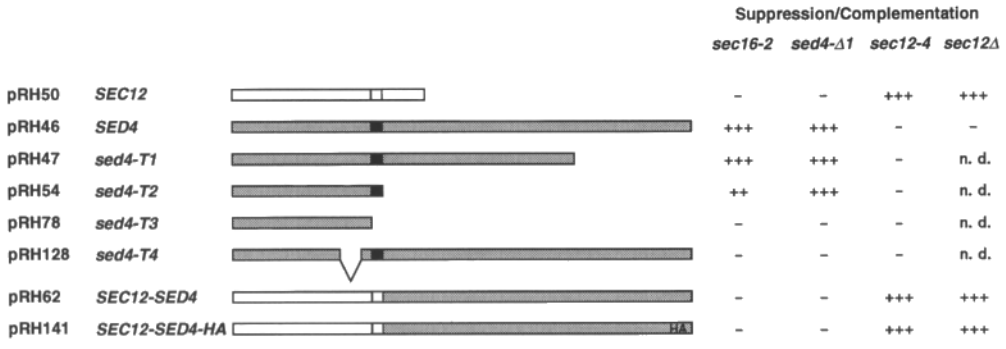

Figure 3. The NH_2-terminal domain of *SED4* is necessary and sufficient for suppression of *sec16-2*. The indicated 2μ plasmids were transformed into CKY50 (*sec16-2*), CKY251 (*sed4-Δ1*), CKY39 (*sec12-4*), and RSY656 (*SEC12/sec12Δ*). Growth of single colonies assayed on selective medium at 33°C (*sec16-2*, *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.

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).

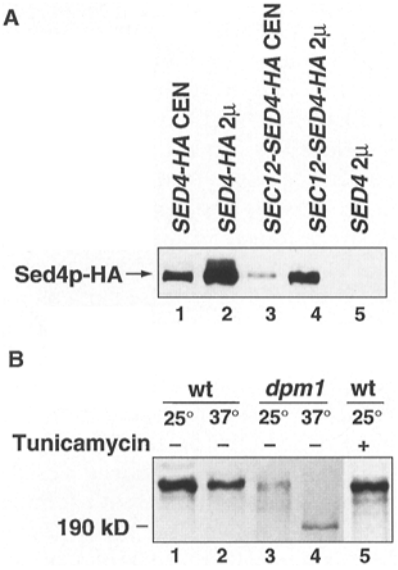

Figure 4. (*A*) Immunodetection of Sed4p-HA and comparison of the protein levels of Sed4p-HA and Sec12-Sed4p-HA fusion proteins. The *SEC12-SED4* fusion construct is described in Fig. 3. Wild-type cells (CKY10) carrying the indicated plasmids (pRH121, pRH120, pRH148, pRH141, and pRH46; lanes *1–5*, respectively) were grown in selective medium. Extracts of 0.2 OD_{600} 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 [^{35}S]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.

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 *sed4* 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 20°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.

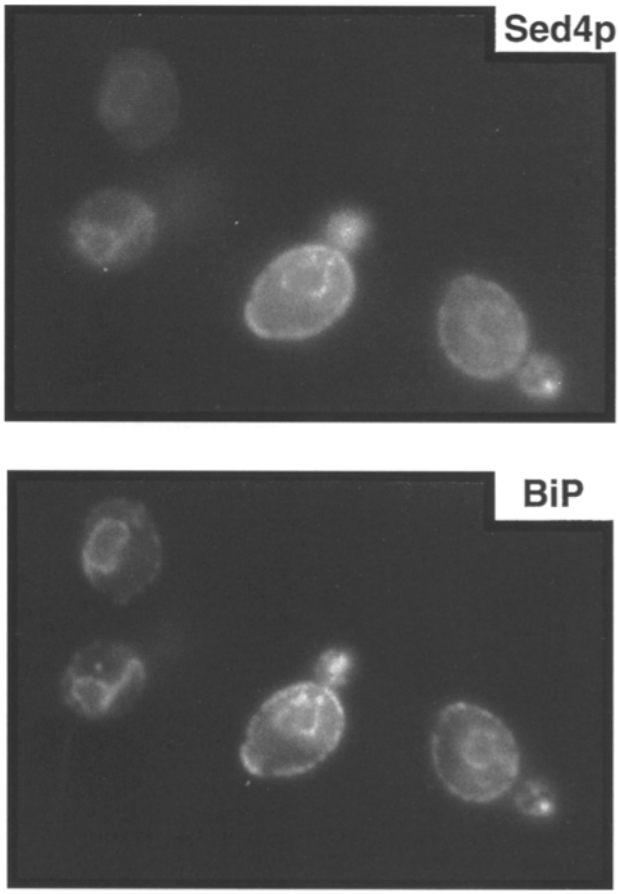

Figure 5. Sed4p-HA is located in the ER. Indirect immunofluorescence of a wild-type diploid strain (CKY8 × CKY10) carrying *SED4-HA* on a 2μ plasmid (pRH120). Fixed cells were incubated with both anti-HA and anti-BiP antibodies. The anti-HA antibody was visualized with rhodamine-coupled secondary antibody (*top*), the anti-BiP antibody was visualized with FITC-coupled secondary antibody (*bottom*).

NH_2-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-

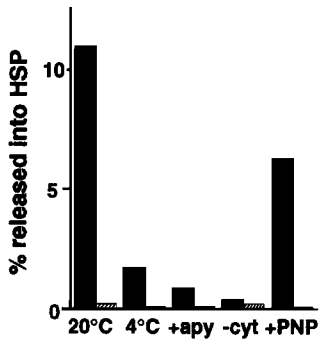

Figure 6. Sed4p-HA is not incorporated into ER-derived vesicles. Vesicles were produced in vitro using ER membranes from a *sed4-Δ1* strain containing *SED4-HA* on a low copy plasmid (CKY295) (see Materials and Methods). The amount of Sed4p-HA or Sec22p in the starting membranes or the vesicle-containing HSP was determined by Western blotting and quantitated by densitometry. The ratio of protein in the HSP to protein in the starting membranes is expressed as percent release. ■, Sec22p; ▨, Sed4p.

tem (Fields and Song, 1989; Gyuris et al., 1993). The NH_2-terminal domain of *SED4* was fused to an acidic transcription activation domain and tested for interaction with each of three overlapping parts of *SEC16* fused to the *lexA* DNA-binding domain. Interaction was scored by the ability of the *lexA* 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 NH_2-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 NH_2-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 Sec16p.

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 NH_2-terminal domains of Sed4p and Sec12p were tagged with the myc epitope and expressed from the *GAL10* 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 NH_2-terminal domain or the Sed4p NH_2-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*a*; 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*. Sed4Np-Myc, but not Sec12Np-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 Sed4Np-Myc was dependent on the presence of Sec16Cp 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.

Table III. NH_2-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 pPE58 (*SEC16C*), pPE59 (*SEC16N*), pPE74 (*SEC16CEN*), pRH151 (*SED4N*), and pRH152 (*SEC12N*).

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-Δ1*). Previously, no growth or secretion defect was found in a *SED4* disruption strain (Hardwick et al., 1992). Consistent with these data, *sed4-Δ1* 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-Δ1* strains did not grow at 41°C, although wild-type strains grew slowly at this temperature. This growth defect of *sed4-Δ1* 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). Complementation of *sed4-Δ1* at 41°C provided another test of *SED4* function and was also used to establish the importance of the NH_2-terminal domain (Fig. 3).

We examined the kinetics of secretion of CPY in *sed4-Δ1* 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 vacuolar (*m*) form after 8 to 10 min of chase (Fig. 8 *A*, lanes *7–12*). In *sed4-Δ1*, 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 p1 CPY to mature form (Fig. 8 *B*) gave a half-life of p1 CPY of 7.1 min in a *sed4-Δ1* strain compared with 4.4 min in wild-type cells. This transport defect in *sed4-Δ1*, although subtle, was highly reproducible, and a 1.6- to 2-fold lower transport rate from the ER to the Golgi of *sed4-Δ1* cells was found in four independent experiments.

Deletion of SED4 Exacerbates Vesicle Formation Mutations

Synthetic lethal interactions between genes that affect the

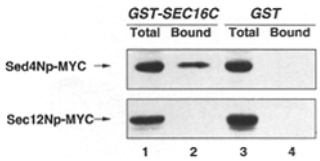

Figure 7. The NH_2-terminal domain of Sed4p and the COOH-terminal domain of Sec16p bind in extracts. The COOH-terminal domain of Sec16p fused to GST (pPE122, lanes *1* and *2*) or GST only (pRD56, lanes *3* and *4*) were expressed in yeast strain CKY289 together with either myc-tagged NH_2-terminal domain of Sed4p (pRH260, *top* row) or myc-tagged NH_2-terminal domain of Sec12p (pRH261, *bottom* row). Tagged proteins were detected by Western blotting after SDS-PAGE on an 8% gel. Lanes *1* and *3*: total extracts from 0.05 OD_{600} U of cells. Lanes *2* and *4*: material bound to glutathione beads from 0.4 OD_{600} U of cells.

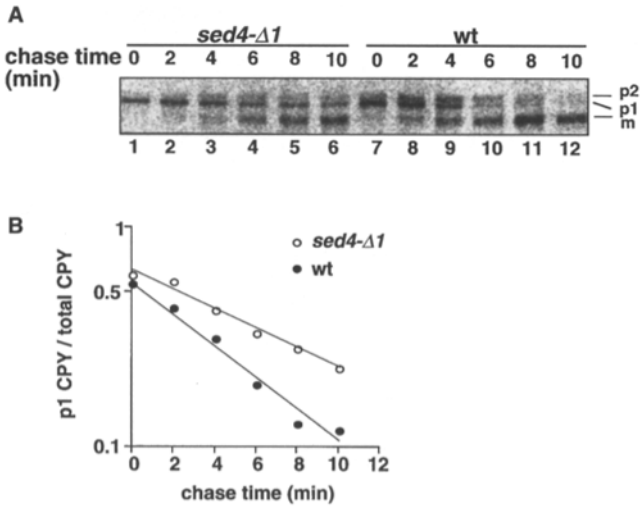

Figure 8. Deletion of *SED4* slows transport of CPY from the ER to the Golgi. (*A*) Pulse–chase analysis of CPY in a *sed4-Δ1* strain (CKY251, lanes *1–6*) or a wild-type strain (CKY252, lanes *7–12*). Cells grown at 38°C were pulse labeled with [^{35}S]methionine for 5 min. The label was chased for the times indicated. The different forms of CPY were immunoprecipitated from extracts and visualized on a phosphorImager after SDS-PAGE. (*B*) Determination of kinetic parameters. The amount of radiolabeled p1 CPY and total CPY was quantitated for each time point using the PhosphorImager software. The ratio p1 CPY/total CPY gives the half-life of p1 from a linear curve-fit on a semilogarithmic plot using Cricket graph (v. 1.0). The half-life of p1 in a *sed4-Δ1* strain is 1.6-fold > wild-type in the experiment shown. Similar results were obtained in four independent experiments.

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*, *sec2-41*, *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. Tetrad 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

Table IV. Deletion of SED4 Exacerbates the Growth Defect of Mutants Defective in Vesicle Formation

Genotype	Incubation temperature		
	28°	30°	33°
Vesicle formation			
sec12-4	+++	+	−
sec12-4 sed4-Δ1::URA3	±	−	−
sec13-1	+++	++	−
sec13-1 sed4-Δ1::URA3	+	±	−
sec16-2	+++	±	−
sec16-2 sed4-Δ1::URA3	±	−	−
sec23-1	+++	−	−
sec23-1 sed4-Δ1::URA3	−	−	−
Vesicle fusion			
sec17-1	+++	+++	±
sec17-1 sed4-Δ1::URA3	+++	+++	±
sec18-1	+	−	−
sec18-1 sed4-Δ1::URA3	+	−	−

Growth of single colonies on YPD after 24–48 h. +++, growth comparable to wild type; −, no growth.

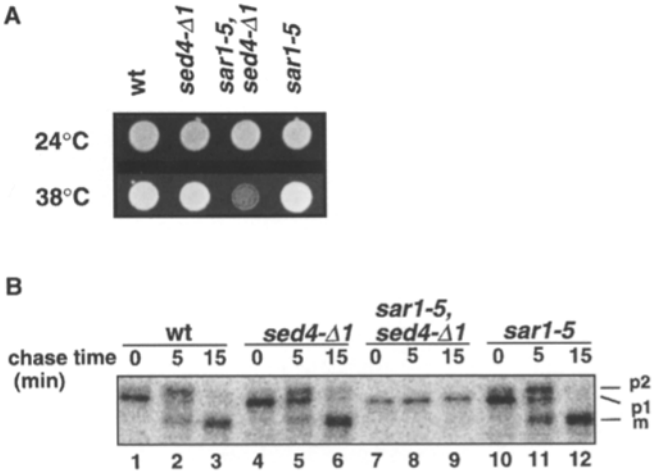

Figure 9. Deletion of *SED4* in combination with a mutation in *SAR1* causes a Ts growth and secretion defect. (*A*) Wild-type (CKY291), *sed4-Δ1::URA3* (CKY292), *sar1-5 sed4-Δ1::URA3* (CKY293), and *sar1-5* (CKY294) cells were spotted on rich medium and incubated for 40 h at 24 or 38°C. (*B*) CPY transport 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 [^{35}S]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 *SAR1* plasmid. The DNA sequence of *sar1-5* 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 sar1-5 Double Mutant Accumulates ER Membranes but Not Vesicles

The finding that deletion of *SED4* in a *sar1-5* 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 sar1-5* 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 sar1-5* double-mutant cells accumulated excess ER membranes, visible as extra layers of membrane throughout the cell (Fig. 10). To determine whether *sed4-Δ1:: URA3 sar1-5* 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 sar1-5* cells was 7.4 ± 1.1. This value is

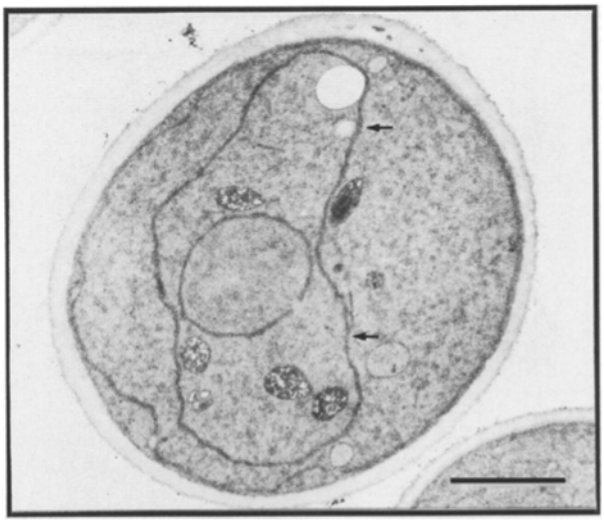

Figure 10. A *sed4-Δ1 sar1-5* double mutant accumulates ER membranes, but not vesicles. Electron micrograph of *sed4-Δ1:: URA3 sar1-5* (CKY293) grown at 25°C and shifted to 38°C for 2 h. Membranes were stained with potassium permanganate. *Arrows*, excess ER. Bar, 1 μm.

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/μm^3 cell volume). This result implies that the *sed4-Δ1:: URA3 sar1-5* 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.

sar1-5 Mutation Disrupts Interaction of SAR1 with SEC16 but Not SEC12

An important test for *SAR1* function is the ability to suppress mutations in other *SEC* genes. *SAR1* was first isolated because overexpression of *SAR1* suppresses *sec12* mutations (Nakano and Muramatsu, 1989). Overexpression of *SAR1* 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 *sar1-5* mutation, we tested *sar1-5* expressed from either a low centromere or a high (2μ) copy plasmid for the ability to suppress different *sec* mutations. The *sar1-5* mutation disrupted the interaction of *SAR1* with *SEC16* and *SEC23*, since *sar1-5* on either low or high copy plasmids did not suppress *sec16-2* or *sec23-1* mutations (Table V). In contrast, *sar1-5* suppressed the temperature-sensitivity of *sec12-4* to the same degree as wild-type *SAR1* (Table V). Thus, the *sar1-5* allele allowed the function of *SAR1* needed to suppress *sec12* mutations to be distinguished from the function(s) needed to suppress *sec16* and *sec23* mutations.

In tests of *sar1-5* for synthetic lethal interactions, *sar1-5* exacerbated the temperature sensitivity of *sec16-2*, *sec13-1*,

Table V. Genetic Interactions of sar1-5 with Vesicle Formation Mutants

Genotype	Incubation temperature				
	24°	27°	30°	33°	38°
sec12-4	+++	+++	+	−	−
sec12-4 sar1-5	+++	+++	+	−	−
sec12-4 (pSAR1)	+++	+++	+++	+++	+++
sec12-4 (psar1-5)	+++	+++	+++	+++	+++
sec13-1	+++	+++	++	−	−
sec13-1 sar1-5	+++	±	−	−	−
sec16-2	+++	+++	±	−	−
sec16-2 sar1-5	+++	±	−	−	−
sec16-2 (pSAR1)	+++	+++	++	++	−
sec16-2 (psar1-5)	+++	+++	±	−	−
sec23-1	+++	+++	−	−	−
sec23-1 sar1-5	++	++	−	−	−
sec23-1 (pSAR1)	+++	+++	++	−	−
sec23-1 (psar1-5)	+++	+++	−	−	−

pSAR1 is pRH259 or pRH280. *psar1-5* is pRH262 or pRH279. Growth of single colonies on YPD after 24–48 h. +++, growth comparable to wild type; −, no growth.

and *sec23-1* (and of *sed4-Δ1* as described above), but had no effect on the growth of *sec12-4* (Table IV). Again, these results indicate that *SAR1* has at least two different functions. One function involves interaction with *SED4*, *SEC16*, and *SEC23* and is disrupted by *sar1-5*, while the other function involves interaction with *SEC12* and is not affected by *sar1-5*.

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 *sar1-5* 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 Muramatsu, 1989), and *sec16-2* is lethal at 27°C when combined with *sar1-5*. *SAR1* also interacts genetically with *SED4*. We show that increased dosage of *SAR1* suppresses the temperature sensitivity caused by *sed4* deletion, while combination of *sar1-5* and *sed4-1* causes a strong transport block. These multiple genetic interactions argue that the functions of Sec16p, Sed4p, and Sar1p 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 COPII proteins Sec13p/Sec31p, Sec23p/Sec24p, and Sar1p (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 Sar1p 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 COPII protein, Sec23p, binds to the COOH-terminal domain of Sec16p. The genetic interactions between *SEC16* and

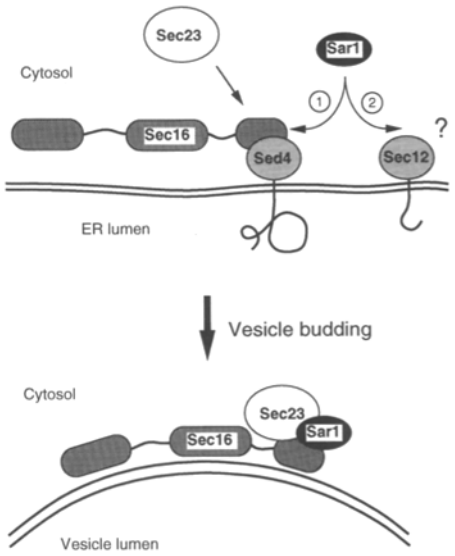

Figure 11. Proposed protein interactions early in vesicle formation. Interaction of Sar1p with Sec16p, Sec23p, and Sed4p (*1*) is interrupted by *sar1-5*. Interaction of Sar1p with Sec12p (*2*) is not affected by *sar1-5*. Interaction of Sec16p with Sed4p and Sec23p is based on genetic interactions and binding studies.

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 (Yosihisha 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 NH_2-terminal domain of Sec12p has been shown to have such activity (Barlowe and Schekman, 1993), but parallel experiments using the partially purified NH_2-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 NH_2-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 compex. Sec12p has been proposed to act similarly in the initial phases of vesicle formation by recruiting Sar1p to the membrane (d'Enfert et al., 1991*b*). 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 NH_2-terminal domains of Sec12p and Sed4p are also different: the NH_2-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 *SAR1* 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 *SAR1* 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 *SAR1*, 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 *SAR1* engages in two independent processes, and that *sar1-5* selectively disrupts the processes that involve *SEC16*. The two ways that *SAR1* 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*, *SAR1*, 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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