SCD5, a Suppressor of Clathrin Deficiency, Encodes a Novel Protein with a Late Secretory Function in Yeast

Karen K. Nelson, Michelle Holmer, and Sandra K. Lemmon*

Department of Molecular Biology and Microbiology, Case Western Reserve University School of Medicine, Cleveland, Ohio 44106-4960

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Clathrin and its associated proteins constitute a major class of coat proteins involved in vesicle budding during membrane transport. An interesting characteristic of the yeast clathrin heavy chain gene (*CHC1*) is that in some strains a *CHC1* deletion is lethal, while in others it is not. Recently, our laboratory developed a screen that identified five multicopy suppressors that can rescue lethal strains of clathrin heavy chain-deficient yeast (*Chc⁻ scd1-i*) to viability. One of these suppressors, *SCD5*, encodes a novel protein of 872 amino acids containing two regions of repeated motifs of unknown function. Deletion of *SCD5* has shown that it is essential for cell growth at 30°C. *scd5-Δ* strains carrying low copy plasmids encoding C-terminal truncations of Scd5p are temperature sensitive for growth at 37°C. At the nonpermissive temperature, cells expressing a 338-amino acid deletion (Scd5p-Δ338) accumulate an internal pool of fully glycosylated invertase and mature α -factor, while processing and sorting of the vacuolar hydrolase carboxypeptidase Y is normal. The truncation mutant also accumulates 80- to 100-nm vesicles similar to many late *sec* mutants. Moreover, at 34°C, overexpression of Scd5p suppresses the temperature sensitivity of a *sec2* mutant, which is blocked at a post-Golgi step of the secretory pathway. Biochemical analyses indicate that ~50% of Scd5p sediments with a 100,000 $\times$ g membrane fraction and is associated as a peripheral membrane protein. Overall, these results indicate that Scd5p is involved in vesicular transport at a late stage of the secretory pathway. Furthermore, this suggests that the lethality of clathrin-deficient yeast can be rescued by modulation of vesicular transport at this late secretory step.

INTRODUCTION

The transfer of proteins along the secretory and endocytic pathways occurs by vesicular transport. The formation of transport vesicles involves the recruitment of coat proteins to the cytoplasmic face of the membrane to facilitate budding. In addition, such coat proteins can mediate the selection of vesicle cargo. A number of different types of coated vesicles have been characterized including clathrin-coated vesicles (Pearse and Robinson, 1990), COP-coated vesicles (Rothman and Orci, 1992), and COPII-coated vesicles (Barlowe *et al.*, 1994). Clathrin and its associated proteins play a key role in the selective con-

centration and sorting of proteins during receptor-mediated endocytosis (for reviews, see Brodsky, 1988; Pearse and Robinson, 1990). In addition, they are involved in sorting of lysosomal hydrolases via mannose-6-phosphate receptors from the *trans*-Golgi network to a pre-lysosomal compartment (Campbell and Rome, 1983; Brown and Farquhar, 1984) and they are involved in the formation of secretion granules, e.g. in hormone or neuropeptide-secreting cells (Tooze and Tooze, 1986; Burgess and Kelly, 1987).

Although most early information on clathrin coat protein structure and function was derived from biochemical and cytological studies using animal cells, in the last several years yeast has provided genetic approaches to the study of the clathrin-mediated vesicular transport machinery. The yeast clathrin heavy

* Corresponding author.

chain (HC) and light chain (LC) have been purified, the genes have been cloned and sequenced (Payne and Schekman, 1985; Lemmon and Jones, 1987; Lemmon *et al.*, 1988, 1991; Silveira *et al.*, 1990), and gene deletions have been generated. In addition, several genes encoding proteins with homology to associated protein subunits have been characterized (Kirchhausen, 1990; Kirchhausen *et al.*, 1991; Nakayama *et al.*, 1991; Phan *et al.*, 1994; Rad *et al.*, 1995; Stepp *et al.*, 1995). It is striking that in some strains a clathrin HC gene (*CHC1*) deletion is lethal, while in others it is not (Payne and Schekman, 1985; Lemmon and Jones, 1987; Munn *et al.*, 1991). In strains studied in this laboratory, this difference results from an independently segregating gene, called suppressor of clathrin deficiency (*SCD1*) (Lemmon and Jones, 1987). In the presence of the *scd1-i* allele, cells lacking clathrin HCs (*Chc*⁻) are inviable; in the presence of the *scd1-v* allele, *Chc*⁻ cells are viable (Lemmon and Jones, 1987).

Viable strains of *Chc*⁻ yeast (*chc1-Δ scd1-v*) exhibit a number of characteristic phenotypes. They grow slowly at 30°C and are temperature sensitive at 37°C (Payne and Schekman, 1985; Lemmon and Jones, 1987). *Chc*⁻ haploids are genetically unstable. These cells tend to acquire faster growing segregants (Lemmon *et al.*, 1990) and their genome copy number increases, apparently resulting from aberrant nuclear divisions. *Chc*⁻ cells are also larger, rounder, and contain multivesiculated secretory organelles; they grow in aggregates, and they display a dramatic increase in the thickness of their cell wall (Lemmon and Jones, 1987; Payne *et al.*, 1987; Nelson, 1994).

The yeast HC is required for the retention of several Golgi membrane proteins. For example, two late Golgi proteins, Kex2p and DPAP A, and a Golgi protein that resides in both medial and late Golgi compartments, α 1,3 mannosyltransferase, are mislocalized to the plasma membrane in *Chc*⁻ cells (Payne and Schekman, 1989; Seeger and Payne, 1992b; Graham *et al.*, 1994). Clathrin HC is involved in, but not essential for, the endocytosis of the seven transmembrane segment pheromone receptors (Payne *et al.*, 1988; Tan *et al.*, 1993). In addition, soluble vacuolar protein sorting is transiently affected by loss of clathrin function in a *chc1-ts* mutant (Seeger and Payne, 1992a).

Although *Chc*⁻ yeast are viable in the presence of the *scd1-v* allele, the availability of a genetic background in which *Chc*⁻ yeast are inviable provided the opportunity to apply suppression analysis to the study of clathrin. Multicopy suppressors of essential genes have been shown in a number of cases to exhibit a similar function or act in the same pathway as the gene they are able to suppress (Bender and Pringle, 1989; Dascher *et al.*, 1991; Hardwick and Pelham, 1992). Suppression analysis has been particularly useful in the analysis of the secretory pathway (Salminen

and Novick, 1987; Hardwick and Pelham, 1992; Hardwick *et al.*, 1992; Brennwald *et al.*, 1994).

We therefore devised a screen to isolate genes whose overexpression could suppress the lethality of inviable strains of clathrin-deficient yeast. Previously, we reported on our identification of five distinct genetic loci whose overexpression rescues *chc1-Δscd1-i* cells to viability (Nelson and Lemmon, 1993). One of the suppressor loci, *SCD2*, was shown to be *UBI4*, the polyubiquitin gene (Nelson and Lemmon, 1993). Considering ubiquitin's function in protein degradation and the fact that *Chc*⁻ strains have unusually small pools of free ubiquitin, our results suggested that overexpression of ubiquitin may rescue *Chc*⁻ cells by promoting the degradation of proteins that are mis-sorted or misprocessed due to lack of clathrin.

In the studies presented in this article, we report the characterization of another suppressor of clathrin deficiency gene, *SCD5*, which encodes a novel protein that is essential for viability of yeast. We also show that *Scd5p* is a peripheral membrane protein that plays a role in the late secretory pathway. These data suggest that modulation of vesicular transport at a late secretory step can also rescue *Chc*⁻ cells to viability.

MATERIALS AND METHODS

Strains, Media, and Genetic Methods

Strains used in this paper are listed in Table 1 and were derived from the S288C background (*gal2 SUC2*) (Mortimer and Johnston, 1986). YEP-dextrose (YEPD), YEP-gal, minimal synthetic medium (MV), and dropout media were prepared as described by Nelson and Lemmon (1993). Yeast mating, sporulation, and tetrad analyses were carried out as described (Guthrie and Fink, 1991). Bacterial strain DH5 α (Hanahan, 1983) was used for plasmid propagation. Yeast were transformed using the lithium acetate method (Ito *et al.*, 1983).

Plasmid Constructions

The isolation of the YEpSCD plasmids that can rescue the lethality of *Chc*⁻ *scd1-i* strains has been described previously (Nelson and Lemmon, 1993). Upon further characterization of the original six classes of high copy suppressors, *SCD7* and *SCD5* were shown to encode the same gene and, therefore, we refer to this locus as *SCD5*. The YEpSCD5 plasmid from the YEp24 library that was analyzed in the most detail is shown in Figure 1. YEp*scd5-Δ32* was constructed by ligating the 3.3-kb *Bam*HI-*Pst*I fragment of YEpSCD5 into YEp352 (Hill *et al.*, 1986). This encodes an *Scd5* protein with a 32-amino acid (aa) C-terminal truncation and an extension of 53 aa due to the in-frame insertion of the restriction fragment in the vector. YCp*scd5-Δ32* was constructed by ligating the 3.3-kb *Bam*HI-*Pst*I fragment of YEpSCD5 into pRS315 (Sikorski and Hieter, 1989). This encodes an *Scd5* protein with a 32-aa C-terminal truncation and an additional 27-aa extension coded for by vector sequences. YEp*scd5-Δ251* contains the 2.6-kb *Bam*HI-*Eco*RV fragment of YEpSCD5 ligated into YEp24 cut with *Bam*HI and *Sma*I. This yields a 251-aa truncation of *Scd5p* with an additional 4 aa coded for by vector sequences. The 1.7-kb *Eco*RI fragment of YEpSCD5 was ligated into the *Eco*RI site of YEp352 and pUN70 (Elledge and Davis, 1988), to create YEp*scd5-Δ338* and YCp*scd5-Δ338*, respectively. Both YEp*scd5-Δ338* and YCp*scd5-Δ338* encode 338-aa truncations of *Scd5p* with no additional aa. YCpSCD5, which contains the complete *SCD5* gene

Table 1. *Saccharomyces cerevisiae* strains

Number	Genotype
SL214	MATa GAL1:CHC1 <i>scd1-i leu2 ura3-52 trp1 his1 GAL2</i>
SL694	MATa/MATα CHC1/CHC1 <i>scd1-i/scd1-i leu2/leu2 trp1/trp1 ADE6/ade6 his1/HIS1 ura3-52/ura3-52 GAL2/gal2</i>
SL782	MATa/MATα CHC1/CHC1 <i>scd1-v/scd1-v leu2/leu2 trp1/trp1 ade6/ADE6 ura3-52/ura3-52 GAL2/gal2</i>
SL794	MATa/MATα CHC1/CHC1 <i>SCD5/scd5-Δ::TRP1 scd1-i/scd1-i GAL2/gal2 ura3-52/ura3-52 ADE6/ade6 his1/HIS1</i>
SL795	MATa/MATα CHC1/CHC1 <i>SCD5/scd5-Δ::TRP1 scd1-v/scd1-v GAL2/gal2 leu2/leu2 ura3-52/ura3-52 ade6/ADE6 trp1/trp1</i>
SL1089 ^a	MATα <i>scd5-Δ::TRP1 CHC1 scd1-i leu2 ura3-52 trp1 his1 YCpScd5-Δ338</i>
SL1517	MATα <i>scd5-Δ::TRP1 CHC1 pep4::HIS3 leu2 ura3-52 YCpSCD5-flu</i>
SL1719 ^a	MATα <i>scd5-Δ::TRP1 CHC1 scd1-i leu2 ura3-52 trp1 his1 YCpSCD5(LEU2)</i>
SL1720 ^a	MATα <i>scd5-Δ::TRP1 CHC1 scd1-i leu2 ura3-52 trp1 his1 YCpScd5-Δ32</i>
SL1818 ^a	MATα <i>scd5-Δ::TRP1 CHC1 scd1-i leu2 ura3-52 trp1 his1 YCpSCD5</i>
NY3	MATa <i>sec1-1 ura3-52</i>
NY17	MATa <i>sec6-4 ura3-52</i>
NY57	MATa <i>sec9-4 ura3-52</i>
NY61	MATa <i>sec10-2 ura3-52</i>
NY64	MATa <i>sec15-1 ura3-52</i>
NY130	MATa <i>sec2-41 ura3-52</i>
NY405	MATa <i>sec4-8 ura3-52</i>
NY402	MATa <i>sec5-24 ura3-52</i>
NY410	MATa <i>sec8-9 ura3-52</i>
NY412	MATa <i>sec3-2 ura3-52</i>
NY431	MATa <i>sec18-1 ura3-52</i>
XW451-3B	MATa <i>mn1-3</i>

All SL strains listed were generated for these studies except SL214 (Nelson and Lemmon, 1993). *sec* strains were from P. Novick and the *mn1* mutant was from C. Ballou.

^a These strains are an isogenic set and differ only by the plasmid they carry.

was constructed by ligating the 4.6-kb *Bam*HI–*Cla*I fragment of YEpSCD5 into the *Bam*HI–*Cla*I sites of YCp50 (Rose *et al.*, 1987). YCpSCD5(LEU2), which contains the complete SCD5 gene in a LEU2, *CEN* vector, was constructed by ligating the *Bam*HI–*Sph*I (in vector) fragment of YEpSCD5 into the *Bam*HI–*Sph*I sites of pUN100 (Elledge and Davis, 1988).

Transposon Mutagenesis

Insertions were isolated using the standard “λ hop” protocol previously described (Way *et al.*, 1984) and the transposon λ mini-Tn10-LUK (Huisman *et al.*, 1987). YEpSCD5 was used as the target plasmid. Transpositions into YEpSCD5 were specifically obtained from the total collection of transposition events by pooling Kan^R colonies, preparing plasmid DNA, transforming a λ-resistant bacterial strain, and selecting for Kan^R and Amp^R. These transformants were checked for sensitivity to tetracycline to ensure that the transposase-coding plasmid was no longer present and no further hopping would be possible. Final selection was done by digesting with *Bam*HI and *Sph*I to identify transformants in which transposition had occurred within the insert of YEpSCD5 and not in the vector. Approximately 85 transformants were screened, of which 23 were found to be in the insert. A sampling of these are shown in Figure 1.

Rescue Assay

To test for rescue of a *GAL1:CHC1 scd1-i* strain depleted of clathrin, a single colony of SL214 carrying the plasmid to be tested was inoculated into YEPD and grown at 30°C for 16 h before equal numbers of cells were plated on YEPD and YEP-gal plates. YEPD plates were incubated for 6 days, and YEP-gal plates were incubated for 4 days at 30°C. Percent rescue was calculated as follows: (number of colonies on glucose/number of colonies on galactose) × 100.

Sequencing SCD5

DNA sequencing was performed by the dideoxy-chain termination procedure (Sanger *et al.*, 1977), using the Sequenase sequencing kit (United States Biochemical, Cleveland, OH) and [α -³⁵S]dATP (Amersham, Arlington Heights, IL). The sequence was assembled and repeated motifs were identified using the MacVector sequence analysis software (International Biotechnologies, New Haven, CT). GenBank was searched by using the BLAST Server at NCBI (Atschul *et al.*, 1990). Prosite (Bairoch and Bucher, 1994) was searched using the Blocks program (Henikoff and Henikoff, 1994).

Gene Disruption

The integrating plasmid, YIpSCD5, was created by ligating the 6.5-kb *Bam*HI–*Sph*I fragment from YEpSCD5 into YIp5-ΔRI, a *URA3* integrating vector (Botstein *et al.*, 1979) in which the *Eco*RI site was deleted. The SCD5 deletion disruption construct (pMH28) was then made by replacing the 1.7-kb *Eco*RI fragment of SCD5 in YIpSCD5 with the 1.4-kb *Eco*RI *TRP1* fragment from pCEN3-*TRP1* (Futcher and Carbon, 1986). This deletes the SCD5 gene from 100 bases upstream of the start of translation to 1600 bases into the coding region, removing codons 1–532. pMH28 was cut with *Bam*HI and *Cla*I to release the *scd5-Δ::TRP1* fragment and transformed into diploid strains SL694 and SL782 to generate *scd5-Δ::TRP1/SCD5* heterozygotes SL794 and SL795, respectively. Integration was confirmed by DNA blot analysis.

Thin Section Electron Microscopy

Cells were prepared for electron microscopy as described by Govindan *et al.* (1995). Thin sections were analyzed on a JEOL 100CX transmission electron microscope at 80 kV.

Epitope Tagging *Scd5p*

A triple influenza hemagglutinin (flu) epitope (YPYDVPDYA) was introduced into *Scd5p* following amino acid 78. YCp-*SCD5-flu* was constructed by first digesting pSM491 (from Bruce Futcher, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) with *NotI* to release the 118-bp fragment that codes for the triple flu tag. This fragment was then blunted with the Klenow fragment of DNA polymerase and inserted into the *SacI* site of YCp-*SCD5*, which had been blunted with T4 DNA polymerase. In this way, the triple flu epitope was subcloned in the sense (YCp-*SCD5-flu*) and antisense (YCp-*SCD5-ulf*) orientations. In the flu-tagged protein, *Scd5p-flu*, one serine (normally aa 79) was replaced by GRIF(*flu*)G(*flu*)GS(*flu*)AQCQCG.

scd5-Δ strains carrying the YCp-*SCD5-flu* and YCp-*SCD5-ulf* plasmids grew as well as wild-type cells at all temperatures, indicating that this internal insertion did not interfere with the function of *Scd5p*. The anti-flu monoclonal antibody (12CA5) (Niman *et al.*, 1983) was shown to be specific because it recognized *Scd5p-flu* but not *Scd5p-ulf* on Western blots (our unpublished results). *Scd5p-flu*, which has a predicted molecular weight of 101,600, had an electrophoretic mobility of ~130 kDa on SDS polyacrylamide gels. The basis for this aberrant migration of *Scd5p* is not known.

Cell Fractionation

The cell fractionation protocol was adapted from Walworth *et al.* (1989). Yeast strain SL1517, which harbors plasmid YCp-*SCD5-flu*, was grown at 30°C to mid log phase, 10^9 cells were harvested, and cells were resuspended in 6 ml spheroplast buffer (1.2 M sorbitol, 50 mM KPi, pH 7.5, 10 mM Na₂N₃, 40 mM β-mercaptoethanol, and 50 U/10⁷ cells of Oxalyticase [ENZOGENETICS, Corvallis, OR]). Cells were spheroplasted for 30 min at 30°C and then protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 5 mM N-ethylmaleimide, 100 μM TPCK, 500 μM E64, 1 mM benzamide HCl, 25 μM pepstatin A, 4 μM leupeptin) were added. Spheroplasts were harvested by centrifugation at $900 \times g$ for 10 min and resuspended in 1.5 ml lysis buffer (0.8 M sorbitol, 1 mM EDTA, 10 mM triethanolamine) plus protease inhibitors. The spheroplast suspension was Dounce homogenized 20 times and the resultant lysate was centrifuged at $450 \times g$ for 3 min to remove unlysed cells. The supernatant (S450) was removed to a new tube. The pellet (P450) was washed once with 1.5 ml lysis buffer plus protease inhibitors, spun again at $450 \times g$, and the supernatant was combined with the previous S450. The P450 was resuspended in 3 ml lysis buffer plus protease inhibitors and set aside on ice. Two hundred microliters of the combined S450 were removed for immunoblot analysis and the remaining S450 was spun at $10,000 \times g$ for 10 min to generate a supernatant (S10k) and pellet (P10k) fraction. The P10k was resuspended in 2.8 ml lysis buffer plus protease inhibitors, and 200 μl of S10k were removed for immunoblot analysis. The S10k fraction was then subjected to a $100,000 \times g$ spin for 1 h, to generate a supernatant (S100k) and a pellet (P100k) fraction. The P100k fraction was resuspended in 2.6 ml lysis buffer plus protease inhibitors. Equal volumes of these fractions were separated on a 7.5% SDS polyacrylamide gel, and transferred to nitrocellulose for immunoblotting. *Scd5p-flu* was detected with culture supernatant of monoclonal cell line 12CA5 (Niman *et al.*, 1983) followed by horseradish peroxidase-conjugated goat anti-mouse IgG (1/10,000 dilution, Kirkegaard and Perry Laboratories, Gaithersburg, MD) and chemiluminescence detection (Amersham). Kex2p was detected with rabbit anti-Kex2p antibodies (gift of Robert Fuller, University of Michigan, Ann Arbor, MI) followed by horseradish peroxidase-conjugated goat anti-rabbit IgG (1/10,000 dilution, Zymed, San Francisco, CA) and chemiluminescence detection.

To determine the nature of the association of *Scd5p* with the P100k fraction, 10^9 cells of yeast strain SL1517 were subjected to the same fractionation procedure described above, except the S450 fraction was centrifuged directly at $100,000 \times g$ for 1 h. Equal aliquots of the P100k fraction were adjusted to 6 M urea, 1% Triton X-100, or

left untreated and incubated for 10 min on ice. The samples were then subjected to a $100,000 \times g$ spin for 1 h to generate a supernatant and a pellet fraction. Pellets were resuspended in lysis buffer to the same volume as the soluble fraction. Equal volumes of these fractions were loaded onto a 7.5% SDS polyacrylamide gel for immunoblotting with 12CA5 antibodies as described above.

Membrane-associated proteins were separated from insoluble protein aggregates and large sedimentable complexes by a sucrose flotation method (Horazdovsky and Emr, 1993). The P100k fraction generated as described for extraction above was suspended in 2.5 ml 60% sucrose (w/w) in 10 mM MES, pH 6.5, plus protease inhibitors, and transferred to the bottom of a Beckman 5.0 ml ultraclear tube. The 60% solution was overlaid with a 35% (w/w) sucrose solution (in 10 mM MES, pH 6.5, plus protease inhibitors) to a total volume of 5 ml. The sucrose step gradient was then centrifuged at $170,000 \times g$ for 18 h. Fractions (0.4 ml) were collected from top to bottom at a flow rate of 0.2 ml/min using an ISCO density gradient fractionator 640. Proteins were precipitated from each fraction with trichloroacetic acid at a final concentration of 5%, washed two times with acetone, resuspended in SDS sample buffer, and then separated on a 7.5% SDS polyacrylamide gel and transferred to nitrocellulose for immunoblotting. *Scd5p-flu* and Kex2p were detected as described above.

α-Factor Antiserum

α-Factor antiserum was produced using a β-galactosidase-α-factor fusion protein as an antigen (Ag). Protein was expressed in *Escherichia coli* carrying the pEX-α6 (a *lacZ* MfaI fusion construct) and purified on preparative SDS gels as described in (Rothblatt and Meyer, 1986). Antiserum was obtained from a rabbit that had been immunized with ~300 μg Ag in complete Freund's adjuvant, and then boosted 5 times with ~50 μg Ag in incomplete Freund's adjuvant at 3- to 4-wk intervals.

Pulse Labeling and Immunoprecipitation

Cells were grown to log phase at 25°C in MV plus requirements for nutritional markers. Cells, 8×10^7 (α-factor) or 4×10^7 (carboxy peptidase Y; CPY), were harvested by centrifugation, washed once, and resuspended in fresh growth medium (2.0 ml for α-factor or 1.1 ml for CPY). Cells were shifted to 37°C for 30 min and then bovine serum albumin (BSA) was added to 1 mg/ml. Labeling was initiated by addition of 135–160 μCi/ml TRANS³⁵S (ICN, Costa Mesa, CA). After a pulse of 5 min (CPY) or 7 min (α-factor) at 37°C, labeling was quenched by the addition of (NH₄)₂SO₄ to 3 mM and methionine and cysteine to 0.01%. After indicated chase periods, 485 μl (α-factor) or 200 μl (CPY) of labeled cells were removed and the chase was terminated by the addition of Na₂N₃ to 0.04% and placing samples on ice. BSA was added to all samples to a final concentration of 1 mg/ml.

For α-factor immunoprecipitation, samples were spun in a microfuge at $12,000 \times g$ for 1 min to separate cells and culture medium. Supernatant was transferred to a new tube and brought up to a volume of 500 μl and pellets were resuspended in 500 μl medium containing 1 mg/ml BSA. All samples were adjusted to 5% trichloroacetic acid and subjected to the double immunoprecipitation procedure of Klionsky *et al.* (1988) using 3 μl of α-factor antiserum. Samples were run on 12.5% SDS gels, and the gels were fixed in 15% methanol/10% acetic acid, soaked in 1 M salicylic acid, dried, and exposed to x-ray film at -90°C. Densitometry was performed on autoradiograms using a Sci Scan 5000 (United States Biochemical).

For pulse chase analysis of CPY, cells were sedimented and cell pellets were resuspended in 2% SDS. Cells were lysed by agitation with glass beads for 2 min, boiled, and diluted to 1.0 ml with immunoprecipitation buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.5% Tween 20, 0.1 mM EDTA) and 1 mg/ml BSA. Immunoprecipitations using anti-CPY antiserum (gift of E.W. Jones, Carnegie Mel-

Ion University, Pittsburgh, PA) were performed as described by Stepp *et al.* (1995).

Invertase Assay

Cells were grown to log phase at 25°C in c-ura (SL1089) or c-leu (SL1719) plus 5% glucose (Glc). Cells ($2-6 \times 10^8$) were washed once in c-ura or c-leu plus 0.05% glucose and then resuspended in 50 ml of c-ura or c-leu plus 0.05% glucose (to derepress synthesis of secreted invertase), which had been prewarmed at 37°C. Cultures were incubated for 1 h at 37°C and samples were removed at various times. At each time point, three volumes of cells were harvested (1 volume = $\sim 5 \times 10^7$ cells), resuspended in 10 mM NaN₃, stored on ice, and then processed for invertase assay as follows. One volume of cells was left untreated to assay for external invertase (unlysed cells). Glass beads were added to the second volume of cells and then the sample was lysed by vortexing on high for 3 min to assay for total invertase activity. The third volume of cells was washed once in spheroplast buffer (1.2 M sorbitol, 50 mM potassium phosphate, pH 7.5, 10 mM NaN₃) and resuspended in spheroplast buffer plus β -mercaptoethanol (4 μ l/ml) and Oxalyticase (100 U/ 10^7 cells). Samples were incubated for 1 h at 37°C and then sedimented in a microcentrifuge to remove external (periplasmic) invertase. Spheroplasts were washed twice in spheroplast buffer and resuspended in 1% Triton X-100. Glass beads were added and the sample was lysed by vortexing on high for 3 min. The lysed spheroplasted sample was used for determination of internal invertase. All samples were assayed for invertase activity as described by Goldstein and Lampen (1975). One unit of invertase activity is defined as the amount of enzyme at pH 4.9 that hydrolyzes sucrose to produce 1 μ mol of glucose per min at 30°C. Activity is expressed as milliunits per milligram total protein. Protein was determined on the total lysed samples by the bicinchoninic acid protein assay (Pierce, Rockford, IL). The percentage of the internal pool of invertase was calculated by dividing the internal invertase activity by the sum of the activities of the internal and external invertase. The sum of the internal and external invertase activity was similar to the activity of the total invertase control sample.

The glycosylation of invertase was examined by native gel electrophoresis followed by staining for invertase activity. Cells were grown to log phase in complete synthetic medium (c) + 5% Glc (BJ3638 and BJ3748), c-ura + 5% Glc (SL1089), or c-leu + 5% Glc (SL1719). Cells (10^8) were harvested, washed once in c + 0.05% Glc, c-ura + 0.05% Glc, or c-leu + 0.05% Glc, and resuspended in 10 ml of medium containing 0.05% Glc prewarmed to 25°C or 37°C. Cells were incubated at 25°C or 37°C for 1 h. Protein extracts were prepared as described (Carlson *et al.*, 1981) and were immediately fractionated on a 4.5% polyacrylamide gel as described (Laemmli, 1970), except SDS was omitted. Electrophoresis was carried out at 4°C until the tracking dye reached the bottom of the gel. Invertase activity was detected by incubating the gel in 0.1 M sucrose, 0.1 M sodium acetate, pH 4.6, at room temperature for 15 min and then staining with 0.1% 2,3,5 triphenyl tetrazolium chloride in 0.5 N NaOH at 100°C (Gabriel and Wang, 1969).

RESULTS

SCD5 Encodes a Novel Protein with Two Repeated Motifs

YEpSCD5 was previously identified as a high copy plasmid that could rescue inviable strains of clathrin HC-deficient yeast to viability (Nelson and Lemmon, 1993). The minimum rescuing region in YEpSCD5 was narrowed by subcloning regions of the insert and by Tn mutagenesis (Figure 1), followed by assaying the new plasmids for their ability to

restore growth of a *GAL1:CHC1 scd1-i* strain (SL214) on glucose. The minimal rescuing region was a 3.3-kb segment between the *Bam*HI and *Pst*II sites. Although this fragment gave full rescue of *GAL1:CHC1 scd1-i* yeast on glucose, subclones or Tn insertions demarcating smaller segments also gave at least partial rescue (see below).

Although high copy YEpSCD5 could rescue clathrin-deficient yeast from lethality, SCD5 could not rescue when present in a low copy *CEN* vector (YCpSCD5) (Figure 1). This suggested that SCD5 is not allelic to the *SCD1* locus, because previous genetic analysis indicated that *scd1-v* should rescue *Chc⁻ scd1-i* yeast in single or low copy (Nelson, 1994). Furthermore, segregation analysis, performed as described previously for SCD2 (Nelson and Lemmon, 1993), confirmed that SCD5 is not allelic to *SCD1* (our unpublished results).

Sequencing in from the ends of the YEpSCD5 insert revealed that the 5' end of the region codes for the C-terminal end of the *MIP1* gene, which encodes the catalytic subunit of a mitochondrial DNA polymerase (Foury, 1989). This indicated that the SCD5 gene is just downstream of *MIP1* (on chromosome XV). The SCD5 region was sequenced and found to contain an open reading frame for a protein of 872 aa with a predicted molecular weight of 97,300 kDa and a pI of 7.6 (Figure 2). A search of GenBank did not reveal homology to any known proteins. The predicted SCD5 product is rich in serine (11.7%) and the C-terminal end (last 339 aa) of the protein is rich in asparagine (12.4%), proline (11.5%), and glutamine (21.2%). Scd5p has no obvious N-terminal signal sequence and there are no other particularly hydrophobic regions, indicating that it is not likely to be an integral membrane or secreted protein. Scd5p has two regions of notable repeated motifs (see Figure 3); one region contains a 20-aa sequence repeated three times and the other shows a 12-aa sequence repeated nine times. However, neither of the repeats match any motifs found in the Prosite data base. These repeated motifs contain conserved Ser and Thr residues and may therefore be targets for phosphorylation.

As illustrated in Figure 1, some plasmids containing Tn insertions within the coding region of SCD5 were able to rescue *GAL1:CHC1 scd1-i* strains on glucose. These plasmids were only screened for growth and not for percentage of rescue and, therefore, it remains possible that they do not rescue as well as the full length gene. However, the 3.3-kb *Bam*HI-*Pst*II fragment (YEp*scd5*- Δ 32), which did rescue as well as full length SCD5, encodes a 32-aa truncation of Scd5p. Furthermore, partial rescue was obtained by a plasmid encoding a 251-aa truncation of Scd5p (YEp*scd5*- Δ 251), indicating that Scd5 proteins containing large C-terminal deletions retain function to some extent in the clathrin HC-deficient yeast rescue assay.

Figure 1. Restriction map of *SCD5* and growth rescue by *SCD5* constructs. Rescue of *GAL1:CHC1 scd1-i* on glucose: DNA fragments from YEpsCD5 were subcloned into the indicated vectors and tested for the ability to rescue growth of SL214 (*GAL1:CHC1 scd1-i*) on glucose as described in MATERIALS AND METHODS. Tn insertion mutagenesis of YEpsCD5 was also used to narrow down the rescuing region (triangles). The rescue of SL214 on glucose was scored based on the % rescue with +++ indicating > 50% rescue, -/+ indicating ~10% rescue, - indicating < 1% rescue, and ND indicating not determined. The transposon insertions were only screened + or - and not for degree of rescue. Rescue of *scd5-Δ::TRP1* knockout in a *Chc⁺* host: An *scd5-Δ/SCD5* diploid strain (SL794) was transformed with single- and multi-copy plasmids carrying *SCD5* truncations as shown. Transformants were sporulated, tetrads dissected, and spores grown up at room temperature. Growth of *Trp⁻* spore clones (*scd5-Δ::TRP1*) at 30°C was taken as evidence that the plasmid rescued *scd5-Δ* lethality. Rescue of *scd5-Δ* was scored based on growth of the strain relative to wild type with +++ indicating wild-type growth and - indicating no growth. Restriction endonuclease sites: B, *Bam*HI; C, *Cla*I; K, *Kpn*I; P, *Pvu*II; Pst, *Pst*I; R, *Eco*RI; S, *Sac*I; V, *Eco*RV; and X, *Xba*I. Stippled box below the coding sequence, 12-aa repeat region; open box, 20-aa repeat region.

SCD5 Is Essential for Cell Growth at 30°C

A gene deletion was generated in which the 1.7-kb *Eco*RI fragment of the *SCD5* gene was replaced with the *TRP1* gene (see Figure 1). The disruption was generated in *CHC1/CHC1 SCD5/SCD5* diploid strains of either the *scd1-i/scd1-i* (SL794) or *scd1-v/scd1-v* (SL795) genotype and tetrads were dissected for phenotypic analysis of haploid progeny. At 30°C, all tetrads (n = 99) yielded two viable (*Trp⁻*) and two inviable spores. However, at 23°C all tetrads (n = 32) yielded two large (*Trp⁻*) colonies and from zero to two very small spore clones. These small colonies presumably contained the *scd5-Δ::TRP1* allele, but they did not grow well enough on synthetic medium for absolute confirmation. These results indicate that *SCD5* is essential at normal growth temperatures in strains of either *scd1-i* or *scd1-v* genotype, but very slow growing spore segregants can survive at 24°C.

SCD5 Truncation Mutants Are Temperature-Sensitive for Growth

Full length clones and deletions of *SCD5*, cloned into high and low copy vectors, were tested for their ability to rescue the lethality of the *scd5* null allele. As shown in Figure 1, three *Scd5p* truncations (Δ32, Δ251, and Δ338) rescued *scd5-Δ* at high efficiency when encoded by a multicopy vector (YEps). The *scd5-Δ* strains carrying low copy (YCp) versions of the Δ32 and Δ338 truncations grew reasonably well at room temperature (see Figures 1 and 4) or 30°C (Nelson, unpublished observations), although not quite as well as an *scd5-Δ* strain carrying YCpSCD5 or high copy versions of the truncation plasmids. However, the *scd5-Δ* strains carrying the Δ32 and Δ338 truncation mutations in low copy grew very poorly or were essentially dead at 37°C (Figure 4). Doubling times of *Scd5p* C-terminal deletion mutants grown in liquid YEPD at 25°C were 2.5–3 h (Figure 4A), which was only slightly slower

GCAAAATCTGCATTAGCTTTTCGCATTGTATTATATATACAGCACATTATAATATTATTTTATTTTATTTTCTACTTACTTTTTCGCCAAATGAGA	100
AAACAAAAGTAAGTTGCCCTTATTAGAAAAAGAGTTAACATTAGGAAACGGGGACAAACAAAAGGTGAATTCAAAATAGGAATTCAAAACCCCAATTA	200
TCGTGCATCTTCTCGTTAGCTTAAGCTTTTATAGATCATTTGCAGGCTCGATAAAATTTACATGGTAACGAAACATGTCGTTTGATTGGCTTAATGTTCCGG	300
	9
GATTGGACTTAAGCAGCGGGGACCAAGCAGAAAAAGGCCATCGAATGGATTAGGACCACCCAGTGTGCTTTTGGATTTTGGTATAAATCTGCTGCTCC	400
G L D L S S G D Q A E K R P S N G L G P P S V S F D F G I N T A A P	43
ACATGATTCAGCTTTTGGGATCAGGGATCAAGAAGTCACAGTGATACAACCTTTGTCTTACAGAAAATACACTCAAATACAGCAGCTGATAATGCCACT	500
H D S S F W D Q G S R S H S D T T L S Y R N N H S N T A A D N A T	76
AACGTGAGCTTCCTCAAAGGATAATCCACCTAATGGGGAGGTACGAACACTTTCTGGAGGCGATGTGTACGCGGAGTACCAGAAGACATGCAAGTCC	600
N V S S P Q K D N P P N G E V R T L S G G D V Y A E S P E D M Q V	109
CATTGTCTTTATCAAAAACCACTTACCCATGAAGAAATAAGGCATATCTAAGATGGTACCACATATATGTTTGAACCCATGGAAAACTAGTAAG	700
P L S L S Q N Q L T H E E I R T Y L R W Y H Y I C L R T H G K L V R	143
ATTGAACGACGTGTTAGTTCCTTACCAATTTTAAATTTATCAGAAAAGTAAAGATAGAATTGTGGAATTTTCGCTAGCTGTAAATTCGGTTAAAT	800
L N D V F R F L T N F N L S Q K V K D R I V E I F R S C K N A L N	176
ATTGGTCAATTTTTCGCTGTTTAAAGATTAGTTCTAGAGCTATTATATATGTCATCTTACCTCTAAGACGCATGATCTTGGAAAAAGCACCTGTTCCCA	900
I G Q F F A V L R L V S R A I I Y G I L P L R R M I L E K A P V P	209
AACCACGCTCTATCTTAAGTAGTGAGAATCAGGAAGGTTACGAGGAGGTGGAAGACGATGATAGTTCTGCCAAAACCTGGTATCAAAAGGTGATTT	1000
K P R P I L S S E N H E E V Y E E V E D D D S S A K T K V D F	243
TGACTCATTTGCTTCATTGCTGTTGACTGGTAAGACAACGAGAAAAAGAGTCAGGAGAAGAATCAAAAATTTGAATTTTAAAGTAGAAGGTAGTGT	1100
D S F A S L L L T G K T T R K R V R R I K N L N F K S K K V R F	276
TCAGAGCATATAACATTTCAAGACCCCTCAAACCTCAATCAAGAATCATTAATAATAGTGAAGCAAGAAAGCAAGATCCTGATGCAGAAAGATGAAGATC	1200
S E H I T F Q D P P N L N Q E S S N N S E A R K Q D P D A E D E D	309
AGGATTCATAATGACAGTCTTTGGATTTCACATTGCCCATGGATCAATTATTAAGAGGCTATATAAGCGGAGAAAAACAGCGGATAGTTTCGAG	1300
Q D S N N D S P L D F T L P M D Q L L K R L Y K R R N S G L V S S	343
TTTGCCCGCAACAACAAGAACTGAGGAGGAGAAGAGTTTGGAGGATTAAGAGGATTCCTTGTCCCATTTTCAACAAATACAGACTGTTGATTCT	1400
L P S E Q Q E T E E E K K V L E D M K D S L S H F K Q I Q T V D S	376
GCCTCATTTGCCAATTTTATCGGTATTTTACAGAATGGCAATCTCTACCAACTAGCAATGTGAATAACACCACTGTTCCACAGCAACTTCCGTTAGAAC	1500
A S L P I S S V F L Q N G N T L P T S N V N N T T V P Q Q L P L E	409
CTTTGAAGCAACCGCCACAGGTTACGCTAATCACTGGTCCGAGAAGAATATAATCAGGGGTTGCATCCAGCAACCGTGCATCCAAACAGGTTTACA	1600
P L K P T A T G S A N H L V R E E Y N Q G L H P S N G A I Q T G L Q	443
ACCTTTAAACCAACAGCAACGGGCTCTGCAAAATTTATGATGCGAAGTCATATGGAACAACCTCAGTCTATCAAACTTTCGAGTACACAGAACAGTT	1700
P L K P T A T G S A N Y L M R S H M E Q P Q S P S T P E T V	476
ACAACTCTGGGGACTTCAACCTTTGAAACCTACCGCCACAGGTTCTGCAAAATTAATGATGAACAGCATATCTCACCTTCAGTTAATAACCCAGTAT	1800
T N S G G L Q P L K P T A T G S A N Y L M K Q H I S P S V N N P V	509
CTTCGATGTTTCAGGCGCAATTTACGAACCAATCTTCATCTCCACAAAGTACAGGACCGCATTTCTGAATTCCTCGCAACATCTTCTCCTCAAGCAA	1900
S S M F Q A Q F T N Q S S S P Q S T G P A F L N S P N I T L P O S N	543
TCAACAGCAACCTTACCAAGAGGTTAATCCGACTCAAGCGAAAAATGAACCATCTAACATATCGCCTCAACACACTACTCTAACATATGTTGGGATTAAT	2000
Q Q Q P Y Q E V N P T Q A K I E P S N I S P O H T Y S N N V R I N	576
AATGGTAACATCGTATCCATGCCAAGTAGAATAAATGTTGCAATCCCCCTCAAAACACTCTTCTCAACATCAGCAATCGCATTGCTTTCTCTCTC	2100
N G N I V S M P K V E I T G A F P P O N T L P O H O O S H L L S P	609
AAAATACGATCCCGCAGCACCAACGGTCCGAATTTGATATCTCCACAAAATACCTTCACTCAAAATCAGCCCATCTTCTCTCGCAACATCTATTCCAA	2200
Q N T I P O H O R S Q L I S P O N T F T O N O P I L S P O H T Y S N	643
TAATCAAGCAACAATGATATCCCCCAGAAATACGTATATAATCAACAGCAGCCCCAACATCTTCCACCTCTCCACCTCCACGCGTCAGCAGCAG	2300
N Q A T M I S P O N T Y T N N Q O Q P Q H L P P P P P P R A Q Q Q	676
CAACAAGGGGCCATTGTGCCACCTCAACATATGTACTCTAACGTGCAAAAGCAGAATAATTTGGTGCCGACCAACCTCTATACAAATTCACCAAGCA	2400
Q Q G A I V P P O H M Y S N V O K Q N N L V P T Q P S Y T N S P S	709
TACAATCTCAAAATTTTGTCACTCAGATGCTGCTAATAGTTACTTTCAATCTTACTTTCTTCTTCCATCTCCAAATCTACGCCATCAAAATGC	2500
I Q S P N F L S P O N A A N S Y F Q S L L S S P S P S P T P S N A	743
GTCTACTGTTAATGGCAATAACGCCAGTAATGGCATAAGTTTCAATCCAAATACTAGTGCGGCAATGAACAATACTCAATCTCATCAGACGTATATTAG	2600
S T V N G N N A S N G I S S F Q N T S A A M N N T Q S H Q T Y I Q	776
CAGCAACAACAACAACCACTCAACAACGAATATATGGTGCCAACTTTCTCAAAATGCAACAACATCCGGGGCACTTCACTTAAACAACAGTGATATAC	2700
Q Q Q Q Q Q T Q Q R I Y G G Q L S Q M Q Q H P G Q L H L N N S D I	809
ATAGCCAACTTAAACGCCAATACGGAATGCTAGGGCAGCAAGTTCACTCAACAGCAACAGCAGCAGCAATTTCCATCTCACTCAGAGTGTTAA	2800
H S Q P N K P N Y G M L G Q Q V H Q Q Q Q Q Q Q Q Q Q F P F T A D V N	843
TAGATCCAATTTCAAGCGATATTTTGGTAACTTCAGTCATTGCGAGCAGCAAGTCATCAACAGCAACAGCAGCAGCAATTTCCATCTCACTCAGAGTGTTAA	2900
R S N S S D I L G N L Q S L Q Q V D A L Q I Q Y N R R P	872
CTAAACAGAGGTGATAGTTTGTAAAGATAATATATGATAGTATATGTATCAATAAAGTCACAAAAGTGATGAATTTATGAACACCTGTTTACTAGCATT	3000
ATTTATGTCCTGATTATTAGCTAGTCTTTTATGTTTACCAA	3043

Figure 2. Sequence of *SCD5*. The nucleotide sequence of *SCD5* is shown with the predicted amino acid sequence directly below. The amino acid (single letter code) is centered under the codon to which it corresponds. Scd5p contains three stretches of a 20-aa repeat (dashed line) and nine stretches of a 12-aa repeat (underlined). Pertinent restriction endonuclease sites are underlined. This sequence has been deposited in the GenBank data base (accession no. U03492).

than cells containing wild-type Scd5p (doubling times were ~2 h for cells carrying YCp*SCD5*). When the

strain carrying YCp*scd5*-Δ338 was grown in liquid YEPD at 37°C, its growth was arrested within one

405	QLPLEPLKPTATGSANHLVR	424
439	QTGLQPLKPTATGSANYLMR	458
479	SGGLQPLKPTATGSANYLMK	498
534	S P N I T L P Q S N Q Q	8/12
564	S P Q H T Y S N N V R I	11/12
593	P P Q N T L P Q H Q Q S	11/12
608	S P Q N T I P Q H Q R S	12/12
623	S P Q N T F T Q N Q P I	11/12
636	S P Q H T Y S N N Q A T	11/12
650	S P Q N T Y T N N Q Q Q	12/12
683	P P Q H M Y S N V Q K Q	9/12
717	S P Q N A A N S Y F Q S	7/12
	S P Q N T O U Q N Q Q U	CONSENSUS
	H N H B	

Figure 3. Repeated motifs in Scd5p. The vertical bars indicate identities for the 20-aa repeat (upper). The consensus for the 12-aa repeats was defined by amino acids that appear most often in a given position amongst the repeats or that represent conservative substitutions. The number of residues in each repeat that fit the consensus are indicated to the right. O, hydrophobic or aromatic aa; U, uncharged aa; and B, basic aa.

generation after temperature shift. The strain with the smaller truncation continued to grow slowly at 37°C, consistent with its sparse growth on plates (Figure 4B).

Scd5p Has a Late Secretory Function

Because *SCD5* was isolated as a high copy suppressor of clathrin-deficient yeast, it was of interest to determine whether Scd5p is involved in vesicular transport. We took advantage of the temperature sensitivity of *scd5*-Δ strains carrying the *scd5*-Δ338 truncation plasmid and assayed for specific sorting and processing defects after shift to the nonpermissive temperature.

We first examined general secretion by monitoring invertase biosynthesis. The *SUC2* gene encodes two forms of invertase; the cytoplasmic form is constitutively expressed, while the external form is derepressed by growth of cells in low concentrations of glucose (Carlson *et al.*, 1981). During synthesis, secreted invertase undergoes core glycosylation in the endoplasmic reticulum (ER) followed by extensive addition of mannose in the Golgi to yield the mature high molecular weight species (Esmon *et al.*, 1981). The

Figure 4. Scd5p C-terminal truncations are temperature sensitive for growth. (A) *scd5*-Δ strains carrying either YCPSCD5, YCPSCD5-Δ32, or YCPSCD5-Δ338 grown to log phase in YEPD were diluted into YEPD medium at 25°C (open symbols) or 37°C (closed symbols). At the times indicated, cell number was determined by hemacytometer counting. Squares, SL1089 (*scd5*-Δ YCPSCD5-Δ338); circles, SL1720 (*scd5*-Δ YCPSCD5-Δ32); and triangles, SL1818 (*scd5*-Δ YCPSCD5). (B) *scd5*-Δ strains carrying either YCPSCD5 (WT; SL1818), YCPSCD5-Δ32 (Δ32; SL1720), or YCPSCD5-Δ338 (Δ338; SL1089) were streaked for single colonies on YEPD and incubated at either 26°C for 3 days or 37°C for 2 days.

mature invertase is then released into the periplasm. Isogenic *scd5*-Δ strains carrying YCPSCD5(*LEU2*) (SL1719) or YCPSCD5-Δ338 (SL1089) were grown in medium containing 5% glucose at 25°C and then

Figure 5. Scd5p-Δ338 cells accumulate internal invertase at 37°C. Cells were grown overnight at 25°C in MV plus 5% glucose and required nutritional supplements and then shifted to MV plus 0.05% glucose and required nutritional supplements and incubated at 37°C. At the indicated times cells were harvested and internal (open symbols) and external (closed symbols) invertase activity was assayed as described in MATERIALS AND METHODS. Squares, SL1089 (*scd5*-Δ YCp*scd5*-Δ338); circles, SL1719 (*scd5*-Δ YCp*SCD5*(LEU2)).

shifted to medium containing 0.05% glucose at 37°C to derepress invertase and inactivate Scd5p-Δ338. Samples were taken at various times after derepression and secretion of invertase was assessed by measuring the internal and external invertase enzymatic activity. Although virtually all newly synthesized invertase was rapidly secreted from *scd5*-Δ strains carrying the wild-type *SCD5* plasmid, cells carrying the *SCD5* truncation plasmid accumulated significant amounts of internal invertase. For example, 37% of the total invertase activity remained within the cell after 1 h of derepression at 37°C in the cells carrying YCp*scd5*-Δ338, as compared with only 10% for the *scd5*-Δ strain carrying the YCp*SCD5* plasmid (Figure 5). In the *scd5*-Δ strain carrying YCp*scd5*-Δ32, 14.6% of the invertase remained internal after 1 h of derepression at 37°C. At 25°C, 8–10% of the total invertase was internal after 1 h of derepression in both the wild-type and Scd5p C-terminal mutant strains. A second treatment of spheroplasts with lyticase did not release the invertase activity accumulated in the *scd5* truncation mutants. This indicated that the mutants were not more resistant to cell wall digestion and verified that the invertase was intracellular.

To determine the glycosylation state of invertase in the *scd5*-Δ338 strain after shift to 37°C, we used native

Figure 6. Glycosylation of invertase in Scd5p-Δ338 cells. Cultures were derepressed for invertase synthesis for 1 h at 25°C or 37°C as described in the legend to Figure 5. Cell extracts were subjected to nondenaturing gel electrophoresis and the gel was stained for invertase activity (see MATERIALS AND METHODS). The band near the bottom of the gel is the nonglycosylated cytoplasmic form of invertase. Strains are as follows: WT (SL1719, *scd5*-Δ YCp*SCD5*(LEU2)); Δ338 (SL1089, *scd5*-Δ YCp*scd5*-Δ338); *mnn1* (XW451-3B); and *sec18* (NY431).

gel electrophoresis, which can resolve various forms of the enzyme. Fully glycosylated invertase penetrates the gel poorly, forming a broad band near the top of the gel, while incompletely glycosylated lower molecular weight forms (such as observed in control *sec18* or *mnn1* mutants) migrate more rapidly (see Figure 6). In *sec18* strains (ER to Golgi transport block) only core glycosylation occurs, but several discrete lower molecular weight invertase species are observed on native gels due to oligomerization of the enzyme (Esmon *et al.*, 1981). *mnn1* mutants are defective in terminal mannose addition due to a defective α -1,3-mannosyltransferase (Graham *et al.*, 1992). In contrast, the migration of invertase extracted from the *scd5*-Δ338 strain, incubated at room temperature or 37°C for 1 h, was identical to that of an isogenic wild-type strain. Because ~40% of the total invertase was internal in the *scd5*-

Figure 7. CPY processing and sorting are normal in *Scd5p* mutants. *scd5-Δ* cells carrying either YCpSCD5 (WT; SL1818) or YCp-*scd5-Δ338* (Δ338; SL1089) were grown at room temperature to mid-log phase and then shifted to 37°C for 30 min. The cells were pulse labeled for 5 min with [³⁵S]methionine and [³⁵S]cysteine and then chased for the times (in min) indicated. CPY was immunoprecipitated, and the different forms were resolved on a 10% SDS polyacrylamide gel and visualized by autoradiography. The ER form of CPY is labeled as p1, the late Golgi form is labeled as p2, and the mature form is labeled as mCPY (see text for details).

Δ338 strain after 1 h at 37°C, any accumulation of more rapidly migrating, incompletely glycosylated forms should have been detected if present. These data indicated that the invertase that had accumulated in *Scd5p-Δ338* cells had transited the Golgi, suggesting that *Scd5p* might function at a late stage of the secretory pathway.

Because transport to the cell surface was impaired in a C-terminal *Scd5p* truncation mutant, we next examined whether transport to the vacuole is affected using pulse chase analysis of the soluble vacuolar protein, CPY. CPY is core glycosylated in the ER, yielding the p1 (67 kDa) form. Next, p2 CPY (69 kDa) is produced by extension of the core oligosaccharides in the Golgi complex. Upon export from the Golgi, the p2 CPY is sorted from the secretory pathway and transported to the vacuole, where it is converted to the mature active form (mCPY, 61 kDa). Cultures of *scd5-Δ* YCpSCD5 (SL1818) and *scd5-Δ* YCp*scd5-Δ338* (SL1089) were shifted to 37°C for 30 min and then subjected to a pulse chase regimen followed by immunoprecipitation with anti-CPY antibodies. In the *Scd5p* truncation strain, CPY was glycosylated and processed to the mature form with kinetics similar to wild type (Figure 7). An independent experiment also confirmed that all of the CPY was retained within the cell. These results indicate that *Scd5p* is not essential for vacuolar protein sorting and provide further evidence that glycosylation and transit through the ER and Golgi is normal in *Scd5p* mutants.

To further characterize the secretion defects of *Scd5p* mutant cells, we examined the processing and export of the mating pheromone α -factor, a peptide that is normally secreted into the medium. α -Factor is synthesized as a large precursor polypeptide (~19 kDa) that contains four copies of the mature pheromone sequence. After translocation into the ER, the signal sequence is cleaved and the core oligosaccharides are added to yield a species of ~30 kDa. In the Golgi, the core oligosaccharides are heterogeneously

extended. This highly glycosylated precursor (~125 kDa) is converted to mature α -factor (3.5 kDa) by a series of late Golgi-localized proteases encoded by the *KEX2*, *STE13*, and *KEX1* genes (Fuller *et al.*, 1988; Redding *et al.*, 1991; Cooper and Bussey, 1992; Roberts *et al.*, 1992). In *Chc⁻* strains, although the rate of transit of proteins through the secretory pathway is relatively normal, the inactive highly glycosylated precursor form of α -factor is secreted. This results from the mislocalization of the membrane-bound Golgi-localized processing enzymes to the plasma membrane (Payne and Schekman, 1989; Seeger and Payne, 1992b).

α -Factor secretion was examined in isogenic *scd5-Δ* strains carrying YCpSCD5 or YCp*scd5-Δ338*. After shift to 37°C for 30 min, cells were pulse labeled with [³⁵S]methionine and [³⁵S]cysteine for 7 min and chased for various times. Samples were separated into medium and cellular fractions and α -factor was immunoprecipitated. The results of this analysis are shown in Figure 8. The α -factor precursor was processed efficiently by both strains to the mature form, further supporting our conclusion that transit through the Golgi is normal in *Scd5p* mutant strains. When wild-type *Scd5p* was present, 85% of the α -factor was extracellular by 10 min of chase. In contrast, in the strain containing the C-terminal truncation of *Scd5p*, only 30% of the α -factor was extracellular after the 10-min chase and even after a 15-min chase the extracellular level was only 38%. These data indicate that *Scd5p* plays a role in secretion from the late Golgi to the plasma membrane but not in pro- α -factor maturation.

SCD5 Is a High Copy Suppressor of a sec2 Temperature-Sensitive Mutant

A number of *SEC* gene products have been implicated in the final stage of the yeast secretory pathway (Novick *et al.*, 1980). In addition, multi-copy suppression of many of the late *sec* mutants by other late-acting *sec* genes has been demonstrated (Salminen and Novick, 1987; Brennwald *et al.*, 1994). We therefore tested whether overexpression of *SCD5* could suppress the temperature sensitivity of any of the late-acting *sec* mutants that are blocked at a post-Golgi step of the secretory pathway. Temperature-sensitive mutants of late-acting *SEC* genes (*sec1-1*, *sec2-41*, *sec3-2*, *sec4-8*, *sec5-24*, *sec6-4*, *sec8-9*, *sec9-4*, *sec10-2*, and *sec15-1*) were all tested for growth at 26, 30, 34, and 37°C after transformation with YEpSCD5. Significantly, we found that overexpression of *SCD5* rescued *sec2-41* cells at 34°C (Figure 9). Overexpression of *SCD5* did not allow growth of the *sec2-41* strain at 37°C and did not suppress the temperature sensitivity of any of the other *sec* mutants tested.

Figure 8. Scd5p- $\Delta 338$ cells accumulate an internal pool of mature α -factor at 37°C. *scd5*- Δ cells carrying either YCpSCD5 (WT; SL1818) or YCp*scd5*- $\Delta 338$ ($\Delta 338$; SL1089) were grown at room temperature to mid-log phase and then shifted to 37°C for 30 min. Because α -factor synthesis is repressed at 37°C (Seeger and Payne, 1992b), both strains also carried pSL762, a plasmid containing α -factor under control of the heterologous promoter TP1 (Bender and Sprague, 1989). The cells were pulse labeled for 7 min with [35 S]methionine and [35 S]cysteine (0 time) and then chased for the times (in min) indicated. Labeled cultures were separated into intracellular (I) and extracellular (E) fractions and α -factor was immunoprecipitated for analysis by SDS-PAGE and autoradiography. Percentages of secreted α -factor as reported in the text were determined by densitometry of autoradiograms and are the average of two experiments. Molecular weight markers in kilodaltons are indicated on the right. Mature α -factor migrates at ~ 3.5 kDa, and the core glycosylated ER form migrates at ~ 30 kDa.

scd5- $\Delta 338$ Cells Accumulate Cytoplasmic Vesicles

The classic morphological phenotype of late *sec* mutants is their accumulation of post-Golgi secretory vesicles within their cytoplasm. Wild-type yeast cells accumulate few secretory vesicles, because transport to and fusion with the plasma membrane is rapid and efficient. To further establish that Scd5p functions at a late stage of the secretory pathway, we examined *scd5*- $\Delta 338$ cells by thin section electron microscopy after shift to the nonpermissive temperature (Figure 10). Although wild-type cells contained few intracellular vesicles, numerous vesicles of 80–100 nm accumulated in the *scd5*- $\Delta 338$ strain. The size of the vesicles was very similar to those seen in late *sec* mutants, such as *sec2*-41 (Figure 10C).

Subcellular Localization of Scd5p

To localize Scd5p in cells, a short oligonucleotide encoding a triple influenza hemagglutinin (flu) epitope

Figure 9. SCD5 is a high copy suppressor of *sec2*-41 at 34°C. *sec2*-41 strains carrying either YEp24 or YEpSCD5 were streaked on c-ura plates and incubated for 3 days at 30°C or 34°C.

tag was introduced into the SCD5 gene between codons 78 and 79. When SCD5-*flu* was expressed from a CEN plasmid as the sole source of Scd5p (SL1517), the tagged protein was found to be fully functional and was specifically recognized with monoclonal antibody 12CA5 (see MATERIALS AND METHODS). The subcellular fractionation protocol used to localize Scd5p (Walworth *et al.*, 1989) involves spheroplasting and gentle lysis of cells, followed by three sequential centrifugations: a 3-min spin at $450 \times g$, a 10-min spin at $10,000 \times g$, and a 1-h spin at $100,000 \times g$. Under these conditions, unlysed cells pellet at $450 \times g$ (P450), whereas most of the mitochondria, plasma membrane, vacuole, nuclei, and ER pellet at $10,000 \times g$ (P10k) (Goud *et al.*, 1988; Hurt *et al.*, 1988; Walworth *et al.*, 1989; Herman *et al.*, 1991). At $100,000 \times g$ Golgi and small secretory vesicles sediment (Walworth *et al.*, 1989; Redding *et al.*, 1991). When the differential centrifugation was carried out for an *scd5*- Δ YCpSCD5-*flu* strain (SL1517), Scd5p-*flu* remained predominantly in the supernatant (S10k) following the $10,000 \times g$ spin, as did the Golgi integral membrane protein, Kex2p (Figure 11A). After S10k was subjected to centrifugation at $100,000 \times g$, Scd5p-*flu* was divided equally between the S100k and the P100k fractions, while Kex2p was entirely in the P100k fraction, as expected.

The sedimentation of Scd5p at $100,000 \times g$ could have been due to interaction of this protein with small membrane structures, such as the Golgi or small vesicles, or due to interaction with a large nonmembrane-bound complex. To determine the nature of the association of Scd5p with the high speed pellet, we first treated a P100k fraction with 6 M urea or 1% Triton X-100 (Figure 11B). Urea disrupted the association of

Figure 10. Electron microscopy of *scd5*- Δ 338 cells shifted to 37°C. Strains indicated were grown at room temperature to mid-log phase and then shifted to 37°C for 2 h before processing for electron microscopy as described in MATERIALS AND METHODS. (A) A typical wild-type cell (SL1818) that does not accumulate secretory vesicles. (B) A representative *scd5*- Δ 338 cell (SL1089) showing the accumulation of numerous vesicles of 80–100 nm. (C) A *sec2*-41 cell (NY130) showing the accumulation of vesicles similar to those seen for the *scd5* truncation mutant. Bar, 1 μ m.

Figure 11. Subcellular fractionation of Scd5p. (A) An *scd5*- Δ YCp-SCD5-*flu* strain (SL1517) was spheroplasted, lysed (total), and then subjected to a $450 \times g$ spin to remove unlysed cells (P450). The supernatant (S450) was then centrifuged at $10,000 \times g$ for 10 min to generate a supernatant (S10k) and pellet (P10k) fraction. The S10k fraction was then centrifuged for 1 h at $100,000 \times g$ to generate a supernatant (S100k) and pellet (P100k) fraction. Samples were run on a 7.5% SDS polyacrylamide gel for transfer to nitrocellulose, and Scd5p-*flu* and Kex2p were detected using 12CA5 monoclonal and anti-Kex2p antibodies, respectively. (B) Cells were subjected to the same fractionation procedure described above, except the supernatant (S450) was centrifuged directly at $100,000 \times g$ for 1 h to generate a supernatant (S100k) and pellet (P100k) fraction. Equal aliquots of the resuspended P100k fraction were adjusted to 6 M urea, 1% Triton X-100, or left untreated and incubated on ice for 10 min. The samples were then subjected to a second $100,000 \times g$ spin and supernatant (S) and pellet (P) fractions were generated. Equal volume equivalents were loaded on a 7.5% SDS gel for immunoblotting of Scd5p-*flu* with 12CA5 antibodies.

Scd5p with the P100k fraction almost quantitatively, while Triton X-100 treatment was less efficient, but typically resulted in release of 30–50%. Sodium carbonate, pH 11, also efficiently liberated Scd5p. As expected, a parallel analysis showed Kex2p could not be released into the supernatant fraction by treatment with urea or sodium carbonate, but Triton X-100 efficiently solubilized it. These results suggest that, if Scd5p is associated with membranes, it must be a peripheral membrane protein.

To verify that Scd5p was associated with membranes but is inefficiently extracted from the P100k fraction with Triton X-100, a high speed pellet was subjected to a single step sucrose flotation gradient (Horazdovsky and Emr, 1993). In this two-step sucrose gradient (35%/60%), membranes resuspended in a 60% sucrose solution will float to the 35%/60% sucrose interface after equilibrium centrifugation, while insoluble aggregates and large protein complexes pellet. When the *scd5*- Δ YCpSCD5-*flu* strain

Figure 12. Scd5p concentrates with the membrane fraction in a sucrose flotation gradient. Cells from strain SL1517 (*SCD5-flu*) were processed as in Figure 11B except the P100 fraction was resuspended in 2.5 ml 60% sucrose solution, transferred to the bottom of a centrifuge tube, and overlaid with 35% sucrose solution. The sucrose step gradient was then centrifuged at $170,000 \times g$ for 18 h. Fractions were collected, proteins were precipitated, run on two identical 7.5% SDS polyacrylamide gels, and separated proteins were transferred to nitrocellulose. Transfers were probed with 12CA5 monoclonal antibodies to detect Scd5p or with anti-Kex2p antiserum. The arrow indicates the boundary between the 35% and 60% sucrose layers.

(SL1517) was subjected to this analysis (Figure 12), both Scd5p-*flu* and Kex2p migrated out of the 60% sucrose layer and concentrated near the interface between the 60% and 35% sucrose layers. Overall, these data indicate that Scd5p has a peripheral association with a small membrane organelle/vesicle fraction.

DISCUSSION

To gain insight into clathrin-mediated vesicular transport, we isolated five genes whose overexpression can suppress the lethality of clathrin-deficient yeast carrying the *scd1-i* allele (Nelson and Lemmon, 1993). We postulated that these suppressors might fall into at least three classes: 1) proteins involved in vesicular transport; 2) essential proteins whose localization or function is perturbed by clathrin deficiency; or 3) proteins whose overexpression can counteract the lethal effects of other proteins that accumulate in an inappropriate compartment due to lack of clathrin function. As reported previously, one of our suppressors, *SCD2*, is identical to *UBI4*, the polyubiquitin gene (Nelson and Lemmon, 1993). Further analysis showed that free ubiquitin is depleted and novel ubiquitin conjugates accumulate in clathrin-deficient yeast. This suggested that higher levels of ubiquitin are required for turnover of mislocalized or improperly processed proteins that accumulate in the absence of clathrin, thereby placing *SCD2/UBI4* in suppression class 3. Consistent with our expectations, the studies reported here indicate that we have also identified at least one class 1 suppressor, because Scd5p plays a role in vesicular transport.

Scd5p is a completely novel protein with two noteworthy repeated motifs: one of 20 aa present in three copies and one of 12 residues present in nine copies.

The 251- and 338-aa Scd5p truncations we have studied remove five or all nine of the 12-aa repeats, respectively. Our finding that *scd5-Δ338* functioned quite well in low copy to complement the *scd5-Δ* null mutation at 25°C and 30°C could indicate that the 12-aa repeat region is not absolutely required for Scd5p function. However, in the clathrin HC deficiency rescue assay, high copy *scd5-Δ251* rescued only weakly and *scd5-Δ338* was nonfunctional. In addition, this repeat region was shown to be essential to complement the disruption of *SCD5* at elevated temperatures, because an *scd5-Δ YCpsc5-Δ338* strain displayed temperature-sensitive growth and secretion defects. These latter observations suggest that the 12-aa repeat region is important for some aspect of Scd5p function. The reason for the difference in the ability of the truncation mutants to rescue *Chc⁻* yeast as compared with *scd5-Δ* cells is not clear. It could be that truncated Scd5p is inefficient in its rescue of *Chc⁻* yeast. Alternatively, Scd5p could be performing a unique function in *Chc⁻* yeast, because it must be overexpressed to suppress the lethality of *scd1-i* cells devoid of the clathrin HC. Also, the C-terminal repeats might be crucial for rescue of clathrin HC mutants, but not absolutely necessary for normal Scd5p function in cells. Further studies to understand the mechanism of suppression of the *Chc⁻* lethal phenotype are underway and should clarify these questions.

Recently we learned that *SCD5* was also isolated as a high copy suppressor of a temperature-sensitive allele of *IPL1* (increase in ploidy) (Chan and Botstein, 1993), which encodes a putative protein-serine/threonine kinase (Clarence Chan, personal communication). Temperature-sensitive alleles of *IPL1* result in severe chromosome missegregation at 37°C. This is interesting because *Chc⁻* cultures have a tendency to accumulate polyploid cells (Lemmon *et al.*, 1990). In addition, *Chc⁻* cells have been shown to undergo aberrant nuclear divisions, in which both progeny nuclei are retained within the mother cell or the nucleus prematurely migrates into the bud (Lemmon and Jones, 1987). However, the chromosome gain defect observed in *Chc⁻* cells is quite distinct from *ipl1* mutants. At 37°C, *ipl1^{ts}* cells show chromosome non-disjunction, which often results in aneuploidy, but there do not appear to be any defects in nuclear location or migration as seen in *chc1-Δ* cells (Francisco *et al.*, 1994). We do not yet understand how overexpression of *SCD5* can suppress both the chromosome non-disjunction associated with the *ipl1* temperature-sensitive mutation and the lethality of *chc1-Δ scd1-i* yeast. However, due to the rapid onset of the secretion defect associated with the *scd5-Δ338* mutation, as early as 15 min after shift to the nonpermissive temperature, and because a large percentage of Scd5p is associated with a $100,000 \times g$ membrane fraction, we conclude that there is a direct role for Scd5p in vesicular transport.

We have obtained several lines of evidence indicating that Scd5p is involved in a post-Golgi secretory step. First, an *scd5-Δ* strain carrying the truncation plasmid, YCp*scd5-Δ338*, accumulated substantially more internal invertase at 37°C when compared with an *scd5-Δ* strain carrying a plasmid encoding full length Scd5p. Second, a large internal pool of mature α -factor accumulated at 37°C in *scd5-Δ* strains expressing Scd5p- Δ 338. Although the secretion of α -factor and invertase was impaired in the *scd5-Δ338* strain, processing kinetics of α -factor and CPY were indistinguishable from wild-type cells and invertase appeared to be fully glycosylated. Therefore, transit of proteins through the Golgi was normal and the secretion defect that occurred in the C-terminal truncation mutant was not simply the result of slower growth at 37°C. Third, *scd5-Δ338* mutants accumulated many 80- to 100-nm vesicles at the nonpermissive temperature. This morphological defect is characteristic of the late *sec* mutants (Novick *et al.*, 1980).

Finally, genetic evidence also supports a role for Scd5p in a late step of the secretory pathway. *SCD5* was found to be a high copy suppressor of a temperature-sensitive allele of the *SEC2* gene, which encodes a protein that is required at a post-Golgi stage of secretion (Novick *et al.*, 1980). Distinct patterns of multicopy suppression have previously been observed among the late-acting *SEC* genes (Salminen and Novick, 1987; Brennwald *et al.*, 1994) and, in at least some cases, physical interactions between gene products have been established (Bowser *et al.*, 1992; TerBush and Novick, 1995). At the present time, the molecular basis for the observed genetic interaction between *SCD5* and *SEC2* is not known. *SEC2* itself encodes a protein of 759 aa containing an N-terminal region predicted to form an α -helical, coiled-coil. The Sec2 protein is present in a high molecular weight complex with an apparent molecular mass of ~500–750 kDa (Nair *et al.*, 1990). Preliminary gel filtration analysis indicates that Scd5p is present in an oligomeric complex of similar size (Nelson, unpublished observations). Therefore, it is possible that Sec2p and Scd5p exist in a complex with one another and overexpression of Scd5p stabilizes that complex or facilitates oligomeric assembly in the *sec2-41* mutant. However, using identical cell fractionation protocols, Sec2p was previously found predominantly in the 100,000 $\times$ g supernatant (Nair *et al.*, 1990), while we found Scd5p equally partitioned between the 100,000 $\times$ g supernatant and pellet. Although this suggests that Scd5p and Sec2p may not coexist in a complex, they may still physically interact within the cell, but that interaction may be more dynamic to regulate some stage of post Golgi secretory vesicle transfer, docking, or fusion. Alternatively, Scd5p overexpression could bypass the *sec2* defect by substituting for Sec2p or by efficiently recruiting other components of the late secretory

transport machinery, perhaps to a vesicle docking complex, thus enabling them to perform their function even in the absence of Sec2p. Further genetic and biochemical analyses are underway to examine the basis for the Scd5p suppression of the *sec2* temperature-sensitive mutant and will provide new information on the exact function of these two proteins in late secretory vesicle transport.

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