ϵ -COP is a structural component of coatomer that functions to stabilize α -COP

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We isolated a novel yeast α -COP mutant, *ret1-3*, in which α -COP is degraded after cells are shifted to a restrictive temperature. *ret1-3* cells cease growth at 28°C and accumulate the ER precursor of carboxypeptidase Y (p1 CPY). In a screen for high copy suppressors of these defects, we isolated the previously unidentified yeast ϵ -COP gene. ϵ -COP (Sec28p) overproduction suppresses the defects of *ret1-3* cells up to 34°C, through stabilizing levels of α -COP. Surprisingly, cells lacking ϵ -COP (*sec28Δ*) grow well up to 34°C and display normal trafficking of carboxypeptidase Y and KKXX-tagged proteins at a permissive temperature. ϵ -COP is thus non-essential for yeast cell growth, but *sec28Δ* cells are thermosensitive. In *sec28Δ* cells shifted to 37°C, wild-type α -COP (Ret1p) levels diminish rapidly and cells accumulate p1 CPY; these defects can be suppressed by α -COP overproduction. Mutant coatomer from *sec28Δ* cells behaves as an unusually large protein complex in gel filtration experiments. The *sec28Δ* mutation displays allele-specific synthetic-lethal interactions with α -COP mutations: *sec28Δ ret1-3* double mutants are unviable at all temperatures, whereas *sec28Δ ret1-1* double mutants grow well up to 30°C. Our results suggest that a function of ϵ -COP is to stabilize α -COP and the coatomer complex.

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Introduction

Two types of non-clathrin coats, COP I and COP II, regulate vesicular transport of proteins in between early compartments of the secretory pathway in all eukaryotic cells (reviewed in Schekman and Orci, 1996). COP II is thought to mediate the bulk of protein export from the endoplasmic reticulum (ER) to the early Golgi, whereas the role of COP I is less straightforward (reviewed in Bednarek *et al.*, 1996). The COP I coat is composed of a small GTPase, ARF, and coatomer, a conserved cytosolic ~700 kDa protein complex consisting of seven subunits: α -, β -, β' -, γ -, δ -, ϵ - and ζ -COP (reviewed in Rothman and Wieland, 1996). Coatomer purified from mammals

and yeast show very similar fractionation properties and subunit composition (Waters *et al.*, 1991; Hosobuchi *et al.*, 1992). Coatomer and ARF are the only cytosolic proteins necessary to produce COP I-coated vesicles *in vitro*, either from mammalian Golgi-enriched membranes or yeast ER membranes (Orci *et al.*, 1993; Bednarek *et al.*, 1995). In mammalian cells, the COP I coat is associated predominantly with the *cis*-side of the Golgi complex and with membranes of the intermediate compartment (*cis*-Golgi network) (Duden *et al.*, 1991), but COP I has also been detected on the ER (Orci *et al.*, 1994). Furthermore, in the presence of GTP- γ -S coatomer can also be recruited to endosomal membranes *in vitro*, and may possibly have a role in endosome function (Whitney *et al.*, 1995; Aniento *et al.*, 1996).

Evidence obtained in both yeast and mammalian cells clearly shows that COP I function is, directly or indirectly, essential for ER-to-Golgi traffic. Several yeast coatomer subunit mutants accumulate ER precursor forms of secreted proteins under non-permissive conditions (Hosobuchi *et al.*, 1992; Duden *et al.*, 1994; Cosson *et al.*, 1996). Furthermore, microinjection of anti- β -COP antibodies into mammalian cells inhibits ER-to-Golgi transport *in vivo*, but not transport from the TGN to the plasma membrane (Pepperkok *et al.*, 1993). Genetic and biochemical evidence obtained in yeast strongly supports a role of coatomer in the Golgi-to-ER retrieval of KKXX-tagged membrane proteins (reviewed in Rothman and Wieland, 1996; Schekman and Orci, 1996). First, coatomer binds directly to the KKXX retrieval motif (Cosson and Letourneur, 1994). Second, a genetic selection for yeast mutants defective in retrieval (*ret* mutants) yielded α -, γ -, δ -, and ζ -COP (Letourneur *et al.*, 1994; Cosson *et al.*, 1996). Several of the coatomer subunit mutants isolated in the selection, e.g. *ret1-1* (α -COP) and *sec21-2* (γ -COP), confer temperature-sensitive growth and show severe defects in the ER-retrieval of KKXX-tagged proteins *in vivo*, but show no accompanying defect in secretion. On the basis of these data it has been argued that COP I may function solely in retrograde Golgi-to-ER transport, continually recycling proteins necessary for ER budding or targeting of COP II vesicles (Pelham, 1994). New temperature-sensitive *sec21* alleles (γ -COP) exhibit cargo-selective defects in ER-to-Golgi transport suggesting that either COP I is involved in anterograde transport of only a subset of proteins, or its involvement in forward traffic is indirect due to a failure to retrieve proteins (Gaynor and Emr, 1997).

Analysis of the precise function of COP I in membrane traffic is complicated by the fact that the coat seems intimately involved in the maintenance of Golgi complex integrity. For example, mammalian cells of the temperature-sensitive mutant CHO cell line *ldlf*, which is defective in ϵ -COP, display a disruption of Golgi structure and

perturbations of membrane traffic when shifted to the non-permissive temperature (Guo *et al.*, 1994). In these mammalian cells ϵ -COP is degraded at a restrictive temperature and cells cease growth, on the basis of which it was suggested that ϵ -COP itself is essential for cell viability (Guo *et al.*, 1994, 1996).

Work from several groups has focused intensely on the structure and biosynthesis of the coatomer complex. cDNAs encoding all coatomer subunits have been cloned and sequenced from mammalian cells (see Kreis *et al.*, 1995 for review and references). In yeast, six out of seven genes encoding coatomer subunits have now been analyzed and all are essential for cell viability at all temperatures. *RET1*, *SEC26*, *SEC27*, *SEC21*, *RET2*, *RET3* encode yeast α -, β -, β' -, γ -, δ - and ζ -COP, respectively (Hosobuchi *et al.*, 1992; Duden *et al.*, 1994; Letourneur *et al.*, 1994; Cosson *et al.*, 1996). The last remaining yeast coatomer subunit gene, encoding ϵ -COP and named *SEC28* here, is characterized in this study. Surprisingly, *SEC28* gene function is non-essential for cell viability. *sec28 Δ* mutant cells entirely lacking ϵ -COP grow well at temperatures up to 34°C, and cease growth only at a higher temperature, 37°C. Our data show that at this elevated temperature ϵ -COP function is required for the stability of another coatomer subunit, α -COP, and thus the functional integrity of the coatomer complex.

Results

Identification of *ret1-3*, a restrictive new α -COP allele

We previously described a genetic screen for temperature-sensitive yeast mutants that accumulate unprocessed forms of the secretory protein α -factor (Wuestehube *et al.*, 1996). One novel α -COP allele isolated in this screen, designated *ret1-3*, is characterized here. *ret1-3* mutant cells display a restrictive phenotype: cells grow at wild-type rates at 24°C, but cease growth at 28°C. We initially became interested in *ret1-3* because this mutant displayed synthetic lethality in a cross with *sec21-1*, a γ -COP mutant. The pattern of spore viability showed that *ret1-3 sec21-1* double mutants were unviable at 24°C (33 tetrads analyzed). The synthetic-lethality was specific because no genetic interaction was observed in crosses with several non-coatomer *sec* mutants, including *sec12-4*, *sec13-1*, *sec16-2*, *sec17-1*, *sec18-1*, *sec20-1* and *sec22-3*. To test whether the *ret1-3* mutation may correspond to a coatomer subunit, we transformed cells with plasmids harboring *SEC21*, *SEC26*, *SEC27* or *RET1* (γ -, β -, β' - or α -COP), or a control plasmid. CEN- and 2 μ m *RET1* plasmids encoding α -COP (but none of the other plasmids) suppressed the thermosensitive growth defect of *ret1-3* cells completely at 37°C (not shown). We next crossed the mutant with two known α -COP alleles, *ret1-1* (Letourneur *et al.*, 1994) and *sec33-1* (Wuestehube *et al.*, 1996). Resulting diploids from both crosses were temperature-sensitive, showing that the mutations fall into the same complementation group. Diploids of the *ret1-1* $\times$ *ret1-3* cross were sporulated, tetrads were dissected and spores grown on YPD plates at 24°C. Only Ts⁻ spores were observed in 79 tetrads analyzed, demonstrating that the two loci are closely linked or identical. We concluded that *ret1-3*

corresponds to a restrictive new allele of the *RET1* locus encoding α -COP.

Mutant α -COP in *ret1-3* cells is unstable at elevated temperatures

Although *ret1-3* cells ceased to grow above 28°C, the onset of a transport defect was slow. In *in vivo* pulse-chase labeling experiments using a 1 h shift to 37°C only a weak defect for the ER-to-Golgi transport of carboxypeptidase Y (CPY) was observed. CPY, a soluble vacuolar hydrolase, is modified in the ER and the Golgi before arrival in the vacuole where it is proteolytically cleaved to the 61 kDa mature (m) enzyme. The ER and Golgi forms of CPY, p1 (67 kDa) and p2 (69 kDa) accumulate in mutants with a secretory block at these stages (Stevens *et al.*, 1982). Increasing the preshift time to 2 h induced a nearly complete block in transport of the p1 (ER) form of CPY in *ret1-3* cells (data not shown). In addition we observed a slow onset of a transport defect of KKXX-tagged proteins in pulse-chase experiments performed as described by Letourneur *et al.* (1994). These experiments were performed as described by Gaynor *et al.* (1994), making use of a chimeric protein (Inv.-Wbp1p) consisting of the invertase protein fused to the transmembrane and cytoplasmic domains of Wbp1p, a KKXX-tagged ER resident protein. We thus considered the possibility that the *ret1-3* mutation may primarily affect the stability of coatomer subunits and coatomer.

To test this we grew *ret1-3* cells, and cells of the isogenic wild-type control strain RSY255, in YPD medium to early log phase at 25°C and then examined coatomer subunits by immunoblot of cells shifted to a restrictive temperature, 37°C. Total cell proteins of timed samples were separated on 10% SDS-PAGE gels and immunoblotted with specific antibodies that recognize all seven COPs (see Materials and methods). Figure 1A shows the growth curves obtained from the two strains used in this experiment. At the 0 h timepoint no differences in the immunoblot signals of the COPs were detected in wild-type and *ret1-3* cells, implying that at a permissive temperature *ret1-3* cells have normal levels of all coatomer subunits. However, over the time course of the experiment, the α -COP immunoblot signal decreased rapidly. After 2 h the α -COP signal was reduced to ~50%, and by 4 h α -COP was virtually undetectable (Figure 1B). The decrease in the α -COP signal was dramatic, but we also noticed a slower decrease in the signal for ϵ -COP. At 4 h only ~50% of the ϵ -COP blot signal remained. However, the other five COPs appeared to be stable throughout the experiment. We conclude that the *ret1-3* mutation renders α -COP unstable at elevated temperatures, thus presumably causing the transport defect and growth arrest. ϵ -COP, but none of the other COPs, is also destabilized at high temperature in *ret1-3* cells, albeit to a lesser degree.

Isolation of *SEC28* as a high copy suppressor of the *ret1-3* mutation

In order to identify genes that may influence the stability of mutant α -COP, we conducted a search for multicopy suppressors of *ret1-3*. We performed our screen at 34°C, i.e. 6°C above the lowest restrictive temperature for *ret1-3*, to select only very strong suppressors. *ret1-3* cells were transformed with a yeast genomic library constructed in

Fig. 1. Mutant α -COP is unstable in *ret1-3* cells at a restrictive temperature. The *ret1-3* mutant and the isogenic wild-type control strain RSY255 were grown to log phase at 24°C, and then shifted to a restrictive temperature, 37°C. (A) Growth curves of strains *ret1-3* and RSY255 at 0–6 h after shift to 37°C are shown. (B) At the timepoints indicated cell extracts were made and analyzed by immunoblotting with antisera recognizing all coatomer subunits (see Materials and methods). The positions of individual COPs are indicated by their Greek symbols. Note the dramatic depletion of α -COP visible at 2 h after shift, a slower depletion of ε -COP, and the relative stability of the other five COPs.

the high copy plasmid YEP24 (Carlson and Botstein, 1982). Among 20 000 Ura⁺ transformants screened, several colonies showed plasmid-dependent growth at 34°C (these colonies were unable to grow on media containing 5-FOA at 34°C). Plasmids were isolated and retransformed into fresh *ret1-3* cells to reconfirm the suppression phenotype. Suppressor plasmids (five) corresponding to two different genomic regions were identified: four related plasmids supported growth of *ret1-3* cells at 34°C, but not 37°C, and the remaining plasmid supported vigorous growth at 34°C and 37°C. Restriction analysis and end-sequencing of genomic inserts followed by database comparisons revealed that the plasmid promoting wild-type growth at 37°C contained the authentic *RET1* gene, as expected. The four related plasmids all harbored the same novel suppressor. Figure 2A shows the suppression of the growth defect of *ret1-3* cells afforded by the novel suppressor gene at 34°C, compared with the control plasmid YEP24 and the authentic gene, *RET1*, present in high copy. Based on our analysis of a deletion mutant of this suppressor gene which displayed a *sec* phenotype (see below), we named the gene *SEC28*.

To test whether the suppressor gene could also correct the CPY transport defect in *ret1-3* cells we performed pulse-chase experiments. *ret1-3* cells were transformed with either the control plasmid YEP24, or 2 μ m plasmids expressing *SEC28* or *RET1* and were grown at a permissive

Fig. 2. *SEC28*-dependent suppression of the growth and CPY transport defects of *ret1-3* cells. (A) The multicopy plasmid YEP24 containing either the *SEC28* gene (2 μ m-*SEC28*), the *RET1* gene (2 μ m-*RET1*), or no insert (YEP24) was introduced into *ret1-3*. Transformants were grown on minimal medium lacking uracil at 24°C, streaked out on the same medium and incubated at 34°C for 3 days. (B) Pulse-chase analysis of CPY transport in *ret1-3* cells transformed with 2 μ m-*SEC28*, 2 μ m-*RET1*, or YEP24 as a control is shown. Cells were shifted for 3 h to the restrictive temperature, 34°C, and then radiolabeled with ³⁵S-Promix label for 10 min at 34°C. Chase time points were taken at 0, 15 and 60 min as indicated. Cell extracts were immunoprecipitated with anti-CPY antiserum. The precipitates were separated on 7.5% SDS-PAGE gels and analyzed using a PhosphorImager. Positions of the p1, p2, and mature forms of CPY are indicated. Maturation of CPY to the mature form is quantified at 60 min chase.

temperature, 24°C, to mid-logarithmic growth phase in selective minimal media. Cells were then shifted to 34°C for 3 h, pulse-labeled for 10 min and chased for 0, 15 and 60 min at 34°C. Total cell extracts were subjected to immunoprecipitation with an antiserum to CPY, proteins were separated by SDS-PAGE and analyzed using a PhosphorImager (Figure 2B). Cells harboring YEP24 accumulated p1 CPY, whereas the presence of the *SEC28* plasmid clearly resulted in suppression. CPY (74%) appeared as the mature form after a 60 min chase in *SEC28*-transformed cells, compared with only 26% in YEP24-transformed cells. For comparison the near identical suppression afforded by the authentic *RET1* gene present on a 2 μ m plasmid is shown (Figure 2B). Suppression afforded by either 2 μ m plasmid was not complete, because even under selective conditions ~20% of cells in a population no longer contain the plasmid. Thus, the *SEC28* suppressor gene rescues the growth defect in *ret1-3* cells and restores normal CPY transport at an intermediate temperature, 34°C.

***SEC28* encodes ε -COP whose overproduction stabilizes α -COP in *ret1-3* cells**

DNA subcloning localized the *SEC28* suppressor activity to a 3.8 kb *Bam*HI/*Eag*I genomic DNA fragment contained in all four plasmids. This 3.8 kb complementing DNA fragment contained only one complete open reading frame (ORF) (DDBJ/EMBL/GenBank designation Y1L076W) which is localized to chromosome IX. The ORF-encoded protein giving rise to suppression was identified by immunoblotting. *ret1-3* cells were transformed with either a control plasmid, CEN- or 2 μ m *RET1* plasmids, or the plasmid harboring the high copy suppressor. Transformants were first grown at the permissive temperature, 24°C, and then temperature-shifted for 3 h to a restrictive temperature

Fig. 3. SEC28-dependent overproduction of ϵ -COP stabilizes α -COP in *ret1-3* cells. The multicopy plasmid YEP24 containing either the SEC28 gene (2 μ m-SEC28), the RET1 gene (2 μ m-RET1), or no insert (YEP24) was introduced into *ret1-3*. A single copy plasmid containing the RET1 gene (CEN-RET1) was used as a control. Transformants were grown to log phase in selective minimal medium at 24°C, and then shifted to the restrictive temperature, 30°C, for 3 h. Cell extracts were prepared and proteins separated on 10% SDS-PAGE gels were analyzed by immunoblotting with antisera against coatomer subunits. Purified coatomer is loaded ('Coat') to define positions of all COPs; individual COPs are denoted by their Greek symbols. Note ϵ -COP overproduction in 2 μ m-SEC28 transformants (arrowhead), and a higher α -COP level relative to YEP24 transformants (black arrow), but comparable signals for the other COPs.

for *ret1-3*, 30°C. Cell lysates were prepared, total cell proteins were separated by SDS-PAGE and again immunoblotted with antisera recognizing all COPs. The presence of the suppressor plasmid resulted in the overproduction of a ~35 kDa protein which was recognized by an anti-coatomer antiserum (described in Duden *et al.*, 1994), and this band comigrated with the ϵ -COP band of purified yeast coatomer (see Figure 3). We conclude that it is ϵ -COP overproduction which suppressed the *ret1-3* (α -COP) mutant and that SEC28 represents the previously uncharacterized yeast ϵ -COP gene. Interestingly, we also noticed an increase in the α -COP blot signal in extracts from cells overexpressing ϵ -COP, whereas the blot signals for the other COPs were roughly equal from all four transformants (see Figure 3; to be further discussed below).

Sequencing of the ϵ -COP ORF contained in the suppressor plasmids using synthetic oligonucleotide primers showed it to be 100% identical to the unidentified ORF YIL076W on chromosome IX (cosmid clone; DDBJ/EMBL/GenBank accession number Z37997). The ϵ -COP ORF is annotated in the databases as encoding a 'hypothetical 41.5 kDa protein in THS1-SEN3 intergenic region' (SWISSPROT accession number P40509). This conceptual protein has been translated from the first ATG contained in the ORF YIL076W, which we show here is incorrect. The context of the first ATG is GTGCGTATG, a highly unfavorable translation initiation context in yeast (Hinnebusch and Liebman, 1991), and translation from it would result in a larger protein of 359 residues. The second ATG in the ORF, however, lies in an appropriate context for translation initiation in yeast (Hinnebusch and Liebman, 1991), namely AAAATCATG, resulting in a protein of 296 residues. We have determined experimentally that only the second ATG is used for translation initiation *in vivo*. We constructed a mutant SEC28 single-copy

plasmid using PCR mutagenesis, changing the first ATG to the ineffective initiation codon AAG (Hinnebusch and Liebman, 1991). The presence of the mutation in the plasmid construct was verified by DNA sequencing. The mutant plasmid encoded functional Sec28p, because it restored vigorous growth of *sec28* Δ cells at a restrictive temperature, 37°C, indistinguishable from the growth afforded by a wild-type SEC28 single-copy plasmid. Total cell proteins from *sec28* Δ cells harboring the wild-type SEC28 single-copy plasmid, the AAG-mutant SEC28 plasmid, or vector alone as a control, were separated on a 12% SDS-PAGE gel, transferred to nitrocellulose and probed with anti-coatomer antiserum. Sec28p expressed from mutant and wild-type plasmids comigrated exactly; as expected Sec28p was undetectable in *sec28* Δ cells transformed with vector alone (data not shown). Our data demonstrate that the first ATG in the SEC28 ORF is not used, and Sec28p shown in Figure 4 was accordingly translated from the second ATG.

The predicted yeast ϵ -COP gene product is thus a hydrophilic protein of 296 amino acids, corresponding to a molecular mass of 33.8 kDa, consistent with its migration on SDS-PAGE gels, and a calculated *pI* of 4.27. Thus, Sec28p is four amino acids longer than *Caenorhabditis elegans* ϵ -COP and 14 residues shorter than cow (*Bos taurus*) ϵ -COP. A multiple sequence alignment of yeast ϵ -COP with its homologs from cow (Hara-Kuge *et al.*, 1994) and the nematode *C. elegans* is shown in Figure 4. In pairwise alignments, yeast and mammalian ϵ -COP showed 33% identity and 60% similarity in an optimized sequence alignment introducing 35 gaps. Only 20% identity and 42% similarity were observed when default program settings were used (introducing 11 gaps). Searching the databases with Sec28p using TBLASTN yielded both ϵ -COP from cow and hamster with a significant, albeit quite low score (55). However, the yeast ORF encoding Sec28p could not be identified in TBLASTN database searches with the same default program settings when mammalian ϵ -COP was used as the search sequence. Clearly, the evolutionary conservation of ϵ -COP from yeast to mammals is significantly lower than that of all other coatomer subunits.

Mutants lacking ϵ -COP are viable, but display temperature-sensitive defects

To characterize SEC28 (ϵ -COP) function we deleted the gene using a polymerase chain reaction (PCR)-based approach (see Baudin *et al.*, 1993). Oligonucleotides corresponding to the 3' and 5' regions of SEC28 and HIS3 were used to direct a 1.1 kb PCR fragment containing the selectable marker gene HIS3 to the SEC28 locus. Our ϵ -COP gene deletion is predicted to remove codons 1–290 (97% of the predicted ϵ -COP gene product as shown in Figure 4) and 160 bp upstream of the initiation ATG. One copy of the ϵ -COP gene was deleted in the SEC *his3* Δ 200 diploid strain YPH501 and the genotype of heterozygous diploid (SEC28/*sec28* Δ ::HIS3) transformants was verified by PCR (see legend of Figure 5C for details). Cells of several independent His⁺ transformants were sporulated and tetrads dissected.

Surprisingly, four viable spores were observed at 24°C in all 32 tetrads analyzed. All four spores in these tetrads formed colonies at temperatures up to 34°C after replica-

Fig. 4. Sequence alignment of yeast ϵ -COP (Sec28p; DDBJ/EMBL/GenBank ORF Y1L076W) with mammalian ϵ -COP from cow (*B.taurus*; DDBJ/EMBL/GenBank accession number X76980) and the nematode *C.elegans* (cosmid; DDBJ/EMBL/GenBank accession number Z93382) were aligned using the CLUSTAL method. In an optimized pairwise alignment, yeast ϵ -COP shows 33% identity (60% similarity) with cow ϵ -COP.

plating, but two out of four of each tetrad were unable to grow at 37°C (Figure 5A). The temperature-sensitive spore-derived colonies in each tetrad were phenotypically His⁺ (Figure 5A). To test for the absence or presence of ε-COP, cell lysates prepared from colonies corresponding to two complete tetrads were analyzed by immunoblotting. As expected, ε-COP was detected in lysates of His⁻ segregants using anti-coatomer antiserum, but no ε-COP band was detected in cell lysates prepared from His⁺/Ts⁻ segregants (Figure 5B). Furthermore, PCR was used to verify that the His⁺ spores carried the correct *sec28Δ* gene deletion (see legend of Figure 5C for details). We conclude that cells harboring the *sec28Δ* mutation grow like wild-type at temperatures up to 34°C, but cease growth at an elevated temperature, 37°C. No other gene product related to Sec28p is present in the fully sequenced yeast genome. Thus, unlike the other six genes encoding COPs in yeast, ε-COP (*SEC28*) gene function is non-essential for yeast cell growth.

Wild-type α -COP is unstable in *sec28 Δ* mutant cells at a restrictive temperature

To test whether the coatomer subunits are stable at a restrictive temperature in cells lacking ϵ -COP, we grew *sec28 Δ* cells, and isogenic wild-type cells as a control, to early log phase at 25°C in YPD medium and then shifted to 37°C in a time course. Total cell proteins of timed cell

samples were separated by SDS-PAGE and immunoblotted for the coatomer subunits (see Figure 6). Figure 6A shows the growth curves obtained from the strains used in this experiment. We noticed that the immunoblot signal for α -COP diminished rapidly after temperature shift. After 2 h the α -COP blot signal was reduced to 30% and by 4 h α -COP was virtually undetectable (Figure 6B). The immunoblot signal for β -, β' -, γ -, δ - and ζ -COP remained roughly constant over the time course, and ϵ -COP was undetectable in *sec28 Δ* cells. Thus, a lack of ϵ -COP (in *sec28 Δ* cells) renders wild-type α -COP unstable at restrictive temperatures, but does not affect the stability of the other five COPs.

We also analyzed *sec28Δ* cells, and *ret1-3* cells, by electron microscopy at the permissive temperature, 24°C, and 2.5 h after shift to a restrictive temperature, 37°C. Both mutants accumulated ER membranes at the restrictive temperature as expected for *sec* mutants (see Kaiser and Schekman, 1990). More interestingly, a sizable fraction of temperature-shifted cells from either mutant also accumulated aggregates of a dense fibrillar material that stained darkly with potassium permanganate (data not shown). These structures may be enriched in COP I proteins, perhaps similar to the aggregates observed in BFA-treated or ATP-depleted mammalian cells (Hendricks *et al.*, 1993), but immunoelectron microscopy experiments will be necessary to address this question.

Fig. 5. (A) Tetrad analysis of a PCR-verified *SEC28/sec28Δ::HIS3* diploid [for the PCR strategy see (C) below]. Of the four spores, only the two carrying the wild-type *SEC28* allele can grow at 37°C; all *His*⁺ segregants fail to grow at 37°C (compare ‘-His’ and ‘37°C’). All four spores in a tetrad grow at temperatures up to 34°C. (B) Extracts of cells corresponding to two complete tetrads [3 and 4 from (A) above] were prepared, proteins separated on a 10% SDS-PAGE gel and analyzed for the presence of ε-COP (ε-), as well as ζ-COP (ζ-) as a loading control, using anti-coatomer antiserum. Note the absence of an ε-COP band in segregants 3b, 3d, 4b and 4d, which corresponds well to their temperature-sensitive, *His*⁺ phenotypes. (C) Schematic of the PCR strategy used to verify the *sec28Δ* mutation. The *SEC28* ORF was replaced by the *HIS3* gene. The forward (fw) and reverse (rev) oligonucleotide primers used for PCR are depicted. Oligos SEC28fw and SEC28rev were derived from sequences outside the *SEC28* ORF and yielded a predicted 1100 bp product in PCR reactions, using DNA from either wild-type cells or cells carrying the *sec28Δ* mutation as a template. Oligos HIS3fw and HIS3rev were derived from sequences of the *HIS3* marker gene. The pairs SEC28fw + HIS3rev, and SEC28rev + HIS3fw yielded a predicted 900 bp PCR product only in *sec28Δ* cells, but no product from wild-type cells, and were thus diagnostic for the *sec28Δ* mutation.

α-COP overproduction rescues the CPY defect in temperature-shifted *sec28Δ* cells

Temperature-shifted *sec28Δ* cells showed a slow onset of a transport defect for CPY in pulse-chase experiments (data not shown). After a 1 h pre-shift to a restrictive temperature, 37°C, only a partial block at the p1 CPY transport stage was observed; a 2 h pre-shift was necessary to induce an almost complete transport block. This showed that a lack of ε-COP renders yeast cells temperature-sensitive not only for growth but also for the ER-to-Golgi trafficking of proteins. We reasoned that if the temperature-sensitive growth defects in *sec28Δ* cells are caused solely by lowered α-COP levels, then overproduction of wild-type Ret1p should restore normal growth. This is indeed what we observed. Mild overproduction of either ε-COP or α-COP from 2 μm plasmids harboring the *SEC28* or *RET1* genes fully suppressed the thermosensitive growth defect in *sec28Δ* cells at 37°C. The control plasmid YEP24 did not suppress (see Figure 7A), and neither did 2 μm overproduction of Sec21p, Sec26p, or Sec27p (γ-, β, or β'-COP) afford any suppression (data not shown). Overproduction of Sec28p or Ret1p also suppressed the

Fig. 6. Wild-type α-COP is unstable in *sec28Δ* cells at restrictive temperature. The *sec28Δ* mutant and an isogenic wild-type control strain (wt) were grown to log phase at 24°C, and then shifted to a restrictive temperature, 37°C. (A) Growth curves of the *sec28Δ* strain and the wild-type strain at 0–6 h after shift to 37°C. (B) At the timepoints indicated cell extracts were made and analyzed by immunoblotting with antisera recognizing the seven COPs (see Materials and methods). Purified coatomer is loaded in the first lane to define the positions of all seven COPs; the individual COPs are indicated by their Greek symbols. Note a depletion of α-COP visible at 2 h after shift, the absence of ε-COP in *sec28Δ* cells and the relative stability of the other COPs.

CPY transport defect in *sec28Δ* cells at 37°C (see Figure 7B). *sec28Δ* cells were shifted to 38°C for 1 h in selective medium, then pulse-labeled for 10 min and chased for 0, 15 and 60 min. The maturation of CPY was quantified: 23% mature CPY was observed at 60 min chase for YEP24 control transformants, compared with 69% in 2 μm *SEC28* transformants, and 75% in 2 μm *RET1* transformed cells (Figure 7B). Thus, 2 μm overproduction of the authentic gene *SEC28* (ε-COP) and the high copy suppressor gene *RET1* (α-COP) rescue the thermosensitive growth and CPY transport defects in *sec28Δ* cells to comparable extents. We conclude that ε-COP acts to stabilize both mutant α-COP (as shown above by high copy suppression in *ret1-3* cells) and wild-type α-COP (as evidenced by the α-COP instability in *sec28Δ* cells, lacking ε-COP, at a restrictive temperature, 37°C).

sec28Δ cells show normal transport of KKXX-tagged proteins at a permissive temperature

We tested the ability of *sec28Δ* cells to transport CPY as well as a KKXX-tagged membrane protein at permissive temperatures. For CPY, pulse-chase experiments were conducted as described above; no CPY transport defect was observed at 30°C (data not shown). Possible retrieval defects for KKXX-tagged proteins were analyzed as described by Gaynor *et al.* (1994), making use of a chimeric protein (Inv.-Wbp1p) consisting of the invertase protein fused to the transmembrane and cytoplasmic domains of Wbp1p, a KKXX-tagged ER resident protein.

Fig. 7. *RET1*-dependent suppression of the growth and CPY transport defects of *sec28Δ* cells. (A) The multicopy plasmid YEP24 containing either the *SEC28* gene (2 μ m-*SEC28*), the *RET1* gene (2 μ m-*RET1*), or no insert (YEP24) was introduced into *sec28Δ*. Transformants were grown on minimal medium lacking uracil at 24°C, streaked out on the same medium and incubated at 37°C for 3 days. (B) Pulse-chase analysis of CPY transport in *sec28Δ* cells transformed with 2 μ m-*SEC28*, 2 μ m-*RET1*, or YEP24 as a control is shown. Cells were shifted for 1 h to a restrictive temperature, 38°C, and then radiolabeled with ³⁵S-Promix label for 10 min at 38°C. Chase time points were taken at 0, 15 and 60 min as indicated. Cell extracts were immunoprecipitated with anti-CPY antiserum. The precipitates were separated on 7.5% SDS-PAGE gels and analyzed using a PhosphorImager. Positions of the p1, p2 and mature forms of CPY are indicated. Maturation of CPY to the mature form is quantified at 60 min chase.

In wild-type cells this protein is at steady state localized to the ER by virtue of its KKXX motif, which leads to the continuous Golgi-to-ER recycling of any protein escaping from the ER. In coatomer mutants defective in KKXX retrieval, e.g. *ret1-1* (α -COP), this protein escapes to the vacuole where it is proteolytically degraded in a manner dependent on the *PEP4*-encoded protease. *sec28Δ* cells were pulse-labeled for 10 min and chased for 0 h and 1 h. The fusion protein was recovered by immunoprecipitation with antibodies against the invertase moiety, treated with endoglycosidase H (Endo H), and resolved on SDS-PAGE (Figure 8). As a control, an Inv.-Wbp1p fusion protein harboring the non-functional signal QKXX was used. Efficient cleavage of the QKXX fusion protein at 60 min chase indicates that forward passage through the Golgi and to the vacuole is normal (80% for *ret1-1* cells, and 67% for *sec28Δ* cells, respectively; see Figure 8). The values obtained for cleavage of the KKXX fusion protein (which can be retrieved) were very similar in *sec28Δ* and isogenic wild-type cells (31 and 34%, respectively). We conclude that Golgi-to-ER retrieval of a KKXX-tagged protein is not impaired in *sec28Δ* cells at a permissive temperature, 30°C.

We also studied the binding of coatomer from *sec28Δ* cells to KKXX-motifs *in vitro* as described by Letourneur *et al.* (1994). A KKXX-containing Wbp1p peptide, and a control peptide containing the non-functional QQXX-motif, were immobilized on Sepharose beads. Whole cell extracts were prepared from *sec28Δ* cells grown at a permissive temperature, 30°C, and congenic control cells, to serve as a source of coatomer. Mutant coatomer present in *sec28Δ* cell extracts, as well as wild-type coatomer in extracts from the control cells, specifically interacted with the KKXX-peptide beads. Coatomer from either source was unable to bind to the QQXX-control beads (data not

Fig. 8. Intracellular transport of a KKXX protein in *sec28Δ* cells is normal at permissive temperature. *sec28Δ* cells (*sec28Δ*) and cells of an isogenic wild-type strain (wt), as well as *ret1-1* cells (*ret1-1*), were transformed with plasmids expressing Inv.-Wbp1 fusion protein carrying either the functional KKXX motif (KK), or a non-functional motif, QKXX (QK). Cells were grown to log phase at 24°C, shifted for 2.5 h to the permissive temperature, 30°C, and pulse-labeled with ³⁵S-Promix for 10 min at 30°C. Chase time points were taken at 0 and 1 h as indicated. Inv.-Wbp1p-(KK) or (QK) fusion protein was immunoprecipitated from the labeled cells, treated with endoH and analyzed on SDS-PAGE gels. Intact and *PEP4*-dependent processed (proc.) fusion protein migrate at 70 and 56 kDa, respectively. The percentage of processing at 1 h is quantified. The asterisk (*) marks a band that may represent nonspecific cross-reactive material and was not included in the quantitation.

shown). These experiments provide further evidence that coatomer lacking ϵ -COP in *sec28Δ* cells grown at a permissive temperature is functional.

Coatomer from *sec28Δ* cells behaves as a large protein complex

Coatomer from *sec28Δ* cells grown at a permissive temperature, 24°C, and from congenic wild-type cells as a control, was analyzed by gel filtration (Figure 9). Immunoblot analysis of fractions obtained from Superose 6 fast-performance liquid chromatography (FPLC) gel filtration experiments (see Materials and methods) showed that coatomer from wild-type cells eluted in a peak corresponding to an apparent molecular mass of 700–800 000 Da (fractions 17 and 18 in Figure 9), as previously described by Hosobuchi *et al.* (1992). Coatomer in cytosol prepared from *ret1-3* cells grown at a permissive temperature, 24°C, showed a peak in fractions 17 and 18 indistinguishable from wild-type cytosol (data not shown). Coatomer from *sec28Δ* cells, however, consistently eluted earlier in a broad peak around fractions 13 and 14, which corresponds to an apparent molecular mass of >1000 kDa. Upon sedimentation in continuous 10–45% (w/v) sucrose gradients coatomer from *sec28Δ* cells also sedimented with a higher S value than coatomer from wild-type cells (13–14 S) (data not shown; see Duden *et al.*, 1991 for methods). The size of this very large protein complex may thus be consistent with a dimer of the coatomer complex, but it is also possible that this complex represents an aggregate, or may contain other proteins in addition to the well-characterized coatomer subunits. The precise protein composition of this complex is not known so far and will be analyzed in future experiments.

The *sec28Δ* and *ret1-3* mutations display specific synthetic-lethal interactions

To test for any synthetic-lethal interactions between the *sec28Δ* mutation and α -COP mutations, we crossed cells of the *sec28Δ* strain with *ret1-3* and *ret1-1* mutants and performed tetrad analysis. The pattern of spore viability showed that a *ret1-3 sec28Δ* double mutant was unviable at 24°C (33 tetrads) and also 16°C (33 tetrads). In contrast,

Fig. 9. Gel filtration chromatography of cytosolic proteins from *sec28Δ* cells (*sec28Δ*) and wild-type cells (wt) (for details see Materials and methods). Top panel: immunoblots of fractions obtained from gel filtration using anti-coatomer serum. Note that wild-type coatomer elutes in a peak at the expected apparent molecular mass of 700–800 kDa (fractions 17+18). Bottom panel: immunoblots of equivalent fractions obtained from gel filtration of *sec28Δ* cytosol using anti-coatomer serum. Note that *sec28Δ* coatomer elutes as a larger complex with a peak at an apparent molecular mass of >1000 kDa (fractions 13+14). V_0 =void volume. Fraction numbers and positions of marker proteins are indicated: T=Thyroglobulin (669 000), A= β -amylase (200 000), C=Carboanhydrase (29 000).

ret1-1 sec28Δ double mutants grew well at 24°C, and most inferred double mutants were even viable at 30°C. We conclude that *ret1-3* shows a strong and allele-specific synthetic-lethal interaction with the *sec28Δ* mutation. Consistent with these results, suppression of the temperature-sensitive growth defects in *ret1* mutants by the 2 μ m *SEC28* plasmid was also allele-specific. Unlike *ret1-3* cells, no suppression at any temperature by 2 μ m *SEC28* was observed in two other α -COP mutants, *ret1-1* (Letourneur *et al.*, 1994) and *sec33-1* (Wuestehube *et al.*, 1996) (data not shown). The most likely interpretation of our genetic and biochemical results is that the *ret1-3* mutation, but not the *ret1-1* mutation, affects a domain on the α -COP protein (Ret1p) responsible for direct interactions with ϵ -COP (Sec28p).

Discussion

This study characterizes the yeast ϵ -COP gene, named *SEC28* here, and reports genetic and functional interactions between mutations in the ϵ -COP and α -COP genes. ϵ -COP was the last of the yeast coatomer subunit genes remaining uncharacterized. Surprisingly, unlike the other six COP genes reported previously, ϵ -COP (Sec28p) is non-essential for yeast cell viability. *sec28Δ* mutant cells entirely lacking ϵ -COP grow well at temperatures up to 34°C, but cease growth at a higher temperature, 37°C, and only then display defects in protein trafficking. At a permissive temperature, *sec28Δ* cells show normal trafficking of carboxypeptidase Y and a KKXX-tagged membrane protein, suggesting that forward and a retrograde transport in between the ER and the Golgi is normal. Our data demonstrate that a coatomer consisting of only

six subunits (i.e. lacking ϵ -COP) is fully functional at permissive temperatures and suggest that ϵ -COP is not necessary for the *in vivo* assembly of coatomer. At elevated temperatures, however, ϵ -COP function is required for the stability of another coatomer subunit, α -COP, and thus the functional integrity of the coatomer complex.

We arrived at these conclusions based on several lines of evidence. First, the ϵ -COP gene was identified as a high copy suppressor of a novel α -COP mutant, *ret1-3*, in which α -COP is unstable at restrictive temperatures. In temperature-shifted *ret1-3* cells the mutant α -COP, but none of the other COPs, is rapidly degraded. Overproduction of ϵ -COP from a 2 μ m *SEC28* plasmid strongly suppresses the growth and transport defects of *ret1-3* at an intermediate temperature, 34°C, and stabilizes cellular α -COP levels to near wild-type.

Second, a *SEC28* gene deletion mutant lacking ϵ -COP which we constructed is viable, but shows temperature-sensitive defects for α -COP stability, cell growth and protein trafficking. In *sec28Δ* cells shifted to the restrictive temperature, 37°C, the wild-type α -COP, but none of the other COPs, is rapidly degraded. If α -COP instability was the primary defect in these cells, one would expect that α -COP overproduction should rescue their growth and transport defects. Indeed, mild overproduction of α -COP from a 2 μ m *RET1* plasmid fully suppressed the growth and transport defects in *sec28Δ* cells.

Third, the *ret1-3* mutation (which renders mutant α -COP unstable) and the *sec28Δ* mutation (which destabilizes wild-type α -COP) display an allele-specific synthetic-lethal interaction: *sec28Δ ret1-3* double mutants are unviable at any temperature, whereas *sec28Δ ret1-1* double mutants grow well up to 30°C. *ret1-1*, the first α -COP mutant to be isolated, served as a control because α -COP is stable in this mutant after temperature-shift although coatomer loses the ability to interact with KKXX motifs (Letourneur *et al.*, 1994; and data not shown). Similarly, allele-specific genetic interactions were observed using high copy suppression. *SEC28* overexpression suppressed *ret1-3* cells but not two other known α -COP mutants, *ret1-1* (Letourneur *et al.*, 1994) and *sec33-1* (Wuestehube *et al.*, 1996). We suggest that the *ret1-3* mutation impairs a domain on α -COP responsible for physical interactions with ϵ -COP, thus destabilizing α -COP.

The assembly of mammalian coatomer has been studied using native immunoprecipitations from pulse-chase labeled cells (Lowe and Kreis, 1996). Assembly of coatomer *in vivo* occurs post-translationally, takes ~1–2 h, and occurs in a stepwise fashion with specific subunit interactions occurring before others (Lowe and Kreis, 1996). Consistent with our data, Lowe and Kreis (1996) demonstrated that ϵ -COP is the last subunit to be added on to coatomer during its biosynthetic assembly. Once formed, coatomer is a stable particle with a $t_{1/2}$ of ~28 h and is incorporated *en bloc* into the COP I coat during vesicle formation (Hara-Kuge *et al.*, 1994; Lowe and Kreis, 1996). Under physiological conditions ζ -COP is the only coatomer subunit for which a significant monomeric pool can be detected (Kuge *et al.*, 1993). The coatomer complex can be reversibly disassembled *in vitro* by altering the buffer salt concentration (Lowe and Kreis, 1995). This approach revealed a subcomplex of coatomer

consisting of α -, β' - and ϵ -COP from both yeast and animal cells which still retains the ability to bind KKXX motifs *in vitro* (Cosson and Letourneur, 1994; Lowe and Kreis, 1995), whereas a β -, γ - and ζ -COP subcomplex interacts with a novel phenylalanine-containing motif present in members of the EMP24 family of putative cargo receptors (Fiedler *et al.*, 1996). Additionally γ -COP has been implicated in the KKXX-coatome interaction by a photo-crosslinking approach (Harter *et al.*, 1996). Inter-subunit interactions within the mammalian coatome have also been investigated using the two-hybrid system (Faulstich *et al.*, 1996). This approach revealed direct interactions between α - and ϵ -COP and α - and β' -COP, as well as between β - and δ -COP and γ - and ζ -COP. Thus, there is a precedent for physical interactions between α - and ϵ -COP. Our study shows that this interaction is necessary for α -COP stability, and thus the functional integrity of coatome, at high temperature. The exact mode of suppression afforded in *ret1-3* cells by overexpressing Sec28p is unknown, but we wish to suggest a simple model. Since ϵ -COP is the last component of coatome to be added during biosynthesis (Lowe and Kreis, 1996), it may normally form a stable 'keystone' which, when present, protects α -COP against degradation. The *ret1-3* mutation may lead to a lowered affinity of α -COP to ϵ -COP, thus ϵ -COP may be able to leave the coatome complex after its assembly. In *ret1-3* cells, raising cellular ϵ -COP levels then may compensate for the lowered affinity of the α -COP/ ϵ -COP interaction, restoring high ϵ -COP occupancy (i.e. an ~1:1 ϵ -COP/ α -COP ratio in the coatome population) which in turn would stabilize the coatome complex.

A mammalian CHO mutant cell line, *ldlF*, which harbors a temperature-sensitive version of ϵ -COP exhibits interesting defects in Golgi structure and membrane traffic at a non-permissive temperature. On the basis of its temperature-sensitive, conditional-lethal phenotype it has been concluded that ϵ -COP gene function is essential (Guo *et al.*, 1994, 1996). Our conclusions based on *sec28Δ* mutant cells which entirely lack ϵ -COP are somewhat in contrast to those of Guo *et al.* (1994, 1996), *ldlF* mutant cells exhibit two defects in membrane traffic at the non-permissive temperature (39.5°C): rapid degradation of LDL receptors, possibly caused by endocytic mis-sorting, and disruption of Golgi structure accompanied by a defect in ER-to-Golgi transport (Guo *et al.*, 1994). At a permissive temperature (34°C) in *ldlF* cells, the steady-state level of ts- ϵ -COP is ~50% that of wild-type ϵ -COP in control CHO cells. The point mutation (a glutamic acid residue at position 251 changed to a lysine) renders mammalian ts- ϵ -COP unstable at high temperature, but does not affect its biosynthesis (Guo *et al.*, 1996), leading to a slow decline in cellular ϵ -COP levels after ts-shift. The $t_{1/2}$ for ts- ϵ -COP is > 6 h at 34°C, and is reduced to ~1–2 h at 39.5°C (Guo *et al.*, 1996). All these defects can be rescued by transfection with an expression vector for wild-type ϵ -COP. Clearly, mutant ϵ -COP in *ldlF* cells is temperature-sensitive for both function and stability. α -COP stability in *ldlF* cells has not yet been analyzed. The only coatome subunit that has been tested is β -COP, which both Guo *et al.* (1996) (in *ldlF* cells) and we in this study (in *sec28Δ* cells) find to be stable after ϵ -COP inactivation. It will be important to test α -COP stability in *ldlF* cells.

In summary, our data clearly show that ϵ -COP gene function in yeast is non-essential for yeast cell viability. Rather, its important role lies in stabilizing the functionality of another subunit, α -COP, and thus maintaining the integrity of coatome at elevated temperatures. Given the high evolutionary conservation of coatome structure and function, we would expect these conclusions to be true for both mammals and yeast. We speculate that in both mammals and yeast a temperature-shift in ϵ -COP mutant cells (i.e. *ldlF* or *sec28Δ*) may result in some conformational changes within the coatome complex that render coatome non-functional and α -COP susceptible to proteolytic degradation. Obviously, in the absence of ϵ -COP, α -COP may either be inactivated by a conformational change which precedes, and leads to, its degradation or it may be proteolysis as such that renders α -COP non-functional. Conceivably, the onset of any observable α -COP degradation (all other things being equal), may be slower in cells harboring a ts- ϵ -COP (such as *ldlF* cells) than in cells entirely lacking ϵ -COP (such as our *sec28Δ* cells). This is because even at restrictive temperatures some ts- ϵ -COP will persist for several hours and may contribute to α -COP stability.

Based on the interaction of coatome with KKXX motifs it is thought that coatome selects its specific cargo in a manner analogous to the clathrin coat, i.e. involving interactions with cytoplasmic tails of cargo proteins or domains on cargo adaptors. Several other proteins are likely to be retrieved from the Golgi to the ER in a COPI-dependent fashion, including v-SNAREs, Erd2p, HDEL-tagged proteins, cargo receptors, and the Sec12p protein required for COP II vesicle budding (see Lewis and Pelham, 1996; Boehm *et al.*, 1997; Gaynor and Emr, 1997). Although ϵ -COP clearly functions to stabilize α -COP and thus coatome, it is possible that *sec28Δ* mutant cells may also have cargo-selective transport defects at the permissive temperature. Coatome lacking ϵ -COP may have altered affinities for certain cargo proteins, thus affecting their inclusion into COP I vesicles and transport kinetics. It will be interesting to study the binding characteristics of mutant coatome lacking ϵ -COP, and of any partial complexes that may form in temperature-shifted *sec28Δ* cells. Furthermore, the *sec28Δ* mutant itself may prove a valuable tool for genetically identifying novel membrane proteins that interact with coatome.

Materials and methods

Yeast strains, genetic techniques and plasmids

The strains used in this study are listed in Table I. Yeast was grown in rich medium consisting of 1% Bacto-Yeast extract (Difco Laboratories, Detroit, MI), 2% Bacto-Peptone (Difco) and 2% glucose (YP-Glu). In some experiments, minimal medium containing 0.7% yeast nitrogen base (Difco) with 2% glucose and the appropriate amino acids (Sherman, 1991) was used. Genetic manipulation of yeast was performed using standard methods (Rose *et al.*, 1989). Transformations into *sec* strains were done using lithium acetate (Ito *et al.*, 1983). For suppression analysis, transformants were grown on selective minimal plates and growth at 24, 30, 34 or 37°C was scored after 3–4 days. Plasmids pEG1-KK and pEG1-QK, encoding Inv.-Wbp1p fusion proteins with, or without, a functional retrieval motif were used (Gaynor *et al.*, 1994). 2 μ m plasmids harboring *SEC26*, *SEC27* (Duden *et al.*, 1994), and *SEC21* (Hosobuchi *et al.*, 1992), and a CEN-*RET1* plasmid (Letourneur *et al.*, 1994) were used.

Table I. Yeast strains

Strain	Genotype	Source or reference
RSY255	MAT α <i>ura3-52 leu2-3,-112</i>	D.Botstein
RSY259	MAT α <i>lys2-801</i>	D.Botstein
RSY263	MAT α <i>sec12-4 ura3-52</i>	Kaiser and Schekman, 1990
RSY265	MAT α <i>sec13-1 ura3-52 his4-619</i>	Kaiser and Schekman, 1990
RSY267	MAT α <i>sec16-2 ura3-52 his4-619</i>	Kaiser and Schekman, 1990
RSY269	MAT α <i>sec17-1 ura3-52 his4-619</i>	Kaiser and Schekman, 1990
RSY271	MAT α <i>sec18-1 ura3-52 his4-619</i>	Kaiser and Schekman, 1990
RSY275	MAT α <i>sec20-1 ura3-52 his4-619</i>	Kaiser and Schekman, 1990
PC70	MAT α <i>ret1-1 ura3 leu2 trp1</i>	Letourneur <i>et al.</i> , 1994
RSY277	MAT α <i>sec21-1 ura3-52</i>	Kaiser and Schekman, 1990
RSY279	MAT α <i>sec22-3 ura3-52 his4-619</i>	Kaiser and Schekman, 1990
RSY281	MAT α <i>sec23-1 ura3-52 his4-619</i>	Kaiser and Schekman, 1990
RSY956	MAT α <i>sec33-1 lys2-801</i>	Wuestehube <i>et al.</i> , 1996
RSY1317	MAT α <i>ret1-3 ura3-52 leu2-3,-112</i>	this study
RSY1318	MAT α <i>ret1-3 ura3-52 leu2-3,-112 lys2</i>	this study
RSY1319	MAT α <i>ura3 leu2 his3-Δ200 trp1 lys2 ade2 sec28Δ::HIS3</i>	this study
RSY1320	MAT α <i>ura3 leu2 his3-Δ200 trp1 lys2 ade2 SEC28</i>	this study

DNA cloning, sequencing and computer analysis

Escherichia coli strain DH5 α was used to amplify plasmid DNA. Plasmid isolation from transformed bacteria and recombinant DNA manipulations were done using standard procedures (Sambrook *et al.*, 1989). Restriction enzymes and other molecular biological reagents were purchased from Boehringer Mannheim. Taq DNA polymerase (Boehringer Mannheim) was used for PCR (Sambrook *et al.*, 1989). Dideoxy DNA sequencing was performed using the Applied Biosystems 477 sequencer following the manufacturer's instructions. Sequence data were compiled and analyzed using the UWGCG package (Devereux *et al.*, 1984). Homology searches in the DDBJ/EMBL/GenBank databases were performed using the program TBLASTN. Pairwise alignments were made using the 'GAP' program, and the multiple alignment in Figure 4 was produced with the program MEGALIGN using the CLUSTAL method.

Electron microscopy

Yeast cells were fixed by osmium or potassium permanganate and embedded, sectioned, and poststained as described (Kaiser and Schekman, 1990). Vesicles were counted using well-stained cell sections that were selected at random as described by Kaiser and Schekman (1990).

Radiolabeling, immunoprecipitations and immunoblotting

For preparation of total cell extracts, equal OD₆₀₀ of cells grown in either YPD or selective minimal media were centrifuged and washed once with ice-cold water. Cell pellets were taken up in SDS sample buffer (2% SDS, 5% β -ME, 80 mM Tris, pH 6.8) at 40 μ l/OD₆₀₀ and heated for 2 min at 95°C. An equal volume of glass beads (0.5 mm; Sigma) was added and cells lysed by vortexing for 2 min at highest speed. An additional 60 μ l sample buffer were added and extracts again heated for 3 min at 95°C. Total cell proteins were normalized to equal OD₆₀₀ equivalents and were analyzed by SDS-PAGE and immunoblotting. Radiolabeling of cells and immunoprecipitations of denatured proteins from radiolabeled extracts was performed as described (Duden *et al.*, 1994). Immunoprecipitates were analyzed on 7.5% SDS-polyacrylamide gels, followed by quantitation using a Molecular Dynamics (Sunnyvale, CA) PhosphorImager. Immunoblotting of proteins transferred to nitrocellulose and detection of filter-bound antibodies were performed using the enhanced chemiluminescence method (ECL, Amersham Corp., Arlington Heights, IL) as described by Duden *et al.* (1994).

For semi-quantitation of ECL immunoblots, exposed films were scanned with an Epson GT-9000 scanner using Adobe Photoshop run on a PowerMac computer. The density of bands was measured using the NIH Image 1.60 program. The following antisera were used to detect yeast coatomer subunits in immunoblots: Ret1p (α), Sec28p (ϵ) and Ret3p (ζ -COP) were followed using an anti-coatomer antiserum (Duden *et al.*, 1994). An antiserum raised against a Sec21p-GST fusion protein was used to follow Sec21p (γ) (Hosobuchi *et al.*, 1992); antisera against Sec26p (β) and Sec27p (β') have been described by Duden *et al.* (1994). Ret2p (δ) was detected using an antiserum raised against a C-terminal peptide (the antiserum was a generous gift from Dr Pierre Cosson, Institute of Immunology, Basel).

Preparation of cytosolic extracts and gel filtration

RSY1320 (SEC28), RSY1319 (sec28 Δ), or RSY1317 (ret1-3) were grown to 1–2 OD₆₀₀ unit of cells/ml with vigorous agitation at 24°C. The cultures were harvested by centrifugation for 5 min at 5000 g in a GSA rotor (Sorvall, DuPont, Wilmington, DE). The cell pellet was washed once with B88 (20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid-KOH, pH 6.8, 0.15 M KOAc, 250 mM sorbitol, 5 mM MgOAc) and then resuspended at 1000 OD₆₀₀ units cells/ml B88 with 1 mM DTT and a protease inhibitor mix [1 mM AEBSE, and 1 tablet of 'CompleteTM, Mini' protease inhibitors (Boehringer Mannheim, FRG)]. Cells were lysed by the addition of an equal volume of acid-washed glass beads (0.5 mm in diameter; Sigma) and vortexing twice for 90 s at maximal speed. The resulting lysate was clarified by centrifugation for 5 min in a refrigerated microcentrifuge (Model Eppendorf 5417R) and then for 30 min at 100 000 g in a TLA45 rotor (Beckman Instruments, Palo Alto, CA).

Gel filtration of soluble proteins from sec28 Δ cells, ret1-3 and congenic wild-type cells was performed in B88 at 4°C with a flow rate of 0.2 ml/min using a Superose 6 column and the FPLC system of Pharmacia-LKB. Fractions (800 μ l) were collected and proteins precipitated with 10% TCA (see Duden *et al.*, 1991). Samples were analyzed on 12% SDS-PAGE gels, transferred to nitrocellulose, and probed with anti-coatomer serum (Duden *et al.*, 1994). The following reference proteins were used for *M_r* calibration: thyroglobulin (670 000), apoferritin (440 000), β -amylase (200 000), alcohol dehydrogenase (150 000), and carboanhydrase (29 000), all from Pharmacia-LKB.

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