The EMBO Journal vol.11 no.11 pp.4187-4195, 1992

Genes that allow yeast cells to grow in the absence of the HDEL receptor

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Communicated by H.R.B.Pelham

The *ERD2* gene of *Saccharomyces cerevisiae* encodes the HDEL receptor that sorts ER proteins; it is essential for growth. In the absence of Erd2p the Golgi apparatus is both functionally and morphologically perturbed. Here we describe the isolation of four *SED* genes (*s*uppressors of the *e*rd2-*d*eletion) which, when present in multiple copies, allow cells to grow in the absence of *ERD2*. The suppressed strains secrete the ER protein BiP and their internal membranes show a variety of morphological abnormalities. Sequence analysis indicates that all these *SED* genes encode membrane proteins: *SED1* encodes a probable cell surface glycoprotein; *SED2* is identical to *SEC12*, a gene required for the formation of ER-derived transport vesicles; *SED4* encodes a protein whose cytoplasmic domain is 45% identical to that of Sec12p; *SED3* is *DPM1*, the structural gene for dolichol-P-mannose synthase. We suggest that the absence of *ERD2* causes an imbalance between membrane flow into and out of the Golgi apparatus, and that the *SED* gene products can compensate for this either by slowing transport from the ER or by stimulating vesicle budding from Golgi membranes.

Key words: ER/*ERD2* gene/Golgi apparatus/*S.cerevisiae*

Introduction

The secretory pathway of eukaryotic cells consists of a number of distinct organelles. Its compartmentalization allows for the efficient synthesis, folding, modification and export of proteins from cells. Passage from one compartment to another is thought to be mediated by transport vesicles (Palade, 1975; Rothman and Orci, 1992). Genetic and biochemical analyses have identified molecular components involved in the budding, transfer, targeting or fusion of these vesicles.

Components of the secretory pathway often have precise structural and/or regulatory roles and to carry them out efficiently they must be targeted to their sites of action. Targeting signals on both luminal and membrane-bound components have been identified and studied in detail (Jackson *et al.*, 1990; Pelham, 1990; Kornfeld and Mellman, 1989; Machamer, 1991). It seems that forward transport through the secretory pathway does not require a specific sorting signal and is the default route, while the sequences mentioned above act as retention, recycling or diverting signals within or from this 'bulk' flow.

Not only must the passage of proteins through the secretory pathway be efficiently regulated, but the pathway itself must also be maintained as a series of discrete organelles. Certain components, such as those that form part of the transport vesicles, will inevitably move between compartments and if they are to be used efficiently must be continually retrieved. Resident luminal proteins of the ER are involved in the early modification and folding of secretory proteins. They are freely diffusible within the ER and as a result can enter vesicles budding from the ER to take secretory proteins to the Golgi complex. It has been shown that luminal ER proteins can reach the Golgi complex where they receive characteristic carbohydrate modifications and they subsequently can be recycled back to the ER (Pelham, 1988, 1989; Dean and Pelham, 1990). A C-terminal tetrapeptide (KDEL) was identified as a sorting signal in mammalian cells and shown to mediate ER retention (Munro and Pelham, 1987). In the budding yeast *Saccharomyces cerevisiae* the sorting signal is HDEL and the receptor for this signal has been identified as the *ERD2* gene product (Lewis *et al.*, 1990; Semenza *et al.*, 1990).

To date there is no evidence that recycling of HDEL proteins is an essential process, although it has been found that cells were unable to grow in the absence of the HDEL receptor. Studies of cells depleted of Erd2p suggested that the main defects were not in the ER but rather within the Golgi complex (Semenza *et al.*, 1990). To try to understand the role of Erd2p in the Golgi and why it is essential for growth, we have carried out a screen for multicopy suppressors that allow cells to grow in the absence of *ERD2*. Here we report the isolation of four *SED* genes (*s*uppressors of the *e*rd2 *d*eletion) and present an analysis of the suppressed strains growing in the absence of Erd2p and of the *SED* genes. Our results support the view that Erd2p helps to maintain the balance of membrane traffic to and from the Golgi apparatus.

Results

Screen for SED genes

The *ERD2* gene has been shown to be essential for growth. Studies on cells depleted of Erd2p indicated that forward transport through the Golgi was defective, but the role of the HDEL receptor in this organelle remained unclear (Semenza *et al.*, 1990). To allow the study of cells growing in the complete absence of the HDEL receptor, we employed a 'plasmid-shuffle' technique to select genes which when present on a multicopy plasmid, would allow such cells to continue growing. Such plasmids must encode proteins which when produced at a higher level than normal, can either substitute for the essential function of Erd2p or bypass its requirement. Such proteins might themselves be important components of the secretory pathway.

A sectoring assay was used as the primary screen for

suppressor plasmids. When a plasmid containing an *ADE3* gene is present in *ade2 ade3* cells they become pink due to the intracellular accumulation of an adenine precursor. As *ADE3* is required for the formation of this precursor, cells that lose the plasmid remain white. Colonies containing some cells without plasmid have a pink and white 'sectored' appearance (Koshland *et al.*, 1985). If the plasmid also has a copy of the *URA3* gene, cells that have lost the plasmid will be able to grow on medium containing 5-fluoro-orotic acid (FOA), which is toxic only to cells containing a wild type copy of *URA3* (Boeke *et al.*, 1984).

We constructed a strain in which the chromosomal *ERD2* gene was disrupted and a copy of the *Kluyveromyces lactis ERD2* homologue was present on a centromere plasmid. This vector contained the markers *URA3* and *ADE3*, allowing us to follow its loss in the two ways mentioned above. The cells remained pink and unable to grow on plates containing FOA because, as previously shown, an *ERD2* gene is required for growth. A multicopy genomic library lacking *ERD2* was constructed in a plasmid vector (see Materials and methods) and used to transform this strain. Sectoring colonies were identified visually and streaked on FOA plates to ensure that the *ERD2* plasmid had been lost. DNA was purified from these strains and individual library plasmids isolated by transformation into *Escherichia coli*. Rescued plasmids were re-transformed into the original yeast strain to determine which plasmid contained the suppressing activity. In this manner several plasmids were isolated that allowed cells to grow in the absence of *ERD2*. A combination of Southern blotting and restriction mapping showed that they contained four different regions of genomic DNA, none of which were homologous to *ERD2*. The corresponding genes were designated *SED1*–*SED4*; of these only *SED3* and *SED4* were obtained more than once, which suggests that the screen was not exhaustive and that there remain other genes that can suppress an *erd2* deletion.

Analysis of strains growing in the absence of Erd2p

Table I shows that the suppressed strains grew well in solution, although only those with plasmid-borne *SED2* or *SED4* grew as rapidly as an isogenic strain carrying *ERD2* on a plasmid. To assess the efficiency of various steps of the secretory pathway, we immunoblotted cell extracts and culture medium using antibodies specific to the vacuolar protein carboxypeptidase Y (CPY) and the luminal ER protein BiP. Defects within the secretory pathway can readily be visualized through the accumulation of CPY precursors with modifications characteristic of the ER or Golgi complex. Suppressed strains were grown to log phase, washed twice and resuspended in fresh medium. Two hours later the cells and medium were analysed by immunoblotting. As expected all four strains had a strong *erd* phenotype: in the 2 h of log phase growth they secreted about as much BiP into the medium as a control strain where the HDEL retrieval signal had been removed from the BiP gene (Figure 1A). Thus although the *SED* genes suppress the growth defect of *erd2Δ* cells, they cannot restore the sorting function which depends upon the receptor activity of Erd2p.

When unsuppressed strains are depleted of Erd2p and stop growing, there is an accumulation of the p2 (Golgi-modified) form of CPY, indicating that transport of this protein from the Golgi complex to the vacuole, where it is processed to its mature form, is blocked (Semenza *et al.*, 1990); there is sometimes also an accumulation of the p1 (ER) precursor. The *SED1*-suppressed *erd2Δ* strain showed a similar but weaker phenotype: relative to the *ERD2*-containing strain there was on average a 1.8-fold increase in p2 and a 1.5-fold increase in p1 (Figure 1B and data not shown). In contrast, strains suppressed by the other *SED* genes showed little or no increase in p2 CPY. However, the p1 form was increased 1.5- to 1.9-fold in the *SED4* strain and 3-fold in the *SED2* strain, indicating that in these strains transport out of the ER has become relatively inefficient. Similar results were obtained by pulse–chase analysis, although the defect in the *SED1* strain was less apparent: after a 20 min chase ~20% of pulse-labelled CPY had been processed to the mature form

Table I. Growth of strains lacking the chromosomal *ERD2* gene

Rescuing gene on plasmid	Doubling time (min)
ERD2 (*S.cerevisiae*)	75
ERD2 (*K.lactis*)	92
SED1	118
SED2	90
SED3	124
SED4	79

Derivatives of strain JCB102 containing the indicated plasmids were grown in YPD with 2% glucose at 30°C and their doubling time measured in the exponential growth phase. The *ERD2* genes were present on centromere vectors, the *SED* genes on multicopy ones.

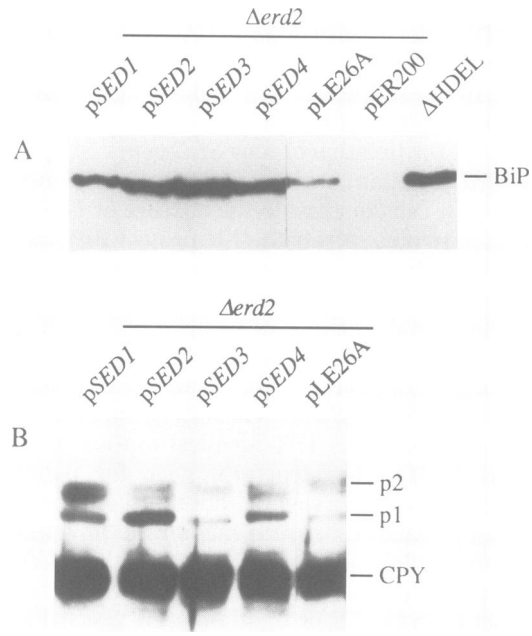

Fig. 1. Phenotypes of suppressed strains. **A.** Cells were suspended in fresh culture medium and after 2 h the BiP content of the medium was analysed by immunoblotting. The first six lanes represent medium from *erd2Δ* cells (strain JCB102) containing plasmids with the indicated genes; pLE26A carries *K. lactis ERD2*, pER200 *S.cerevisiae ERD2*. The last lane represents the material secreted from a strain in which the chromosomal BiP gene had been modified to remove the HDEL coding sequence (strain YFGR; Hardwick *et al.*, 1990). **B.** Cell extracts were analysed for their content of CPY and its p1(ER) and p2(Golgi) precursors by immunoblotting. Lanes contain strain JCB102 with the indicated plasmids. In experiments similar to the one shown here, cells containing pER200 gave the same CPY pattern as those containing pLE26A.

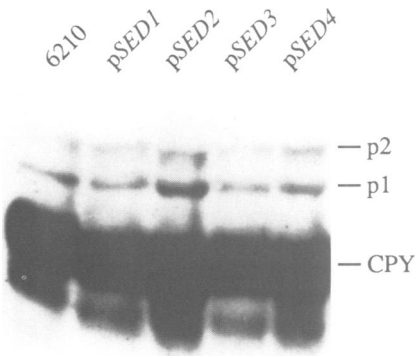

Fig. 2. Effects of *SED* genes on CPY transport in *ERD2*-containing cells. The wild type strain 6210 was transformed with *SED* gene-containing plasmids (as in Figure 1) or with the plasmid vector alone and grown in medium lacking histidine to maintain the plasmids. Cell extracts were then immunoblotted with anti-CPY antibodies, as in Figure 1.

in cells containing *ERD2*, *SED1* or *SED3*, whereas only ~7% was processed in the *SED4* strain and a negligible proportion in the *SED2* strain (data not shown). Thus overexpression of the *SED* genes can substantially reduce the Golgi transport defect that is apparent in Erd2p-depleted cells, but in some cases they hinder an earlier step in the secretory pathway.

We also tested the effects of the *SED* genes in a strain (SEY6210) containing a wild type *ERD2* gene. Overexpression of *SED1*, *3* and *4* did not have any obvious effect on the levels of CPY precursors (as determined by densitometry of immunoblots), but overexpression of *SED2* led to a slight accumulation of p1 (Figure 2). This result indicates that *SED2* can affect the export of proteins from the ER whether or not Erd2p is present, although whether its ability to slow this step is either necessary or sufficient to compensate for the absence of *ERD2* remains unclear.

Electron microscopy of cells overexpressing SED genes

The suppressed *erd2*Δ strains were analysed by thin-section electron microscopy after fixation with 1.5% potassium permanganate to accentuate membrane structures (Figure 3). When cells stop growing due to depletion of Erd2p, they accumulate long strands of membrane that are not obviously connected to the ER; these may be derived from one or more Golgi compartments (Semenza *et al.*, 1990). Growing cells from the suppressed strains did not have this appearance, but they all showed some accumulation of intracellular membranes, the precise phenotype depending on the *SED* gene present. With *SED1* the ER appeared normal, but there was an increase in the number of short membrane strands and vesicles in the cytoplasm (Figure 3B). Given the accumulation of p2 CPY in these cells, it seems likely that at least some of these are derived from the Golgi complex. With *SED2* there was a noticeable increase in the amount of peripheral ER (Figure 3C), consistent with the slowing of ER–Golgi transport in this strain; in addition, there were two other kinds of structure. In some cells there were numerous short membranous strands that were not visibly connected to either the peripheral ER or the nuclear envelope and which often formed loose clusters or parallel arrays. More striking was the appearance of clusters of tubules and vesicles that had a more distended appearance than that of the ER: the diameter of the vesicles was ~80–100 nm, which is the size reported for post-Golgi transport vesicles (Novick *et al.*, 1981). Identical structures were observed in the *SED4*-suppressed strain (Figure 3E). These membranes showed a propensity to associate with each other, occasionally forming a stacked array (for a striking example see inset in Figure 3E). Such structures were never observed in cells expressing Erd2p, even when *SED2* or *SED4* was overexpressed. Our tentative interpretation is that they represent an exaggerated form of one or more Golgi compartments. If so, their appearance when Erd2p is removed would be consistent with the view that Erd2p normally stimulates the retrieval of membrane from the Golgi apparatus and thus regulates its size.

A different picture was presented by suppressed strains carrying *SED3* (Figure 3D). These showed a spectacular disorganization: the ER was frequently fragmented and the cells filled with a mixture of patches of membrane and small vesicles (serial sectioning indicated that the latter were unlikely to be cross-sections of long tubules). This disorganization was, at least in part, a consequence of the overexpression of the *SED3* gene, because even in cells with a normal *ERD2* gene some fragmentation of the ER was apparent (Figure 4). It seems that Sed3p can perturb membrane traffic severely and that this somehow compensates for the lack of Erd2p.

Isolation and sequence analysis of SED genes

The varied and dramatic effects of the *SED* genes encouraged us to investigate the nature of their protein products. Such proteins must presumably compensate for the lack of the HDEL receptor either by carrying out some of its functions or by bypassing its requirement. It therefore seemed possible, although by no means certain, that these proteins could themselves be important components of the secretory pathway.

To map the genes, portions of the plasmid inserts were subcloned into another multicopy yeast expression vector. Alternatively, restriction sites were filled in to disrupt potential reading frames within the genomic fragments. The resulting plasmids were tested for their ability to suppress *erd2*Δ using the sectoring assay, and growth on FOA-containing plates, as described above. Sequencing of the functional regions revealed a single open reading frame for each gene, and for *SED1*, *SED2* and *SED4* a PCR-amplified version of the reading frame that was cloned into a suitable expression vector was shown to be sufficient to suppress *erd2*Δ (data not shown).

The sequence of *SED1* is shown in Figure 5. The gene encodes a 34 kDa protein with a typical signal peptide and a hydrophobic C-terminus, lacking any distal charged residues, which is likely to be exchanged for a GPI membrane anchor within the ER (Ferguson and Williams, 1988). It is extremely rich in serine and threonine residues (41.6%) and contains seven potential N-linked glycosylation sites, suggesting that it may receive extensive carbohydrate modifications. Much of the sequence is repetitive: there is a perfect 43 amino acid duplication and many short repeats (Figure 5). These features are characteristic of a number of *O*-glycosylated, GPI-anchored cell surface glycoproteins in *S.cerevisiae*, for example the *AGA1* and *KRE1* products (Boone *et al.*, 1990; Roy *et al.*, 1991). Sed1p may therefore

also be a cell surface protein. The codon bias index of the *SED1* reading frame is 0.7, suggesting that the gene is expressed at a very high level (Bennetzen and Hall, 1982).

A derivative of *SED1* in which the sequence corresponding to amino acids 58–137 was excised by *Kpn*I digestion, thus removing the short repeats and four of the N-linked

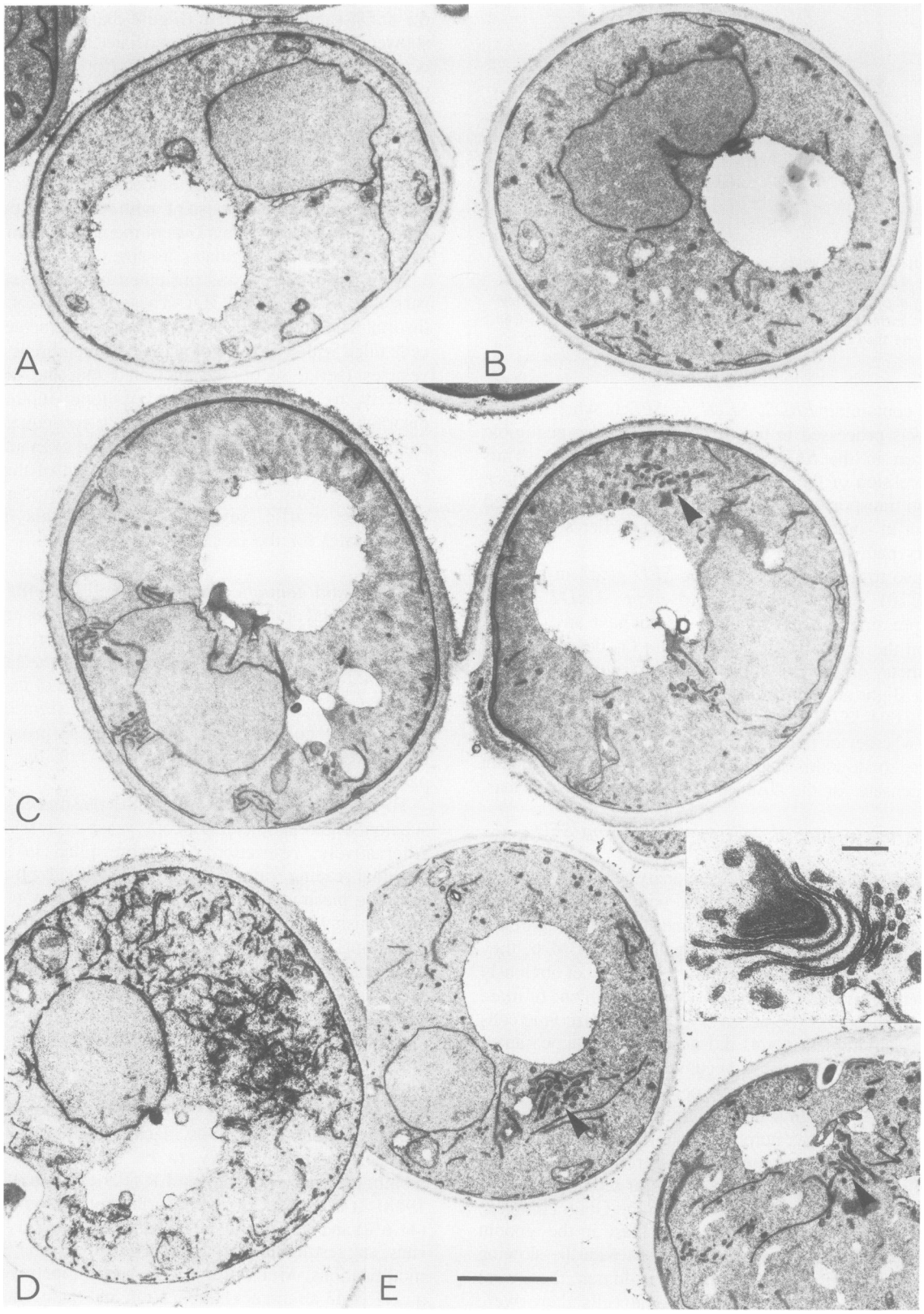

Fig. 3. Membrane morphology in suppressed strains. Electron micrographs of permanganate-stained cells are shown. All are derivatives of strain JCB102 (*erd2*Δ), with plasmid-borne copies of the following genes: **A**, *K.lactis ERD2*; **B**, *SED1*; **C**, *SED2*; **D**, *SED3*; **E**, *SED4*. Arrows indicate structures that may correspond to a late Golgi compartment; a higher magnification view of one such structure (from a *SED4*-containing cell) is shown in the inset panel in E. Bar in E is 1 μm and in the inset panel is 0.3 μm.

glycosylation sites, was also able to support the growth of *erd2Δ* cells. This suggests that the precise structure of Sed1p is not crucial for its suppressor activity.

To determine whether Sed1p is essential for growth a disruption construct was generated such that sequences corresponding to amino acids 15−321 were replaced by a fragment containing the *LEU2* gene. A haploid strain was transformed with this construct and *sed1*-disrupted strains identified by PCR amplification and Southern blotting. The disruptants showed no decrease in growth rate relative to the parental strain, at either 16, 30 or 37°C. Analysis of the secretory pathway by EM and immunoblotting of CPY failed to reveal any defects (data not shown). We therefore conclude that Sed1p either does not play an important role in cell growth or performs a function that can be efficiently carried out by the product of another gene. Its ability to compensate for the lack of Erd2p when expressed from a multicopy plasmid may result from a relatively non-specific effect: the overloading of the secretory pathway by the massive synthesis of a single membrane protein.

SED2 is SEC12

SED2 was mapped in a similar manner to *SED1*; sequencing identified it as the *SEC12* gene. *SEC12* encodes a ~70 kDa type II integral membrane glycoprotein. Analysis of temperature-sensitive mutants *in vivo* and *in vitro* has shown that it is required for the formation or budding of transport vesicles from the ER (Kaiser and Schekman, 1990; Rexach and Schekman, 1991). This function involves the cytoplasmic domain of the protein, which is thought to interact directly with the small GTP-binding protein Sar1p (d'Enfert *et al.*, 1991; Oka *et al.*, 1991). Although biochemical fractionation indicates that the majority of Sec12p is found in the ER, analysis of its carbohydrate side chains indicates that it receives Golgi-specific modifications, implying that the protein can reach the Golgi complex (Nakano *et al.*, 1988). It has been suggested that Sec12p cycles between ER and Golgi, although it is not known whether this is important for its function. Sec12p is a rare protein in wild type cells: the *SEC12* reading frame has a codon bias index of 0.06 and quantification of Northern blots suggested that it is represented by 2−4 mRNA molecules per cell (Nakano *et al.*, 1988).

SED4 encodes a protein related to Sec12p

We isolated two plasmids with overlapping sequences derived from the *SED4* gene. Mapping and sequencing of the first identified a reading frame that was able to suppress *erd2Δ*. This reading frame extended to the junction between the genomic DNA insert and the vector and the protein it encoded ended with a short amino acid stretch derived from vector sequences. Subsequently, a second plasmid with suppressing activity was isolated and proved to contain the entire *SED4* reading frame. While sequencing the additional portion of the gene, we discovered that it resides on

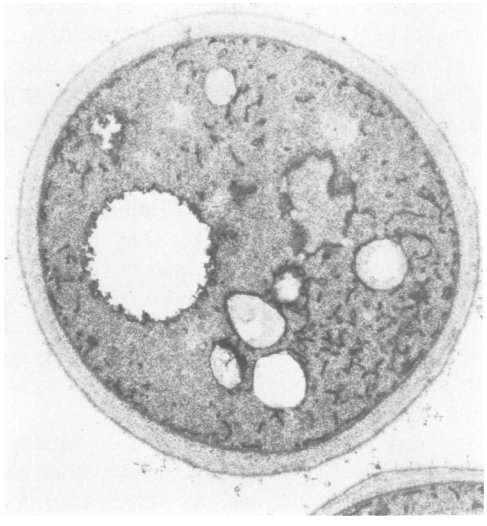

Fig. 4. Fragmentation of intracellular membranes by *SED3*. The figure shows an electron micrograph of a cell from strain 6210 (wild type for *ERD2*) transformed with the multicopy *SED3* plasmid. Note the disruption of ER morphology and the presence of vesicular structures. Cells containing *SED1*, *2* or *4* on multicopy plasmids were indistinguishable from the parent strain.

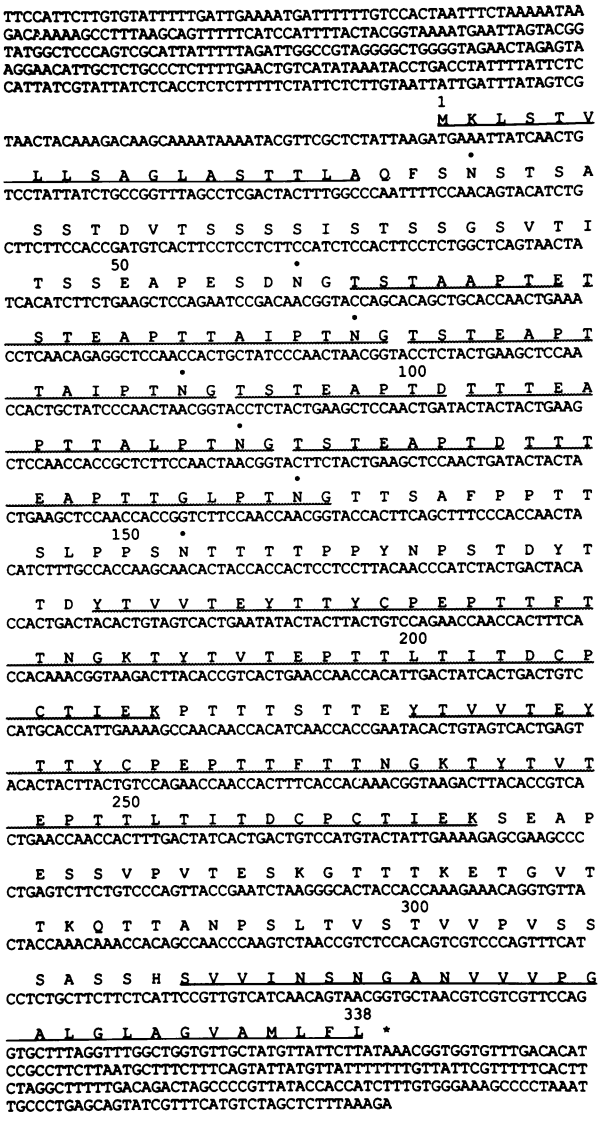

Fig. 5. Sequence of the *SED1* gene. The hydrophobic signal peptide and C terminus of the protein are indicated by solid underlining, and repeated sequences by dotted underlining. Solid dots indicate potential N-linked glycosylation sites. The DNA sequence has been submitted to the EMBL database (accession number X66838).

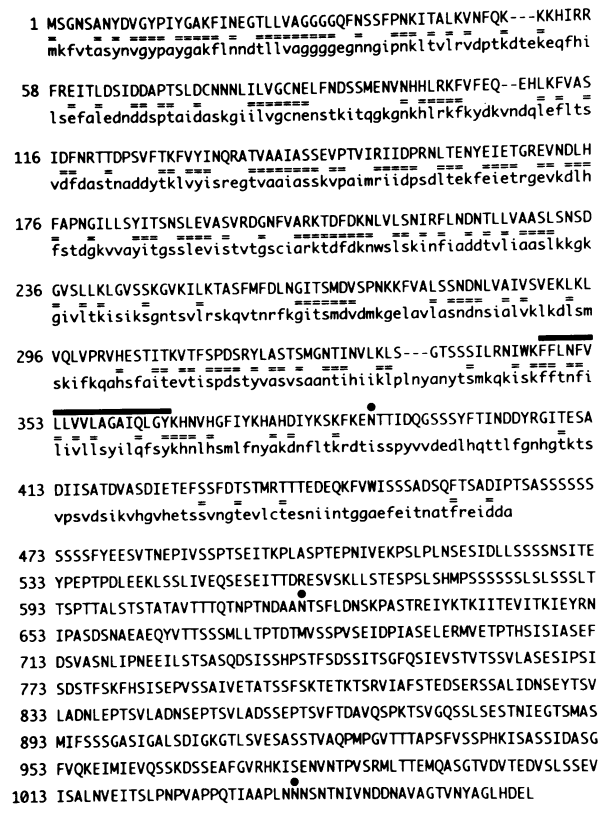

Fig. 6. Alignment of the amino acid sequences of Sed4p and Sec12p. The Sed4p sequence is shown in capital letters, with the Sec12p sequence below it in lower case. Identities are indicated by =. The transmembrane domain is overlined and sites for N-linked glycosylation in the luminal domain of Sed4p are indicated by dots.

chromosome III, whose entire sequence has recently become available (Oliver *et al.*, 1992). The nucleotide sequence we obtained for *SED4* was identical to that of the YCR67c reading frame found on chromosome III.

Sed4p is predicted to be a type II membrane protein with a single transmembrane domain. The N-terminal cytoplasmic domain shows a striking similarity (45% identical residues) to the corresponding portion of Sec12p. An alignment of the two sequences in shown in Figure 6. The luminal domains of the two proteins differ markedly in size and sequence, although both are rich in serine and threonine residues and have sites for N-linked glycosylation. Since the version of *SED4* that we first isolated encoded only the first 395 amino acids of Sed4p, it is clear that most, if not all, of the luminal domain is dispensible for suppression of *erd2Δ*; the luminal domain of Sec12p also appears to lack function in vesicular transport (d'Enfert *et al.*, 1991). Like Sec12p, Sed4p is predicted to be a rare protein, with a codon bias index of 0.09.

Strikingly the C-terminus of Sed4p bears the ER retention signal HDEL. One other integral membrane protein, Sec20p, is known to carry an HDEL signal, and has been shown to be sorted by the *ERD2*-encoded receptor (Sweet and Pelham, 1992). The presence of the ER retention signal strongly suggests that Sed4p can cycle between the ER and Golgi complex, as has been proposed for Sec12p.

These similarities between *SED4* and *SEC12*, and the similar phenotypes of *erd2Δ* strains suppressed by these genes (Table I, Figure 3), suggest that the two genes have related functions. However, *SEC12* is an essential gene and the temperature-sensitive allele *sec12-4* was not complemented by *SED4* even when the latter was expressed from the strong TPI promoter on a multicopy plasmid. On the contrary, such expression slowed the growth of a *sec12-4* strain on plates incubated at 30°C, though it had no effect on the growth of an isogenic *SEC12* strain (data not shown). This observation suggests that Sed4p can specifically interfere with the function of Sec12p, perhaps by competing for an interacting protein such as Sar1p, and underlines the fact that Sed4p cannot perform all the functions of Sec12p.

To investigate further the role of *SED4*, a construct was prepared in which DNA encoding the first 266 amino acids of the protein, together with 164 bp of 5′ flanking sequence, was replaced by the *HIS3* gene; this was used to disrupt the gene in a diploid strain. Haploid derivatives of this strain with a disrupted chromosomal copy of *SED4* appeared unaffected as judged by growth rate, the steady-state level of CPY precursors and the appearance of intracellular membranes in the EM (data not shown). We conclude that the function of *SED4* is either too subtle to be detected in these assays or can be performed adequately by another gene. Southern blotting did not reveal any closely related *SED4* homologue; there might, however, be another more distantly related gene or it may be that *SEC12* can substitute for *SED4*, even though the converse is not true.

SED3 is DPM1

Mapping and preliminary sequencing of *SED3* indicated that it was identical to the *DPM1* gene. *DPM1* is an essential gene that encodes dolichol-phosphate-mannose (Dol-P-Man) synthase, which catalyses the conversion of dolichol-phosphate and GDP-mannose to Dol-P-Man (Orlean *et al.*, 1988). Dol-P-Man is used in the synthesis of N-linked core oligosaccharides, donating the last four mannose residues of the man_9 core-oligosaccharide; it is the sugar donor for the first mannose of O-linked oligosaccharides attached to serine and threonine residues and it also donates the mannose used in the synthesis of GPI anchor precursor lipids used to attach a number of plasma membrane proteins to the lipid bilayer (Orlean, 1990). To confirm that the *erd2Δ* suppressing activity resides in the *DPM1*-encoded protein, we tested two temperature-sensitive alleles of *DPM1*, *dpm1-4* and *dpm1-6*. These alleles were generated by *in vitro* mutagenesis of the cloned *DPM1* gene and have very little enzymatic activity even at low temperature, as determined by *in vitro* assay (Orlean, 1990). They were unable to suppress *erd2Δ* when present on a multicopy vector, whereas the unmutagenized parent plasmid did suppress, as expected. It seems that only a moderate increase in functional Dpm1p is required for suppression, because the wild type gene was found to suppress even when present on a centromere-containing vector (data not shown).

Dpm1p is a 30 kDa protein with a stretch of 25 hydrophobic residues at its C-terminus, which is thought to anchor it in the ER membrane. As judged from its codon bias index (0.3), it is likely to be moderately abundant. It is possible that its ability to generate vesicles when overexpressed is not a consequence of its enzymatic activity, but is a separate functional property of the protein. However, our current data do not allow a clear distinction between these possibilities.

Not all membrane proteins can suppress the growth defect of erd2Δ cells

The varied nature of the *SED* gene products suggests that at least some of them may suppress the *erd2Δ* defect in an indirect way. Since all of them are membrane proteins that are associated with the secretory pathway, we tested several other such proteins to see how general this suppression phenomenon might be. When present on a multicopy vector or placed under the control of the strong TPI promoter, neither *ERD1*, which encodes a probable Golgi resident (Hardwick *et al.*, 1990), *ERS1*, which partially suppresses the *erd* phenotype of *erd1* (Hardwick and Pelham, 1990), *MNT1*, which encodes a Golgi mannosyltransferase (Häusler and Robbins, 1992), *SEC20*, which is required for transport between ER and Golgi (Sweet and Pelham, 1992), nor *WBP1*, which encodes a component of oligosaccharyl transferase (te Heesen *et al.*, 1992), were able to suppress. Thus, the *SED* genes must have special properties that are not shared by most membrane proteins.

Discussion

Why is ERD2 required for growth?

We have previously found that the HDEL receptor, encoded by the *ERD2* gene, is required for growth of yeast cells. Since viable *erd2* mutants exist that show no retention of HDEL-containing proteins, the essential role of *ERD2* can be distinguished from its sorting function. Indeed, depletion of Erd2p does not impair ER function; rather, it causes a defect in transport through the Golgi complex (Semenza *et al.*, 1990). In this paper we have shown that cells can grow in the complete absence of Erd2p, provided that one of four *SED* genes is overexpressed. Examination of such cells and of the *SED* genes themselves, gives further information about the normal role of the receptor.

The common feature of all the strains that lack *ERD2* is that the organelles of the secretory pathway are morphologically abnormal. In some cases the peripheral ER appears unaffected, but in each strain there is an accumulation of membranes and/or vesicles. In the strains expressing *SED2* (*SEC12*) or *SED4*, some of these membranes have an appearance that clearly distinguishes them from the ER and suggests that they represent an enlarged Golgi compartment. These structures do not closely resemble the 'Berkeley bodies' observed in *sec7* and *sec14* temperature-sensitive mutants at the restrictive temperature, which are also thought to represent an abnormal Golgi apparatus (Novick *et al.*, 1981); however, the underlying defects in the different mutants are unlikely to be identical. The lack of good criteria for identifying Golgi-derived membranes by EM makes any interpretation speculative, but it seems likely that the Golgi apparatus is morphologically abnormal in most, if not all, of the suppressed *erd2Δ* strains. *SED1*, *2* and *4* did not significantly affect membrane morphology when they were overexpressed in cells containing Erd2p, so it would appear that the perturbation of Golgi membranes is a consequence of the absence of Erd2p.

Our current view is that Erd2p, as a major recycling protein, helps to maintain the rate of membrane retrieval from the Golgi complex: retrograde carriers will contain not only Erd2p and bound ligand, but also other membrane proteins and lipid molecules. If recycling is reduced, ER proteins and lipids will tend to accumulate within Golgi membranes. In cells depleted of Erd2p, transport out of the ER continues; thus their growth arrest is unlikely to be caused by the loss of a crucial ER component that is required for forward transport. However, the Golgi complex may become 'poisoned' by the presence of ER components, either protein or lipid, and fail to carry out its normal transport functions efficiently. In such a model the HDEL receptor would regulate the size and composition of the Golgi complex. It could stimulate reverse transport simply by providing a structural component of the retrograde carriers or it might play a more subtle regulatory role in their formation. In its absence, the Golgi compartments would enlarge, because more membrane was entering than leaving, to the point where their function was impaired. Interestingly, overexpression of Erd2p in mammalian cells appears to have the opposite effect, causing disruption of the Golgi apparatus and the appearance of Golgi markers in the ER (Hsu *et al.*, 1992). This could be explained by an excessive stimulation of retrograde transport by Erd2p, although more indirect effects cannot be ruled out.

The ability of cells to grow without *ERD2* under some circumstances implies either that the receptor is not essential for retrograde transport or that such transport is not absolutely essential for the maintenance of a functional secretory pathway. On the other hand, since all the *erd2Δ* strains have an abnormal membrane organization it seems that the full role of Erd2p in membrane transport cannot easily be played by other proteins.

How do the SED genes compensate for the lack of ERD2?

If the defect in *erd2Δ* cells is caused by an imbalance of traffic into and out of the Golgi complex, then expression of any protein that can perturb this balance in an appropriate way may be sufficient to compensate for the defect. In principle, balance could be restored by slowing the influx of membrane into the Golgi apparatus from the ER or by stimulating either forward or reverse transport out of the Golgi compartments. Recent experiments with the *SEC20* gene suggest that slowing import may indeed be sufficient to solve the problem. Sec20p is a type II membrane protein that is required for ER–Golgi transport (Sweet and Pelham, 1992) and expression of a soluble form of its cytoplasmic domain results in the competitive inhibition of this transport step (D.Sweet, unpublished observations). Strikingly, expression of this domain also allows cells lacking Erd2p to grow, albeit slowly. This suggests that any protein that can specifically slow export from the ER might be capable of suppressing *erd2Δ* strains.

Whether any of the *SED* genes act in this way remains to be seen. The *SED1* product is likely to be an abundant cell surface glycoprotein and its expression at a very high level may interfere (directly or indirectly) with the kinetics of the secretory pathway. However, it did not have a dramatic effect on ER–Golgi transport in either wild type or *erd2Δ* cells, as judged by the appearance of the ER in electron micrographs. Perhaps high levels of cargo, in the form of Sed1p, can stimulate the budding of vesicles from the Golgi complex. Alternatively, overexpression might hinder the processing of Sed1p and the resulting abnormal molecules stimulate recycling to the ER, following a pathway

analogous to that proposed for unassembled class I MHC molecules in animal cells (Hsu *et al.*, 1991).

SED3 (*DPM1*) failed to cause a significant accumulation of the ER precursor of CPY, but it clearly had an effect on membrane organization: surprisingly, overexpression of this gene caused numerous small vesicles to accumulate in both wild type and *erd2Δ* cells. Whether this effect requires the enzymatic activity of Dpm1p, which encodes dolichol-P-Man synthase, or merely the presence of a relatively abundant protein that can interact with the transport machinery, remains to be determined. Dpm1p is thought to be a constituent of the ER membrane (Orlean *et al.*, 1988), but it is possible that it can recycle through the Golgi complex, in which case it may influence the budding (or fusion) of vesicles derived from both ER and Golgi compartments. Alternatively, overexpression of Dpm1p may perturb the regulation of dolichol metabolism and thereby alter the properties of ER and Golgi membranes indirectly—it is known for example that dolichol phosphate can affect membrane fluidity and fusion activity in model systems (van Duijn *et al.*, 1986).

The genes that are likely to have the most specific effects are *SED2* or *SEC12* and *SED4*. These encode rare proteins, yet allow *erd2Δ* strains to grow as rapidly as those complemented with *ERD2*. The two proteins have related cytoplasmic domains and each induces the same unusual morphology of presumptive Golgi membranes in suppressed strains, suggesting that they act in a similar manner. However, whereas overexpression of Sec12p slows ER−Golgi transport, as shown by the accumulation of the p1 form of CPY and of ER membranes, Sed4p has only a small effect. Thus while inhibition of forward transport is a plausible mechanism by which *SEC12* could suppress, this seems less likely for *SED4*; it is possible that both genes have some other effect on the secretory pathway that contributes to their suppressor activity.

What could this effect be? Sec12p is known to be involved in the budding of vesicles from the ER and is also known to receive Golgi-specific carbohydrate modifications, implying that it can reach the Golgi apparatus (Nakano *et al.*, 1988). Sed4p carries an HDEL signal and is therefore likely to cycle between ER and Golgi compartments in wild type cells, and may travel even further in the absence of Erd2p. Perhaps overexpression of either protein is sufficient to stimulate budding of transport vesicles from the Golgi complex and thus to compensate for the absence of Erd2p.

Such a model is difficult to prove genetically. Loss of *SEC12* function blocks transport from the ER and this would mask any possible role in the Golgi apparatus. *SED4* cannot substitute for *SEC12*, implying that it has a distinct function, but deletion of the *SED4* gene from wild type cells did not result in any strong phenotype. This would be explicable if Sec12p has multiple functions, only some of which are shared with Sed4p. Alternatively, Sed4p might merely serve to stimulate a transport step that is not rate limiting in *ERD2*-containing strains under laboratory conditions. A third possibility is that there is a larger family of *SEC12*-like genes that mediate budding from various compartments and an undiscovered member of this family can substitute for *SED4*; however, our attempts to identify additional related genes by PCR and cross-hybridization have so far been unsuccessful. Clearly, further work will be required to define the precise roles of *SED4* and *SEC12*.

Although it is not easy to deduce the function of each *SED* gene, our genetic screen for *erd2Δ* suppressors may nevertheless prove a useful way to identify proteins that regulate the secretory pathway—in particular, those that stimulate retrograde transport. Indeed, we have recently characterized a new *SED* gene (*SED5*), which encodes a novel membrane protein required for vesicular transport (Hardwick and Pelham, 1992). Further suppressor genes have been isolated, and are currently under investigation.

Materials and methods

Strains, library construction and SED gene isolation

We mated strains CH1304 (provided by C.Holm) and D209 (Semenza *et al.*, 1990), sporulated the resulting diploid and screened haploid progeny for the following genotype: *MAT***a** *ade2 ade3 his3-Δ200 leu2-3,-112 ura3-52 TRP1 erd2Δ*; pJS209 (*ERD2 URA3*). This strain (named JCB102) was then transformed with pLE25A (TPI-driven *Kluveromyces lactis ERD2*, *CEN6*, *LEU2*, *ADE3*) and after growth on plates lacking leucine, but containing uracil, single colonies were screened to identify those that could no longer grow in the absence of uracil and thus had lost pJS209. These were then transformed with pLE26A (TPI-driven *K.lactis ERD2*, *CEN6*, *URA3*, *ADE3*) and cells that had lost pLE25A were identified in the same way. These derivatives of strain JCB102 were used for all sectoring assays and for isolation of the *SED* genes. To assess the effects of *SED* genes in wild type cells, we used strain SEY6210 (*MATα ura3-52 his3-Δ200 leu2-3,-112 trp1-Δ901 lys2-801 suc2-Δ9*). *SED4* and *SEC12* constructs were also tested in strains RSY263 (*MATα sec12-4 ura3-52*) and RSY255 (*MATα ura3-52 leu2-3,-112*), obtained from C.Kaiser.

To construct a library lacking the *ERD2* gene, genomic DNA was prepared from a derivative of JCB102 that carried the *K.lactis ERD2* gene on a *CEN* plasmid, pJB11. DNA was digested with *Not*I, which cuts pJB11 to give a 1.7 kb fragment containing *ERD2*. The DNA was then cut partially with *Sau*3A and fragments >2 kb were gel purified, thereby eliminating *ERD2* sequences. These fragments were ligated into the unique *Bam*HI site of the multicopy yeast vector HUC13, which was made from the 2μ−*LEU2* vector ZUC13 by replacing the *Bam*HI−*Cla*I fragment of *LEU2* with a 1.7 kb *Bam*HI−*Cla*I *HIS3* fragment.

To isolate *SED* genes, the library was transformed into the sectoring strain JCB102, transformants being selected on plates lacking histidine. After 2−3 days growth, the transformants were pooled and replated on plates containing 20 μg/ml adenine sulphate. Low adenine concentrations enhance the red colour of *ade2 ADE3* strains. Sectoring did not occur on the original transformation plates, because *ADE3* is required for growth in the absence of added histidine. White (*ade3*) colonies and sectors were picked and streaked on plates containing 5-fluoro orotic acid (FOA), which selects against *URA3* (Boeke *et al.*, 1984); only cells that had lost pLE26A (and thus the *URA3* gene) could grow on these plates.

Expression plasmids

*Eco*RI sites were introduced at the 5′ ends and *Bam*HI sites at the 3′ ends of the open reading frames encoding *SED1*, *SEC12*, *SED4* and *SEC20* using PCR-mediated, oligonucleotide-directed mutagenesis (Higuchi, 1989). Where possible, portions of the PCR-amplified DNA were replaced with cloned genomic DNA to avoid possible sequence errors introduced during the PCR reaction; regions that were not replaced were checked by sequencing. For high level expression, the *Eco*RI−*Bam*HI fragments containing the coding sequences were inserted into plasmid JS209 (Semenza *et al.*, 1990) in place of the *ERD2* gene. This plasmid contains the TPI promoter, a 2μ origin of replication and the *URA3* marker. *ERS1* and *ERD1* were expressed from their own promoters, in the multicopy vector ZUC13 (containing *LEU2*). *Bam*HI−*Nru*I fragments containing the wild type *DPM1* gene from pDM8 and temperature-sensitive alleles *dpm1-4* and *dpm1-6* from mutagenized pDM8 derivatives (Orlean, 1990) were subcloned into ZUC13 before testing for suppressor activity. The *MNT1* coding sequence (isolated from genomic DNA by PCR) was also expressed from the TPI promoter in a construct integrated at *URA3*. Disruption of *SED1* and *SED4* was performed by the one-step method (Rothstein, 1983), and checked by Southern blotting. For *SED1*, sequences corresponding to amino acids 15−321 were replaced by the *LEU2* gene; for *SED4*, 164 bp of 5′ flanking sequence and the first 266 codons were replaced by the *HIS3* gene.

Other procedures

Protein extracts for western blotting were prepared from log phase cells by glass bead lysis as described by Blumer *et al.* (1988). Rabbit antibodies

to CPY and BiP were provided by N.Dean and J.Vogel respectively; they were detected using the ECL detection kit (Amersham) and peroxidase-conjugated goat anti-rabbit IgG (Sigma). Sequence was obtained from alkali-denatured DNA using Sequenase kits (United States Biochemical Corporation). Initial sequence of one strand was obtained from nested deletions (Yanisch-Perron *et al.*, 1985) made in Bluescript vectors (Promega) and confirmed by sequencing the other strand using synthetic oligonucleotide primers. Plasmid DNA was isolated from yeast cells by the rapid method described by Ward (1990). For EM, yeast cells were fixed with 1.5% potassium permanganate, sectioned and post-stained as described by Sweet and Pelham (1992). Other yeast manipulations were performed by standard methods (see Guthrie and Fink, 1991).

Acknowledgements

We thank Debbie Sweet for plasmids expressing intact and truncated *SEC20*, and for unpublished information and advice; Connie Holm for plasmids and yeast strains for the sectoring assay and Peter Orlean for *dpm1*-containing plasmids and strains. We are also grateful to Douglas Kershaw for help with the electron microscopy, Neta Dean and Joe Vogel for antibodies, and Marcus Aebi, Rowan Chapman and Sean Munro for plasmids encoding *WBP1* and *MNT1*. Alison Pidoux, Gabriella Frigerio and Debbie Sweet provided helpful comments on the manuscript. J.C.B. was supported in part by a Fogarty Senior International Fellowship and the Buroughs Wellcome Fund; K.H. is a Junior Research Fellow of Churchill College, Cambridge. A.D.R. was supported in part by a Robert Bates Travelling Fellowship.

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Received on 12 June, 1992; revised on 22 July, 1992