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ERD1, a yeast gene required for the retention of luminal endoplasmic reticulum proteins, affects glycoprotein processing in the Golgi apparatus

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We have previously shown that the C-terminal sequence HDEL acts as a retention signal for luminal endoplasmic reticulum (ER) proteins in *Saccharomyces cerevisiae*, and that it is possible to isolate mutants that fail to retain an invertase fusion protein bearing this signal. Analysis of many such mutants defines two genes, *ERD1* and *ERD2*. Cells lacking the *ERD1* gene secrete the endogenous ER protein, BiP. Under normal growth conditions, the rate of secretion is equivalent to the rate at which wild-type cells secrete a modified form of BiP that lacks the HDEL signal altogether. Thus, *erd1* cells show a profound disruption of the retention system. The mutant cells have no gross abnormality of their intracellular membrane system, but show defects in the Golgi-dependent modification of glycoproteins. We suggest that sorting of luminal ER proteins normally occurs in the Golgi, and that the function of *ERD1* is required for the correct interaction of an HDEL receptor with its ligands. The sequence of *ERD1* predicts a membrane protein with several transmembrane domains, a conclusion supported by analysis of *ERD1-SUC2* fusion proteins.

Key words: endoplasmic reticulum/protein sorting/secretion/*Saccharomyces*

Introduction

The targeting of proteins to their correct locations in eucaryotic cells involves the recognition of signals encoded in their amino acid sequences. Specific cellular components must interact with these signals and direct the proteins to their destinations.

One such signal is found at the C-termini of resident luminal endoplasmic reticulum (ER) proteins. Although they reside within the secretory pathway, these proteins are not secreted; their continued presence in the ER appears to depend only upon the C-terminal sequence KDEL (in animal cells) or HDEL (in the yeast *Saccharomyces cerevisiae*) (Munro and Pelham, 1987; Pelham *et al.*, 1988). There is evidence that the proteins can leave the ER by vesicular transport but are specifically retrieved from a subsequent salvage compartment and returned to the ER. This retrieval is presumed to involve a membrane-bound KDEL (HDEL) receptor that cycles between the salvage compartment and the ER (Pelham, 1988, 1989).

In an attempt to learn more about the HDEL retention system in yeast, we have isolated mutants that are defective in the retention of HDEL-containing invertase fusion proteins (Pelham *et al.*, 1988). Here we describe the characterization of these *erd* (ER retention defective) mutants and present the DNA sequence of *ERD1*, which encodes a non-essential integral membrane protein. Cells lacking *erd1* show defects in the Golgi-dependent processing of several glycoproteins, which supports the idea that the Golgi is involved in the sorting of yeast ER proteins.

Results

Antisera specific for yeast ER proteins

To generate a reagent suitable for localizing endogenous ER proteins in wild-type and mutant cells, we prepared antisera against the synthetic peptide YTFEHDEL, the last six amino acids of which correspond to the C-terminus of the yeast homologue of the luminal ER protein BiP (Bole *et al.*, 1986; Munro and Pelham, 1986; Normington *et al.*, 1989; Rose *et al.*, 1989; L.Moran, personal communication). The sera obtained were affinity purified on a column containing the peptide GSDVHDEL, to ensure that they recognize only the terminal HDEL sequence; however, similar results were obtained with antisera purified on the original YTFEHDEL peptide.

When the anti-HDEL antibodies were used to probe blots of total yeast extracts several proteins were specifically bound (Figure 1), the most prominent of which had an apparent molecular weight of about 83 kd. We were able to confirm that this band corresponds to BiP, using a cloned copy of the BiP gene which we isolated from an expression library using the anti-HDEL antibodies. The cloned gene was used to introduce a mutation into the 3′ end of the chromosomal gene (see Materials and methods), which converted the C-terminus of BiP from FEHDEL to FGR. As expected, the strain expressing this altered form of BiP lacked the 83 kd anti-HDEL reactive protein band (Figure 1b).

A second prominent band, labelled X in Figure 1, showed an increased gel mobility after endo H digestion, indicating that it is a glycoprotein; its apparent molecular weight after deglycosylation was 60 kd. Both proteins were present at higher levels in extracts made from cells exposed to a heat shock (30 min at 40°C followed by 60 min recovery at 30°C). Two other proteins, Y and Z, were detected when high levels of protein were loaded on the gels (Figure 1b). Y is a glycoprotein whose size after endo H treatment was ~97 kd; it may correspond to the mammalian ER protein GRP94 (for review see Pelham, 1989). Z, which is barely visible in Figure 1b but was detected reproducibly, is a non-glycosylated protein of ~40 kd.

Protein X showed a marked increase in apparent abundance after endo H digestion. This suggests that some of the protein normally exists in a Golgi-modified form—such proteins have large and heterogeneous carbohydrate side chains and run as a smear on SDS-containing gels which is hard to detect by immunoblotting. We have previously reported that invertase fusion proteins bearing the HDEL signal undergo some

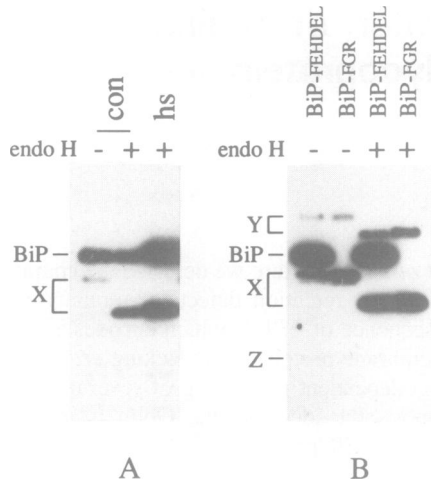

Fig. 1. Detection of HDEL-containing proteins by immunoblotting. (**A**) Whole cell extracts from cells grown at 30°C (con) or from heat-shocked cells were analysed with or without endo H digestion. (**B**) Cells from strains containing the wild-type BiP gene (BiP-FEHDEL) or a modified BiP gene in which the last five codons have been altered (BiP-FGR) were analysed. More protein was loaded on this gel, to facilitate detection of minor immunoreactive bands.

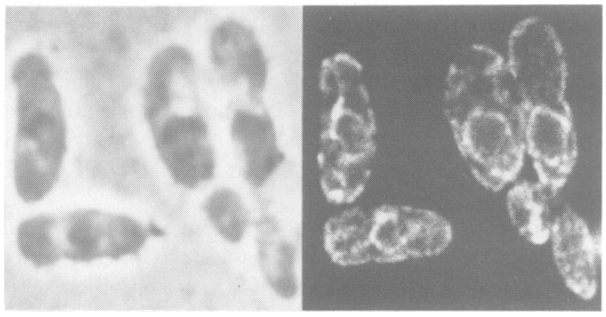

Fig. 2. Immunofluorescent staining of yeast (*S.uvarum*) cells with anti-HDEL serum. Phase-contrast (**left**) and immunofluorescent (**right**) images were obtained with a confocal microscope. Note staining of the nuclear envelope, material close to the plasma membrane and some strands of presumptive ER in the cytoplasm. Full width of the images corresponds to 20 μm.

Table I. Summary of *erd* mutants obtained

Complementation group	Number of mutants		
	a	α	total
erd1	14	19	33
erd2	3	2	5
unclassified[a]	7	5	12

[a]Only mutants with a strong phenotype are included. All of the **a** mutants complemented all of the α mutants.

Golgi-specific modifications, even though they are not secreted from cells (Pelham *et al.*, 1988).

Figure 2 shows the immunofluorescence pattern obtained when yeast cells were stained with the anti-HDEL antibodies. For this figure, we used a strain of *S.uvarum* which gives particularly clear morphology (Kilmartin and Adams, 1984), but similar results were obtained with *S.cerevisiae*. Examination of optical sections obtained with a confocal laser-scanning microscope, together with three-dimensional image reconstruction, shows that the staining was restricted mainly to the nuclear envelope (identified by double labelling of nuclear DNA with DAPI) and to a fenestrated shell close to the plasma membrane, with some staining in linear structures connected to these. This is the typical distribution of the ER as seen in electron micrographs of yeast cells (e.g. see Figure 8a). We conclude that the antibodies recognize a small family of proteins which are, as expected, normally resident in the ER.

Isolation of mutants that secrete endogenous ER proteins

The isolation of the *erd* mutants was based on their ability to secrete an HDEL-bearing invertase fusion protein, as previously described (Pelham *et al.*, 1988). UV-induced mutants were detected either by an *in situ* assay for external invertase activity, or by their ability to form large colonies on plates which contain sucrose as the sole carbon source (growth on sucrose requires extracellular invertase to convert sucrose to glucose and fructose, because sucrose itself cannot be taken up by yeast cells). To identify mutants that are truly defective in the HDEL retention system, we devised a secondary screen that detects secretion of endogenous resident ER proteins.

Mutant cells were grown in contact with a nitrocellulose filter. Secreted ER proteins, bound to the filter, were then detected by probing with the anti-HDEL antibodies followed by ^{125}I-labelled protein A (see Figure 6 for an example of the method). Many, but not all, of the mutants secreted significant quantities of HDEL-containing proteins. In principle, this assay should detect the invertase fusion protein itself, but the level of expression of this protein was much lower than that of BiP and protein X, and examination of mutant strains from which the fusion gene had been removed by recombination showed that it did not contribute significantly to the anti-HDEL signal (data not shown). Initial screening revealed that a subset of the mutants showed a strong phenotype both by this assay (as judged by densitometry) and by quantitation of the secreted invertase activity. We selected for further study only those mutants that gave a signal at least half as strong as this apparent maximum in both assays.

Mutants were isolated from both **a** and α strains. They were tested for dominance by back-crossing to the parental strain of the appropriate mating type, and the few dominant mutants were not characterized further. The remainder were crossed with mutants of the opposite mating type, and the resulting diploids assayed for secretion of invertase activity and HDEL-containing proteins.

This analysis defined two complementation groups of mutants with strong phenotypes (*erd1* and *erd2*, see Table I). Of the remaining mutants, none secreted ER proteins as efficiently as the strongest alleles of *erd1* or *erd2*, and they complemented all mutants with the opposite mating type. Further studies of *ERD1* are described below; *ERD2* will be described in a subsequent paper.

Cloning of ERD1

The wild-type *ERD1* gene was isolated by transforming mutant cells with a library of yeast genomic DNA in a centromere-containing vector and screening for colonies that were negative in the *in situ* assay for secreted invertase activity. Plasmid DNAs were isolated from these negative colonies and tested for their ability to complement *erd1* mutations upon re-transformation. Three independent plasmids, with overlapping restriction maps, were identified in this way.

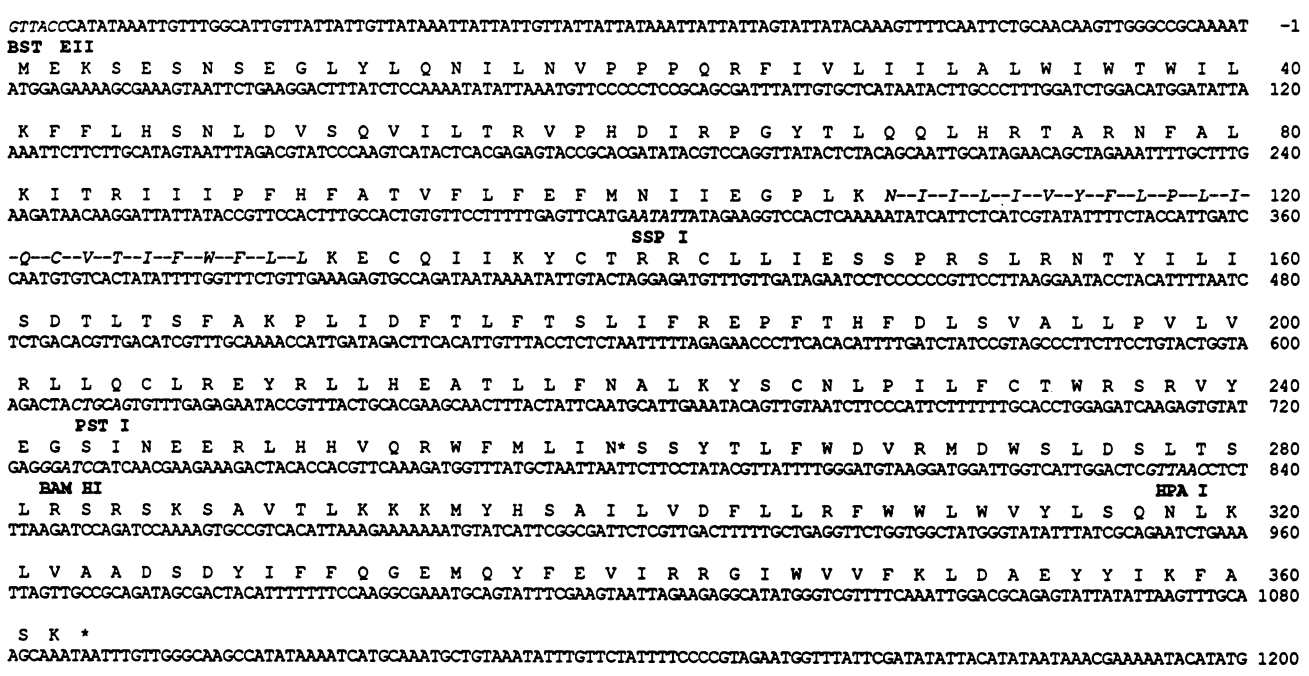

Fig. 3. Sequence of the *ERD1* gene. Hyphens in the amino acid sequence indicate the longest stretch of uncharged residues (corresponding to the black rectangle in Figure 9). The potential glycosylation site at amino acid 260 is marked by an asterisk. The positions of relevant restriction sites are indicated below the nucleotide sequence.

Subcloning showed that the complementing activity resided on a 2.2 kb *Kpn*I−*Nco*I fragment, and this was sequenced.

The sequence revealed a single long open reading frame (Figure 3), with no obvious homology to other proteins in the current sequence databases. Expression of this reading frame under the control of the triose phosphate isomerase promoter was sufficient to complement an *erd1* mutation, whereas insertion of four bases at a *Bam*HI site within the reading frame abolished the complementing activity of the cloned fragment. A portion of the coding region was deleted from the chromosome by the two-step insertion−excision method (see Materials and methods). The resulting strain had a strong *erd* phenotype and was unable to complement *erd1* strains of the opposite mating type. We therefore define the cloned gene as *ERD1*; all subsequent phenotypic studies used mutants generated by disruption of this gene.

Disruption of the ERD1 gene

To determine the null-phenotype of an *erd1* mutation, we made a deletion of the cloned gene that removed all but the 3′ 253 bp of the coding region, and introduced this deletion into the genome of the invertase-negative strain SEY2102 by the two-step disruption method (Boeke *et al.*, 1984). The resultant strain grew as rapidly as its parent at 30°C. The nitrocellulose blot assay showed that it secreted HDEL-containing proteins, as expected.

A quantitative estimate of the efficiency of retention of HDEL proteins in the absence of Erd1p was obtained by expressing the invertase fusion proteins bearing the C-terminal sequences FEHDEL and SEKDEL in the *erd1* deletion strain. In wild-type strains the HDEL protein is retained whereas the KDEL version is secreted; in the mutant no significant discrimination between these sequences could be detected by enzyme assay (Table II).

Secretion of BiP by erd1 deletion mutants

To estimate the importance of the *ERD1* gene in the retention of endogenous ER proteins, we examined the synthesis and secretion of BiP in the mutant cells. For comparison, we analysed in parallel the strain expressing BiP without the HDEL signal (BiP-FGR); in this strain there should be no HDEL-dependent retention of BiP at all.

Table II. Effect of *erd1* deletion on secretion of invertase fusion proteins

C-terminus of fusion protein	Percent secretion[a]	
	ERD1$^+$	*erd1*$^-$
FEHDEL	2	35
SEKDEL	29	32

[a]Figures are calculated from the averages of duplicate assays of total and external invertase activity.

Figure 4a shows an experiment in which BiP secretion was assayed by immunoblotting. Cells growing in log phase in rich medium were washed, suspended in fresh medium, and incubated for 2 h. After centrifugation, cells and medium were subjected to gel electrophoresis, and BiP detected with an antibody raised against the C-terminal 216 amino acids of the protein (Rose *et al.*, 1989). The results show first, that the intracellular levels of BiP are essentially identical in wild-type, BiP-FGR and *erd1* cells and second, that BiP-FGR and *erd1* cells secrete BiP into the medium at comparable rates. After 2 h, ~12% of the total BiP in both the *erd1* and BiP-FGR cultures was found in the medium.

We also examined the kinetics of BiP secretion by pulse-labelling cells with $^{35}SO_4$. Cells were labelled for 20 min, chased for 40 min, their cell walls removed and the intracellular and external BiP immunoprecipitated. Figure 5a shows that in the BiP-FGR strain, the rate of synthesis of BiP was higher than in the wild-type strain, and BiP was secreted from the cells. Surprisingly, the *erd1* cells showed neither increased synthesis of BiP nor a substantial amount of secretion under these conditions. It appears that when grown in

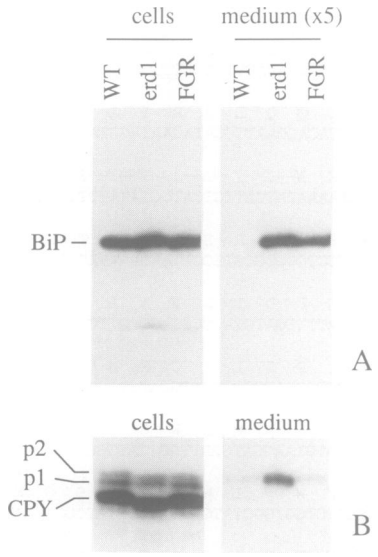

Fig. 4. Immunoblot analysis of the secretion of BiP and CPY from mutant strains. Immunoblots of cells and medium from wild-type (WT), *erd1* and BiP-FGR (FGR) strains were probed with antibodies against BiP (**A**) or CPY (**B**). Cells were grown in rich medium, in log phase, and the medium was changed 2 h prior to harvesting. Bands corresponding to BiP, mature CPY, the core-glycosylated ER form of proCPY (p1) and the Golgi-modified form of proCPY (p2) are indicated. Note that the autoradiograph of the medium samples in A was exposed for 5 times longer than the corresponding autoradiograph of the cell samples.

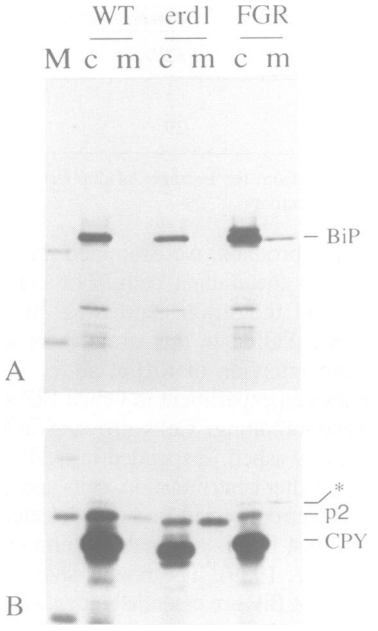

Fig. 5. Pulse-chase analysis of BiP and CPY. Cells from wild-type (WT), *erd1* and BiP-FGR (FGR) strains were grown in low-sulphate medium, pulse-labelled with $^{35}SO_4$ for 20 min and chased for 40 min. Cell walls were digested and cell extracts (c) and media (including periplasm) (m) were immunoprecipitated sequentially with anti-BiP (**A**) and anti-CPY (**B**). Some residual BiP–anti-BiP complexes were precipitated during the second round of immunoprecipitation, resulting in minor amounts of BiP in (B) (asterisk). M indicates mol. wt markers (92.5, 69 and 43 kd).

the defined, low-sulphate medium used for the labelling experiments, *erd1* cells do not show their normal phenotype. So far, we have been unable to identify any one constituent

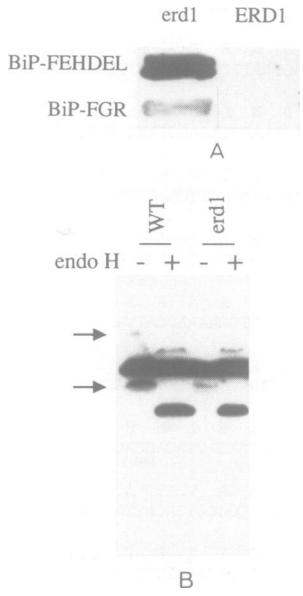

Fig. 6. Secretion of HDEL-containing proteins by *erd1* deletion mutants. (**A**) Proteins secreted by cells grown in contact with nitrocellulose were probed with anti-HDEL antibodies. Strains contained either the wild-type *ERD1* gene or a deletion of the gene, and either the normal BiP gene (BiP-FEHDEL) or one that encodes an altered BiP protein lacking HDEL (BiP-FGR). (**B**) Total proteins from wild-type (WT) and *erd1* deletion strains were analysed by immunoblotting with anti-HDEL serum as in Figure 1. Arrows indicate the ER-glycosylated forms of proteins X and Y, which are present at lower levels in the mutant.

of the medium that is responsible for this effect, but we note that both wild-type and mutant cells grow substantially slower in the labelling medium than in rich medium (a doubling time of 4 h instead of 2 h).

These experiments revealed another apparent anomaly in the secretion of BiP. From the amount of BiP detected in the medium, either by labelling or immunoblotting, and the known doubling time of the cells, the apparent half-time for BiP secretion from the BiP-FGR strain can be calculated. In the pulse–chase experiment, ~8% of the BiP was outside the cells after the chase, which indicates an apparent half-time for secretion of 7–8 h; the immunoblotting experiment gave a half-time in the same range (7.3 h). However, it is also possible to estimate the half-time of loss of BiP from the cells from the observation that the BiP-FGR strain synthesizes BiP 4.3 times as fast as the wild-type strain, as judged by densitometry of the autoradiogram in Figure 5, yet accumulates no more protein as estimated by immunoblotting. Taking the doubling time of the cells into account, the half-time is ~1.4 h. The discrepancy between this and the previous figure can be explained if a substantial proportion of the BiP that leaves the ER does not reach the medium, but instead is diverted to the vacuole and degraded. This would be consistent with the observation that an invertase-BiP fusion protein lacking HDEL is inefficiently secreted: only ~30% of the invertase activity can be detected outside cells (Table II). Invertase is protease-resistant, and if delivered to the vacuole retains its activity (Johnson *et al.*, 1987).

In conclusion, it seems that without HDEL, BiP leaves the ER with a half-time of ~1.5 h. This slow rate is reminiscent of the behaviour of BiP in animal cells, which without its retention signal is secreted with a half-life of ~3 h (Munro and Pelham, 1987). *Erd1* deletion mutants secrete BiP, in rich

medium, as though it had no retention signal. By analogy with the BiP-FGR strain, it is likely that the loss of BiP from *erd1* cells is compensated for by an increased rate of synthesis.

At least one other HDEL-containing protein is secreted from the mutant cells. Although such a protein could not be detected by immunoblot analysis of the medium, the nitrocellulose filter assay showed that a strain carrying both the *erd1* deletion and the BiP-FGR mutation still secreted some anti-HDEL reactive material (Figure 6a). However, analysis of *erd1* cells indicated that the levels of protein X and Y were not substantially reduced, although a slight decrease in the levels of the ER-modified forms of these glycoproteins was apparent (Figure 6b).

Deletion of ERD1 does not prevent vacuolar protein sorting

The secretion of HDEL-containing proteins from *erd1* mutants could, in principle, merely reflect a more general disruption of protein targeting within the secretory pathway. We therefore examined the effect of the *erd1* deletion on the transport of the protease carboxypeptidase Y (CPY) to the vacuole. CPY is normally synthesized as an inactive precursor that enters the secretory pathway but is diverted from a 'late Golgi' compartment to the vacuole, where it is activated by a proteolytic cleavage catalysed by the product of the *PEP4* gene; more than 50 genes that affect this targeting process have been identified (Hemmings *et al.*, 1981; Stevens *et al.*, 1982; Banta *et al.*, 1988; Rothman *et al.*, 1989).

The cell and medium samples used for the assay of BiP secretion were similarly analysed for CPY content. In both rich medium (Figure 4b) and the low-sulphate medium (Figure 5b), *erd1* cells showed an increase in secretion of pro-CPY relative to wild-type cells. The magnitude of this increase varied between experiments, and sometimes no difference was detectable; in all experiments the bulk of the CPY was retained in the cells and processed in the vacuole. The mature and ER-modified (p1) forms of CPY were never observed in the medium, indicating that the mutant cells did not lose proteins as a result of random lysis.

In both assays, a small but reproducible difference between the gel mobility of CPY in *erd1* and wild-type cells was detected. Close examination of the autoradiographs showed that the ER precursor form (p1) was unaltered, whereas both the Golgi-modified precursor (p2) and the mature CPY had slightly increased mobilities (Figure 4b). Thus, deletion of the *erd1* gene has both a small quantitative effect on the targeting of CPY to the vacuole, and an apparent qualitative effect on its processing in the Golgi. Nevertheless, the bulk of the CPY is correctly delivered to the vacuole.

Protein glycosylation in erd1 mutants

The altered mobility of CPY in *erd1* cells suggested a defect in oligosaccharide processing. To investigate this further, we studied the maturation of invertase by pulse-labelling and immunoprecipitation (Figure 7a). To check the specificity of the antiserum, we examined both strains that lacked invertase altogether, and the same strains containing a plasmid with the coding region for the secreted form of invertase under the control of the triose phosphate isomerase promoter. After a 25 min pulse, wild-type invertase-expressing cells contained a small amount of the ER-glycosylated form of the protein (arrowed in lane 2 of Figure 7a), and rather more of the Golgi-modified form that has large, heterogeneous, mannose-rich 'outer chains'. After a 45 min chase, all the protein was

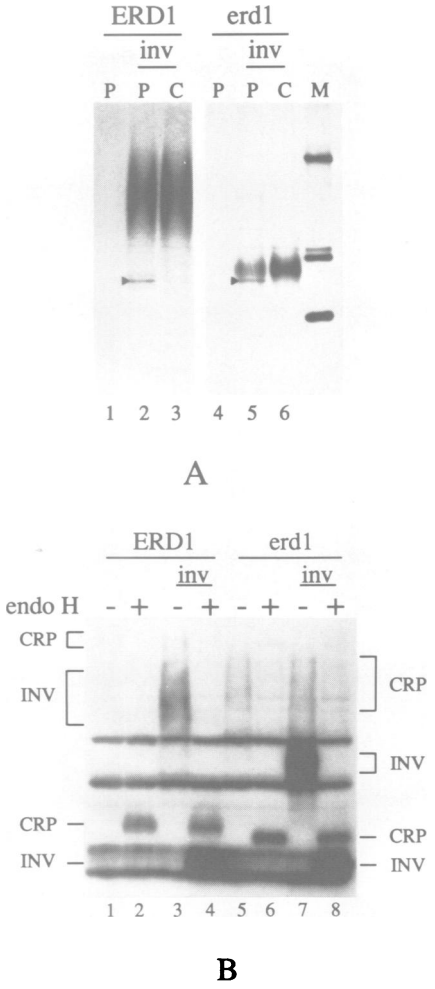

Fig. 7. Protein glycosylation in an *erd1* deletion mutant. Wild-type and mutant invertase-deficient strains carried either a centromere plasmid expressing invertase (inv), or a control plasmid. (**A**) Cells were pulsed-labelled with $^{35}SO_4$ for 25 min (P), and then chased for a further 45 min (C), and labelled invertase analysed by immunoprecipitation and gel electrophoresis. The ER-glycosylated form of invertase (the most prominent band of a triplet visible on the autoradiogram) is indicated by an arrowhead in lanes 2 and 5. The markers (M) have molecular weights of 200, 92.5 (lower band of the doublet) and 69 kd. All lanes are from the same gel, but exposures have been normalized to compensate for variations in labelling efficiency. (**B**) Immunoblot of unlabelled samples corresponding to those in (A), with and without endo H digestion. The anti-invertase serum used reacts with a number of proteins including one glycoprotein (which is also recognized by other anti-invertase sera). The positions of undigested and endo H-digested invertase and the cross-reacting glycoprotein (CRP) are indicated on the left for the wild-type strain and on the right for the *erd1* mutant strain.

in this Golgi-modified form. In contrast, in the *erd1* deletion mutant the invertase accumulated in a form that was only slightly larger than the ER form (Figure 7a, lane 6). The ER form, visible after the pulse, appeared normal both in size and relative amount (lane 5). It appears, therefore, that the Golgi-dependent outer-chain modification of oligosaccharide side chains is severely impaired in an *erd1* strain.

These results were confirmed by immunoblotting of cells grown in rich medium. The analysis was complicated by the cross-reaction of the anti-invertase antibodies with other proteins, but it is clear from Figure 7b that the bulk of the invertase in the mutant strain has a much higher gel mobility than usual (lanes 3 and 7); this difference is entirely due to

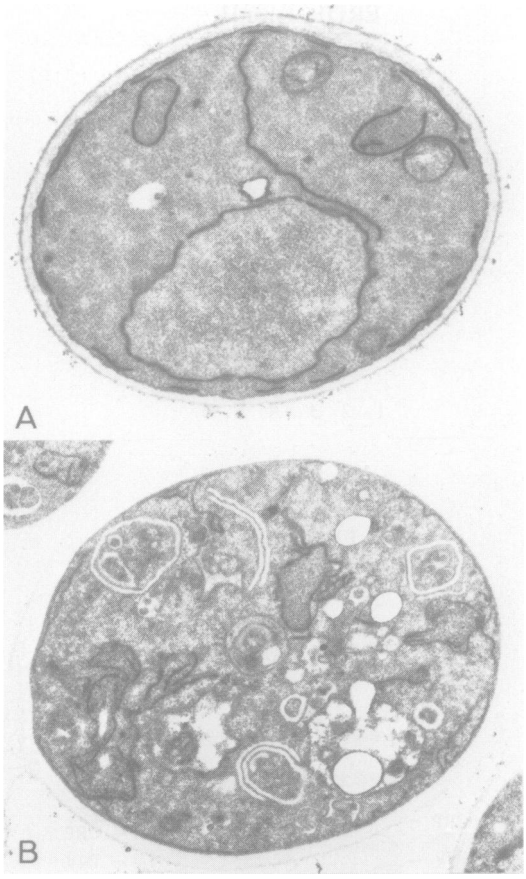

Fig. 8. Electron microscopy of *erd1* cells. (**A**) A thin section of an *erd1* cell stained with potassium permanganate; the cell is indistinguishable from cells of the parental wild-type strain. The nuclear envelope and ER are stained black. (**B**) A section of an *erd1 sec7*^{ts} double mutant cell that had been incubated for 3 h at the non-permissive temperature. Putative Golgi stacks and Berkeley Bodies are visible as poorly-staining (white) cisternae that are curved or circular. Identical structures were seen in the parental *ERD1*, *sec7*^{ts} strain. Total widths of the images correspond to 3 μm.

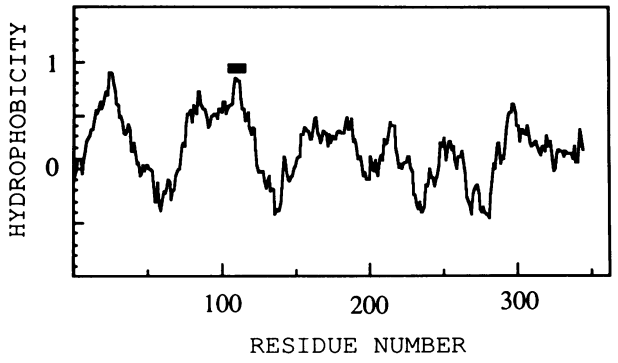

Fig. 9. Hydropathy plot of Erd1p. Hydrophobicity was calculated according to the method of Eisenberg *et al.* (1984), using a window of 18 amino acids. Transmembrane segments usually have a score of more than 0.42 on this scale. The black rectangle indicates the stretch of 22 uncharged amino acids marked in Figure 3.

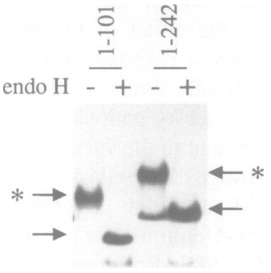

Fig. 10. Glycosylation of erd1-invertase fusion proteins. Genes encoding the first 101 or 242 residues of Erd1p fused to the mature portion of invertase were expressed in yeast, and the fusion proteins detected by immunoblotting with anti-invertase serum. ER-glycosylated (*) and non-glycosylated forms of the fusion proteins are indicated by arrows; lower bands probably represent proteolytic fragments.

N-linked carbohydrate modifications, because it is abolished by endo H digestion (lanes 4 and 8). The anti-invertase antiserum also recognized a second glycoprotein on the blots. This cross-reacting protein (CRP) was also altered in the *erd1* mutant (Figure 7b), apparently because of reduced Golgi modification. Moreover, a difference in mobility persisted even after endo H digestion (compare lanes 2 and 4 with lanes 6 and 8). The precise reason for this difference is unknown, but it could be explained by an impairment of *O*-linked glycosylation in the mutant strain; at least some modification to *O*-linked oligosaccharides occurs in the Golgi (Kukuruzinska *et al.*, 1987).

These results indicate that Erd1p is required for correct processing of at least three glycoproteins. The defect appears to be in the Golgi, since this is known to be the site of outer-chain oligosaccharide synthesis. The initial *N*-linked glycosylation of CPY (Figure 4b), invertase (Figure 7a) and the HDEL-containing protein X (Figure 6b), which occurs in the ER, is unaffected by the *erd1* mutation.

Electron microscopy of erd1 mutants

To determine whether the defects in oligosaccharide processing were accompanied by any gross abnormality of the intracellular membrane system, the *erd1* deletion mutant was examined by thin-section electron microscopy of permanganate-fixed cells. No differences from the *ERD1*$^{+}$ parental strain were detected. Figure 8a shows a typical micrograph of a mutant cell. The appearance of the Golgi was checked in *sec7*^{ts} strains, with or without an *erd1* deletion, after incubation for 3 h at the non-permissive temperature (37°C). Typical Golgi stacks and 'Berkeley Bodies' (Novick *et al.*, 1980) were seen (Figure 8b), and again, no obvious differences between the *erd1*$^{-}$ and *ERD1*$^{+}$ strains were detected. We conclude from these studies that mutation of *ERD1* does not substantially alter the morphology of either ER or Golgi.

The ERD1 product appears to be an integral membrane protein

A hydropathy plot of the *ERD1* protein (Erd1p) is shown in Figure 9. The protein does not have an obvious N-terminal signal sequence, but has several hydrophobic regions; one of these, indicated in Figures 3 and 9, contains a sequence of 22 uncharged amino acids, but others are interrupted by one or more charged residues. There is a single potential site of *N*-glycosylation at amino acid 260.

To test the possibility that Erd1p traverses the ER membrane, we expressed, in yeast, invertase fusion proteins in which the invertase signal peptide was replaced by the first 101 or 242 amino acids of Erd1p. The glycosylation state

of the invertase was assayed by immunoblotting total protein from the transformed strains, with or without endo H digestion. Figure 10 shows that the presence of the first 101 amino acids of Erd1p is sufficient to allow transfer of the invertase portion of the fusion protein into the ER, where it is glycosylated. Invertase was also glycosylated when fused to the first 242 amino acids of Erd1p, although some of this fusion protein remained in an unglycosylated state (Figure 10).

It is commonly believed that charged residues within the lipid bilayer are stabilized by interaction with each other, or are located within aqueous pores. If so, it is hard to predict the orientation of fusion proteins containing an incomplete set of transmembrane domains, because these domains may not cross the membrane in the absence of their partners. Thus, while the sequence data and the properties of the fusion proteins suggest that Erd1p is an integral membrane protein, determination of its precise topology will require a more detailed biochemical analysis.

Discussion

The HDEL retrieval system

We have shown that several proteins in yeast are recognized by an antibody specific for the HDEL signal, and that the HDEL-containing proteins can be localized to the ER by immunofluorescence. Two of the HDEL-containing proteins are glycosylated, and at least one of them appears to be partially modified by the outer-chain synthesizing enzymes of the Golgi. This is consistent with our previous evidence for retrieval of ER proteins from the Golgi (Pelham *et al.*, 1988). However, substantial proportions of the glycoproteins remain in the ER-modified form, implying that retrieval may usually occur prior to outer-chain glycosylation.

The HDEL system does not appear to be essential for maintenance of a functional secretory pathway. We found that removal of HDEL from BiP had no deleterious effects on growth, even though BiP is essential for viability (Nicholson *et al.*, 1989; Normington *et al.*, 1989; Rose *et al.*, 1989). Furthermore, *erd* mutants that showed no obvious HDEL-dependent retention grew well and did not lack luminal ER proteins. In the case of BiP, maintenance of protein levels in the absence of the HDEL signal can be explained by the twin observations that secretion occurs slowly, and that loss of the protein from the cells is compensated for by an increase in the rate of its synthesis.

The non-essential nature of the HDEL system has allowed us to isolate viable mutants that do not efficiently retain HDEL proteins. Extensive screening has thus far identified only two genes whose mutation can abolish HDEL-dependent retention. This suggests that few proteins are dedicated to this task, although our screens would not identify genes whose functions are redundant.

Possible functions of ERD1

Our current model for the retrieval system envisages a membrane-bound receptor that cycles between the ER and a salvage compartment located on the *cis* side of the Golgi, binding HDEL in the salvage compartment and releasing it in the ER (Munro and Pelham, 1987; Pelham, 1988, 1989). Proteins specifically required for this process might include the receptor itself, components involved in the recycling process, and others involved in the maintenance of the appropriate conditions in the ER and salvage compartment that are required for binding and release of HDEL.

A possible function for Erd1p is suggested by the presence of at least two defects in the maturation of glycoproteins in an *erd1* deleted strain. Golgi-dependent modification of the *N*-linked oligosaccharides of invertase and at least one other glycoprotein is altered, and the putative *O*-linked glycosylation of the second glycoprotein is also abnormal. In addition, Golgi processing of carboxypeptidase Y is affected. The existence of multiple defects argues against the simple possibility that *ERD1* encodes a glycosyl transferase. The defects are unlikely to be a mere consequence of the *erd* phenotype, because they are also observed when the mutant cells are grown in low-sulphate medium, conditions that suppress the secretion of BiP and presumably other HDEL-containing proteins. Nor is a glycosylation defect itself sufficient to cause secretion of ER proteins, because two other mutants that are defective in outer-chain glycosylation, *mnn9* (Gopal and Ballou, 1987) and *pmr1* (Rudolf *et al.*, 1989) do not secrete significant quantities of BiP (our unpublished observations). A simple explanation could be that Erd1p is required to maintain the structural integrity or appropriate aqueous environment of the Golgi. Alteration of the Golgi could have a pleiotropic effect on the activities of several enzymes, as well as on the binding of HDEL to its receptor and/or the recycling of the receptor to the ER. It could also account for the slight defect in CPY sorting, which normally occurs in a late region of the Golgi.

If this explanation is correct, it seems surprising that a change in the growth conditions can suppress the *erd* phenotype of the mutant cells without correcting either their glycosylation defects or the mis-sorting of CPY. However, recent studies of the glycosylation events in the Golgi have led to the suggestion that multiple distinct compartments exist; outer-chain synthesis does not occur in the first of these, but in a later compartment (Franzusoff and Schekman, 1989). Addition of the first few mannose residues, which is not prevented in *erd1* cells, occurs in an early compartment. We have shown previously that a significant fraction of an HDEL-bearing invertase fusion protein lacks outer chains (Pelham *et al.*, 1988), which suggests that it has not passed beyond this early Golgi compartment. The properties of *erd1* mutants can thus be explained if the first part of the Golgi remains fully functional, allowing efficient retrieval of HDEL proteins under some growth conditions. When growth is rapid, a higher proportion of the luminal ER proteins may reach the defective region of the Golgi and escape.

A major conclusion from these studies is that a mutation that affects primarily the later compartments of the Golgi complex has a profound effect on the retention of luminal ER proteins. This strongly supports the idea that sorting of these proteins occurs not in the ER, but in the Golgi.

Materials and methods

Yeast strains

All data in this paper, except Figures 2 and 8b, were obtained with isogenic derivatives of strains SEY2101 (*MAT*a *suc2*-d9 *ura3*-52 *leu2*-3, −112 *ade2*-1) and SEY2102 (*MAT*α *suc2*-d9 *ura3*-52 *leu2*-3, −112 *his4*-519), as previously described (Pelham *et al.*, 1988). Mutants were isolated from strains carrying a *PRC1*-invertase−rat BiP-HDEL fusion gene integrated at the *PRC1* locus. Figure 8b shows strain ND721 (*sec7*-1 *erd1*-d2 *ura3*-52 *trp1*-d901 *his3*-d200 *leu2*-3, −112 *suc2*-d9), produced for this work. A strain of *S.uvarum* (NCYC 74; Kilmartin and Adams, 1984) was used in Figure 2.

Antibodies

To prepare anti-HDEL antibodies, the synthetic peptide YTFEHDEL was coupled to keyhole limpet hemocyanin by glutaraldehyde cross-linking, and used to immunize rabbits. Sera were affinity purified on Affi-gel 15 columns

to which either YTFEHDEL or GSDVHDEL had been coupled, via their N-termini. Anti-CPY antibodies were a gift from T.Stevens, and anti-BiP antibodies were a gift from J.Vogel and M.Rose. Antibodies against denatured invertase were prepared by immunizing rabbits with invertase (Sigma) that had been deglycosylated with endo F (Boehringer); the sera were preabsorbed against intact invertase-deficient yeast cells to remove any anti-carbohydrate activity. Initial samples of anti-invertase were kindly provided by R.Schekman.

Preparation of gel samples, endo H digestion and immunoblotting were performed as previously described, using ^{125}I-labelled protein A to detect the antibodies (Pelham *et al.*, 1989). Analysis of secreted proteins was essentially as described by Rothman *et al.* (1986); briefly, freshly-grown cells were streaked thinly on plates and covered with a nitrocellulose filter for 12–16 h. The filter was then washed and treated as for a normal immunoblot. Cell labelling with $^{35}SO_4$, immunoprecipitation and analysis of invertase, BiP and CPY was performed as described by Stevens *et al.* (1982). To ensure efficient recovery of secreted proteins, labelling was performed in the presence of 0.5 mg/ml bovine serum albumin (Stevens *et al.*, 1986).

Cloning of ERD1 and BiP

ERD1 was isolated by transforming a heteroallelic diploid *erd1* mutant strain with a genomic library in a centromere vector (Sengstag and Hinnen, 1987). A pool of transformed colonies was replated, and colonies that failed to secrete invertase activity detected by staining of filter paper replicas (Klionsky *et al.*, 1988; Pelham *et al.*, 1988). Three independent plasmids were recovered from such colonies. The gene was located by testing the ability of fragments of the genomic insert, subcloned into the centromere vector YCP86 (Hope and Struhl, 1986), to suppress the *erd1* phenotype.

The BiP gene was isolated by screening a λgt11 library (Clontech) with anti-HDEL antibodies. A fragment containing the 3′ half of the gene was identified by sequence homology to rat BiP, and by the presence of the expected C-terminal sequence.

Genes were sequenced by the dideoxy method after subcloning into Bluescript, nested deletions being generated by the exonuclease III–exonuclease VII technique (Yanisch-Perron *et al.*, 1985).

Gene fusions and disruptions

For expression of the *ERD1*-invertase fusions, a 900 bp fragment containing the TPI promoter was joined to a deletion endpoint generated during sequencing of *ERD1*, such that the junction was just upstream of the *ERD1* open reading frame. The *ERD1* gene was then joined at the *Ssp*I site (amino acid 101) or the *Bam*HI site (amino acid 242) to the *Bam*HI site of a version of the *SUC2* gene that has a polylinker at the beginning of the reading frame of the mature protein (from plasmid SEYC306, Johnson *et al.*, 1987). The *Bam*HI fusion was then digested with *Bam*HI and *Hin*dIII and the ends filled in and rejoined to generate the correct reading frame. The fusion genes were then cloned into the integration vector YIP56X (Pelham *et al.*, 1988), and integrated at the *URA3* locus of strain SEY2102.

For disruption of *ERD1*, sequences between the *Bam*HI and *Pst*I sites in the open reading frame, or between the *Bst*EII and *Hpa*I sites (see Figure 3) were removed, and the gene cloned into the *URA3* vector YIP56. All phenotypic studies used the larger deletion. To modify the BiP gene, the synthetic oligonucleotide CGGCCGTTAACGGC was inserted into a *Bst*BI site close to the termination codon; this has the effect of converting the C-terminus of the encoded protein from FEHDEL to FGR. The altered gene was subcloned into YIP56. Disruptions were performed by the two-step procedure: plasmids were linearized at a site within the gene, and integrants selected on plates lacking uracil. Recombination between the wild-type and altered copies of the gene in these transformants, resulting in loss of the *URA3* gene, was selected for by exposing the cells to 5-fluoroorotic acid (Boeke *et al.*, 1984). Strains that had lost the wild-type gene were identified by screening for secretion of HDEL-containing proteins (for *erd1* disruptions), or by performing immunoblots of whole cell extracts (for the BiP gene), and by Southern blotting. In the case of BiP, the 5′ end of the gene was not present in the integrating plasmid, and the primary integrant contained one altered and one 5′-truncated (inactive) copy of the gene. For the experiment in Figure 6a, these *URA3*-containing integrants were used without further manipulation.

Microscopy

Immunofluorescent staining with anti-HDEL antibodies was performed as described by Kilmartin and Adams (1984). Stained cells were visualized with an MRC-500 confocal laser-scanning microscope (BioRad).

For thin-section electron microscopy, log-phase cells grown in the presence of fructose were washed in a freshly-prepared solution of potassium permanganate (15 mg/ml), incubated in the permanganate solution for 45 min at 4°C, washed several times with water and embedded in 1% agar. Araldite sections were prepared and post-stained with 5% uranyl acetate for 10 min at 60°C followed by lead citrate (0.25% in 0.1 M NaOH) for 5 min at room temperature.

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