Cell, Vol. 61, 1349–1357, June 29, 1990, Copyright © 1990 by Cell Press

ERD2, a Yeast Gene Required for the Receptor-Mediated Retrieval of Luminal ER Proteins from the Secretory Pathway

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Summary

Resident proteins of the ER lumen carry a specific tetrapeptide signal (KDEL or HDEL) that prevents their secretion. We have previously described the isolation of yeast mutants that fail to retain such resident proteins within the cell. Here we describe *ERD2*, a gene required for retention. It encodes a 26 kd integral membrane protein whose abundance determines the efficiency and capacity of the retention system. Reduced expression of *ERD2* leads to secretion of proteins bearing the HDEL signal, whereas overexpression of *ERD2* improves retention both in wild-type cells and in other mutants. These results are consistent with other evidence that *ERD2* encodes the HDEL receptor (see accompanying paper). The gene is also required, perhaps indirectly, for normal protein transport through the Golgi, and hence for growth. We discuss possible roles for *ERD2* in the secretory pathway.

Introduction

In eukaryotic cells, newly synthesized secretory proteins are first inserted into the endoplasmic reticulum (ER) and are then transported in vesicles to the Golgi and eventually to the cell surface (Palade, 1975). However, such proteins comprise only a small fraction of the total protein content of the ER lumen. The most abundant polypeptides are resident soluble proteins such as BiP (a member of the hsp70 family), grp94 (a relative of hsp90), and protein disulfide isomerase (for review see Pelham, 1989). The main role of these proteins seems to be to aid the translocation, folding, and maturation of secretory proteins.

Transport of secretory proteins from the ER to the cell surface appears to occur in a nonselective fashion: no specific transport signal has yet been identified (Pfeffer and Rothman, 1987; Rose and Doms, 1988). In contrast, the soluble residents of the ER are distinguished by a C-terminal tetrapeptide sequence, which in animal cells is usually KDEL. This sequence is both necessary and sufficient for retention in the ER (Munro and Pelham, 1987; Pelham, 1988; Zagouras and Rose, 1989; Mazzarella et al., 1990).

Proteins bearing the KDEL signal are not immobilized in the ER. Examination of the carbohydrate modifications that occur to a KDEL-tagged lysosomal enzyme, cathepsin D, suggests that they can be transported to a subsequent compartment on the secretory pathway by the same nonselective transport vesicles that carry proteins destined for secretion (Pelham, 1988). ER proteins are then thought to be recovered by a receptor-mediated process. They are assumed to bind to a KDEL receptor in a "salvage compartment" (which in animal cells probably corresponds to the "15°C compartment") and to be incorporated into specialized vesicles that carry them back to the ER (Pelham, 1989).

To identify essential components of this recycling pathway, we have used a genetic approach. Our strategy has been to isolate mutants of the yeast Saccharomyces cerevisiae that are defective in the retention system. Previous work has established that yeast uses HDEL, rather than KDEL, as an ER retention signal; that HDEL-containing proteins can leave the ER, receive Golgi-specific carbohydrate modifications, and then return; and that the retrieval system is saturable, as expected for a receptor-mediated process (Pelham et al., 1988; Dean and Pelham, 1990). Extensive mutagenesis resulted in the identification of two genes required for the retention in the ER of HDEL-tagged invertase fusion proteins; these were named *erd1* and *erd2* (for "ER retention defective"). We have recently shown that deletion of the *ERD1* gene causes a pleiotropic defect in part of the Golgi apparatus, which evidently results in inefficient retrieval from this organelle (Hardwick et al., 1990).

In this paper we describe the characterization of the *ERD2* gene. This gene encodes an integral membrane protein that is required both for retention of ER proteins and, perhaps indirectly, for normal traffic of proteins through the Golgi. Strikingly, the capacity of the retention system is determined by the level of expression of the *ERD2* gene. This observation, together with evidence that *ERD2* determines the signal specificity of the retention system (Lewis et al., 1990 [accompanying paper]), leads us to propose that *ERD2* encodes the HDEL receptor.

Results

erd2 Mutants Secrete BiP

The *erd2* mutants were selected for their ability to secrete an invertase fusion protein bearing the HDEL signal (Pelham et al., 1988; Hardwick et al., 1990). To see whether an endogenous ER protein was also secreted, an *erd2* strain was pulse-labeled with $^{35}SO_4$ and cells and media were subjected to immunoprecipitation with an antibody specific for BiP. As a control, we also examined a strain (YFGR) in which the BiP gene had been modified such that it encoded a protein with the C-terminal sequence FGR instead of FEHDEL (Hardwick et al., 1990). Since it lacks the HDEL signal, this protein is not a substrate for the retention system. Figure 1A shows that pulse-labeled BiP was secreted from the *erd2* strain as efficiently as from the YFGR strain.

Secretion of BiP from the *erd2* strain was confirmed by immunoblotting of cells and media (Figure 1B): the *erd2* and YFGR strains released equivalent amounts of BiP

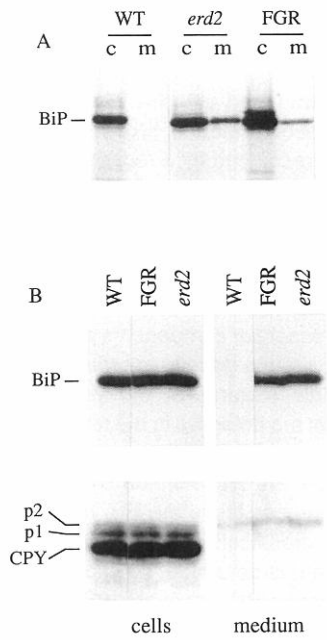

Figure 1. *erd2* Cells Secrete BiP

(A) Cells were labeled with $^{35}SO_4$ for 20 min and chased for 40 min. BiP was then immunoprecipitated from cells (c) and media (m). Strains analyzed were wild-type (WT), an isogenic strain whose BiP gene has been modified to delete the HDEL sequence (FGR), and an *erd2* mutant (*erd2*). The band slightly larger than BiP in the FGR cell sample is unprocessed pre-BiP, which tends to accumulate when expression levels are high (Rose et al., 1989).

(B) The same strains were analyzed by immunoblotting of proteins from cells and media with anti-BiP (upper panel) or anti-CPY (lower panel); p1 and p2 refer to the ER and Golgi forms of CPY, respectively. The autoradiogram of the anti-BiP medium samples was exposed for 5 times as long as the cell samples, for clarity.

into the medium, whereas the wild-type strain did not. Nevertheless, cells of all three strains contained similar levels of the protein. As we have shown previously, the loss of BiP from the YFGR strain is compensated for by an increase in its rate of synthesis (Figure 1A). Although it is not obvious in the experiment shown in Figure 1A, other experiments have shown a similar induction of BiP synthesis in *erd2* strains.

Also shown in Figure 1B is an immunoblot of the vacuolar enzyme carboxypeptidase Y (CPY). Three forms of the CPY glycoprotein can be identified: the mature vacuolar form, the core-glycosylated ER form of the proenzyme (p1), and the more extensively modified p2 form, which is found in the Golgi (Stevens et al., 1982). Small amounts of the p2 form fail to reach the vacuole and are secreted into the medium. In contrast to *erd1* mutants (Hardwick et al., 1990), *erd2* cells showed no obvious abnormality in the glycosylation or targeting of CPY (Figure 1B). Thus, the *erd2* defect appears specific to the ER retention system.

Cloning of *ERD2*

A plasmid that complemented the *erd2* phenotype was isolated from a genomic library. Subclones of the 10 kb insert were tested, and the activity was localized on a 3.3 kb HindIII fragment that was then sequenced (Figure 2). The sequence contains two open reading frames. One is present on the strand complementary to that shown in Figure 2, between bases 2496 and 2960; it potentially encodes a 155 amino acid protein, but fragments containing this open reading frame were unable to complement *erd2* mutations. The second open reading frame encodes 219 amino acids, interrupted by a single intron close to the N terminus with perfect consensus splice donor, branchpoint, and acceptor sequences (Figure 2; Vijayraghavan et al., 1986). S1 nuclease mapping confirmed that the region was transcribed and that splicing occurred in the expected position (data not shown). An intronless version of this open reading frame, prepared by oligonucleotide-directed mutagenesis and inserted into an expression vector (see Experimental Procedures), was sufficient both to complement an *erd2* mutation and to provide other *ERD2*-associated functions (see below).

To confirm that this open reading frame corresponds to the gene that is mutated in the *erd2* strains, we isolated the corresponding gene from four independently isolated *erd2* alleles using polymerase chain reaction (PCR) amplification. The PCR products were then cloned and sequenced. Individual clones frequently contained two or more base changes, but comparison of several independent clones from each mutant revealed that in each case only one change was consistently observed, and in three of the four genes this could be checked by digestion of the uncloned PCR products with restriction enzymes (Table 1). Two of the mutants, isolated in different genetic backgrounds, had the same base change that generates a termination codon 12 amino acids from the C terminus. The other two had single amino acid changes elsewhere in the protein (Table 1, Figure 2). Thus, all the *erd2* mutant strains analyzed carry mutations in the gene that we have cloned. We therefore refer to this gene as *ERD2*.

ERD2 Encodes an Integral Membrane Protein

The predicted *ERD2* protein sequence contains no cysteine residues, nor any potential N-glycosylation sites. Data base searches revealed no significant homology to other known proteins, but there is a striking similarity between residues 26–40 and 46–60 (Figure 2), which suggests an ancient intragenic duplication.

A notable feature of the protein is its hydrophobicity, which can be seen from the hydropathy plot in Figure 3. There is no obvious N-terminal signal sequence, but there is a stretch of 17 uncharged amino acids between residues 66 and 82, and hydropathy analysis suggests the presence of several (2–4) transmembrane segments. We therefore sought evidence that the *ERD2* protein is membrane associated.

To facilitate detection of the protein, we tagged the C terminus of the open reading frame with a sequence encoding a 10 amino acid epitope from the human c-*myc* gene, which is recognized by the monoclonal antibody 9E10 (Evan et al., 1985; Munro and Pelham, 1986). The tagged gene remained functional, as judged from its ability to complement an *erd2* mutation (not shown, but see Figure

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AAGCTTCCTGACGGAATGTTAATTCTCGTTGACCCTGAGCAGGCTGTTGAGCCCGGGGATTTCTGCATAGCCAGACTTGGGGGTGATGAGTTTACCTTCAAGAAACTGATCCATCCAAAC   120
CATTTTTGGGTTTAGTTGCTGCTTCCGCTGGTATCCTTCAAGATGTCATTGAAGGTAAGTACGATCTTGAGGCCGGCGAAAACAAATTCAACTTTTAAATCAAAATTTTAGAATAAAAGT   240
CTACTGCATTAACAAAGATAAATTTCTAATTTTTTTATGTATATTATATTTTTTGGAAGAGAAAATATTTGTAGTGCTTCTCTACACTGTTTTTTTTTTTTCGTTCATGTTGTCTTTCCT   360
CTCCTGAATATATTTAATTTAATAAATCTATCTTATCTGACATATAGAAGCTGTTTCGATAGAGTTTTCGACTGGCCAACAGTTGTTACTGACTGGATAAAAATTTACGATTTTTCCTCC   480
AATCTTGGCTTTCAAAATGAGAAATAAGAGTCTTGAAATTCAAAGAATGTATCTATGTATGAATATAGAATATGCTCAAATATTTAGTTTTGTATAAGGAAGTTCTAAATAATTTTCCAT   600
GGCGTGCTTTTACCAAAGTACTGAACAGGGAGATTATTTATCATTTTTTTTCTTACTCTTCGAGTTGAAGTCGATTGTAGGTGATGACATGAACATTAAATAAAATGTACTTGAACAAAT   720
CAGACCAGCAAACACCCACATCGCAATGGTACTGGGTTTTCTCGTTATTCTGTGGAAGAATTCAATTACCGGATTATTGTTAAAAAAGCCCATTAGAAGTATCCTAACGATTGATTAAAG   840
TAAATTGTTATTCACGCTACTATCGCAATCGTCCAACTGAACCTTATTCTTTATTGTATATAATCTCTGCCGAGAAATTCTTATACTTATGTATCTGGGAATTCACCAGGTTAGAATACC   960
TTCCCCTCCTCAATTTCTAATTTTTTTTTCTACCCTTATTTTTCTCTCTTGCTTCATCGCTCTCAAAGCTAAAAGCTAATACAGCAAACAAAAGTGTTATTAGGAATTCAATTGAGTCGT  1080
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ACCCGGACTGAAAACGACGTTTTAGCTAAATATTGAACACGTTAAAAATAAGTTCAAATTTCGAGGCAGATGAATTTGCTGAGGGCACTGTTCAAGAGTGAGATATACGCGCAAGTTGAG  1320

                M  N  P  F  R  I  L
GCATAATCAGTAAGCAATGAATCCGTTTAGAATCTTAGGTATGTTACTATTTGGAGTTTCATGAGGCTTTTCCCGCCGTAGATCGAACCCAATCTTACTAACAGAGAAAGGGCTTTTTCC  1440

                G  D  L  S  H  L  T  S  I  L  I  L  I  H  N  I  K  T  T  R  Y  I  E  G  I  S  F  K  T  Q  T  L  Y  A  L  V   43
CGACCATCAAGACAGGTGATTTATCACATCTAACCAGTATACTGATCCTGATTCATAATATCAAGACCACAAGGTACATTGAAGGTATTTCTTTCAAGACCCAAACGTTGTACGCTTTGG  1560
                N
   F  I  T  R  Y  L  D  L  L  T  F  H  W  V  S  L  Y  N  A  L  M  K  I  F  F  I  V  S  T  A  Y  I  V  V  L  L  Q  G  S  K   83
TTTTCATAACACGATACTTGGATCTCTTGACTTTTCACTGGGTATCCCTATACAATGCTCTAATGAAAATATTTTTCATTGTATCTACCGCTTACATTGTAGTGCTATTACAAGGGTCTA  1680

   R  T  N  T  I  A  Y  N  E  M  L  M  H  D  T  F  K  I  Q  H  L  L  I  G  S  A  L  M  S  V  F  F  H  H  K  F  T  F  L  E  123
AAAGAACCAACACCATTGCGTATAATGAAATGCTTATGCATGATACCTTTAAGATCCAGCATTTACTAATTGGGAGTGCTCTAATGAGTGTTTTTTTCCATCACAAGTTCACTTTTCTTG  1800
                                                                                                P
   L  A  W  S  F  S  V  W  L  E  S  V  A  I  L  P  Q  L  Y  M  L  S  K  G  G  K  T  R  S  L  T  V  H  Y  I  F  A  M  G  L  163
AATTAGCATGGAGTTTTTCTGTATGGTTGGAGAGTGTGGCTATTCTACCTCAATTGTACATGCTATCTAAGGGTGGGAAGACTAGAAGTCTAACTGTTCATTATATTTTTGCCATGGGAT  1920

   Y  R  A  L  Y  I  P  N  W  I  W  R  Y  S  T  E  D  K  K  L  D  K  I  A  F  F  A  G  L  L  Q  T  L  L  Y  S  D  F  F  Y  203
TATACAGAGCATTGTATATTCCTAACTGGATTTGGAGGTACAGCACGGAAGATAAAAAATTGGACAAGATTGCCTTCTTCGCGGGACTTTTGCAAACTCTGTTGTACTCTGATTTCTTTT  2040
                *
   I  Y  Y  T  K  V  I  R  G  K  G  F  K  L  P  K  *
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AAAAGAAAAAAAAAAATACATAAAATTGATGTTGATATTGGTCTATTTCTTTTTATACTATGATATGTATGCATTAATCTCTTTTTAGCTCATAAAATTCTTTCCTTACACCATCCTTGGT  2280
GACAATAAGGATTTCCAGCCCATCACCCACTTGTATATGTCTTTCTGTAGCAGAAGTGAACGAGTCTCTCACCAGTTTGATGACTTCTTCCACGGACAAGTATTTCAAAGGCTTTTTGAC  2400
TTTACCGTTTGTACCTGGCTCATATTGAATTTTTGAAATTAACCTGATTGTCCAAAAATGGCATGATCAATGATGCCGCAGCACCACCTGCTCTACACTGTTCTCTTTCGTAGGAGCCAA  2520
ACTGGGTCGAACGAATAGACAGCGCCCTTACCATCTTCGTCAAGACCCGCAATGATCGTATGAACGTAGTAAGGGAAAAACCTCTTCCCGTACAGAAGATGTTGAATGTTCCTTGCTGCA  2640
GAGTTTATAGATAGTTTTTTGTCGTTGTGGTCGAAATGGTACCATTTTACACTATTTTTGAATCTTTTTACTAAAGCGTCGCCGTCTGCTGCAAATCCATTCGCCGACATGACTATGTTA  2760
TCACCACAATCAAAAACCTTGGGTTCATAACGAGAATTAATTGAGTAATCGGTGATATTTCTTGTATCGCCTGCTAACACTGCGAAATCTTCACCTGCAATGCCCAGGATTGTACCACCA  2880
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CTTTCCAACTCAGTTCACCTTGTTATTATACCGCTTGTTTTTGCCACCCTTTGAGTTTCTCGATCCTTTAAGTTGGAAAAGATAAAAACAATTATCGCTCCGTACGAACACCAAATCTCT  3120
GCGACGCAGAGACGGCAATACAAAATCAGAAAAAAAAAACAATTTATCCTATGCTTCACTAGTCACAGGCTCTACGAAACATTCTATCGCCTCACTGCCTTTACATCACTTAAGCAAGAA  3240
GTTTTAAATATTACGGCAATTCCTTTGCAGCAATACCTTTACTTTATTTCGAGAGAAAATAGATCGTAAAATGCCTACTCCAAGCTT
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Figure 2. Sequence of the *ERD2* Gene

The consensus splice donor, branchpoint, and acceptor sequences are indicated by double underlining. Dotted lines mark two related 15 amino acid sequences near the N terminus of the protein. Amino acid changes caused by the mutations listed in Table 1 are shown in boldface above the wild-type sequence.

5 for an example of its activity). Immunoblotting of proteins from a strain expressing this gene revealed a 9E10-reactive band at the expected position for a 26 kd protein, which was absent from a strain lacking the tagged protein (Figure 4A).

The tagged *ERD2* protein sedimented with membranes during centrifugation. It could not be extracted from microsomes with sodium carbonate at pH 11.5, a treatment that is considered to remove all nonintegral proteins from membranes (Fujiki et al., 1982) and that resulted in efficient release of BiP (Figure 4B). Furthermore, a substantial portion of the *ERD2* protein partitioned into the detergent phase when cell extracts were extracted with Triton X-114 (Figure 4C). Finally, boiling of cell extracts in SDS prior to gel analysis resulted in a failure of the protein to enter the gel, a commonly observed phenomenon with membrane proteins (Figure 4A, lane 1). Together, these results strongly suggest that the *ERD2* gene product is an integral membrane protein.

Table 1. *erd2* Mutations

Allele	Nucleotide Change	Amino Acid Change	Restriction Site
B25	1581 G→A	50 D→N	Sau3A lost
D15	1891 T→C	153 L→P	—
B36, R93	2055 A→T	208 K→stop	AflII gained

In preliminary studies, we have used indirect immunofluorescence to locate the tagged protein within cells. The staining observed was punctate, and associated with neither nucleus nor vacuole. It was similar to that obtained with antibodies to the Golgi-associated *YPT1* protein (Segev et al., 1988) and distinct from the ER pattern seen with anti-HDEL antibodies (Hardwick et al., 1990). Thus, although caution is required in interpreting the distribution of a protein that has been modified by tagging, it seems likely that much of the *ERD2* protein is normally present in a post-ER, Golgi-like compartment. Further analysis will be required to identify its precise location.

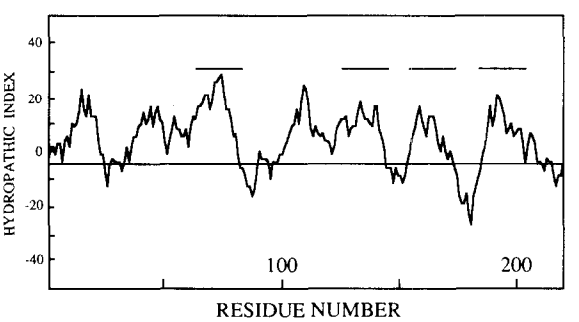

Figure 3. Hydropathy Plot of the Predicted *ERD2* Protein

The algorithm of Kyte and Doolittle (1982) was used, with a window of 11 amino acids. Hydrophobic portions are above the horizontal line. Bars above the plot indicate potential transmembrane segments.

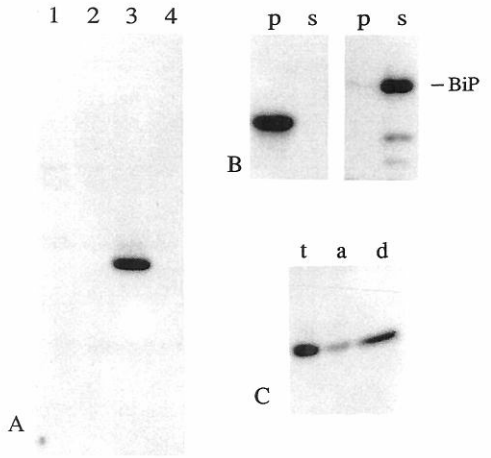

Figure 4. Detection and Membrane Association of the *ERD2* Protein

(A) Total protein from a wild-type strain (lanes 2 and 4) and from a strain lacking the chromosomal *ERD2* gene but carrying a multicopy plasmid (JS209) that expresses *ERD2* protein tagged with the 9E10 epitope (lanes 1 and 3) was analyzed by immunoblot using the 9E10 monoclonal antibody. Samples in lanes 1 and 2 were boiled in SDS sample buffer before gel electrophoresis. The band in lane 3 had the mobility expected for a 26 kd protein, as judged from markers run on the same gel.

(B) Membrane fractions enriched for ER were prepared from the strain expressing tagged *ERD2*, extracted with sodium carbonate (pH 11.5), and the membranes then pelleted by centrifugation. Pellet (p) and supernatant (s) fractions were immunoblotted with 9E10 antibody (lefthand panel) or with anti-BiP (righthand panel). Note that *ERD2* protein remained in the pellet, while BiP was extracted.

(C) Proteins from the same strain were partitioned between an aqueous phase and the detergent Triton X-114, and samples of the total extract (t), the aqueous phase (a), and the detergent phase (d) were blotted and probed with the 9E10 antibody. A substantial proportion of the tagged *ERD2* protein was recovered in the detergent phase.

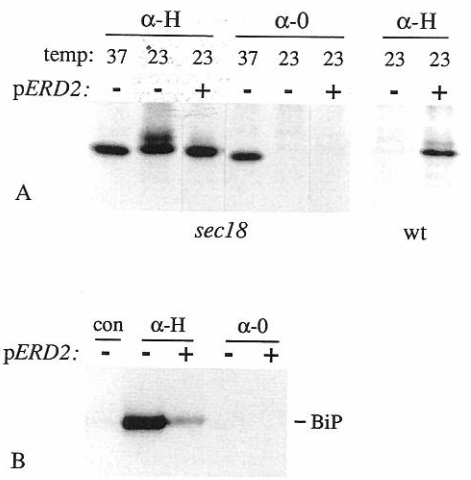

Figure 5. *ERD2* Overexpression Increases the Efficiency and Capacity of the Retention System

(A) *sec18* and *sec*$^+$ (wt) cells expressing pro-α factor fusion proteins tagged with HDEL (α-H) or not (α-0), and carrying a multicopy *ERD2* plasmid where indicated, were pulse-labeled for 10 min and chased for 10 min, and intact pro-α factor was isolated by immunoprecipitation. Labeling was carried out either at the *sec18* permissive temperature (23°C) or at the nonpermissive temperature (37°C). Note that the main band visible is the ER-modified form of the fusion protein; the pulse-labeled protein in the band above this has been subjected to Golgi modification, which does not occur in *sec18* cells at 37°C. Overexpression of *ERD2* reduces this modification in *sec18* cells and reduces proteolytic processing of the protein in wild-type cells.

(B) The BiP content of the medium in various cultures was revealed by immunoblotting. Cells were *sec*$^+$ and expressed the α-H construct, the α-0 construct, or neither (con). Overexpression of *ERD2* reduced the secretion of BiP that was induced by the α-H construct.

All experiments used a high copy plasmid carrying the normal *ERD2* gene (plasmid PER220).

ERD2 Expression Level Regulates the Capacity of the HDEL Retention System

To investigate the role of *ERD2*, we studied the effect of its overexpression on the retention of a pro-α factor–HDEL fusion protein (this protein also carries the c-*myc* epitope, to allow specific immunoprecipitation). This fusion protein has previously been used to demonstrate the recycling pathway in yeast cells (Dean and Pelham, 1990). It is normally efficiently exported from the ER, and has two advantages for our purposes: it is a small glycoprotein, which allows even minor Golgi modifications to be detected by changes in its electrophoretic mobility, and when it reaches the later part of the Golgi it is proteolytically processed to the 13 amino acid α factor (Julius et al., 1984); thus, the modification state of pro-α factor retained in the ER can be examined without interference from secreted molecules.

Figure 5A shows the results obtained when *sec18* strains expressing pro-α factor fusion proteins were labeled for 10 min, chased for 10 min, and then analyzed by immunoprecipitation and gel electrophoresis. Cells carrying the *sec18-1* mutation have a temperature-sensitive lesion in vesicular transport between ER and Golgi; thus at the nonpermissive temperature (37°C), fusion proteins that either contained HDEL (α-H) or lacked it (α-0) were retained in the ER. At the permissive temperature (23°C) the α-0 construct was rapidly lost from the ER, while the α-H protein was retained. Some of the retained protein had been exposed to the Golgi α(1–6)mannosyl transferase, and showed a characteristically reduced mobility. Cells containing *ERD2* on a multicopy plasmid also specifically retained the α-H protein, but there was considerably less of the Golgi-modified form. This result is consistent with the idea that high levels of *ERD2* either slow the exit of HDEL proteins from the ER or promote their retrieval from a compartment prior to the one that contains α(1–6)mannosyl transferase.

In a *sec*$^+$ strain, high level expression of the α-H construct resulted in its secretion (Figure 5A), a phenomenon that reflects the saturation of the HDEL retention system (Dean and Pelham, 1990). Overexpression of *ERD2*, however, allowed retention to occur, suggesting that the capacity of the system had been increased. This interpretation was confirmed by examination of BiP secretion from the same strains. Immunoblots of the culture medium (Figure 5B) showed that, whereas the parental strain or one expressing α-0 did not secrete significant quantities of BiP, the strain expressing α-H did, as expected if the

Table 2. Retention of HDEL-Tagged Invertase Fusion Protein in Strains with Different *ERD2* Content

ERD2 Gene Content	Invertase Activity Secreted (% total)	
	+ HDEL	– HDEL
Chromosomal + high copy vector[a]	5	31
Chromosomal[b]	14	33
Low copy expression vector only[c]	28	30

Numbers are the average of four or more determinations. The control fusion protein (– HDEL) had the C-terminal sequence EDLN.
[a] Plasmid PER220 in a strain derived from SEY2102.
[b] Strain as above, but with ZUC13 vector.
[c] *erd2* deletion strain D214.

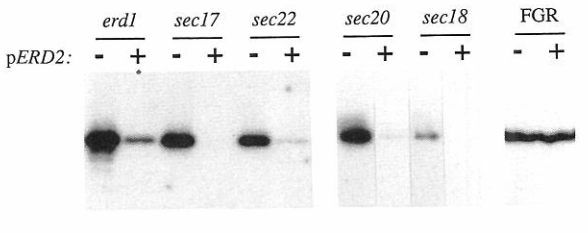

Figure 6. *ERD2* Suppresses the *erd* Phenotype of Other Mutants

Mutant strains were transformed with an *ERD2*-containing plasmid or with a control plasmid, and samples of medium from the various cultures were analyzed for their BiP content by immunoblotting. For this experiment, a multicopy plasmid (JS209) expressing a tagged, intronless copy of the *ERD2* gene was used, although similar results were obtained with the normal gene. The *erd1* strain carries a deletion of the *ERD1* gene. All *sec* mutants, which are temperature-sensitive, were grown at the permissive temperature (23°C). The FGR strain has its chromosomal BiP gene modified to remove the HDEL coding sequence; note that *ERD2* had no effect on the secretion of this modified BiP.

retention system is saturated. BiP secretion was reduced when *ERD2* was overexpressed, again implying an increase in the capacity of the HDEL system.

As an additional test of the relationship between *ERD2* levels and the retention capacity of cells, we measured the efficiency of secretion of an HDEL-tagged invertase fusion protein in strains engineered to contain different levels of *ERD2*. In the wild-type strain, secretion of the HDEL protein was clearly reduced relative to a control protein that lacked HDEL, but some secretion was observed (Table 2). In a strain carrying *ERD2* on a multicopy vector, retention of the HDEL protein was improved. Conversely, in a strain that had the chromosomal copy of *ERD2* deleted and carried only a cDNA copy of the gene on a centromere-containing expression vector, the HDEL protein was secreted almost as efficiently as the control. These cells must contain some *ERD2* protein because it is required for their growth (see below). However, the mere presence of the wild-type protein is evidently not sufficient for the retrieval system to function effectively.

We conclude from these experiments that the capacity of the HDEL retention system is primarily determined by the level of *ERD2* protein. Overexpression of *ERD2* increases the efficiency of retention, while mutation of *ERD2*, or low expression of the wild-type protein, severely reduces the ability of cells to retrieve ER proteins from the Golgi.

ERD2 Overexpression Suppresses the *erd* Phenotype of Other Mutants

During our search for mutants with an *erd* phenotype, we screened a variety of *sec* mutants that are known to affect vesicular traffic between ER and Golgi (*sec12*, *sec13*, *sec16*, *sec17*, *sec18*, *sec19*, *sec20*, *sec21*, *sec22*, *sec23*). These mutants are temperature-sensitive for growth, and fail to export proteins from the ER at the nonpermissive temperature (Novick et al., 1980, 1981). Our expectation was that some of them might show a partial defect at the permissive temperature, and that this might interfere with the recycling of ER proteins from the Golgi to the ER.

Of the mutants tested, four (*sec17*, *sec18*, *sec20*, and *sec22*) showed detectable secretion of HDEL-containing proteins at the permissive temperature, although all had a weaker phenotype than *erd2* mutants, and the *sec18* signal was very low (Figure 6). These four *sec* genes represent a specific class, in that they all accumulate pre-Golgi transport vesicles when incubated at the nonpermissive temperature and have elevated levels of these vesicles at the permissive temperature (Kaiser and Schekman, 1990). Mutants that fail to generate transport vesicles did not have an *erd* phenotype. This suggests that some component necessary for recycling is sequestered in transport vesicles and becomes limiting when they accumulate, slowing the retrieval process and allowing ER proteins to escape.

Figure 6 shows that overexpression of *ERD2* prevented the secretion of BiP from all four of the *sec* mutants, suggesting that it is the limiting component of the recycling system that is present in transport vesicles. *ERD2* did not, however, overcome the temperature-sensitive phenotype of these mutants (nor of any of the other *sec* mutants tested), nor did it prevent the secretion of BiP molecules that lacked the HDEL signal from the YFGR strain (Figure 6). Thus, the effects of *ERD2* overexpression are restricted to the HDEL retrieval system—the normal process of secretion is unaffected.

ERD2 also suppressed the secretion of BiP from an *erd1* deletion mutant (Figure 6). This mutant has a defect that prevents the addition of "outer-chain" mannose residues to glycoproteins in later Golgi compartments, although the early part of the Golgi appears to function normally (Hardwick et al., 1990). Examination of glycoproteins in the suppressed strain showed that *ERD2* did not correct the glycosylation defect (not shown); its action can be most easily explained by an increase in the efficiency of retrieval of ER proteins from the early, nondefective Golgi compartments. This is consistent with our previous observation that the extent of Golgi modification of a pro-α factor–HDEL fusion protein is reduced by overexpression of *ERD2* (Figure 5).

ERD2 Is Essential for Growth

To investigate the effects of a complete absence of the *ERD2* protein, we deleted from the cloned gene an NcoI

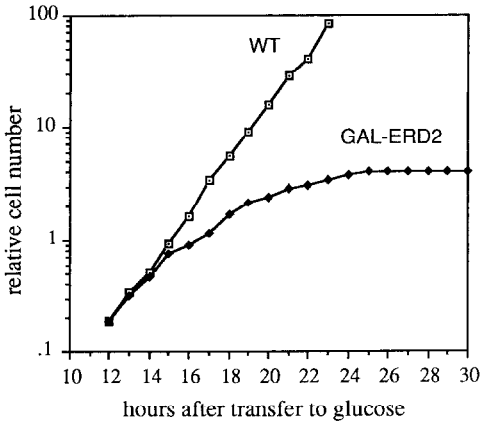

Figure 7. *ERD2* Expression Is Essential for Growth

A strain was constructed that lacked the normal *ERD2* gene but carried an integrated copy of the *ERD2* coding sequence under the control of the *GAL1* promoter (see Experimental Procedures). This strain (solid symbols) and an *ERD2*$^+$ control (open symbols) were maintained in galactose-containing medium, then transferred to glucose medium and their subsequent growth monitored. The cultures were diluted at 17 hr to maintain logarithmic growth conditions.

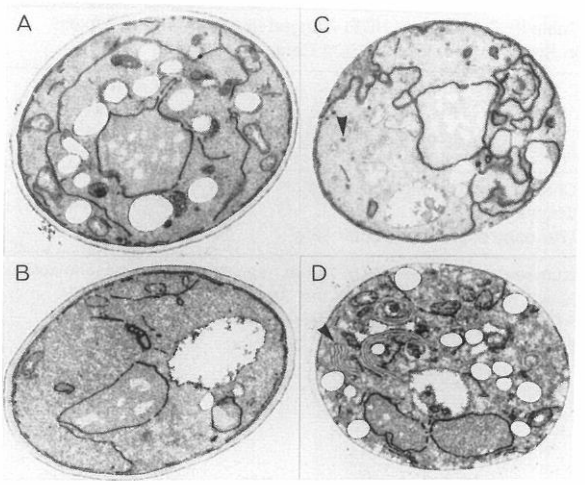

Figure 8. Accumulation of Membranes in *ERD2*-Depleted Cells

Electron micrographs of permanganate-fixed cells are shown. Total width of each panel corresponds to 3 μm.

(A) A cell carrying the *GAL1–ERD2* fusion gene, 24 hr after transfer to glucose; note abundant ER-like membranes. Clear white areas are lipid droplets.

(B) An *ERD2*$^+$ control, treated as in (A).

(C) A *sec20* cell after 3 hr at 37°C; note accumulation of continuous strands of ER, together with vesicles (arrowhead).

(D) A *sec7* cell after 3 hr at 37°C; note Golgi-like structures with poorly stained lumen (arrowhead).

fragment that includes all but the last 59 codons of the open reading frame and some 700 bp of 5′ flanking sequence (bases 600 to 1915 in Figure 2), and introduced this deletion into one chromosome of a diploid strain by the two-step procedure of Boeke et al. (1984). Deletion of the fragment was confirmed by Southern blot analysis (not shown). Sporulation of this strain revealed a recessive lethal mutation. Ten tetrads showed a segregation pattern of two viable and two nonviable spores; the latter germinated and divided three times on average, but then ceased to grow.

Confirmation that *ERD2* protein is essential for growth was provided by an additional experiment. The *ERD2* open reading frame was fused to the inducible *GAL1* promoter and integrated at the *URA3* locus of the diploid strain that carried the *erd2* deletion. Sporulation of this strain gave rise to some haploid cells whose only copy of *ERD2* was under *GAL1* control. These cells were grown in medium containing galactose to activate transcription from the *GAL1* promoter (Johnston and Davis, 1984); under these conditions, they divided as rapidly as wild-type cells. However, when the *GAL1* promoter was inactivated by transferring the cells to glucose-containing medium, the strain lacking the normal *ERD2* gene gradually ceased to grow (Figure 7). The cells remained quiescent but did not die: 2 days after they had ceased to grow, 80% of the cells were still capable of forming colonies on galactose-containing plates. Thus, growth can be reversibly halted by depletion of the *ERD2* protein.

Protein Traffic through the Golgi Is Defective in Cells Lacking *ERD2*

The growth defect of cells lacking *ERD2* was unexpected, because previous studies had suggested that the HDEL retention system is dispensable (Hardwick et al., 1990), and indeed the growth-arrested cells had normal levels of BiP (not shown). It seemed therefore that *ERD2* must perform a second function; to identify this function, we examined the properties of *ERD2*-depleted cells.

Figure 8A shows an electron micrograph of a cell containing the *GAL1–ERD2* fusion gene that had been grown on glucose for 24 hr. For comparison, wild-type cells (Figure 8B) and *sec20* and *sec7* cells that had been incubated for 3 hr at the nonpermissive temperature, which induces the accumulation of ER and transport vesicles (*sec20*; Figure 8C) or Golgi-like structures (*sec7*; Figure 8D) (Novick et al., 1980; Kaiser and Schekman, 1990), are also shown. The *erd2*-deficient strain accumulated higher than normal amounts of intracellular membranes. This phenotype was detected in some cells as early as 14 hr after transfer to glucose; after longer times it was apparent in most cells. The membranes seemed more fragmented than the ER that accumulates in mutants such as *sec20*, which typically show much longer continuous ribbons of ER than are seen in wild-type cells. There was no significant accumulation of vesicles, nor of the characteristically bloated Golgi structures seen in the *sec7* mutant. However, since the distended cisternae seen in *sec7* cells are not present in wild-type cells, which nevertheless have Golgi compartments, it remains possible that some of the membranes in the *erd2*-depleted cells are Golgi derived. All the mutants accumulated lipid droplets, visible as white areas in the micrographs.

These results are consistent with a block in the secretory pathway in *erd2*-depleted cells, but the phenotype appeared different from that of any known *sec* mutant. To

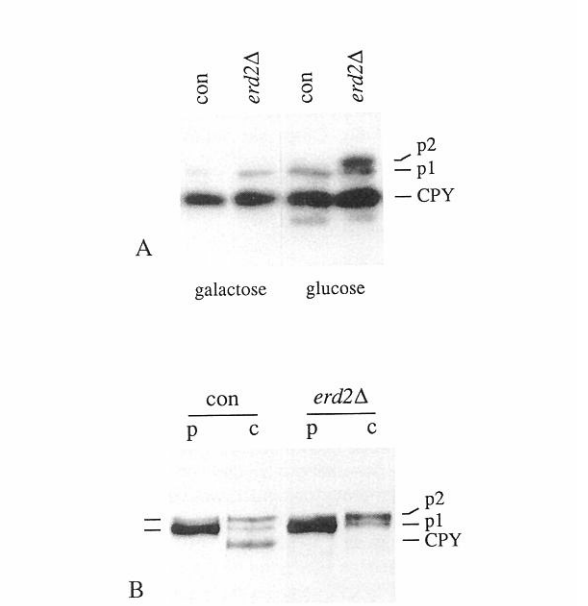

Figure 9. *ERD2*-Deficient Cells Accumulate the Golgi Form of CPY

(A) Cells carrying the *GAL1–ERD2* fusion gene (*erd2*Δ) and *ERD2*$^+$ controls (con) were grown in galactose medium, or transferred to glucose medium for 15 hr, washed, and their CPY content analyzed by immunoblotting. Similar results were obtained after 24 hr in glucose.

(B) Control and *ERD2*-deficient cells, after 15 hr growth in glucose, were pulse-labeled for 10 min (p) and chased for 30 min (c). Intracellular CPY was then isolated by immunoprecipitation. The Golgi precursor of CPY (p2) accumulated in the mutant cells in both experiments, while the ER form (p1) appeared normal.

identify the step that becomes rate limiting, we examined the processing of CPY in the absence of *ERD2*. Cells carrying the *GAL1–ERD2* fusion gene were grown for 15 hr in glucose medium and their CPY content was analyzed by immunoblotting (Figure 9A). At 15 hr, growth of the cells was just beginning to slow down (Figure 7); similar results were obtained after 24 hr, when growth had ceased entirely. Relative to the control cells, there was no change in the level of the p1 ER form of CPY, but there was a marked increase in the amount of the p2 Golgi form, suggesting that passage of CPY through the Golgi was inhibited.

The transport defect was confirmed by pulse-chase analysis (Figure 9B). Synthesis, translocation into the ER, and conversion of the ER form of CPY to the Golgi form occurred with normal kinetics in the arrested cells, but vacuolar processing was substantially delayed or inhibited (Figure 9B). In some experiments the accumulated p2 form in the mutant cells appeared to have a very slightly increased mobility relative to the control cells, suggesting that its modification state might be different. However, at least some of this precursor was precipitable with antibodies that recognize α(1–3)mannose (not shown), as is normal p2 (Franzusoff and Sckekman, 1989). Since these residues are added in a late Golgi compartment, transport through most of the Golgi must have occurred, though perhaps more slowly than usual. The p2 CPY remained associated with the cells and therefore was not simply mistargeted to the cell surface. It also seems unlikely that it was transported to the vacuole but failed to be processed, since the cells still contained high levels of mature CPY (Figure 9A) and presumably also other vacuolar proteases.

These results imply that *ERD2* function is required, directly or indirectly, for normal transport of CPY (and presumably other proteins) through the Golgi apparatus. Since there is an accumulation of intracellular membranes in the absence of *ERD2*, it would appear that the defect is due to inefficient vesicular transport.

Discussion

Our genetic analysis of the retention of HDEL proteins in yeast has so far identified only two genes that are required for this purpose. The *ERD1* gene is not required when cells are grown in certain minimal media (Hardwick et al., 1990), but the *ERD2* gene appears to be needed under all conditions. The *ERD2* product is therefore likely to be closely involved in the retention mechanism. The results reported here indicate that it is a 26 kd integral membrane protein that possibly contains multiple membrane-spanning segments. Preliminary immunofluorescence studies suggest that much of it is found in a post-ER, Golgi-like compartment.

The Role of *ERD2* in Retention of Luminal ER Proteins

A striking observation is the correlation between the level of *ERD2* expression and the capacity of the HDEL retention system. Thus, while mutation or underexpression of the gene causes secretion of ER proteins such as BiP, overexpression improves their retention in wild-type cells without affecting the secretion of other proteins. In particular, when the HDEL system is saturated by the abundant synthesis of an HDEL-bearing fusion protein, high levels of *ERD2* alleviate the problem and reduce secretion of both the fusion protein and endogenous ER proteins. This ability to increase capacity suggests that *ERD2* controls the concentration of the HDEL receptor in the sorting compartment; the simplest interpretation is that *ERD2* encodes the receptor.

Although this argument is suggestive, it is not conclusive. However, in the accompanying paper it is shown that replacement of the *ERD2* gene with the equivalent gene from Kluyveromyces lactis (another budding yeast) changes the signal specificity of the retention system (Lewis et al., 1990). Thus *ERD2* determines both the capacity and specificity of the retention system, providing strong evidence that it is indeed the receptor.

If so, the ability of *ERD2* to suppress the weak *erd* phenotype of the *sec* mutants that accumulate pre-Golgi transport vesicles is readily explained. Presumably, HDEL receptor is present in the accumulated vesicles and is correspondingly depleted from the sorting compartment. Providing more receptor would compensate for this effect. It could also encourage retrieval from early compartments on the secretory pathway, thus accounting for the suppression of BiP secretion from *erd1* mutants, which are defective only in the later regions of the Golgi (Hardwick et al., 1990).

The Role of *ERD2* in Secretion

The fact that *ERD2* is essential for growth suggests that it has a second function, distinct from its role in the targeting of soluble ER proteins. This function seems to be required for the correct operation of the secretory pathway. Specifically, transport of CPY through the Golgi is impaired when the *ERD2* protein is depleted from cells, and intracellular membranes accumulate.

It seems very unlikely that this defect is merely a consequence of the failure to retain resident soluble proteins in the ER. Retention of BiP by the HDEL system is certainly not essential for growth, because a strain whose only BiP gene lacks the sequences encoding HDEL grows normally. Viable *erd2* mutants secrete BiP at the same rate as this strain, and thus show no detectable HDEL-dependent retention (Figure 1). Moreover, any residual receptor activity can be saturated by overexpression of a pro-α factor–HDEL fusion protein, with no obvious ill effect. Finally, if the growth defect were due to the reduction of the level of some hypothetical luminal ER protein below a critical threshold, one might expect to find a defect in ER function. In contrast, we find that translocation of CPY into the ER, its glycosylation, and transport out of the ER all appear normal in the absence of *ERD2*; rather, it is traffic through the Golgi that is affected, at least initially.

What might be the role of *ERD2* in Golgi transport? One possibility is that this small protein has two quite separate functions, one being to bind HDEL proteins and the other being essential for the maintenance of Golgi structure or physiology. A more attractive possibility is that the two functions are related. If the *ERD2* protein is the HDEL receptor, it follows that it must cycle between Golgi and ER. It seems likely to be a major component of the vesicles that return from the Golgi, perhaps the most abundant membrane protein in them. It would not be surprising if it were required for such vesicles to form. For example, by clustering in the membrane and associating with a suitable coat protein, *ERD2* protein could help to form a structure analogous to a coated pit. If it plays such a role, then in its absence no proteins, either soluble or transmembrane, could return from the Golgi to the ER. It is not clear whether any ER membrane proteins normally escape to the Golgi and require retrieval, but recycling of the *SEC12* protein, and perhaps other membrane proteins involved in vesicular traffic, has been suggested (Nakano et al., 1989). *ERD2* might be necessary to maintain the supply of such components in the compartments from which vesicles bud. Alternatively, their accumulation in an inappropriate compartment might cause mistargeting of transport vesicles. In either case, a failure to retrieve these membrane proteins could be sufficient to reduce the flow of the secretory pathway to a trickle.

Experimental Procedures

The strains used in Figure 1 (and part of Table 2, as indicated) were derivatives of SEY2102 (*MATα ura3-52 leu2-3,-112 his4-519 suc2-d9*). Other constructed strains (except for *sec* strains) were derived from SEY6210 (*MATα ura3-52 his3-d200 leu2-3,-112 trp1-d901 lys-801 suc2-d9*) or, for the *erd2* deletion strains, from a diploid obtained by crossing SEY6210 with SEY6211 (*MATa ura3-52 his3-d200 leu2-3,-112 trp1-d901 suc2-d9 ade2-101*). The *sec18* strain used in Figure 5 is ND1811 (*MATa sec18-1 ura3-52 suc2-d9*) (Dean and Pelham, 1990). The *sec7* strain used for electron microscopy was ND72 (*sec7-1 ura3-52 trp1-d901 his3-d200 leu2-3,-112 suc2-d9*). Other *sec* mutant strains were kindly provided by Chris Kaiser: RSY263 (*sec12-4*), RSY265 (*sec13-1*), RSY267 (*sec16-2*), RSY269 (*sec17-1*), RSY271 (*sec18-1*), RSY273 (*sec19-1*), RSY275 (*sec20-1*), RSY277 (*sec21-1*), RSY279 (*sec22-3*), RSY281 (*sec23-1*) (Kaiser and Schekman, 1990).

ERD2 Plasmids

The *ERD2* gene was isolated by complementation, using a library constructed in a centromere vector (Sengstag and Hinnen, 1987). An intronless version of the open reading frame was prepared by PCR (Higuchi, 1989), using a primer encoding the first 12 amino acids of the protein. A PstI site was introduced immediately after the last codon by similar means and was then used to fuse the open reading frame to DNA encoding the epitope (EQKLISEEDLN) recognized by the 9E10 monoclonal antibody, generating a tagged version of the gene. The structure of the PCR-generated constructs was verified by sequencing. Mutant *erd2* alleles were isolated from genomic DNA by PCR, using primers that flank the coding sequence.

Four expression plasmids were used for the experiments described in this paper. Plasmid PER220 contains the HindIII fragment whose sequence is shown in Figure 2, inserted into the *LEU2*-containing 2μm vector ZUC13. JS209 contains a tagged, intronless copy of the *ERD2* coding sequence fused to the triose phosphate isomerase (*TPI*) promoter, on a *URA3*-containing 2μm vector. JS214 contains an intronless but untagged gene, also fused to the *TPI* promoter, on a vector with *TRP1*, *ARS1*, and *CEN3*. HP208 contains the untagged, intronless gene fused to the *GAL1* promoter, on a vector (YIP56X) that allows integration at the *ura3-52* locus (Pelham et al., 1988).

To delete the chromosomal *ERD2* gene, the NcoI fragment that extends from nucleotide 597 to 1912 was deleted from the HindIII fragment (Figure 2) and the deleted fragment inserted into the *URA3*-containing integration vector YIP56. This was linearized at the single remaining KpnI site (nucleotide 2682) and integrated at one copy of the *ERD2* locus in a diploid strain (generated by crossing SEY6210 and SEY6211). After 5-fluoro-orotic acid selection (Boeke et al., 1984), a strain carrying one deleted copy of the gene was identified by Southern blot analysis. This was transformed, separately, with JS209, JS214, and HP208. The resulting strains were sporulated, and spores lacking the chromosomal gene identified by Southern blot analysis (and, in the case of HP208, by their dependence on galactose medium for growth). The resulting strains were designated D209, D214, and D208, respectively. Other *ERD2* expression plasmids were introduced into the deletion background by transfection of strain D209 followed by 5-fluoro-orotic acid selection to eliminate JS209.

Plasmids containing the invertase and prepro-α factor fusion genes have been described elsewhere (Pelham et al., 1988; Dean and Pelham, 1990). The α factor constructs include the coding sequence for the 9E10 epitope, allowing specific immunoprecipitation of the expressed protein with monoclonal antibody 9E10 without interference from endogenous α factor. Invertase–BiP fusion proteins were expressed from the *PRC1* promoter, prepro-α factor constructs from the *TPI* promoter; both were integrated at *URA3*, and the number of integrated copies was checked either by Southern blot analysis or by quantitative invertase assays.

Detection of Tagged *ERD2* Protein

Crude ER fractions were prepared using a sucrose step gradient as described by Dean and Pelham (1990). Samples were diluted 10-fold in 0.1 M sodium carbonate (pH 11.5), incubated for 30 min at 0°C, and then centrifuged for 1 hr at 245,000 × g in a Beckman TLA-100.2 rotor, essentially as described by Fujiki et al. (1982). Triton X-114 extraction of whole spheroplasts was performed as described by Lewis et al. (1985).

Other Techniques

Immunoblotting of cells and media, pulse-labeling with $^{35}SO_4$ and immunoprecipitation, invertase assays, and electron microscopy were performed as previously described (Dean and Pelham, 1990; Hardwick et al., 1990; Pelham et al., 1988).

Acknowledgments

K. G. Hardwick and J. C. Semenza made equal contributions to the work described in this paper. We thank Deborah Sweet for the data shown in Table 2, Mark Rose, Tom Stevens, and Gerard Evan for antibodies, Albert Hinnen for the genomic library, Chris Kaiser for yeast strains, Mike Lewis for plasmids, Nichol Thomson and Douglas Kershaw for help with electron microscopy, and Sean Munro, Mark Bretscher, and Barbara Pearse for comments on the manuscript. N. D. was supported by a fellowship from the American Cancer Society, and J. C. S. by the Roche Research Foundation.

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Received May 10, 1990.

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GenBank Accession Number

The accession number for the sequence reported in this paper is M34777.