Retrieval of HDEL Proteins Is Required for Growth of Yeast Cells

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Abstract. The *ERD2* gene of *Saccharomyces cerevisiae* encodes the receptor which retrieves HDEL-containing ER proteins from the Golgi apparatus. Viable *erd2* mutants have been isolated that show no obvious HDEL-dependent retention of the luminal ER protein BiP, suggesting that retrieval of HDEL proteins is not essential for growth. However, cells that lack Erd2p completely have a defective Golgi apparatus and cannot grow. This observation led to the suggestion that the receptor had a second function, possibly related to its ability to recycle from Golgi to ER.

In this paper we investigate the requirements for Erd2p to support growth. We show that mutations that block its recycling also prevent growth. In addition, we show that all mutant receptors that can support growth have a residual ability to retrieve BiP, which is detectable when they are overexpressed. Mere recycling of an inactive form of the receptor, mediated by a cytoplasmic KKXX sequence, is not sufficient for growth. Furthermore, saturation of the receptor by expression of an HDEL-tagged version of pro-α factor inhibits growth, even of strains that do not show obvious BiP retention. We conclude that growth requires the HDEL-dependent retrieval of one or more proteins, and that these proteins can be recognized even under conditions where BiP is secreted. Genetic screens have failed to identify any one protein whose loss could account for the Erd2p requirement. Therefore, growth may require the retention of multiple HDEL proteins in the ER, or alternatively the removal of such proteins from the Golgi apparatus.

RESIDENT soluble ER proteins, and some type II membrane proteins, have a COOH-terminal tetrapeptide sorting signal, typically KDEL or HDEL, that is both necessary and sufficient to retain them in this compartment (reviewed by Pelham, 1989, 1990). Retention is thought to be mediated by continual retrieval from a post-ER compartment, as soluble ER proteins can acquire carbohydrate modifications characteristic of the Golgi apparatus (Pelham, 1988; Dean and Pelham, 1990; Peter et al., 1992; Jackson et al., 1993). A membrane-bound receptor in the Golgi apparatus or an intermediate compartment binds to these proteins, and then enters a retrograde transport pathway to return them to their source.

The HDEL receptor was identified by genetic means in yeast and is the product of the *ERD2* gene (Lewis et al., 1990; Semenza et al., 1990). Receptor homologues have since been identified in a wide variety of other species including humans (Lewis and Pelham, 1990, 1992*b*; Hsu et al., 1992), cows (Tang et al., 1993), *Arabidopsis* (Lee et al., 1993), *Plasmodium* (Elmendorf and Haldar, 1993), *Drosophila* and *Caenorhabditis* (Banfield, D., unpublished observations). Sequence comparisons indicate a highly conserved seven transmembrane domain (TM)[1] structure (see Townsley et al., 1993). A human receptor has been shown to bind KDEL and HDEL sequences in vitro (Wilson et al., 1993). Optimal binding occurs at acid pH, suggesting that selective binding of ligands in the Golgi and their release in the ER may be facilitated by a pH difference between these organelles. Ligand binding also controls the movement of the receptor: when expressed in COS cells, the human receptors are normally concentrated in or near the Golgi apparatus, but their steady-state distribution can be shifted to the ER by high level expression of an appropriate ligand such as a KDEL-tagged version of hen lysozyme (Lewis and Pelham, 1992*a*; Townsley et al., 1993).

A puzzling feature of the HDEL receptor is that its presence is required for yeast cell growth, even though viable *erd2* strains exist that show no obvious HDEL-dependent retention—they secrete the ER protein BiP as efficiently as wild-type cells secrete an HDEL-deleted version of BiP (Semenza et al., 1990). Cells depleted of Erd2p accumulate intracellular membranes, and protein transport through the Golgi apparatus is impaired. The requirement for the recep-

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1. *Abbreviations used in this paper*: FOA, 5-fluoroorotic acid; TM, transmembrane.

tor is not absolute, because six genes (termed *SED* genes) have been identified which, when overexpressed, allow growth of an *erd2*-deletion strain (Hardwick et al., 1992; Hardwick and Pelham, 1992, 1994). Some of these genes encode membrane proteins which themselves play an essential role within the secretory pathway, but it is uncertain how they compensate for the loss of Erd2p; none of them restores the membrane organization of *erd2Δ* strains to normal. These results indicate that Erd2p is somehow required to maintain the structure and function of the Golgi apparatus, and that this task cannot be performed by other proteins. One possibility suggested previously is that Erd2p directly modulates the retrograde transport pathway, and thus controls the balance of membrane flow between the ER and Golgi compartments (Hardwick et al., 1992).

We made a number of mutations to locate functional residues in the human KDEL receptor (Townsley et al., 1993). These experiments showed that ligand binding is dependent upon charged residues within the TM domains and that retrograde transport of occupied receptor to the ER is critically dependent upon an aspartic acid residue in the seventh TM domain. We have used this information to make selected mutations in the *Saccharomyces cerevisiae* HDEL receptor, and hence to define the properties of Erd2p that are essential for growth. The results suggest that both ligand binding and recycling of the receptor are necessary. Furthermore, we find that all mutant receptors that support growth can also retain BiP when expressed at a sufficiently high level, and that saturation of the receptor by overexpression of an α factor–HDEL fusion protein can impair growth. These results strongly suggest that the ability to recognize and retrieve one or more endogenous HDEL ligands is crucial for normal growth. Since genetic screens have failed to identify any single protein whose retention is essential, growth may require the retrieval of multiple HDEL proteins from the Golgi apparatus.

Materials and Methods

Plasmids

To facilitate mutagenesis, a 2.1-kb PstI-SalI fragment from plasmid HP210 containing the intronless, untagged *ERD2* gene fused to the *TPI* promoter was cloned into pBluescript (Stratagene Corp., San Diego, CA). Mutations were introduced into the gene by site-directed mutagenesis using the method of Kunkel et al. (1987) and checked by sequencing. The same method was used to add the residues KSL to the precise carboxyl terminus of the *ERD2* gene (Lys 219), creating a KKXX signal. Other coding sequences were added to the COOH terminus of the receptor as described previously (Townsley and Pelham, 1994). For expression in yeast, the *TPI*-driven modified *ERD2* genes were cloned as a 2.1-kb PstI-SalI fragment into vectors pRS315 (*LEU2*, *CEN6*; Sikorski and Hieter, 1989), or YEp351 (*LEU2*, 2μ; Hill et al., 1986).

Multicopy plasmids (*URA3*, 2μ) expressing *TPI*-driven pro-α factor fusion proteins (Dean and Pelham, 1990) were a gift from Debbie Sweet (this laboratory). The fusion proteins terminate with the *c-myc* epitope tag followed by FEHDEL (α-H), YFDDEL (α-D), or no additional sequences (α-O).

For the isolation of *SED4*-dependent mutants, a multicopy plasmid carrying the *URA3* and *ADE3* genes and a truncated copy of *SED4* under the control of the *TPI* promoter was constructed. Sequences encoding the *c-myc* epitope were fused to a 1.3-kb EcoRI/NheI fragment, producing a protein that lacked most of the luminal domain of Sed4p.

Yeast Strains and Yeast Viability Assay

To assess the viability of yeast containing mutant Erd2 proteins, plasmids with a *LEU2* marker expressing *TPI*-driven mutant *ERD2* genes were transformed into the sectoring strain ΔLE26A (*MATα ade2 ade3 erd2Δ ura3 leu2 his3*, pLE26A [*CEN6, ADE3, URA3, TPI-K. lactis ERD2*]; Townsley and Pelham, 1994) and transformants were selected on plates lacking leucine. After two days of growth the transformants were streaked on rich plates containing 20 μg/ml adenine sulphate (low adenine concentrations enhance the red color of *ade2 ADE3* strains) and onto plates containing 5-fluoroorotic acid (FOA) which selects against *URA3* (Sikorski and Boeke, 1991). If a mutant Erd2p could support growth, then cells could lose pLE26A and transformants grew on FOA and produced red/white sectoring colonies on rich medium (Koshland et al., 1985). In most cases when a mutant protein was able to support growth, sectoring colonies were evident throughout the streak and all transformants could grow on FOA. In some cases mutant proteins appeared to have minimal activity, and occasional sectoring colonies were found, or a few colonies sectored at the edges, and only some transformants could grow on FOA. This phenotype is designated +/− in the Tables.

For subsequent experiments, plasmids expressing *ERD2* derivatives were transformed into the strain ΔJS209 (*Mata ura3-52 leu2-3, -112 his3-Δ200 trp1-Δ901 suc2-Δ9 lys2-801 ade2-101 erd2Δ*; pJS209 [2μ, *URA3, TPI-ERD2*]; Semenza et al., 1990) and colonies that had lost plasmid JS209 were selected on FOA. The resultant strains were used directly to monitor BiP secretion. Isogenic strains containing a modified version of the BiP gene (*KAR2*) which converts the COOH terminus of BiP from FEHDEL to FGR were made as described by Hardwick et al. (1990). ΔJS209-derived *erd2* strains were transformed with the plasmids expressing pro-α factor fusion proteins to be tested in the halo assays (Sprague, 1991). These were performed on plates lacking leucine as well as uracil, to slow down growth and increase halo size. ΔLE26A containing a vector with the *LEU2* marker was used as the control α strain.

Isolation of SED4-dependent Mutants

A yeast strain was constructed with the genotype *MATa ade2 ade3 ura3 leu2 lys2 sed4*::*LEU2*, and transformed with the 2μ *URA3 ADE3 TPI-SED4* plasmid described above. Cells were spread on sectoring plates (as above) and mutagenized with UV light (95–97% killing). Nonsectoring colonies were picked, checked twice more for the nonsectoring phenotype and then tested on FOA plates to confirm that loss of the plasmid was lethal. Proof that the mutations lay in the *erd2* gene was provided either by transformation with an *ERD2*-containing plasmid, which restored the ability to form sectors and also allowed growth on FOA, or by crossing with the *erd2* deletion strain ΔLE26A, which produced diploids that were unable to grow on FOA.

Immunofluorescence

Cells were fixed and mounted on slides as described by Hardwick and Pelham (1992). Antibody incubations were carried out in PBS + 2% dried milk. Primary antibody incubations were carried out overnight at 4°C and secondary antibody incubations for 2 h at room temperature. mAb 9E10 (Evan et al., 1985) was used at 3 μg/ml. Secondary antibody (FITC-conjugated sheep anti-mouse Ig) was obtained from Amersham International (Amersham, UK) and diluted 1/50.

Colony Blotting Analysis of Secreted BiP

Analysis of secreted BiP was essentially as described by Hardwick et al. (1990). Otherwise isogenic strains expressing BiP-HDEL or BiP-FGR were always assayed together. Briefly, freshly grown cells were streaked thinly onto rich plates and covered with a 0.45-μm nitrocellulose filter, then grown at 30°C for 12–16 h. The filter was washed in PBS and then treated as for a normal immunoblot. Antibody incubations were carried out in PBS + 2% dried milk for 1 h at room temperature. Anti-BiP antiserum was diluted 1/20,000, and the secondary antibody, peroxidase-conjugated anti-rabbit Ig (Sigma Chem. Co., St. Louis, MO), was diluted 1/4,000. Secreted protein was detected by chemiluminescence (ECL kit; Amersham International, Amersham, UK) and autoradiography, and quantitation performed using a model 300A densitometer (Molecular Dynamics, Sunnyvale, CA).

Results

Recycling Is Necessary for the Essential Function of Erd2p

To establish the structural requirements for the essential

function of yeast Erd2p we made a number of different point mutations, selected on the basis of our previous analysis of the human receptor, and tested the mutant proteins in vivo using a plasmid shuffle assay (see Materials and Methods for details). Mutant genes were transformed into a strain whose chromosomal copy of *ERD2* is disrupted, but which carries a plasmid encoding the *Kluyveromyces lactis ERD2* gene (pLE26A). If a mutant receptor was able to support growth then cells could spontaneously lose the *K. lactis ERD2* plasmid. The genotype of the cells allowed this loss to be monitored in two ways: pLE26A contains an *ADE3* gene, whose loss leads to the formation of white sectors in otherwise pink colonies; it also contains a *URA3* gene, and cells that lack this plasmid are able to grow on medium containing FOA, which is toxic only to cells that carry a wild-type copy of *URA3*.

As a major recycling protein, we imagined that Erd2p could be required to stimulate retrograde transport from the Golgi apparatus; in its absence ER components would accumulate in the Golgi and this might be detrimental to growth (Hardwick et al., 1992). To test whether recycling is required for the essential function of the receptor, we chose a mutation that specifically affects recycling in COS cells. Alteration of residue D193 in the human receptor to an asparagine has no effect on ligand binding in vitro, but prevents retrograde transport of occupied receptor to the ER in vivo (Townsley et al., 1993). We made the equivalent mutation in yeast (D200-N, for the proposed structure of the receptor; see Fig. 1) and found that even at high levels of expression this mutant protein was incapable of supporting growth (Table I). This strongly suggests that recycling is necessary for the essential function of the yeast receptor.

To further investigate the importance of receptor recycling we asked whether an alternative recycling signal could suppress the lethal effects of the D200-N mutation. We have shown that a COOH-terminal KKXX sequence can mediate retrieval of a type I integral membrane protein from the Golgi to the ER in yeast; this signal is also sufficient to redistribute wild-type Erd2p from the Golgi to the ER (Townsley and Pelham 1994). We added the *c-myc* epitope and the sequence KKSL to the COOH terminus of the D200-N mutant receptor; as a control, the KKSL sequence was omitted, or replaced with KLSK. As expected, the KKXX signal redistributed the mutant receptor from the Golgi to the ER as judged by immunofluorescence (Fig. 2); it also restored the growth-promoting function of the D200-N mutant (Table II), but only when the receptor was expressed from a multicopy vector. However, suppression did not result entirely from KKXX-mediated recycling, because addition of other amino acids to the COOH terminus of the receptor also rescued the lethal effects of this mutation. These sequences did not have to provide an autonomous recycling signal—the *c-myc* epitope, or the tripeptide LSK could suffice (Table II). Presumably, the extra amino acids alter the normal structure of the receptor, and indirectly suppress the recycling defect of the D200-N mutant.

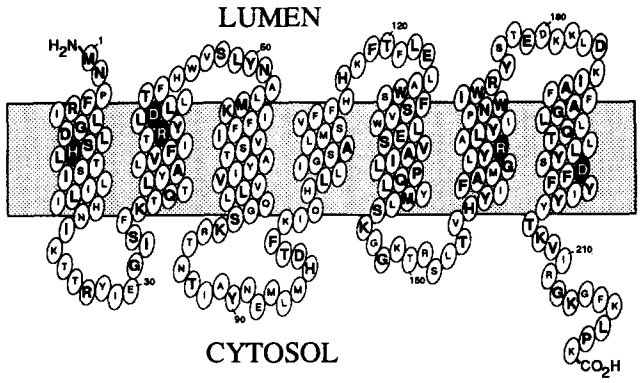

Figure 1. Schematic diagram of the proposed structure of *S. cerevisiae* Erd2p. Bold letters indicate residues identical to the human receptor previously analyzed by mutagenesis. Filled ovals correspond to mutated residues discussed in the text. This model is based on previous work (Townsley et al., 1993); an alternative configuration for the first two TM domains has recently been proposed based on the analysis of fusion proteins (Singh et al., 1993), but this seems less likely; the fusion method can give incorrect results where there are interactions between TM domains (Hennessey and Broome-Smith, 1993).

Table I. Viability of Yeast Cells Expressing Mutant Erd2 Proteins

Mutant	Viability of yeast when mutant expressed from *CEN* plasmid*	Viability of yeast when mutant expressed from 2 μ plasmid*
Wild-type	+	+
H12-A	+/−	+
R47-Q	−	−
D50-N	+	+
R165-N	−	−
D200-N	−	−

* Viability was tested using the plasmid swap assay described in Materials and Methods.

Suppression of the growth phenotype of the D200-N mutation by COOH-terminal sequences allowed us to confirm that the presence of this mutation does not prevent ligand recognition in vivo. Previous work has shown that measurement of the intracellular level of BiP does not give an indication of retention efficiency, because cells regulate the rate of BiP synthesis to maintain a constant steady-state level in the ER (Hardwick et al., 1990; Semenza et al., 1990). However, the rate of BiP secretion gives an inverse estimate of the retention efficiency. We therefore monitored BiP secretion in strains bearing mutant receptors and, for comparison, analyzed in parallel otherwise isogenic strains expressing BiP without the HDEL signal (BiP-FGR; Hardwick et al., 1990). Table III shows that the myc-tagged D200-N mutant receptor could retain BiP. Retention was not as efficient as for the wild-type receptor, presumably because recycling of this mutant is inefficient. Addition of the KKXX signal improved BiP retention to some extent, which suggests that the activity of the D200-N mutant is limited by its inefficient recycling, and that the KKXX signal can indeed partially compensate for this. Further evidence that the KKXX signal stimulates the activity of the D200-N mutant is presented below.

Mere Recycling of Erd2p Is Not Sufficient for Growth

The above results indicate that recycling of Erd2p is required for yeast cell growth, but since the D200-N mutation does not prevent ligand recognition, we cannot discern whether

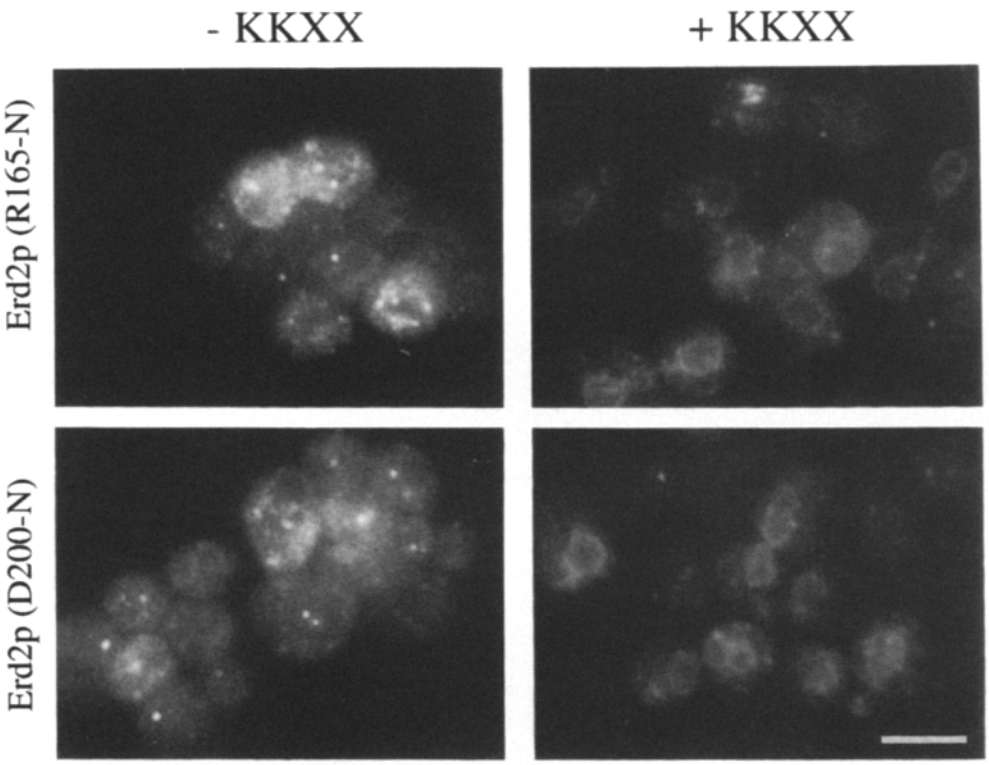

Figure 2. KKXX-mediated redistribution of mutant receptors. Mutant versions of Erd2p, carrying a *myc* tag and the KKSL sequence as indicated, were expressed from a multicopy vector and visualized by immunofluorescence. Note staining of the nuclear envelope with the KKXX-containing proteins. Bar, 5 μm.

ligand binding is also necessary. We made mutations in conserved residues that are known to be important for ligand binding by the human receptor (H12-A, R47-Q, and R165-N; see Fig. 1) and tested the mutant receptors for their ability to support growth in our assay. The H12-A mutant receptor retained some activity when expressed at low levels, but efficient growth required expression from a multicopy plasmid (Table I). Strains containing this mutant generally showed poor BiP retention (Table III), but in some experiments more significant retention was observed. Further experiments described below indicate that this mutant is indeed able to recognize HDEL ligands. In contrast, the mutants R47-Q and R165-N failed to keep cells alive even when expressed from a multicopy plasmid (Table I); by analogy with the human receptor, these mutations are likely to abolish ligand binding completely.

We added the *c-myc* epitope and a KKXX signal to the COOH terminus of the R47-Q and R165-N mutant receptors and expressed them from a multicopy vector. KKXX was sufficient to redistribute the receptors from the Golgi to the ER (Fig. 2), but it was not able to restore their growth-promoting function (Table II). Thus mere recycling of an inactive form of the molecule is not sufficient to support yeast cell growth. It follows that ligand recognition, or some other property dependent on the normal structure of the receptor, is important for growth. Ligand binding cannot simply be required to promote recycling.

Table II. Effects of the KKXX Signal on the Intracellular Distribution and Function of Erd2p Mutants

Mutant	COOH-terminal addition	Distribution*	Ability to support growth‡
Wild-type§	*myc*	Golgi	+
	myc-KKSL	ER	+
D200-N	*myc*	Golgi	+
	*myc*KLSK	Golgi	+
	*myc*KKSL	ER	+
	(K)LSK‖		+
	(K)KSL‖		+
R47-Q	*myc*	Golgi	−
	myc-KKSL	ER	−
R165-N	*myc*	Golgi	−
	myc-KKSL	ER	−

* Mutant proteins were expressed from a multicopy vector, and their distribution assessed in *erd2Δ* strains carrying *K. lactis ERD2*.
‡ Ability to support growth when expressed from a multicopy vector was determined by the plasmid shuffle assay, as in Table I.
§ See Townsley and Pelham, 1994.
‖ The K residue in parenthesis is the COOH-terminal amino acid of Erd2p.

Table III. Secretion of BiP by Cells with Mutant Receptors

Mutant	BiP secretion (percent control)*
Wild-type	21
erd2Δ; p*SED5*	102
D200-N *myc*	48
D200-N *myc*-KKSL	33
D200-N KLSK	49
D200-N KKSL	36
H12-A	94
D50-N	23

* The amount of BiP secreted was assayed as described in Materials and Methods, and in each case is expressed as a percentage of the BiP secreted by an isogenic strain that has been modified to remove the HDEL sequence from BiP. Numbers are the average of three experiments.

The Original erd2 Alleles Can Recognize HDEL Ligand

The results described so far strongly suggest that ligand binding is required for yeast cell growth, yet strains carrying the original *erd2* mutations showed no HDEL-dependent retention of BiP (Semenza et al., 1990). This apparent discrepancy prompted us to test whether any binding activity could be detected when the receptors from such strains were overexpressed. Of the three alleles that have been characterized in detail, two (B36 and R93) contain a termination codon 12 amino acids from the COOH terminus (Semenza et al., 1990). However, it is unlikely that a receptor truncated at this point is active, because complete deletion of the last 12 codons from the *erd2* gene results in a nonviable allele (Semenza 1991); presumably, the point mutants are viable because a small amount of effectively wild-type protein is provided by readthrough of the termination codon. Such a readthrough product is likely to retain binding activity, and indeed, overexpression of the R93 allele partially restores BiP retention (Semenza, 1991).

The third mutant allele of *erd2* which fails to retain BiP (B25) contains a D50-N change in TM2. We expressed a receptor containing this mutation from the strong *TPI* promoter, on a multi-copy plasmid. As expected, strains lacking the chromosomal *ERD2* gene but carrying this plasmid could grow (Table I). In contrast to cells containing only a single copy of the mutant gene, they also showed quite efficient retention of BiP (Table III). We conclude that all viable *erd2* alleles that have been characterized so far retain some residual binding activity, which can be revealed by overexpression. Growth presumably requires only a low level of binding, which allows the isolation of viable mutants that secrete BiP.

Saturation of the HDEL Retention System Can Inhibit Growth

We have found that mutant receptors that support yeast cell growth also retain some ability to recognize HDEL ligand. Thus we cannot exclude the possibility that the essential function of the HDEL receptor is to retain one or more HDEL proteins. Alternatively, some feature of the normal ligand-induced retrograde transport mechanism might be required to maintain Golgi structure and function. To distinguish these two possibilities we attempted to saturate the HDEL binding capacity of the receptor with an artificial ligand; this should stimulate retrograde transport, but competitively inhibit the retention of endogenous HDEL proteins. We used the previously described pro-α factor–HDEL fusion protein which, when expressed at moderate levels, competes for the receptor and causes secretion of BiP (Dean and Pelham, 1990; Semenza et al., 1990). As controls, equivalent constructs in which the HDEL sequence was absent or replaced with DDEL (a sequence that is much less efficiently recognized by the receptor) were used. Wild-type and mutant *erd2* strains were transformed with multicopy plasmids bearing the fusion genes under the control of the strong TPI promoter, to maximize expression.

When the pro-α factor–HDEL fusion protein (α-H) was expressed in a wild-type strain there was a striking inhibition of growth (as judged by colony size) relative to the same strain expressing a fusion protein that lacked HDEL (α-O or α-D; Table IV). Similar results were obtained with an *erd2* deletion strain carrying low levels of the wild-type receptor (*ERD2* on a centromere vector). Thus, it seems that saturation of the HDEL retention system can inhibit growth.

Table IV. Growth of Yeast Cells Expressing Pro-αFactor Fusion Proteins

Strain	Growth of yeast when expressing*		
	α-H	α-O	α-D
Wild-type	+	++	++
*erd2*Δ; p*ERD2* (*CEN*)	+	++	++
*erd2*Δ; p*ERD2* (2μ)	++	++	++
*erd2*Δ; p*SED1*‡	+++	+++	
*erd2*Δ; p*SED2*‡	+++	+++	
*erd2*Δ; p*SED3*‡	+++	+++	
*erd2*Δ; p*SED4*‡	+++	+++	
*erd2*Δ; p*SED5*‡	+++	+++	
B25§	++	+++	++
B36§	++	+++	+++
H12-A	+	++	++
H12-A, BiP-FGR	+	++	++
D200-N *myc*	+	++	
D200-N *myc*KLSK	+	++	
D200-N *myc*KKSL	++	++	
D200-N KLSK	+	++	
D200-N KKSL	++	++	

* Strains were transformed with plasmids expressing pro-α factor fusion proteins and selected on appropriate medium. After 48 h at 30°C growth was assessed by colony size (for examples see Fig. 3).
‡ Strains that lack Erd2p but are kept alive by overexpression of one of the *SED* genes (Hardwick et al., 1992); these are not isogenic with the wild-type and other mutant strains and grow somewhat faster.
§ Strains carrying the original *erd2* mutations (Semenza et al., 1990); these strains are not isogenic with the wild-type strain and grow somewhat faster.

Control experiments demonstrated the specificity of this effect. When the capacity of the retention system was increased by expression of *ERD2* from a multicopy vector, the resultant strain was not affected by α-H (Table IV). Furthermore, strains that lacked *ERD2* but whose growth defect was suppressed by one of the *SED* genes (*SED1*, *2*, *3*, *4* or *5*; Hardwick et al., 1992; Hardwick and Pelham, 1992) were also resistant to α-H (Fig. 3, Table IV). This is consistent with our previous conclusion that the *SED* genes can bypass the requirement for HDEL-mediated retention.

We also transformed the fusion proteins into strains carrying the original *erd2* alleles. Expression of α-H inhibited growth of both the B25 and B36 *erd2* strains (Fig. 3 and Table IV), which confirms that the mutant receptors in these strains are still capable of ligand recognition in vivo. Interestingly, growth of the B25 strain was also inhibited when a pro-α factor–DDEL construct was overexpressed (Table IV). Although DDEL is recognized much less efficiently than HDEL as a retention signal in *S. cerevisiae*, both are equally effective in *K. lactis*. The B25 mutation (D50-N) lies in a region that is known to be important for determining ligand specificity (Semenza and Pelham, 1992; Lewis and Pelham, 1992*a*; Wilson et al., 1993), and it creates a substitution that is found naturally in the *K. lactis* receptor. Evidently, this change alters the specificity of the *S. cerevisiae* protein so that DDEL can compete effectively with HDEL. This result again shows that the toxic effects of the α-H construct are a consequence of its interaction with Erd2p.

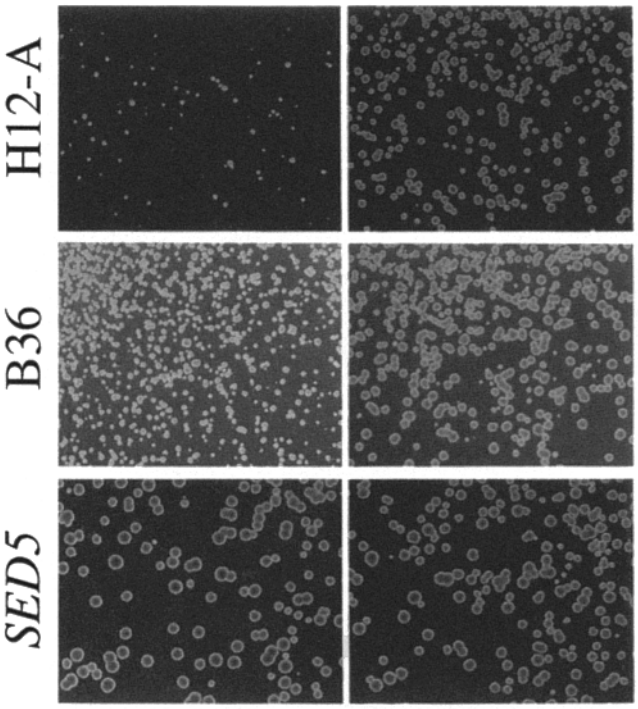

Figure 3. Effects of α factor fusion proteins on growth. Plasmids expressing fusion proteins with or without HDEL were transformed into the indicated strains, and the resultant colonies photographed after 2 days growth. *H12-A*, an *erd2*Δ strain expressing the H12-A mutant; *B36*, an original *erd2* mutant strain, with a stop codon at position 208; *SED5*, an *erd2*Δ strain maintained by overexpression of *SED5*. The three strains differ in their genetic background, and the faster growth of the *SED5* strain is a consequence of this, rather than the lack of Erd2p.

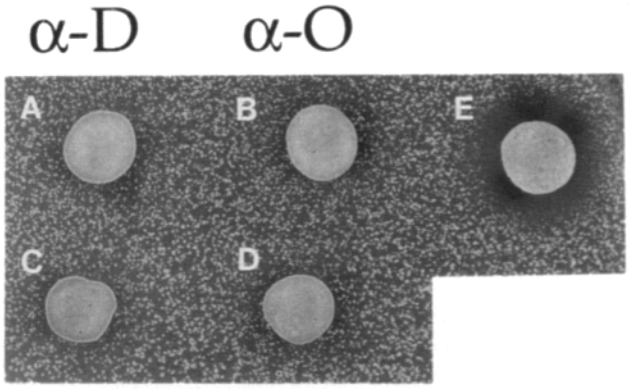

Figure 4. Halo assay for secretion of α factor. *A–D* show the halos produced by patches of cells expressing nonretained pro-α factor fusion proteins; all are *MAT* a and lack the chromosomal *ERD2* gene, *A* and *B* carry a 2μ plasmid with wild-type *ERD2*, *C* and *D* express the H12-A mutant. *E* is a strain that is *MAT* α, lacks the chromosomal *ERD2* gene and carries *ERD2* on a plasmid. Note that this strain secretes more α factor than is produced by the fusion constructs.

The effects of the constructs were also tested in strains expressing the H12-A and D200-N mutant receptors. Such strains were sensitive to α-H, confirming that they could still recognize HDEL (Fig. 3, Table IV). Despite being expressed at a high level, these mutant receptors did not have the protective effect of wild-type Erd2p, presumably because they retained HDEL proteins inefficiently. In the case of the D200-N mutant, addition of a KKXX signal was sufficient to make the cells insensitive to α-H (Table IV). Our interpretation of this result is that the KKXX signal, by providing an alternative recycling mechanism, partially corrects the functional defect of the D200-N mutant, and that the activity of the receptor is then sufficient to alleviate the competition from the α-H protein. This shows that when the retention system is overloaded, growth can be restored by improving HDEL retention, regardless of the precise mechanism used to recycle the receptor.

We considered the possibility that overexpression of the fusion proteins might increase the requirement for ER resident proteins and thus create a need for HDEL-mediated retention that would not otherwise exist. However, α factor is normally produced in large amounts by α strains, and using a halo assay (Sprague, 1991) we found that a cells expressing the α-O construct secreted significantly less α factor than a normal α strain (Fig. 4). Since there is no evidence that the presence of a short COOH-terminal extension impairs the transport or processing of pro-α factor (Dean and Pelham, 1990), it seems unlikely that this level of expression would place a significant extra burden on the resident ER proteins. In agreement with this, we found that α and a strains were equally sensitive to α-H.

In conclusion, these experiments strongly suggest that even in *erd2* strains that show no obvious retention of BiP, some receptor-mediated retrieval of HDEL proteins is occurring, and that this retrieval is important for growth. The data do not support the alternative model in which ligand-stimulated recycling of Erd2p is the process that is required for growth.

Is There a Single Erd2p Ligand Whose Retrieval Is Essential?

Our results argue that retrieval of one or more HDEL proteins is essential for growth, but all the HDEL-containing proteins that have been studied are either completely dispensable or do not require the HDEL sequence to support growth. This suggests two possibilities: either there is an undiscovered protein which does require HDEL-mediated retention, or else loss of multiple HDEL proteins results in growth inhibition.

As one approach to this problem, we re-examined the possibility that some residual retention of BiP might be necessary under conditions where other HDEL proteins become limiting. To do this, we expressed the proalpha factor fusion proteins in an *erd2* strain whose BiP lacked the HDEL signal (H12-A, BiP-FGR; Table IV). There was no significant difference in growth rate between this strain and an isogenic strain expressing normal BiP, even when growth was inhibited by the α-H construct. Thus HDEL-dependent retention of BiP is not essential for growth even when the retention system is saturated.

A second strategy would be to search for HDEL proteins which, when overexpressed, compensate for the loss of Erd2p. If a single protein becomes limiting, then such an approach should identify it. However, although a screen of this

kind identified six *SED* genes, only one of them had an HDEL sequence. This gene (*SED4*) encodes a membrane protein related to Sec12p (a protein required for vesicle budding from the ER), but it is not essential for growth (Hardwick et al., 1992). One trivial explanation for this could be that a second *SED4* homologue exists that can substitute for it, but which was missed in the *SED* screen. We therefore set up a synthetic lethal screen to search for such a protein. This was based on the sectoring method described earlier: a strain carrying a chromosomal deletion of *sed4* and a *SED4*-expressing plasmid was mutagenized, and colonies that were incapable of losing the plasmid (i.e., that did not form white sectors) were selected (see Materials and Methods for details). If *SED4* were one of a pair of genes, each of which could provide some essential function, then mutations in the other should have a nonsectoring phenotype.

A second purpose of the screen was to isolate mutations in genes other than *erd2* that made cells dependent on the overexpression of a *SED* gene. If there were a single HDEL protein whose level in the ER is crucial, then mutations that remove its HDEL sequence, or lower its activity, would be expected to have such a phenotype. The same would be true of mutations in any protein required exclusively for recycling of the HDEL receptor.

78 mutants were isolated. 30 of them were transformed with a plasmid carrying a wild-type *ERD2* gene, and in each case they regained the ability to lose the *SED4* plasmid, suggesting that the mutation lay in the *erd2* gene. The remaining 48 were crossed to an *erd2* deletion strain carrying an *ERD2* plasmid, and the diploids tested for their ability to lose both the *ERD2* and the *SED4* plasmids. In no case was this possible, indicating that these mutations are also likely to lie in the *erd2* gene. We also tested the *erd* phenotype of some of the mutants, and found that they secreted BiP. Thus, all 78 mutants recovered in the screen appear to be alleles of *erd2*. We conclude that the dispensability of *SED4* cannot be explained by a second *SED4*-like gene. Furthermore, we found no candidate for a single HDEL protein that requires Erd2p in order to function, nor one that might be required for Erd2p to operate. It therefore seems likely, though by no means certain, that Erd2p is required to retrieve more than one HDEL protein from the Golgi apparatus.

Discussion

Previous studies, while establishing that the *ERD2* gene encodes the HDEL receptor required for the retrieval of ER proteins from the Golgi apparatus, also suggested that the receptor has a second function in the secretory pathway. This second function was proposed in order to account for an apparent paradox. Viable *erd2* mutants exist that secrete the ER protein BiP at a rate indistinguishable from the rate of secretion of a BiP derivative lacking the HDEL retrieval signal, and thus appear to lack the ability to recognize HDEL. Despite this, depletion of Erd2p from yeast cells inhibits transport through the Golgi apparatus, and prevents growth (Semenza et al., 1990). In this paper we have argued that, despite appearances, all viable *erd2* mutants retain some ability to recognize and retrieve HDEL proteins, and that it is this residual retrieval activity that is required for normal growth.

There are two main lines of evidence that support this conclusion. One is that the analysis of Erd2p mutants shows a good correlation between the ability both to recognize HDEL and to recycle between Golgi and ER, and the ability to support growth. In particular, all mutant receptors capable of supporting growth can, when expressed at a high level, retain BiP to some extent. This includes the original mutants which, when expressed from a single chromosomal gene, did not appear to retain BiP at all. The second line of evidence is that the deliberate saturation of the HDEL retrieval system by expression of an HDEL-tagged version of pro-α factor inhibits growth. This strongly suggests that it is the specific retrieval of endogenous proteins that is required, even in mutant strains that do not appear to retain BiP. Models in which the act of ligand-induced recycling of the receptor provides the important function, for example by stimulating the retrograde transport of bulk membrane, are not supported by these results.

The conclusion from this is that secretion of BiP, or of an HDEL-tagged invertase fusion protein (Semenza et al., 1990), is not a definitive measure of the ability of an *erd2* mutant to function as a retrieval receptor. Other proteins may have a higher affinity for the mutant receptor and be preferentially bound. For example, they may have additional interactions with the receptor which allow them to remain bound even when the HDEL interaction is weak. Membrane proteins, being already constrained in their movement, will also require less binding energy to form a complex. Selective retrieval of such tightly bound proteins could be sufficient to support growth.

What are the proteins for which retention is critical? Their identity remains a mystery, although BiP is clearly not one of them. Indeed, so far there is no single known protein whose HDEL-mediated retention is essential. Some of the proteins known to bear this signal are not required for growth: Eug1p (Tachibana and Stevens, 1992), cyclophilin D (Frigerio and Pelham, 1993), and Sed4p (Hardwick et al., 1992). Others are essential, but the signal itself can be deleted without loss of viability. These include BiP (Hardwick et al., 1990), protein disulphide isomerase (LaMantia et al., 1991), Sec20p (Sweet and Pelham, 1992) and Kre5p (Meaden et al., 1990). Recently, a new HDEL protein (a distant relative of BiP) has been revealed by genome sequencing (EMBL database accession number X75780), but we have found that the HDEL sequence of this protein is also dispensable for growth.

If there were only one protein that had to be retained in the ER, then it might have been identified as a multicopy suppressor of an *erd2* deletion, that is, a *SED* gene. Moreover, a mutation that reduced its activity should mimic an *erd2* deletion, and be suppressible by the *SED* genes. However, our genetic screens failed to reveal any single protein with the appropriate characteristics. The simplest explanation for this is that multiple HDEL proteins have to be retained. These may be proteins that have not yet been discovered, or it may simply be that, although loss of individual proteins can be tolerated, the simultaneous loss of all the HDEL proteins is incompatible with growth. Alternatively, growth inhibition in the absence of Erd2p might be a consequence not of the loss of proteins from the ER, but of the accumulation of inappropriate proteins in the Golgi apparatus. We cannot at present distinguish these possibilities.

A remaining question concerns the mechanism by which

the *SED* genes can overcome the requirement for *ERD2*, at least insofar as they allow growth. This is not easy to understand, because the six known *SED* genes have varied functions and may not all act in the same way (Hardwick et al., 1992; Hardwick and Pelham, 1992, 1994). In principle, they could either reduce the loss of HDEL proteins from the ER, or compensate for the Golgi defect that results, directly or indirectly, from this loss. There is reason to believe that the former is sufficient. For example, overexpression of the cytoplasmic domain of Sec20p, which causes a nonspecific slowing of transport from ER to Golgi (Sweet and Pelham, 1993), can allow growth of an *erd2* null mutant (Hardwick et al., 1992). We have also noticed that very high expression of *SED4* both improves its ability to suppress an *erd2* null and strongly reduces the amount of BiP secreted. The mechanism by which this occurs remains obscure, but it may be another instance in which reduced loss of HDEL proteins permits growth.

In conclusion, we have shown that the genetics of the yeast HDEL receptor can be explained without the need to invoke any function for this protein other than HDEL retention. We also conclude that the HDEL system itself plays a much more important role in the growth of yeast cells than has previously been appreciated.

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