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Sorting of soluble ER proteins in yeast

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In animal cells, luminal endoplasmic reticulum (ER) proteins are prevented from being secreted by a sorting system that recognizes the C-terminal sequence KDEL. We show that yeast has a similar sorting system, but it recognizes HDEL, rather than KDEL: derivatives of the enzyme invertase that bear the HDEL signal fail to be secreted. An invertase fusion protein that is retained in the cells is partially modified by outer-chain mannosyl transferases, which reside in the Golgi element. This supports the view, based on studies in animal cells, that ER targeting is achieved by continuous retrieval of proteins from the Golgi. We have used an invertase fusion gene to screen for mutants that are defective in this sorting system. Over 60 mutants were obtained; eight of these are alleles of a single gene, *erd1*. The mutant strains grow normally at 30°C, but instead of retaining the fusion protein in the cells, they secrete it.

Key words: ER/glycosylation/protein sorting/secretion/yeast

Introduction

Maintenance of the complex architecture of eucaryotic cells requires that newly-synthesized proteins are delivered to their appropriate locations and retained there during their lifetime. A striking example of this is provided by a class of soluble proteins that reside in the lumen of the endoplasmic reticulum (ER). Although these proteins have the same location as newly-synthesized secretory proteins, they are not secreted. The three whose sequences are known [GRP78(BiP), GRP94 and protein disulphide isomerase] share a common C terminal peptide, KDEL, and we have shown that the presence of this sequence is essential for retention of GRP78 in the ER of COS cells (Munro and Pelham, 1987). Moreover, addition of the sequence SEKDEL to lysozyme, a secretory protein, is sufficient to prevent its secretion. Current evidence suggests that proteins bearing this signal are not simply held in the ER, but are selectively retrieved from a post-ER compartment (possibly the *cis* Golgi) and returned to their normal location (Pelham, 1988; Ceriotti and Colman, 1988).

As one way to identify proteins involved in this ER sorting system, we have set out to isolate yeast mutants that are specifically defective in the retention of luminal ER proteins. The feasibility of this approach depends on the existence of similar retention systems in yeast and vertebrate cells. We show here that the yeast *Saccharomyces cerevisiae* has an ER sorting system similar to that of animal cells, but it recognizes the sequence HDEL rather than KDEL. We also report the identification of at least one gene that is required for the correct localization of a fusion protein containing the HDEL signal.

Results

A presumptive yeast ER retention signal does not work in animal cells

By analogy with the vertebrate system, we expected an ER retention signal to be present at the C terminus of a yeast ER protein. A major ER protein in mammals is GRP78 (also known as BiP) which is a member of the hsp70 family (Munro and Pelham, 1986). A candidate for a gene encoding yeast GRP78 has been identified amongst clones which hybridize to a heterologous hsp70 probe; it encodes a protein with clear homology to rat GRP78, and it has the C terminal sequence HDEL (Moran *et al.*, 1983; L.Moran, personal communication). This sequence differs from the KDEL motif found in all the mammalian and chicken luminal ER proteins whose sequences are known. The difference suggests one of three possibilities: that the conservative Lys to His change does not prevent normal recognition by the KDEL system; that yeast has a similar sorting system to animal cells, but with a different sequence specificity; or that ER targeting is achieved in a different way in yeast, allowing divergence of the C terminal sequence.

To see whether HDEL is a functional alternative to KDEL in animal cells, we fused a sequence coding for FEHDEL (the last six amino acids of yeast GRP78) to the lysozyme gene. The construct also contains a short epitope from the human c-*myc* gene that can be detected with a monoclonal antibody against c-*myc* (Munro and Pelham, 1986,1987). The gene was transfected into COS cells, and after 2 days the cells were transferred to fresh medium, incubated for a further 4 h, harvested, and the expressed protein in the cells and medium detected by immunoblotting (Figure 1). Most of the protein was in the medium, showing that it was efficiently secreted. A control construct with SEKDEL at the C terminus was also assayed; as expected, this protein was efficiently retained in the cells (Figure 1). We conclude that HDEL is not an efficient ER retention signal in animal cells.

FEHDEL is a functional signal in yeast

To test the HDEL sequence in yeast, we chose to make fusion proteins based on the enzyme invertase. Invertase fusions have been used previously as the basis for a vacuolar targeting assay, and to isolate mutants that fail to divert the fusion proteins to the vacuole (Bankaitis *et al.*, 1986; Johnson *et al.*, 1987). The enzyme is normally secreted from yeast cells, but remains trapped under the cell wall. Because its substrate (sucrose) cannot pass through the plasma membrane, it is possible to assay secreted invertase using

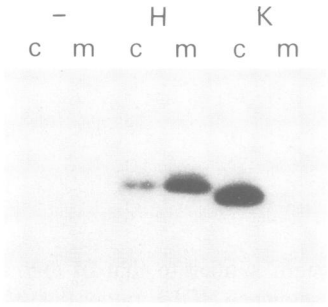

Fig. 1. HDEL does not function as a retention signal in COS cells. Cells were transfected with plasmids expressing lysozyme derivatives with the C-terminal sequence FEHDEL (H) or SEKDEL (K), or were mock-transfected (−). The medium was changed 4 h before harvesting. The expressed proteins in the cells (c) and medium (m) were detected by immunoblotting using a monoclonal antibody that recognizes the lysozyme construct. The proteins migrate slightly differently in SDS-containing gels, despite differing in only two amino acids.

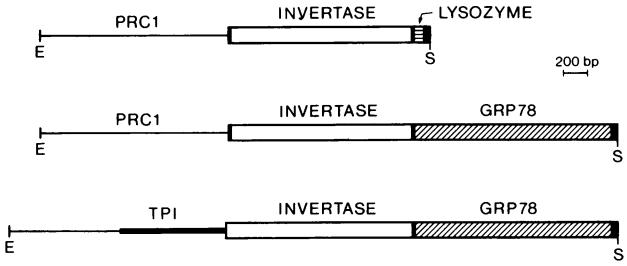

Fig. 2. Outline of the invertase fusion genes. The maps are drawn to scale, with wide bars indicating coding sequences. Each construct is flanked by *Eco*RI (E) and *Sal*I (S) sites, and they were cloned into appropriate vectors for replication or integration. Each fusion gene encodes at its C terminus the sequence EQKLISEEDLN, followed by either SEKDEL, FEHDEL, FEHDELS or SAEARRL as specified in the text or legends. The underlined sequence is the c-*myc* epitope recognized by the monoclonal antibody which is used to detect the proteins.

Table I. Secretion of invertase fusion proteins

Construct[a]	% secreted	
	log phase	stationary phase
Centromere plasmids		
Invertase−lysozyme−SAEARRL	85	23
Invertase−lysozyme−SEKDEL	77	24
Invertase−lysozyme−FEHDEL	60	9
Invertase−GRP78−SEKDEL	64	22
Invertase−GRP78−FEHDEL	59	4
Invertase−GRP78−FEHDELS	65	13
Integrated genes[b]		
Invertase−GRP78−SEKDEL	50	52
Invertase−GRP78−FEHDEL	5	3

[a]All fusion genes were under the control of the *PRC1* promoter.
[b]Single copies of the genes were integrated at the *URA3* locus.

intact cells; total invertase activity can be determined after lysis of the cell (Novick and Schekman, 1979).

Initial experiments in which FEHDEL was joined directly to the C terminus of invertase showed no effect of this sequence on secretion. This could be due to masking of the C terminus by the structure of the protein, making it

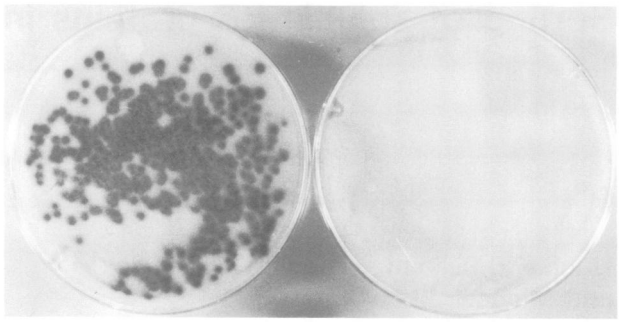

Fig. 3. Plate assay for secreted invertase. Yeast colonies with a copy of the *PRC1*-driven invertase−GRP78 fusion gene integrated at *URA3* were assayed for external invertase activity after transfer to a filter, as described in Materials and methods. The filters were photographed from the underside. The fusion constructs had the C-terminal sequences indicated.

Table II. Effect of promoter strength on secretion of invertase−GRP78 fusion proteins[a]

Promoter	C terminus	% Secreted	Total activity[b]
TPI	SEKDEL	34	500
	FEHDEL	16	530
PRC1	SEKDEL	43	78
	FEHDEL	6	85

[a]Genes were integrated at *URA3*.
[b]Activity is in nmoles of glucose produced per minute per OD_{600}.

inaccessible to the putative HDEL receptor. We therefore fused larger protein fragments to invertase: either 44 amino acids from the C terminus of lysozyme, or most of the rat GRP78 coding region (without its signal peptide). These sequences were followed by the c-*myc* epitope (to aid subsequent immunological detection of the fusion protein), and then by either FEHDEL, SEKDEL or an unrelated sequence (see Figure 2). Both the GRP78 and lysozyme molecules had previously been shown to be retained in the ER in COS cells, when carrying the KDEL sequence, and thus must have an exposed C terminus (Munro and Pelham, 1987). The fusion genes were assayed on centromere-containing plasmids, under the control of the weak *PRC1* promoter.

Clear differences in the extent of invertase secretion were apparent when cells grown to stationary phase were assayed (Table I). A fusion protein bearing the sequence FEHDEL was inefficiently secreted compared to an analogous construct with an unrelated C terminal sequence. In contrast, the mammalian sequence SEKDEL did not reduce secretion. Similar results were obtained with the invertase−GRP78 and invertase−lysozyme fusions (Table I). In addition, a construct with the terminal sequence FEHDELS was retained poorly, although the level of secretion of this protein was reproducibly less than that of the SEKDEL derivative. These results show that a mechanism does exist in yeast which prevents secretion of proteins with an appropriate C terminus. The sequence recognized is HDEL rather than KDEL and, as in animal cells, it is important that it occurs at the extreme C terminus. However, the residual activity of FEHDELS shows that this location is not absolutely essential.

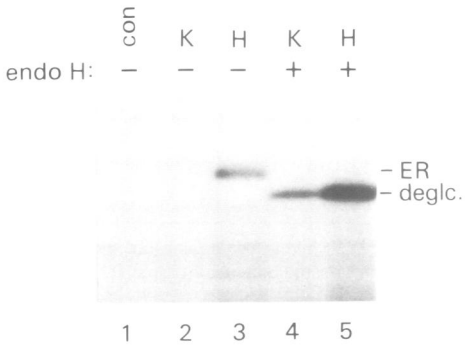

Fig. 4. Immunoblotting of invertase–GRP78 fusion proteins. The fusion genes were integrated at *URA3*, and were under the control of the *PRC1* promoter. 'Con' indicates the parental strain, lacking a fusion gene. The C-terminal sequences were either SEKDEL (K) or FEHDEL (H), and samples were treated with endo H as indicated. The positions of the ER-glycosylated protein and the deglycosylated form are indicated. Cells from overnight cultures were used in this experiment.

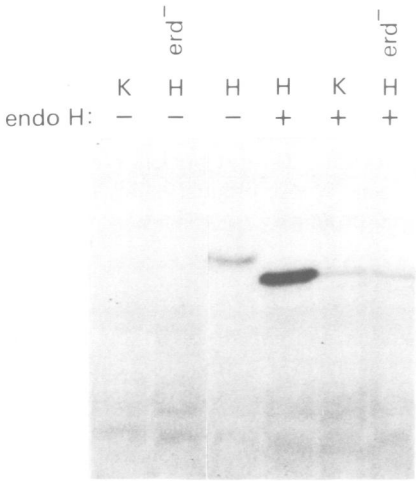

Fig. 5. Analysis of fusion protein in an *erd1*$^-$ strain. Proteins were analysed as in Figure 4, except that log phase cultures were used. *ERD*$^+$ strains expressing fusion proteins ending in SEKDEL (K) or FEHDEL (H) were compared to an *erd1*$^-$ strain expressing an FEHDEL-bearing fusion protein.

A puzzling feature of these experiments was the striking difference between log and stationary phase cells (Table I). In log phase cells, secretion in general appeared more efficient but specific retention of the HDEL-containing constructs was barely detectable. We reasoned that this might be due in part to imprecise copy-number control or some other feature of the centromere vectors. We therefore integrated the invertase–GRP78 fusion constructs, which seemed to give the clearest results, into the genome. The resultant strains showed specific retention of the HDEL constructs in both log and stationary phase cells (Table I), and the effect was particularly clear when individual colonies were assayed on plates (Figure 3). We conclude that the retention system is effective in both growing and resting cells. It is not clear why plasmid-borne and integrated genes give such different results in log phase cells, but we used integrated constructs for all subsequent experiments.

The retention system is saturable

To see whether the HDEL sorting system was saturable, we examined the effect of replacing the *PRC1* promoter with the much stronger triose phosphate isomerase (TPI) promoter, again integrating the constructs into the genome. Table II shows that the TPI constructs expressed 6- to 7-fold more invertase activity than the *PRC1* constructs, and that this over-expression resulted in secretion of a higher proportion of the HDEL fusion protein.

We attempted to demonstrate specific competition for a putative HDEL receptor by co-expressing low levels of an invertase–GRP78–FEHDEL construct with high levels of a preproalpha-factor–GRP78 fusion protein bearing either HDEL or KDEL sequences. The presence of an HDEL-bearing competitor specifically increased the amount of invertase secretion as judged by a plate assay, but the effect was small (data not shown). One problem may have been insolubility of the alpha factor–GRP78 fusion protein in the ER, which might prevent it from reaching the HDEL receptor, but we have not investigated this in detail. Taken together, our results suggest that the ER retention system in yeast is saturable, but that efficient ER localization is possible when fusion proteins are expressed at a low level from a chromosomal gene.

Glycosylation state of the retained fusion proteins

The site at which secretion of the retained fusion proteins is blocked can be deduced from their glycosylation state. Invertase itself is a well-studied substrate for N-linked glycosylation: 10–12 core oligosaccharides containing nine mannose residues are added in the ER, and these are extensively modified in the Golgi complex, resulting in the addition of up to 150 mannose residues per oligosaccharide (Schekman, 1985; Kukuruzinska *et al.*, 1987). The non-glycosylated and ER-modified forms of the protein can easily be separated on SDS-containing gels; the mature form is considerably larger, but is harder to detect because of its heterogeneity (see, e.g. Johnson *et al.*, 1987). If secretion of a fusion protein carrying HDEL is blocked prior to the Golgi modification step, it should accumulate in the simply glycosylated ER form. On the other hand, if it can reach the Golgi it should be more extensively modified. These modifications will occur only on the invertase part of the fusion protein, there being no sites for N-linked glycosylation in the GRP78 sequence.

The glycosylation state of the constructs with C-terminal FEHDEL or SEKDEL (a secreted control) was determined by immunoblotting of total cell extracts, which contain both the intracellular and the secreted, periplasmic forms of the proteins. For detection, we used the monoclonal antibody which recognizes the c-*myc* epitope near the C terminus of all the constructs (see Figure 2 legend).

The secreted fusion protein was not readily detectable on the blots (Figure 4, lane 2). This is probably a reflection of the low abundance, large size and heterogeneity of the fully-glycosylated protein—it either fails to enter the gel or is present at too low a concentration per unit area to be detectable. In agreement with this, we found that treatment of the cell extracts with endoglycosidase (endo) H or *N*-glycanase, which remove the oligosaccharide side chains, allowed the detection of a discrete protein band of the expected size for the fusion protein (125 kd; Figure 4, lane 4). In contrast, at least some of the retained fusion protein was detectable without endo H treatment, migrating as expected for a polypeptide with the ER form of glycosylation (Figure 4, lane 3). This material has presumably not

reached the region of the Golgi element that contains the outer-chain mannosyl transferases.

After endo H treatment of the retained protein, a band of 4- to 8-fold greater intensity appeared in the position corresponding to deglycosylated protein (Figure 4, lane 5; see also Figure 5). The higher intensity of this band implies that it cannot simply be derived from the ER-glycosylated material. However, the results can be explained if most (>75%) of the retained protein is extensively modified: this material would be undetectable in undigested samples, but would be visible after endo H treatment. Indeed, faint traces of immunoreactive species larger than the ER-modified form can be discerned on heavily-loaded blots of both retained and secreted proteins (data not shown). A more trivial explanation could be that the ER-glycosylated form is for some reason poorly detected in the assay and its abundance therefore under-estimated. This is unlikely, because some mutant strains (see below) show only a slight increase in apparent abundance of the fusion protein after endo H treatment; if the increase were due to improved detection efficiency, it should not be strain-dependent. We conclude that the retained protein can reach the Golgi and be modified by enzymes that reside there. By analogy with the animal cell system, it seems likely that the mature protein returns to the ER, and is maintained there by a continual process of retrieval from the Golgi. Efficient retrieval would limit the action of Golgi enzymes, and thus account for the relative abundance of the ER-glycosylated form of the protein.

A further feature of the retained protein is that, even after deglycosylation, it appears much more abundant than the secreted version (Figures 4 and 5), even though the invertase activity in the two strains is comparable (Table II). This can most easily be explained by proteolytic trimming of the secreted protein in the periplasmic space, which contains proteases. Proteolysis would remove the epitope recognized by the monoclonal antibody, but invertase itself is known to be resistant to degradation (Johnson *et al.*, 1987). [A similar result would be expected if some of the 'secreted' protein ends up in the vacuole, a possibility that could explain the rather low level of secretion estimated from invertase assays.]

Isolation of mutants

Having established conditions for efficient retention of invertase fusion proteins, we mutagenized appropriate strains and screened for mutants that fail to retain the protein, using the colony assay for external invertase activity (see Figure 3). Mutants were tested for a strong phenotype, stability and recessiveness when crossed with invertase-deficient strains. The latter test eliminated those that have a lesion in the fusion gene, and thereby lack the retention signal.

Complementation analysis was performed by crossing mutants of opposite mating type. Analysis of about 30 recessive mutants of each type has revealed one well-defined complementation group with eight alleles. We have called the mutant gene defined by this group *erd1* (for ER Retention Defective). A preliminary characterization of a typical *erd1* allele is presented in Table III and Figure 5. This strain behaves as though the invertase fusion protein lacked a retention signal, both in its efficiency of secretion (Table III) and in the state of the protein within the cells (Figure 5). The cells grow normally at 30°C, and show no signs of excessive lysis or proteolysis of the fusion protein within the cell.

Table III. Effect of the *erd1* mutation on secretion of invertase–GRP78 fusion proteins[a]

Genotype	C terminus	% Secreted	Total activity
ERD1$^+$	SEKDEL	33	44
	FEHDEL	5	50
erd1$^-$	FEHDEL	34	56

[a]Strains contained *PRC1*-driven fusion genes integrated at the *PRC1* locus. Activity is in the same units as in Table II.

From the remaining mutants, three possible complementation groups each with two members could be identified, but most mutants were either complemented in all crosses or showed a complex pattern of interactions rather than discrete complementation groups. These strains may contain multiple lesions that contribute to their phenotype. In general, they show a weaker phenotype than the *erd1* alleles. Some express the fusion protein at an elevated level, and in at least two cases this is the only detectable difference from the parental strain. Others do secrete a high proportion of the fusion protein, but immunoblotting has shown that they also contain high levels of the ER-modified form of the protein. We suspect that these mutants have a reduced rate of ER–Golgi transport, and that their *erd* phenotype is a consequence of this. Thus at present *erd1* is the best candidate for a gene whose product is devoted entirely to the sorting of ER proteins.

Discussion

An ER retention system in yeast

We have shown that the C-terminal six amino acids of a yeast ER protein, when added to an invertase derivative, are sufficient to prevent secretion. The yeast retention system appears to be similar to the ER protein sorting system of vertebrates: the signal is similar in sequence and located at the same position. However, the conserved animal sequence KDEL is replaced by HDEL in yeast, and neither sequence is functional in the other species. Interestingly, the nematode *Caenorhabditis elegans* has two GRP78-like genes, one with the C terminal sequence KDEL and one with HDEL, and presumably both are functional signals in this species (M.Heschl, personal communciaton). As with the vertebrate system (Munro and Pelham, 1987), extension of the C terminus of the yeast signal, even by one amino acid, reduces its efficacy. However, there is some residual activity of the extended sequence, showing that a C-terminal location is not absolutely essential.

A further difference between the yeast and mammalian systems is that while the latter is very hard to saturate, the yeast system seems quite leaky and easily saturated. It is not clear whether this is an inherent property of the cells, or whether the particular sequence we have used provides a sub-optimal signal. Nevertheless, this saturability, together with the sequence specificity of the sorting system, strongly suggests that a specific HDEL receptor exists in yeast.

Our current view of the retention system in animal cells is that KDEL-bearing proteins are not immobilized in the ER, but remain soluble and can begin to pass down the secretory pathway. At some subsequent point, in or near the *cis* Golgi, a receptor recognizes the KDEL sequence and ER proteins are selectively retrieved, being returned to the ER by vesicular transport. In support of this model, we find

that a lysosomal enzyme bearing the KDEL signal accumulates in the ER, but can still be modified by GlcNAc-phosphotransferase, which is thought to be a *cis* Golgi enzyme (Pelham, 1988). In yeast, the Golgi element is less well defined, but the extensive addition of mannose residues to the oligosaccharide side chains of invertase is thought to be an 'early' Golgi function, because it is blocked by mutations that prevent ER-to-Golgi transport, but not by *sec7*, which causes accumulation of Golgi stacks (Schekman, 1985). HDEL-bearing invertase fusion proteins appear to be substrates for this modification. We cannot formally rule out the possibility that they accumulate in a Golgi or post-Golgi compartment, but by analogy with the animal system, we suggest that they are retrieved from an early Golgi compartment and returned to the ER. As with cathepsin D in animal cells (Pelham 1988), the modification of the retained protein by Golgi enzymes seems to occur more slowly than with the corresponding secreted version, as judged by the higher steady-state level of the ER-modified form. This could be explained if retrieval from the Golgi is rapid, and frequently occurs before the protein has had time to reach, or be a substrate for, the Golgi enzymes.

Mutants defective in the retention of luminal ER proteins

We have isolated a number of mutants which fail to retain proteins carrying the HDEL signal. Some of these behave as though they completely lack the retention system, at least under the conditions we have tested. It seems likely that the mutated genes encode essential components of the ER sorting system, such as the putative HDEL receptor. The best candidate for such a gene is *ERD1*, and we are in the process of isolating and characterizing this gene.

The mutant strains are viable, grow normally at 30°C and appear to have an unimpaired secretion pathway. It seems surprising that such cells, which presumably secrete their own ER proteins, do not suffer ill effects. However, studies with GRP78 in animal cells have shown that even when the KDEL sequence is removed, secretion is very slow (Munro and Pelham, 1987). Thus although the steady-state levels of luminal ER proteins may be reduced in the mutants, they are unlikely to be zero. Furthermore, synthesis of at least the major proteins GRP78 and GRP94 is induced when under-glycosylated or otherwise abnormal proteins accumulate in the ER of animal cells (for references see Munro and Pelham, 1987; Lee, 1987). It may be that deleterious effects of the *erd* mutations in yeast are alleviated by increased expression of luminal ER proteins.

Certain secretory proteins that cannot fold correctly are known to bind to GRP78 (BiP) in the ER, and they may be kept in the cell by their association with this protein (Gething *et al.*, 1986; Pelham, 1986; Hendershot *et al.*, 1987). An example is provided by the immunoglobulin heavy chains, which are known to bind GRP78 (BiP) and are very inefficiently secreted in the absence of light chains. In the yeast mutants such a restraint should not apply, because GRP78 itself can be secreted; thus it may be possible to use *erd*$^-$ strains to secrete otherwise intractable proteins as complexes with GRP78. Indeed, in preliminary experiments we have found that a fusion protein containing a segment of immunoglobulin heavy chain is more efficiently secreted from *erd*$^-$ strains than from normal strains.

Materials and methods

Yeast strains

The results in the tables were obtained with *S.cerevisiae* strain SEY2102 (*MAT*α *suc2*-Δ9 *ura3*-52 *leu2*-3,-112 *his4*-519). For mutant isolation we also used SEY2101 (*MAT*a *suc2*-Δ9 *ura3*-52 *leu2*-3,-112 *ade2*-1). Both strains were provided by G.Ammerer, from S.Emr (Johnson *et al.*, 1987). Some early experiments used strain MCY881 (*MAT*a, *ade2*-101, *ura3*-52, *suc2*-Δ9), kindly provided by Marian Carlson. Results were essentially identical in all three strains.

Fusion genes, plasmids and yeast transformation

The structure of the fusion genes is indicated in outline in Figure 1. The *PRC1*−invertase fusion, with a polylinker at the 3′ end of the coding sequences was derived from plasmid SEYC352, a gift from Scott Emr. The signal-peptide-deleted version of the GRP78 gene was derived from the plasmid described by Munro and Pelham (1986). The sequences attached to the 3′ end of the GRP78 sequences were derived from plasmid SAGM2 (Munro and Pelham, 1987). The C-terminal portion of chick lysozyme, tagged with the c-*myc* sequence and SEKDEL was derived from plasmid SAYMK2 (Munro and Pelham, 1987). The SEKDEL coding sequences were replaced with synthetic oligonucleotides encoding FEHDEL or other sequences as indicated. A 900 bp fragment containing the TPI promoter, joined to the normal invertase signal peptide coding region was derived from plasmid GA1643 (a gift from Gustav Ammerer). These plasmids all encode exclusively the signal peptide containing version of invertase, not the cytoplasmic form. The amino acid sequences at the various junctions (see Figure 2) are as follows: amino acid (aa)-1 of carboxypeptidase Y (the *PRC1* product) is joined to aa3 of invertase via a 2-aa linker, . . . LAKA/MF/TNET . . .; aa512 of invertase is joined to aa84 of lysozyme via a 4-aa linker, . . . VREV/NPGD/LSSD . . . , or to aa14 of GRP78 via a 17-aa linker, . . . VREV/NPGDPSTCSQACTMPAI/GIDL . . . ; aa127 of lysozyme is joined to c-*myc* and synthetic sequences via a 3-aa linker, . . . IRGC/PCM/EQKLISEEDLNFEHDEL; aa575 of GRP78 is joined directly to c-*myc* sequences, . . . KETM/EQKLISEEDLNFEHDEL.

For expression from plasmids, the *Eco*RI−*Sal*I region covering the fusion genes was inserted into the corresponding sites in plasmid SEYC306 (Johnson *et al.*, 1987; provided by Scott Emr). This contains the *URA3* gene, ARS1 and CEN4. For integration at the *PRC1* locus, *URA3* and CEN4 sequences were deleted by excision of a *Xho*I−*Nru*I fragment from the vector. The plasmids were then linearized at the *Sac*II site within the *PRC1* promoter and co-transfected into strains SEY2101 and SEY2102 together with a circular *URA3*/CEN4/ARS1 plasmid. Colonies that could grow without uracil were screened for invertase activity. Positives were passaged on non-selective plates, plated out, and colonies lacking the *URA3* plasmid identified by replica plating. Integration of a single copy of the fusion gene into the *PRC1* locus was confirmed by Southern blotting. This procedure resulted in strains with simple insertions (i.e. with the entire plasmid in the genome, and the *PRC1* promoter duplicated), which retained the *ura3* marker of the parent.

For integration at the *URA3* locus, the *Eco*RI−*Sal*I region of the fusion genes were inserted into a polylinker in the plasmid YIp56X. This is a derivative of the *URA3*/pBR322 plasmid YIp56 (a gift from Kevin Struhl), in which an *Xho*I linker has been inserted at the *Stu*I site in the *URA3* gene, inactivating this gene. The plasmids were linearized with *Xho*I, transfected into SEY2102, and transformants selected on plates lacking uracil. Integration of one or two copies was confirmed by Southern blotting. We used this approach because the fusion genes have weak ARS activity which allows the persistence of religated, non-integrated plasmid. With the *Xho*I linker in the *URA3* gene, URA3 activity can only be generated by recombination between the plasmid and the non-functional chromosomal copy of the gene.

Invertase assays

Liquid assays were performed as described by Johnson *et al.* (1987), using the method of Goldstein and Lampen (1975). Yeast was grown in rich medium containing 2% fructose. Typically, 100 μl of an overnight culture was centrifuged, the cells washed with 0.1% sodium azide, resuspended in 75 μl of 0.1M Na acetate, pH 5.4, and split into two portions, one for external invertase and one for total activity. Both were made up to 75 μl in the acetate buffer, but Triton X-100 (1% final) was added to the total activity sample, and it was frozen on dry ice and then thawed. A 25 μl aliquot of 0.5M sucrose was added to each sample, and they were incubated for 10 min at room temperature. An equal volume of potassium phosphate buffer, pH 7.0, was added, the mixture heated to 100°C for 2 min and centrifuged. Aliquots (3−30 μl) were added to 500 μl of 100 mM K phosphate, pH 7, 10 μg/ml horseradish peroxidase, 8.4 U/ml glucose

oxidase, 0.6 mg/ml *o*-dianisidine and incubated for a further 10 min. A 0.9 ml aliquot 6M HCl was added, and the absorbance of 540 nm measured. Activities given in the tables are in nanomoles of glucose released per minute per OD_{600} of the culture. It is not clear whether the assay of live yeast gives meaningful absolute values for external invertase, particularly when the activity is low. Possible sources of error include lysis of some of the cells, and uptake and metabolism of the glucose produced. The values for percentage secretion obtained should thus be regarded as approximate.

To assay individual colonies or patches, filter paper or nitrocellulose sheets were pressed over the colonies (which were grown on plates containing fructose rather than glucose), removed with the yeast stuck to it, and placed (yeast upwards) on a filter paper soaked in a freshly-prepared solution of 0.1M Na acetate, pH 5.4, 0.125 M sucrose, 10 μg/ml peroxidase, 8.4 U/ml glucose oxidase, 0.6 mg/ml *o*-dianisidine. Red color developed over a period of 10–30 min. Activity was visually estimated by inspection of the underside of the lower filter, and viable yeast could be recovered from the upper filter. Total activity was checked by first lysing the cells with chloroform vapour.

Mutagenesis and mutant isolation

Derivatives of SEY2101 and SEY2102 containing fusion genes integrated at the *PRC1* locus were mutagenized by exposure of freshly-plated cells to a germicidal UV lamp (90% killing). Mutant colonies (~1 per 500–1000) were identified by the filter assay, replated and single positive colonies picked. For testing of dominance and complementation mapping, appropriate strains were patched together on plates lacking adenine and histidine. Diploids and the corresponding haploid strains were then patched out on non-selective plates and assayed by the filter method.

Detection of fusion proteins by immunoblotting

Typically 5 ml of an overnight culture was centrifuged, and the cells taken up in 0.5 ml of SDS sample buffer containing 1 mM PMSF, 0.5 mM TPCK, 0.025 mM TLCK, 2 μg/ml pepstatin A. The solution was immediately frozen as droplets in liquid nitrogen. The frozen material was ground in a pestle and mortar, transferred to a tube and boiled for 2 min. For endo H treatment, a 20 μl sample was then diluted 4-fold with 0.1 M Na acetate, pH 5.4 and incubated with 2 mU endo H (Boehringer) overnight at 37°C. The protein was precipitated with 4 volumes of acetone at −20°C and taken up in SDS sample buffer. Control treatments without endo H showed that the protein was completely stable under these conditions. Gel electrophoresis and immunoblotting with monoclonal antibody 9E10 was performed as described previously (Munro and Pelham, 1987).

COS cell expression

The plasmid HYK is a slight modification of plasmid SAYMK2 described previously (Munro and Pelham, 1987). It contains the adenovirus major late promoter, the chick lysozyme coding sequence followed by the c-*myc* epitope and SEKDEL, the herpes virus *tk* gene polyadenylation site and the SV40 origin of replication. Plasmid HYH is similar, but encodes FEHDEL instead of SEKDEL; it was generated by cloning an appropriate double-stranded synthetic oligonucleotide between the *Eco*RI and *Xba*I sites that flank the SEKDEL coding sequence in HYK. The C terminal sequence of the plasmid used for transfection was confirmed by DNA sequencing. Transfection into COS cells and immunoblot analysis of the expressed proteins was performed as described previously (Munro and Pelham, 1987).

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Note added in proof

We have recently analysed the glycosylation state of the retained HDEL-bearing invertase fusion protein using antibodies against α1→6-Man. These react only with the outer-chain mannose residues added in the Golgi [Esmon,B., Novick,P. and Schekman,R. (1981) *Cell*, **25**, 451–460; D.Baker, L.Hicke, M.Rexach, M.Schleyer and R.Schekman, submitted]. Two-thirds of the invertase activity was antibody-precipitable, confirming our conclusion that the retained protein is able to reach the Golgi apparatus.