Recycling of Proteins from the Golgi Compartment to the ER In Yeast

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Abstract. In the yeast Saccharomyces cerevisiae, the carboxyl terminal sequence His-Asp-Glu-Leu (HDEL) has been shown to function as an ER retention sequence (Pelham, H. R. B., K. G. Hardwick, and M. J. Lewis. 1988. EMBO (Eur. Mol. Biol. Organ.) J. 7:1757-1762). To examine the mechanism of retention of soluble ER proteins in yeast, we have analyzed the expression of a preproalpha factor fusion protein, tagged at the carboxyl terminus with the HDEL sequence. We demonstrate that this fusion protein, expressed in vivo, accumulates intracellularly as a precursor containing both ER and Golgi-specific oligosaccharide modifications. The Golgi-specific carbohydrate modification, which occurs in a SEC18-dependent manner, consists of α1-6 mannose linkages, with no detectable α1-3 mannose additions, indicating that the transit of the HDEL-tagged fusion protein is confined to an early Golgi compartment. Results obtained from the fractionation of subcellular organelles from yeast expressing HDEL-tagged fusion proteins suggest that the Golgi-modified species are present in the ER. Overexpression of HDEL-tagged preproalpha factor results in the secretion of an endogenous HDEL-containing protein, demonstrating that the HDEL recognition system can be saturated. These results support the model in which the retention of these proteins in the ER is dependent on their receptor-mediated recycling from the Golgi complex back to the ER.

The complex organization of eukaryotic cells requires mechanisms that direct proteins from their site of synthesis to their site of function. Most secreted and membrane bound proteins are initially directed to the secretory pathway by their cotranslational translocation into the ER. While most of these proteins are subsequently routed to the Golgi body and eventually compartmentalized or secreted, certain proteins remain and function in the ER. Many of the soluble ER proteins in mammalian cells bear a carboxyl terminal tetrapeptide sequence, Lys-Asp-Glu-Leu (KDEL), which has been demonstrated to be both necessary and sufficient for the retention of at least one of these proteins (BiP) in the ER (Munro and Pelham, 1987). The growing list of luminal ER proteins that bear this sequence (for review, see Pelham, 1989) implicates it as the distinctive feature that signals retention in the ER.

While it is clear that the KDEL sequence functions as an ER retention signal, the mode by which the cellular secretory apparatus recognizes it is less clearly understood. The current model favored for the accumulation of luminal ER proteins envisages a recycling mechanism, in which these proteins are retrieved from a post-ER compartment. The best evidence for this model comes from experiments in animal cells, where it was shown that cathepsin D-KDEL fusion proteins are accessible to post-ER enzymes, and yet accumulate in the ER (Pelham, 1988).

Analyses of many aspects of the secretory pathway in the yeast, Saccharomyces cerevisiae, indicate a high degree of conservation with higher eukaryotes. Rather than the sequence KDEL, the carboxyl terminal HDEL sequence functions as an ER retention sequence in yeast (Pelham et al., 1988). Invertase–HDEL fusion proteins accumulate intracellularly and are partially modified by Golgi-specific mannosyltransferases. By analogy with results from ER targeting studies in animal cells, these results support a model in which the accumulation of luminal ER proteins is mediated, at least in part, by retrieval from the Golgi apparatus.

To identify more precisely the site at which sorting occurs in yeast, we have undertaken a detailed analysis of the retention process using HDEL-tagged preproalpha factor fusion proteins. The biosynthetic pathway of alpha factor has been studied in great detail and is well understood (Julius et al., 1984b; for review, see Fuller et al., 1988). Alpha factor is synthesized as a precursor that contains four tandem copies of the 13 amino acid mature pheromone, separated by spacer peptides. The primary translation product is a 165 amino acid protein with a molecular mass of ~18.6 kD. Cleavage of the signal sequence (Waters et al., 1988) and the acquisition of three N-linked core oligosaccharides occur after translocation into the ER, resulting in a species with molecular mass of ~26.5 kD. After transport into the Golgi apparatus, the core oligosaccharides are modified by Golgi apparatus-specific mannosyltransferases, resulting in a highly glycosylated heterogenous form. Both endoproteolytic and exopro-
teolytic processing are required for the production of mature alpha factor. The first cleavage, carried out by the KEX2 gene product, cuts the precursor after dibasic amino acid pairs to generate the four copies of alpha factor and is thought to be a late Golgi event (Julius et al., 1984a). Subsequent exopeptidase by dipeptidyl amino peptidase A, product of the STE13 gene (Julius et al., 1983) and by carboxypeptidase, product of the KEX1 gene (Dmochowska et al., 1987), trim the amino and carboxy extensions, resulting in mature alpha factor.

As it passes through the secretory pathway, preproalpha factor is processed into discrete electrotopically distinctable forms, which can be precisely correlated with its intracellular location. Here, we demonstrate that addition of a carboxy terminal HDEL tetrapeptide results in the intracellular accumulation of preproalpha factor in two forms, indicative of processing by both ER and Golgi-specific enzymes. Both of these forms cofractionate with the ER, as does the Golgi-modified form of an HDEL-tagged inverase fusion protein. Overexpression of an HDEL-containing fusion protein results in the secretion of an endogenous luminal ER protein BiP. These results are consistent with a receptor-mediated retrieval mechanism for retention of these proteins in the ER.

Materials and Methods

Yeast Strains

Saccharomyces cerevisiae strain J8311 (leu2; trp1; ura3-52; pbr1-J12; pep8-3; prc1-407) (kindly provided by P. Sorger, Medical Research Council Laboratory of Molecular Biology, Cambridge, UK) was used for the preparation of yeast translation lysates. SEY 6210 (MATa; ura3-52; his3-200; trpl-901; lys2-201; suc2-a9) (this work) was used as the parental strain for examining BiP secretion. ND1811 (MATa; sec18-1; ura3-52; suc2-a9) (this work) was used as the parental strain for all immunoprecipitations and fractionations except for the experiment shown in Table II. This experiment used strains 1002 and 1012, which are derivatives of SEY2102 (MATa; suc2-a9; ura3-52; leu2-3;112; his4-S19) carrying PRC1-invertase-rat BiP fusion genes integrated at the PRC1 locus (Hardwick et al., 1990).

Plasmids, Cloning, and Mutagenesis

To facilitate subsequent manipulation and mutagenesis, the 614bp Bam HI fragment from pDJ100 (kindly provided by G. Waters, Rockefeller University, New York), containing the entire protein coding region and flanking sequences of the preproalpha factor gene, was cloned into Bluescript (Pro-mega Biotec, Madison, WI). A unique Nco I site was introduced at the extreme 3' end of the coding region (position 497, relative to +1), using PCR-mediated, oligonucleotide-directed mutagenesis (Higuchi, 1989). The plasmid pSH was constructed by insertion of an Nco I/Xho I fragment containing the human c-myc epitope followed by the HDEL sequence and translation stop codons. This resulted in an in-frame fusion gene encoding the protein sequence SMEQLISEEDLNFHDEL directly abutting the pre- cise carboxy terminus of preproalpha factor (following tyr 165). The plasmid pPOH was constructed in an analogous manner, and contains a fusion gene encoding the sequence SMEQKLISEEDLN, followed by a transla tional stop codon. SPsPH and SPsPO were constructed by ligating Bam HI/Sal I fragments of pPOH and pPOH, which contain the entire coding regions, into the vector SP65. SPsPH and SPsPO were used as templates for all in vitro assays.

For the construction of plasmids appropriate for expression in yeast, Eco RI/Sal I fragments of pSPsPH or pSPsPO were inserted into a derivative of the integration vector, YIP56X (Pelham et al., 1988), such that expression of these genes is under the control of the triose phosphate isomerase (TPI) promoter. This vector contains an Xho I linker at the Stu I site of the URA3 gene. URA3 activity requires a recombination event between the inactive chromosomal gene and the plasmids. Yeast cells (ura3-52) were transformed with plasmids linearized with Xho I and transformants selected for uracil auxotrophy.

For analysis of invertase fusion proteins, a YIP56X derivative encoding an invertase-GFP78 fusion protein terminating with either FEHDEL or SEKDEL, under the control of the TPI promoter (Pelham et al., 1988) was integrated at the ura3 locus as above.

In Vivo Radiolabeling and Immunoprecipitation

Strains were grown at 24°C (for labeling of ts strains at permissive temperature) or 30°C in sulfate-free Wickerham's media (Wickerham, 1946) supplemented with 100 μM ammonium sulfate and 2% glucose to an OD600 of 1-3. Cells (2 OD U) were washed once and resuspended in 150 μl of sulfate-free Wickerham's media plus 0.5 mg/ml BSA. Radiolabeling was initiated by the addition of 100-150 μCi [35S]sulfate (Amersham International, Amersham, Buckinghamshire, UK). For labeling of temperature sensitive cells at the nonpermissive temperature, cells were incubated at 37°C for 5 min before the addition of isolate. After a 10-min pulse, ammonium sulfate was added to 25 mM to prevent further labeling. Reactions were terminated by the addition of ice cold 10 mM NaCl. Cells were sedimented, washed once with 10 mM NaCl, and converted to spheroplasts using a 30-45 min incubation at 0°C in 1X spheroplasting buffer (1.4 M sorbitol; 50 mM Tris pH 7.5; 5 mM MgCl2; 20 mM β-mercaptoethanol) plus 25 μl tylicase (Sigma Chemical Co., St. Louis, MO) per OD U. Spheroplasts formed during this time were centrifuged and resuspended in 200 μl of ice cold 1% Triton X-100 in PBS containing 0.5 mM PMSF, 0.1 mM TLCK (n-tosyl-L-lysine chloromethy ketone), 0.2 mM TPCK (n-tosyl-L-phenylalanine chloromethyl ketone). 200 μl PBS was added and unlysed cells, and debris were removed by centrifugation for 10 min at 4°C in an appandor centrifuge.

Samples were immunoprecipitated by addition of 1 μl of 9E10 monoclonal antibody followed by addition of 25 μl of 30% suspension of rabbit anti-mouse IgG bound to protein A Sepharose (prepared by incubating a 50% suspension of protein A Sepharose in PBS and 100 mM Tris pH 7.4 with 0.2 mg/ml rabbit anti-mouse IgG) (Sigma Chemical Co.) and 1% BSA for 1 h at room temperature). Samples were rotated at room temperature for 2 h. Immunoprecipitates were washed twice with 1 ml RIPA buffer (0.5 M NaCl; 50 mM Tris pH 7.4; 0.5% NP-40; 0.5% deoxycholate; 0.1% SDS), followed by two washes with PBS. Samples were then resuspended in SDS sample buffer, heated for 5 min at 95°C and analyzed by electrophoresis on 12% SDS-polyacrylamide gels (Laemmli, 1970), followed by fluorography.

For analyses of α-6 mannose or α-3 mannose reactive species, cell lysates were first immunoprecipitated with the 9E10 antibodies as described above. After washing extensively, 9E10 reactive proteins were eluted from the resin by adding 50 μl of 1% SDS and heating for 5 min at 95°C. Eluted proteins were then immunoprecipitated with anti-α-6 mannose antibody (prepared as described by Nakajima and Ballou, 1975) or anti-α-3 mannose antibody (kindly provided by A. Franzusoff, University of Colorado, Denver, CO), washed, and analyzed as described (Baker et al., 1988).

Labeling and analysis of invertase fusion proteins was essentially as described above. Cells were labeled for 10 min and chased for 30 min. Fusion proteins were precipitated with antiinvertase (obtained from R. Schekman, University of California, Berkeley, CA), redissolved, and reprecipitated with the appropriate antiamnose antibodies. To eliminate residual background, the proteins were analyzed by gel electrophoresis, and quantitated by densitometry of fluorograms.

Western Immunoblotting Analysis of Secreted BiP

15 OD600 U of an overnight culture, grown in YEP (1% bacto-yeast extract, 2% bacto-peptone) with 2% glucose were spun down and the supernatants precipitated with 4 vol of ice cold acetone for 30 min at -20°C. Proteins were sedimented by centrifugation at 9.5 Krpm in a tabletop centrifuge (International Equipment Co., Dunstable, Bedfordshire, England). Pellets were resuspended by vortexing in 100 μl of Laemmli's sample buffer, heated for 5 min at 95°C and 5 μl electrophoresed on an 8% SDS polyacrylamide gel. Proteins were transferred to nitrocellulose, and detection of BiP was performed by incubation in anti-BiP serum (1:5,000, kindly provided by Mark Rose) and secondarily in I25I-protein A (Amersham International) followed by autoradiography at ~70°C.

Fractionation of Subcellular Organelles

Yeast cells were metabolically labeled as described above, except the reac-

1. Abbreviations used in this paper: CPY Y, carboxypeptidase Y; TPI, triose phosphate isomerase.

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tion was scaled up 10-fold. For a typical experiment 20 OD U were radiola-
beled. After a 10-min pulse and 10-min chase, cells were washed with 10
mM Na3H, and mixed with 80 OD U of unlabeled cells, which served as
carrier during the subsequent fractionation. Cells were converted to spher-
oplasts as described above, except cells were resuspended in a total of 2 ml
spheroplasting buffer. When >90% of the cells had formed spheroplasts,
they were spun down, resuspended in 1.0 ml lysis buffer (0.8 M Sorbitol;
10 mM triethanolamine [pH 7.2]; 1 mM EDTA), and dounced 15-20 times.
The lystate was spun at 14 Krpm (10 kg), 4°C in a rotor (TL100.3 Beckman
Instruments, Palo Alto, CA) in an ultracentrifuge (TL100; Beckman Instru-
ments). The resulting pellet was resuspended in 0.1 ml of lysis buffer and
applied to a two step sucrose gradient (0.6 ml each of 1.1 and 1.2 M sucrose
in 50 mM potassium acetate; 2 mM EDTA; 1 mM DTT; 1 mM PMSF; 20
mM Hepes, pH 7.4) and spun for 2 h in a swinging bucket rotor (TL 5.55;
Beckman Instruments) at 35 Krpm (81.5 kg) in the ultracentrifuge (Beck-
man Instruments). 30-μl fractions were collected; the third, containing the
interface between 1.2 and 1.5 M sucrose, corresponds to the RER frac-
tion. The supernatant from the 14 Krpm spin was centrifuged in a rotor
(TL100.2; Beckman Instruments) at 4°C for 15 min at 50 Krpm (0.2 kg)
to generate a high speed supernatant and high speed pellet (Golgi fraction),
which was resuspended in 300 μl of 1% Triton X-100 in PBS.

For the fractionation of semisemlant yeast cells (Fig. 4 C), an in vitro tra-
slocation and transport reaction (see below) was scaled up 10-fold. Subcellu-
ar organelles were fractionated during a low speed (2 kg) medium speed
(7.6 kg), and high speed (100 kg) spin as described (Baker et al., 1988).
The medium and high speed pellets constituted the crude ER and Golgi frac-
tions, respectively. For the experiment shown in Table II, the low speed spin
was omitted; the cells were diluted in transport buffer and dounced (three
strokes) before fractionation. The medium speed pellet was further fraction-
ated on a two-step sucrose gradient as described above. Fractions were precipitated with Con A Sepharose or anti-c-myc mnmnose (Baker et al.,
1988), the Sepharose beads assays for invertase activity (Pelham et al.,
1988), then recovered by centrifugation. Bound proteins were eluted with
2% SDS and radioactivity determined by scintillation counting.

In Vitro Reactions

Yeast translation lysates were prepared by a modification of the method de-
scribed by Gasior et al. (1979). 4-6 liters of the S. cerevisiae protase
deficient strain JB118 was grown in YEP medium containing 2% glucose,
to an OD600 of 2, harvested, washed once with H2O and converted to
spheroplasts by digestion with lyticase (10 units/OD) for 30 minutes at
30°C. Spheroplasts were allowed to resume metabolism during a 60-min
incubation in 1.0 M sorbitol, 0.75× YEP, containing 1% glutamate. All fol-
lowing steps were done at 4°C. Regenerated spheroplasts were harvested
and washed once with ice cold 1.0 M sorbitol, and resuspended in 10 ml
lysis buffer (100 mM potassium acetate; 2 mM magnesium acetate; 20 mM
Hepes [pH 7.4]; 2 mM DTT; 0.5 mM PMSF). Cells were dounced 15-20
times, and the lystate was centrifuged 15 min at 27 K. The supernatant
was collected and centrifuged at 100 kg for 30 min. The resulting supernatant
was passed over a Sephadex G-25 column equilibrated in lysis buffer plus
25% glycerol. Fractions on the leading edge of the protein peak were pooled,
 aliquoted, and stored at -70°C.

In vitro transcription of plasmid pSSpO (linearized with Sal I) with SP6
polymerase was as described (Hansen et al., 1986). For small scale analy-
sis, in vitro translation assays were performed in 15 μl reactions, containing
a final concentration (including salt contributed by the addition of yeast lys-
ate) 40 mM Hepes (pH 7.4); 180 mM potassium acetate; 2 mM magnesium
acetate; 20 mM creatine phosphate; 80 μg/ml creatine kinase; 1 mM ATP;
0.1 mM GTP; 30 μM amino acids (-met); 1,500 U/ml RNase inhibitor; 10
μl yeast lysate; mRNA (1 μl transcription mixture), and were incubated at
30°C for 2 h. The reactions were scaled up to 1 ml for the preparation of
labeled protein substrate used for the in vitro translocation and transport
reaction, and desalted over 5 ml G-25 Sephadex columns before aliquoting
and freezing at -70°C.

Permeabilization of yeast cells and in vitro translocation and transport
assays were performed as described (Baker et al., 1988).

Results

Expression of Preproalpha Factor Fusion Proteins

Preproalpha factor fusion genes were constructed using polymerase
chain reaction-mediated mutagenesis (see Materials and Methods) to
introduce a unique Nco I site at the 3' end of the coding region. A DNA fragment encoding a human
c-myc epitope (Munro and Pelham, 1987) followed by the
yeast ER retention sequence HDEL was cloned into this site
resulting in a preproalpha fusion gene that encodes an addi-
tional carboxyl terminal peptide, SMEKLIISEDDELFHED-
DEL. As a control, a similar fusion gene was constructed,
but, in this gene, the fragment cloned at the unique Nco I site
encodes the sequence SMEKLIISEDDEL; i.e., lacking the
HDEL sequence. The proper translational frame was main-
tained, as demonstrated by the immunoprecipitability of both fusion
proteins by the monoclonal antibody 9E10, which
recognizes the c-myc epitope (Evan et al., 1985), whereas
only the HDEL-tagged version was reactive with antisera
specific for the HDEL epitope. Each fusion gene was cloned
into a yeast integration vector, under the control of the TPI
promoter (see Materials and Methods) and transformed into
various yeast strains.

HDEL-tagged Preproalpha Factor Receives
Golgi Modifications

Fusion proteins were analyzed from yeast that had been metaboli-
cally radiolabeled with 35S, followed by immu-
nonprecipitation of cell lysates with the 9E10 monoclonal
antibody. Fig. 1 shows a kinetic analysis of the expression of the fusion proteins in cells carrying a temperature sensitive lesion in the sec18 gene product. At the nonpermissive tem-
perature (37°C) all vesicular traffic is blocked in this mutant,
and newly synthesized proteins cannot leave the ER (Novick
et al., 1981; Beckers et al., 1989; Wilson et al., 1989). Fu-
sion proteins synthesized at this temperature had sizes con-
sistent with those predicted for the primary translation produc-
tions plus three N-linked core oligosaccharides. The fusion
protein containing the HDEL tag had a slower electropho-
retic mobility, as compared to that lacking the additional hex-
apeptide sequence (Fig. 1, compare alpha-H and alpha-O).

At the permissive temperature, as expected, cells expressing
preproalpha factor bearing a COOH-terminal HDEL se-
cue accumulated the fusion protein, while those expressing
the control fusion protein lacking HDEL did not. Cells
were pulse labeled for 10 min, followed by a chase for vari-
able lengths of time. At very early times after the pulse, the
HDEL-tagged preproalpha factor accumulated as an ER-spe-
cific species, identical to that which accumulates when cells
are radiolabeled at the nonpermissive temperature (compare
in Fig. 1, lanes 2 and 3). With increasing times of chase, the
ER form of HDEL alpha factor decreased in abundance,
and was almost undetectable after 5 min of chase (Fig. 1,
lanes 9, 10, and 11). Presumably, it was a substrate for clea-
gage by the KEX2 endoprotease, resulting in loss of the c-myc
epitope required for its immunodetection.

These results demonstrate several important points. First,
the accumulation of precursor was dependent upon the addi-
tional FEHDEL hexapeptide sequence. Second, a substan-
Figure 1. Pulse-chase analysis of proalpha factor fusion proteins. Sec18 strains expressing HDEL-tagged (alpha-H) or control fusion proteins (alpha-O) were pulse labeled for 10 min and chased for the indicated times (minutes) at 24°C (permissive temperature) or, where indicated, at 37°C (nonpermissive temperature). Fusion proteins were immunoprecipitated with 9E10 antibody, and analyzed by gel electrophoresis and autoradiography. The outer lanes show immunoprecipitates from the parental sec18 cells, not expressing fusion proteins, labeled at the permissive and restrictive temperatures.

Figure 2. HDEL-tagged alpha factor receives α1-6, but not α1-3-linked mannose additions in vivo. Sec18 cells expressing the alpha factor-HDEL fusion protein were pulse labeled and chased for 10 min either at the permissive (24°C) or restrictive temperature (37°C). Equal aliquots of cell lysates were immunoprecipitated with either 9E10 antibody (lanes labeled 9E10) or 9E10 antibody followed by a second precipitation with antibodies directed against α1-6-linked mannoses or α1-3-linked mannoses (lanes labeled α1-6 or α1-3). Immunoprecipitates were analyzed by electrophoresis on SDS-polyacrylamide gels followed by autoradiography. Lanes 6 and 7 show a longer exposure of lanes 4 and 5.

Do HDEL-containing Proteins Reach Late Golgi Compartments?

Recent evidence suggests the yeast Golgi apparatus is composed of discrete compartments. Studies employing the sec7 mutant, which accumulates Golgi-like subcellular organelles at the nonpermissive temperature, demonstrate that at least two stages in mannoprotein assembly can be separated. These are the addition of α1-6-linked mannoses, shown to be an early Golgi event, and the addition of α1-3-linked mannose residues, shown to be a later event (Franzusoff and Schekman, 1989). Determination of the type of mannosyl linkages formed on the HDEL-tagged fusion protein could thus delineate the boundary of its distribution.

Sec18 cells expressing HDEL-tagged preproalpha factor were pulse labeled for 10 min, and chased for 10 min at the permissive temperature. Radiolabeled proteins in cell extracts were immunoprecipitated with 9E10 antibody, washed extensively, and then immunoprecipitated with antibodies specific for either the α1-6-linked mannoses or the α1-3 linkage. Protein extracted from cells labeled at the nonpermissive temperature (Fig. 2, lane 1) showed no reactivity with antibody against the α1-6 mannosylation linkage (Fig. 2, lane 2) as expected. These results confirm the Golgi specificity of the α1-6 mannosyltransferase, as well as acting as a control for the efficiency of blockage at the restrictive temperature. Protein extracted from cells radiolabeled at the permissive temperature had acquired the α1-6 mannosylation addition, as evidenced by the precipitation of α1-6 mannosyl-containing material (Fig. 2, lanes 4 and 6). No α1-3 mannosyl immunoreactive material could be detected when an equal aliquot of 9E10 precipitable protein was immunoprecipitated with the appropriate antibody (Fig. 2, lane 5), even upon a prolonged exposure of the gel (Fig. 2, lane 7).

These results suggest that HDEL-containing proteins do not reach the α1-3 mannosyl transferase. However, it is also possible that this transferase is located in the same region of the Golgi complex as the KEX2 endoprotease. If this is so, any HDEL-tagged fusion protein that had acquired α1-3-linked mannose would fail to be detected because the cleavage by Kex 2 would remove the c-myc epitope. We therefore repeated the experiments, using invertase fusion proteins instead of the alpha factor constructs. As shown in Table I, invertase constructs bearing either HDEL or KDEL (a nonfunctional signal) were precipitable with antibodies specific for α1-6 mannosyl linkages, but the HDEL-tagged protein received far less α1-3 linked mannose than the KDEL-tagged one. These results suggest that the HDEL-containing fusion protein had reached the early Golgi modifying enzymes, but had not proceeded to the compartment that contains α1-3 mannosyltransferase activity.

The Retention System Is Saturable

If retrieval was efficient and continuously occurring from an early Golgi compartment, one would predict that with longer times of chase, all of the HDEL-tagged proalpha factor should be converted to the Golgi form. While the ratio of Golgi to ER form does increase during relatively short times of chase with very long chase periods (1 h or more), we ob-

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two proteins were obtained in this step. They were then reprecipitated with the cd-6 mannose protein from the first step that was precipitated in this second step was estimated.

Invertase fusion proteins bearing either HDEL or KDEL at their COOH termini were precipitated with anti-invertase from cells that had been labeled with HDEL/KDEL (percentage of input) and chased for 30 min. Approximately equal amounts of the two proteins were obtained in this step. They were then reprecipitated with the appropriate mannose antibody, and the percentage of the radioactive fusion protein from the first step that was precipitated in this second step was estimated.

Table 1. Modification of Invertase Fusion Proteins

<table>
<thead>
<tr>
<th>Antibody specificity</th>
<th>Amount precipitated (percentage of input)</th>
<th>Ratio (HDEL/KDEL)</th>
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</thead>
<tbody>
<tr>
<td>α1-6 mannose</td>
<td>30</td>
<td>1.1</td>
</tr>
<tr>
<td>α1-3 mannose</td>
<td>2</td>
<td>0.2</td>
</tr>
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Invertase fusion proteins were metabolically labeled, converted into spheroplasts, and then solubilized and half of it subjected to reprecipitation with anti-α6-6 mannose antibody.

To reduce potential complications resulting from saturation of the retention system, we used a yeast strain that overexpresses a gene, ERD2, that is required for retention of HDEL proteins (Hardwick et al., 1990). As will be described elsewhere, overexpression of ERD2 increases the efficiency of retention without otherwise affecting the operation of the secretory pathway. Yeast cells expressing the fusion proteins were metabolically labeled, converted into spheroplasts, and then fractionated by differential centrifugation to separate the various subcellular organelles (see Materials and Methods). Each of the fractions was immunoprecipitated with the 9E10 antibody. The precipitate was then solubilized and half of it subjected to reprecipitation with anti-α6-6 mannose antibody.

The efficiency of separation of ER from Golgi was monitored by the use of carboxypeptidase Y (CPY) as a fraction marker. CPY has distinct forms as it passes through the secretory pathway. Species of 67, 69, and 61 kD can be observed corresponding to the ER, Golgi, and vacuolar forms, respectively (Hasilik and Tanner, 1978; Stevens et al., 1982; see Fig. 4 a). The same fractions assayed for 9E10 and α6-6 reactive materials were also immunoprecipitated with antibody directed against CPY.

The ER fraction contained both the ER form of the HDEL-tagged fusion protein and some anti-α6-6 mannose-precipitable material (Fig. 4 b, lanes 1 and 3). In the strain used, the amount of Golgi-modified material was rather lower than that seen in Fig. 1 and 2, but prolonged exposure of the α6-6 mannose-containing material clearly showed it to be present (Fig. 4 b, lane 3). In contrast, the Golgi fraction (Fig. 4 b, lanes 2 and 4) contained little of either form. In this fraction, the only material seen in the anti-α6-6 mannose precipitate had a high apparent molecular weight, and represents either more extensively modified forms of the protein or, more likely, a background of nonspecifically bound material.

The fractionation procedure used in this experiment did result in the resolution of Golgi membranes from ER membranes, as seen by the separation of the Golgi and ER forms of CPY, precipitated from the same fractions (Fig. 4 a, compare lanes 1 and 2), and also by the specific enrichment of BiP in the ER fraction (data not shown). The method used for cell lysis also resulted in little release of the Golgi and ER forms of CPY into the cytoplasmic fraction (Fig. 4 a, lane 3), indicating that these membrane complexes had retained their integrity.

These results suggest that the bulk of the Golgi-modified form of the HDEL-tagged protein is localized in the ER.
Both the ER and α1-6-linked mannosylated forms of HDEL-tagged alpha factor sediment in an ER-enriched fraction. Cells expressing alpha factor-HDEL were labeled, lysed, and fractionated as described in Materials and Methods to separate the ER from the Golgi apparatus. (A) Immunoprecipitation of CPY from the ER, Golgi, and high speed supernatant fraction (sup.). The ER, Golgi, and mature forms of CPY are indicated as p1, p2, and m, respectively; the supernatant fraction contains mature CPY from lysed vacuoles. (B) Equal portions of the same ER and Golgi fractions as in A were immunoprecipitated with either the 9E10 antibody (lanes 1 and 2) or with 9E10 followed by antibody specific for α1-6-linked mannose (lanes 3 and 4). Note that the second two lanes are from a different gel than the first two, and were exposed to x-ray film for ~10 times as long. The arrow indicates the position of migration of the Golgi-modified form of the fusion protein as seen in Fig. 1 and 2. (C) Fractionation of preproalpha factor transported from ER to Golgi apparatus in vitro. After the transport reaction, the permeabilized cells were fractionated as described in Materials and Methods, to separate the ER and Golgi components. Portions of each fraction were then precipitated with either 9E10 or anti-α1-6 mannose antibodies.

However, it is difficult to rule out the possibility that it resides in some intermediate compartment that cofractionates with the ER. The p2 form of CPY contains α1-3-linked mannose residues, and thus is presumably present in a late Golgi compartment (Franzusoff and Schekman, 1989). No convenient marker for the earlier part of the Golgi compartment exists. However, it has been observed that transport of proalpha factor in vitro, using permeabilized cells, does not proceed beyond the stage of α1-6 mannose addition (Franzusoff and Schekman, 1989). We therefore sought to demonstrate that this in vitro-modified material could be separated from the ER by differential centrifugation.

Fig. 4 c shows results obtained when radiolabeled preproalphafactor (tagged with the c-myc sequence) was incubated with permeabilized cells as described by Baker et al. (1988). After translocation and transport, the cells were fractionated by sequential centrifugation steps, and the fractions precipitated with either the 9E10 monoclonal antibody or α1-6 mannose-specific antibodies. The bulk of the ER form of proalpha factor sedimented during centrifugation at 8 kg (Fig. 4 c, lanes 1 and 2), whereas the majority of the α1-6 mannose-containing material remained in the supernatant, although it could be sedimented by centrifugation at 100 kg (Fig. 4 c, lanes 3 and 4). These results indicate that the early Golgi compartment can readily be separated from the ER under conditions similar to those used to fractionate the cells labeled in vivo.

We have also attempted to demonstrate recycling of HDEL-tagged proalpha factor from the Golgi to the ER in permeabilized cells, but have not so far been successful. Transport of the HDEL-containing protein to the Golgi occurs normally, but the return step does not appear to occur in vitro (data not shown).

Recycling of Invertase Fusion Proteins

To confirm the existence of the recycling process, we also examined the intracellular distribution of an invertase fusion protein bearing the HDEL signal. As a control, we studied a version of the protein that terminates with KDEL, which is not efficiently recognized as a retention signal in S. cerevisiae (Pelham et al., 1988). The fusion protein includes the rat BiP sequence, and even without the HDEL signal is secreted very slowly, as is BiP itself (Table I; Hardwick et al., 1990). In the steady state, a substantial quantity of the protein is present in the ER whether or not the retention signal is present; our expectation was that this ER material would be Golgi-modified in the case of the HDEL tagged protein, but not for the KDEL version. A further advantage of this fusion protein is that, provided it is expressed at a low level, the HDEL version is efficiently retained (Pelham et al., 1988). We have confirmed that under these conditions the retention system is not saturated, as indicated by the absence of BiP secretion from the cells (data not shown).

To provide an internal marker for the early Golgi compartment, cells expressing the HDEL and KDEL proteins were permeabilized and incubated with radiolabeled preproalpha factor. After the transport reaction, the cells were gently homogenized, and centrifuged at moderate speed to separate Golgi body from ER. The pellet, containing the ER, was then fractionated on a sucrose gradient to provide a purified ER fraction as in Fig. 4 a. A second, more slowly sediment-
Table II. Subcellular Fractionation of Strains Expressing Invertase Fusion Proteins

<table>
<thead>
<tr>
<th>Strain 1012 (SEKDEL)</th>
<th>Invertase Con A (U)*</th>
<th>α-1-6 Con A (kcpm)</th>
<th>α-1-6 (% of Con A)</th>
<th>Strain 1002 (FEHDEL)</th>
<th>Invertase Con A (U)</th>
<th>α-1-6 Con A (kcpm)</th>
<th>α-1-6 (% of Con A)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ER(1)†</td>
<td>4.6</td>
<td>10</td>
<td>4.3</td>
<td>7.1</td>
<td>68</td>
<td>3.6</td>
<td>18</td>
</tr>
<tr>
<td>ER(2)†</td>
<td>4.8</td>
<td>16</td>
<td>5.3</td>
<td>7.2</td>
<td>69</td>
<td>3.6</td>
<td>10</td>
</tr>
<tr>
<td>Golgi complex</td>
<td>13.8</td>
<td>73</td>
<td>51.3</td>
<td>20.5</td>
<td>70</td>
<td>48.6</td>
<td>39</td>
</tr>
<tr>
<td>Supernatant</td>
<td>7.1</td>
<td>58</td>
<td>28.7</td>
<td>8.6</td>
<td>50</td>
<td>30.6</td>
<td>3</td>
</tr>
<tr>
<td>Pellet</td>
<td>73.0</td>
<td>32</td>
<td>59.8</td>
<td>102.3</td>
<td>51</td>
<td>49.1</td>
<td>11</td>
</tr>
</tbody>
</table>

Cells from strains expressing invertase fusion proteins with COOH terminal SEKDEL or FEHDEL were permeabilized and added to an in vitro transport reaction with 35S-labeled preproalpha factor. The cells were then fractionated as described in the text and Materials and Methods. Portions of each fraction were precipitated with Con A or anti-α(1-6) mannose, and the precipitates assayed for invertase activity and radioactivity.

§ Material bound to Con A-Sepharose.

* Material precipitable with antibodies to α(1-6)-linked mannose residues, expressed as a percentage of the Con A-precipitable material.

† Invertase activity is expressed in arbitrary units.

ER(1) is the more slowly sedimenting band (possibly smooth ER); ER(2) corresponds to rough ER, as analyzed in Fig. 4 a.

Discussion

Retention and Recycling

The results in this paper confirm earlier studies indicating that the COOH-terminal sequence HDEL is a sufficient signal for retention of proteins within the secretory pathway of S. cerevisiae. A proalpha factor fusion protein bearing this signal accumulated intracellularly, whereas one lacking HDEL was rapidly processed and/or secreted. Fractionation studies demonstrated that the accumulated protein was, as expected, in the ER. Similar results were obtained with invertase fusion proteins. As predicted for a receptor-mediated process, retention was not only specific but also saturable, as shown by the secretion of an endogenous ER protein, BiP, from cells overexpressing an HDEL fusion protein.

We have also shown that portions of the retained proalpha factor and invertase constructs were modified by the addition of α1-6 mannose residues, a reaction that is thought to occur in the Golgi apparatus (Kukuruzinska et al., 1987). Modification of alpha factor-HDEL required the activity of the SEC78 gene product, which is required for the fusion of transport vesicles with their target compartment (Beckers et al., 1989; Wilson et al., 1989); thus, it undoubtedly occurs outside the ER.

Kinetic studies showed that in addition to this Golgi-modified form of the fusion protein, the ER form also accumulated relative to a non-HDEL containing control. This raises the possibility that the primary role of the HDEL signal is to slow the initial exit of proteins from the ER, and that the Golgi-modified form represents molecules that have escaped and are destined for secretion or destruction. The alternative model, which we prefer, is that no discrimination of HDEL-containing molecules occurs until after they have left the ER, but only some of them acquire Golgi-specific modifications before returning. This model is consistent with the idea that binding of HDEL to its receptor and release occur in topologically distinct compartments that presumably have different ionic environments to regulate this change in the receptor’s activity.

In support of the recycling model, our biochemical data indicate that Golgi-modified HDEL proteins cofractionate with the ER, implying that they can return there. Moreover, in permeabilized cells, which apparently lack the capacity to recycle proteins from the Golgi to the ER, the rate of transport of fusion proteins from ER to Golgi is unaffected by the presence of the HDEL signal (our unpublished observations).

Sorting within the Golgi Complex

How far can HDEL-containing proteins get before they are...
The majority of the protein remained a discrete "outer-chain" of 6-linked residues, and became significantly smaller in two steps. In the first, a small number of residues (probably less than four) is added. Subsequently, many pro-alpha factor fusion proteins may penetrate further into the Golgi compartment when cells are grown in rich media than when they are grown in minimal media (Hardwick et al., 1990).

Addition of 6-linked mannose residues is thought to occur in two steps. In the first, a small number of residues (probably less than four) is added. Subsequently, many proteins (including proalpha factor) receive large numbers of "outer-chain" 6-linked residues, and become significantly larger and more heterodisperse (Kukuruzinska et al., 1987).

A significant proportion of the proalpha factor fusion protein underwent the first of these steps but apparently not the second, since the majority of the protein remained a discrete size, only slightly larger than the ER form. It seems that the bulk of the protein is retrieved before it reaches the compartment where outer chains are added. In agreement with this, we have observed that the majority of an invertase fusion protein bearing the HDEL signal becomes only slightly larger than the ER species even after a prolonged chase, as judged by immunoprecipitation and gel electrophoresis of radiolabeled material (our unpublished observations).

These results differ slightly from our previous conclusion that an HDEL-tagged invertase fusion protein can undergo at least some outer-chain modification, based on immunoblot analysis of the steady-state material in cells grown in rich medium (Pelham et al., 1988). This apparent discrepancy probably results from the different growth media used: as discussed elsewhere, HDEL-tagged proteins may penetrate further into the Golgi compartment when cells are grown in rich media than when they are grown in minimal media (Hardwick et al., 1990).

Later processing events occurring in the Golgi compartment include the addition of mannose residues to the outer chain via 1-2 and 1-3 linkages, and the KEX2-dependent processing of proalpha factor (Julius et al., 1984a). The accumulation of intact proalpha factor-HDEL protein in pulse-chase experiments indicates that much of the retrieval must occur before the KEX2 compartment. We also failed to detect significant addition of 1-3-linked mannose to either proalpha factor or invertase HDEL fusion proteins. Thus, retrieval of luminal ER proteins from the later Golgi compartments probably occurs rarely, if at all, under the conditions we have used.

In conclusion, the results presented in this paper provide strong evidence that sorting events in the early part of the Golgi compartment play a crucial role in the maintenance of resident proteins in the lumen of the ER.

Sec7 cells accumulate some glycoprotein precursors that appear to be identical to the ER forms. Because the cells do not accumulate ER as such, this led to the suggestion of a "pre-Golgi" compartment in which few carbohydrate modifications would occur (Franzusoff and Schekman, 1989). Retrieval of some HDEL proteins from this compartment would satisfy the requirement that sorting occurs outside the ER (in an environment where HDEL-receptor interactions are strong) while accounting for the retention of ER-specific forms of the proalpha factor fusion protein.

Addition of 6-linked mannose residues is thought to occur in two steps. In the first, a small number of residues (probably less than four) is added. Subsequently, many proteins (including proalpha factor) receive large numbers of "outer-chain" 6-linked residues, and become significantly larger and more heterodisperse (Kukuruzinska et al., 1987).

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**Figure 5.** Schematic outline of proposed events in the yeast Golgi compartment. Each box represents a functionally distinct compartment, but is not meant to imply any particular physical form or arrangement. They correspond in general to the compartments proposed by Franzusoff and Schekman (1989). The carbohydrate additions characteristic of each one are indicated. Downward-pointing arrows indicate forward vesicular transport. Upward-pointing arrows indicate vesicular traffic from a "pre-Golgi" and an "early Golgi" compartment to the ER; the thin arrow corresponds to reverse flow from a later compartment, which may occur when cells are grown in rich medium (see text).


