Signal-mediated Retrieval of a Membrane Protein from the Golgi to the ER in Yeast

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Abstract. The *Saccharomyces cerevisiae* Wbpl protein is an endoplasmic reticulum (ER), type I transmembrane protein which contains a cytoplasmic dilysine (KKXX) motif. This motif has previously been shown to direct Golgi-to-ER retrieval of type I membrane proteins in mammalian cells (Jackson, M. R., T. Nilsson, and P. A. Peterson. 1993. *J. Cell Biol.* 121: 317–333). To analyze the role of this motif in yeast, we constructed a *SUC2-WBP1* chimera consisting of the coding sequence for the normally secreted glycoprotein invertase fused to the coding sequence of the COOH terminus (including the transmembrane domain and 16-amino acid cytoplasmic tail) of Wbplp. Carbohydrate analysis of the invertase-Wbpl fusion protein using mannose linkage-specific antiserum demonstrated that the fusion protein was efficiently modified by the early Golgi initial α1,6 mannosyltransferase (Och1p). Subcellular fractionation revealed that >90% of the α1,6 mannose-modified fusion protein colocalized with the ER (Wbplp) and not with the Golgi Och1p-containing compartment or other membrane fractions. Amino acid changes within the dily sine motif (KK→QK, KQ, or QQ) did not change the kinetics of initial α1,6 mannose modification of the fusion protein but did dramatically increase the rate of modification by more distal Golgi (elongating α1,6 and α1,3) mannosyltransferases. These mutant fusion proteins were then delivered directly from a late Golgi compartment to the vacuole, where they were proteolytically cleaved in a *PEP4*-dependent manner. While amino acids surrounding the dilysine motif played only a minor role in retention ability, mutations that altered the position of the lysines relative to the COOH terminus of the fusion protein also yielded a dramatic defect in ER retention. Collectively, our results indicate that the KKXX motif does not simply retain proteins in the ER but rather directs their rapid retrieval from a novel, Och1p-containing early Golgi compartment. Similar to observations in mammalian cells, it is the presence of two lysine residues at the appropriate COOH-terminal position which represents the most important feature of this sorting determinant.

In eukaryotic cells, the secretory pathway provides a common biosynthetic route not only for proteins destined to be secreted from the cell but also for proteins that reside in the various organelles of the pathway itself. This ability to discriminate between these different protein populations and thus selectively retain or target resident proteins away from the bulk flow of vesicle-mediated secretory traffic ensures that the unique compartmental structure of the pathway can be maintained (Palade, 1975; Pfeffer and Rothman, 1987). All proteins entering the secretory pathway are first translocated into the endoplasmic reticulum (ER).[1] To maintain residence, ER proteins must therefore either be sequestered away from anterograde vesicular transport (by either an active or passive mechanism) or be specifically retrieved from distal compartments in the pathway if they escape from the ER.

Lumenal ER proteins typically contain a COOH-terminal, four-amino acid retention/retrieval signal. The mechanism for retention of these soluble proteins has been quite well-characterized: both the mammalian KDEL (Munro and Pelham, 1987) and the *Saccharomyces cerevisiae* HDEL (Pelham et al., 1988) COOH-terminal sequences are thought to confer ER residence to proteins by interacting with specific, Golgi-localized receptors (Lewis and Pelham, 1990; Lewis et al., 1990; Semenza et al., 1990) which mediate recycling of these proteins from the Golgi complex (Dean and Pelham, 1990; Lewis and Pelham, 1992). While retrieval of mammalian KDEL-containing proteins may occur from several distinct Golgi regions (Lewis and Pelham, 1992), yeast cells appear to recycle soluble ER proteins only from an early Golgi compartment (Dean and Pelham, 1990).

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1. *Abbreviations used in this paper*: CPY, carboxypeptidase Y; endo H, endoglycosidase H; ER, endoplasmic reticulum; OTase, *N*-oligosaccharyltransferase; PNGase F, *N*-Glycosidase F; SD, synthetic dextrose medium; YPD, yeast extract, peptone, and dextrose medium.

Type I integral ER membrane proteins in mammalian cells contain a COOH-terminal dilysine consensus motif (i.e., KKXX) which, like KDEL for soluble proteins, specifies their ER retention/recycling. Deletion of the COOH-terminal DEKKMP sequence from the E3/19K (E19) adenovirus protein results in its cell surface expression (Pääbo et al., 1987), and chimeric proteins consisting of either CD8 or CD4 cell surface proteins fused to the COOH-terminal tail of E19 are retained in the ER (Nilsson et al., 1989). However, the CD8- and CD4-E19 chimeras acquire Golgi-specific carbohydrate modifications consistent with a role for KKXX in the specific retrieval of these proteins from the Golgi complex (Jackson et al., 1993); as with the KDEL system, retrieval is thought to occur from several distinct Golgi compartments. Although the precise mechanism involved in this recycling process is still largely unknown, the two lysine residues cannot be substituted by other basic amino acids and must be in either the -3,-4 or the -3,-5 positions relative to the COOH terminus to function efficiently as a retrieval motif (Jackson et al., 1990). Recently, it was found that the KKXX motif specifically binds coatomer proteins in vitro (Cosson and Letourneur, 1994), which may suggest a role for non–clathrin-coated vesicles in dilysine-mediated Golgi-to-ER recycling.

In yeast, only one protein containing the COOH-terminal KKXX motif has thus far been identified. Wbplp is an essential, 45-kD type I ER membrane protein which functions as a component of the yeast *N*-oligosaccharyltransferase (OTase) complex (te Heesen et al., 1991, 1992, 1993). As such, its ER localization is crucial for proper functioning of the gene product. We report here that the dilysine motif of Wbplp functions as an ER retrieval signal in yeast. Using a series of invertase-Wbpl fusion proteins to study the recycling process, we found that the wild-type COOH terminus of Wbplp is both necessary and sufficient to elicit recycling of an invertase-Wbpl chimera from a very early Golgi compartment back to the ER. This Golgi compartment is likely to contain Ochlp, the initial α1,6 mannosyltransferase, but not elongating α1,6 or α1,3 mannosyltransferases. Specific mutations within the KKXX motif result in transit of the fusions through later stages of the secretory pathway. Surprisingly, fusion proteins not recycled to the ER are ultimately delivered to the vacuole where they are proteolytically cleaved in a vacuolar protease-dependent manner.

Materials and Methods

Strains and Media

Escherichia coli strains used in this study were XL1-Blue [*supE44 thi-1 lac endA1 gyrA96 hsdR17 relA1* (F' *proAB lacI*^{q} ZΔ*M15* Tn*10*)] and BMH 71-18 [*thi supE* Δ(*lac-proAB*) (*mutS*::Tn*10*) (F'*proAB, lacI*^{q} ZΔ*M15*)]. Bacterial strains were grown on standard media (Miller, 1972). *S. cerevisiae* strains used were SEY6210 (*MAT*α *leu2-3,112 ura3-52 his3*Δ*200 trp*Δ*901 lys2-801 suc2*Δ*9*) (Robinson et al., 1988), TVY1 (SEY6210 Δ*pep4::LEU2*) (T. Vida, unpublished), YG0117 (*WBP1-KK::URA3* Δ*wbp1::HIS3 ade2-101 his3*Δ*200 tyr1*), YG0119 (*WBP1-QQ::URA3* Δ*wbp1::HIS3 ade2-101 his3*Δ*200 tyr1*), TH453 (*MATa/MAT*α *WBP1/*Δ*wbp1::HIS3 ade2-101/ade2-101 his3*Δ*200/ his3*Δ*200 ura3-52/ura3-52 lys2-801/LYS2 tyr1/TYR1*) (te Heesen et al., 1991), SEY5188 (*MAT*α *sec18-1 suc2*Δ*9 leu2-3,112 ura3-52*) (Graham and Emr, 1991), and YS57-2C (*MAT*α *och1::LEU2 leu2 ura3 trp1 his1 his3*) (Nakanishi-Shindo et al., 1994). Yeast strains were grown on yeast extract, peptone and dextrose (YPD) or synthetic dextrose (SD) medium supplemented as necessary (Sherman et al., 1979).

Plasmid Construction and Site-Directed Mutagenesis

The plasmid pBS(*WBP1*/HII-HII) was generated by inserting a 2-kb HindII-HindII fragment from the original p45-11 *WBP1* clone (te Heesen et al., 1991) into the HindII site of pBluescript(KS)+ (Stratagene Inc., La Jolla, CA); this resulted in a new SalI site at one end of the fragment. Plasmids YIp5[*WBP1-KQ*], YIp5[*WBP1-QK*], and YIp5[*WBP1-QQ*] and were generated as follows. First, site-directed mutagenesis of pBS(*WBP1*/HII-HII) was used to generate mutant *wbp1* sequences encoding the predicted K→Q amino acid changes and creating a new SpeI site. Then, the following three fragments were ligated into the yeast integrating plasmid YIp5 (*URA3*) (Rothstein, 1991), which had been cleaved with HindIII and SalI: (*a*) a 3.1-kb HindIII-BamHI fragment from p45-11, (*b*) a 332-bp BamHI-SpeI fragment encoding the indicated K→Q amino acid substitutions, and (*c*) a 678-bp SpeI-SalI fragment extending past the 3′ end of the WBP1-coding region. Finally, the *CEN V* sequence (located ~2 kb from the WBP1 open reading frame) was removed from these YIp5[*WBP1-XX*] constructs by cleavage with HindIII and HpaI, the ends were blunted using the Klenow enzyme, and the plasmids were ligated. YIp5[*WBP1-KK*] was constructed by replacing the BamHI-SalI fragment of YIp5[*WBP1-QQ*] with the BamHI-SalI fragment of pBS(*WBP1*/HII-HII). YIp5[*WBP1-KKmyc*] was constructed from Yip5[*WBP1-KK*] and contains sequences encoding a 12-amino acid c-myc epitope tag (Evan et al., 1985) at the 3′ end of the gene. The predicted amino acid sequence of the modified COOH terminus of this construct was KKTNEQKLISEEDLN (placing the lysine residues at -14,-15). All mutations were verified by sequence analysis.

Plasmid pSEYC350 (*URA3, CEN*) containing the *PRC1* promoter and signal sequence followed by codons 3–512 of the *SUC2* gene was generated from pCYI-20 (Robinson et al., 1988) by digestion by pCYI-20 with BamHI and HindIII, exonuclease treatment of the 5′ overhanging ends (thus destroying the HindIII and BamHI sites), and ligation of the plasmid. SmaI and BamHI sites were then introduced between codons 512 and 514 (514 is the stop codon) of *SUC2* by oligonucleotide-directed mutagenesis. Plasmid pSEYC68 (*URA3, CEN*) was constructed by subcloning the polylinker from pUC18 (Norrander et al., 1983) into pSEYC58 (Emr et al., 1986).

Plasmids pEG1-KK, pEG1-KQ, pEG1-QK, pEG1-QQ, and pEG1-KKmyc (encoding the KK, KQ, QK, QQ, and KK+myc invertase-Wbp1 fusion proteins, respectively) were constructed by subcloning the following two fragments into the plasmid pSEYC68 (*URA3 CEN*), which had been cleaved with EcoRI and SalI: (*a*) the 3.2-kb EcoRI-BamHI fragment from pSEYC350 containing the *PRC1* promoter and signal sequence and the *SUC2* gene, and (*b*) the appropriate 1-kb BamHI-SalI fragment from the corresponding YIp5[*WBP1-XX*] constructs described above. Plasmids pEG1-KK3, pEG1-KK4, and pEG1-KK5 (encoding the TFKKSS, SSKKTN, and TFKKT invertase-Wbp1 fusion proteins, respectively) were generated by oligonucleotide-directed mutagenesis of pEG1-KK, using a mutagenizing oligonucleotide and a selection oligonucleotide as described in the Transformer Site Directed Mutagenesis Kit (Clontech Laboratories, Inc., Palo Alto, CA). The drOST construct (pSWdrOST) was generated from pEG1-QQ by replacing the 3′ sequences (encoding the 16 COOH-terminal amino acids) of *wbp1* with sequences encoding the COOH-terminal 20 amino acids of the *Drosophila WBP1* homologue (drOST).

Yeast and Bacterial Methods

E. coli transformations were performed using the method of Hanahan (1983). Yeast transformations were performed by the alkali cation method (Ito et al., 1983). Standard yeast genetic techniques were used throughout (Sherman et al., 1979). To construct yeast strains YG0117 and YG0119, plasmids YIp5[*WBP1-KK*] and YIp5[*WBP1-QQ*], respectively, were linearized with StuI and integrated into the *ura3-52* locus of TH453 to yield diploid strains. After sporulation, *Ura+His+* haploid segregants were isolated: these segregants had a deleted *wbp1* gene and carried the *WBP1-KK* (YG0117) or *WBP1-QQ* (YG0119) genes at *ura3-52*. Invertase activity was determined by the method of Goldstein and Lampen (1975).

Cell Labeling and Immunoprecipitation

Cells were grown to mid-logarithmic phase (A_{600} = 0.8–1.0 OD units) in SD medium supplemented with the appropriate amino acids and 0.2% yeast extract. Cells were labeled in SD medium (without yeast extract) at 5 OD equivalents/ml with 20 μCi of Tran ^{35}S-label (ICN Biochemicals, Inc. Costa Mesa, CA) per OD equivalent of cells. Chase was initiated by adding a 10× chase solution (50 mM methionine, 10 mM cysteine, 4% yeast extract, 20% glucose) to a 1× final concentration. Chase was terminated by

adding TCA to a final concentration of 10%. Immunoprecipitation of the samples was carried out as previously described (Klionsky et al., 1988), except that the buffer used to resuspend the dried pellet before glass bead lysis also contained 6 M urea. Antigens to be reimmunoprecipitated with a secondary antibody were dissociated from the first antibody by boiling for 5 min in 100 μl of 1% SDS, 20 mM Tris-Cl (pH 7.5), then diluted in 900 μl of Tween 20-IP buffer and subjected to a second immunoprecipitation as described above. Immunoprecipitated samples to be treated with endoglycosidase H (endo H) were boiled for 5 min in 32 μl 0.5% SDS, 1% β-mercaptoethanol then incubated in 40 μl 0.05 M NaCitrate (pH 5.45) with 200 units of endo H (New England BioLabs, Beverly, MA) at 37°C for 1 h. Untreated samples from the same immunoprecipitates were similarly prepared, except that endo H was not added to the incubation.

To prepare invertase antiserum, commercially prepared invertase (Boehringer Mannheim Biochemicals, Indianapolis, IN) was deglycosylated with *N*-Glycosidase F (PNGase F) (New England BioLabs), gel purified, and used to immunize New Zealand white rabbits as previously described (Klionsky et al., 1988). Antiserum directed against Wbplp (wheat germ agglutinin-binding protein) was prepared as previously described (te Heesen et al., 1991). α1,6 and α1,3 mannose-specific antisera were a gift from Randy Schekman. Kex2p (Kex2 protease) antiserum was provided by William Wickner. Ochlp antiserum was a gift from Yoshifumi Jigami. ALP (alkaline phosphatase) antiserum was prepared as previously described (Klionsky et al., 1988).

Subcellular Fractionation

Cells were grown in SD medium as described for immunoprecipitation and converted to spheroplasts as previously described (Vida et al., 1990). Spheroplasts were labeled for 10 min and chased for 60 min as described above, except that the labeling medium also contained 1 M sorbitol. NaN_3 and NaF were then added to a 20 mM final concentration each, and the spheroplasts were harvested by centrifugation at 4°C (all further procedures were also performed at 4°C or on ice). Spheroplasts were resuspended in a hypoosmotic lysis buffer (0.2 M sorbitol, 50 mM KOAc, 2 mM EDTA, 20 mM Hepes-KOH [pH 6.8], 1 mM DTT, phenylmethylsulfonyl fluoride [20 μg/ml], antipain [5 μg/ml], aprotinin [1 μg/ml], leupeptin [0.5 μg/ml], pepstatin [0.7 μg/ml], α_2-macroglobulin [10 μg/ml]) and dounced ~20 times with a glass tissue homogenizer. The crude lysate was centrifuged at 300 *g* to remove unlysed spheroplasts. The 300 *g* supernatant was centrifuged at 13,000 *g* for 15 min to generate intermediate speed pellet (P13) and supernatant (S13) fractions; the S13 was centrifuged at 100,000 *g* for 1 h to generate high speed pellet (P100) and supernatant (S100) fraction (Fig. 6 *A*). 5 OD-equivalents for each of the P13 and P100 fractions were precipitated by resuspending the pellet in lysis buffer and adding TCA to 10% final concentration. Sucrose step gradient procedures were performed essentially as described by Dean and Pelham (1990), with the following modifications. 20 OD-equivalents of the P13 material were resuspended in 200 μl of lysis buffer and loaded onto the top of a 1.2/1.5 M sucrose gradient (Fig. 6 *B*), consisting of 800 μl each of 1.5 and 1.2 M sucrose in sucrose gradient buffer (50 mM KOAc, 2 mM EDTA, 20 mM Hepes [pH 6.8], 1 mM DTT, 1 mM PMSF). The gradient was spun at 85,000 *g* for 1 h in a TLS55 swinging bucket rotor. Five equivalent fractions were removed from the gradient, diluted in 500 μl lysis buffer, and precipitated by the addition of TCA to 10% final concentration. All TCA-precipitated proteins were processed for immunoprecipitation and/or second immunoprecipitations as described above.

Results

Effect of Dilysine Motif Mutations on Wbp1 Protein Localization

We first addressed the question of whether the KKXX motif is necessary and/or sufficient for retention of Wbplp in the yeast ER. If this is the case, then specific disruption of the dilysine region might be expected to yield a detectable phenotype (i.e., inviability or Wbplp mislocalization). A set of *wbp1* point mutants was constructed by site-directed mutagenesis in which either one or both of the lysine residues at the -3, -4 COOH-terminal positions were replaced with glutamine. As mentioned earlier, Wbplp is an essential subunit of the yeast *N*-oligosaccharyltransferase complex; loss of Wbplp function is lethal (te Heesen et al., 1991). The *wbp1-KQ, wbp1-QK, wbp1-QQ* mutant alleles or the *WBP1-KK* wild-type control carried in a *URA3* integrating plasmid were integrated at the *ura3-52* locus of TH453, a diploid yeast strain in which one of the chromosomal copies of *WBP1* was disrupted with the *HIS3* gene. Following sporulation, viable haploid progeny were recovered that contained both the Δ*wbp1::HIS3* allele (His$^+$) and the *wbp1* point mutant alleles (*KQ, QK,* or *QQ*) (Ura$^+$). The *wbp1* mutant alleles thus complemented a lethal Δ*wbp1* null allele, indicating that the KKXX motif is not absolutely required for proper Wbplp function.

To test directly if wild-type Wbplp or the K→Q point mutants transit out of the ER and into the Golgi complex, we determined if the Wbp1 proteins received Golgi-specific carbohydrate modifications. In yeast, core oligosaccharides added in the ER to appropriate asparagine residues are extended first by α1,6 mannosyltransferases in early (*cis*) Golgi compartments and then by α1,2 and α1,3 mannosyltransferases in middle and late (*medial* and *trans*) Golgi compartments (Franzusoff and Schekman, 1989; Graham and Emr, 1991). The presence of specific mannose linkages on a protein serves as an indicator of how far that protein has progressed in the yeast secretory pathway. The lumenal domain of Wbplp contains two potential N-linked glycosylation sites. To determine if these sites are utilized, we pulse-labeled the yeast strains YG0117 (*WBP1-KK*) and YG0119 (*wbp1-QQ*) for 10 min with Tran^{35}S-label, chased with cold methionine and cysteine for various times, and immunoprecipitated Wbplp. Treatment of Wbplp recovered from each strain with endo H to remove N-linked oligosaccharides resulted in a ~4 kD faster mobility on SDS–polyacrylamide gels. This indicated that Wbplp receives at least core N-linked oligosaccharide modification (data not shown). To test specifically for the presence of α1,6 mannosyl (early Golgi) modifications on both wild-type Wbplp and the Wbp1-QQ mutant protein, YG0117 (*WBP1-KK*) and YG0119 (*wbp1-QQ*) cells were pulse-labeled with Tran^{35}S-label for 10 min and chased for 60 min. Equal aliquots of cells were removed after 0 and 60 min and processed for immunoprecipitation with Wbp1-specific antiserum. Wbplp was eluted from the primary antibody, and half of each sample was reimmunoprecipitated with either anti-Wbp1 antiserum (Fig. 1, lanes *1* and *3*) or antiserum specific to α1,6 mannose linkages (Fig. 1, lanes *2* and *4*). Less than 5% of either the wild-type or mutant Wbp1 protein was recovered with the linkage-specific antiserum (Fig. 1, lanes *3* and *4*), indicating that they do not acquire Golgi-specific modifications.

Changing the COOH-terminal dilysine sequence of Wbplp from KK to QQ did not disrupt protein function (as evidenced by the ability of the point mutants to complement an otherwise lethal *WBP1* deletion mutation) nor did it result in Golgi-specific modification of Wbplp. These results can be interpreted in several ways. First, Wbplp may cycle through a post-ER compartment which does not contain α1,6 mannosyltransferase activity; alternatively, the N-linked glycosylation sites of Wbplp may somehow become masked after core modification and are thus unable to serve as a substrate for α1,6 mannosyltransferase(s). It is also possible that the KKXX sequence does not serve as an ER retention motif in yeast despite its demonstrated importance in the retrieval of

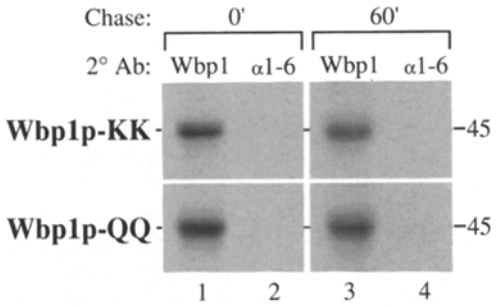

Figure 1. Immunoprecipitation and α1,6 mannose modification of Wbplp-KK and Wbplp-QQ. Strains YG0117 (WBP1-KK, *top panels*) and YG0119 (*wbp1-QQ, bottom panels*) were labeled for 10 min with Tran^{35}S-label and chased for 0 or 60 min. Wbp1-KK and Wbp1-QQ proteins were recovered by immunoprecipitation. Each Wbp1 protein was then split into two equal aliquots which were subjected to a second immunoprecipitation with antisera to either Wbplp (lanes *1* and *3*) or α1,6 mannose residues (lanes *2* and *4*).

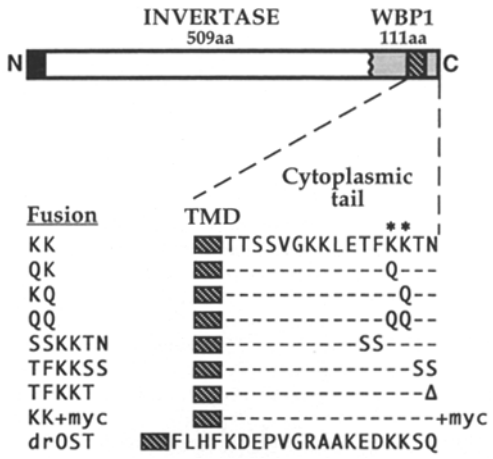

Figure 2. Invertase-Wbp1 fusion constructs. The 20-amino acid signal sequence of CPY (*black box*) and codons 3-512 of the *SUC2* gene encoding external invertase (*open box*) were fused to the COOH-terminal 111 codons of *WBP1* (*gray box*) as described in Materials and Methods. The 16-amino acid COOH-terminal cytoplasmic tail sequence of Wbplp is shown in entirety for the wild-type (*KK*) fusion. Asterisks indicate the lysine residues of the KKXX motif and are shown above the -3 and -4 COOH-terminal positions. Each of the other fusion constructs also encode the CPY signal sequence, invertase, and the lumenal and transmembrane (*TMD*) domains of Wbplp. For these fusions, specific amino acid or tail length changes are indicated (the Δ denotes an amino acid which was removed from the COOH terminus). Dashed lines represent amino acid residues which are the same as wild-type (*KK*). The 20-amino acid COOH-terminal cytoplasmic tail sequence of the drOST (*Drosophila* OTase homologue) construct is also shown in entirety.

type I membrane proteins in mammalian cells. Finally, Wbplp may be retained in the ER by at least two distinct mechanisms, one that keeps the protein in the ER and one that recycles the protein from more distal compartments if it escapes the ER. If the lumenal domain of Wbplp either contains an efficient primary ER retention signal or interacts with other subunits of the OTase complex which may themselves contain ER retention signals, then the entire complex may rarely, if ever, escape the ER. Consequently, the KKXX motif, while functional, could simply be rendered nonessential in the context of this particular protein.

Construction of Invertase-Wbp1 Chimeras

To test the possibility that the retention/recycling role of the dilysine motif may be masked in Wbplp by another retention mechanism, we fused the transmembrane domain and cytoplasmic COOH-terminal tail of Wbplp to invertase, a reporter enzyme not normally retained in the ER. Secreted invertase is a well-characterized glycoprotein which localizes to the periplasmic space between the plasma membrane and cell wall. Invertase contains 10–13 N-linked oligosaccharides (Trimble and Maley, 1977) that are extensively and heterogeneously modified by the Golgi mannosyltransferases as the protein transits through the secretory pathway (Ballou, 1976; Trimble et al., 1983). Secreted invertase migrates as a high molecular mass smear on SDS-polyacrylamide gels as a result of the heterogeneous glycosylation. The invertase-Wbp1 fusion construct consists of the *PRC1* (encoding carboxypeptidase Y) promoter and signal sequence, the *SUC2* gene encoding enzymatically active invertase, and the COOH-terminal 111 codons of *WBP1* (Fig. 2). Carboxypeptidase Y (CPY) is an abundant and constitutively expressed vacuolar hydrolase (Hasilik and Tanner, 1978). Use of the CPY promoter and signal sequence ensures that the chimera will be both expressed at reasonable levels (0.05–0.1% of total cell protein) and appropriately directed into the secretory pathway. The CPY signal sequence is cleaved after translocation into the ER (Johnson et al., 1987), generating the invertase-Wbp1 fusion protein. Eight additional chimeras were constructed which differ from the wild type only with respect to the specific amino acid sequence of their COOH-terminal cytoplasmic tail domains (Fig. 2). These tail sequences were designed to test the effects of changing the position and sequence of both the dilysine motif and its surrounding amino acids as well as the effects of exchanging the yeast Wbp1 tail for that of its *Drosophila* homologue, which also contains the KKXX motif (I. Stagljar and M. Aebi, unpublished observations). These chimeras were subcloned into a low copy number (*CEN*) vector and will be referred to as the "KK fusion," "QK fusion," etc.

Dilysine Motif Mutations Affect Glycosylation and Retention of Invertase-WBP1 Fusion Proteins

If the dilysine motif functions in yeast in a manner similar to that observed in mammalian cells, then mutations within the motif should result in a defect in recycling of the fusion from the Golgi back to the ER. Transit through the Golgi complex would subject the invertase portion of the hybrid to modification by the Golgi-specific glycosyltransferases; consequently, a recycling defect should yield a concomitant change in fusion glycosylation characteristics. To characterize the glycosylation state of the fusion proteins, yeast strains (Δ*suc2*) harboring low copy number (*CEN*) plasmids containing either the KK (wild type) or QK (single point mutant) invertase-Wbp1 fusion constructs were pulse-labeled for 10

min with Tran[35]S-label and chased by the addition of cold methionine and cysteine. Cells were removed at various timepoints and processed for immunoprecipitation with invertase-specific antiserum. The immunoprecipitated fusion proteins were eluted from the primary antibody and split into four equal aliquots. Two of these aliquots were reimmunoprecipitated with anti-invertase antiserum (Fig. 3 *A*, and *B*). To determine if Golgi-specific N-linked carbohydrates had been added to the chimeras, the remaining two aliquots were reimmunoprecipitated with either α1,6 mannose- (Fig. 3 *C*) or α1,3 mannose- (Fig. 3 *D*) specific antiserum. One of the invertase immunoprecipitates (Fig. 3 *B*) as well as the α1,6 mannose and α1,3 mannose immunoprecipitates (Fig. 3, *C* and *D*) were treated with endo H to remove N-linked sugars so that the amount of fusion protein modified by the Golgi-specific mannosyltransferases could be determined. The remaining invertase immunoprecipitate was left untreated to observe the extent of hyperglycosylation of the fusion proteins (Fig. 3 *A*).

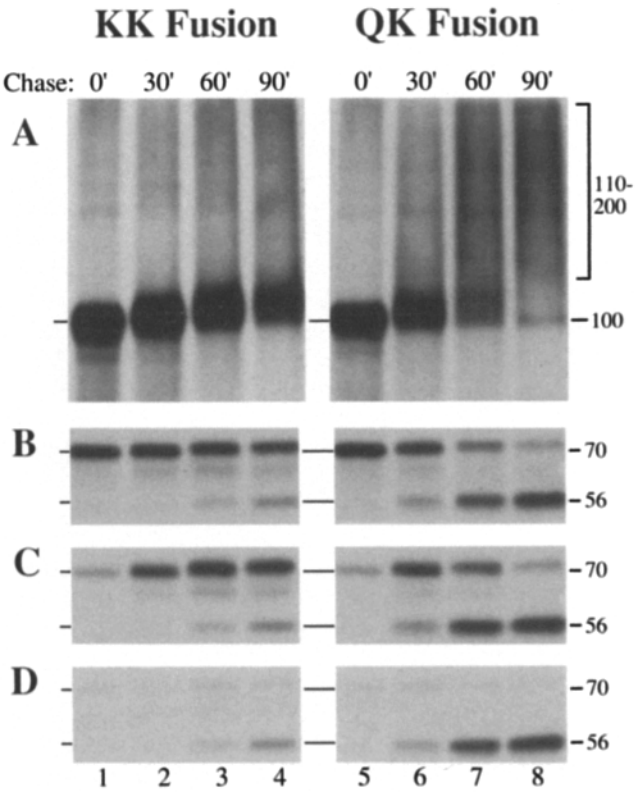

Figure 3. Glycosylation and processing phenotypes of the KK vs. QK invertase-Wbp1 fusion proteins. Wild-type (SEY6210) cells harboring either pEG1-KK (KK fusion *CEN*, lanes *1–4*) or pEG1-QK (QK fusion *CEN*, lanes *5–8*) were pulse-labeled for 10 min with Tran[35]S-label and chased for 90 min. At the indicated timepoints, equal amounts of cells were removed. KK and QK fusion proteins were recovered from each sample by quantitative immunoprecipitation with invertase-specific antiserum and subsequently eluted from the primary antibody by boiling in 1% SDS. The eluates were divided into four equal aliquots. Two of the aliquots were reimmunoprecipitated with invertase-specific antiserum (*A* and *B*), one of which was subsequently treated with endo H (*B*) to remove N-linked sugars. The remaining two aliquots were reimmunoprecipitated with antisera specific to either α1,6- (*C*) or α1,3- (*D*) linked mannose residues and then treated with endo H. All samples were resolved by SDS-PAGE. Apparent molecular masses (in kD) are shown to the right of the figure. In *A*, core-glycosylated fusion protein migrated at ~100 kD, while hyperglycosylated fusion protein migrated as a ~110–200-kD smear. After deglycosylation (*B–D*), intact fusion protein migrated at ~70 kD, and proteolytically processed fusion protein migrated at ~56 kD.

Before the chase, both the KK and QK fusions migrated as a ~100 kD band (Fig. 3 *A*, lanes *1* and *5*). During a 90-min chase, the KK hybrid protein showed only a slight increase in apparent molecular mass (Fig. 3 *A*, lanes *1–4*), while the QK fusion was converted to a high molecular mass heterogeneous smear (Fig. 3 *A*, lanes *5–8*) similar to wild-type invertase. These changes were due largely to N-linked mannose modification, as endo H treatment converted the smear into a tight band (Fig. 3 *B*; see below). These data suggest that unlike the KK fusion, the majority (>85%) of which did not reach Golgi compartments in which N-linked core oligosaccharides could be extensively glycosylated, the QK fusion rapidly traveled to more distal Golgi compartments containing transferases responsible for the addition of outer-chain (i.e., elongated α1,6 and α1,3) N-linked mannose residues.

Endo H treatment to remove N-linked oligosaccharides converted the 100 kD band to ~70 kD, the approximate predicted molecular mass for the unglycosylated fusion protein (Fig. 3 *B*, lanes *1* and *5*). Endo H treatment also revealed a proteolytic processing event (Fig. 3 *B*) which occurred in the vacuole (see below). The processing event, marked by the appearance of a 56-kD band and the concomitant disappearance of the 70-kD band, occurred with kinetics similar to those observed for hyperglycosylation (Fig. 3 *A*). Phosphorimager quantitation of endo H-treated fusion protein bands in this and other experiments with long chase times indicated that the half time for proteolytic processing for the QK fusion is approximately fourfold faster ($t_{1/2}$ ~50′) than for the KK fusion ($t_{1/2}$ ~200′).

After 60 min of chase, both the KK and QK fusions were quantitatively reimmunoprecipitated with α1,6 mannose-specific antiserum (Fig. 3 *C*), indicating that all the labeled fusion protein had acquired at least initial α1,6 mannose modification. Interestingly, the KK fusion was quantitatively α1,6-modified but not hyperglycosylated at the 60- and 90-min timepoints (Fig. 3, *A* and *C*, lanes *3* and *4*), while the QK fusion had acquired significant and extensive outer chain modification after the same amount of chase (Fig. 3 *A*, lanes *7* and *8*). In addition, α1,6 modification of these fusions was found to be both *SEC18*- and *OCH1*-dependent (data not shown). Sec18p is required for vesicle-mediated ER-to-Golgi transport (Novick et al., 1980; Kaiser and Schekman, 1990) and consequently for conversion of core-glycosylated secretory and vacuolar proteins to their Golgi-modified forms (Stevens et al., 1982; Julius et al., 1984; Graham and Emr, 1991). Och1p has recently been identified as the mannosyltransferase responsible for adding the initial α1,6-linked mannose residue onto core N-linked oligosaccharides (Nakanishi-Shindo et al., 1994). Taken together, these data suggest that (*a*) the fusions exit the ER and transit to an early (Och1p-containing) Golgi compartment in a manner independent of the dilysine motif, and (*b*) initial α1,6 and elongating α1,6 mannosyltransferases may reside in distinct Golgi compartments.

As mentioned above, the α1,6 mannose-specific antiserum detected both the intact (70 kD) and processed (56 kD) fusions equally well (i.e., compare Figs. 3, *B* with *C*, lanes *2–4* and lanes *6–8*). In contrast, only the proteolytically processed fusion protein was quantitatively immunoprecipitated with α1,3 mannose-specific antiserum (Fig. 3 *D*). Accordingly, the QK fusion was much more rapidly α1,3 mannosylated than the KK fusion, and the kinetics of appearance of the processed fusion directly correlated with the appearance of the α1,3-modified protein (compare Fig. 3, *B* and *D*). α1,3 mannose modification and proteolytic processing were also nearly coincident with the appearance of the hyperglycosylated smear (compare Fig. 3, *A* and *D*); in fact, we have observed that >85% of the hyperglycosylated fusion protein migrating at ~110–200 kD in Fig. 3 *A* can be reimmunoprecipitated by the α1,3 mannose-specific antiserum (data not shown). α1,3 mannose modification of the fusion protein thus appeared to be kinetically coupled to elongation of the α1,6 mannose chains which in turn was tightly coupled to the proteolytic processing event (Fig. 3 *D*). In summary, while initial α1,6 mannose modification occurred at the same rate for the KK and QK fusions, a single point mutation within the dilysine motif significantly increased the kinetics of α1,6 mannose elongation, α1,3 mannosylation, and proteolytic processing of the fusion protein. These results demonstrate that the COOH-terminal KKXX motif does not affect exit of the fusion from the ER or its subsequent transit to the early Golgi but does significantly delay its transport to later Golgi compartments.

Dilysine Motif Mutant Fusion Proteins Are Delivered to the Vacuole

If the invertase-Wbpl fusion proteins travel to the plasma membrane when the dilysine motif is disrupted (analogous to their mammalian CD4- and CD8-E19 counterparts [Jackson et al., 1990]), then cells containing the mutant fusions should express cell surface invertase activity. Liquid invertase assays were performed on SEY6210 (Δ*suc2*) cells harboring either the KK, QK, KQ, or QQ fusions to compare internal versus external (plasma membrane) enzyme activity. Although these cells expressed high levels of invertase activity, <5% of the total invertase activity for any of these strains was detected at the cell surface; instead, >95% of the invertase activity was retained in some intracellular compartment (data not shown). Together with the observed proteolytic processing (Fig. 3), this suggested that the mutant fusion proteins may be delivered to the vacuole, where vacuolar proteases present in this compartment could cleave off the Wbpl portion of the fusion and release soluble, active invertase. Similar events had been observed previously with CPY-invertase fusions: when directed to the vacuole via a specific vacuolar targeting sequence in the NH_2-terminus of CPY, these fusions underwent a vacuolar proteolytic cleavage to yield fully active, intact invertase (Johnson et al., 1987).

To determine if processing of the invertase-Wbpl fusion protein was being catalyzed by vacuolar proteases, we transformed the KK and QK fusions into a protease-deficient Δ*pep4* strain. The *PEP4* gene (Ammerer et al., 1986) encodes the vacuolar hydrolase proteinase A (PrA), which activates several protease zymogens within the vacuole. Isogenic wild-type and Δ*pep4* cells harboring the KK and QK fusions were pulse-labeled with Tran^{35}S-label then chased for 60 min. Cells were removed after 0 and 60 min of chase, processed for immunoprecipitation with anti-invertase antiserum, and treated with endo H after immunoprecipitation so the fusion proteins would migrate as a tight band in the SDS gel. We found that processing of the QK fusion as well as the minor processing of the KK fusion was almost completely abolished in the Δ*pep4* strain (Fig. 4). In addition, the processed invertase-Wbpl fusion migrated at the approximate molecular mass predicted for unglycosylated invertase (~56 kD) (Fig. 4, lane *4*). Subcellular fractionation experiments have indicated that the processed (56 kD) fusion protein localizes to a soluble, osmotically sensitive intracellular compartment (i.e., the vacuole) (data not shown). Therefore, the processing event appears to be a *PEP4*-dependent proteolytic cleavage at or near the invertase-Wbpl junction.

Finally, to test whether the fusion proteins first go the cell surface and are then delivered to the vacuole via an endocytic pathway, we examined the KK and QK fusions in a *sec1* ts mutant strain. At the nonpermissive temperature of 37°C, *sec1* mutants are blocked specifically in the delivery of secretory vesicles from the Golgi to the cell surface (Novick and Schekman, 1979). Pulse-chase experiments at 37°C showed that the *sec1* mutation did not block or delay the proteolytic processing of either the wild-type or mutant fusion proteins (data not shown). This indicates that the chimeras most likely travel directly from the *trans*-Golgi to the vacuole and thus follow a route which is similar to that of native vacuolar proteins.

The Dilysine Motif Is Essential for Retention/Recycling of Invertase-Wbpl Fusions

The results shown thus far indicate that the cytoplasmic dilysine motif dramatically delays transport of the invertase-Wbpl fusion from an early Golgi compartment to the vacuole. We reasoned that the degree of *PEP4*-dependent processing could be used as a simple assay to measure the relative efficiency of various COOH-terminal sequences to retain/recycle the invertase-Wbpl chimera. For this experiment, a 60-min chase point was chosen as a convenient timepoint due to the difference in observed processing of the

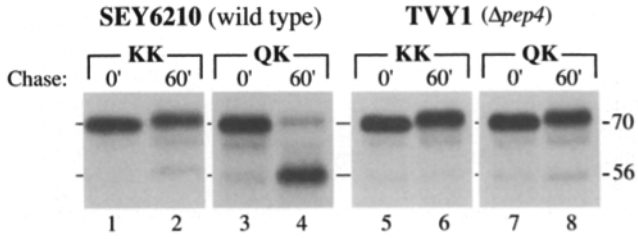

Figure 4. PEP4-dependent processing of invertase-Wbpl fusion proteins. Wild-type (SEY6210 lanes *1–4*) and Δ*pep4* (TVY1, lanes *5–8*) cells harboring either pEG1-KK (lanes *1*, *2*, *5*, and *6*) or pEG1-QK (lanes *3*, *4*, *7*, and *8*) were pulse-labeled for 10 min with Tran^{35}S-label and chased for 0 or 60 min. Equal amounts of cells were removed, and the KK and QK fusion proteins were recovered by immunoprecipitation with invertase antiserum and treated with endo H to remove N-linked oligosaccharides. Apparent molecular masses (in kD) are shown to the right of the figure. Intact and processed fusion proteins migrated at 70 and 56 kD, respectively.

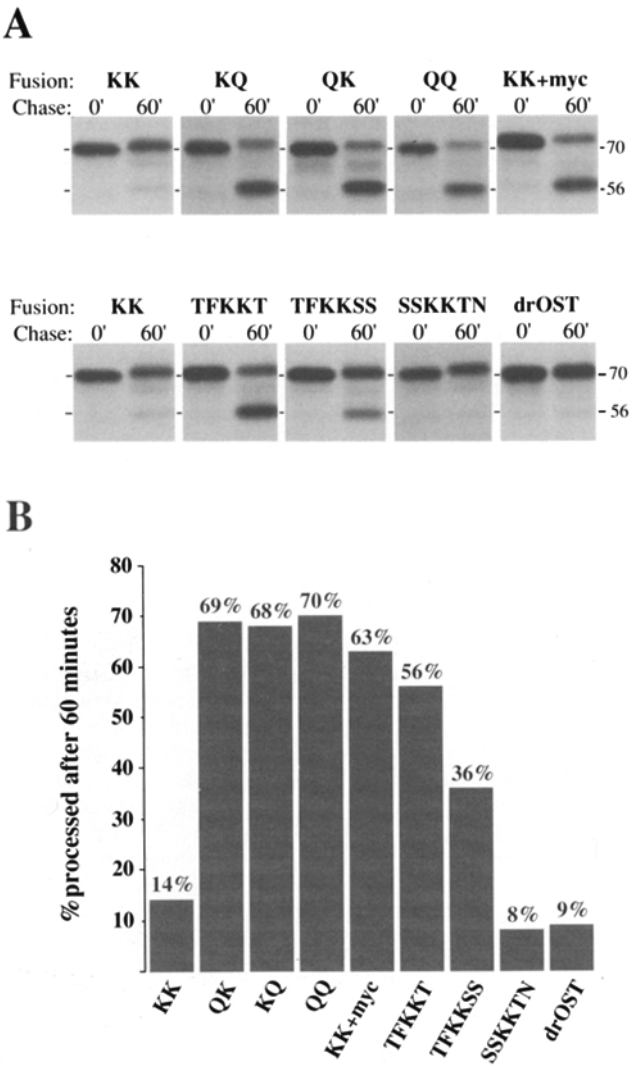

Figure 5. Proteolytic processing of invertase-Wbpl fusion proteins. Wild-type cells (SEY6210) harboring each of the invertase-Wbpl fusions described in Fig. 2 (see Materials and Methods) were pulse-labeled, chased, immunoprecipitated, and endo H-treated as described in the legend for Fig. 4. (*A*) Fusion proteins were recovered after 0 and 60 min of chase. Fusion names (see Fig. 2) are shown above each panel. Apparent molecular masses are shown to the right. Intact and processed fusion proteins migrated at 70 and 56 kD, respectively. (*B*) Percent processed after 60 min was determined by phosphorimager quantitation and is shown in the bar graph for each fusion. Percentages shown represent the average of three separate experiments; <10% variability in percent processed was observed. At 0 min, all fusions were ~5% processed.

wild-type and mutant fusions (see Fig. 3 *B*). Cells expressing each fusion protein shown in Fig. 2 from a low copy number vector were pulse-labeled for 10 min and chased for 60 min. At 0 and 60 min, the fusion proteins were recovered by immunoprecipitation with invertase-specific antiserum, treated with endo H, and resolved on SDS-polyacrylamide gels (Fig. 5 *A*). The gels were then subjected to phosphorimager analysis to quantitate the degree to which each fusion is processed after 60 min of chase (Fig. 5 *B*).

Consistent with a critical role for the dilysine motif in retention/recycling, the KQ, QK, and QQ fusions yielded the most severe retention defect when compared with the wild-type KK fusion (Fig. 5). Further experiments have shown that the processing and glycosylation (similar to those shown in Fig. 3) phenotypes of all of the K→Q mutant chimeras are essentially identical regardless of whether one or both lysine residues are changed to glutamine (data not shown). Shifting the position of the lysine residues from their -3, -4 positions by either extending (KK+myc) or shortening (TFKKT) the COOH-terminal tail also rendered the motif less effective for retention/recycling than wild type (Fig. 5). However, neither of these fusions was quite as defective as the K→Q point mutants. Finally, the remaining three fusions (TFKKSS, SSKKTN, and drOST) might be expected to exhibit wild-type behavior: the TFKKSS and SSKKTN constructs change amino acids surrounding the dilysine motif to serines, and the drOST construct exchanges the Wbplp cytoplasmic COOH-terminal tail for that of its *Drosophila* homologue (I. Stagljar, F. Simmen, S. te Heesen, and M. Aebi, unpublished), which also contains the KKXX motif (see Fig. 2 for the exact COOH-terminal sequence of the drOST construct). The TFKKSS chimera displayed an intermediate processing phenotype (Fig. 5); that is, the rate of transport to the vacuole was faster than the wild-type KK construct but slower than the K→Q point mutant fusions. Interestingly, both the SSKKTN and drOST chimeras were processed more slowly than the wild-type KK fusion (Fig. 5). To test if these fusions were simply retained in the ER due to improper folding, we performed pulse-chase experiments which showed that both the SSKKTN and drOST fusions could be quantitatively reimmunoprecipitated with anti-α1,6-specific antiserum after 60 min of chase (data not shown); these fusions thus appeared to be α1,6 mannosylated with kinetics similar to those observed for the wild-type KK fusion (Fig. 3 *C*). Clearly, the ability of KKXX to elicit retention/recycling in yeast is both lysine and position specific; however, the amino acids surrounding the motif (both before and after the KK) also appear to play a role in its function.

The Dilysine Motif Signals Retrieval to the ER

In mammalian cells, the KKXX COOH-terminal sequence is thought to serve as a retrieval rather than retention signal for type I ER membrane proteins. The most convincing evidence for this comes from studies which localize Golgi-modified CD8-KKXX chimeras to the ER via immunofluorescent staining with lectin-specific antibodies (Jackson et al., 1990; Jackson et al., 1993). Our data indicate that in yeast, the KKXX motif either retains the invertase-Wbpl fusion in an early (pre-α1,3 mannosyltransferase-containing) Golgi compartment or it functions as a recycling signal that directs the return of proteins back to the ER. In an effort to distinguish between these possibilities, we investigated the subcellular distribution of the KK fusion protein. If the KKXX COOH-terminal sequence does in fact elicit Golgi-to-ER retrieval of type I membrane proteins in yeast, then α1,6-modified KK fusion protein should localize primarily to the ER rather than to the Golgi complex.

Fig. 6, *A* and *B* illustrate the protocol we employed to fractionate yeast cells in a manner which would effectively distinguish endoplasmic reticulum membranes from Golgi and vacuolar membranes as well as from soluble vacuolar and cytosolic material. Wild-type cells harboring the KK invertase-Wbpl fusion were converted to spheroplasts, labeled for

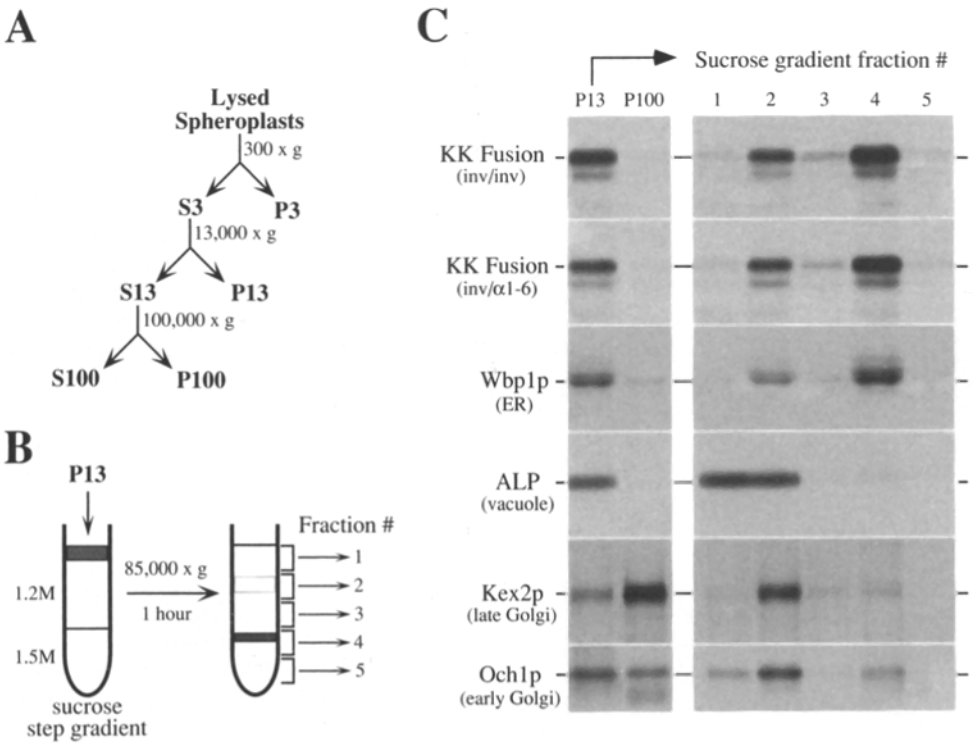

Figure 6. Subcellular fractionation of the invertase-Wbp1 (KK) fusion protein. Wild-type (SEY6210) spheroplasts harboring pEG1-KK (KK fusion) were labeled for 10 min with Tran^{35}S-label, chased for 60 min, and osmotically lysed. (*A*) Lysed spheroplasts were subjected to differential centrifugation as described in the text and in Materials and Methods. 5 OD-equivalents (20% of the total yield) of the P13 and P100 fractions were processed for immunoprecipitation. (*B*) 80% (20 OD-equivalents) of the P13 material was resolved on a 1.2/1.5 M sucrose gradient. After an 85,000 *g* spin for 1 h, five equivalent fractions were removed and processed for immunoprecipitation. (*C*) P13, P100, and P13-sucrose gradient fractions described above were subjected to quantitative immunoprecipitation with invertase antiserum to recover the invertase-Wbp1 fusion protein. Half of each invertase immunoprecipitate was subjected to a second immunoprecipitation with α1,6 mannose-specific antiserum (inv/α1,6), while the other half was reimmunoprecipitated with invertase antiserum (inv/inv). The minor band observed just beneath the KK fusion band corresponds to nonspecific cross-reactive material, as a band of this molecular mass was also detected in invertase immunoprecipitates from SEY6210 spheroplasts not harboring a fusion protein. The marker proteins Wbplp, ALP, Kex2p, and Ochlp were also recovered from the fractions by quantitative immunoprecipitation. Because Wbplp immunoprecipitates often appear as a doublet, both bands in the Wbplp panels correspond to Wbp1 protein.

10 min with Tran^{35}S-label, and chased for 60 min. At this chase point, the KK fusion can be quantitatively immunoprecipitated with α1,6 mannose-specific antiserum (see Fig. 3 *C*), indicating that all of the labeled fusion protein has traveled to the early Golgi. After gentle osmotic lysis and a 300 *g* spin to remove unlysed spheroplasts, the lysates were subjected to differential centrifugation as shown in Fig. 6 *A*. This generated 13,000 and 100,000-*g* pellet (P13 and P100) fractions, from which cell-equivalent amounts were precipitated with TCA and processed for immunoprecipitation. The remaining P13 material, where ER membranes would be expected to fractionate, was further resolved on a sucrose step (1.2/1.5 M) gradient (Fig. 6 *B*). After centrifugation at 85,000 *g* for 1 h, five equivalent fractions were removed from the sucrose gradient and precipitated with TCA. Membranes which migrated to the interface of this gradient appeared as a sharp band and were collected in fraction 4, while fraction 2 consisted of lower density membranes which migrated as a diffuse band in the 1.2 M sucrose (Fig. 6 *B*). The KK chimera as well as various marker proteins for specific organelles were recovered from each fraction by quantitative immunoprecipitation. To determine whether labeled fusion protein had indeed traveled to the early Golgi, each invertase immunoprecipitate was split and subjected to a second immunoprecipitation with either anti-invertase or anti-α1,6 mannose-specific antiserum before treatment with endo H and resolution by SDS-PAGE.

The KK fusion protein was found to be quantitatively modified by α1,6 mannose residues and localized primarily (>80%) to the P13 fraction (Fig. 6 *C*). The P13 fraction also contained ~80% of Wbplp (marking the ER) (te Heesen et al., 1991, 1992) and ~95% of alkaline phosphatase (ALP), a resident vacuolar membrane protein (Klionsky and Emr, 1989). In contrast, the majority (80%) of the late Golgi marker Kex2p (Redding et al., 1991) was found in the P100 fraction, while Ochlp (Nakayama et al., 1992), an early Golgi marker protein, was split between the P13 and P100 fractions. Immunoprecipitates from the soluble (S100) fraction are not shown: all proteins in question are membrane-bound, and the only material found specifically in this fraction was the small amount (~10%) of fusion protein which had been proteolytically processed to the 56-kD form in the vacuole.

To localize the intact KK fusion more definitively to the ER and to separate it from other membranes which also pelleted at 13,000 *g*, the P13 fraction was further resolved on a sucrose step gradient as described above. In this gradient, ER, Golgi, and vacuole membrane proteins exhibited distinctly different migration profiles (Fig. 6 *C*). α1,6-modified KK fusion protein comigrated with the ER resident Wbp1 marker protein: as determined by phosphorimager analysis, 60% of each protein localized to fraction 4, 24% was in fraction 2, and the remaining 14% was mostly in fraction 3. Another ER resident protein, protein disulfide

isomerase (which localized primarily to the P13 fraction), yielded a sucrose gradient migration pattern similar to that of the KK fusion and Wbplp (data not shown). In contrast, ~100% of the vacuolar membrane marker ALP was found in fractions 1 and 2, and the majority (70–80%) of the early and late Golgi markers, Ochlp and Kex2p, migrated to the upper, lower density membrane band (fraction 2) (Fig. 6 *C*). When the α1,3 mannosyltransferase Mnnlp (Yip et al., 1994), which resides in the *medial* Golgi (T. Graham, S. Emr, unpublished observations; Graham and Emr, 1991), was also immunoprecipitated from these fractions, we found that the fractionation profile and sucrose gradient distribution of Mnnlp was similar to that of Kex2p and Ochlp and distinct from that of the KK fusion and Wbplp (data not shown). Membrane proteins of the plasma membrane are also known to pellet at 13,000 *g* (Marcusson et al., 1994). It is very unlikely that the KK fusion protein is localized to the plasma membrane for several reasons: (*a*) the intact KK fusion was not modified with α1,3 mannose (Fig. 3 *D*), (*b*) these cells exhibited <5% surface invertase activity, and (*c*) a *SEC1*-independent delivery to the vacuole appeared to be the fate for nonretained fusions. Therefore, the KK fusion fractionated like an ER protein even though it was subject to early Golgi modification: α1,6 mannose-modified KK fusion cofractionated with Wbplp, and the majority of these proteins migrated to a region of the sucrose gradient which was depleted for Golgi and vacuolar membranes. Taken together with data presented above, these results strongly support a model in which the KKXX motif elicits recycling of type I membrane proteins from an early Golgi compartment to the ER in yeast.

Discussion

A COOH-terminal, cytoplasmic dilysine motif has been shown in mammalian cells to mediate retrieval of type I membrane proteins from the Golgi to the ER (Jackson et al., 1993). *WBP1* is the only yeast gene currently in the literature which encodes an ER-resident, type I membrane protein containing a COOH-terminal KKXX sequence (te Heesen et al., 1991, 1992). In this work, we present the first evidence that this dilysine motif also functions to mediate retrieval of type I membrane proteins from the Golgi to the ER in yeast. We have used a series of invertase-Wbp1 fusion proteins to show that the dilysine motif does not simply retain these fusions in the ER immediately following their synthesis but rather mediates their rapid retrieval from a very early *cis*-Golgi compartment. Efficiency of retrieval not only depends on the presence and the position of the COOH-terminal lysine residues but is also influenced by amino acids surrounding the motif. Fusion proteins with disrupted KKXX motifs transit through the *medial*- and *trans*-Golgi regions and are efficiently shunted off to the vacuole, where they are proteolytically cleaved in a *PEP4*-dependent manner.

The wild-type Wbp1 protein does not appear to be transported to the Golgi and recycled back to the ER. Wpblp does not require the dilysine motif for proper function/localization nor does it acquire α1,6 mannose modification indicative of transit to an early Golgi compartment (Fig. 1). It appears that many mammalian ER-resident membrane proteins (including at least one KKXX-containing protein; Jackson et al., 1993) also remain in the ER without first passing through an intermediate or "salvage" compartment (Ivessa et al., 1992; Ahn et al., 1993; Szczesna-Skorupa and Kemper, 1993). Wbplp has been shown to form a protein complex with at least one other member of the OTase complex, Swplp (te Heesen et al., 1993). For Wbplp-Swplp complex formation, lumenal Wpbl sequences encoded proximal of the BamHI site used to generate the invertase-Wbp1 fusion constructs are essential (L. Wyder, S. te Heesen, and M. Aebi, unpublished observations). It is possible that this complex formation is responsible for the primary ER retention of Wbplp. If Wbplp, Swplp, or another OTase subunit contains an efficient ER retention signal, then the entire complex may never leave the ER. The invertase-Wbp1 fusions allowed for specific analysis of the effect of the dilysine motif on ER localization of membrane proteins in yeast, presumably because the fusion proteins lack sequence determinants in Wbplp that allow for OTase complex formation.

Model for KKXX-mediated Recycling from the Early Golgi to the ER

Comparison of the glycosylation characteristics of the wild-type invertase-Wbp1 fusion (KK fusion) with a fusion in which one of the lysine residues had been changed to glutamine (QK fusion) showed that the fusion proteins acquire initial α1,6-linked mannose modification with similar kinetics ($t_{1/2}$ ~18 min for both the KK and QK fusions) (Fig. 3 *C*). α1,6 modification of the chimeras was *SEC18*-dependent, indicating that the fusions exit the ER and subsequently undergo vesicle-mediated transport to the Golgi complex (Novick et al., 1980; Wilson et al., 1989; Kaiser and Schekman, 1990). α1,6 modification was also dependent on the *OCH1* gene product, which has recently been identified as the mannosyltransferase responsible for adding the initial α1,6-linked mannose residue onto Asn-linked core oligosaccharides (Romero and Herscovics, 1989; Nakanishi-Shindo et al., 1994). Ochlp activity represents the first enzymatic event required for synthesis of outer chain mannose linkages on yeast secretory proteins and thus must reside in a very early Golgi compartment. Transit of the fusions to an Ochlp-containing Golgi compartment therefore occurs independently of the dilysine motif. However, the α1,6 chains on the KK fusion were not significantly extended by elongating mannosyltransferase(s), while the QK fusion acquired outer chain Golgi modifications (Fig. 3). The dilysine motif thus dramatically delays the kinetics of transport of the fusion protein through the secretory pathway, and the lack of elongated α1,6 and α1,3 mannose residues on the KK fusion is not due to an inability of the KK fusion to receive this modification.

We utilized differential centrifugation and sucrose gradient analysis to show that the α1,6-modified KK fusion protein localized to an ER-enriched and relatively vacuole- and Golgo-free membrane fraction (Fig. 6 *C*). Together, our glycosylation and fractionation results support the recycling model shown in Fig. 7: in an early, Ochlp-containing compartment, KKXX-containing type I membrane proteins (i.e., the KK fusion) are first selectively sorted away from proteins destined for further anterograde movement (i.e., the QK fusion) and are then rapidly returned to the ER. If the KK fusion is subjected to repeated rounds of cycling through the

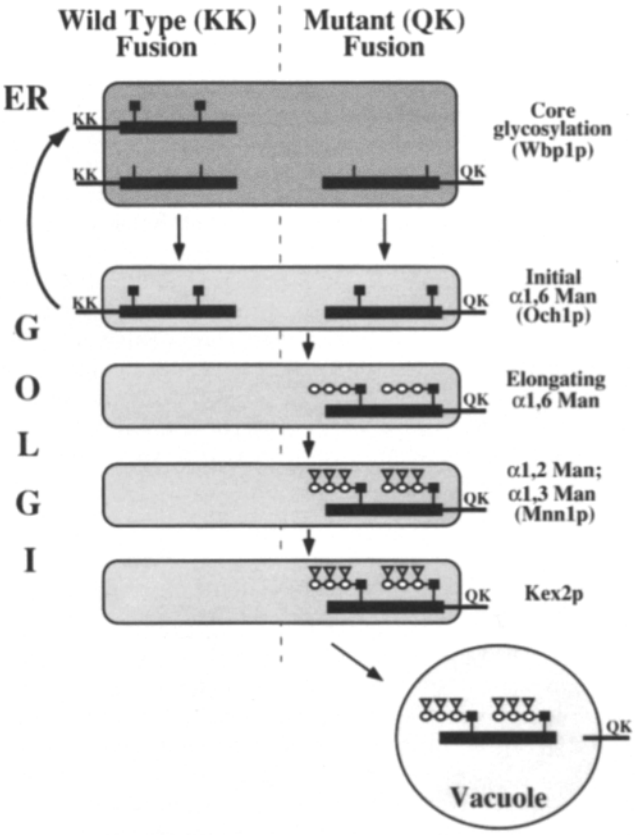

Figure 7. Model for KKXX-mediated recycling of the invertase-Wbp1 fusion protein. Both the wild-type (KK) and mutant (QK) invertase-Wbp1 fusion proteins exit the ER and transit to an early Golgi compartment which contains Och1p but not elongating α1,6 mannosyltransferase(s). In this compartment, the wild-type (KK) fusion protein acquires initial α1,6 mannose residues and then recycles back to the ER. The mutant (QK) fusion does not recycle but instead transits through the rest of the Golgi complex (thereby acquiring outer chain modification) and on to the vacuole, where it is cleaved at the invertase-Wbp1 junction. Core oligosaccharides are represented by a vertical line, initial α1,6 mannose moieties by a solid square, elongated α1,6 mannose residues by linked circles, and α1,3 residues by inverted triangles.

Och1p-containing compartment, each pass through the compartment could result in the acquisition of additional α1,6 mannose residues on the >10 potential N-linked core oligosaccharides of invertase. This would account for our observation that the KK fusion gradually increased in molecular mass over the course of a 90-min chase yet did not exhibit the hyperglycosylation indicative of modification by elongating α1,6 and α1,3 mannosyltransferases (Fig. 3). While we cannot exclude the possibility that recycling may occur from more distal Golgi compartments, the tight coupling of elongated α1,6 mannose modification (hyperglycosylation), α1,3 mannose modification, and vacuole-localized processing observed for the QK fusion makes this unlikely. The very slow outer chain modification and processing observed for a small pool of the wild-type KK fusion (long chase points) is thus likely to reflect a gradual escape from the retrieval mechanism (possibly due to the expression level of the fusion proteins) rather than retrieval from distal Golgi compartments.

Our results are also consistent with a model in which Och1p undergoes Golgi-ER cycling and consequently initiates α1,6 mannose modification of the KK fusion in the ER rather than in the Golgi. This model seems very unlikely for several reasons. First, we have shown that Och1p does not cofractionate with ER membranes (Fig. 6 *C*). Second, we observe that α1,6 mannose modification of the fusions is *SEC18*-dependent, consistent with a requirement for ER-to-Golgi transport of the fusions (in fact, the *sec18* block should result in the accumulation of newly synthesized Och1p molecules in the ER; however, these proteins are clearly unable to catalyze α1,6 mannosylation). Third, Wbp1p, which does not appear to leave the ER, is core glycosylated but not α1,6 modified (Fig. 1). Finally, it has previously been shown that the ER resident glycoprotein protein disulfied isomerase (LaMantia et al., 1991) does not receive α1,6 mannose modification either in the presence or absence of brefeldin A (Graham et al., 1993). Together with our Wbp1p results, this suggests that even if Och1p does recycle, it is not likely to be functional in the ER. α1,6 mannose modification of the KK fusion therefore first requires anterograde transport into the Golgi complex.

Och1p Defines a Novel Early Golgi Compartment

Previous studies have shown that α1,6 mannosyltransferase, α1,3 mannosyltransferase, and Kex2 endopeptidase activities can be used to separate and define distinct Golgi compartments (Franzusoff and Schekman, 1989; Graham and Emr, 1991). Initiation and elongation of α1,6 mannose chains are known to depend on the activity of at least two distinct enzymes (reviewed in Herskovics and Orlean, 1993). The elongating α1,6 mannosyltransferase(s) are unable to extend N-linked carbohydrate chains lacking the initial α1,6 mannose residue (Ballou et al., 1990; Nakayama et al., 1992; Yip et al., 1994). Our observation that the α1,6 mannose-modified KK fusion recycles without acquiring elongated α1,6 mannose moieties (Fig. 3 *A*, and Fig. 6 *C*) extends this work and provides evidence that initiation and elongation of α1,6-linked mannose chains are also likely to occur in functionally distinct Golgi compartments. As Fig. 7 illustrates, our results indicate that Och1p activity is likely to define an additional early Golgi compartment. Consistent with this notion, in vitro work has shown that conversion of pro-alpha factor from its 26-kD ER form to a 28-kD form precedes and is distinct from Golgi-specific outer chain carbohydrate modification yet does not occur until after transport vesicles are released from the ER (Bacon et al., 1989; Groesch et al., 1990). Since the 28-kD form of pro-alpha factor is α1,6 modified (Graham and Emr, 1991), this 26–28 kD conversion may also occur in a Golgi compartment containing Och1p but not elongating α1,6 mannosyltransferase(s). Consistent with our recycling model, retrieval of soluble HDEL-tagged proteins in yeast is also thought to occur from either a *cis*- or a pre-Golgi compartment (Dean and Pelham, 1990). Unlike mammalian cells, which can recycle ER proteins from *cis*-, *medial*-, and *trans*-Golgi regions (Pelham, 1988; Peter et al., 1992, Jackson et al., 1993), retrieval of both soluble and membrane proteins in yeast may thus be confined to the same early Golgi compartment which is likely to contain Och1p but not elongating α1,6 mannosyltransferases.

Retrieval-defective Fusion Proteins Are Delivered to the Vacuole

An invertase-Wbpl fusion protein with a single K→Q amino acid substitution in the dilysine motif was not recycled back to the ER; instead, it acquired outer chain α1,6- and α1,3-linked carbohydrate modification (Fig. 3) and was then rapidly transported to the vacuole where it underwent a *PEP4*-dependent proteolytic cleavage (Figs. 3 and 4). This vacuolar default transport is somewhat surprising and is distinct from events observed in mammalian cells, where disruption of the dilysine motif leads to plasma membrane expression of CD8- and CD4-KKXX chimeras (Jackson et al., 1990). Soluble yeast proteins also appear at the cell surface if they lack critical retention regions or if they are not properly targeted to the appropriate subcellular destination (Johnson et al., 1987; Hardwick et al., 1990; Valls et al., 1990). Recently, however, the vacuole has been suggested to be the default destination for several yeast late Golgi membrane proteins (e.g., Kex2p, Wilcox et al., 1992; Kex1p, Cooper and Bussey, 1992; DPAP A, Roberts et al., 1992) whose Golgi targeting/retention signals have been either altered or eliminated. Like these Golgi proteins, the invertase-Wbpl mutant chimeras contain no known vacuolar targeting signal yet are rapidly and efficiently transported to the vacuole. Because a *sec1* mutant, which blocks Golgi to plasma membrane traffic (Novick and Schekman, 1979), does not block vacuolar delivery of the invertase-Wbpl fusions, vacuolar transport is likely to occur directly from the late Golgi and not via endocytosis.

Using the *PEP4*-dependent cleavage of the invertase-Wbpl fusions to assess the relative ability of various COOH-terminal sequences to direct recycling, we have found that, similar to the mammalian dilysine recycling system (Jackson et al., 1990): (*a*) both lysine residues appear to be critical for proper functioning of the retrieval motif, and (*b*) correct positioning of the lysines relative to the COOH terminus is required for efficient recycling (Fig. 5). Changing the amino acids surrounding the motif resulted in near wild-type behavior; however, two constructs (SSKKTN and drOST) appeared to be recycled slightly more efficiently than the wild-type KK fusion, and one construct (TFKKSS) showed an intermediate phenotype. Together, these data indicate that while properly positioned lysine residues are required for recycling, efficiency of retrieval is also influenced by neighboring amino acids.

The data presented here naturally lead to speculation about both the precise role of the KKXX motif in yeast and the nature of the machinery involved in KKXX-mediated Golgi-ER recycling. Assuming that Wbplp may rarely, if ever, leave the ER following its synthesis, its KKXX motif may represent a "secondary" or redundant ER targeting signal which ensures that this essential protein, if it escapes the ER, can be retrieved to its appropriate site of function. In addition, there may be as yet unidentified yeast dilysine-containing proteins which, like the mammalian E19 adenovirus protein (Nilsson et al., 1989), are much more dependent than Wbplp on KKXX-mediated retrieval for their proper ER localization.

The retrieval mechanism which directs recycling of the invertase-Wbpl fusions is likely to involve factors which initially recognize the dilysine motif and then mediate recycling of these and other type I KKXX-containing membrane proteins back to the ER. It has recently been shown that the yeast *RER1* gene is involved in Golgi-ER retrieval of Sec12p, a type II ER membrane protein which does not contain a KKXX motif (Nishikawa and Nakano, 1993). We have found that *rer1* mutants are not defective in retrieval of the invertase-Wbpl fusion proteins (E. Gaynor and S. Emr, unpublished observations), suggesting the KKXX-specific factors may be involved in the Golgi-ER recycling pathway. Both the E19 and Wbplp cytoplasmic domains have recently been shown to bind coatomer proteins in vitro; mutations which change the lysine residues to serine abolish the interaction (Cosson and Letourneur, 1994). In mammalian systems, binding of the coatomer complex to membranes is thought to initiate budding of COP-coated vesicles implicated in ER-to-Golgi (Pepperkok et al., 1993; Peter et al., 1993) and intra-Golgi anterograde traffic (reviewed in Rothman and Orci, 1992). Retrograde vesicular transport represents a likely mechanism by which proteins could recycle between the Golgi and ER; however, this in turn requires mechanisms able to distinguish Golgi-derived vesicles involved in anterograde traffic from those participating in ER protein retrieval. The yeast γ-COP homologue, Sec21p (Hosobuchi et al., 1992; Stenbeck et al., 1992), has been shown to play a role in ER-to-Golgi protein transport (Kaiser and Schekman, 1990). Preliminary experiments analyzing the *sec21-1* ts mutant at a semipermissive temperature indicated that this coatomer mutant allele may exhibit a modest defect in Golgi-to-ER retrieval of the invertase-Wbpl fusion protein (E. Gaynor and S. Emr, unpublished observations). Further experiments will be required to address the role of coatomer in KKXX-mediated Golgi-to-ER recycling.

In this study, we have shown that the KKXX motif functions efficiently as an early Golgi-to-ER retrieval signal in yeast. We therefore hope to take advantage of the invertase-Wbpl fusion system to develop a genetic selection allowing us to screen for mutants which are specifically defective in this retention/recycling process. Identification of the genes involved and biochemical analysis of their products should help elucidate the mechanism by which the dilysine motif directs retrieval of type I membrane proteins to the ER.

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References

Ahn, K. E. Szczensa-Skorupa, and B. Kemper. 1993. The amino-terminal 29 amino acids of cytochrome P450 2C1 are sufficient for retention in the endoplasmic reticulum. *J. Biol. Chem.* 268:18726–18733.

Ammerer, G., C. P. Hunter, J. H. Rothman, H. C. Saari, L. A. Valls, and T. H. Stevens. 1986. *PEP4* gene of *Saccharomyces cerevisiae* encodes proteinase A, a vacuolar enzyme required for processing of vacuolar precursors. *Mol. Cell. Biol.* 6:2490–2499.

Bacon, R. A., A. Salminen, H. Ruohola, P. Novick, and S. Ferro-Novick. 1989. The GTP-binding protein Ypt1 is required for transport in vitro: the Golgi apparatus is defective in *ypt1* mutants. *J. Cell Biol.* 109:1015–1022.

Ballou, C. 1976. Structure and biosynthesis of the mannan component of the

yeast cell envelope. *Adv. Microb. Physiol.* 14:93–158.
Ballou, L., L. M. Hernandez, E. Alvarado, and C. E. Ballou. 1990. Revision of the oligosaccharide structures of yeast carboxypeptidase Y. *Proc. Natl. Acad. Sci. USA.* 87:3368–3372.
Cooper, A., and H. Bussey. 1992. Yeast Kex1p is a Golgi-associated membrane protein: deletions in a cytoplasmic targeting domain result in mislocalization to the vacuolar membrane. *J. Cell Biol.* 119:1459–1468.
Cosson, P., and F. Letourneur. 1994. Coatomer interaction with di-lysine endoplasmic reticulum retention motifs. *Science (Wash. DC).* 263:1629–1631.
Dean, N., and H. R. B. Pelham. 1990. Recycling of proteins from the Golgi compartment to the ER in yeast. *J. Cell Biol.* 111:369–377.
Emr, S. D., A. Vassarotti, J. Garrett, B. L. Geller, M. Takeda, and M. G. Douglas. 1986. The amino terminus of the yeast F_1-ATPase β-subunit precursor functions as a mitochondrial import signal. *J. Cell Biol.* 102:523–533.
Evan, G. I., G. K. Lewis, G. Ramsay, and J. M. Bishop. 1985. Isolation of monoclonal antibodies specific for human c-myc proto-oncogene product. *Mol. Cell. Biol.* 5:3610–3616.
Franzusoff, A., and R. Schekman. 1989. Functional compartments of the yeast Golgi apparatus are defined by the *sec7* mutation. *EMBO (Eur. Mol. Biol. Organ.) J.* 8:2695–2702.
Goldstein, A., and O. J. Lampen. 1975. β-D-Fructofuranoside fructohydrolase from yeast. *Methods Enzymol.* 42:504–511.
Graham, T. R., and S. D. Emr. 1991. Compartmental organization of Golgi-specific protein modification and vacuolar protein sorting events defined in yeast *sec18* (NSF) mutant. *J. Cell Biol.* 114:207–218.
Graham, T. R., P. A. Scott, and S. D. Emr. 1993. Brefeldin A reversibly blocks early but not late protein transport steps in the yeast secretory pathway. *EMBO (Eur. Mol. Biol. Organ.) J.* 12:869–877.
Groesch, M. E., H. Ruohola, R. Bacon, G. Rossi, and S. Ferro-Novick. 1990. Isolation of a functional vesicular intermediate that mediates ER to Golgi transport in yeast. *J. Cell. Biol.* 111:45–53.
Hanahan, D. 1983. Studies on transformation of *Escherichia coli* with plasmids. *J. Mol. Biol.* 166:557–580.
Hardwick, K. G., M. J. Lewis, J. Semenza, N. Dean, and H. R. B. Pelham. 1990. *ERD1*, a yeast gene required for the retention of luminal endoplasmic reticulum proteins, affects glycoproteins processing in the Golgi apparatus. *EMBO (Eur. Mol. Biol. Organ.) J.* 9:623–630.
Hasilik, A., and W. Tanner. 1978. Biosynthesis of the vacuolar yeast glycoprotein carboxypeptidase Y. Conversion of precursor into the enzyme. *Eur. J. Biochem.* 85:599–608.
Herskovics, A., and P. Orlean. 1993. Glycoprotein biosynthesis in yeast. *FASEB (Fed. Am. Soc. Exp. Biol.) J.* 7:540–550.
Hosobuchi, M., T. Kreis, and R. Schekman. 1992. *SEC21* is a gene required for ER to Golgi protein transport that encodes a subunit of yeast coatomer. *Nature (Lond.).* 360:603–605.
Ito, H., Y. Fudada, K. Murata, and A. Kimura. 1983. Transformation of intact yeast cells treated with alkali cations. *J. Bacteriol.* 153:163–168.
Ivessa, N. E., C. De Lemos-Chiarandini, Y. S. Tsao, A. Takatsuki, M. Adesnik, D. D. Sabatini, and G. Kreibich. 1992. O-glycosylation of intact and truncated ribophorins in brefeldin A-treated cells: newly synthesized intact ribophorins are only transiently accessible to the relocated glycosyltransferases. *J. Cell Biol.* 117:949–958.
Jackson, M. R., T. Nilsson, and P. A. Peterson. 1990. Identification of a consensus motif for retention of transmembrane proteins in the endoplasmic reticulum. *EMBO (Eur. Mol. Biol. Organ.) J.* 9:3153–3162.
Jackson, M. R., T. Nilsson, and P. A. Peterson. 1993. Retrieval of transmembrane proteins to the endoplasmic reticulum. *J. Cell Biol.* 121:317–333.
Johnson, L. M., V. A. Bankaitis, and S. D. Emr. 1987. Distinct sequence determinants direct intracellular sorting and modification of a yeast vacuolar protein. *Cell.* 48:875–885.
Julius, D., R. Schekman, and J. Thorner. 1984. Glycosylation and processing of prepro-α-factor through the yeast secretory pathway. *Cell.* 36:309–318.
Kaiser, C. A., and R. Schekman. 1990. Distinct sets of *SEC* genes govern transport vesicle formation and fusion early in the secretory pathway. *Cell.* 61:723–733.
Klionsky, D. J., L. M. Banta, and S. D. Emr. 1988. Intracellular sorting and processing of a yeast vacuolar hydrolase: proteinase A propeptide contains vacuolar targeting information. *Mol. Cell Biol.* 8:2105–2116.
Klionsky, D. J., and S. D. Emr. 1989. Membrane protein sorting: biosynthesis, transport and processing of yeast vacuolar alkaline phosphatase. *EMBO (Eur. Mol. Biol. Organ.) J.* 8:2241–2250.
LaMantia, M., T. Miura, H. Tachikawa, H. A. Kaplan, W. J. Lennarz, and T. Mizunaga. 1991. Glycosylation site binding protein and protein disulfide isomerase are identical and essential for cell viability in yeast. *Proc. Natl. Acad. Sci. USA.* 88:4453–4457.
Lewis, M. J., and H. R. B. Pelham. 1990. A human homologue of the yeast HDEL receptor. *Nature (Lond.).* 348:162–163.
Lewis, M. J., and H. R. B. Pelham. 1992. Ligand-induced redistribution of a human KDEL receptor from the Golgi complex to the endoplasmic reticulum. *Cell.* 68:353–364.
Lewis, M. J., D. J. Sweet, and H. R. B. Pelham. 1990. The *ERD2* gene determines the specificity of the luminal ER protein retention system. *Cell.* 61:1359–1363.
Marcusson, E. G., B. F. Horazdovsky, J. L. Cereghino, E. Gharakhanian, and S. D. Emr. 1994. The sorting receptor for yeast vacuolar carboxypeptidase Y is encoded by *VPS10*. *Cell.* 74:579–586.
Miller, J. 1972. Book experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 431–435.
Munro, S., and H. R. B. Pelham. 1987. A COOH-terminal signal prevents secretion of luminal ER proteins. *Cell.* 48:899–907.
Nakanishi-Shindo, Y., K. Nakayama, A. Tanaka, Y. Toda, and Y. Jigami. 1994. Structure of the N-linked oligosaccharides that show the complete loss of α-1,6-polymannose outer chain from *och1*, *och1 mnn1*, and *och1 mnn1 alg3* mutants of *Saccharomyces cerevisiae*. *J. Biol. Chem.* 268:26338–26345.
Nakayama, K. N. Takeshi, Y. Shimma, J. Kuromitsu, and Y. Jigami. 1992. *OCH1* encodes a novel membrane bound mannosyltransferase: outer chain elongation of asparagine-linked oligosaccharides. *EMBO (Eur. Mol. Biol. Organ.) J.* 11:2511–2519.
Nilsson, T., M. Jackson, and P. A. Peterson. 1989. Short cytoplasmic sequences serve as retention signals for transmembrane proteins in the endoplasmic reticulum. *Cell.* 58:707–718.
Nishikawa, S. and A. Nakano. 1993. Identification of a gene required for membrane protein retention in the early secretory pathway. *Proc. Natl. Acad. Sci. USA.* 90:8179–8183.
Norrander, J., T. Kempe, and J. Messing. 1983. Construction of improved M13 vectors using oligodeoxynucleotide-directed mutagenesis. *Gene (Amst.).* 26:101–106.
Novick, P., C. Field, and R. Schekman. 1980. Identification of 23 complementation groups required for post-translational events in the yeast secretory pathway. *Cell.* 21:205–215.
Novick, P., and R. Schekman. 1979. Secretion and cell-surface growth are blocked in a temperature-sensitive mutant of *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. USA.* 76:1858–1862.
Pääbo, S., B. M. Bhat, W. S. M. Wold, and P. A Peterson. 1987. A short sequence in the COOH-terminus makes an adenovirus membrane glycoprotein a resident of the endoplasmic reticulum. *Cell.* 50:311–317.
Palade, G. 1975. Intracellular aspects of the process of protein synthesis. *Science (Wash. DC).* 189:347–357.
Pelham, H. R. B. 1988. Evidence that luminal ER proteins are sorted from secreted proteins in a post-ER compartment. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:913–918.
Pelham, H. R. B., K. G. Hardwick, and M. J. Lewis. 1988. Sorting of soluble ER proteins in yeast. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:1757–1762.
Pepperkok, R., J. Scheel, H. Horstmann, H. P. Hauri, G. Griffiths, and T. E. Kreis. 1993. β-COP is essential for biosynthetic membrane transport from the endoplasmic reticulum to the Golgi complex *in vivo*. *Cell.* 74:71–82.
Peter, F., P. Nguyen Van, and H. D. Soling. 1992. Different sorting of Lys-Asp-Glu-Leu proteins in rat liver. *J. Biol. Chem.* 267:10631–10637.
Peter, F., H. Plutner, H. Zhu, T. E. Kreis, and W. E. Balch. 1993. β-COP is essential for transport of protein from the endoplasmic reticulum to the Golgi in vitro. *J. Cell Biol.* 122:1155–1167.
Pfeffer, S. R., and J. E. Rothman. 1987. Biosynthetic protein transport and sorting by the endoplasmic reticulum and Golgi. *Annu. Rev. Biochem.* 56: 829–852.
Redding, K., C. Holcomb, and R. S. Fuller. 1991. Immunolocalization of Kex2 protease identifies a putative late Golgi compartment in the yeast *Saccharomyces cerevisiae*. *J. Cell Biol.* 113:527–538.
Roberts, C. J., S. F. Nothwehr, and T. H. Stevens. 1992. Membrane protein sorting in the yeast secretory pathway: evidence that the vacuole may be the default compartment. *J. Cell Biol.* 119:69–83.
Robinson, J. S., D. J. Klionsky, L. M. Banta, and S. D. Emr. 1988. Protein sorting in *Saccharomyces cerevisiae*: isolation of mutants defective in the delivery and processing of multiple vacuolar hydrolases. *Mol. Cell. Biol.* 8:4936–4948.
Romero, P. A., and A. Herscovics. 1989. Glycoprotein biosynthesis in *Saccharomyces cerevisiae*. Characterization of alpha-1,6-mannosyltransferase which initiates outer chain formation. *J. Biol. Chem.* 264:1946–1950.
Rothman, J. E., and L. Orci. 1992. Molecular dissection of the secretory pathway. *Nature (Lond.).* 355:409–415.
Rothstein, R. 1991. Targeting, disruption, replacement, and allele rescue: integrative DNA transformation in yeast. *Methods Enzymol.* 194:281–301.
Semenza, J. C., K. G. Hardwick, N. Dean, and H. R. B. Pelham. 1990. *ERD2*, a yeast gene required for the receptor-mediated retrieval of luminal ER proteins from the secretory pathway. *Cell.* 61:1349–1357.
Sherman, F., G. R. Fink, and L. W. Lawrence. 1979. Book methods in yeast genetics: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Stenbeck, G., R. Schreiner, D. Herrmann, S. Auerbach, F. Lottspeich, J. E. Rothman, and F. T. Wieland. 1992. γ-COP, a coat subunit of non-clathrin-coated vesicles with homology to Sec21p. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 314:195–198.
Stevens, T., B. Esmon, and R. Schekman. 1982. Early stages in the yeast secretory pathway are required for transport of carboxypeptidase Y to the vacuole. *Cell.* 30:439–448.
Szczesna-Skorupa, E., and B. Kemper. 1993. An N-terminal glycosylation signal on cytochrome P450 is restricted to the endoplasmic reticulum in a luminal orientation. *J. Biol. Chem.* 268:1757–1762.
te Heesen, S., B. Janetzky, L. Lehle, and M. Aebi. 1992. The yeast *WBP1* is

essential for oligosaccharyl transferase activity *in vivo* and *in vitro*. *EMBO (Eur. Mol. Biol. Organ.) J.* 11:2071–2075.

te Heesen, S., R. Knauer, L. Lehle, and M. Aebi. 1993. Yeast Wbp1p and Swp1p form a protein complex essential for oligosaccharyl transferase activity. *EMBO (Eur. Mol. Biol. Organ.) J.* 12:279–284.

te Heesen, S., R. Rauhut, R. Aebersold, J. Abelson, M. Aebi, and M. W. Clark. 1991. An essential 45 kD yeast transmembrane protein reacts with anti-nuclear pore antibodies: purification of the protein, immunolocalization and cloning of the gene. *Eur. J. Cell Biol.* 56:8–18.

Trimble, R. B., and F. Maley. 1977. Subunit structure of external invertase from *Saccharomyces cerevisiae*. *J. Biol. Chem.* 252:4409–4412.

Trimble, R. B., F. Maley, and F. K. Chu. 1983. Glycoprotein biosynthesis in yeast. Protein conformation affects processing of high mannose oligosaccharides on carboxypeptidase Y and invertase. *J. Biol. Chem.* 258:2562–2567.

Valls, L. A., J. R. Winther, and T. H. Stevens. 1990. Yeast carboxypeptidase Y vacuolar targeting signal is defined by four propeptide amino acids. *J. Cell Biol.* 111:361–368.

Vida, T. A., T. R. Graham, and S. D. Emr. 1990. In vitro reconstitution of intercompartmental protein transport to the yeast vacuole. *J. Cell Biol.* 111: 2871–2884.

Wilcox, C. A., K. Redding, R. Wright, and R. S. Fuller. 1992. Mutation of a tyrosine localization signal in the cytosolic tail of yeast Kex2 protease disrupts Golgi retention and results in default transport to the vacuole. *Mol. Biol. Cell.* 3:1353–1371.

Wilson, D. W., C. A. Wilcox, G. C. Flynn, E. Chen, W. J. Kuang, W. J. Henzel, M. R. Block, A. Ullrich, and J. E. Rothman. 1989. A fusion protein required for vesicle-mediated transport in both mammalian cells and yeast. *Nature (Lond.)*. 339:355–359.

Yip, C. L., S. K. Welch, F. Klebl, T. Gilbert, P. Seidel, F. Grant, P. J. O'Hara, and V. L. MacKay. 1994. Cloning and analysis of the Saccharomyces cerevisiae *MNN9* and *MNN1* genes required for complex glycosylation of secreted proteins. *Proc. Natl. Acad. Sci. USA*. 91:2723–2727.