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Kex2-dependent invertase secretion as a tool to study the targeting of transmembrane proteins which are involved in ER→Golgi transport in yeast

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Mutants were isolated that are defective in the retention of a transmembrane protein in the early secretory compartments in yeast. A series of hybrid proteins was tested for their use in the selection of such mutants. Each of these hybrid proteins consisted of a type II transmembrane protein (N_{in}/C_{out}) and invertase (Suc2) as a reporter separated by a peptide linker containing a cleavage site for the Golgi protease Kex2. The integral membrane proteins which were used—Sec12p, Sec22/Sly2p or Bet1/Sly12p—are all known to be required for ER→Golgi transport in yeast. Invertase was readily cleaved from the fusions containing Sec22/Sly2p or Bet1/Sly12p as the membrane anchoring part. In contrast, Sec12–invertase expressing transformants required mutations in either of two different genes for Kex2-dependent invertase secretion. The mutant showing the stronger retention defect (*rer1*) was used to clone the corresponding gene. *RER1* represents the first reading frame left of the centromere of chromosome III. Cells carrying a disruption of the *RER1* gene are viable and show the same mislocalizing phenotype as the original mutants. The Rer1 protein, as deduced from the nucleotide sequence, contains four transmembrane domains. It has been suggested before that Sec12p cycles between the ER and the *cis*-Golgi compartment. Some results obtained by using Sec12–invertase and the *rer1* mutants resemble observations on the retention of Golgi-resident glycosyltransferases and viral proteins in mammalian cells. For instance, retention of Sec12–invertase is non-saturable and the membrane-spanning domain of Sec12p seems to constitute an important targeting signal.

Key words: hybrid genes/*RER1*/retention mutants/*Saccharomyces cerevisiae*/transmembrane proteins

Introduction

Proteins residing in the endoplasmic reticulum (ER) or the Golgi complex must be sorted from proteins that are transported to the plasma membrane, the lysosome or storage vesicles. It is widely accepted that vesicles serve as carriers during these transport events (Rothman and Orci, 1992). Therefore, exclusion of proteins from the sites of vesicle budding may prevent resident components from leaving their normal position. Pelham and co-workers, however, showed that soluble ER-resident proteins cycle between ER and the Golgi compartment (Pelham, 1988; Dean and Pelham, 1990). The signal for retrieval from the Golgi is a conserved C-terminal tetrapeptide segment: KDEL in mammals or HDEL in *Saccharomyces cerevisiae* (Munro and Pelham, 1987; Pelham *et al.*, 1988). The receptor for this targeting signal is an integral membrane protein called Erd2p in yeast and hERD2 in humans (Lewis and Pelham, 1990; Semenza *et al.*, 1990).

It is conceivable that the targeting of integral membrane proteins of the ER or the late Golgi also involves retrieval from later compartments (Payne and Schekman, 1989; Pearse and Robinson, 1990; Hsu *et al.*, 1991; Lewis and Pelham, 1992; Jackson *et al.*, 1993; Wilsbach and Payne, 1993). Particular sequence motifs required for recycling of integral membrane proteins were identified in several cases. Type I (N_{out}/C_{in}) and type II (N_{in}/C_{out}) transmembrane proteins located in the ER of mammalian cells require either two lysine residues close to their C-terminus or two arginines close to their N-terminus for proper localization (Jackson *et al.*, 1990; Nilsson *et al.*, 1991; Schutze *et al.*, 1994). The critical sequence in the cytoplasmic domains of proteins cycling between late Golgi and endosomes or late Golgi and plasma membrane always contain important aromatic residues (Pearse and Robinson, 1990; Wilcox *et al.*, 1992; Humphrey *et al.*, 1993; Nothwehr *et al.*, 1993). Recently, two integral membrane proteins, the products of the *SEC20* and the *SED4* gene, were identified in yeast. They carry the HDEL signal and may therefore cycle between the ER and the Golgi (Hardwick *et al.*, 1992; Sweet and Pelham, 1992).

A large number of transmembrane proteins are thought to direct the attachment of transport vesicles to the proper target membrane either as vesicle-specific or target membrane-specific receptors (Söllner *et al.*, 1993). The vesicle-specific receptors known so far belong to the family of VAMP/synaptobrevin-like proteins, while many of the target membrane-specific receptors belong to the syntaxin family. Members of both groups carry a single C-terminal membrane-anchor segment, and the lack of a potential N-terminal leader sequence suggests that they are type II transmembrane proteins (Kutay *et al.*, 1993). Accessibility to proteases was used to confirm this topology experimentally (Südhof *et al.*, 1989; Newman *et al.*, 1992). All members of these two families of membrane proteins are in fact essential for distinct steps of vesicle transport (Bennett and Scheller, 1993).

We wanted to compare the possible targeting mechanism of two of the putative vesicle-specific receptors in yeast, Sec22/Sly2p and Bet1/Sly12p, with that of a structurally similar protein, Sec12p. Sec22/Sly2p and Bet1/Sly12p share some similarities with synaptobrevin/VAMP and are required for the fusion of ER-derived transport vesicles

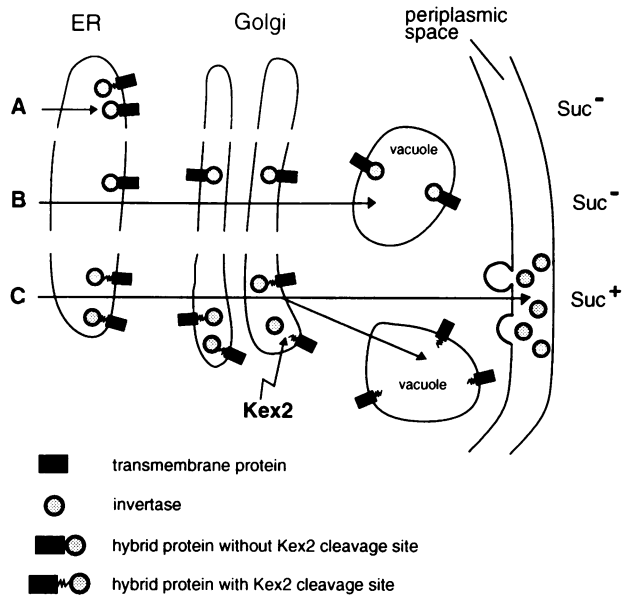

Fig. 1. Outline of the intracellular targeting and transport events that either prevent or give way to invertase secretion from the different Sec12−, Sec22− or Bet1−invertase hybrid proteins. (**A**) Proteins are maintained in the ER and cannot reach the Kex2 compartment; no invertase secretion (examples: Sec12−invertase and Sec12−a·f−invertase in wild-type cells). (**B**) Kex2-resistant chimeric proteins that reach the Kex2 compartment do not result in invertase appearing at the cell surface. The uncleaved proteins are transported along the default pathway for transmembrane proteins to the vacuole (examples: Sec22−invertase and Bet1−invertase in wild-type cells, and Sec12−invertase in *rer* mutants). (**C**) Trimeric proteins with Kex2 cleavage site and reaching the Kex2 compartment lead to invertase secretion (examples: Sec22− and Bet1−a·f−invertase in wild-type cells and Sec12−a·f−invertase in *rer* mutants).

with the Golgi membranes (Novick *et al.*, 1981; Newman and Ferro-Novick, 1987; Dascher *et al.*, 1991; Ossig *et al.*, 1991; Newman *et al.*, 1992). In contrast, *SEC12* is required for the formation of transport vesicles at the ER *in vivo* as well as *in vitro* (Kaiser and Schekman, 1990; Oka *et al.*, 1991; Rexach and Schekman, 1991). It acts as a nucleotide exchange factor for the small GTP-binding protein Sar1 (Barlowe and Schekman, 1993). Sec12p is a type II transmembrane protein localized in an early compartment of the yeast secretory pathway, presumably cycling between the Golgi compartment and the ER (Nakano *et al.*, 1988; d'Enfert *et al.*, 1991).

We constructed a series of trimeric genes consisting of either *SEC22/SLY2, BET1/SLY12* or *SEC12*, the invertase gene (*SUC2*) and an intervening segment that encodes a peptide with a cleavage site for the late Golgi-localized Kex2 protease. We reasoned that retention of any of these hybrid proteins in an early secretory compartment may prevent Kex2 from cleaving the invertase part from these chimeras. Cleavage of the hybrid proteins by Kex2 and subsequent secretion of the reporter enzyme would indicate the appearance of the hybrid protein in the late Golgi. Thus one would be able to select mutants carrying a retention defect by means of their Suc$^+$ phenotype (see Figure 1 for an outline of the approach).

We found that in cells expressing the *SEC12*−α·*f*−*SUC2* hybrid gene, Kex2 cleavage could be used very conveniently to select mutants that fail to retain the hybrid protein in an early secretory compartment. Reversal of the Suc$^+$ phenotype of one of these mutants was used to clone the corresponding gene from a genomic library. Since the extent of the retention defects could be assessed very easily, some results obtained with different hybrid genes may in addition give some clues on the actual retention mechanism involved.

Results

Characterization of the hybrid proteins

Figure 2 shows schematic representations of the hybrid proteins used in this study. The *SEC12*−*SUC2* hybrid gene (Figure 2C) was used previously by d'Enfert *et al.* (1991) who demonstrated that the hybrid protein can functionally replace the wild-type Sec12 protein. Analogous chimeric genes were constructed using *SEC22/SLY2* and *BET1/SLY12* (see Figure 8 for details of the plasmid construction).

For the convenient detection of the different hybrid proteins by Western blot analysis, we planned to use the monoclonal antibody 9E10. This antibody recognizes a 10 residue long peptide segment derived from the c-*myc* oncogene (Evan *et al.*, 1985; Pelham, 1988). A pair of annealed oligonucleotides encoding this stretch of amino acids plus a Kex2 cleavage site were inserted into the *Hin*dIII site in front of the *SUC2* sequences (see Figure 8). The sequence encoding the Kex2 cleavage site (-Tyr-Lys-Arg↓-Glu-Ala-Glu-Ala-) was derived from the *MFα1* gene. *MFα1* encodes a precursor protein which is translocated through the ER membrane and proteolytically processed by several proteases to give the mature α-factor pheromone (Fuller *et al.*, 1988). The Kex2-mediated excision of the four tandem repeats from this precursor occurs in a late Golgi compartment (Redding *et al.*, 1991). The Kex2 endopeptidase cleaves on the carboxyl side of the Lys-Arg sequence present in the spacer between the different α-factor repeats (Julius *et al.*, 1984; Achstetter and Wolf, 1985). Cleavage by this protease was shown to be a suitable indicator for the passage of a vacuole-targeted hybrid protein through the late Golgi compartment (Graham and Emr, 1991). Therefore, the analogous proteolytic processing of transmembrane proteins, like those shown in Figure 2, should release the reporter enzyme invertase as soon as it arrives in the late Golgi (see also Figure 1). To obtain reasonable amounts of protein that could be detected by immunoblot analysis and for the easy quantification of invertase activities, the *SEC12*- and *BET1/SLY12*-derived hybrid genes were placed under the control of the *CYC1* promoter (columns marked with *c* and *d* in Figure 2; see also Figure 8 and Materials and methods for details). The products of these chimeric genes containing the segments coding for the c-*myc* tag and the Kex2 cleavage site were named Sec12−, Sec22− and Bet1−α·f−invertase, respectively.

Sec12−α·f−invertase appeared as a single protein of 135 kDa in wild-type cells (Figure 3A, first lane). This is the expected molecular mass of the core-glycosylated form of this protein (d'Enfert *et al.*, 1991). In extracts from wild-type cells expressing the *SEC22*−α·*f*−*SUC2* hybrid gene, only the small product of the Kex2 cleavage (~25 kDa) consisting of Sec22/Sly2p and the c-*myc* epitope was visible on Western blots (Figure 3A, second lane). This band is also recognized by anti-Sec22/Sly2p antibodies.

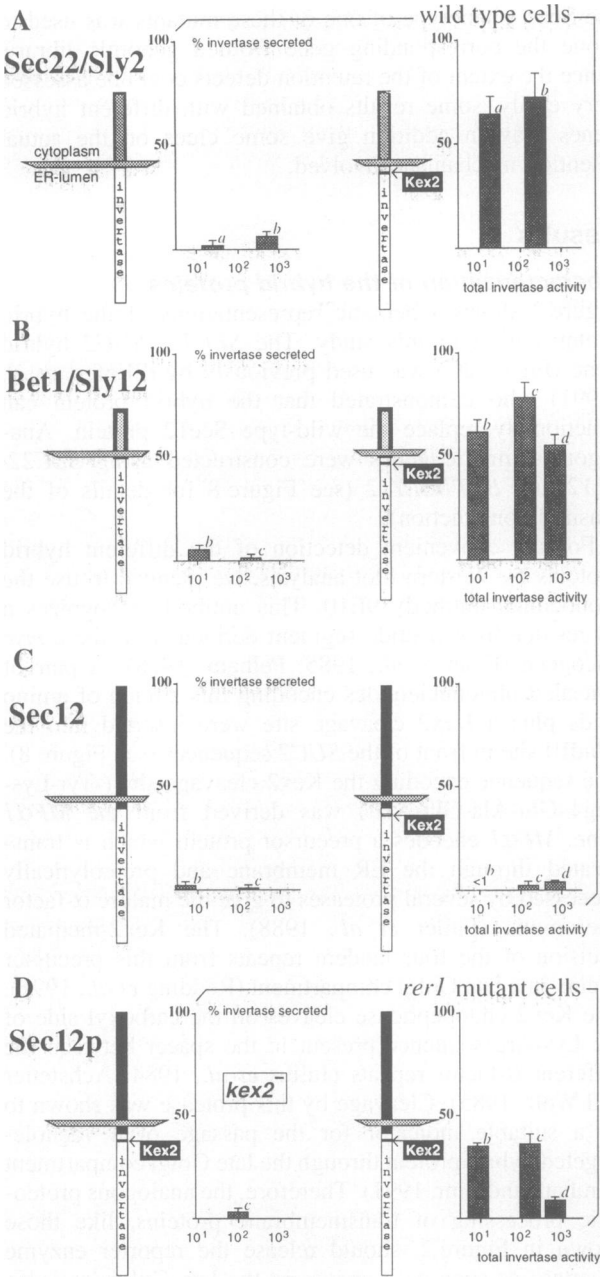

Fig. 2. Kex2-dependent invertase secretion of wild-type (**A**–**C**) and mutant cells (**D**) producing various hybrid proteins at different levels. Schematic representations of the different chimeric proteins are shown on the left side of each panel. The results (*y*-scale) are presented as a percentage of total invertase that is secreted. Each value represents the mean of at least three independent measurements. The standard deviation is also indicated. The *x*-scale shows the total invertase activity obtained with the different expression systems. One invertase unit is defined as the amount (in nmol) of glucose produced per min by 1 OD_{600} unit of cells. The type of vector used to express the different hybrid genes in wild-type strains or *rer1* mutants is indicated by letters above each column (*a*: centromeric vector, original promoter; *b*: multicopy vector, original promoter; *c*: centromeric vector, *CYC1* promoter; *d*: multicopy vector, *CYC1* promoter). *kex2*$^-$ indicates that a *kex2* null mutant was used.

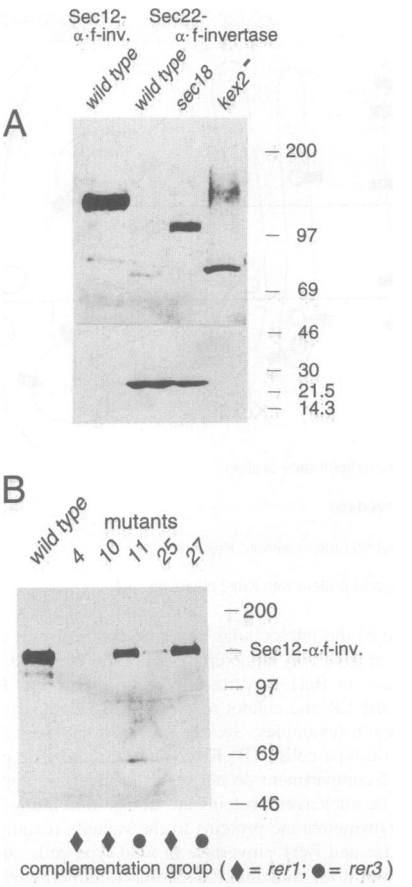

Fig. 3. (**A**) Comparison of Sec12– and Sec22–a·f–invertase hybrid proteins by immunoblot analysis. Total protein extracts were prepared from wild-type cells (SEY6210; first and second lane), a *sec18* mutant strain shifted to the restrictive temperature of 37°C for 2 h (third lane) and *kex2*$^-$ cells (SEY6210; *kex2*::*TRP1*; fourth lane). These strains contained either plasmid pCSEC12^{KR} (CEN4-ARS1, *URA3*, *CYC1*–*SEC12*–α·*f*–*SUC2*; first lane) or pSSE14^{KR} (2μ, *URA3*, *SEC22*–α·*f*–*SUC2*; second to fourth lanes). The equivalent of 1 OD_{600} unit of cells was analysed by immunoblotting using the monoclonal antibody 9E10. Samples were fractionated by SDS–PAGE with 8 or 12% polyacrylamide (upper and lower part, respectively). The molecular mass is shown in kDa. (**B**) Initial characterization of Sec12–a·f–invertase mislocalizing mutants by immunoblot analysis. Total cell extracts from wild-type and mutant cells containing plasmid pCSEC12^{KR} were analysed as in (A). Strains, relevant genotypes and the percentage of total invertase secreted were as follows: SEY6210 (wild-type; 2%), C22.*4*-4A (*rer1-4*; 42%), C21.*10*-5A (*rer1-10*; 39%), C21.*11*-5C (*rer3-11*; 11%), C21.*25*-1C (*rer1-25*; 34%) and C21.*27*-2D (*rer3-27*; 6%). Note that mutants 10 and 25 and mutants 11 and 27 each originated from the same experiment. Therefore, we cannot rule out that the mutants are identical.

To detect full-length Sec22–α·f–invertase it was either necessary to express the hybrid gene in a *sec18* mutant or *kex2*$^-$ cells. Blocking ER→Golgi transport in *sec18-1* cells at the non-permissive temperature led to the accumulation of the core-glycosylated hybrid protein (110 kDa; Figure 3A, third lane). In *kex2*$^-$, the fully glycosylated form can be detected as a heterogeneous smear at ~150 kDa as well as the unglycosylated protein that may have failed to be translocated properly (Figure 3A, fourth lane). Even at the highest expression levels, it was very difficult to detect Bet1–α·f–invertase by immunoblot analysis, consistent with the finding of Newman *et al.* (1992) who observed that Bet1/Sly12 is easily susceptible to proteolysis in wild-type cells. Again, only in *sec18* mutants incubated at 37°C could a band be seen that represents the core-glycosylated form of Bet1–α·f–invertase (data not shown). These initial findings suggested that of the three hybrid proteins, only Sec12–α·f–invertase is efficiently retained in an early secretory compartment.

Kex2-dependent invertase secretion

To confirm the results obtained by the immunoblot analysis, the percentage of invertase secreted by transformants expressing the hybrid genes at different levels was measured. In the absence of the peptide segment that encodes the Kex2 cleavage site, very little invertase was secreted (Figure 2A, B and C, left panels). Different results were obtained in the case of the Kex2-sensitive chimeras. About 70% of total invertase was secreted by cells expressing *SEC22–α·f–SUC2* or *BET1–α·f–SUC2* (Figure 2A and B, right panels), but <4% by those expressing *SEC12–α·f–SUC2* (Figure 2C, right panel). The total amounts of invertase activities varied considerably with the different promoters or vectors. However, in wild-type cells the ratio of secreted to total invertase was almost constant (Figure 2A, B and C).

The level of Kex2-dependent invertase secretion by Sec22– and Bet1–α·f–invertase expressing cells is comparable to that observed with a soluble hybrid protein that consists of the targeting sequence of the vacuolar hydrolase CPY, a segment of the α-factor precursor containing the Kex2 cleavage site and invertase (Graham and Emr, 1991). Very likely, Kex2 cleavage of CPY–α·f–invertase as well as Sec22– and Bet1–α·f–invertase, followed by secretion of soluble invertase, compete with the delivery of the uncleaved protein to the vacuole (compare Figure 1B and C). According to a recently established model, transport to the vacuole may represent the default pathway for integral membrane proteins in yeast (Cooper and Bussey, 1992; Roberts *et al.*, 1992; Wilcox *et al.*, 1992). Thus, without Kex2 cleavage all chimeric transmembrane proteins mislocalized beyond the Golgi would be delivered to the vacuole, and no invertase would arrive in the periplasm (see Figure 2A and B). In summary, Kex2-dependent invertase secretion may represent a simple method to distinguish between transmembrane proteins that are retained in the early part of the yeast secretory system and those which reach the late Golgi.

Isolation of mutants that fail to retain Sec12–α·f–invertase in the ER

We used Kex2-dependent invertase secretion to select mutants that mislocalize Sec12–α·f–invertase. Since yeast cannot take up sucrose, invertase must be secreted to enable the cells to ferment this carbon source. Cells carrying a deletion of the chromosomal *SUC2* gene (*suc2-Δ9*) and producing Sec22– or Bet1–α·f–invertase in sufficient amounts are able to grow with sucrose as sole carbon source (Suc^+). In contrast, *suc2-Δ9* cells expressing the *SEC12–α·f–SUC2* hybrid gene from the *CYC* promoter on a centromeric plasmid are still Suc^-. *SEC12–α·f–SUC2* expressing cells were subjected to UV mutagenesis. In several independent experiments, mutants were selected that were able to grow with sucrose as sole carbon source. Genetic analysis demonstrated that these isolates fell into two complementation groups. All mutations were recessive. Invertase secretion was measured to estimate the extent of the retention defect. Mutants belonging to complementation group I secreted between 34 and 42% of total invertase. The amount of invertase secreted by members of complementation group II varied between 6 and 11%. These data correlate well with the amount of core-glycosylated Sec12–α·f–invertase hybrid protein detectable by immunoblot analysis (Figure 3B). In mutants that secreted up to 40% of the total invertase almost no core-glycosylated hybrid protein could be detected, while a significant amount of the core-glycosylated Sec12–α·f–invertase hybrid protein was still present in those mutants that secreted less invertase. Invertase secretion by both types of mutants was strictly Kex2 dependent. First, mutants harbouring the *SEC12–SUC2* instead of the *SEC12–α·f–SUC2* gene were Suc^-. Second, disrupting the *KEX2* gene in two representative mutants reduced the invertase secretion to 4 and 1%, respectively (Figure 2D, left panel). The *KEX2* deletion did not restore the normal amount of Sec12–α·f–invertase that is found in wild-type cells (data not shown). We took this as first evidence that it is the hybrid protein that is mislocalized in the mutants and not the protease.

While this work was in progress, Nishikawa and Nakano (1993) isolated retention mutants by a similar approach. Their selection scheme involved a Sec12–α-factor hybrid protein. Defects in two different genes (*RER1* and *RER2*) resulted in the secretion of the α-factor pheromone. We crossed an *rer1-2* and an *rer2-2* mutant which carry a *suc2* null allele with our mutants. Diploids made from a mutant of complementation group I (C21.25-1C) and an *rer1-2* mutant (SNHS6-12B; Nishikawa and Nakano, 1993) grew well on sucrose plates and secreted invertase. This indicates that the same gene is affected in these mutants. Final proof for this came from the cloning of the *RER1* gene (see below). In contrast, mutants belonging to our complemention group II (strain C21.27-2D) can complement the *rer2-2* mutant (HS023-3B; Nishikawa and Nakano, 1993) showing that different genes are defective in these mutants. In accordance with the nomenclature used by Nishikawa and Nakano (1993), we would like to name the corresponding genes *RER1* and *RER3*. Many of the experiments described below were performed using *rer1* as well as *rer3* mutants. However, we will concentrate on *rer1* since its stronger retention defect made it easier to characterize the retention defect and to clone the gene.

RER1 encodes a novel protein with four putative transmembrane domains

Several plasmids were obtained from a genomic library which were able to suppress the Kex2-dependent invertase secretion of a *SEC12–α·f–SUC2* expressing *rer1-25* mutant. The library was constructed using the yeast multicopy vector YEp13 and yeast genomic DNA partially digested with *Sau*3A (Broach *et al.*, 1979; Dascher *et al.*, 1991). Two of the plasmids we obtained induced an accumulation of core-glycosylated Sec12–α·f–invertase protein as found in wild-type cells. Deleting parts of the original inserts and subcloning showed that the complementing activity was located on a 1.7 kb *Sau*3A–*Nhe*I fragment (Figure 4A). Sequencing revealed that this fragment was fully contained in a 2.7 kb *Xba*I–*Nhe*I fragment derived from the second isolate. Both fragments maintained their *rer1* complementing activity on the centromeric vector pRS315 (Sikorski and Hieter, 1989) and were able to reduce the invertase secretion of *rer1* mutants to ~8%. This incomplete complementation may be due to the fact that even under selective conditions a significant part of the cell population contains no plasmid (Sikorski

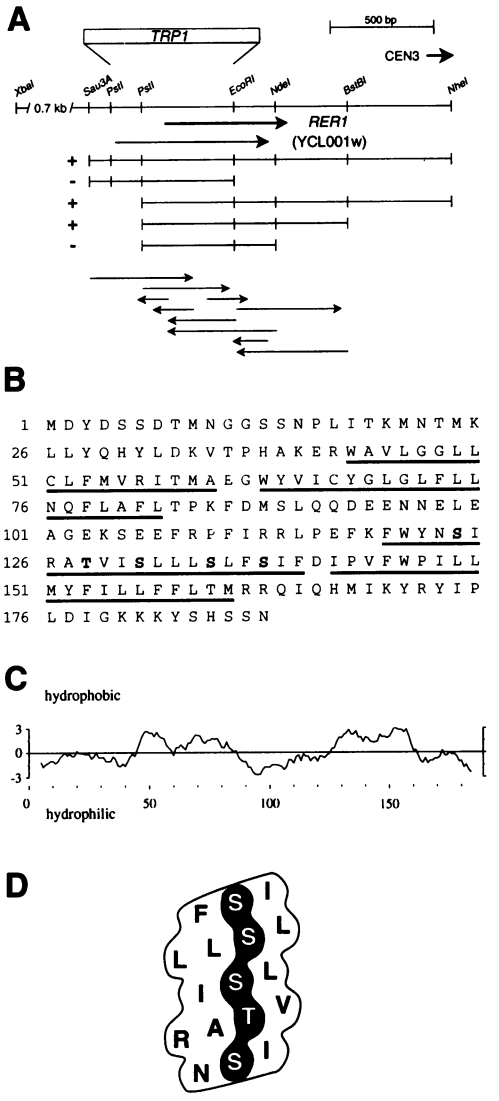

Fig. 4. (**A**) Restriction map of the *RER1* gene and schematic representation of the deletions that were made to localize the complementing activity. + indicates complementation of the *rer1-25* defect (resulting in a Suc$^-$ phenotype of transformants expressing *SEC12−α-f−SUC2*); − indicates lack of complementation (Suc$^+$ phenotype). Arrows below the restriction map show the *RER1* coding region and the position of the YCL001w reading frame (Oliver *et al.*, 1992). Codon 1 of the *RER1* reading frame corresponds to codon 80 of YCL001w. The 3′ end of *RER1* extends 89 bp further downstream of YCL001w. Arrows below the deletion maps illustrate the sequencing strategy. (**B**) Amino acid sequence of the predicted Rer1 protein. Apart from the differences at the ends of the gene, the deduced Rer1 protein differs from the YCL001w ORF from position 109 to 124, at position 136 and 141, and from position 145 to 160. The putative membrane-spanning domains are indicated by underlining. Bold letters represent the serine and threonine residues that are highlighted in (D). The DNA sequence was submitted to the EMBL database (accession number D28552; K.Sato and A.Nakano, personal communication). (**C**) Hydropathy blot of the putative Rer1 protein according to Kyte and Doolittle (1985) with a window setting of 9. (**D**) View of part of the third transmembrane domain of the Rer1 protein showing the ordered array of four serine residues and one threonine residue along one side of the putative α-helix.

and Hieter, 1989). Screening the databases with the initial sequencing results showed that the *rer1* complementing fragment resides on chromosome III, whose entire sequence is known (Oliver *et al.*, 1992). The 1.7 kb *Sau*3A−*Nhe*I fragment contains only one open reading frame, called YCL001w, the first reading frame on the left arm of this chromosome.

Tetrad analysis confirmed that *RER1* is located near the centromere of chromosome III and it is linked to the *LEU2* gene, confirming the observations made by Nishikawa and Nakano (1993). Further evidence that YCL001w represents *RER1* came from gene disruption experiments and the standard genetic tests described in Materials and methods. Cells carrying the disrupted YCL001w reading frame (see Figure 4A) behave like the original *rer1* mutants in all tests we performed. Obviously, this gene is neither necessary for vegetative growth nor for the germination of spores.

Attempts to express the part of YCL001w encoding the hydrophilic N-terminus in bacterial expression systems failed. Sequencing of the subcloned polymerase chain reaction (PCR) products revealed that in each case bp 233 of YCL001w was missing. At the same time we were informed that there are several discrepancies between the *rer1* complementing sequence and the published sequence of chromosome III (K.Sato and A.Nakano, personal communication). A number of sequencing errors were clustered around the 3′ end of YCL001w. Sequencing of the original clone confirmed that the *rer1* complementing sequences isolated in A.Nakano's and our laboratory were identical (K.Sato and A.Nakano, personal communication). We also established that the *rer1* complementing activity lies closer to the centromere of chromosome III than YCL001w (Figure 4A).

This reading frame encodes a predicted protein of 188 amino acids (Figure 4B). Analysis of the primary structure of this putative protein according Kyte and Doolittle (1982) suggests that Rer1p carries four transmembrane domains (Figure 4C). Since no N-terminal signal sequence is present, the three larger hydrophilic segments of the protein may be oriented towards the cytoplasm, and only very few residues may face the luminal side of the membrane compartments. The Rer1p protein exhibits no significant sequence similarity to any protein in the databases.

Characterization of the mislocalization defect

The specificity of the retention defects was first examined by following BiP/Kar2p secretion. Two ER retention mutants (*erd1* and *erd2*) were isolated by Pelham and co-workers (Pelham *et al.*, 1988; Hardwick *et al.*, 1990). These mutants secrete ER-resident soluble proteins like BiP/Kar2p. Figure 5A shows that the *rer1* and also the *rer3* mutants do not secrete BiP/Kar2p in amounts detectable by immunoblotting. A sample of medium from a *sec22-3* culture served as a positive control in this experiment. Semenza *et al.* (1990) previously demonstrated that mutants defective in the consumption of ER-derived vesicles secrete BiP/Kar2p at the permissive temperature to an extent comparable to *erd1* mutants. The comparison indicated that the Sec12−invertase retention mutants are different from the mutants that are defective in the sorting of soluble ER proteins.

The results presented in Figure 2C indicated that the retention of Sec12−α-f−invertase is equally effective at all three expression levels that were tested. We also looked at the extent of mislocalization in *rer1* mutants expressing the hybrid gene at different levels. At the lowest levels (7.5 and 153 U of total invertase, respectively) *rer1-25*

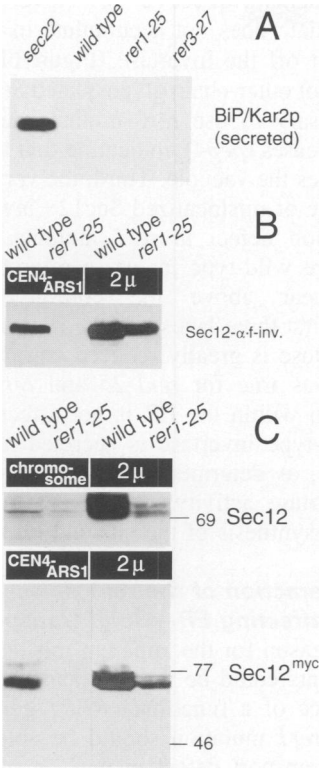

Fig. 5. (**A**) Sec12−a·f−invertase mislocalizing mutants do not secrete BiP/Kar2p. Proteins are precipitated from the medium of growing cultures of strain SHC22-12A (*sec22-3*), SEY6210 (wild-type), C21.25-1C (*rer1-25*) and C21.27-2D (*rer3-27*), and analysed by immunoblotting with BiP/Kar2p-specific antibody as described in Materials and methods. (**B**) Immunoblot analysis of Sec12−a·f−invertase protein in wild-type and *rer1* mutant cells strongly overproducing this hybrid protein. Total cell extracts were prepared from wild-type (SEY6210) or mutant cells (C21.25-1C, *rer1-25*) containing plasmid pCSEC12^{KR} (CEN4-ARS1, *URA3*, *CYC1−SEC12−α·f−SUC2*) or pMCSEC12^{KR} (2μ, *URA3*, *CYC1−SEC12−α·f−SUC2*). (**C**) Immunoblot analysis of wild-type Sec12 protein (upper panel) and a tagged Sec12 variant (lower panel) expressed at different levels in wild-type and *rer1* mutant cells (strains PS42-1A, *RER1*, *pep4* and PR41–4B, *rer1-25*, *pep4*). For high-level expression of the wild-type *SEC12* gene and the tagged variant, plasmid ANY1-9 was used (2μ, *URA3*; Nakano *et al.*, 1988). To express the *myc*-tagged allele at lower levels, it was subcloned into a centromeric vector. Note that the detection of Sec12^{myc} at low copy number required an ~10 times longer exposure during the ECL chemiluminescence reaction.

mutants secreted 36 ± 7% or 37 ± 6% of the total invertase activity (Figure 2D). Surprisingly, the percentage of secreted invertase fell to values between 11 and 16% when the mutant cells expressed the *SEC12−α·f−SUC2* construct from the strong *GAL1* promoter on a centromeric plasmid (700 U of total invertase) and to values between 8 and 11% when expressed from the *CYC1* promoter on a multicopy vector (750 U). *rer1* null mutants (*rer1*::*TRP1*) behaved like the original mutants, ruling out the possibility that the original mutant represents a leaky defect. In addition, in extracts from mutant cells strongly overproducing the hybrid protein, the core-glycosylated form of this protein could be detected (Figure 5B). This suggests that there is an inverse relationship between the expression level of the hybrid protein and the extent of the retention defect. Increasing the gene dosage of *KEX2* by transforming the mutants with plasmid pRS-KEX2 did not change the ratio of secreted to total invertase in these cells.

The targeting of Sec12−invertase chimeras may be different from that of the authentic Sec12 protein. To exclude this possibility, we determined the steady-state level of Sec12p in wild-type cells and in mutants with different levels of this protein. Western blot analysis using Sec12p-specific antibodies showed that the *rer1* mutation led to a strong decrease in the amount of Sec12p (Figure 5C, upper panel). This experiment was repeated using a version of Sec12p that was tagged with the c-*myc* epitope to facilitate the detection of Sec12p expressed at a low level (see Materials and methods). Sec12p^{myc} protein cannot complement the *sec12-4* defect. In fact, it interferes with the growth of the *sec12-4* mutant cells at 30°C, suggesting that it can compete with the mutant Sec12 protein. When expressed from centromeric or multicopy plasmids, the amount of Sec12^{myc} protein is strongly reduced in samples from *rer1* mutants compared with those from wild-type cells (Figure 5C, lower panel).

The dramatic effect of the *rer1* mutation on the steady-state level of Sec12p and the Sec12-derived hybrid proteins raised the question of whether a proteolytic event may induce the mislocalization. To rule out this possibility, we performed pulse−chase experiments (see Figure 6A). Wild-type and *rer1* mutant cells producing Sec12−invertase without Kex2 cleavage site were radioactively labelled for 15 min, and samples were taken after 0, 1.5 and 3 h of chase. The hybrid protein was precipitated from the extracts by invertase-specific antibodies. In wild-type cells the mobility of the hybrid protein did not change (Figure 6A). In *rer1* mutants, however, a significant part of the hybrid protein received outer-chain glycosylation, as shown by digestion of the immunoprecipitate with Endo H and by re-precipitation with antibodies directed against outer-chain α1→6-linked mannose (Figure 6A). The uniform migration rate of the Endo H-treated hybrid protein indicates that the mislocalization of the protein is not initiated by a proteolytic trimming of the cytoplasmic domain. Only the higher molecular weight form of the hybrid protein accumulating in the mutant was recognized by the anti-α1→6 mannose serum. In wild-type cells, a small part of core-glycosylated Sec12−invertase was also recognized by the anti-α1→6 mannose serum. In this respect, the hybrid protein resembles the behaviour of the wild-type Sec12 protein (d'Enfert *et al.*, 1991; Nishikawa and Nakano, 1993). A species migrating above 200 kDa was visible when Sec12−invertase was precipitated from extracts of wild-type cells. This putative complex may be specific because such bands were not found in cells overexpressing wild-type *SUC2* (see Figure 6C). In several experiments using three different wild-type and four mutant strains (*rer1*, *rer1*$^{-}$ or *rer3*) as well as cells expressing *SEC12−SUC2* or *SEC12−α·f−SUC2* chimeras, this high molecular weight species was only observed in extracts from wild-type cells. Similar bands present in extracts from mutants immediately after the labelling disappeared later. In wild-type cells, the amount and size of the putative complex increase with time, Endo H treatment had only a slight effect on its mobility in sodium dodecyl sulphate (SDS) gels, and it can be re-precipitated with anti-α1→6 mannose serum.

Secreted invertase migrates as a disperse band (Esmon *et al.*, 1981; Figure 6C), while mislocalized Sec12−invertase receives only little outer-chain glycosylation (Figure 6A, sixth lane). This suggests that 3 h after the

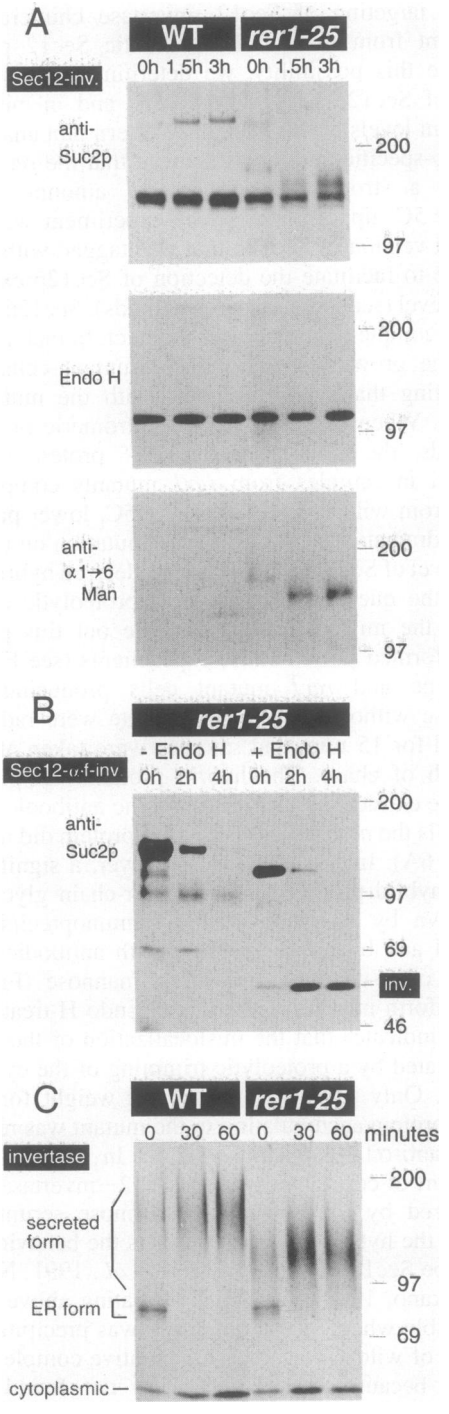

Fig. 6. Pulse–chase labelling of Sec12–invertase (**A**), Sec12–a·f–invertase (**B**) and wild-type invertase (**C**) in wild-type cells (strain PS42-1A) and an *rer1-25* mutant (strain PR41-4B). Cells were labelled for 15 min with [^{35}S]methionine and chased for the times indicated. After preparing crude extracts, the proteins were precipitated using an anti-invertase serum. Aliquots of the precipitate were treated with Endo H (A and B, lanes 3–6) or re-precipitated with an anti-α1→6 mannose serum (A). The hybrid proteins were produced from vector pCSEC12^{WT} (CEN4-ARS1, *URA3*, *CYC1–SEC12–SUC2*) or pCSEC12^{KR} (CEN4-ARS1, *URA3*, *CYC1–SEC12–α·f–SUC2*). To detect invertase, the *suc2-Δ9* cells were transformed with a multicopy plasmid containing the wild-type *SUC2* gene (YEp24-SUC2; a gift from S.Hohmann).

metabolic labelling the hybrid protein may still be retained in an early Golgi compartment of *rer1* mutants. However, this is unlikely for the following reasons. First, pulse–chase experiments with Sec12–α·f–invertase showed that this intermediate does not accumulate in *rer1* mutants if Kex2 can cut off the invertase (Figure 6B). Second, for the detection of outer-chain glycosylated Sec12–invertase, it was necessary to use *rer1* mutants deficient in most vacuolar proteases (*pep4*), indicating that the mislocalized protein reaches the vacuole. Third, the very homogeneous migration rate of mislocalized Sec12–invertase is due to a glycosylation defect in *rer1* mutants. As mentioned above, mature wild-type invertase migrates as a heterogeneous smear above the core-glycosylated form (Figure 6C, first three lanes). In the *rer1* mutant, the final size of invertase is greatly reduced compared with wild-type. This was true for *rer1-25* and *rer1* null mutants. Glycosylation within the ER is not affected to the same extent. Wild-type invertase is secreted normally by the *rer1* mutants, as determined by measuring external and internal invertase activity at different time points after inducing the synthesis of the enzyme (not shown).

Genetic interaction of the rer1 defect with mutations affecting ER→Golgi transport

One trivial reason for the apparent mislocalization defect in *rer1* mutants could be that ER and Golgi simply fuse in the absence of a functional *RER1* gene. If this were correct, the *rer1* mutation should be able to bypass the ER→Golgi transport defect in *bet1* or *sec22* mutants or in cells deficient in Ypt1p. These three defects can be suppressed rather easily by mutations in a second gene or by dosage-dependent suppressors (Rudolph *et al.*, 1989; Newman *et al.*, 1990; Dascher *et al.*, 1991). Double mutants containing either the *bet1-1*, *sec22-3* or *GAL10-YPT1* alleles and the *rer1* defect were constructed (for the convenient detection of the *rer1* defect in most of these genetic tests, we used *rer1::TRP1* strains). In none of these double mutants could the *rer1* defect improve the growth at temperatures >30°C (*bet1-1*; *sec22-3*) or on glucose-containing medium (*GAL10-YPT1*).

A genetic interaction was observed when the *rer1*$^-$ defect was combined with mutations leading to a conditional defect in vesicle budding from the ER (Kaiser and Schekman, 1989). *sec12-4*, *sec13-1*, *sec16-2* and *sec23-1* mutations show some residual growth at 29°C on agar plates, while double mutants containing any of these mutations and the *rer1* defect fail to grow at this temperature. In liquid culture, the doubling time of *sec12* mutants at 29°C is almost like that of wild-type cells or *rer1* single mutants (120 min compared with 90 min). Two hours after the transfer to 29°C, the doubling time of *rer1 sec12* double mutants increases to >10 h. This effect was not due to a 'modifier' that is closely linked to *rer1*, since introducing an intact *RER1* allele on a centromeric plasmid could restore normal growth of *sec12 rer1* double mutants at 29°C. Taken together, these results suggest that the *rer1* defect does not induce a short circuit of the protein transport between ER and Golgi. Instead of this, *rer1* specifically interferes with the growth of mutants affected in the budding of vesicles from the ER.

Sequences within Sec12p required for retention

The Sec12 protein does not contain any of the known signals for ER retention. To identify the signal required for retention, we first removed parts of the *SEC12* sequences from the *CYC1*-controlled *SEC12–α·f–SUC2*

hybrid gene. On the luminal side, 27 residues were deleted so that the *myc*-tag/Kex2 segment is placed next to the transmembrane domain of Sec12p. In a second construct, residues 19–322 were removed from the 357 residue long cytoplasmic domain. Invertase secretion from transformants expressing these *SEC12ΔL−* or *SEC12ΔCyt-α·f−SUC2* constructs was as low as in case of the original construct. Immunoblot analysis confirmed that these proteins accumulated in their core-glycosylated forms (not shown).

We also tested whether these deletions influenced the extent of mislocalization in *rer1* mutants. While the mislocalization of the ΔL protein was comparable to that of the original protein, the retention defect was less evident in the case of ΔCyt (<10% of the total invertase was secreted). This suggested that there may be an additional mechanism besides the *RER1*-dependent system that retains the ΔCyt protein in the ER. For the further analysis of the retention signal, it would therefore be necessary either to make smaller deletions or to transplant parts of Sec12p to a non-ER-resident reporter protein. The results obtained with the ΔL and ΔCyt constructs had already suggested that the transmembrane domain (TMD) could be important for the retention of Sec12p. In a first attempt to test this, we replaced the TMD of Sec22−α·f−invertase (SQ**YAPIVIVAFFFVFLFWWIFL**K) with that of Sec12p (K**FFTNFILIVLLSYILQFSY**K). When this *SEC22/12−α·f−SUC2* hybrid was expressed from multicopy plasmids, only 22 ± 2% of the total invertase was found in the periplasm. The percentage measured with the original *SEC22−α·f−SUC2* construct was 67 ± 6%. In an attempt to improve the observed retention, a second construct was made in which the five residues preceding the Sec12p TMD were also included in such a Sec22/Sec12 hybrid. It was shown by Munro (1991) and Nilsson *et al.* (1991) that sequences flanking the TMD of Golgi proteins can influence their ability to localize proteins to the Golgi. Placing these five additional residues (KQKISkfftnf..; see above) in front of the transplanted Sec12 TMD did not result in further reduction of invertase secretion (15 ± 3%) and thus had no strong effect on the retention. The retention conferred to the Sec22-derived hybrid protein by the TMD of Sec12p was also *RER1* dependent since *rer1* mutants again secreted between 46 and 52% of the invertase. Immunoblot analysis confirmed that the retained hybrid protein was in its core-glycosylated form (Figure 7A, compare with Figure 3A, third lane). The apparent ER retention also prevented the accumulation of the 27 kDa Sec22/Sly2 fragment that results from the cleavage of the hybrid protein by Kex2. In the *rer1*$^-$ cells, this cleavage product was visible again.

Replacing the Sec12 TMD with that of Bet1/Sly12 can strongly decrease retention. (Of the sequence ..IS**KFFTNFILIVLLSYILQFSYK**MD.. the bold residues were substituted by the sequence TWLIIFFMVGVLFFWVWIT.) In fact, this change led to a strong increase in the amount of invertase secreted by wild-type cells (31 ± 6% compared with 2 ± 1.5%). Surprisingly, invertase secretion was almost twice as high if this construct was expressed in *rer1* mutants, suggesting that there is still considerable *RER1*-dependent retention of this Sec12/Bet1−α·f−invertase triple fusion. Immunoblot analysis again confirmed that the increase in invertase secretion goes along with a decrease in the amount of core-glycosylated hybrid protein (Figure 7B).

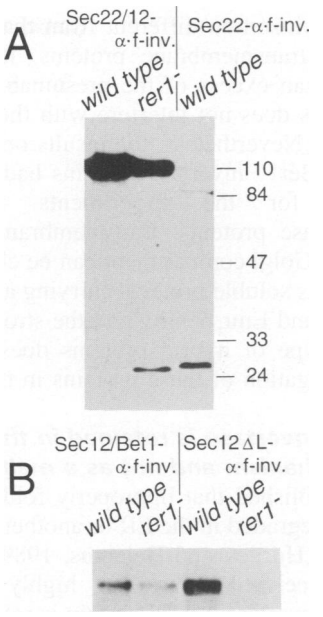

Fig. 7. (**A**) Immunoblot analysis of Sec22−a·f−invertase carrying the TMD of Sec12p. The chimeric genes were expressed from multicopy vector pSSE14^{KR} (2μ, *URA3*) in strain JBD2-2A (*RER1*, first lane) or JBD2-2D (*rer1::TRP1*, second lane). For comparison, the third lane shows a sample from cells expressing the original *SEC22−α·f−SUC2* hybrid gene. (**B**) Immunoblot analysis of Sec12ΔL−a·f−invertase carrying the TMD of Bet1/Sly12p (first and second lanes) or the genuine TMD of Sec12p (third and fourth lanes). The different chimeras were expressed from the *CYC1* promoter on a centromeric plasmid (pCSEC12KR) in strains JBD2-2A (*RER1*) and JBD2-2D (*rer1::TRP1*).

Discussion

We have shown in this report that Sec12−invertase carrying a Kex2 cleavage site could be used very conveniently to isolate retention mutants, to quantitate the extent of the retention defects and to clone the genes involved. The first gene cloned, called *RER1*, was also identified by A.Nakano and co-workers using α-factor pheromone as a reporter (Nishikawa and Nakano, 1993; K.Sato and A.Nakano, personal communication). The function of the second gene, *RER3*, is not clear. In contrast to the *rer2* defect identified by Nishikawa and Nakano (1993), *rer3* mutants are not defective in the retention of the soluble ER protein BiP/Kar2p. Pulse−chase experiments showed that Sec12−invertase is unstable in *rer3* mutants, but immunoblot analysis did not reveal a significant decrease in the amount of the wild-type Sec12 protein. Therefore, these mutations may only affect the hybrid protein.

Using the Kex2-dependent invertase secretion as a tool, we were not able to observe a retention of Sec22−α·f−invertase or Bet1−α·f−invertase in an early compartment of the yeast secretory pathway. Recent results from our laboratory (W.Ballensiefen, unpublished results) suggest that at least for Sec22/Sly2p this may be an artefact caused by the large invertase part of the chimeric proteins. As proposed by Kutay *et al.* (1993), tail-anchored proteins like Sec22/Sly2p or Bet1/Sly12p may follow a pathway

for membrane insertion different from that used by classical type II transmembrane proteins. It is surprising, however, that an excess of the presumably mislocalized hybrid proteins does not interfere with the growth of the transformants. Nevertheless, the results obtained with the Sec22− and Bet1−invertase proteins had two important implications for the experiments involving the Sec12−invertase proteins: transmembrane proteins that reach the late Golgi compartment can be cleaved by Kex2 as efficiently as soluble proteins carrying a Kex2 cleavage site (Graham and Emr, 1991), and the strong overproduction of this type of hybrid proteins does not lead to a massive aggregation of these proteins in the ER.

Sec12−α·*f*−*invertase is retained in the ER by a specific mechanism and not as a malfolded protein*

It is well established that improperly folded proteins are retained and degraded in the ER or another non-lysosomal compartment (Hurtley and Helenius, 1989). Therefore, it may be conceivable that the highly overproduced Sec12−α·f−invertase hybrid protein is retained in the ER by a system that recognizes malfolded proteins. We think that this is unlikely for the following reasons. First, the invertase part of the ER-retained hybrid protein is fully active (total invertase activities were almost the same when *SEC12*−α·*f*−*SUC2* and *BET1*−α·*f*−*SUC2* were expressed from the same promoter in the same vector). Results obtained by Reddy and Maley (1990) showed that the active site of invertase is close to the N-terminus and thus not far from the site where invertase was fused to Sec12p. Second, for enzymatic activity of the invertase the assembly into dimers is required (Chu *et al.*, 1983). Therefore, the high activity of the ER-retained hybrid protein rules out the possibility that the hybrid protein accumulates in the ER in an unassembled form. Third, we found that the regulated and very strong expression of the *SEC12*−α·*f*−*SUC2* hybrid gene placed under the control of the *GAL1* promoter did not increase BiP/Kar2p synthesis (data not shown). Tokunaga *et al.* (1992) had demonstrated that the synthesis of a single heterologous protein from the *GAL7* promoter can indeed be sufficient to increase the amount of the ER-resident chaperon protein Bip/Kar2p.

Perhaps the most convincing evidence arguing against a non-specific aggregation of the hybrid protein in the ER came from our search for the retention signal within the Sec12 protein. The luminal domain of Sec12ΔL-α·f−invertase is indistinguishable from that of Sec22−α·f−invertase. While both proteins were expressed at similar levels, only the former of these chimeras was retained in the ER. Evidence for a specific retention mechanism was also obtained from the analysis of the *rer1* mutants. Immunoblot analysis showed that the *rer1* retention defect affected the stability not only of the chimeric protein, but also of the authentic Sec12 protein. Consistent with the results of Nishikawa and Nakano (1993), the *rer1* (and *rer3*) mutants do not affect the retention of the soluble ER protein BiP/Kar2p.

What is the fate of the mislocalized protein?

The main reason for following the processing of the mislocalized protein by pulse−chase experiments was to exclude the possibility that a proteolytic event triggered or mimicked the apparent mislocalization. The finding that Sec12−invertase received outer-chain glycosylation in the *rer1* mutant confirmed that the hybrid protein reached the Golgi apparatus. This proved that the release of invertase from Sec12−α·f−invertase was not due to Kex2 protease that had become active in the ER.

The fact that invertase secretion from the hybrid proteins is Kex2 dependent suggests that the cleavage of the chimeras is necessary for the invertase to escape from the transport to the vacuole, the likely default pathway for transmembrane proteins in yeast (Cooper and Bussey, 1992; Roberts *et al.*, 1992; Wilcox *et al.*, 1992). As a result of targeting to the vacuole, the mislocalized Sec12 protein may be very unstable (see Figure 5C). As the experiments presented in Figures 5C, 6 and 7 were performed using proteinase A-deficient strains lacking most soluble vacuolar hydrolases (Jones, 1991), the mislocalized Sec12p may be degraded by the proteases CpS and DPAP-B. These two enzymes do not require maturation steps catalysed by proteinase A. Since the Sec22/Sly2 part cleaved from the hybrid protein is more stable than Sec12p, it might be possible to identify signals in the cytoplasmic domain of Sec12p that contribute to the instability of the mislocalized protein.

Some differences in the glycosylation of Sec12p and Sec12−invertase in *rer1* mutants were noted. Nishikawa and Nakano (1993), who had used a Sec12−Mfα1 hybrid protein for the isolation of retention mutants, showed that upon mislocalization in an *rer1-2* mutant the authentic Sec12 protein receives very heterogeneous outer-chain glycosylation. In our *rer1-25* mutants, the oligosaccharide side chains of mislocalized Sec12−invertase were extended to a lower degree than expected. Insufficient outer-chain glycosylation by *rer1-25* and *rer1* null mutants was also observed in the case of soluble wild-type invertase, and is therefore not specific for the chimeric proteins. The differences in the results obtained by Nishikawa and Nakano (1993) and us may be due to the fact that the authentic Sec12 protein receives O-linked oligosaccharides (Nakano *et al.*, 1988), while the hybrid protein is only *N*-glycosylated.

A puzzling observation concerns the different stabilities of Sec12−invertase without or with a Kex2 cleavage site in *rer1* mutants. In wild-type cells, both proteins were stable and remained in their core-glycosylated forms for several hours (Figure 6A and data not shown). In *rer1* mutants that were deficient in several vacuolar proteases (*pep4*), Sec12−invertase was also rather stable. Part of it was mislocalized and received outer-chain glycosylation. In contrast, the core-glycosylated form of the Kex2-sensitive hybrid protein was very rapidly cleaved in *rer1* cells (Figure 6B) and little of it remained in the ER. This observation made by immunoprecipitation correlates well with the results obtained by immunoblot analysis (Figure 3B). Taken together, this implies that the cleavage by Kex2 can increase the mislocalization of the hybrid protein by a kind of feedback mechanism. Results obtained with *PEP4*$^+$ strains and hybrid proteins that contain a mutated Kex2 cleavage site suggested that degradation in the vacuole has the same effect. A feedback inhibition of the mislocalization may also account for the observation that in *rer1* cells the hybrid protein accumulates in its core-glycosylated form if it is expressed at very high

levels. Alternatively, *rer1* mutants may be unable to maintain the ER, the Golgi apparatus and the vacuole as separate compartments. This seems unlikely for the following reasons. First, the *rer1* defect does not lead to a bypass of ER→Golgi transport defects that can otherwise be suppressed very easily (*sec22*, *bet1*, *ypt1*$^-$). Instead of this, it increases the growth defect caused by the mutations that affect the budding of vesicles from the ER. Second, the retention of other membrane-bound hybrid proteins accumulating in the ER is not affected by the *rer1* defect (R.Kölling, personal communication). Third, electron microscopy showed that neither the *rer1* mutation nor the high-level expression of the Sec12-invertase hybrid protein (*CYC1* promoter, multicopy plasmid) has a strong effect on the cellular membranes (H.-H.Trepte and J.Boehm, unpublished results). This also rules out the possibility that the proteins simply accumulate in exaggerated membrane structures like those induced by the overproduction of other integral membrane proteins (Wright *et al.*, 1988; Schunck *et al.*, 1991). Fourth, Nishikawa and Nakano (1993) showed that Sec12p mislocalization in an *rer1* mutant still requires vesicular traffic catalysed by the *SEC18* gene product. Therefore we believe that our observations can best be explained by a back-up of excess hybrid protein into the ER, as observed with highly overproduced Golgi-resident proteins (Munro, 1991; Nilsson *et al.*, 1991; Machamer, 1993).

The targeting of Sec12p resembles the retention of Golgi-resident viral proteins and glycosyltransferases

In wild-type cells, Sec12p and Sec12−invertase receive α1→6-linked mannose residues, although to a very low extent (d'Enfert *et al.*, 1991; Nishikawa and Nakano, 1993; this work). Since this modification occurs in an early Golgi compartment (Franzusoff and Schekman, 1989), it suggests that the targeting of Sec12p involves a continuous recycling between Golgi and ER.

Retrieval systems known so far rely on the recognition of a molecular signal by a specific receptor or carrier protein. Receptors can be saturated with ligands, suggesting that their number becomes limiting (Stevens *et al.*, 1986; von Figura and Hasilik, 1986; Pelham *et al.*, 1988; Dean and Pelham, 1990; Roberts *et al.*, 1992; Humphrey *et al.*, 1993; Nothwehr *et al.*, 1993). However, within the range of *SEC12*−α*f*−*SUC2* expression achieved (7, 150 and 750 U of total invertase activity), no dramatic increase in the portion of secreted invertase was observed. *SEC12* is known to be a very weakly expressed gene (Nakano *et al.*, 1988). Assuming that the lowest expression level using a pSEY304-derived vector may roughly correspond to the expression level of the wild-type protein (d'Enfert *et al.*, 1991), we may have obtained a 100-fold overproduction. The apparently non-saturable retention of the Sec12p−invertase hybrid protein resembles that of glycosyltransferases and viral proteins in the Golgi of mammalian cells (Munro, 1991; Nilsson *et al.*, 1991; Swift and Machamer, 1991; Teasdale *et al.*, 1992; Wong *et al.*, 1992).

Another analogy between the retention of Sec12−invertase and the retention of Golgi-resident glycosyltransferases and viral proteins concerns the retention signal. In both cases, the main targeting determinant resides in the membrane-spanning domains of these proteins. The TMD of Sec12p differs from that of Sec22/Sly2p or Bet1/Sly12p by the presence of five hydrophilic residues. Several hydrophilic residues in the TMD of membrane IgM are required for ER retention (Williams *et al.*, 1990), while ER retention and degradation of unassembled subunits of the T-cell antigen receptor depend on charged residues within their TMDs (Cosson *et al.*, 1991). In these examples, the retention signal must be masked by the formation of specific complexes to overcome retention and achieve transport. In contrast, the assembly into specific aggregates is necessary for the retention of Golgi-resident viral proteins and glycosyltransferases. According to a recent model (Nilsson *et al.*, 1994), glycosyltransferases of the *medial*-Golgi and *trans*-Golgi form separate 'kin oligomers', each of them including a different set of related proteins. The various homodimers may contact the neighbouring dimers via their TMDs. Oligomers that are SDS resistant were observed by Weisz *et al.* (1993) using VSV G-derived hybrid protein (Gm1) that contained the first TMD of the Golgi-retained viral M glycoprotein. Notably, polar residues within the TMD of this hybrid protein were critical for Golgi retention and the formation of SDS-resistant oligomers (Machamer *et al.*, 1993; Weisz *et al.*, 1993). High molecular weight bands which may represent SDS-resistant oligomers were also observed in pulse−chase experiments using extracts from wild-type cells expressing the *SEC12*−*SUC2* or *SEC12*−α*f*−*SUC2* hybrid genes (Figure 6A and data not shown). However, as in the case of Gm1, these SDS-resistant oligomers were not observed with wild-type protein (Nishikawa and Nakano, 1993). Therefore, the significance of the oligomer formation for the retention of the wild-type protein remains unclear.

Our results neither establish nor rule out that the TMD of Sec12p is the only retention signal. For instance, Sec22−α·f−invertase carrying the TMD of Sec12p is not entirely retained in the ER. Possibly, the TMD of Sec12p is not able to overcome completely the propensity of Sec22/Sly2p to be selectively packaged into transport vesicles (Lian and Ferro-Novick, 1993). Likewise, replacing the Sec12p TMD with that of Bet1/Sly12p did not entirely abolish ER retention. As discussed above, polar residues within the TMD of Sec12p may play an important role in the process. In our Sec12/Bet1 hybrid the lysine residues normally flanking the TMD of Sec12p were replaced by the threonine residues that flank the Bet1/Sly12 TMD. These two polar residues may now be part of the membrane-spanning α-helix and contribute to the still present *RER1*-dependent retention of Sec12/Bet1−α·f−invertase. Changing certain residues in the TMD of Sec12p will be necessary to find the crucial residues within the membrane-spanning α-helix, to test the importance of the flanking lysine residues, and to examine whether the TMD is the only retention signal.

Where does the sorting of Sec12p from transported proteins take place?

Localization of a membrane protein within a specific cellular compartment can be achieved by retention, recycling or a combination of both mechanisms. The analogies to Golgi retention described above suggest that the localization of Sec12p is mainly due to prevention of its exit

from the ER. Retention may involve Rer1p and the TMD of Sec12p, but neither the deletion of *RER1* nor the replacement of the TMD of Sec12p with the TMD of Bet1/Sly12p made Sec12−α·f−invertase behave like Sec22− or Bet1−α·f−invertase. Thus, a second retention mechanism may exist. As proposed by Rose and Doms (1988) and Jackson *et al.* (1993), targeting of a transmembrane protein may be mediated by a combination of two systems. Transport is still slow if the recycling of ER proteins is prevented by destroying a retrieval signal (Munro and Pelham, 1987; Nilsson *et al.*, 1993). Two pools of Sec12p may therefore exist in the cell. Excess Sec12p may be fixed in specialized membrane subdomains, while Sec12p that is needed for vesicle budding would be recruited from these subdomains. Retrieval may be necessary to bring back those molecules which escaped the first retention mechanism during the budding process. During their passage through the first Golgi compartment, these few molecules may encounter α1→6 mannosyltransferase, thus accounting for the rather low α1→6 mannosylation of Sec12p and Sec12−invertase.

Of course, other models can be envisaged. For instance, receptor-mediated recycling could also involve the formation of oligomers. The high efficiency of the Sec12p retention system may be due to the ability of a putative receptor to bind and retrieve larger oligomers. Townsley *et al.* (1993) recently showed that Golgi→ER retrieval of the human KDEL receptor depends on a residue that lies within the seventh membrane-spanning domain. This suggests that the sorting of a protein to the ER can in fact depend on interactions within the Golgi membrane. The type of aggregates implicated in this targeting process should be different from the 'kin oligomers' described by Nilsson *et al.* (1994) which are thought to be excluded from vesicular transport due to their size. If oligomers do in fact recycle, they must either be smaller or their recycling may not involve carrier vesicles or thin tubular extensions.

If the targeting of Sec12p indeed involves the self-assembly into larger complexes, then Rer1p may either promote the formation of certain oligomers or it may sort different proteins into separate aggregates and thereby keep different oligomeric structures apart from each other. The third of the four transmembrane domains of the deduced Rer1 protein has a rather interesting structure. Four serine residues and one threonine residue all reside on one face of the putative α-helix (Figure 4D). Thus, Rer1p may ideally be suited for interacting with other integral membrane proteins that carry polar residues at different positions within their transmembrane domains. Locating the Rer1 protein within the cell and the identification of interacting proteins may help to resolve its primary function.

Materials and methods

Yeast strains and growth conditions

Yeast strains are listed in Table I. Genetic techniques were performed as described by Sherman *et al.* (1986). Leu^+ and Trp^+ transformants were selected and maintained on minimal media (SD) containing 2% glucose and lacking either leucine or tryptophan. Ura^+ transformants were selected and maintained on SD plates containing 0.5% peptone 140 (Gibco, Gaitersburg). Suc^+/Suc^- phenotypes were assayed after replica plating of colonies onto YEP or synthetic medium containing 2% sucrose, 10 μg/ml antimycin A and 30 μg/ml bromocresol purple. pH was adjusted to 6.8 in this Suc assay medium.

Mutant isolation

Separate samples of strain MSUC-2C containing plasmid $pCSEC12^{KR}$ (CEN4-ARS1, *URA3, SEC12−α·f−SUC2*) were grown in SD medium lacking uracil and washed with 50 mM KPO_4 buffer (pH 6.5). Approxi-

Table I. Genotypes of strains used

Strain	Genotype	Source[a]
SEY6210	*MATα leu2 ura3 his3 trp1 lys2 suc2-Δ9*	Robinson *et al.*, 1988
MSUC-2C	*MATα leu2 ura3 his3 lys2 suc2-Δ9*	
MSUC-6D	*MATa leu2 ura3 his3 trp1 ade2 ade8 suc2-Δ9*	
C22.4-4A	*MATa rer1-4 leu2 ura3 his3 lys2 ade2 suc2-Δ9*	
C21.10-5A	*MATα rer1-10 leu2 ura3 his3 ade2 suc2-Δ9*	
C21.11-5C	*MATα rer3-11 leu2 ura3 his3 ade2 suc2-Δ9*	
C21.25-1C	*MATa rer1-25 leu2 ura3 his3 trp1 lys2 ade2 ade8 suc2-Δ9*	
C21.27-2D	*MATa rer3-27 leu2 ura3 his3 trp1 ade8 suc2-Δ9*	
C21.25-3A	*MATa leu2 ura3 his3 trp1 ade2 ade8 suc2-Δ9*	
C21.25-4C	*MATα leu2 ura3 his3 trp1 ade2 suc2-Δ9*	
C21D0	*MATa/MATα rer1-25/RER1* (C21.25-1C × C21.25-4C)	
PS42-1A	*MATa ura3 his3 trp1 leu2 lys2 suc2-Δ9 pep4::HIS3*	
PR41-4B	*MATα rer1-25 ura3 his3 leu2 lys2 suc2-Δ9 pep4::HIS3*	
JBD2-2A	*MATa ura3 his3 lys2 trp1 lys2 ade2 ade8 suc2-Δ9*	
JBD5-1B	*MATα rer1::TRP1 ura3 his3 lys2 trp1 ade2 ade8 suc2-Δ9*	
JBD2-2D	*MATa rer1::TRP1 ura3 his3 trp1 ade2 suc2-Δ9*	
MB18	*MATα sec18-1 leu2 ura3 his4 suc2-Δ9*	M.Bielefeld (Düsseldorf)
RH220-7D	*MATa sec12-4 ura3 leu2 his4 lys2*	H.Riezman (Basle)
SHC22-12A	*MATα sec22-3 ura3 leu2 his3 lys2 suc2-Δ9*	
BSH-7C	*MATα bet1-1 ura3 his3 trp1 suc2-Δ9*	
GFUI-6D	*MATa GAL10-YPT1-HIS3 ura3 leu2 his3 trp1*	Dascher *et al.*, 1991
STS13-4C	*MATa sec13-1 ura3 leu2 his4 lys2 trp1 ade2*	
STS16-6D	*MATα sec16-2 leu2 trp1 suc2-Δ9*	
STS23-6D	*MATa sec23-1 ura3 leu2 trp1 ade2 ade8 suc2-Δ9*	

[a]Unless otherwise indicated, the strains listed are from this study.

mately 2×10^7 cells were plated on YEP sucrose and treated with UV light for 20 s (viability was ~50%). After several days, ~50 yellow colonies appeared on each plate. They were re-tested on Suc-assay medium. Suc^+ mutants were crossed to strain MSUC-6D and the Suc phenotype of the diploids was determined. All of them were Suc^-, indicating that the mutations were recessive. *MAT***a**, Suc^+ spores derived from these diploids were crossed to the original isolates for complementation assays. This initial test was repeated later with representative clones originating from another round of back-crossing. Mutants screened for loss of $pCSEC12^{KR}$ were transformed again with this plasmid and tested for the Suc phenotype to rule out that the Suc^+ phenotype was due to mutations within the reporter construct.

Plasmids and nucleic acid techniques

DNA manipulations, such as *Escherichia coli* transformation, restriction enzyme digestions and ligations, were performed as described by Sambrook *et al.* (1989). Vectors pSEY304 (2μ, *URA3*, *'suc2*; Bankaitis *et al.*, 1986) and pSEYC306 (CEN4-ARS1, *URA3*, *'suc2*; Johnson *et al.*, 1987) were used for the construction of hybrid proteins (see Figure 8). The construction of the pSEY304-derived plasmid pCEY2 (2μ, *URA3*, *SEC12−SUC2*) was described by d'Enfert *et al.* (1991). pSEY304 and pSEYC306 contain a truncated *SUC2* gene downstream of the *Hin*dIII site of the pUC8-derived polylinker region. The *SUC2* coding sequence in these vectors starts with the triplet encoding the third amino acid of the secreted invertase. To create in-frame fusions, *SEC22/SLY2* and *BET1/SLY12* sequences were subcloned as 934 and 999 bp *Eco*RI−*Bam*HI fragments into the polylinker region of pSEY304 and pSEYC306. The appropriate restriction sites were either created by PCR amplification or by insertion of a pair of synthetic oligonucleotides. The *Eco*RI site

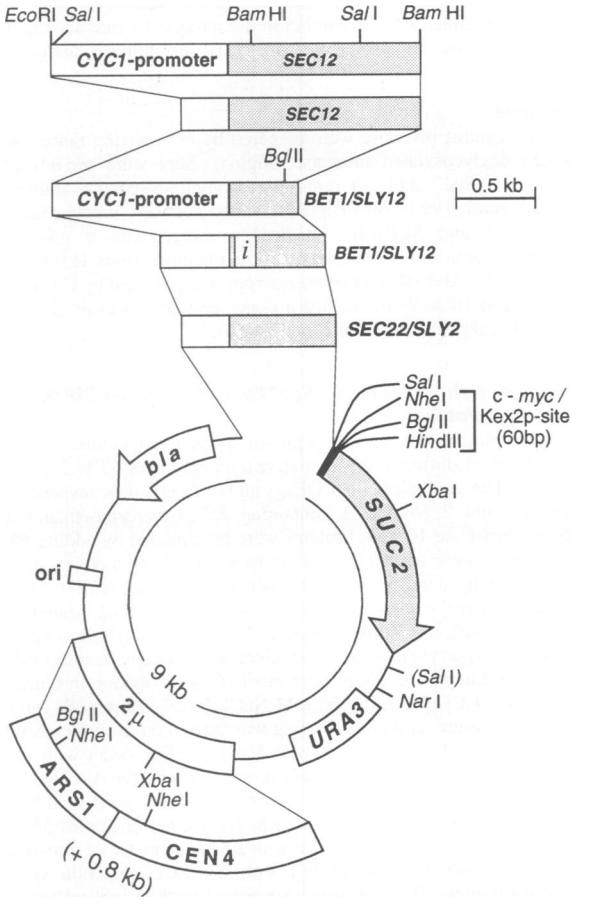

Fig. 8. Construction of plasmids. pSEY304 (2μ; Bankaitis *et al.*, 1986) and pSEYC306 (CEN4-ARS1; Johnson *et al.*, 1987) were used as vectors (lower part). Sequences encoding the transmembrane proteins fused to *SUC2* are shown in the upper part as *Eco*RI−*Bam*HI inserts. Grey boxes: sequences encoding the hybrid proteins; open boxes: 5′ untranslated and intron sequences (*i*); closed box: linker encoding the c-*myc* epitope and the Kex2 cleavage site; *URA3*, CEN4-ARS1 and 2μ sequences as indicated. The approximate positions of some restriction sites are shown. (The second *Sal*I site within the vector sequences is absent from pSEY304-derived plasmids.)

5′ of the *BET1/SLY12* gene (at position −460 from the translation start site) originates from the polylinker present in plasmid pSPT18-SLY12 (Dascher *et al.*, 1991). The polylinker region derived from pSEYC306 and pSEY304, and present between the two parts of the hybrid genes, encodes an 11 amino acid spacer ...GDPCSTCSQAF.... The *Bam*HI sites were introduced immediately after the last codon of the *SEC22/SLY2* and *BET1/SLY12* genes, without changing the amino acid sequence. The plasmids obtained were named pSSC4 (CEN4-ARS1, *URA3*, *SEC22-SUC2*), pSSE14 (2μ, *URA3*, *SEC22-SUC2*) and pCD^{WT} (2μ, *URA3*, *BET1-SUC2*). A 60 bp linker encoding the c-*myc* epitope (italic) and the *MFα1* sequence's Kex2 cleavage site (bold) was introduced into the hybrid genes by insertion of the following pair of annealed synthetic oligonucleotides into the *Hin*dIII site of pCEY2: ($MTKR^+$) agc*t-AGCATGGAACAAAAGCTAATTTCTGAAGAAGATCTGAAT*TACAAG-AGAGAAGCTGA and ($MTKR^-$) AGC**TTCAGCTTCTCTCTTGT-**A*ATTCAGATCTTCTTCAGAAATTAGCTTTTGTTCCATGCT*. This linker contains a *Bgl*II and a *Nhe*I site which were used for the construction of several plasmid derivatives described below. The insertion of the 60 bp linker leaves one *Hin*dIII site in front of the *SUC2* sequences intact. The *SEC12*-containing clone which we selected was named $pCEY2^{KR}$ (CEN4-ARS1, *URA3*, *SEC12−α·f−SUC2*). A 3 kb *Bam*HI−*Nar*I fragment of $pCEY2^{KR}$ containing this linker and the adjacent *SUC2* sequences was transferred to pSSC4, pSSE14 and pCD^{WT} to create plasmids pRO^{KR} (CEN4-ARS1, *URA3*, *SEC22−α·f−SUC2*), $pSSE14^{KR}$ (2μ, *URA3*, *SEC22−α·f−SUC2*) and pCD^{KR} (2μ, *URA3*, *BET1−α·f−SUC2*). In summary, we obtained clones with either amino acid 400 of Sec12p (plus a glycine residue encoded by a *Sma*I site) or the last residue of Sec22/Sly2p and Bet1/Sly12p joined to the third residue of the secreted form of Suc2p by the spacer DPSTCSQA*SMEQKLISEE-DLN***YKREAEA**F (c-*myc* sequence in italics, *MF*α1-derived sequence bold).

For joining the *SEC12−SUC2* hybrid to the *CYC1* promoter, a *Bgl*II site was created by PCR at the 5′ end of the *SEC12* sequence. The *Bgl*II-digested PCR product was ligated to the *Bam*HI site that joins *CYC1* and *lacZ* sequences in vector pCSSZ-100 (a gift from Warren Kibbe). The *CYC1* sequences are present in pCSSZ-100 as a 1.1 kb *Eco*RI−*Bam*HI fragment; the *Bam*HI site joins the *lacZ* coding sequence to the first two codons of the *CYC1* gene (W.Kibbe, personal communication). The *CYC1−SEC12* fusion was transferred to plasmid pRO^{KR}, thus creating $pCSEC12^{KR}$ (CEN4-ARS1, *URA3*, *CYC1−SEC12−α·f−SUC2*). A 1.06 kb *Bam*HI fragment from vector $pCEY2^{KR}$ containing most of the *SEC12* coding region was used to replace the corresponding fragment from $pCSEC12^{KR}$ to avoid PCR mutations. Sequencing confirmed that the PCR-derived parts of *SEC12* sequences did not contain mutations. Plasmids derived from this construct were $pCSEC12^{WT}$ (CEN4-ARS1, *URA3*, *CYC1−SEC12−SUC2*) and $pMCSEC12^{KR}$ (2μ, *URA3*, *CYC1−SEC12−α·f−SUC2*). PCR was also used to create a *Bam*HI site 3′ of the TMD encoding sequence. Thus, we could delete the rest of the luminal part of Sec12p that was still present in the original chimeric proteins. The ΔCyt construct was obtained by deleting a 915 bp *Bsi*WI−*Bsp*EI fragment from plasmid $pCSEC12^{KR}$. The *CYC1−BET1* fusion was obtained as follows. An intronless *BET1/SLY12* allele carrying a *Bcl*I site at the 5′ end was created by PCR using the oligonucleotides GAGCC*TGATCA*AGCTCGAGATTTGCAGGGGGAAACGCGTAT-CAACGTG and $MTKR^-$ as primers, and pCD^{KR} as a template. The *Bcl*I/*Nhe*I-digested PCR product was used to replace a *Bam*HI/*Nhe*I fragment in pCSSZ-100. A 1.15 kb *Eco*RI/*Bam*HI fragment containing the *CYC1−BET1* fusion was transferred to the different pSEYC306- and pSEY304-derived vectors to create $pCSLY12^{KR}$ (CEN4-ARS1, *URA3*, *CYC1−BET1−α·f−SUC2*), $pCSLY12^{WT}$ (CEN4-ARS1, *URA3*, *CYC1−BET1-SUC2*) and $pMCSLY12^{KR}$ (2μ, *URA3*, *CYC1−BET1−α·f−SUC2*).

To obtain the ΔCyt version of the *CYC1−SEC12−α·f−SUC2* hybrid, plasmid $pCSEC12^{KR}$ was cut with *Bsi*WI and *Bsp*EI, ends were filled using Klenow polymerase and ligated. The *SEC22−α·f−SUC2* hybrid that carries the sequences encoding TMD of *SEC12* was obtained as follows. PCR was used to create *Bcl*I sites in front of the TMD-encoding sequence of *SEC12−α·f−SUC2*. Thus, the *SEC12−α·f−SUC2* sequences starting with codon 349 or 354 of *SEC12* could be fused to codon 191 of *SEC22/SLY2*. To replace the TMD of Sec12−α·f−invertase with that of Bet1/Sly12p, we first used PCR to replace the TMD-encoding stretch of *SEC12−α·f−SUC2* with a DNA segment that carries unique *Afl*II and *Cla*I sites. This should facilitate the insertion of artificial membrane-spanning α-helices or those derived from other proteins. A pair of oligonucleotides encoding the TMD of Bet1/Sly12p was inserted. It carried an artificial *Bsa*HI site for the convenient detection of the recombinant clones. The amino acid sequence encoded by these segments is shown in Results.

SEC12 wild-type gene was overexpressed using plasmid ANY1-9 (Nakano *et al.*, 1988). The c-*myc*-tagged variant was obtained by inserting a pair of oligonucleotides encoding 13 amino acid residues (SMEQKLISEEDLAY) into the unique *Bsi*WI site 41 bp from the translation start of the *SEC12*. For low copy number expression, a 3.3 kb *Eco*RI−*Pst*I fragment was transferred from the ANY1-9^{myc} plasmid to the centromeric vector pRS314 (Sikorski and Hieter, 1989).

For disruption of the *KEX2* gene, the *KEX2* coding region and flanking sequences were cloned by PCR amplification, thereby creating a *Xho*I site at position −257 5′ of the translation start site and a *Bam*HI site 215 bp 3′ of the *KEX2* reading frame. The *Xho*I- and *Bam*HI-digested PCR product was subcloned into pRS315 (Sikorski and Hieter, 1989) to create pRS-KEX2 and into pBluescript II KS+ (Stratagene). A 0.85 kb *Eco*RI−*Bgl*II fragment containing the *TRP1* marker gene was used to replace a 1.1 kb *Bgl*II−*Eco*RI fragment from the *KEX2* coding region in the pBluescript subclone. The resulting plasmid was cut with *Xho*I and *Bam*HI, and the DNA was used to transform yeasts. The absence of Kex2p in extracts of the transformants was confirmed by immunoblotting using anti-Kex2p serum.

Cloning of RER1

Our attempts to obtain an *rer1*-complementing plasmid from a DNA library constructed by using a centromeric vector failed because of the close linkage of *RER1* to the centromere of chromosome III. A yeast DNA library based on vector YEp13 (2μ, *LEU2*; Broach *et al.*, 1979; Dascher *et al.*, 1991), however, yielded several Suc^- transformants which were identified by replica plating onto sucrose minimal medium. Two of these transformants contained core-glycosylated Sec12−α-f− invertase protein, as confirmed by immunoblotting. Plasmid DNA from these candidates was isolated. The size of the yeast DNA insert present in the first clone was ~3.5 kb. Subcloning into YEp13 and pRS315 showed that the *rer1* complementing activity was contained in a 1.9 kb *Nhe*I fragment (0.15 kb of this fragment represented vector sequences adjacent to the *Sau*3A site indicated in Figure 4). From the second plasmid (17 kb insert), three *Nhe*I fragments (a total of ~10 kb) could be deleted without an effect on the *rer1*-complementing activity. A 2.7 kb *Xba*I−*Nhe*I was inserted into YEp13 and pRS315. Again, both plasmids complemented the *rer1-25* defect.

Disruption of the RER1 gene and genetic tests to verify the cloning of RER1

Disruption of *RER1* was carried out by insertion of the 2.7 kb *Xba*I−*Nhe*I fragment into pBluescript II KS^+-derived vector from which the *Eco*RI and *Pst*I sites were deleted. A *Pst*I−*Eco*RI fragment ranging from 20 bp 5′ of the YCL001w reading frame to codon 155 was replaced by a 0.83 kb *Pst*I−*Eco*RI fragment containing the *TRP1* gene, followed by cleavage in the polylinker region to recover yeast sequences and transformation of the strains C21.*25*-3A (haploid; *RER1*) and C21D0 (diploid; *RER1/rer1-25*). To verify the correct integration of the DNA fragment, DNA was prepared from wild-type cells, a diploid and a haploid transformant. YCL001w and flanking sequences were amplified by PCR starting at sites outside the *Xba*I−*Nhe*I DNA fragment. PCR products, 2.8 kb (wild type) and 3 kb (disrupted allele), were purified by agarose gel electrophoresis and further analysed by using different restriction enzymes.

All haploid transformants were Suc^+, and those which were tested secreted between 31 and 36% of the total invertase. The Kex2p-dependent invertase secretion was suppressed again (8% of invertase secreted) when the intact YCL001w reading frame was present on a centromeric plasmid. Accordingly, no core-glycosylated Sec12−α-f− invertase hybrid protein could be detected in the disrupted strains and plasmids containing the YCL001w reading frame restored normal levels of core-glycosylated hybrid protein (data not shown). The diploid transformants were either Suc^+ or Suc^-, depending on which of the two chromosomal alleles was replaced by the *rer1*::*TRP1* construct. The proper integration was confirmed by PCR followed by restriction analysis of the different PCR products. The diploid transformants were allowed to sporulate and tetrads were dissected. All spores derived from a Suc^+ diploid gave rise to Suc^+ colonies provided that they contained the plasmid carrying the reporter construct (a total of 25 spores were tested). Only the Trp^+ spores were Suc^+ when a Suc^- diploid transformant was sporulated (29 spores were tested). This indicated that the genotype of Suc^+ diploid transformant was *rer1-25/rer1*::*TRP1*, while that of Suc^- was *RER1/rer1*::*TRP1*.

Sequencing of RER1

The strategy for sequencing the *RER1* gene is outlined in Figure 4. Deletions ranging into the *rer1* complementing sequence were made starting from the pBluescript II KS^+ polylinker region present in the pRS316-*RER1* subclone and ranging to the different restriction sites indicated in Figure 4. These plasmids were used for the complementation assay for the *rer1* mutant as well as for the sequencing.

Assay for internal and periplasmic invertase

Overnight cultures in SD media were collected by centrifugation and washed twice with ice-cold sodium azide (10 mM). The cell density was adjusted to 10 OD_{600}/ml and samples were split into two portions. The equivalent of 0.01–0.2 OD_{600} units of cells were assayed for periplasmic invertase at 30°C for 30 min in 0.1 M Na acetate (pH 4.9) with 0.1 M sucrose in a total volume of 250 μl (Goldstein and Lampen, 1975). The reaction was stopped by the addition of 300 μl of 0.2 M KPO_4 (pH 10) and subsequent incubation in a boiling water bath. The amount of glucose produced was determined by adding 2 ml 0.1 M KPO_4 (pH 7) containing 20 μg/ml glucose oxidase, 2.5 μg/ml horseradish peroxidase, 150 μg/ml *o*-dianisidine and 0.1 mM *N*-ethylmaleimide. After 30 min incubation at 30°C, 2 ml 6 M HCl were added and absorbance at 540 nm was measured. The basal level determined with cells not expressing invertase (SEY6210) was subtracted from each value. One invertase unit was defined as the amount (in nmol) of glucose produced per minute by 1 OD_{600} unit of cells. Intracellular invertase was assayed after preparing sphaeroplasts as described by Schauer *et al.* (1985). Sphaeroplasts were washed with 1 M sorbitol and lysed in the presence of 0.1% Triton X-100. In addition, assays for total invertase were performed as described by Johnson *et al.* (1987). Samples were suspended in 200 μl of Na acetate buffer, split into two parts as described above, and one portion of cells was permeabilized in the presence of 0.5% Triton X-100 by freezing in an ethanol/dry ice bath. The extracts obtained from sphaeroplasts as well as the permeabilized cells were assayed for invertase activity parallel to the intact cells (see above). The results obtained by the two different methods were almost identical.

Antibodies

Antibodies against invertase were prepared by immunizing rabbits with denatured deglycosylated invertase (Sigma). Sera were pre-adsorbed against intact Suc^- cells to remove carbohydrate-specific antibodies (Hardwick *et al.*, 1990). Anti-Bip/Kar2 antibodies were kindly provided by A.Hinnen and M.Benli, anti-Sec12p serum was a gift from R.Schekman, anti-Kex2p antibodies were obtained from H.Behr and Dieter Gallwitz. Anti-α1→6 mannose serum was provided by C.Dascher. Monoclonal 9E10 antibody against a c-*myc* epitope (Evan *et al.*, 1985) was from Cambridge Research Biochemicals.

Analysis of hybrid proteins, Sec12p and secreted BiP/Kar2p by immunoblotting

For immunoblot analysis cells were grown in selective medium overnight. *sec18* cells were shifted to the restrictive temperature of 37°C 2 h before harvesting. The equivalent of 5 OD_{600} units of cells was suspended in 0.5 ml ice-cold 2 M NaOH containing 5% 2-mercaptoethanol and incubated on ice for 10 min. Proteins were precipitated by adding 55 μl 100% trichloroacetic acid (TCA). After 10 min incubation on ice, protein was collected by centrifugation, the pellet was rinsed with 1 M Tris base, pellets were suspended in SDS sample buffer and heated in a boiling water bath for 2 min. Samples (20 μl) were applied to an 8 or 12% SDS−polyacrylamide gel and electrophoretically transferred to nitrocellulose filters. The filters were blocked by incubation in buffer A [50 mM Tris−HCl (pH 7.4), 150 mM NaCl, 0.05% Tween-20] for 1 h at room temperature. Antibody binding was carried out in buffer A with 10 μl of 9E10 for 1 h or buffer A with 5% dry milk powder with 10 μl anti-Sec12p serum. Filters were washed once with buffer A, twice with buffer B (0.2% SDS, 0.9% NaCl, 0.5% Triton X-100), again with buffer A for 10 min each, and then incubated in 100 ml buffer A plus 5% dry milk powder and 10 μl peroxidase-conjugated anti-mouse or anti-rabbit Ig for 1 h. Bands were visualized with the ECL detection system (Amersham-Buchler, Braunschweig) according to the supplier's recommendations.

For the immunoblot analysis of secreted BiP/Kar2p, 30 OD_{600} of cells from an overnight culture grown at 30 or 25°C (*sec22* strain) were harvested, and proteins were precipitated from the supernatant by the addition of 1/10 vol. of 100% TCA. After 10 min on ice, proteins were collected by centrifugation. The resulting pellet was neutralized with 1 M Tris base and suspended in 0.25 ml SDS sample buffer. After heating, 30 μl were subjected to SDS−PAGE (8%) and transferred to a nitrocellulose filter. The filter was treated as described above. Peroxidase-conjugated anti-rabbit Ig was from Amersham-Buchler (Braunschweig).

Radiolabelling of cells, immunoprecipitation and Endo H treatment

For immunoprecipitation of invertase, transformants were grown overnight to exponential phase in SD medium containing 2% glucose. Cells were washed, resuspended in minimal medium with 0.5% glucose and without sulfate, and pre-incubated for 3 h in this medium for sulfur starvation. The equivalent of 3 OD_{600} units of cells was harvested and resuspended in 1 ml of this medium; 160 μCi [^{35}S]methionine (Amersham/Buchler, Braunschweig) per OD_{600} unit of cells were added. After 15 min, labelled cells were chased by the addition of 0.01 vol. of a solution containing 100 mM ammonium sulfate, 0.3% L-cysteine and 0.4% L-methionine (Rothblatt and Schekman, 1989). Incubation was terminated by adding one-third of the cells to 1 ml of a suspension that contained 10 mM sodium azide, 0.5 mM PMSF and 10 OD_{600} equivalents of non-labelled Suc$^-$ cells. Cells were broken by agitating for 2 min with 0.2 g glass beads, immunoprecipitation with invertase serum, washing of the Protein A–Sepharose (Pharmacia) was performed as described by Rothblatt and Schekman (1989). Aliquots of radioactively labelled Sec12p–invertase hybrid protein were treated with Endo H (Boehringer) in the presence of 1% SDS and 1% 2-mercaptoethanol for 2 h at 37°C. Re-precipitation of Sec12–invertase protein with anti-α1→6 mannose serum was carried out for 2 h at room temperature. During the final washing step of these precipitates, IP buffer was used (Rothblatt and Schekman, 1989) that contained 1% 2-mercaptoethanol.

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