

Sec12p requires Rer1p for sorting to coatomer (COPI)-coated vesicles and retrieval to the ER

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SUMMARY

In *Saccharomyces cerevisiae* cells lacking the Rer1 protein (Rer1p), the type II transmembrane protein Sec12p fails to be retained in the ER. The transmembrane domain of Sec12p is sufficient to confer Rer1p-dependent ER retention to other membrane proteins. In *rer1* mutants a large part of the Sec12-derived proteins can escape to the late Golgi. In contrast, *rer3* mutants accumulate Sec12-derived hybrid proteins carrying early Golgi modifications. We found that *rer3* mutants harbour unique alleles of the α COP-encoding *RET1* gene. *ret1* mutants, along with other coatomer mutants, fail to retrieve KKXX-tagged type I transmembrane proteins from the Golgi back to the ER. Surprisingly *rer3-11(=ret1-12)* mutants do not affect this

kind of ER recycling. Pulse-chase experiments using these mutants show that α COP and Rer1p function together in a very early Golgi compartment to initiate the recycling of Sec12p-derived hybrid proteins. Rer1p protein may be directly involved in the retrieval process since it also recycles between the early Golgi and ER in a coatomer (COPI)-dependent manner. Rer1p may act as an adapter coupling the recycling of non-KKXX transmembrane proteins like Sec12p to the coatomer (COPI)-mediated backward traffic.

Key words: Coatomer, Endoplasmic reticulum, Recycling/RER1/SEC12

INTRODUCTION

The endoplasmic reticulum (ER) is the site of protein translocation and quality control while in the Golgi complex proteins coming from the ER are sorted to their different cellular destinations (Helenius, 1994; Palade, 1975). During the passage through these organelles proteins are processed in a highly ordered fashion which has been conserved throughout the evolution of eukaryotic cells (Hirschberg and Snider, 1987). To work properly, permanent residents of the ER and the Golgi must continuously be singled out from the massive, on-going traffic within and between these and later organelles. It is widely accepted that the transport between the compartments is mediated by vesicles (Rothman and Orci, 1992). The budding of different types of vesicles requires the assembly of distinct coats. Non-clathrin-coated vesicles, the COPI vesicles, carry the coatomer complex consisting of seven subunits (α , β , β' , γ , δ , ϵ , and ζ COP). COPI vesicles have been proposed to mediate intra-Golgi transport as well as ER-to-Golgi transport (Rothman and Orci, 1992; Pepperkok et al., 1993; Duden et al., 1994). A second type of coat, COPII, was first identified in yeast. It is composed of the Sec23p-Sec24p and Sec13p-Sec31p complexes, the small GTPase Sar1p and Sec16p (Barlowe et al., 1994; Espenshade et al., 1995). Although both types of vesicles, COPI and COPII, bud from the nuclear envelope of yeast at least in vitro, COPII coated vesicles are

mainly involved in the transport of cargo molecules from ER to Golgi (Bednarek et al., 1995).

Protein transport is not unidirectional (Pelham, 1988; Lewis and Pelham, 1996). Since vesicles mediate this transport, sorting can occur in two distinct ways: specific barriers may prevent proteins and lipids from entering sites of vesicle formation and thus from leaving a compartment. This mechanism represents true retention. Alternatively, recycling systems can withdraw ER or Golgi-resident proteins from the so-called bulk flow and retrieve these proteins to earlier compartments. Recycling proteins encounter modifying enzymes which are characteristic for the compartments they are passing through (Pelham, 1988; Jackson et al., 1993). Both mechanisms, retention and retrieval, are not exclusive and can function either in parallel or in combination.

Specific signals required for ER retention are conserved among eukaryotes. Soluble ER proteins carry a C-terminal sequence motif, KDEL in mammalian cells and HDEL in yeast (Pelham et al., 1988). Another C-terminal motif, the dilysine or KKXX motif of type I transmembrane proteins, is required for ER retention in both mammalian cells and yeast (Jackson et al., 1990; Nilsson et al., 1989; Gaynor et al., 1994; Townsley and Pelham, 1994). A double-arginine motif has been identified as the retention signal for a type II transmembrane protein (Schutze et al., 1994).

The factors mediating retention are also conserved through-

out evolution. The K(H)DEL signal carried by soluble ER proteins including also a few type II transmembrane proteins is recognized by the Erd2 receptor in yeast and mammals, respectively (Semenza et al., 1990; Lewis and Pelham, 1990). Coatomer from mammalian cells and yeast binds to the KKXX motif carried by ER-resident type I transmembrane proteins (Cosson and Letourneur, 1994). In fact, most mutations causing a defect in the retention of KKXX-tagged membrane proteins lie in genes encoding subunits of the coatomer. Mutations affecting KKXX-recycling were found in *RET1*, *SEC21*, *SEC27*, *RET2* and *RET3* genes encoding α COP, γ COP, β -COP, δ COP and ζ COP, respectively (Letourneur et al., 1994; Duden et al., 1994; Cosson et al., 1996). Coatomer from *ret1* and *sec27* mutants is unable to bind dilysine motifs in vitro. Very recently it was found that in addition to the COP proteins two transmembrane proteins, Ufe1p and Sec20p, are also required for the recycling of both KKXX-tagged proteins and the HDEL-receptor (Lewis and Pelham, 1996; Cosson et al., 1997).

A third retention system in yeast may be specific for type II transmembrane proteins. The ER retention mutants *rer1*, *rer2* and *rer3* mutants were isolated by using Sec12p-derived hybrid proteins (Nishikawa and Nakano, 1993; Boehm et al., 1994). Interestingly, Rer1p-dependent retention can be transferred to other type II membrane proteins simply by replacing their own transmembrane domain (TMD) with that of the Sec12 protein (Boehm et al., 1994; Sato et al., 1996). *RER1* encodes a protein which itself may contain four TMDs (Boehm et al., 1994; Sato et al., 1995).

rer1 and *rer3* mutants show no secretion of the HDEL-tagged BiP/Kar2 protein, suggesting that these mutants are not deficient in the recycling of soluble ER proteins from the Golgi to the ER. Therefore, *rer1* and *rer3* mutants are different from the *erd* mutants which were identified by Pelham and coworkers (Hardwick et al., 1990). Some functional overlap may, however, exist between *rer* mutants and the coatomer (COPI)-dependent retention of type I proteins. Here we show that *rer3* defects represent unique alleles of *RET1* suggesting that coatomer is required to Sec12p recycling to the ER. In

addition, we present evidence that Rer1p may act as an intermediary between Sec12p and coatomer.

MATERIALS AND METHODS

Genetic techniques

Standard techniques were used for mating of haploid strains, complementation analysis, sporulation and the analysis of tetrads (Sherman et al., 1986). The assays to detect retention defects using either Sec12- α f-invertase, Ste2-Wbp1p and invertase-Wbp1 hybrid proteins were described previously (Boehm et al., 1994; Letourneur et al., 1994; Gaynor et al., 1994).

Strains and plasmids

The yeast strains used are listed in Table 1. A schematic representation of the hybrid proteins used in this study is given in Fig. 1. The plasmids used for expressing *SEC12-SUC2*, *STE2-WBP1* and *SUC2-WBP1* hybrid genes were described previously (Boehm et al., 1994; Letourneur et al., 1994; Gaynor et al., 1994). In some experiments transient expression of *SEC12-SUC2* hybrids was achieved by employing *GALI* regulated copies of the hybrid genes. These chimeras were expressed from single copy vectors (pGSEC12^{WT} and pGSEC12^{KR}; *URA3*, *CEN4-ARS1*). These vectors were obtained by replacing the *CYC1-SEC12* region of the pCSEC12 vectors (Boehm et al., 1994) by a corresponding *GALI-SEC12*-containing fragment derived from pCEY6 (d'Enfert et al., 1991).

To place *SEC22* chimeras under the control of the *CYC1* promoter a *Bgl*III site was created at the start codon of *SEC22* by PCR. The PCR product carried an *Afl*III, a *Cla*I and a *Bam*HI site at the 3'-end of *SEC22*. These sites replace the TMD-encoding part of *SEC22*. This made it possible to insert pairs of annealed oligonucleotides encoding different TMDs as described previously (Boehm et al., 1994). The PCR product was first subcloned into pBluescript KS(+) (Stratagene, San Diego) for sequencing and further subcloning. A 1.1 kb *Eco*RI-*Bam*HI fragment was excised from vector pSCCZ-100 (a gift from W. Kibbe) and inserted into this pBluescript clone. Thus the *SEC22* sequence was fused to codon 3 of a *CYC1-lacZ* hybrid gene. A 1.6 kb *Eco*RI-*Bam*HI fragment carrying this *CYC1-SEC22* fusion gene was used to replace the *CYC1-SEC12* hybrid gene in pCSEC12^{KR} to give vector pCSEC22^{KR}. Annealed oligonucleotides fitting into pCSEC22^{KR} cleaved with *Afl*III and *Cla*I were inserted to fuse different TMD-encoding segments to the *SEC22* sequences.

Table 1. Strains used in this study

Yeast strain	Genotype	Source
PS42-1A	<i>MATa ura3 leu2 his3 trp1 lys2 pep4::HIS3 suc2-Δ</i>	Boehm et al., 1994
R1R3-7A	<i>MATa ret1-12(=rer3-11) ura3 leu2 his3 trp1 lys2 ade2 pep4::HIS3 suc2-Δ</i>	This work
R1R3-8B	<i>MATa rer1::TRP1 ura3 leu2 his3 trp1 lys2 ade2 pep4::HIS3 suc2-Δ</i>	This work
R1R3-10A	<i>MATa ret1-12(=rer3-11) rer1::TRP1 ura3 leu2 his3 trp1 lys2 ade2 pep4::HIS3 suc2-Δ</i>	This work
RT1P4-4B	<i>MATa ret1-1 ura3 leu2 his3 pep4::HIS3 suc2-Δ</i>	This work
SC21P4-7D	<i>MATαsec21-2 ura3 leu2 his3 trp1 lys2 pep4::HIS3 suc2-Δ</i>	This work
SC27P4-2D	<i>MATαsec27-1 ura3 leu2 his3 pep4::HIS3 suc2-Δ</i>	This work
JBD2-2A	<i>MATa ura3 leu2 his3 trp1 lys2 ade2 ade8 suc2-Δ</i>	Boehm et al., 1994
C21.27-2D	<i>MATa ret1-27(=rer3-27) ura3 leu2 his3 trp1 ade8 suc2Δ9</i>	Boehm et al., 1994
R3P4-10D	<i>MATa ret1-12(=rer3-11) ura3 leu2 his3 lys2 ade2 suc2-Δ</i>	Boehm et al., 1994
EGY101	<i>MATa ret1-1 ura3 leu2 his3 lys2 trp1 suc2-Δ</i>	Letourneur et al., 1994
FLY35	<i>MATa ret1-27(=rer3-27) ura3 leu2 his3 trp1 ade8 suc2-Δ ste2::LEU2 STE2-WBP1::URA3</i>	This work
FLY37	<i>MATa rer1-25 ura3 leu2 his3 trp1 lys2 ade2 ade8 suc2-Δ ste2::LEU2 STE2-WBP1::URA3</i>	This work
FLY85	<i>MATa ret1-12(=rer3-11) ura3 leu2 his3 lys2 suc2-Δ ste2::LEU2 STE2-WBP1::URA3</i>	This work
FLY62	<i>MATa ura3 leu2 his3 trp1 lys2 ade2 ade8 suc2-Δ ste2::LEU2 STE2-WBP1::URA3</i>	This work
RH3048	<i>MATa sec12-4 ura3 leu2 his4 lys2 myc-EMP47::LEU2</i>	S. Schröder, Basel
SC12R3	<i>MATαsec12-4 ret1-12(=rer3-11) ura3 leu2 his4 lys2 myc-EMP47::LEU2</i>	This work
SC16d	<i>MATα sec16-1 ura3 leu2 (heterozygous: his4/+ lys2/+ ade2/+ ade8/+)</i>	This work
SC16R3d	<i>MATα sec16-1 ret1-12(=rer3-11) ura3 leu2 his3 lys2 (heterozygous: ade2/+ trp1/+)</i>	This work
SC1621a	<i>MATα sec16-1 sec21-1 ura3 leu2 lys2 trp1 (heterozygous: ade2/+)</i>	This work
SC1627a	<i>MATα sec16-1 sec27-1 ura3 leu2 lys2 ade8 ade2 (heterozygous: his3/+ trp1/+)</i>	This work

The *SUC2* sequences in pCSEC22^{KR} (*URA3*, *CEN4-ARS1*, *CYC1-SEC22- α -SUC2*) were replaced by the last of four repeats encoding the α factor peptide as well as 310 bp 3' of the stop codon of the *Mfd* gene (Kurjan and HersHKovitz, 1982). A 1.7 kb *EcoRI* fragment containing the *Mfd* gene was subcloned into pBluescript KS(+). From this vector a 0.35 kb *HindIII-PvuII* fragment was excised and subcloned into *HindIII* and *PvuII* cleaved pCSEC22^{KR}. The resulting plasmid was named pCCSEC22- α (*URA3*, *CEN4-ARS1*, *CYC1-SEC22- α -SUC2*).

The following plasmids were used to express epitope-tagged version of Golgi marker proteins: pL161 (*PMR1::HA*, 2 μ *URA3*; Antebi and Fink, 1992), SN218 (*KEX2::HA*, *CEN6-ARSH1*, *URA3*; Nothwehr et al., 1995), and pJOCH1 (*TPI-OCH1::c-myc*, 2 μ *URA3*; a gift from M. Lewis, Cambridge).

To obtain isogenic pairs of mutant and wild-type strains pBLys-ret1 (Letourneur et al., 1994) was linearized with *SaII*. This cleavage should facilitate the integration of this vector at the *RET1* locus. *ret1 lys2* mutants were transformed to *Lys*⁺. After the transformation the *ret* phenotypes were determined by back crossing to suitable tester strains. Alternatively, strains were transformed with a *CEN*-based vector carrying the *RET1* gene. To obtain such a vector a 5 kb *SacI-SaII* fragment from pBLys-ret1 containing the *RET1* gene was subcloned into vector pRS314 (*TRP1*, *CEN6-ARSH1*; Sikorski and Hieter, 1989).

Antibodies

Antibodies were raised against a peptide representing the 27 last residues of the *Rer1* protein. An additional cysteine residue was introduced for efficient coupling of this peptide to the maleimide activated KLH protein (Pierce). For affinity purification this peptide was coupled to CNBr-activated Sepharose 4B (Pharmacia). This antibody as well as the following polyclonal or monoclonal antibodies was used for immunoblot analysis, immunoprecipitation and immunofluorescence experiments: anti-invertase and anti- α -6 mannose antibodies (Boehm et al., 1994), anti-Sec22p antibodies (Ossig et al., 1991) the monoclonal antibody Y-27B1 directed against the C-terminal part of *Ypt1p* (Haubruck et al., 1987); a serum against *BiP/Kar2p* (M. Benli and D. Gallwitz, Göttingen); the *c-myc* specific monoclonal antibody 9E10 was obtained from Cambridge Research Biochemicals (Northwich); the HA-specific monoclonal antibody 12CA5 was from Boehringer (Mannheim). Cy3-conjugated anti-mouse IgG and FITC-conjugated anti-rabbit IgG from goat (Dianova, Hamburg) were used as secondary antibodies in indirect immunofluorescence experiments. Peroxidase-conjugated anti-rabbit or anti-mouse IgG from donkey were obtained from Amersham-Buchler (Braunschweig).

Indirect immunofluorescence

Immunofluorescence was performed as described by Schröder et al. (1995). Cells were grown overnight to reach A_{600} 1-1.5. An equivalent of 10 A_{600} of cells was collected per each of two incubation temperatures. After adding 10 ml of fresh medium containing 20 μ g/ml of cycloheximide, cells were transferred to 25°C and 35°C water incubators for 1 hour. Then cells were pre-fixed by adding 1.3 ml of 37% formaldehyde over one hour at room temperature and were resuspended in 1 ml paraformaldehyde solution (3.5% paraformaldehyde in PBS/10% sorbitol). After 2 hours cells were washed once in PBS/10% sorbitol, resuspended in 1 ml of this solution. To remove cell walls samples were treated with lyticase (30 μ l of 10 μ g/ml solution) in the presence of 2-mercaptoethanol (5 μ l/ml) for 1 hour. Sphaeroplasts were collected, washed 4 times in PBS/10% sorbitol and resuspended in 400 μ l of PBS/10% sorbitol, then 15 μ l of this suspension were applied to each slide well which was covered before by a poly-lysine solution (Sigma) to allow cell attachment. After 10 minutes the suspension was removed and 15 μ l of blocking solution (PBS/10% sorbitol, 1% Triton X-100, 1% milk powder) were added for 10 minutes. Incubation with the first antibody was carried out overnight at 4°C at appropriate dilutions in blocking solution.

Secondary antibodies (Cy3-conjugated F(ab)' fragment of goat anti-rabbit IgG and anti-mouse IgG; Sigma) were added after 5 times washing of wells with PBS/10% sorbitol solution. The wells were then washed 7 times, stained with DAPI and washed twice again in PBS/10% sorbitol. About 200 μ l of embedding medium was applied on a slide that was then covered by a coverslip.

Western blotting and immunoprecipitation

Western blotting analysis and immunoprecipitation were performed as described by Boehm et al. (1994). Immunoprecipitations using anti-Sec22 antibodies were performed in the presence of a surplus of extract from a *Dec22* strain (Ossig et al., 1991).

RESULTS

rer3 mutants are defective in the retention of KKXX-tagged proteins

To examine whether *rer* mutants alter ER retention of type I transmembrane proteins carrying the KKXX retrieval signal, we performed the *Ste2*-Wbp1-dependent mating assay described previously (Letourneur et al., 1994). *rer1* and *rer3* mutant cells lacking the chromosomal α factor receptor (*Ste2p*) were used for this assay. In these *ste2* cells mating requires that some of the plasmid encoded KKXX-tagged *Ste2* proteins (Fig. 1A) fail to be retained in the ER. This leakage from the ER leads to plasma membrane exposure of the receptor and thus allows mating of the otherwise sterile host cells.

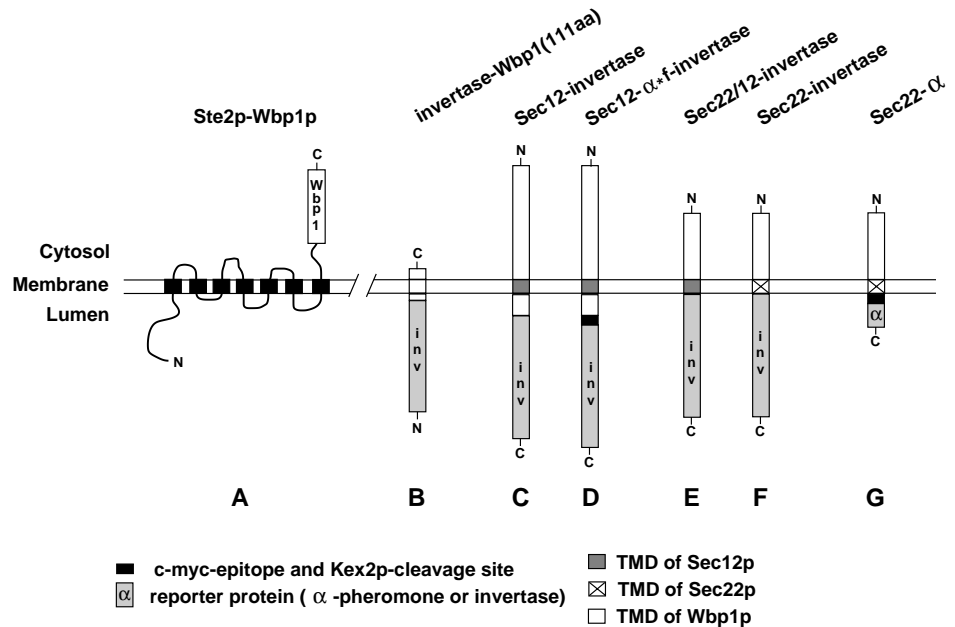
This assay is very sensitive since very few mislocalized proteins are sufficient for mating. The absence of mating observed with cells lacking the *RER1* gene indicates that the *Rer1* protein is not required for retaining KKXX-tagged proteins in the ER. This is consistent with the observation that the *rer1-2* mutant isolated by Nishikawa and Nakano (1993) correctly targets an invertase-Wbp1 hybrid protein (Gaynor et al., 1994).

Different results were obtained for the *rer3* mutants. Two mutant alleles of *RER3* had been obtained, *rer3-11* and *rer3-27*, among which *rer3-11* caused a stronger retention defect (Boehm et al., 1994). With the *Ste2*-Wbp1-based assay efficient mating occurs in *rer3-27* mutants and to a much lower extent in *rer3-11* cells (Fig. 2A). This indicated a defect in the targeting of KKXX-tagged ER-proteins at least in *rer3-27* mutants. These results were confirmed by following the transport of a chimeric protein composed of the invertase protein fused to the TMD and the cytoplasmic tail of Wbp1p (*Inv-Wbp1p*, Fig. 1B; Gaynor et al., 1994). Here the defect in ER retention results in transport of the *Inv-Wbp1* construct to the vacuole and subsequent proteolytic cleavage. Cells producing *Inv-Wbp1p* were pulsed for 10 minutes and chased for 0 or 1 hour at room temperature. The hybrid protein was recovered by immunoprecipitation with an anti-invertase polyclonal antibody, treated with endoglycosidase H and resolved on a 8% polyacrylamide gel. Fig. 2B shows that ER retrieval of *Inv-Wbp1p* is normal in the *rer3-11* mutants while the retention defect in *rer3-27* is quantitatively similar to that observed in *ret1-1* mutants.

As with *ret1-1* mutants, this lack of retention may be due to a defect in KKXX binding of coatomer from *rer3-27* cells. Fig. 2C shows the results of an in vitro binding assay to a KKXX-carrying matrix with extracts from *rer3-27* and *rer3-11* cells (Letourneur et al., 1994). While coatomer from an extract of

Fig. 1. Schematic illustration of the hybrid proteins used in this study. The constructs were used for the experiments shown in the following figures: (A) –Fig. 2A; (B) –Fig. 2B; (C) –Fig. 3; (D) –Fig. 4; (E and F) –Fig. 5A,B; (G) –Fig. 5C.

(A and B) KKXX-tagged versions of the Ste2 receptor and invertase, respectively (Letourneur et al., 1994; Gaynor et al., 1994). The C terminus of these hybrid proteins is derived from Wbp1p a subunit of the oligosaccharyl transferase of the ER (te Heesen et al., 1993). The two versions of Sec12-invertase (C,D) had been used to select and characterize the *rer* mutants, while results obtained with the analogous Sec22 and Sec22/12 chimeras (E,F) had shown that the transmembrane domain of Sec12p mediates Rer1p-dependent ER retention (Boehm et al., 1994). The construction of Sec22- α factor is described in this work. The c-myc epitope present in D and G is represented by a black box. It precedes the sequence YKREAEA which is cleavable by the late Golgi protease Kex2p. Invertase is encoded by the *SUC2* gene. α Factor is a 13 residue long mating pheromone produced by MAT α cells.



rer3-11 mutants binds to the KKXX column as efficiently as coatomer from wild-type cells, coatomer from a *rer3-27* is not able to bind to this affinity matrix. Thus, in *rer3-27* mutants the lack in KKXX retention goes along with a defect in the in vitro binding to the KKXX affinity matrix very much like in *ret1-1* and *sec27-1* mutations.

***rer3* mutations represent unique alleles of the α COP encoding *RET1* gene**

The lack of Ste2-Wbp1p retention indicated that the *rer3* mutations may affect a component of the yeast coatomer or a regulatory element involved in the formation of the yeast coatomer. To reveal possible genetic interactions between *rer3* and other coatomer mutations we tried to create double mutants of the *rer3* mutations and *ret1-1*, *sec21-2* and *sec27-1* mutations, respectively. In the course of these experiments the following evidence for *rer3* mutations being novel alleles of the α COP encoding *RET1* gene were obtained: (i) *ret1-1* mutants cannot complement the defect in Sec12-invertase retention of *rer3* mutants as indicated by the Suc⁺ phenotype of heterozygous *ret1-1 rer3* cells expressing the *SEC12- α -SUC2* hybrid gene (Boehm et al., 1994). (ii) Only one wild-type spore (Ts⁺, Suc⁻) was obtained among 153 spores from crosses between *ret1-1* with either *rer3-27* or *rer3-11*. Therefore, *RET1* and *RER3* are at least very closely linked genes. (iii) The *RET1* gene expressed from a centromeric vector is able to suppress the Kex2p-dependent invertase secretion of *SEC12- α -SUC2* expressing *rer3* mutants as well as the instability of epitope-tagged Sec12 proteins in *rer3-11* mutants (data not shown). In addition, the sequence of the *RET1* gene from the *rer3-11* mutant revealed a specific missense mutation in one of the WD40 repeats of α COP (F. Letourneur, unpublished results).

The genetic experiments also showed that *rer3 sec21-2* and *rer3 sec27-1* double mutants are viable. In this respect, *rer3*

mutations differ from the *ret1-1* allele. This lack of genetic interaction may be due to the fact that, unlike the *ret1-1* allele, none of the *rer3* mutations results in temperature-sensitive growth. As with *ret1-1* mutants, *rer3* mutants did not show a defect in the transport of the lysosomal hydrolase CPY (data not shown). Moreover, *rer3* mutants do not show a defect in outer chain glycosylation of invertase as do, for instance, *rer1* mutants (data not shown).

Taken together, these results suggest that *RER3* and *RET1* are allelic. Especially, the *rer3-11* seems to represent a *RET1* allele with unique properties. Since it interferes with the targeting of Sec12p without affecting the recycling of KKXX-tagged proteins, we renamed this allele *ret1-12*.

The *ret1-12* mutation increases the rate of Golgi-modification of Sec12-invertase

Mislocalization of proteins can be analyzed by using their sugar modifications as an indicator of their progress within the secretory pathway (Franzoso and Schekman, 1989; Gaynor et al., 1994). Pulse-chase experiments were performed to compare the N-linked glycosylation of Sec12-invertase (Fig. 1C). *SEC12-SUC2* was expressed in *ret1-12* cells, *rer1*⁻ single mutants and *ret1-12 rer1*⁻ double mutants (Fig. 3A). As shown before, Sec12-invertase is found in its core-glycosylated form in wild-type cells (d'Enfert et al., 1991; Boehm et al., 1994). It receives outer chain glycosylation in a very slow reaction (Fig. 3A, lane 2, lower panel). In contrast, Sec12-invertase expressed in *rer1*⁻ mutants is strongly glycosylated resulting in a shift in electrophoretic mobility of the protein (Fig. 3A, lane 6). The slower migrating proteins carry Golgi modifications as shown by precipitation with an α -6 mannosylation specific serum (Boehm et al., 1994; see also Fig. 3A, lane 6 and 8, lower panel). As in wild-type cells, no significant increase in the size of Sec12-invertase was seen in *ret1-12* mutants (Fig. 3A, lane 4). However, reprecipitation with an α -

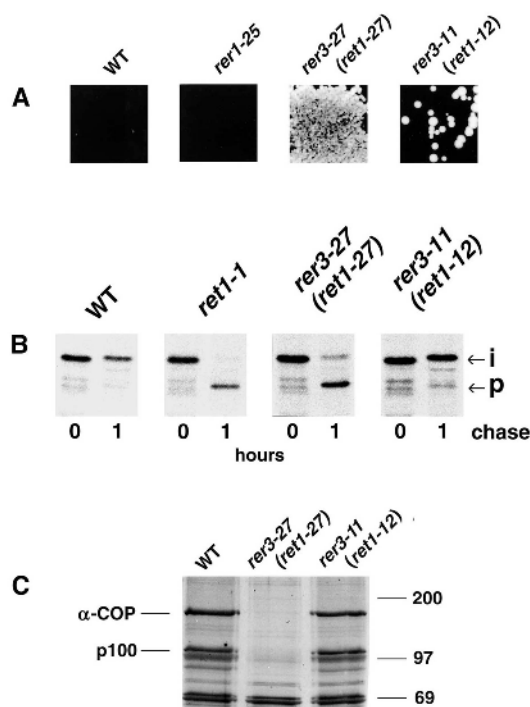


Fig. 2. (A) Mating assay to examine *rer* mutants for defects in ER retention of KKXX-tagged proteins. *MATa*, *ste2* cells expressing the *STE2-WBP1* chimera (WT: FLY62, *rer1-25*:FLY37, *rer3-27*:FLY35, *rer3-11*:FLY85) carrying the C-terminal KKXX retention signal were grown on YEPD plates replica-plated to a lawn of *MAT α* cells (RH311-3D). After 6 hours of mating at 30°C, cells were replica-plated to SD plates selective for growth of diploid cells. (B) Assay for retention defects of KKXX-carrying invertase-Wbp1 hybrids. Wild-type (JBD2-2A), *ret1-1* (EGY101), *rer3-27* (C21.27-2D) and *rer3-11* (R3P4-10A) cells expressing the Inv-Wbp1 fusion proteins were pulsed for 10 minutes with Tran^[35S]-label and chased for 0 or 1 hour at room temperature. Inv-Wbp1p was immunoprecipitated from the labelled cells with an anti-invertase serum, treated with endo H and analyzed on an SDS-polyacrylamide gel. Intact (i) and processed (p) hybrid protein migrated at 70 and 56 kDa, respectively. (C) In vitro binding assay of coatomer to the dilysine motifs. Whole cell lysates from the indicated cells were incubated with WBP1 peptide coupled to Sepharose beads. The adsorbed proteins were separated by SDS-PAGE and revealed by silver staining. The position of α COP and the p100 family (β , β -, and γ COP) are indicated.

6 mannose-specific serum showed that the protein receives α -6 mannose-specific residues to a much higher extent than in wild-type cells (Fig. 3A, lane 4, lower panel). This type of Golgi-specific glycosylation is likely to be due to the initiation of α -6 mannosylation which is catalyzed by the Och1 protein in a very early Golgi compartment of yeast (Nakanishi-Shindo et al., 1993; Gaynor et al., 1994). The absence of extensive outer chain elongation suggests that Sec12-invertase produced in a *ret1-12* mutant is either trapped in the Och1p-containing Golgi compartment or penetrating a little further into the Golgi compartment than in wild-type cells. (The two types of glycosylation described here are more clearly distinguishable when pulse-chase experiments were carried out with a different hybrid protein in a wild-type background as shown in Fig. 5.) Sec12-invertase may receive OCH1-specific glycosylation

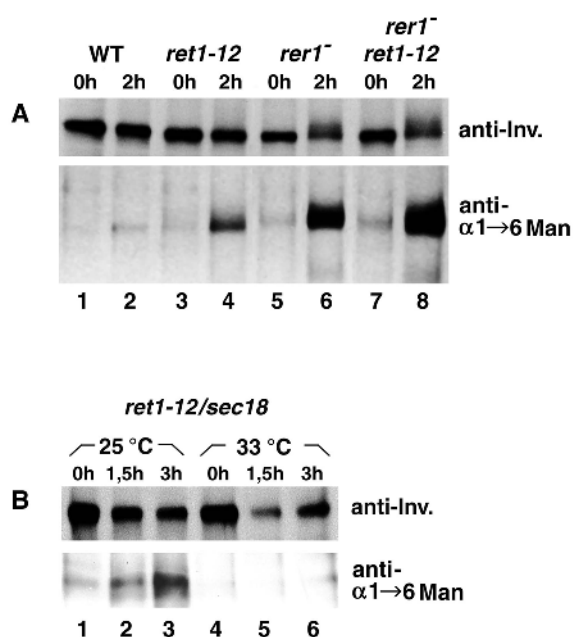


Fig. 3. Outer chain glycosylation of Sec12-invertase hybrids in *ret1-12* and *rer1-* mutants. (A) Pulse-chase experiments were performed using R1R3 strains (Table 1) with genotypes as indicated. Cells were labelled with Tran^[35S]-label for 15 minutes. Extracts were prepared 0 and 2 hours after the labelling of the cells. Sec12-invertase was precipitated from the extract by using an anti-invertase serum. Precipitated proteins were eluted from Protein A-Sepharose beads and aliquots were resolved by SDS-PAGE. Dried gels were exposed to fluorography. (B) Comparison of α -6 mannosylation between wild-type, *ret1-12* *rer1-* and *ret1-12 rer1-* cells. Aliquots of samples presented in A were reprecipitated with an anti- α -6 Man serum. (C) Golgi modification of Sec12-invertase in the *ret1-12* mutants requires ER-to-Golgi transport. A *sec18 ret1-12* double mutant was labelled with Tran^[35S]-label at 25°C. The sample was split at the beginning of the chase period. The two samples were incubated either at the permissive temperature of 25°C or at 33°C to block ER to Golgi transport. Aliquots were taken at the indicated times. Extracts were prepared and treated with anti-invertase serum. After elution from the Protein A-Sepharose beads the samples were split and reprecipitated with anti-invertase antibodies again or with anti- α -6 Man serum as indicated.

upon mislocalization to the Golgi compartment. Alternatively, the *ret1-12* mutation may cause premature α -6 mannosylation in the ER by allowing newly synthesized enzymes to be active while they are passing through the ER. To exclude this possibility the *SEC12-SUC2* hybrid gene was expressed in a *sec18 ret1-12* mutant and the processing of the hybrid protein was followed both at 25°C and at 33°C. At 33°C most vesicular protein transport is blocked in *sec18* mutants (Novick et al., 1981; Graham and Emr, 1991). Labelling of the cells was performed at the permissive temperature. The sample was split and each half of the cells was incubated at either the permissive or nonpermissive temperature, respectively. Fig. 3B shows that the shift to 33°C completely blocked the α -6 mannosylation that was observed at 25°C. This suggests that Sec12-invertase had to leave the ER to receive OCH1-specific glycosylation in *ret1-12* mutants. In addition, experiments presented below show that the Kex2p-dependent invertase secretion in

ret1 mutants is not due to mislocalization of the late Golgi protease.

Besides *ret1-12* mutants, none of the other coatomer mutants expressing *SEC12- α -SUC2* showed detectable Kex2p-dependent invertase secretion. However, mislocalization of Sec12-invertase to the Och1p-containing Golgi compartment may not be confined to *ret1-12* mutants. When grown at 30°C Sec12-invertase from *ret1-1* and *ret3-1* mutants could also be precipitated at levels above wild-type using α -6 Man antibodies. At least in *sec27-1* mutants mislocalization of Sec12-invertase can be induced by an incubation at temperatures above 30°C (data not shown). The amount of hybrid protein that could be reprecipitated with α -6 Man specific serum from a *sec27-1* extract was still lower than in *ret1-12* mutants.

The effects of *ret1-12* and *rer1* are not additive

rer1 mutants cause a stronger Sec12-invertase defect in Sec12-invertase retention than *ret1-12* mutants (Boehm et al., 1994). Accordingly, using α -6 mannose specific antibodies less Sec12-invertase could be precipitated from extracts of *ret1-12* mutants compared to extracts of *rer1*⁻ mutants (Fig. 3A). We also examined whether the two defects are additive (Fig. 3, lanes 6 and 8). Variable results were observed in different pulse-chase experiments. To avoid errors due to variable efficiencies of labelling and precipitation, Kex2p-dependent invertase secretion was used to reveal subtle differences between *rer1*⁻ single and *rer1*⁻*ret1-12* double mutants. This assay employed the transient expression of a *SEC12-SUC2* hybrid gene encoding a protein in which the Sec12 and invertase parts were separated by a Kex2p cleavable site (SEC12- α -invertase; Fig. 1D; Boehm et al., 1994). Kex2p efficiently releases invertase from hybrid proteins passing through a late Golgi compartment (Graham and Emr, 1991; Boehm et al., 1994; Chapman and Munro, 1994). Therefore, Kex2p-dependent invertase secretion can indicate mislocalization of hybrid proteins that are normally retained in early compartments of the secretory pathway. Transient expression of the hybrid protein was achieved by the addition of galactose for 30 minutes to induce a *GAL1* promoter controlled *SEC12- α -SUC2* construct. To stop expression of the hybrid gene, cells were collected by centrifugation and resuspended in glucose-containing medium. This pulse gave rise to about 16 units of total invertase activity. At full induction about 80 units of total invertase activity could be reached.

The amount of internal and external invertase activity was determined at different time points before glucose addition to the cells. No significant difference between *rer1*⁻ single mutants and *rer1*⁻*ret1-12* double mutants could be observed (Fig. 4). As a control, the amount of secreted and total invertase was determined when the *GAL1*-regulated *SEC12- α -SUC2* construct was expressed in wild-type cells and *ret1-12* mutants.

Taken together these results indicate that the mislocalization of Sec12-invertase in a *rer1*⁻ background cannot be aggravated by the *ret1-12* mutation.

Sec22(Sly2)p-based chimeras

Several assays using Sec22(Sly2) chimeras served as controls for the experiments shown above. Sec22(Sly2)p is a protein with a similar topology to Sec12p. It is a synaptobrevin-like protein (Dascher et al., 1991) and acts as a vesicle-specific receptor, a so-called v-SNARE in the targeting of ER-derived

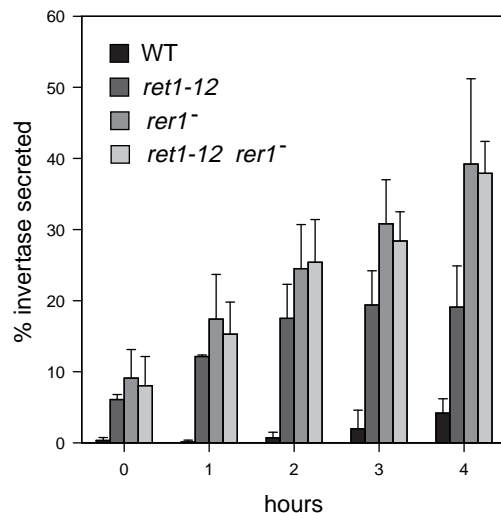


Fig. 4. A 30 minute pulse of hybrid protein expression was achieved by using a *GAL1*-regulated copy of the *SEC12- α -SUC2* hybrid gene. Samples were taken before and at different times after the incubation with galactose. External and total invertase were measured. The percentage of invertase secreted is indicated. Data represent mean values obtained in at least three different experiments.

vesicles (Søgaard et al., 1994). Sec22p is enriched several hundredfold during the budding of transport vesicles at the ER to the Golgi (Rexach et al., 1994). It is generally believed that Sec22p along with other v-SNAREs is retrieved from the Golgi by COPI coated vesicles (Pelham, 1994; Duden et al., 1994). Sec12 protein, in contrast, could not be detected on transport vesicles (Barlowe et al., 1994). Accordingly, the rate of Kex2p-dependent invertase secretion is much higher from cells expressing *SEC22- α -SUC2* than from cells expressing *SEC12- α -SUC2*. Obviously, the fast exit of Sec22-chimeras from the ER also promotes rapid transport to the late Golgi. Sec22-invertase hybrids can become ER resident proteins if their TMD is replaced by that of Sec12p, and the retention of such a Sec22/12-invertase hybrid depends on Rer1p (Boehm et al., 1994).

One important reason for employing Sec22- and Sec22/12-chimeras as a control was the fact that experiments using these chimeras could be carried out in wild-type cells instead of *rer1* mutants. *rer1* mutants exhibit a defect in outer chain glycosylation of invertase. Therefore, there is only a small difference in the mobility of Sec12-invertase from *ret1-12* and *rer1*⁻ mutants (Fig. 3A). The pulse-chase experiment shown in Fig. 5A (lanes 1 to 3) demonstrates that Sec22-invertase rapidly receives outer chain glycosylation. This leads to an extensive smear which is characteristic for secreted invertase. Sec22/12-invertase behaves differently (see Fig. 1E and F for a comparison of the two hybrid proteins). Even 90 minutes after the labelling reaction, Sec22/12-invertase migrates as a sharp band with the same size as at the beginning of the chase period. However, reprecipitation with α -6-man specific serum indicated that a large portion of the Sec22/12-invertase protein had reached an early Golgi compartment where the α -6 mannosylation is initiated by Och1p. Therefore, Sec22/12-invertase very much resembles Sec12-invertase expressed in a *ret1-12* mutant. In the absence of Rer1p Sec12-invertase receives

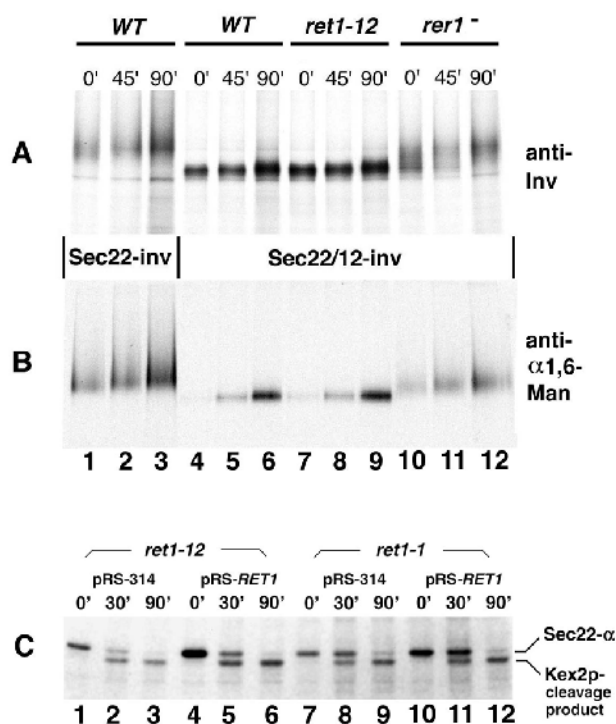


Fig. 5. Pulse-chase experiments using Sec22-derived chimeras. (A) Sec22-invertase and Sec22/12-invertase (lacking a Kex2p-cleavage site) were expressed in a protease-deficient *Δpep4* strain (R1R3-7A-pBLYS-*ret1*, WT; lanes 1 to 6; R1R3-7A, *ret1-12*; lanes 7 to 9; R1R3-10A-pLys-RET1, *Δret1*). Cells were radiolabelled for 15 minutes at 30°C and then chased at the same temperature for 0, 45, and 90 minutes. (B) After the first precipitation using anti-Sec22p antibodies and elution from the Protein A-Sepharose beads, half of the samples were reprecipitated with an anti- α -1,6 man serum. (C) Sec22- α factor cleavage by Kex2p is not increased in *ret1* mutants. To analyze the effect of different *RET1* alleles on the transport of Sec22- α factor, pulse-chase experiments were performed using mutants expressing moderate levels of the hybrid gene (*CYC1* promoter, CEN-based vector). To obtain isogenic strains mutants were transformed either with vector pRS314 or pRS314-*RET1* carrying the wild-type gene. Labelling and chase were performed in synthetic minimal medium selective for both plasmids. Note that the cleaved hybrid protein still carries segments encoded by polylinker regions in addition to the *c-myc*-epitope. Therefore, it still migrates above the Sec22 protein derived from the chromosomal wild-type gene which can be seen as a faint band below the cleavage product.

extensive outer chain glycosylation (Fig. 5A,B; lane 9 to 12) indicating that Rer1p prevents the exit of chimeras carrying the TMD of Sec12 from an early Golgi compartment.

Fig. 5A and B also show that the Golgi modification of Sec22/12-invertase is not accelerated by the *ret1-12* mutation (compare lanes 4 to 6 and 7 to 9). This result was confirmed by assays using the analogous hybrid protein carrying a Kex2-cleavage site. Neither by measuring the amount of invertase secreted, nor by immunoblot analysis were we able to find a significant difference between wild-type and *ret1-12* cells (data not shown).

The much faster transport of Sec22-invertase compared to Sec12-invertase may in part be an artifact caused by the bulky invertase reporter. To test this, a new chimera was constructed, Sec22- α factor (see Fig. 1G). This chimeric protein differs from

the previously used hybrids by carrying a short α factor peptide as a reporter (13 residues; Kurjan and Herskowitz, 1982) instead of invertase (69 kDa). Pulse-chase experiments were performed using wild-type and mutant cells expressing moderate levels of the Sec22- α factor hybrid (*CYC1* promoter, CEN-based vector). To obtain pairs of truly isogenic strains, *ret1-12* mutants were transformed either with the pRS314 vector alone or pRS314 containing the *RET1* gene (pRS314-*RET1*). As shown in Fig. 5C the Sec22- α factor is cleaved with a half-time of about 30 minutes. This cleavage does not occur in Kex2p-deficient cells (not shown). The half time for cleavage by Kex2p of about 30 minutes is rather slow when compared to a half time of 5 minutes for an analogous hybrid protein derived from the *cis*-Golgi glycosyltransferase Och1p (Harris and Waters, 1996). Even longer half times can be obtained when less Sec22- α factor is produced (W. Ballensiefen and H. D. Schmitt, unpublished results). Thus, targeting of Sec22p-derived chimeras is sensitive to the reporter to which it is fused as well as to the rate of expression. In these respects Sec22 hybrids differ greatly from analogous Sec12 hybrids.

No significant difference between wild-type and *ret1-12* and *ret1-1* mutants was observed when the rate of Kex2 processing of Sec22- α factor were compared (Fig. 5C). In these experiments labelling and chase were performed at 30°C. Under these conditions both mutants exhibit the missorting defect as indicated by using the Sec12-invertase and Ste2-Wbp1p test system.

Obviously, *ret1* mutants do not disturb the targeting Sec22p. This, of course, does not rule out the possibility that coatamer is involved in the recycling of Sec22p. The experiment shown in Fig. 5C also indicates that the Kex2p-dependent invertase secretion, the approach by which the '*rer3*' mutants had been selected, is not due to an increased activity of Kex2p in an early secretory compartment.

Rer1p localization

Comparing the mislocalization defects in *rer1* and *ret1* mutants suggested that α COP encoded by *RET1* may act before Rer1p, therefore placing Rer1p into the Golgi. This was confirmed by double labelling immunofluorescence. Formaldehyde-fixed spheroplasts of cells expressing normal levels of Rer1p were incubated with affinity-purified anti-Rer1p antibodies. A monoclonal antibody directed against the C terminus of Ypt1p was used to visualize this Golgi marker (Haubruck et al., 1987; Preuss et al., 1992). To facilitate double labelling with additional marker proteins, transformants were used that expressed tagged versions of these proteins. Och1p was employed as a marker for an early Golgi compartment (Nakanishi-Shindo et al., 1993; Gaynor et al., 1994), Pmr1p as marker for the medial Golgi (Antebi and Fink, 1992; Schröder et al., 1995) and Kex2p as marker for a late Golgi compartment (Redding et al., 1991). These proteins were either *c-myc*- or hemagglutinin(HA)-tagged versions carried by multi-copy plasmids (Och1p-*myc* and HA-Pmr1p; M. Lewis, personal communication; Antebi and Fink, 1992), or expressed from CEN-based single copy plasmids (Kex2p-HA; Nothwehr et al., 1995). Staining with each of these antibodies gave a punctuate pattern (Fig. 6). Overlap in the staining pattern between Rer1p and the different Golgi markers was quantitated as described by Antebi and Fink (1992). All antibodies stained about two to three dots per cell.

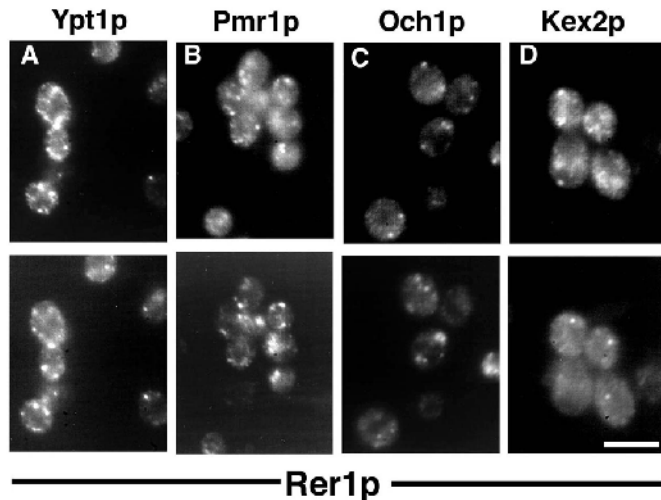


Fig. 6. Double labelling of wild-type cells (JBD2-2A) with anti-Rer1p antibodies and the Golgi markers Ypt1p, Pmr1, and Och1p. Wild-type cells or transformants carrying plasmids for the expression of epitope-tagged Golgi markers were fixed, permeabilized and treated as described in Materials and Methods. Fixed cells were incubated with monoclonal antibodies (upper panels) directed against either Ypt1p, 9E10 antibody directed against the *c-myc*-epitope to visualize *c-myc*-tagged Och1p or 12CA5 antibody directed against the HA epitope to visualize HA-tagged Pmr1p and Kex2p. All samples labelled with an affinity-purified anti-Rer1 antibody (lower panels). Cy3-conjugated anti-mouse IgG and FITC-conjugated anti-rabbit IgG were used as secondary antibodies. Bar, 5 μ m.

Those which were labelled with Rer1p antibodies were checked for staining by the second antibody and vice versa. The dots associated with Rer1p colocalized best with Ypt1p and Pmr1p (Table 2) and to a lower extent with Och1p. Colocalization of 70 to 80% may represent the maximal overlap that can be observed since double staining of Ypt1p with both a polyclonal and a monoclonal antibody did not result in a higher degree of colocalization (data not shown). Very little overlap was found when dots associated with Rer1p and the late Golgi marker Kex2p were compared. This indicated that Rer1p resides in the earlier compartments of yeast Golgi.

Similar punctuate structures were observed when a *ret1-12* mutant was stained for Rer1p and Ypt1p. The number of dots associated with Rer1p remained the same, and also the degree of colocalization did not change significantly (80 and 89%, respectively). These experiments were performed in a Δ *pep4* background as well as in *Pep*⁺ strains (see also Fig. 8G). The *pep4* mutation should prevent degradation of proteins missorted to the vacuole. Since *pep4* mutants exhibit no vacuolar sorting defect (Klionsky et al., 1990) it should be

Table 2. Colocalization of Rer1p with other Golgi markers

Comparison	% Colocalization	Comparison	% Colocalization
Rer1p-Ypt1p	73	Ypt1p-Rer1p	76
Rer1p-Pmr1p	72	Pmr1p-Rer1p	67
Rer1p-Och1p	59	Och1p-Rer1p	53
Rer1p-Kex2p	22	Kex2p-Rer1p	24

Values refer to the percentage colocalization with respect to the antigen that is listed first.

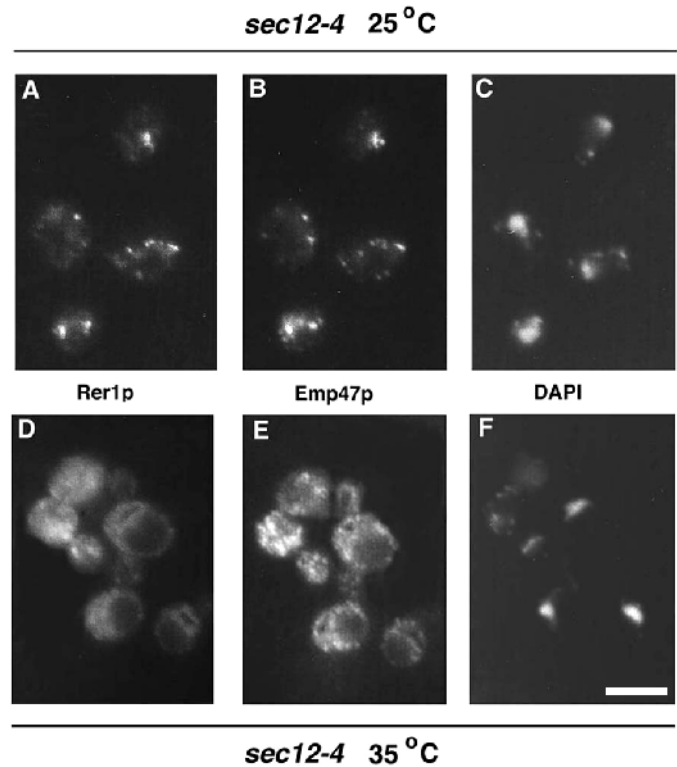


Fig. 7. Immunofluorescence localization to reveal redistribution of Rer1p and Emp47p to the ER. A temperature-sensitive *sec12-4* strain (RH3048) expressing *c-myc-EMP47* integrated at the *LEU2* locus was incubated at 25°C (permissive) or 35°C (nonpermissive) for 1 hour. 20 μ g/ml cycloheximide were added to block protein synthesis. Cells were fixed, permeabilized and stained as described in legend of Fig. 6. In addition to an affinity-purified anti-Rer1p antibody (A and D), the 9E10 monoclonal antibody was used to visualize *myc*-tagged Emp47 protein. Cy3-conjugated anti-mouse IgG and FITC-conjugated anti-rabbit IgG were used to stain the antigens. DAPI (1 μ M) was included in one of the last washing steps to visualize nuclei (C and F). Bar, 5 μ m.

possible to detect missorted proteins that have reached this compartment (Gaynor et al., 1994; Schröder et al., 1995). However, Rer1p antibodies did not stain the vacuole of *ret1-12*. Thus, if *ret1-12* affects Sec12p retrieval by first altering the distribution of Rer1p, this must be too subtle a change to be detected by immunofluorescence techniques.

Rer1p recycles to the ER and this recycling is blocked in coatomer mutants

A high degree of colocalization was also found when the cellular distribution of Rer1p and another Golgi protein, Emp47p, was compared in double immunofluorescence experiments (S. Schröder, personal communication; Fig. 7). Emp47p is a type I transmembrane protein that carries a dilysine motif. It colocalizes best with Pmr1p as determined by cell fractionation and immunofluorescence. Schröder et al. (1995) also demonstrated that Emp47p undergoes retrograde transport from the Golgi to the ER. The assay relies on mutant cells, such as *sec12* mutants, that can be blocked in forward transport without disturbing the retrograde transport. As shown by Schröder et al. (1995) for Emp47p and more recently by Lewis and Pelham (1996) for the HDEL-receptor, Golgi proteins

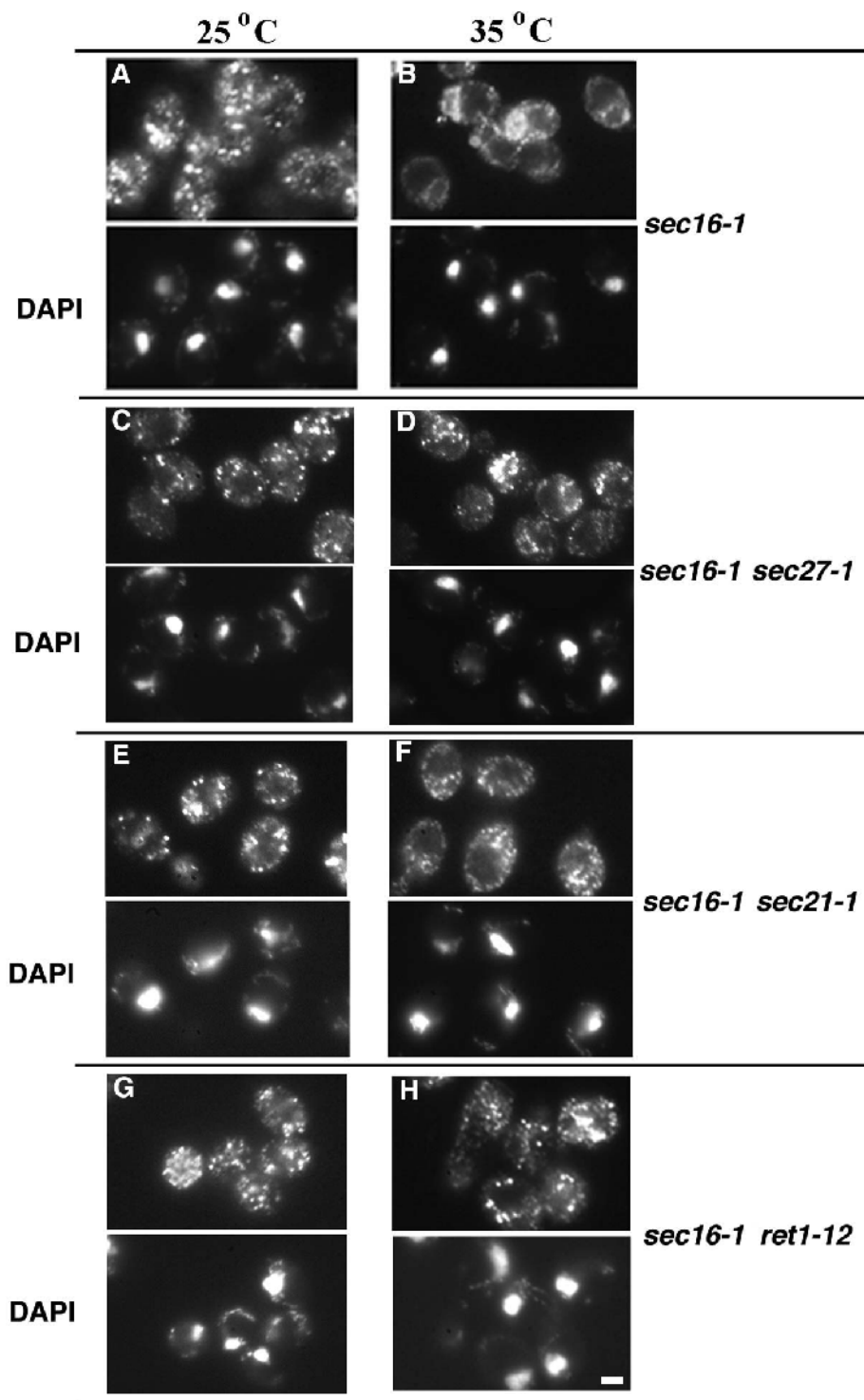


Fig. 8. Immunofluorescence localization of Rer1p in *sec16-1* mutants (A and B) and *sec16-1* mutants carrying additional COPI defects as indicated. Cells were treated as described in Fig. 7. The cells used were diploid to facilitate the visualization of the different structures. DAPI was used to stain nuclei and mitochondria (panels below the immunofluorescence micrographs). Bar, 5 μ m.

which recycle can be trapped in the ER in *sec12* mutants at the nonpermissive temperature. To see whether Rer1p redistributes in a similar way to Emp47p and Erd2p, a *sec12-4* mutant expressing a *myc*-tagged copy of Emp47p from a chromosomally integrated vector was incubated for 1 hour at either 25°C or 35°C. During this incubation further protein synthesis was blocked by the addition of cycloheximide. Cells were fixed and stained for Rer1p and Emp47p. The normal punctuate staining pattern was observed in samples that were incubated at the per-

missive temperature (25°C; Fig. 7A,B). At 35°C perinuclear staining typical for the ER of yeast (Rose et al., 1989) was found when cells were stained for Rer1p and Emp47p (Fig. 7D,E). This result demonstrates that Rer1p recycles between Golgi and ER as shown before for Emp47p and Erd2p.

To investigate whether the recycling of Rer1p is mediated by coatomer (COPI)-coated vesicles, we repeated the redistribution experiment described above using various double mutants. In these mutants a defect blocking the budding of

COPII vesicles from the ER was combined with mutations in coatamer (COPI)-encoding genes. Diploid cells were used in these experiments to facilitate the visualization of various structures. Staining the cells for Rer1p demonstrated that, unlike *sec12-4* single mutants, *sec12-4 ret1-12* double mutants display no ER-like structures when incubated at 35°C (data not shown). Additional immunofluorescence experiments were performed using *sec16-1* mutants rather than *sec12-4* to avoid artifacts due to a possible interaction of Rer1p with mutated Sec12p. The results obtained with *sec16-1* confirmed the observations made with *sec12-4*. At 35°C the *sec16-1* single mutants also displayed an ER-like pattern when stained with anti-Rer1p antibodies (Fig. 8B). The *ret1-12* mutation inhibits the redistribution of Rer1p to the ER since punctuate structures are still visible in *sec16-1 ret1-12* double mutants incubated at 35°C (Fig. 8H). Two additional COPI affecting defects, *sec21-1* and *sec27-1*, were also combined with the COPII mutation *sec16-1* to test the relocalization of Rer1p. Like the *ret1-12* mutation, the *sec21-1* mutation efficiently prevents the relocalization of Rer1p (Fig. 8F). In *sec27-1 sec16-1* mutants some cells still showed a perinuclear ring suggesting that this COPI mutation prevents the recycling of Rer1p to a lower extent (Fig. 8D). Since *sec27-1* mislocalizes Sec12-invertase only at higher temperatures and less efficiently than *ret1-12*, these results indicate that the mislocalization of the Sec12p-derived hybrid protein relates to the slowdown of Rer1 recycling. In summary, Rer1p recycles to the ER via coatamer-coated vesicles as described for Emp47p and Erd2p.

DISCUSSION

ret1 mutants were first identified as defective in the retention of dilysine-tagged membrane proteins in the ER of yeast. In this work we show that mutations in the α COP-encoding *RET1* gene also result in mislocalization of membrane proteins different from the dilysine-tagged type I transmembrane proteins. The targeting defects caused by the *ret1-12* mutant (previously referred to as *rer3-11*) can occur with only little effect on the retention of KKXX-tagged proteins. The finding that *rer3* mutants are alleles of the α COP encoding *RET1* gene suggests that, in addition to the previously characterized Rer1 protein, coatamer contributes to the retention of Sec12p in the ER.

There is certainly a third mechanism that contributes to the targeting of Sec12p. As mentioned before, Sec22p is a protein with a topology similar to that of Sec12p. Unlike Sec12p, however, Sec22p is actively packaged into transport vesicles that bud from the ER (Rexach et al., 1994; Barlowe et al., 1994). Factors that favour active packaging (Sec22p) or delay the exit from the ER (Sec12p) very likely reside in the cytoplasm domain (Sato et al., 1996). The importance of the cytoplasmic domains is also illustrated by the finding that the swapping of the TMDs cannot change Sec12-invertase into a rapidly transported protein like Sec22-invertase, while Sec22/12-invertase is not as efficiently retained in the ER as Sec12-invertase (Boehm et al., 1994; J. Boehm and H. D. Schmitt, unpublished results). As shown here (Fig. 5A), the TMD of the respective hybrid proteins is responsible for determining the rate of exit from a very early Golgi compartment rather than preventing the exit from the ER.

Comparing the *ret1* and *rer1* defects

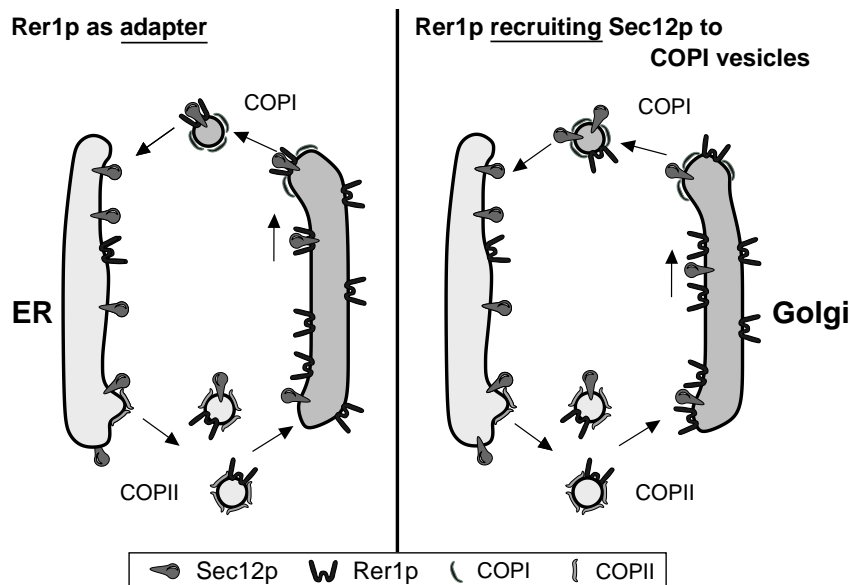
The *ret1-12* mutation and the *rer1* defects which were characterized previously (Nishikawa and Nakano, 1993; Boehm et al., 1994) have several features in common. Both defects lead to mislocalization of Sec12-invertase chimeras and to a reduced steady state level of Sec12p or epitope-tagged Sec12 proteins (Boehm et al., 1994; our unpublished results). This instability may be explained by the transport of the mislocated chimeras to the vacuole. Lack of ER retention was demonstrated in two ways: (i) Sec12-invertase carrying an intervening cleavage site for the late Golgi protease Kex2p is cleaved and invertase is released. (ii) The rate of outer chain glycosylation of Sec12-invertase is increased in the mutants. In *ret1-12* mutants Sec12-invertase receives outer chain glycosylation as shown by precipitation with glycosylation specific antibodies. However, this modification did not change the apparent size of the proteins, indicating that the chimeras had reached the earliest Golgi compartment of yeast where α -6 mannosylation is initiated by the Och1 protein (Nakanishi-Shindo et al., 1993; Gaynor et al., 1994; Lehle et al., 1995). In *rer1* mutants a large part of Sec12-invertase receives extensive outer chain glycosylation, a reaction which requires the elongation of the α -6 mannose chain in later compartments of the Golgi. Different controls showed that cleavage by Kex2p was not due to premature activity of Kex2p in the ER and that the outer chain glycosylation of Sec12p and Sec12-invertase required ongoing ER-to-Golgi transport (Nishikawa and Nakano, 1993; this work).

From these phenotypes one could conclude that α COP and Rer1p both act on the same transport intermediates, Sec12-invertase glycosylated by Och1p. While Rer1p may block the transport of this intermediate to later Golgi compartments, α COP is involved in the recycling to the ER. It should be pointed out that the glycosylation intermediate observed in *ret1* mutants also occurs in wild-type cells, but to a much lesser extent. This suggests that in wild-type cells recycling mediated by COPI vesicles is efficient enough to prevent almost completely the glycosylation of Sec12-invertase by Och1p.

Assuming that Rer1p acts together with or downstream of α COP one would suggest that Rer1p functions in the Golgi. Accordingly, Rer1p was located in the Golgi by fractionation studies as well as by immunofluorescence. There is some overlap between Rer1p and the early Golgi marker Och1p. A higher degree of colocalization was found between Rer1p and the medial Golgi markers Pmr1p and Emp47p. There was only very little overlap in the staining pattern of Rer1p and the late Golgi marker Kex2p. These results are consistent with the findings of Sato et al. (1995) who were able to partially separate HA-tagged Rer1p and Och1p from the late Golgi marker Kex2p by a sucrose step gradient, and who found a good colocalization of Ypt1p and HA-tagged Rer1p expressed from a multi-copy vector. Therefore, rather than being part of a rigid retention system that prevents Sec12p from exiting the ER, Rer1p carries out its retention function in the early and medial Golgi compartments of yeast.

The following observations are also consistent with the interpretation that Rer1p acts in the Golgi of yeast: a partial defect in outer chain glycosylation of invertase was observed with *rer1* mutants (Boehm et al., 1994; see also Fig. 5), and a close homolog of *RER1* was recently isolated from a human cell line. A tagged version of hRer1 localizes to the Golgi and

Fig. 9. Two models illustrating the possible role of Rer1p in the recycling of Sec12p. Both transmembrane proteins cycle between ER and Golgi. Rer1p is a transmembrane protein with four transmembrane domains and only a few residues exposed at the luminal side of the membrane compartment (shown as a 'W'). It is necessary for the retrieval of Sec12p or type II transmembrane proteins carrying the TMD of Sec12p. In the first model, Sec12p binds to Rer1p directly and serves as an adapter by contacting Sec12p and COPI simultaneously. As shown in the second model, Rer1p may direct Sec12p to certain sites within the Golgi where COPI vesicles can form. In this case, the recycling of Rer1p would be a way to keep Rer1p at its normal locations in the cell. Sec12p and Rer1p may exit their regular sites at low rates to achieve different steady state concentrations in ER and Golgi.



overexpression of this protein leads to relocalization to ER-like structures (J. Füllekrug, J. Boehm, T. Nilsson, G. Mieskes and H. D. Schmitt, unpublished results).

The relationship between α COP and Rer1p

In both mutants, *ret1* and *rer1*, the mislocalization is a rather slow process which takes hours to become evident. Therefore, it was quite surprising that the two defects are not synergistic and not even additive. When discussing the possible functional relationship between α COP and Rer1p one has to consider this lack of genetic interaction. At least this makes it unlikely that the *rer1* and *ret1* defects disturb two parallel pathways.

Two models can be envisaged that account for the absence of additive effects (Fig. 9). First, coatomer and Rer1p may cooperate directly in the retention of Sec12p. There is no evidence that COPI interacts with Sec12p. Coatomer may control Sec12p localization through a third protein, possibly Rer1p. The primary defect in the *ret1-12* mutants may lie in the failure of coatomer to bind the complex of Rer1p and Sec12p and to retrieve this complex to the ER. If Rer1p is absent then, of course, the mislocalization of Sec12p cannot be aggravated by a *ret1* defect.

In this model, Rer1p would act as a receptor or adapter analogous to the HDEL receptor, Erd2p (Semenza et al., 1990; Lewis et al., 1990) or as a cargo receptor like Emp24p (Schimmöller et al., 1995). Similar to the HDEL sequence that binds to the Erd2 receptor, the TMD of Sec12p may represent a domain that is recognized by Rer1p. As with Erd2p, Rer1p would then carry Sec12p to the ER while it is recycling via COPI vesicles. Lewis and Pelham (1996) demonstrated that the recycling of Erd2p receptor requires functional γ COP (Sec21p). Accordingly, we also found that Rer1p recycles to the ER in a COPI-dependent fashion. Emp24p, however, is a constituent of COPII vesicles which is involved in the packaging of specific cargo molecules (Schimmöller et al., 1995). Therefore, it was supposed that its primary function lies in the anterograde transport. Surprisingly, Fiedler et al. (1996) could show that Emp24p binds to coatomer although it is lacking a classical retrieval signal. They also showed that

coatomer has more than one binding specificity. This makes it likely that Rer1p which also is lacking any of the known binding motifs for coatomer could be recognized by this coat complex.

In a second model Rer1p may function as sorting factor which is required for the packaging of Sec12p into COPI vesicles. There is no evidence that Sec12p and Rer1p recycle to the ER as a complex. A sorting factor which does not enter vesicles was recently described by Kuehn et al. (1996). Shr3p is required for the packaging of amino acid permeases into COPII vesicles. In contrast to the cargo receptor Emp24p, Shr3p is not found in COPII vesicles which were obtained in an *in vitro* reaction. Accordingly, the recycling of Rer1p to the ER would be a way to keep its specific cellular localization. Mislocalization of Sec12p in *ret1* mutants would in that case be the result of an improper targeting of Rer1p caused by a coatomer defect. Coatomer can in fact be involved in the targeting and possibly the functioning of Golgi proteins. Schröder et al. (1995) showed that the Golgi-localization of Emp47p depends on a C-terminal dilysine motif. So far, however, we were unable to observe differences in the localization of Rer1p when comparing wild-type and *ret1-12* cells. Thus, the defect in *ret1-12* may be due to very subtle differences in the localization or function of Rer1p.

The following observation may disprove these two models. The *ret1-12* mutation has no effect on the targeting of Sec22-invertase chimeras carrying the TMD of Sec12p (Sec22/12-invertase). Retention of this hybrid protein requires Rer1p (Boehm et al., 1994; Sato et al., 1996). If *ret1-12* affects Sec12p targeting through Rer1p, then it should also interfere with the targeting of these proteins. This was not the case. The mechanisms preventing Och1p-dependent glycosylation of Sec12-invertase in wild-type cells may be saturable as shown by experiments using strong expression of *SEC12-SUC2* from the *GALI* promoter (unpublished results). Since the Sec22p leaves the ER much faster than Sec12p (Rexach et al., 1994; Barlowe et al., 1994), the observation that Sec22/12-invertase is unaffected by the *ret1-12* mutation may be due to saturation of the early Golgi-to-ER recycling process. The more efficient

retention within the early Golgi may reflect a higher concentration of Rer1p in the medial Golgi compartment as indicated by its colocalization with Emp47p and Pmr1p, whose steady state localization is between the Och1p and the Kex2p sub-compartments (Schröder et al., 1995).

From the results presented here it is clear that both coatomer and Rer1p are involved in the retrieval of Sec12p. The results add to the growing list of evidence that COPI vesicles are the common route for the recycling of all type proteins from the Golgi to the ER. Further experiments will be required to see whether Rer1p is itself a part of the recycling machinery or whether it has only a sorting function and recycling is just a way to target this protein correctly. In particular, comparisons between different COPI defects are necessary to confirm a close relationship between the recycling of Sec12p and Rer1p as indicated by the comparison of *ret1-12* and *sec27-1* mutants.

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