Journal of Cell Science 111, 1507-1520 (1998)
Printed in Great Britain © The Company of Biologists Limited 1998
JCS1518

Recycling of the yeast *v*-SNARE Sec22p involves COPI-proteins and the ER transmembrane proteins Ufe1p and Sec20p

Wolfgang Ballensiefen*, Dmitri Ossipov and Hans Dieter Schmitt†

Department of Molecular Genetics, Max-Planck-Institute for Biophysical Chemistry, D-37070 Göttingen, Germany
*Present address: MRC UCL, Laboratory for Molecular and Cell Biology, Gower Street, London WC1E 6BT, UK
†Author for correspondence

Accepted 30 March; published on WWW 14 May 1998

SUMMARY

Vesicle-specific SNAP receptors (*v*-SNAREs) are believed to cycle between consecutive membrane compartments. The *v*-SNARE Sec22(Sly2)p mediates the targeting of vesicles between endoplasmic reticulum (ER) and early Golgi of *Saccharomyces cerevisiae.* To analyze factors involved in targeting of Sec22(Sly2)p, an α-factor-tagged Sec22 protein (Sec22-α) was employed. Only on reaching the late Golgi, can α-factor be cleaved from this hybrid protein by Kex2p, a protease localized in this compartment. In wild-type cells Kex2p-cleavage is observed only when Sec22-α is greatly overproduced. Immunofluorescence microscopy and subcellular fractionation studies showed that Sec22-α is returned to the ER from the late Golgi (Kex2p) compartment. When Sec22-α is expressed in wild-type cells at levels comparable to the quantities of endogenous Sec22p, very little of this protein is cleaved by Kex2p. Efficient cleavage, however, occurs in mutants defective in the retrograde transport of different ER-resident proteins indicating that Sec22-α rapidly reaches the late Golgi of these cells. These mutants (*sec20-1, sec21-1, sec27-1* and *ufe1-1*) reveal Golgi structures when stained for Sec22-α and do not show the ER-immunofluorescence observed in wild-type cells. These results show consistently that Sec22p recycles from the Golgi back to the ER and that this recycling involves retrograde COPI vesicles.

Key words: Sec22p, ER-Golgi, *v*-SNARE, Retrograde transport, COPI

INTRODUCTION

Protein transport between secretory compartments is mediated by membrane-vesicles (Rothman and Orci, 1992). In yeast, vesicular transport between the endoplasmic reticulum (ER) and the Golgi involves two different types of coated vesicles, COPI and COPII (Barlowe et al., 1994; Duden et al., 1994; Letourneur et al., 1994). COPI in yeast is equivalent to the mammalian COPI-complex (= coatomer) and homologs of all seven mammalian COPI proteins have also been identified in yeast (Hosobuchi et al., 1992; Duden et al., 1994, 1998; Letourneur et al., 1994; Cosson et al., 1996). The COPI protein complex in general covers Golgi-derived vesicles and is believed to assist the budding process as well as sorting the cargos of this vesicle population. At present, it is not clear whether COPI vesicles function exclusively in retrograde transport or both in anterograde and retrograde transport (Pelham, 1994; Bednarek et al., 1995; Gaynor and Emr, 1997; Rowe et al., 1996; Orci et al., 1997; Scales et al., 1997). COPII complex proteins were first identified in yeast, but recently homologues have also been identified in mammalian cells (Barlowe et al., 1994; Aridor et al., 1995). COPII vesicles bud exclusively from the ER and carry cargo destined for further anterograde transport (Bednarek et al., 1995).

There is strong evidence for COPI-coated vesicles being involved in the recycling of proteins from the Golgi back to the ER: (a) The COPI complex in extracts from mammalian and yeast cells binds proteins carrying the C-terminal di-lysine motif (-*KK*XX) the retrieval signal carried by ER-resident type I transmembrane proteins (Cosson and Letourneur, 1994; Jackson et al., 1990); (b) COPI-complexes from yeast mutants with defects in certain subunits (*ret1-1* and *sec27-1*) loose the ability to bind di-lysine motifs in vitro (Letourneur et al., 1994); (c) *ret1-1* and *sec27-1* mutants as well as mutants defective in other subunits (sec*21-2*, *ret2-1* and *ret3-1*) are unable to retain di-lysine-tagged proteins in the ER (Letourneur et al., 1994; Cosson et al., 1996); (d) the following receptors or sorting factors as well as their ligands or substrates depend on COPI for recycling from the Golgi to the ER: the HDEL-receptor (Erd2p) required for retrograde transport of soluble HDEL-tagged ER proteins (Lewis and Pelham, 1996) and Rer1p which mediates the retrieval of ER transmembrane proteins lacking the KKXX signal (Boehm et al., 1997; Sato et al., 1997).

Coated vesicles have to be uncoated before they can dock and fuse with their target membrane (Rothman, 1994). After uncoating a set of specific receptors is exposed on the surface of the vesicles. These *v*-(vesicular)SNAREs can then interact with their cognate *t*-(target)SNAREs on the acceptor membrane (Söllner et al., 1993; Søgaard et al., 1994). The name SNARE derives from the fact that SNARE proteins also act as receptors for SNAPs (*s*oluble *N*SF (*N*-*e*thylmaleimide-

sensitive *f*actor) *a*ttachment *p*roteins), members of a family of evolutionarily-conserved proteins required for vesicle fusion (Clary et al., 1990). NSF and SNAPs were supposed to function after docking through rearranging SNAREs during the fusion of lipid bilayers (Söllner et al., 1993b). Recent evidence suggests, however, that NSF and SNAP act before docking by activating the SNAREs (Mayer et al., 1996; Otto et al., 1997).

Each type of vesicle fusion involves a specific set of *v*-SNARE and *t*-SNARE proteins (Söllner and Rothman, 1994). Most of them are synaptobrevin-like or syntaxin-like transmembrane proteins. Newly synthesized members of these two protein families are first inserted into the ER membrane before being transported to their cellular destinations via the secretory pathway (Jäntii et al., 1994; Kutay et al., 1995; Ossig et al., 1995).

The SNARE proteins identified as *v*-SNAREs and as *t*-SNAREs involved in ER-to-Golgi transport in yeast (Søgaard et al., 1994) are Sec22(Sly2)p, Bet1(Sly12)p, Bos1p, Ykt6p and Sed5p (Dascher et al., 1991; Newman et al., 1992; Søgaard et al., 1994; Hardwick and Pelham, 1992). Retrograde transport by COPI vesicles depends on two specific transmembrane proteins: Ufe1p, which represents a member of the family of syntaxin-related *t*-SNAREs, and Sec20p (Lewis and Pelham, 1996; Sweet and Pelham, 1992). Similar to COPI mutants, *ufe1-1* and *sec20-1* mutants exhibit defects in retrograde Golgi-ER transport (Lewis and Pelham, 1996; Cosson et al., 1997).

In this work we analyzed the targeting of Sec22p, a *v*-SNARE involved in ER-Golgi transport of yeast. Tagged versions of Sec22p were constructed to monitor the cellular fate of this tail-anchored membrane protein by different methods and at various expression levels. Processing by proteases and visualizing of the stable cleavage products by western blotting can be used to monitor the arrival of hybrid proteins in specific target compartments, e.g. in the late Golgi or in the vacuole (Graham and Emr, 1991; Nishikawa and Nakano, 1993; Boehm et al., 1994; Chapman and Munro, 1994; Gaynor et al., 1994; Harris and Waters, 1996). Proteolytic processing of epitope-tagged Sec22p in various mutants and immunofluorescence experiments showed that the targeting of Sec22p involves coatomer (COPI)-coated vesicles.

MATERIALS AND METHODS

Yeast strains and growth conditions

Saccharomyces cerevisiae strains are listed in Table 1. Cells were grown in synthetic minimal medium containing glycerol (3%) and ethanol (2%), galactose (2%) or glucose (2%) as carbon sources and supplemented as necessary with 20 mg/l tryptophan, histidine, adenine or uracil, or 30 mg/l leucine or lysine. To ensure that pairs of isogenic mutant and wild-type cells were used, different approaches were employed: (a) comparisons of COPI-mutants (*sec21-1* and *sec27-1*) with the corresponding wild-type cells were carried out using mutant cells transformed with either a single copy plasmid encoding the particular wild-type protein or the same vector without insert (pRS315-*SEC21*, pRS315-*SEC27*, or pRS315 alone); (b) strains *MLY100* and *MLY101* were used as a pair of isogenic wild-type (*UFE1*) and *ufe1-1* mutant cells. Both strains carried a chromosomal disruption of the *UFE1* gene and a centromeric vector with either the *UFE1* wild-type gene or the *ufe1-1* allele (Lewis and Pelham, 1996); (c) isogenic pairs of mutant strains (*sec20-1* and an *erd2*; RSY275 and B36, repectively), and wild-type strains (RSY255, SEY2102) were obtained from M. Lewis. For cloning purposes *Escherichia coli* RR1 was used.

Table 1. List of yeast strains

Strain	Genotype	Source
WBY-2	*MATα, SEC, mfa1::ADE2, mfa2::TRP1, bar1::HIS3, ura3, lys2, leu2, his3, ade2, trp1*	This study
S27PP-1A	*MAT***a***, SEC, ura3, leu2*	This study
S21PP-6C	*MAT***a***, sec21-1, ura3, leu2*	This study
WBY-8	*MATα, sec21-1, ura3, leu2, lys2*	This study
WBY-10	*MATα, sec27-1, ura3, leu2, his3, pep4::HIS3*	This study
WBY-6	*MATα, sec27-1, ura3, leu2, lys2, ade2*	This study
SHC22-12A	*MAT***a***, sec22-3, ura3, lys2, leu2, his3, suc2-Δ9*	Boehm et al., 1994
RH589-3C	*MATα, sec23-1, ura3, leu2*	S. Schröder-Köhne
RSY255	*MATα, SEC, ura3, leu2*	C. Kaiser
RSY275	*MATα, sec20-1, ura3, his4*	C. Kaiser
MLY-100	*MATα, ura3, ade2, his, trp1, ufe1::TRP1,* containing p*UFE*315	M. Lewis
MLY-101	*MATα, ura3, ade2, his, trp1, ufe1::TRP1,* containing p*UT*1	M. Lewis
SEY2102	*MATα, ura3, leu2, his4, suc2-Δ9*	Emr et al., 1983
B36	*MATα, erd2, ura3, leu2, his4, suc2-Δ9*	M. Lewis

Construction of the *SEC22/SEC12*-α-pheromone hybrid genes

Plasmids used in this study are listed in Table 2. The α-factor-tagged Sec22 proteins were constructed as follows. Plasmid pDA6300 carrying the *MFα1* gene (Kurjan and Herskowitz, 1982) was cleaved with *Eco*RI and a 1.7 kb fragment was cloned into the pBluescript vector. Cleavage of this plasmid with *Hin*dIII and *Pvu*II yields a fragment which contains a cassette encoding one repeat of the mature α-factor, the 3′ non-coding region of the *MFα1* gene and additional sequences derived from the pBluescript vector (*lac*Z gene). The *SUC2* reporter gene encoding the invertase portion of the previously described *SEC22-SUC2* hybrid gene (Boehm et al., 1994) was replaced by this *Hin*dIII/*Pvu*II fragment.

The *SEC22*-α construct was placed under the control of the *GAL1* promoter by creating a *Bgl*II site before the start codon. This site could be joined to a *Bam*HI site downstream of the *GAL1* promoter in vectors derived from pCEY6 (d’Enfert et al., 1991).

TMD (transmembrane domain) substitution in Sec22-α hybrid proteins

The *SEC22-α*f-SUC2* construct (Boehm et al., 1994, 1997) carrying the sequences of the Sec12p-TMD was obtained as follows: PCR was used to create a *Bcl*I site in front of the TMD-encoding sequence of

Table 2. Plasmids used in this study

Plasmid	Genes	Source
pWB-*GALA*α	*GAL1-SEC22-c-myc*-α, *URA3, CEN4/ARS1*	This study
pWB-*GALAC*α	*GAL1-SEC22/12-c-myc*-α, *URA3, CEN4/ARS1*	This study
pWB-Amα	*SEC22-c-myc*-α, *URA3, 2μm*	Ballensiefen and Schmitt, 1997
pWB-Amcα	*CYC1-SEC22-c-myc*-α, *URA3, 2μm*	Boehm et al., 1997
pRS315	*LEU2, CEN6/ARSH4*	Sikorski and Hieter, 1989
pRS315-*SEC21*	*SEC21, LEU2, CEN6/ARSH4*	R. Duden
pRS315-*SEC27*	*SEC27, LEU2, CEN6/ARSH4*	This study
p*UFE*315	*UFE1, LEU2, CEN6/ARSH4*	M. Lewis
p*UT*1	*ufe1-1, LEU2, CEN6/ARSH4*	M. Lewis
p*DA6300*	*MFα1, LEU2*	H. Riezman

*SEC12-α*f-SUC2*. Thus the *SEC12-α*f-SUC2* sequences starting with codon 354 of *SEC12* could be fused to codon 191 of the *SEC22-α*f-SUC2* gene. The invertase gene was replaced by the sequence encoding the α-pheromone as described above. For the *GAL1*-dependent 'pulse-chase' experiments, this construct was fused to the *GAL1* promoter (plasmid pWB*GAL*-ACα; Table 2).

Antibodies

The monoclonal anti-*c-myc* antibody 9E10 (Evan et al., 1985) and a polyclonal anti-*c-myc* antibody (A-14) were obtained from Santa Cruz Biotechnology. Antibodies against Sec22p (R. Ossig and R. Grabowski), Kar2p/BiP, Kex2p, α-factor (M. Benli and D. Gallwitz), Ypt7p (D. Schegelmann and D. Gallwitz) and Sec61p (T. Sommer) were raised in rabbits. Monoclonal antibodies against the 60 kDa subunit of the vacuolar H^+ ATPase (Kane et al., 1992) were from Molecular Probes. For ECL-detection of the hybrid proteins, HRP-coupled secondary anti-rabbit or anti-mouse antibodies were purchased from Jackson Laboratories. Cyanine-(Cy2 or Cy3)-conjugated secondary antibodies were from Amersham and Jackson Laboratories, respectively.

Transient expression using the *GAL1* promoter

All media used were synthetic minimal media selective for the *URA3* and/or *LEU2* marker. The yeast transformants growing exponentially in glucose selective medium (*GAL1* promoter repressed) were transferred to medium containing both 3% glycerol and 2% ethanol (*GAL1* promoter neither repressed nor induced). Equivalents of 1 OD_{600} of the overnight cultures were harvested. Synthesis of Sec22-α was induced by incubating the plasmid-harboring strains for 35 minutes in 2% galactose medium (pulse). Cells were transferred into 2% glucose selective medium to stop the production of hybrid protein (chase).

Protein extraction and immunoblotting

Western blotting analysis was performed as described by Boehm et al. (1994). Aliquots (1 $OD_{600} = 1.7\times10^7$ cells) of transformed cells were lysed in 2 M NaOH, 5% mercaptoethanol and proteins precipitated with 10% trichloroacetic acid (TCA), neutralized with 1 M Tris-base and dissolved in SDS sample buffer. Proteins were resolved on 12.5% SDS-PAGE. To re-use the nitrocellulose filters for different immunoblot assays, they were washed for 30 to 60 minutes with 0.2 M glycine, pH 2.5.

Subcellular fractionation

Extracts from wild-type cells (*WBY-2*) expressing the *SEC22*-α hybrid gene (pWB-*GAL*Aα) were fractionated by velocity sedimentation on sucrose density gradients. The procedure is a modification of the protocol described by Antebi and Fink (1992). The equivalent of 100 OD_{600} of cells was collected by centrifugation. The cells were resuspended in 1 ml of 10 mM Tris-HCl, pH 9.4, 10 mM NaN_3, 30 mM β-mercaptoethanol and incubated at room temperature for 10 minutes. After washing with 10 mM NaN_3 cells were resuspended in spheroplasting buffer (1.4 M sorbitol, 50 mM Na-PO_4, pH 7.5, 10 mM NaN_3, 80 mM β-mercaptoethanol). 2,000 units of lyticase (Sigma) were added and the spheroplasting reaction was carried out by gently shaking the suspension at 30°C. The reaction was monitored by diluting aliquots of the suspension into water. As soon as the OD_{600} dropped to 10% of the initial value the suspension was layered onto a 1.8 M sorbitol cushion and spun at 8,000 ***g*** for 5 minutes in a HB-4 rotor. The pellet was resuspended in lysis buffer (0.8 M sorbitol, 10 mM triethaonolamine, pH 7.2, 1 mM EDTA, 1 mM PMSF) and the cells were broken in a 5 ml Dounce homogenizer. The lysate was cleared twice (10 minutes at 650 ***g***, 4°C).

The supernatant was layered on a 12 step sucrose gradient (10 mM Hepes, pH 7.5, 10 mM $MgCl_2$, 0-50%, w/v, sucrose) and spun at 170,000 ***g*** for 3 hours at 4°C in a SW40 rotor. The gradient was fractionated from the top. Proteins from these fractions were TCA precipitated, separated by SDS-PAGE and transferred onto nitrocellulose filters. Proteins were detected by using the antibodies listed above and visualized using the ECL detection system from Amersham.

Indirect immunofluorescence

Immunofluorescence was performed as described by Schröder et al. (1995). Cells expressing the *GAL1*-controlled *SEC22*-α gene were grown overnight in selective minimal medium containing galactose to reach OD_{600} 1-1.5 (pulse conditions). An equivalent of 10 OD_{600} of cells was collected and resuspended in 10 ml glucose-containing selective medium (chase conditions). After a chase time of 2 hours the cells were pre-fixed by adding 1.3 ml of 37% formaldehyde. After one hour at room temperature fixation was continued in 1 ml of a paraformaldehyde solution for two additional hours (3.5% paraformaldehyde in PBS/10% sorbitol). The cells were then washed with PBS/10% sorbitol, resuspended in 1 ml of this buffer and treated with 60 units zymolase in the presence of 5 μl/ml β-mercaptoethanol for one hour to remove cell walls. Spheroplasts were collected, washed four times in PBS/10% sorbitol and resuspended in 400 μl of PBS/10% sorbitol. 15 μl of this suspension was applied per slide well, previously treated with a poly-lysine solution (Sigma) to allow cell attachment. After 10 minutes the suspension was removed and 15 μl of blocking solution (PBS/10% sorbitol, 1% Triton X-100, 1% milk powder) applied for 10 minutes. Incubation with the first antibody (A-14, 9E10, anti-Sec61p, anti-Ypt7 or anti-60 kDa V-ATPase) was carried out overnight at 4°C at appropriate dilution in blocking solution. After seven consecutive washes of these cells with PBS/10% sorbitol, secondary antibody was added (Cy3-conjugated Fab fragment of goat anti-rabbit or anti-mouse IgG, Cy2-conjugated anti-rabbit or anti-mouse IgG). After 1 hour at 30°C the cells were washed seven times with PBS/10% sorbitol solution, DNA was stained with DAPI (4′,6-diamidino-2-phenylindole), and cells were washed twice in PBS/10% sorbitol. About 200 μl of embedding medium was applied to each slide and then sealed with a coverslip.

RESULTS

Proteolytic processing of α-factor-tagged Sec22p (Sec22-α)

The targeting of Sec22(Sly2)p, a *v*-SNARE protein for ER-to-Golgi transport in yeast was investigated by using Sec22p-derived hybrid proteins and their Kex2p-dependent processing in the late Golgi (Fig. 1). In addition we wanted to use this system to define mutants which are defective in the expected recycling.

Most mammalian *v*-SNAREs, as well as Sec22p from yeast, are tail-anchored transmembrane proteins carrying no or only a very small luminal extension (Fig. 2A; Dascher et al., 1991; Hay et al., 1997; Paek et al., 1997). Consequently, the analysis of the intracellular Sec22p transport requires luminally exposed peptide- or protein-reporters. It was important to ensure that its dynamic localization between ER and Golgi was not affected by the reporter. The overall length of the reporter including α-factor fused to Sec22p through a Kex2p cleavage site and a c-*myc* epitope is only 41 residues (Sec22-α, Fig. 2B; Boehm et al., 1997; Ballensiefen and Schmitt, 1997). The c-*myc* epitope was inserted, to give sufficient space between Kex2p-cleavage site and membrane to guarantee protease activity by Kex2p on Sec22-α. This also enabled anti-c-*myc* antibodies to be used specifically to detect Sec22-α and its proteolytic products in western blot analyses and by indirect immunofluorescence.

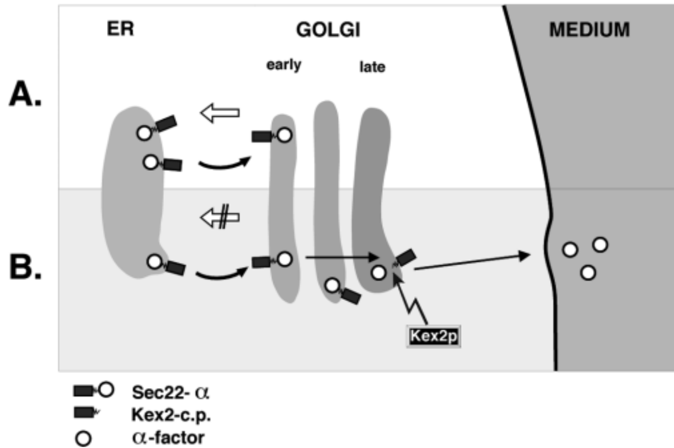
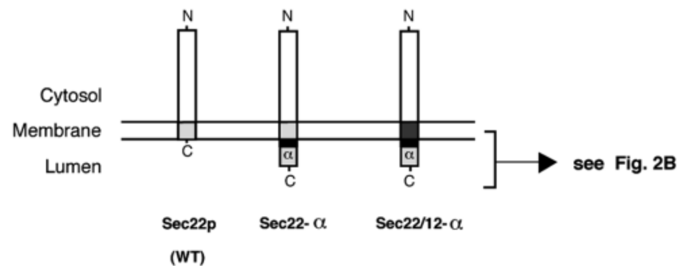


Fig. 1. Schematic illustration of the Kex2p-dependent assay for ER-retention of α-factor-tagged Sec22p (Sec22-α). (A) Due to recycling of Sec22-α (open arrow) Sec22-α does not reach the late Golgi in wild-type cells (Kex2p-containing compartment). (B) The Sec22-α recycling can be disturbed by saturation due to an overexpression or by mutations in genes encoding factors which are crucial for its recycling. Accordingly, Sec22-α escapes to the late Golgi, Kex2p is able to cleave the tagged protein and α-factor peptide is secreted.

To examine whether Sec22-α is functional, a *sec22-3* mutant strain was transformed with plasmids expressing different levels of the *SEC22*-α hybrid gene. We found that *SEC22*-α expressed at wild-type levels (*SEC22* promoter, centromeric plasmid) was sufficient to allow *sec22-3* cells to grow at the non-permissive temperature (37°C). Additionally, overproduced Sec22-α (*GAL1* promoter, centromeric vector) was able to suppress the growth defect of the *ufe1-1* mutation at 37°C, as reported previously for Sec22 protein (Lewis and Pelham, 1996).

Previous experiments with Sec22-α in *MAT***a** haploid strains, indicated that it is rapidly processed by the Bar1 protease (Ballensiefen and Schmitt, 1997); this cleavage occurs in the ER. This finding, as well as the efficient cleavage by Kex2p in mutant cells (e.g. Figs 4, 5), confirms that Sec22-α is correctly inserted into the ER membrane. To avoid cleavage by Bar1p, all immunoblot experiments presented in this study were performed in cells of mating type α.

For most of the experiments shown below, *SEC22*-α was expressed from the inducible *GAL1* promoter (Schneider and Guarente, 1991). Addition of galactose induces the synthesis of Sec22-α from the *GAL1* promoter-controlled construct. To guarantee a rapid induction of the hybrid gene the transformands were preincubated under non-repressive conditions (3% glycerol and 2% ethanol). After induction by galactose, expression could be stopped rapidly by transferring cells to glucose-containing medium. This transient expression allowed us to perform a kind of ‘pulse-chase’ analysis with Sec22-α. Results obtained previously by conventional, radioactive pulse-chase experiments with *SEC22*-α were quite similar to the experiments based on the galactose induction technique (Boehm et al., 1997; Ballensiefen and



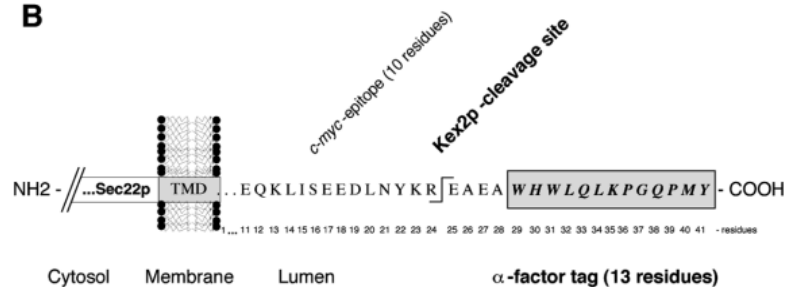


Fig. 2. (A) Diagrammatic representation of the Sec22p wild-type protein and derived α-factor-tagged hybrid proteins (Sec22-α and Sec22/12-α) used in this study. The Sec22/12-α construct harbors the Sec12p transmembrane domain instead of the original one. (B) Amino acid sequence of the luminally exposed α-factor tag fused to Sec22p, including the c-*myc* epitope, the Kex2p and the Bar1p-cleavage site. Note that the overall extension of the Sec22p wild-type protein is only 41 residues. The Kex2p trimmed Sec22-α can be detected in western blot analysis using the anti-Sec22p serum or the anti-c-*myc* antibody but not with antibodies specific for the α-factor.

Schmitt, 1997; this work). Nevertheless, utilization of the inducible *GAL1* promoter had several advantages: first, the quantity of Sec22-α synthesized could be controlled by varying the induction time. This allowed the production of a quantity of protein, which was either below or above the saturation level of the recycling machinery. Second, in contrast to classical pulse-chase experiments using constitutive synthesis, Sec22-α is produced only during the galactose induction (pulse). The amount produced during this time is easily detectable by western blot analysis. Third, it gives an opportunity to examine the time-dependent distribution of Sec22-α and its Kex2p cleavage product by immunofluorescence or subcellular fractionation, avoiding the use of cycloheximide. However, the rate of galactose-dependent induction can vary between different yeast strains and growth conditions. Therefore, it was essential to compare isogenic mutant and wild-type strains which were grown and treated in parallel (see Materials and Methods). As shown below, high expression of *SEC22*-α induces processing of Sec22-α by Kex2p even in wild-type cells. Two observations confirmed that the presence of this cleavage product is in fact related to the late-Golgi protease. First, no cleavage was observed in cells lacking the *KEX2* gene (data not shown). Second, the presumed Kex2-dependent cleavage product could be detected by the monoclonal anti-c-myc antibody 9E10 but not by anti α-factor antibodies (see Fig. 9). This showed the protease had removed the α-factor moiety exposed at the luminal side of the hybrid protein by cleaving between the c-myc epitope and the α-factor.

Cellular distribution of Sec22-α in wild-type cells using a transient expression

To confirm that the subcellular distribution of Sec22-α resembles that of the wild-type protein, the localization of both proteins was analyzed by subcellular fractionation on sucrose gradients. Exponentially growing wild-type cells (*WBY-2*) were shifted to galactose-containing medium for 50 minutes to induce the *GAL1*-controlled *SEC22*-α gene (pulse). Cells were transferred to glucose-containing medium (chase) and incubated for two hours. Extracts were prepared and analyzed by sedimentation on sucrose density gradients as described in Materials and Methods. Fractions were collected and analyzed by immunoblot analysis. The results were quantified by densitometry. Sec22-α, Kex2p-cleaved Sec22-α (Kex2p-c.p.) and wild-type Sec22p were detected by applying an anti-Sec22p serum. The distribution of these proteins was nearly identical (Fig. 3B). They were found in fractions containing the Golgi marker Kex2p as well as the ER markers BiP/Kar2p and Sec61p (Fig. 3A). These results suggest that the α-factor reporter of the Sec22-α does not interfere with the cellular localization of the wild-type Sec22p. The identity of bands was confirmed by analyzing samples collected at different time points after the transfer to glucose medium (data not shown). The amount of uncleaved Sec22-α decreases during the chase time. But even after an extensive chase time of two hours there was still about 50% of uncleaved Sec22-α detectable, indicating that the recycling of Sec22-α before the late Golgi is quite efficient (Kex2p-compartment; Fig. 1). The observation that Kex2p-cleaved Sec22-α was present in fractions containing the ER markers indicated that the return of Sec22-α to the ER is also possible from a late Golgi compartment. This result was confirmed by the immunofluorescence experiments shown below.

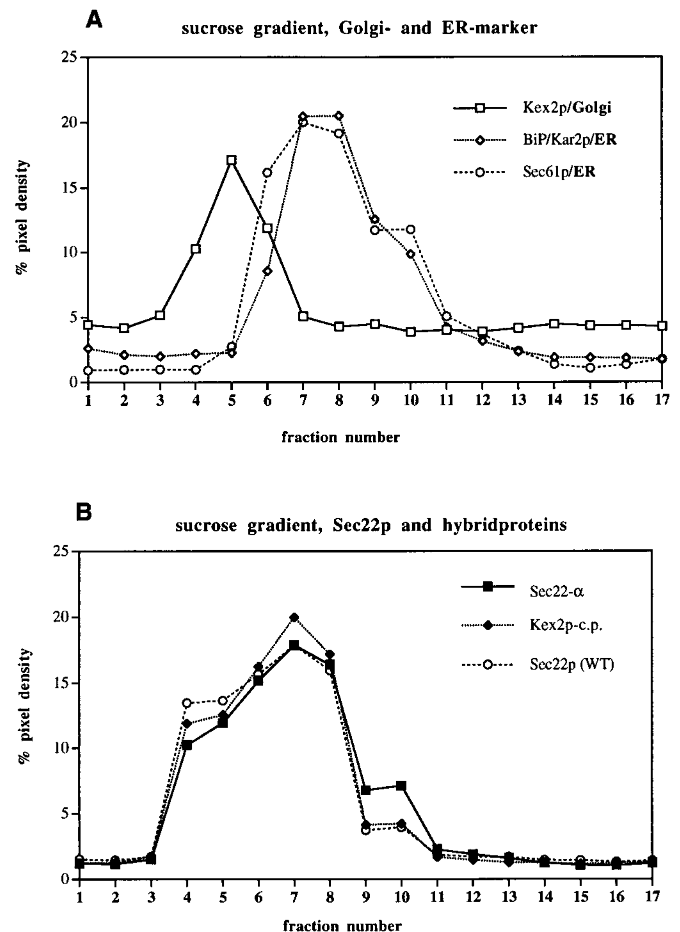


Fig. 3. Subcellular fractionation of cells expressing the *GAL1*-controlled *SEC22*-α. Wild-type cells (WBY-2) carrying plasmid pWB-*GAL*Aα were grown overnight at 30°C in glycerol/ethanol minimal medium. To induce synthesis of Sec22-α they were shifted to galactose minimal medium. After 50 minutes (pulse) cells were transferred to glucose-containing minimal medium to stop further synthesis of Sec22-α (chase). Extracts were prepared 120 minutes after the shift to glucose medium. The low speed supernatant was centrifuged to equilibrium in a 0%-50% (w/v) sucrose gradient and fractions were subjected to western blot analysis. Distribution of the Kex2 wild-type protein indicates the Golgi containing fractions. Kar2p/BiP and Sec61p were employed as ER markers. The distribution of the Sec22p wild-type protein, the Sec22-α hybrid protein and the Kex2p-cleavage product (Kex2p-c.p.) was analyzed using an anti-Sec22p serum. Fractions were numbered from 1 (0%, w/v, sucrose) to 17 (50%, w/v, sucrose). The immunoblots were scanned and pixel density was determined with the phosphoimager software.

In some ER-Golgi recycling mutants Sec22-α is more rapidly cleaved by Kex2p than in isogenic wild-type cells

Immunoblot analysis was used to determine the rate of Kex2 cleavage of transiently expressed Sec22-α. Transient expression was achieved by a 35 minutes incubation of cells in

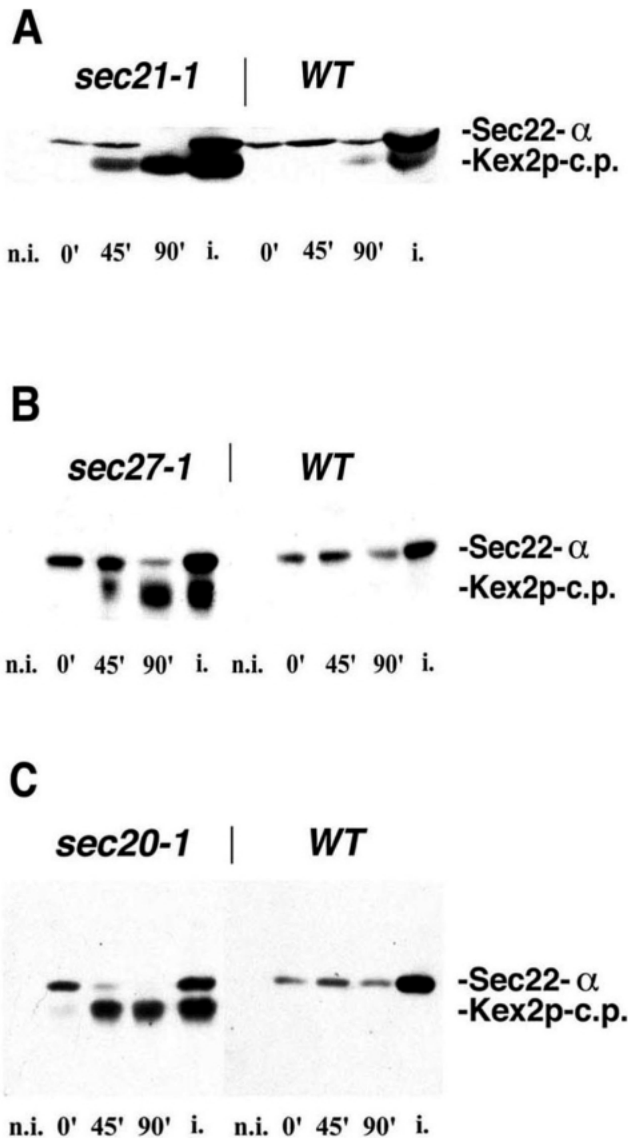


Fig. 4. Immunoblot analysis of transiently produced Sec22-α. The *SEC22*-α hybrid gene on plasmid pWB-*GALA*α was expressed in *sec21-1* (WBY-8; A) *sec27-1* (WBY-6; B) and *sec20-1* (RSY275; C) mutant strains as well as in isogenic wild-type strains (WBY-8 and WBY-6 containing pRS315-*SEC21* or pRS315-*SEC27*, respectively; RSY255). Transformands were grown overnight in glycerol/ethanol medium. To achieve transient expression from the *GAL1*-controlled hybrid gene, cells were incubated for 35 minutes in galactose-containing medium at 25°C (pulse). Then cells were transferred to glucose-containing medium to stop the expression of *SEC22*-α (chase). Incubation was continued at the permissive temperature. Aliquots were taken after 0, 45 and 90 minutes of incubation in glucose-containing medium. Extracts from non-induced cells (n.i.) and cells kept under inducing conditions during the entire pretreatment (2 hours; i.) were included as controls. The Sec22-α chimeras and their Kex2p cleavage product (Kex2p-c.p.) were detected using a polyclonal anti-*c-myc* antibody A-14.

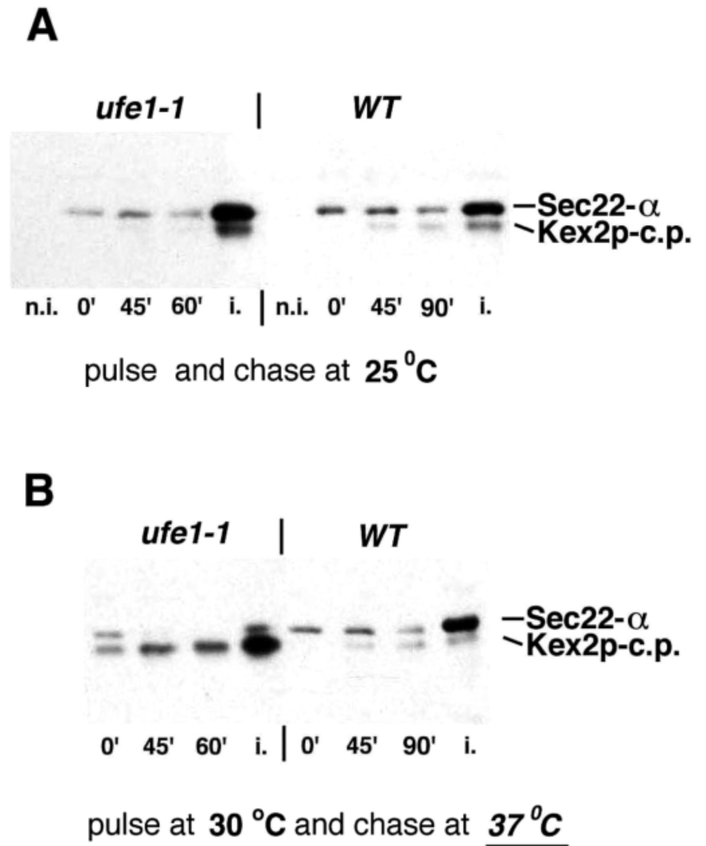


Fig. 5. Immunoblot analysis of transiently produced Sec22-α. The *GAL1*-controlled *SEC22*-α hybrid gene (plasmid pWB-*GALA*α) was expressed in a *ufe1-1* mutant strain and in an isogenic wild-type strain (MLY-100 and MLY101). Transient expression of *SEC22*-α was achieved as described in Fig. 3. (A) Cells were pregrown and incubated at 25°C during the entire experiment. (B) *SEC22*-α expression was induced by incubating the cells in galactose-containing medium at 30°C. Subsequent incubation in the repressing glucose medium was performed at 37°C. Extracts from non-induced cells (n.i.) and cells kept under inducing conditions during the entire pretreatment (2 hours; i.) were included as controls. The Sec22-α chimeras and their Kex2p cleavage product (Kex2p-c.p.) were detected using the polyclonal anti-*c-myc* antibody A-14.

galactose-containing medium (pulse). After this short pulse, *GAL1*-dependent synthesis of Sec22-α was shut down by transferring the cells to glucose-containing medium. The amount of Sec22-α produced during 35 minutes of induction is comparable to the steady state level of the endogenous Sec22p. The chase in glucose-containing medium was performed for different times (0, 45 and 90 minutes). Two controls were included into this assay: extracts from non-induced cells (marked ‘n.i. = not induced’ in Figs 4, 5) and extracts from cells kept under inducing conditions during the chase time (marked ‘i. = induced’ in Figs 4, 5). As can be seen in the right panels of Figs 4 and 5, Sec22-α is very slowly cleaved by Kex2p when the hybrid gene is expressed at low levels in wild-type strains.

Recycling of *v*-SNAREs like Sec22p may involve COPI-coated vesicles. To test this, COPI mutants and two other yeast mutants known to carry defects in the recycling of proteins to the ER were examined for an increase in Kex2p-dependent processing of Sec22-α. Mutant cells carrying the *sec21-1, sec27-1*, *sec20-1* and *ufe1-1* alleles were treated and extracts

analyzed in the same way as the isogenic wild-type cells. In all these mutants cleavage products were already present after 45 minutes chase in glucose medium. After 90 minutes almost all Sec22-α was processed by Kex2p (Figs 4, 5B, left part of each panel). Only after a long period of induction some cleavage could be observed in wild-type cells (two hours in galactose-containing medium, lanes marked 'i.'). As shown below, this processing is likely to be due to the higher expression levels reached in these samples. These samples represent the ratio of uncleaved and cleaved Sec22-α under almost steady state conditions. Even under these conditions the difference between mutants and wild-type is quite evident.

The *sec21-1*, *sec27-1* and *sec20-1* mutants exhibit secretion defects at temperatures above 30°C (Novick et al., 1980; Duden et al., 1994). To observe the increase in processing of Sec22-α by Kex2p it was not necessary to incubate these temperature-sensitive (Ts^-) mutants at temperatures that inhibit the growth of these cells. Our results are consistent with the anterograde transport of these mutants being unaffected at the permissive temperature. Nevertheless, under these experimental conditions, they showed a drastic increase in the rate of Sec22-α cleavage by Kex2p. In case of the *ufe1-1* mutant, however, it was necessary to shift the cells to higher temperatures to observe Kex2p-cleaved Sec22-α. Under these conditions Kex2p-cleaved Sec22-α was already detectable at the end of the induction interval (Fig. 5B).

Several other mutants known to be deficient in the retrieval of various ER proteins were tested for Sec22-α targeting. As published earlier, processing of Sec22-α by Kex2p was not enhanced in either of the *ret1* (α-COP) mutants (Boehm et al., 1997). Likewise, *rer1Δ* mutants or *erd2* recycling mutants did not influence the Sec22-α targeting as determined by the Kex2p-dependent processing of Sec22-α (data not shown; see also Fig. 7). The lack of any observable effect in *ret1* and *erd2* mutants is most certainly due to the fact that these mutants were selected for defects in the recycling of very specific proteins. It does not rule out the possibility that the products of the *RET1* and *ERD2* genes are involved in the recycling of Sec22p. Perhaps other point mutations within these genes may lead to mislocalization of Sec22-α.

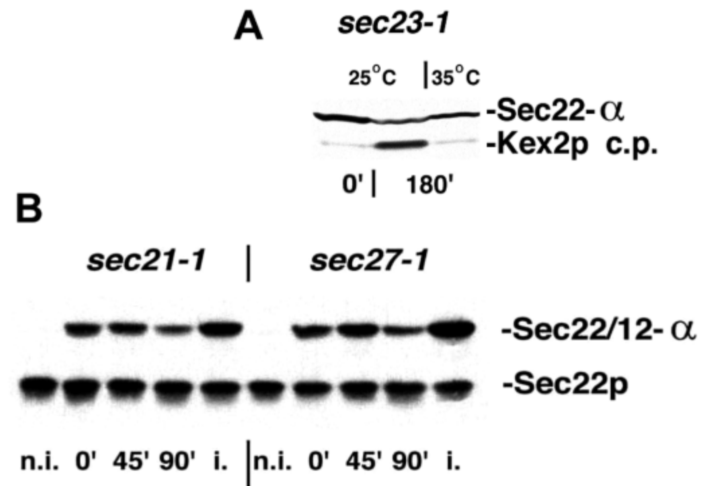


Fig. 6. (A) Cleavage of Sec22-α by Kex2p requires ER-to-Golgi transport. *GAL1*-dependent *SEC22*-α expression was induced by overnight incubation of *sec23-1* cells (strain RH589- 3C) in galactose-containing medium. To block ER-to-Golgi transport and trap Sec22-α in the ER an aliquot of the COPII mutants was incubated at 35°C during the chase in glucose-containing medium. At the indicated time points cells were harvested and analyzed by western blot analysis. Sec22-α was detected using poly-clonal anti-myc antibodies (A-14). (B) Sec22-α protein carrying the TMD of the ER-resident protein Sec12p is not cleaved by Kex2p in the COPI-mutants *sec21-1* and *sec27-1* (strains WBY-8, WBY-6). Western blot analysis of transiently expressed *SEC22/12*-α was performed with extracts from mutant cells containing vector pWB-*GAL*ACα. The pretreatment of cells was done as described in the legend to Fig. 4. Glycerol/ethanol-containing medium was used to grow the cells overnight. To induce the expression of the *SEC22*-α hybrid gene, cells were transferred to galactose-containing medium for 35 minutes (pulse). The temperature was 25°C during this time as well as the following incubation in glucose medium (chase). Samples were taken at different times after the transfer to this medium. Extracts from non-induced cells (n.i.) and cells kept under inducing conditions during the entire pretreatment (2 hours; i.) were included as controls. Wild-type Sec22p and Sec22/12-α were detected using a polyclonal anti-Sec22p serum.

Kex2p cleavage does not occur in the ER of mutants

The results described above could be due to Kex2p being active in the ER of the mutant cells instead of Sec22-α which is mislocalized to the late-Golgi. The following experiments were performed to rule out this possibility. First, ER-to-Golgi transport was blocked in a COPII mutant (*sec23-1*) expressing *SEC22*-α by incubation at a non-permissive temperature. To induce transport of Sec22-α to the late Golgi the protein was overproduced by an overnight induction of the *GAL1*-regulated *SEC22*-α gene (see below). Cells were then incubated in glucose-containing medium for 3 hours. During this chase aliquots were incubated either at permissive or non-permissive temperature (25°C or 35°C). Kex2p-dependent cleavage occurred only when cells were incubated at the permissive temperature (Fig. 6A) proving that ER-to-Golgi transport is necessary to observe the processing by the late-Golgi protease. This is consistent with the previously made observation that newly synthesized Kex2p is not active in the ER of *sec18* cells when incubated at the non-permissive temperature (Graham and Emr, 1991; Harris and Waters, 1996; Boehm et al., 1997). In a second control experiment transport of Sec22-α to the late Golgi was blocked specifically by replacing the Sec22-transmembrane domain by that of the ER-resident Sec12p. The TMD of Sec12p is a very efficient retention signal which confers ER localization to other proteins. Hybrid proteins carrying the TMD of Sec12p are not transported beyond a very early Golgi compartment (Boehm et al., 1994; Sato et al., 1996; Boehm et al., 1997). Sec22-α carrying the TMD of Sec12p (Sec22/12-α, see Fig. 2A) was produced in *sec21-1* and *sec27-1* mutants. These transformands were treated in the same way as those expressing the original *SEC22*-α construct (Fig. 4A,B). As shown in Fig. 6B no cleavage products appeared that migrated between the uncleaved Sec22/12-α protein and the wild-type Sec22 protein indicating that Sec22/12-α is not processed by Kex2p. Apparently, under the conditions used in our assays Kex2p had no access a protein that is likely to cycle between an early Golgi compartment and the ER. This shows that the cleavage of Sec22-α seen

above can not be due to the Kex2p present in the ER or early Golgi. Similar results were obtained with a Sec12-α construct that was highly overexpressed (*CYC1* promoter, 2μ multi-copy vector; data not shown). Taken together the increase in Kex2p-dependent cleavage of Sec22-α in COPI mutants (*sec21-1* and *sec27-1*) as well as in *sec20-1* and *ufe1-1* mutants, is a strong evidence that the retrograde transport of Sec22-α is mediated by COPI-vesicles.

The rate of processing increases with the expression level of *SEC22-α*

We examined whether the level of Sec22-α production influences its targeting and recycling. Overnight incubation of cells expressing the *GAL1*-regulated *SEC22*-α gene in galactose medium was one possibility to achieve a expression level higher than that reached in the experiments presented above. (An even higher expression level was achieved by using a strong promoter in combination with a high-copy number plasmid. The results of these experiments will be is described below.) Aliquots of mutant and wild-type cells containing pWB-*GAL*Aα were taken for immunoblot analysis at the beginning as well as 1 and 2 hours after the transfer of cells to glucose-containing medium. As shown in Fig. 7, most of the hybrid protein is cleaved by the Kex2 protease two hours after incubation in glucose-containing medium. In contrast to the results presented above this cleavage occurred not only in mutants but also in wild-type cells. But still there is a clear differences in the rate of cleavage when *sec21-1* and *sec27-1* mutant cells were compared with isogenic wild-type cells (Fig. 7A,B). This indicates that the mechanism preventing cleavage of Sec22-α by Kex2p is saturable. As expected from the results mentioned above, no difference in the time course of cleavage by Kex2p was observed in wild-type cells and the corresponding *erd2* mutant (Fig. 7C).

Immunolocalization of the Kex2p-processed Sec22-α

Indirect immunofluorescence microscopy was used to determine the localization of Sec22-α in isogenic pairs of wild-type and mutant cells. To induce sufficient quantities of Sec22-α to be detected by immunofluorescence, mutant and wild-type cells were grown overnight at permissive temperature (25°C) in galactose-containing medium (pulse). Before applying the immunofluorescence protocol, the cells were incubated in glucose medium for two hours to shut down the synthesis of the Sec22-α (chase). Note that these were exactly the conditions used for the experiment shown in Fig. 7. There were two reasons to follow this protocol: first, the shut down of Sec22-α synthesis should avoid a contribution of newly synthesized Sec22-α to the immunofluorescence pattern. Second, the results presented in Fig. 7 had shown that most of the Sec22-α protein produced under these growth conditions is cleaved by Kex2p. Thus, by applying these growth conditions one can determine the distribution of Sec22-α which already had passed through the Kex2-compartment. The polyclonal anti-c-*myc* antibody was used to localize Sec22-α since it does not detect the constitutively produced wild-type Sec22 protein.

We first compared the Sec22-α localization in a set of mutants which showed enhanced Sec22-α cleavage by Kex2p with that of isogenic wild-type cells. In all wild-type cells Sec22-α was concentrated near the nucleus (Fig. 8, left panels).

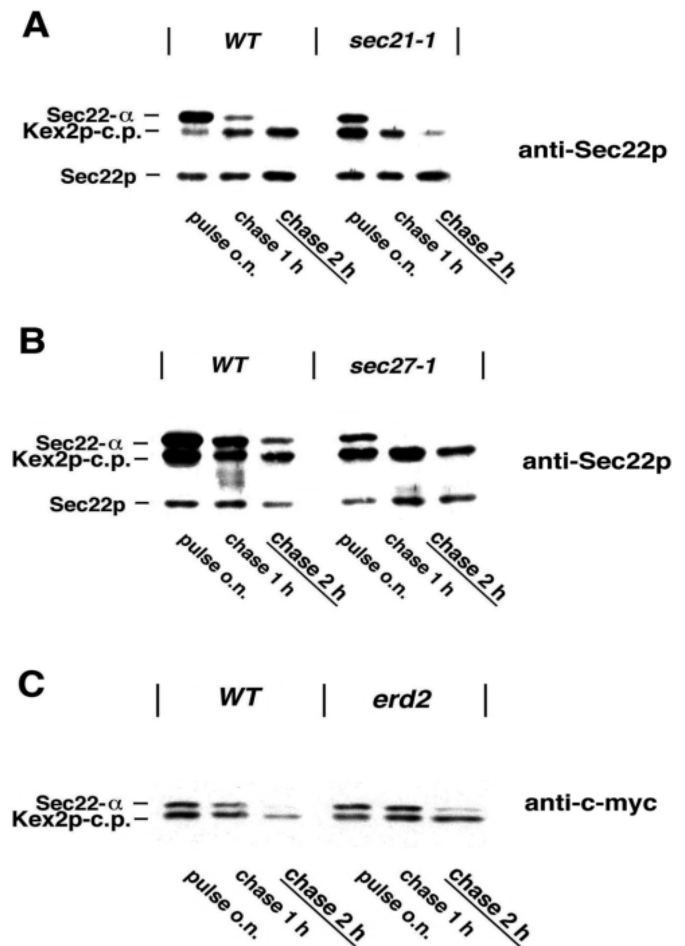


Fig. 7. (A and B) Immunoblot analysis of cellular extracts from *sec21-1* and *sec27-1* (strains WBY-8, WBY-6) transformands (strains WBY-8, WBY-6) containing pWB-*GAL*Aα. The same transformands were used as for Figs 4 and 6. Aliquots of cells were harvested after the overnight incubation in galactose-containing medium (pulse) as well as 1 hour and 2 hours after the transfer to glucose-containing minimal medium (chase). A polyclonal anti-Sec22p serum was used to detect the Sec22p-derived hybrid protein (Sec22-α), its Kex2p cleavage product (Kex2p-c.p) and the Sec22 protein. Aliquots of the samples taken at two hours after the shift to glucose-medium were analyzed by indirect immunofluorescence (first two rows of Fig. 8). (C) Immunoblot analysis of extracts from *erd2* mutant cells (MLY-100) and isogenic wild-type cells (MLY-101). Aliquots of cells were harvested after the overnight incubation in galactose-containing medium (pulse) as well as 1 hour and 2 hours after the transfer to glucose-containing minimal medium (chase). Sec22-α and its Kex2p-processed form (Kex2p-c.p.) were detected using the polyclonal anti-*c-myc* antibody (A-14) which was also applied in the immunofluorescence experiment shown in Fig. 8, fifth row.

This can be seen by comparing the Sec22-α staining pattern with the nuclear staining by the fluorescent dye DAPI (4′,6-diamidino-2-phenylindole). In yeast, ER stained by immunofluorescence methods appears as a ring around the nucleus (Rose et al., 1989; Preuss et al., 1992). The labeling of Golgi proteins results in a punctuate staining. Normally, about 3 to 12 dots per cell are randomly distributed throughout the cytoplasm (Franzusoff et al., 1991; Redding et al., 1991;

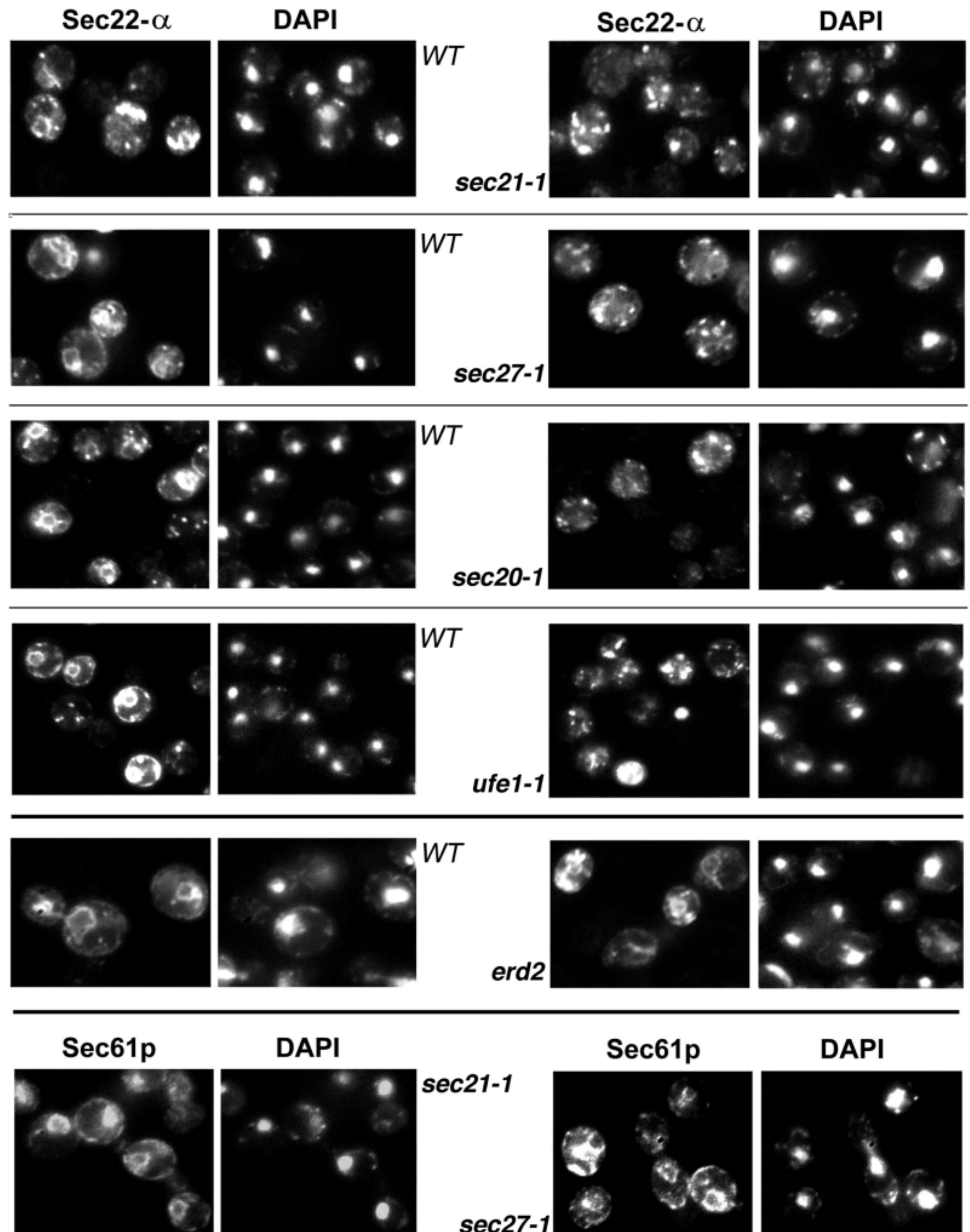


Fig. 8. Indirect immunofluorescence to determine the localization of the Sec22-α hybrid protein in wild-type and isogenic mutant cells as indicated. To express a *GAL1*-controlled *SEC22*-α, cells were transformed with vector pWB-*GALA*α. For the analysis of *sec21-1, sec27-1, sec20-1, ufe1-1 and erd2* mutants and the corresponding wild-type cells (same strains as used for Figs 4, 5 and 7) synthesis of Sec22-α was induced by overnight incubation in selective medium containing galactose (pulse; 25°C), followed by two hours of incubation in glucose-containing medium to stop synthesis of the hybrid protein (chase; 30°C). Fixed and permeabilized cells were stained using a polyclonal anti-*c-myc* antibody (A-14) and a Cy3-conjugated secondary antibody as described in Materials and Methods. DAPI-staining was used to localize the nuclei. In contrast to the other mutants, the *ufe1-1* mutant and the corresponding wild-type strain (*UFE1*) were incubated overnight at 30°C in galactose medium (pulse) and for 2 hours at 37°C in glucose-containing medium (chase) prior to fixation and immunofluorescence staining. In parallel to this analysis aliquots of some samples were taken for the immunoblot analysis shown in Fig. 7.

Antebi and Fink, 1992). In addition to the perinuclear fluorescence a few dots also appeared in wild-type cells suggesting that some Sec22-α was present in the Golgi as well (Fig. 8, left panels). However, the COPI mutants *sec27-1* and *sec21-1* as well as *sec20-1* and *ufe1-1* mutants never showed ER-immunofluorescence when stained for Sec22-α. Instead only dots were visible indicating that the protein was trapped in the Golgi or an endocytic compartment. In contrast to the other mutants, *ufe1-1* cells had to be incubated at temperatures above 30°C during the chase to observe exclusively the punctuate staining pattern.

We also analyzed an *erd2* mutant strain together with a corresponding wild-type strain. As mentioned above, the *erd2* mutant belongs to those recycling mutants which did not show an increase in Kex2p-dependent cleavage of Sec22-α. As expected, Sec22-α is predominantly found in the ER of *erd2* mutant and isogenic wild-type cells (Fig. 8, fifth row). In summary, the results of the immunofluorescence analysis are consistent with those obtained by Kex2p-dependent cleavage of Sec22-α produced at a low level (Figs 4, 5). Both approaches show that COPI as well as Sec20p and Ufe1p are involved in the recycling of Sec22p from the late Golgi back to the ER.

The lack of perinuclear staining could be due to dramatic changes in morphology of the ER in the mutants. Therefore,

an additional immunofluorescence experiment was performed to exclude this possibility. The *sec21-1* and *sec27-1* cells were analyzed for the distribution of Sec61p, an ER-marker protein (Panzner et al., 1995). Staining with anti-Sec61p antibodies showed a typical perinuclear ER-staining pattern (Fig. 8, sixth row). It has been shown by electron microscopy that these mutants, as well as *sec20-1* and *ufe1-1* mutants, accumulate ER structures instead of being depleted of this compartment when incubated at the restrictive temperature (Novick et al., 1980; Duden et al., 1994; Lewis and Pelham, 1996). Therefore, the failure to detect Sec22-α in ER-like structures in these mutants could not due to the absence of typical ER. In summary, the absence of ER-fluorescence when stained for Sec22-α is very likely to be the result of a recycling defect in the mutants.

Sec22-α is retained in the Golgi

Immunolabeling of *GAL1*-dependently produced Sec22-α in *sec21-1*, *sec27-1*, *sec20-1* and *ufe1-1* mutants primarily gave rise to punctuate staining patterns. This suggested that Sec22-α is trapped in the Golgi of these mutants. In addition, no potential vacuolar cleavage product could be observed in the immunoblot analysis shown above. This suggested that Sec22-α may differ from many other mislocalized transmembrane proteins of the ER and Golgi. Sec22-α may be unusual since in yeast transport to the vacuole is considered to be the default pathway for membrane proteins of the ER and Golgi as soon as they fail to be retained correctly (Roberts et al., 1992; Gaynor et al., 1994; Chapman and Munro, 1994; Letourneur et al., 1994). Fig. 9 shows that Sec22-α can, in fact, reach the vacuole. A cleavage product of Sec22-α migrating just above

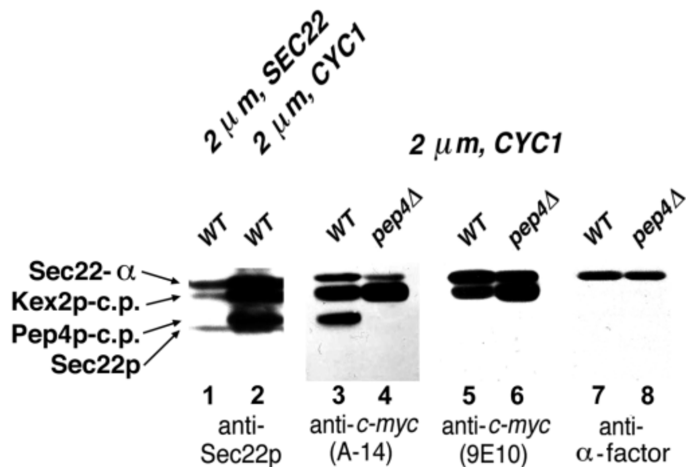


Fig. 9. Only when highly overproduced (*CYC1* promoter, 2μm vector) is Sec22-α processed in a Pep4p-dependent manner. Wild-type cells (WBY-2) were transformed with either plasmid pWB-Amα (lane 1) or pWB-Amcα (lane 2-8). Whole cell extracts of transformands were analyzed by immunoblotting using antibodies specific for Sec22p or Sec22-α: To reveal Sec22-α derivatives as well as the Sec22 wild-type protein anti-Sec22 antibodies were used (lane 1 and 2). Uncleaved Sec22-α, its Kex2- and Pep4-dependent cleavage products (Kex2p-c.p. and Pep4p-c.p.) can be detected with the polyclonal anti-c-*myc* antibodies (A-14, lane 3 and 4), while unprocessed Sec22-α and its Kex2-dependent cleavage product (Kex2p-c.p.) were visualized using a monoclonal anti-c-*myc* antibody (9E10, lane 3 and 4). Anti-α-factor antibody detects only the uncleaved Sec22-α (lane 7 and 8).

the wild-type Sec22 protein was visible in cells constitutively overexpressing *SEC22*-α at a very high level (*CYC1* promoter and multi-copy vector; Fig. 9, lanes 1 and 2). A comparison of wild-type cells and cells deficient in vacuolar hydrolases (*pep4*Δ; Klionsky et al., 1990) was used to prove that this cleavage product originates from partial degradation of Sec22-α in the vacuole. As shown in Fig. 9 the fast migrating band can be detected with Sec22p-specific antibodies or a polyclonal anti-c-*myc* antibody (A-14; lane 3), but not with the monoclonal anti-c-myc antibody (9E10; lane 5) which requires the entire c-*myc*-epitope for binding (Evan et al., 1985). This demonstrates that the short cleavage product seen in the second lane of Fig. 9 is due to additional cleavage of the luminal domain of Sec22-α. The absence of this band from extracts of a *pep4* deletion strain indicated that this cleavage indeed happens in the vacuole. The absence of a Pep4p-dependent cleavage product in Figs 4, 5 and 7 may therefore be due retention of Sec22-α in the Golgi.

The lack of vacuolar cleavage observed at low expression levels is also consistent with the observation that Sec22-α does not localize to ring-like structures in the cytoplasm (Fig. 8). A ring-like staining pattern within the cytoplasm is usually observed if the labeled protein resides on the vacuolar membrane (Raymond et al., 1992; Kane et al., 1992; see also lower part of Fig. 10). Double labeling experiments were performed to determine the localization of the Sec22-α in the *sec21-1* and *sec27-1* mutants in more detail. Cells were labeled with antibodies specific for Sec22-α (9E10; Evan et al., 1985), the Golgi marker Rer1p (Sato et al., 1996; Boehm et al., 1997) and the vacuolar marker Ypt7p (Haas et al., 1995). Rer1p was chosen as a Golgi marker since it is not mislocalized to the vacuole in *sec21-1* and *sec27-1* mutants (Boehm et al., 1997). Cells were treated prior to fixation as described for Fig. 8. Consistent with the results presented above, Sec22-α was localized to perinuclear as well as small punctuate structures in wild-type cells (Fig. 10A, green fluorescence in G). Only punctuate staining was seen in mutant cells (Fig. 10C,E,I, green fluorescence in H). Similarly, anti-Rer1p antibodies exclusively gave rise to punctuate staining patterns in wild-type as well as mutant cells (Fig. 10B,D,F). The staining associated with Rer1p substantially overlaps with the punctuate staining pattern observed for Sec22-α. Similar results were obtained with Kex2p as Golgi marker (data not shown).

Double labeling experiments also showed that the staining patterns of Sec22-α and that of the vacuolar marker Ypt7p are completely distinct (Fig. 10G-K). This proves that Sec22-α does not reach the vacuole or is not associated with a vacuole-associated endosomal compartment (Piper et al., 1995; Hicke et al., 1997). It is important to note that the staining pattern of Sec22-α as seen in Figs 8 and 10 represents the steady state distribution of this protein since the synthesis of the hybrid protein had been stopped 2 hours prior to the fixation of cells. Vacuolar structures should at least be visible in *sec27-1* since the strain used for these labeling experiments lacks vacuolar hydrolases. Moreover, *sec21-1* and wild-type cells were also labeled with a monoclonal antibody specific for the 60 kDa subunit of the vacuolar ATPase (Kane et al., 1992) and the polyclonal anti-c-myc antibody A-14 which is able to detect the vacuolar cleavage product. The result of this double labeling experiment was identical to that shown in Fig. 10G and H (data not shown). Taken together these results indicate

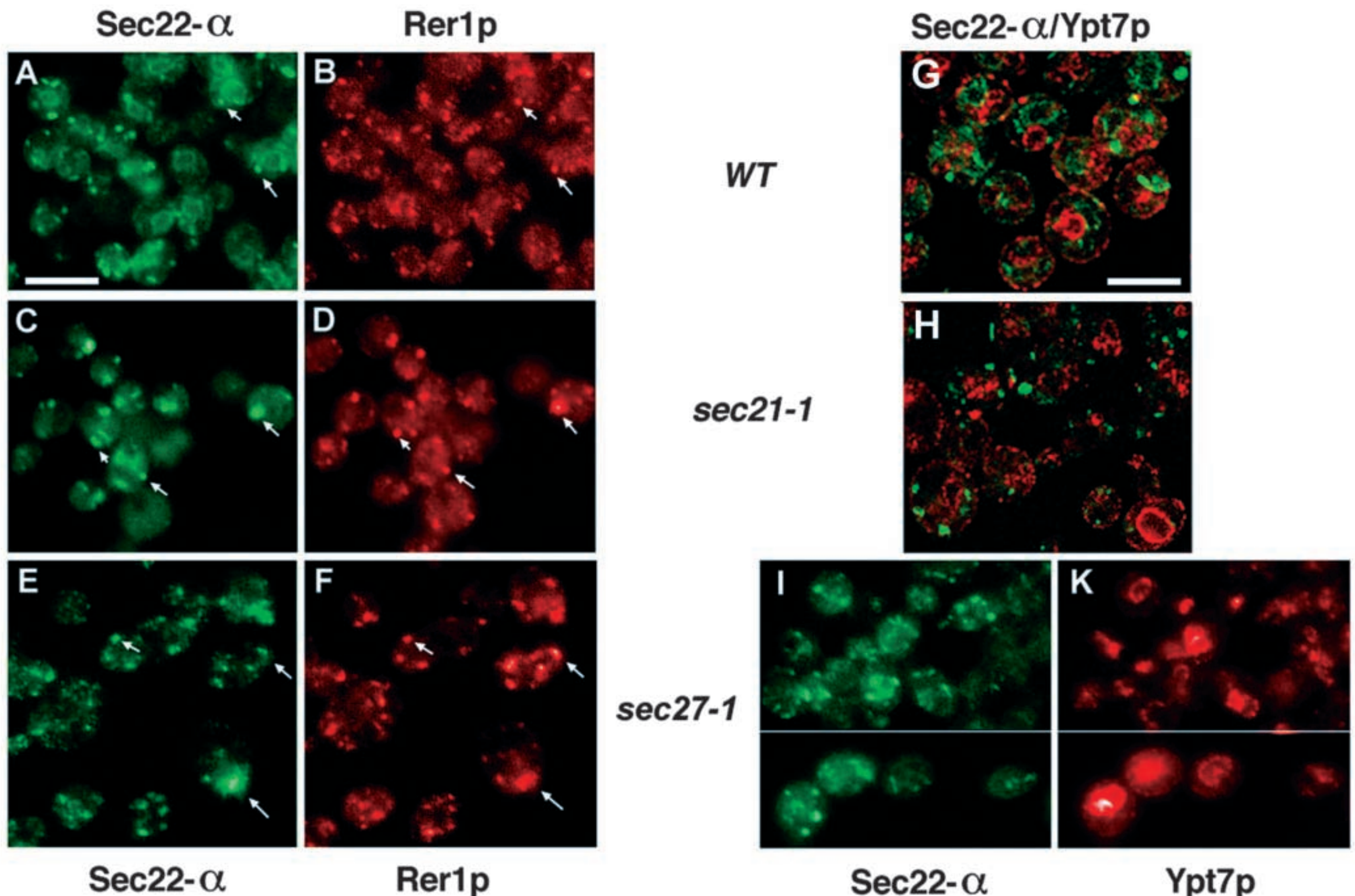


Fig. 10. Immunofluorescence localization of Sec22-α, the Golgi marker Rer1p and the vacuolar marker Ypt7p to reveal possible colocalization in wild-type, *sec21* and *sec27-1* mutant cells. Wild-type (S21PP-1A), *sec21-1* (S21PP-6C) and *sec27-1* (pep4Δ, WBY-10) cells were transformed with vector pWB-*GAL*Aα. To determine the steady state localization of Sec22-α samples were taken and fixed two hours after the transfer to glucose medium to stop further synthesis of Sec22-α. Cells were labeled with anti-c-*myc* antibody (9E10) in combination with Cy2-conjugated anti-mouse antibodies (A,C,E,I, green fluorescence in G and H). Polyclonal antibodies together with Cy3-conjugated secondary antibodies were employed to localize the Golgi marker Rer1p (B,D,F) and the vacuolar marker Ypt7p (K, red fluorescence in G and H).

that Sec22-α does not reach the vacuole or a vacuole-associated compartment in COPI-mutants.

DISCUSSION

In this work direct evidence is provided for a COPI-dependent recycling of a *v*-SNARE involved in ER-to-Golgi transport. Two assays were performed to examine the targeting of Sec22p in wild-type and mutant cells. First, cleavage of Sec22-α by the late Golgi protease Kex2p was used to indicate transport of the tagged protein to this compartment. This assay was performed at expression levels comparable to that of the *SEC22* wild-type gene. Under these conditions Sec22-α did not reach the late Golgi in wild-type cells as indicated by the failure to detect cleavage products of Kex2p from the hybrid protein. Kex2p-dependent cleavage of Sec22-α was observed in *sec20*, *sec21, sec27* and *ufe1-1* mutant cells.

Immunofluorescence analysis demonstrated that a large proportion of the Sec22-α protein was located in the ER even though most of it had been processed by the late Golgi protease Kex2p. In these experiments processing by Kex2p had been induced by the strong overexpression of the hybrid gene. This result confirmed the observation that the distribution of wild-type Sec22p and Kex2p-cleaved hybrid protein in fractions from sucrose gradients was always identical (Fig. 3). Both proteins were present in fractions containing ER and Golgi markers. This distribution resembles that of the C-terminally tagged ERS-24 protein, a Sec22p homolog from hamster (Paek et al., 1997). According to Hay et al. (1997), however, an N-terminally tagged Sec22p homolog from mouse localizes to the ER.

The immunofluorescence experiments showed that the same set of mutants which displayed an enhanced processing of Sec22-α by Kex2p failed to exhibit ER-immunofluorescence when stained for Sec22-α. These mutants were the COPI mutants *sec21-1* (γ-COP) and *sec27-1* (β′-COP) as well as mutants encoding defective Sec20 and Ufe1 proteins. Therefore, it can be concluded that Sec22p follows the same route for recycling to the ER as, for instance, the di-lysine-tagged transmembrane proteins (Letourneur et al., 1994), multiple membrane spanning proteins like Erd2p and Rer1p,

as well as proteins whose retrieval requires the latter two proteins as auxiliary factors (Lewis and Pelham, 1996; Boehm et al., 1997; Sato et al., 1997).

Remarkably, the mutants which did not show an effect on the targeting of Sec22-α (*rer1*, *ret1* and *erd2*) have been selected as mutants affected in the ER retention of Sec12p, KKXX-tagged or HDEL-tagged proteins, respectively. Thus they express alleles which very specifically affect the retention of particular proteins. However, most of the mutants which showed an effect in our assays were isolated as secretion mutants blocked in ER-to-Golgi transport at the restrictive temperature (*sec20-1*, *sec21-1* and *sec27-1*; Novick et al., 1980; Duden et al., 1994). They exhibit the recycling defect at the permissive temperature. This argues in favor of the recycling defect being the primary defect in these mutants (Gaynor and Emr, 1997). As supposed by Pelham (1994), the failure to recycle *v*-SNAREs like Sec22p may reduce the supply of these membrane proteins for further rounds of vesicle fusion with the Golgi membrane and this lack of *v*-SNAREs may block secretion. Unfortunately, we were not able to select specific mutants using a halo-assay which detects Kex2p-dependent α-factor secretion. This assay was not sensitive enough to reveal differences between mutants and wild-type when Sec22-α was expressed constitutively.

Recycling of ER- or Golgi-resident proteins can be initiated from several compartments. This has been shown for several mammalian as well as yeast proteins. Jackson et al. (1993) demonstrated that ER proteins in mammalian cells can be retrieved from the *cis-* and *medial-* Golgi compartments. KDEL-tagged bacterial exotoxins as well as KDEL-tagged peptides are transported from the *trans*-Golgi network to the ER (Seetharam et al., 1991; Miesenböck and Rothman, 1995). In yeast, growth conditions and media have a strong influence on how far HDEL-tagged proteins can reach within the Golgi before they are retrieved (Hardwick et al., 1990). Also, very efficient retrieval of a yeast membrane protein from late Golgi to early Golgi was demonstrated by Harris and Waters (1996). These authors showed that chimeras derived from the early Golgi-mannosyltransferase Och1p, are cleaved by Kex2p with a half time of about 5 minutes while the steady-state localization of Och1p and its tagged derivatives is in the early Golgi. To keep the gradient of Och1p across the Golgi, the apparently fast forward transport must be counteracted by even more rapid recycling. For the *v*-SNARE Sec22p our results indicate that a concentration gradient from ER to late Golgi is maintained which is steep enough to prevent processing by the late Golgi protease. Gradients like this may be maintained by a kind of distillation mechanism consisting of several consecutive recycling steps (Rothman and Wieland, 1996). Two findings presented here are consistent with this hypothesis: First, overproduction induces more rapid transport of Sec22-α to the late Golgi, while overproduction using a strong promoter in combination with multi-copy vectors additionally resulted in transport to the vacuole suggesting that the putative distillation mechanism can be saturated. Second, in wild-type cells overproducing Sec22-α ER-localized Sec22-α is cleaved by a late-Golgi protease. Therefore, retrieval of Sec22p may be initiated in different Golgi compartments.

A second possibility for the shift in the concentration gradient of Sec22-α from ER to Golgi is mutation in genes encoding COPI components. Accordingly, *sec21-1* and *sec27-1* mutants expressing functionally impaired coatomer exhibit enhanced cleavage of Sec22-α by the late Golgi protease Kex2p as well as a lack of ER-fluorescence when stained for Sec22-α. Sec22-α can even reach the vacuole. But this seems to be an artifact of an extremely high expression rate. We never observed vacuolar cleavage when Sec22-α was synthesized transiently in mutants or wild-type cells. Proof for an efficient Golgi retention of Sec22-α in mutant cells came from double immunofluorescence experiments. The Golgi retention of Sec22-α may be due to slow packaging of Sec22p into vesicles destined to the vacuole or the plasma-membrane. This Golgi retention is not simply the result of a block in vacuolar transport of Sec22-α since protease-deficient *sec21-1* and *sec27-1* cells show perfect colocalization of Sec22-α and the vacuolar marker Ypt7p when Sec22-α is overproduced at very high levels (data not shown).

Sec22-α processing in the late Golgi was also enhanced in *sec20-1* and *ufe1-1* mutants. While the COPI complex is implicated in many different kinds of vesicular transport (Schekman and Orci, 1996; Orci et al., 1997), Sec20p and Ufe1p are ER-resident proteins (Sweet and Pelham, 1992; Lewis and Pelham, 1996). Therefore, it is likely that the *sec20-1* and *ufe1-1* mutations affect only the transport back from the earliest Golgi compartment to the ER and not a putative recycling within consecutive Golgi compartments. It remains to be determined whether the increased processing of Sec22-α by Kex2p observed in *sec20-1* and *ufe1-1* mutants is simply the result of an impaired early Golgi-to-ER recycling mechanism or whether these mutants carry defects in intra-Golgi recycling as well.

Our results are consistent with the observation that Sec22p along with other *v*-SNARE proteins is a constituent of both COPI and COPII vesicles derived from ER in vitro (Barlowe et al., 1994; Bednarek et al., 1995; Campbell and Schekman, 1997). Retrograde transport of Sec22p from Golgi to ER is also obligatory for the recently established function of Sec22p as a *v*-SNARE during the fusion of vesicles with the ER membrane (Lewis et al., 1997). Thus, Sec22p may be important for anterograde as well as retrograde transport. Two observations are consistent with this dual function: (a) *SEC22* is a multi-copy suppressor of vesicle-targeting as well as recycling defects (Newman et al., 1990; Ossig et al., 1991; Duden et al., 1994; Sapperstein et al., 1996; Lewis and Pelham, 1996); (b) like other mutants involved in anterograde and retrograde transport, the *sec22-3* mutant is defective in the retention of HDEL-tagged proteins (Semenza et al., 1990). There are at least two other SNARE proteins which may be required for different steps in vesicular transport. Sed5p may represent a yeast *t*-SNARE which functions in anterograde transport from the ER to the Golgi and in the retrograde transport from later Golgi (Banfield et al., 1995). In mammalian cells GOS28/GS28 may function as a *v*-SNARE in intra-Golgi (Nagahama et al., 1996) and in ER-to-Golgi transport (Subramaniam et al., 1996).

The approaches described here will also be applied to other SNARE proteins. If Sec22p functions in retrograde transport as well, it may combine with another set of *v*-SNARE proteins to form a complex functionally different from one active in anterograde transport. To achieve this functional specificity other *v*-SNAREs like Bos1p and Bet1p might combine with another subcomplex of the COPI and therefore undergo

retrieval differently from Sec22p. Our results do not prove that the Sec22p directly interacts with COPI components. As with other proteins recycling to the ER a receptor or adapter may be required to sort or package this *v*-SNARE into COPI vesicles (Semenza et al., 1992; Boehm et al., 1997). Further experiments are required to either identify this adaptor or to proof that COPI and Sec22p interact directly.

We are very grateful to Mike Lewis, Johannes Boehm, Rainer Duden, François Letourneur, M. Gerard Waters, Steven F. Nothwehr and Howard Riezman for providing plasmids and yeast strains. We thank Mike Anson, Sabine Schaertl, Stephan Schröder-Köhne and David Ferrari for critical comments on the manuscript. We also thank Donna Arndt-Jovin for help with the confocal microscope, Rainer Ossig, Reiner Grabowski, Mustafa Benli, Dietrich Scheglmann and Thomas Sommer for supplying antibodies, Hans-Peter Geithe for synthesizing oligonucleotides as well as sequencing, Hannegret Frahm for technical assistance and Dieter Gallwitz for his support. This work was in part supported by the Deutsche Forschungsgemeinschaft (Grant Schm990 and SFB 523).

REFERENCES

Antebi, A. and Fink, G. R. (1992). The yeast Ca^{2+}-ATPase homolog, *PMR1*, is required for normal Golgi function and localizes in a novel Golgi-like distribution. *Mol. Biol. Cell* **3**, 633-654.

Aridor, M., Bannykh, S. I., Rowe, T. and Balch, W. E. (1995). Sequential coupling between COPII and COPI vesicle coats in endoplasmic-reticulum to Golgi transport. *J. Cell Biol.* **131**, 875-893.

Ballensiefen, W. and Schmitt, H. D. (1997). Periplasmic Bar1 protease of Saccharomyces cerevisiae is active before reaching its extracellular destination. *Eur. J. Biochem.* **247**, 142-147.

Banfield, D. K., Lewis, M. J. and Pelham, H. R. B. (1995). A SNARE-like protein required for traffic through the Golgi-complex. *Nature* **375**, 806-809.

Barlowe, C., Orci, L., Yeung, T., Hosobuchi, M., Hamamoto, S., Salama, N., Rexach, M. F., Ravazzola, M., Amherdt, M. and Schekman, R. (1994). COPII: a membrane coat formed by Sec proteins that drive vesicle budding from the endoplasmic reticulum. *Cell* **77**, 895-907.

Bednarek, S., Ravazzola, M., Hosobuchi, M., Amherdt, M., Perrelet, A., Schekman, R. and Orci, L. (1995). COPI-coated and COPII-coated vesicles bud directly from the endoplasmic reticulum in yeast. *Cell* **83**, 1183-1196.

Boehm, J., Ulrich, H. D., Ossig, R. and Schmitt, H. D. (1994). Kex2-dependent invertase secretion as a tool to study the targeting of transmembrane proteins which are involved in ER-Golgi transport in yeast. *EMBO J.* **13**, 3696-3710.

Boehm, J., Letourneur, F., Ballensiefen, W., Ossipov, D., Démollière, C. and Schmitt, H. D. (1997). Sec12p requires Rer1p for sorting to coatomer (COPI)-coated vesicles and retrieval to the ER. *J. Cell Sci.* **110**, 991-1003.

Campbell, J. L. and Schekman, R. (1997). Selective packaging of cargo molecules into endoplasmatic reticulum-derived COPII vesicles. *Proc. Nat. Acad. Sci. USA* **94**, 837-842.

Chapman, R. E. and Munro, S. (1994). The functioning of the yeast Golgi apparatus requires an ER proein encoded by *ANP1*, a member of a new family of genes affecting the secretory pathway. *EMBO J.* **13**, 4896-4907.

Clary, D. O., Griff, I. C. and Rothman, J. E. (1990). SNAPs, a family of NSF attachment proteins involved in intracellular membrane-fusion in animals and yeast. *Cell* **61**, 709-721.

Cosson, P. and Letourneur, F. (1994). Coatomer interaction with di-lysine endoplasmic reticulum retention motifs. *Science* **263**, 1629-1631.

Cosson, P., Démollière, C., Hennecke, S., Duden, R. and Letourneur F. (1996). δ-COP and ζ-COP, two coatomer subunits homologous to clathrin-associated proteins, are involved in ER retrieval. *EMBO J.* **15**, 1792-1798.

Cosson, P., Schröder-Köhne, S., Sweet, D. S., Démollière, C., Hennecke, S., Frigerio, G. and Letourneur, F. (1997). The Sec20/Tip20p complex is involved in ER retrieval of di-lysine-tagged proteins. *Eur. J. Cell Biol.* **73**, 93-97.

Dascher, C., Ossig, R., Gallwitz, D. and Schmitt, H. D. (1991). Identification and structure of four yeast genes (SLY) that are able to suppress the functional loss of *YPT1*, a member of the ras-superfamily. *Mol. Cell. Biol.* **11**, 872-885.

d'Enfert, C., Barlowe, C., Nishikawa, S., Nakano, A. and Schekman, R. (1991). Structural and functional dissection of a membrane glycoprotein required for vesicle budding from the endoplasmic reticulum. *Mol. Cell. Biol.* **11**, 5727-5734.

Duden, R., Hosobuchi, M., Hamamoto, S., Winey, M., Byers, B. and Schekman, R. (1994). Yeast β- and β′-coat proteins (COP). Two coatomer subunits essential for endoplasmic reticulum-to-Golgi protein traffic. *J. Biol. Chem.* **269**, 24486-24495.

Duden, R., Kajikawa, L., Wuestehube, L. and Schekman, R. (1998). ε-COP is a structural component of coatomer that functions to stabilize α-COP. *EMBO J.* (in press).

Evan, G. J., Lewis, G. K., Ramsay, G. and Bishop, J. M. (1985). Isolation of monoclonal antibodies pecific for human c-myc proto-oncogene product. *Mol. Cell. Biol.* **5**, 3610-3616.

Franzusoff, A., Redding, K., Crosby, J., Fuller, R. S. and Schekman, R. (1991). Localization of components involved in protein transport and processing through the yeast Golgi-apparatus. *J. Cell Biol.* **112**, 27-37.

Gaynor E. C. and Emr, S. D. (1997). COPI-independent anterograde transport: cargo-selective ER to Golgi protein transport in yeast COPI mutants. *J. Cell Biol.* **136**, 789-802.

Gaynor, E. C., Heesen, S. T., Graham, T. R., Aebi, M. and Emr, S. D. (1994). Signal-mediated retrieval of a membrane-protein from the Golgi to the ER in yeast. *J. Cell Biol.* **127**, 653-665.

Graham, T. R. and Emr, S. D. (1991). Compartmental organization of Golgi-specific protein modification and vacuolar protein sorting events defined in a yeast *sec18* (NSF) mutant. *J. Cell Biol.* **114**, 207-218.

Haas, A., Schegelmann, D., Lazar, T., Gallwitz, D. and Wickner, W. (1995). The GTPase Ypt7p of *Saccharomyces cerevisiae* is required on both partner vacuoles for the homotypic fusion step of vacuole inheritance. *EMBO J.* **14**, 5258-5270.

Hardwick, K. G. and Pelham, H. R. B. (1992). *SED5* encodes a 39-kD integral membrane protein required for vesicular transport between the ER and the Golgi complex. *J. Cell Biol.* **119**, 513-521.

Hardwick, K. G., Lewis, M. J., Semenza, J., Dean, N. and Pelham, H. R. B. (1990). *ERD1*, a yeast gene required for the retention of luminal endoplasmic reticulum proteins, affects glycoprotein processing in the Golgi apparatus. *EMBO J.* **9**, 623-630.

Harris, S. L. and Waters, M. G. (1996). Localization of a yeast early Golgi mannosyltransferase, Och1p, involves retrograde transport. *J. Cell Biol.* **132**, 985-998.

Hay, J. C., Chao, D. S., Kuo, C. S. and Scheller, R. H. (1997). Protein interactions regulating vesicle transport between the endoplasmic reticulum and Golgi-apparatus in mammalian-cells. *Cell* **89**, 149-158.

Hicke, L., Zanolari, B., Pypaert, M., Rohrer, J. and Riezman H. (1997). Transport through the yeast endocytic pathway occurs through morphologically distinct compartments and requires and active secretory pathway and Sec18/N-Ethylmaleimide-sensitive fusion protein. *Mol. Biol. Cell* **8**, 13-31.

Hosobuchi, M., Kreis T. and Schekman, R. (1992). *SEC21* is a gene required for ER to Golgi protein-transport that encodes a subunit of a yeast coatomer. *Nature* **360**, 603-605.

Jackson, M. R., Nilsson, T. and Peterson, P. A. (1990). Identification of a consensus motif for retention of transmembrane proteins in the endoplasmic reticulum. *EMBO J.* **9**, 3153-3162.

Jackson, M. R., Nilsson, T. and Peterson, P. A. (1993). Retrieval of transmembrane proteins to the endoplasmic reticulum. *J. Cell Biol.* **121**, 317-333.

Jäntti, J., Keränen, S., Toikkanen, J., Kuismanen, E., Ehnholm, C., Söderlund, H. and Olkkonen, V. M. (1994). Membrane insertion and intracellular transport of yeast syntaxin Sso2p in mammalian cells. *J. Cell Sci.* **107**, 3623-3633.

Kane, P. M., Kuehn, M. C., Howald-Stevenson, I. and Stevens, T. H. (1992). Assembly and targeting of peripheral and integral membrane subunts of the yeast vacuolar H^+-ATPase. *J. Biol Chem.* **267**, 447-454.

Klionsky, D. J., Herman, P. K. and Emr, S. D. (1990). The fungal vacuole: composition, function, and biogenesis. *Microbiol. Rev.* **54**, 266-292.

Kurjan, J. and Herskowitz, I. (1982). Structure of a yeast pheromone gene (mf α): a putative α-factor precursor contains four tandem copies of mature α-factor. *Cell* **30**, 933-943.

Kutay, U., Ahnert-Hilger, G., Hartmann, E., Wiedenmann, B. and Rapoport, T. A. (1995). Transport route for synaptobrevin via a novel

pathway of insertion into the endoplasmic reticulum membrane. *EMBO J.* **14**, 217-223.

Letourneur, F., Gaynor, E. C., Hennecke, S., Démollière, C., Duden,R., Emr, S. D., Riezman, H. and Cosson, P. (1994). Coatomer is essential for retrieval of di-lysine-tagged proteins to the endoplasmic reticulum. *Cell* **79**, 1199-1207.

Lewis, M. J. and Pelham, H. R. B. (1996). SNARE-mediated retrograde traffic from the Golgi-complex to the endoplasmic reticulum. *Cell* **85**, 205-215.

Lewis, M. J., Raynor, J. C. and Pelham, H. R. B. (1997). A novel SNARE complex implicated in vesicle fusion with the endoplasmic reticulum. *EMBO J.* **16**, 3017-3024.

Mayer, A., Wickner, W. and Haas, A. (1996). Sec18p (NSF)-driven release of Sec17p (a-SNAP) can precede docking and fusion of yeast vacuoles. *Cell* **85**, 83-94.

Miesenböck, G. and Rothman, J. E. (1995). The capacity to retrieve escaped ER proteins extends to the trans-most cisterna of the Golgi stack. *J. Cell Biol.* **129**, 309-319.

Nagahama, M., Orci, L., Ravazzola, M., Amherdt, M., Lacomis, L., Tempst, P., Rothman, J. E. and Söllner, T. H. (1996). A v-SNARE implicated in intra-Golgi transport. *J. Cell Biol.* **133**, 507-516.

Newman, A. P., Shim, J. and Ferro-Novick, S. (1990). *BET1*, *BOS1*, and *SEC22* are members of a group of interacting yeast genes required for transport from the endoplasmic reticulum to the Golgi complex. *Mol. Cell. Biol.* **10**, 3405-3414.

Newman, A. P., Groesch, M. E. and Ferro-Novick, S. (1992). Bos1p, a membrane-protein required for ER to Golgi transport in yeast, copurifies with the carrier vesicles and with Bet1p and the ER membrane. *EMBO J.* **11**, 3609-3617.

Nishikawa, S.-I. and Nakano, A. (1993). Identification of a novel gene required for membrane protein retention in the early secretory pathway. *Proc. Nat. Acad. Sci. USA* **90**, 8179-8183

Novick, P., Field C. and Schekman R. (1980). Identification of 23 complementation groups required for post-translational events in the yeast secretory pathway. *Cell* **21**, 205-215.

Ossig, R., Dascher, C., Trepte, H. H., Schmitt, H. D. and Gallwitz, D. (1991). The yeast *SLY* gene-products, suppressors of defects in the essential GTP-binding Ypt1 protein, may act in endoplasmic reticulum-to-Golgi transport. *Mol. Cell. Biol.* **11**, 2980-2993.

Ossig, R., Laufer, W., Schmitt, H. D. and Gallwitz, D. (1995). Functionality and specific membrane localization of transport GTPases carrying C-terminal membrane anchors of synaptobrevin-like proteins. *EMBO J.* **14**, 3645-3653.

Orci L., Stamnes M., Ravazzola, M., Amherdt, M., Perrelet, A., Söllner, T. H. and Rothman J. E. (1997). Bidirectional transport by distinct populations of COPI-coated vesicles. *Cell* **90**, 335-349.

Otto, H., Hanson, P. I. and Jahn, R. (1997). Assembly and disassembly of a ternary complex of synaptobrevin, syntaxin, and SNAP-25 in the membrane of synaptic vesicles. *Proc. Nat. Acad. Sci. USA* **94**, 6197-6201.

Paek, I., Orci L., Ravazzola, M., Erdjument-Bromage, H., Amherdt, M., Tempst, P., Söllner, T. H. and Rothman J. E. (1997). ERS-24, a mammalian v-SNARE implicated in vesicle traffic between the ER an the Golgi. *J. Cell Biol.* **137**, 1017-1028.

Panzner, S., Dreier, L., Hartmann, E., Kostka, S. and Rapoport, T. A. (1995). Posttranslational protein-transport in yeast reconstituted with a purified complex of Sec proteins and Kar2p. *Cell* **81**, 561-570.

Pelham, H. R. B. (1994). About turn for the COPs. *Cell* **79**, 1125-1127.

Piper, R. C., Cooper, A. A., Yang, H. and Stevens., T. H. (1995). VPS27 controls vacuolar and endocytic traffic through a prevacuolar compartment in *Saccharomyces cerevisiae*. *J. Cell Biol.* **131**, 603-617.

Preuss, D., Mulholland, J., Franzusoff, A., Segev, N. and Botstein, D. (1992). Characterization of the Saccharomyces Golgi complex through the cell-cycle by immunoelectron microscopy. *Mol. Biol. Cell* **3**, 789-803.

Raymond, C. K., Howald-Stevenson, I., Vater, C. A. and Stevens, T. H. (1992). Morphological classification of the yeast vacuolar protein sorting mutants: evidence for a prevacuolar compartment in class E *vps* mutants. *Mol. Biol. Cell* **3**, 1389-1402.

Redding, K., Holcomb, C. and Fuller, R. S. (1991). Immunolocalization of Kex2 protease identifies a putative late Golgi compartment in the yeast Saccharomyces cerevisiae. *J. Cell Biol.* **113**, 527-538.

Roberts, C. J., Nothwehr, S. F. and Stevens, T. H. (1992). Membrane-protein sorting in the yeast secretory pathway: evidence that the vacuole may be the default compartment. *J. Cell Biol.* **119**, 69-83.

Rose, M. D., Misra, L. M. and Vogel, J. P. (1989). *KAR2*, a karyogamy gene, is the yeast homolog of the mammalian BiP/GRP78 gene. *Cell* **57**, 1211-1221.

Rothman, J. E. (1994). Mechanism of intracellular protein-transport. *Nature* **372**, 55-63.

Rothman, J. E. and Orci, L. (1992). Molecular dissection of the secretory pathway. *Nature* **355**, 409-415.

Rothman, J. E. and Wieland, F. T. (1996). Protein sorting by transport vesicles. *Science* **272***,* 227-234.

Rowe, T., Aridor, M., McCaffery, J. M., Plutner, H., Nuoffer, C. and Balch, W. E. (1996). COPII vesicles derived from mammalian endoplasmic reticulum microsomes recruit COPI. *J. Cell Biol.* **135**, 895-911.

Sapperstein, S. K., Lupashin, V. V., Schmitt, H. D. and Waters, M. G. (1996). Assembly of the ER to Golgi SNARE complex requires Uso1p. *J. Cell Biol.* **132**, 755-767.

Sato, M., Sato, K. and Nakano, A. (1996). Endoplasmic reticulum localization of Sec12p is achieved by two mechanisms: Rer1p-dependent retrieval that requires the transmembrane domain and Rer1p-independent retention that involves the cytoplasmic domain. *J. Cell Biol.* **134**, 279-293.

Sato, K., Sato, M. and Nakano, A. (1997). Rer1p as common machinery for the endoplasmic reticulum localization of membrane proteins. *Proc. Nat. Acad. Sci. USA* **94**, 9693-9698

Scales, S. J., Pepperkok, R. and Kreis, T. E. (1997). Visualization of ER-to-Golgi transport in living cells reveals a sequential mode of action for COPII and COPI. *Cell* **90**, 1137-1148.

Schekman, R. and Orci, L. (1996). Coat proteins and vesicle budding. *Science* **271**, 1526-1533.

Schneider, C. E. and Guarente, L. (1991). Vectors for expression of cloned genes in yeast: regulation, overproduction , and underproduction. *Meth. Enzymol.* **194**, 373-388.

Schröder, S., Schimmöller, F., Singer-Krüger, B. and Riezman, H. (1995). The Golgi-localization of yeast Emp47p depends on its di-lysine motif but is not affected by the *ret1-1* mutation in α-COP. *J. Cell Biol.* **131**, 895-912.

Seetharam, S., Chaudhary, V. K., FitzGerald, D. and Pastan, I. (1991). Increased cytotoxic activity of Pseudomonas exotoxin and two chimeric toxins ending in KDEL. *J. Biol. Chem.* **266**, 17376-17381.

Semenza, J. C., Hardwick, K. G., Dean, N. and Pelham, H. R. B. (1990). *ERD2*, a yeast gene required for the receptor-mediated retrieval of luminal ER proteins from the secretory pathway. *Cell* **61**, 1349-1357.

Sikorski, R. S. and Hieter, P. (1989). A system of shuttle vectors and yeast host strains for efficient manipulation of DNA in Saccharomyces cerevisiae. *Genetics* **122**, 19-27.

Søgaard, M., Tani, K., Ye, R. R., Geromanos, S., Tempst, P., Kirchhausen, T., Rothman, J. E. and Söllner T. (1994). A rab protein is required for the assembly of SNARE complexes in the docking of transport vesicles. *Cell* **78**, 937-948.

Söllner, T. and Rothman, J. E. (1994). Neurotransmission harnessing fusion machinery at the synapse. *Trends Neurosci.* **17**, 344-348.

Söllner, T., Whitehart, S. W., Brunner, M., Erdjument-Bromage, H., Geromanos, S., Tempst, P. and Rothman, J. E. (1993). SNAP receptors implicated in vesicle targeting and fusion. *Nature* **362**, 318-324.

Söllner, T., Bennett, M. K., Whiteheart, S. W., Scheller, R. H. and Rothman, J. E. (1993b). A protein assembly-disassembly pathway in-vitro that may correspond to sequential steps of synaptic vesicle docking, activation, and fusion. *Cell* **75**, 409-418.

Subramaniam, V. N., Peter, F., Philp, R., Wong, S. H. and Hong, W. J. (1996). GS28, a 28-kilodalton Golgi SNARE that participates in ER-Golgi transport. *Science* **272**, 1161-1163.

Sweet, D. J. and Pelham H. R. B. (1992). The *Saccharomyces cerevisiae SEC20* gene encodes a membrane glycoprotein which is sorted by the HDEL retrieval-system. *EMBO J.* **11**, 423-432.