Journal of Cell Science 110, 2703-2714 (1997)
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JCS9646

Specific requirements for the ER to Golgi transport of GPI-anchored proteins in yeast

Christine Sütterlin[1], Tamara L. Doering[2,*], Frauke Schimmöller[1], Stephan Schröder[1] and Howard Riezman[1,†]

[1]Biozentrum of the University of Basel, CH-4056 Basel, Switzerland
[2]Department of Molecular and Cell Biology, Howard Hughes Research Institute, University of California, Berkeley, CA, 94720, USA
*Present address: Department of Pharmacology, Cornell University Medical College, 1300 York Avenue, New York, 10021, USA
†Author for correspondence (e-mail: Riezman@ubaclu.unibas.ch)

SUMMARY

GPI-anchored proteins are attached to the membrane via a glycosylphosphatidylinositol-(GPI) anchor whose carbohydrate core is conserved in all eukaryotes. Apart from membrane attachment, the precise role of the GPI-anchor is not known, but it has been proposed to play a role in protein sorting. We have investigated the transport of the yeast GPI-anchored protein Gas1p. We identified two mutant strains involved in very different cellular processes that are blocked selectively in the transport of GPI-anchored proteins before arrival to the Golgi. The *end8-1/lcb1-100* mutant is defective in ceramide synthesis. In vitro data suggest a requirement for ceramides after the exit from the ER. We therefore propose that ceramides might function in the fusion of a GPI-containing vesicle with the Golgi, but we cannot exclude a role in the ER. The second mutant that blocks the transport of GPI-anchored proteins to the Golgi is *ret1-1*, a mutant in the α-subunit of coatomer. In both mutants, GPI-anchor attachment is normal and in *ret1-1* cells, the GPI-anchors are remodeled with ceramide to the same extent as in wild-type cells.

Key words: Glycosylphosphatidylinositol, Biosynthetic transport, Ceramide, Coatomer, COP II

INTRODUCTION

Glycosylphosphatidylinositol (GPI)-anchored proteins represent a subclass of cell surface proteins found in all eukaryotic cells. These proteins serve diverse cellular functions such as cell surface protection, cell adhesion, transmembrane signaling and cell wall synthesis (Lisanti et al., 1990; McConville and Ferguson, 1993; Klis, 1994). The attachment of GPI-anchored proteins to the membrane via a glycolipid moiety instead of a proteinaceous transmembrane domain seems to determine the protein's fate during protein sorting and secretion (Brown, 1992; Zurzolo et al., 1994; Futerman, 1995). In yeast, the GPI-anchored protein Gas1p represents a major cell surface glycoprotein (Conzelmann et al., 1988; Nuoffer et al., 1991) that has been ascribed a function in morphogenesis, cell separation and cell wall synthesis (Popolo et al., 1993; Ram et al., 1995). Gas1p is synthesized with two signal sequences: an N-terminal one required for insertion of the protein into the ER, and a C-terminal one directing attachment of the GPI-anchor in the lumen of the ER. The GPI-signal sequence is very similar to those found in GPI-anchored protein precursors in animal cells (Nuoffer et al., 1993). Anchor attachment is thought to occur as a transamidation reaction in which the preformed GPI-precursor is attached to the protein with the concomitant release of a short peptide (Maxwell et al., 1995). The yeast Gaa1 protein is required for the transfer of the anchor precursor to the protein (Hamburger et al., 1995). Anchor attachment is necessary for exit of Gas1p from the ER (Nuoffer et al., 1993; Doering and Schekman, 1996). After attachment of the GPI-anchor, Gas1p leaves the ER and is transported to the Golgi, where its carbohydrate chains are elongated causing a shift in apparent molecular mass from 105 kDa to 125 kDa (Conzelmann et al., 1988). Many GPI anchors in yeast undergo a remodeling process in which a base-sensitive diacylglycerol moiety is replaced with a base-resistant ceramide lipid moiety (Conzelmann et al., 1992). The Gas1p anchor, however, is not remodeled to ceramide and remains base-sensitive (Fankhauser et al., 1993).

Transport of proteins along the secretory pathway is mediated by vesicles carrying distinct protein coats (Mallabiabarrena and Malhotra, 1995; Schekman and Orci, 1996). The COP II coat has been shown to be required for ER to Golgi transport and is composed of the Sec13p/Sec31p, Sec23p/Sec24p protein complexes and the small GTPase Sar1p (Barlowe et al., 1994). The purified components are sufficient to drive vesicle budding from the ER in vitro. The in vitro budding assay produces vesicles that are capable of targeting to and fusing with the Golgi. The pheromone precursor pre pro-α-factor, Gas1p and two amino acid permeases have been shown to be segregated into COP II-coated vesicles in vitro (Barlowe et al., 1994; Doering and Schekman, 1996; Kuehn et al., 1996). Another non-clathrin coat, coatomer, consists of 7 polypeptide subunits that are recruited onto vesicles due to the action of the small GTPase Arf in its GTP-bound form (Orci et al., 1993; Ktistakis et al., 1996). The coat subunits (α through ζ) associate in equimolar ratio and contribute most of

the coat's mass. Several studies suggest that COP I-coated vesicles are also involved in ER to Golgi transport (Peter et al., 1993; Aridor et al., 1995). Although COP I-coated vesicles have been found to be formed on ER membranes, their role in anterograde transport is not clear (Bednarek et al., 1995). Studies from yeast provide evidence for a primary role of coatomer in the retrieval of proteins from the Golgi to the ER (Letourneur et al., 1994; Lewis and Pelham, 1996; Boehm et al., 1997). Moreover, forward transport of a subset of proteins is not dependent on coatomer, consistent with a retrograde role for coatomer-coated vesicles (Gaynor and Emr, 1997).

We have previously provided evidence that ceramide synthesis enhances the transport of GPI-anchored proteins to the Golgi in yeast (Horvath et al., 1994). Incubation of yeast cells with myriocin, an inhibitor of ceramide synthesis, significantly slowed down the maturation of Gas1p from the 105 kDa form to the 125 kDa form of Gas1p. The delayed maturation of Gas1p was a measure of the transport between the ER and the medial Golgi, since the extension of core glycan with mannose in α-1,3 linkage typically occurs in the medial Golgi compartment (Herscovics and Orlean, 1993). It was therefore not clear where between the ER and the medial Golgi compartment the ceramide effect occurred. Evidence from work with mammalian cells suggests that ceramides play a role at a later stage of the secretory pathway, controlling the transport of GPI-anchored proteins from the Golgi to the different surface domains (Zurzolo et al., 1994; Mays et al., 1995). The difference in the site of requirement for ceramides could reflect differences in the localization of the enzymes that modify ceramides (Futerman, 1995).

In this study, we provide evidence that Gas1p transport to the Golgi is strictly dependent on ceramide synthesis. We propose a role for ceramides in the fusion of Gas1p-containing vesicles with the Golgi, but cannot exclude a role in the vesicle budding process. Furthermore, we have found a coatomer mutant, *ret1-1*, that specifically disrupts the transport of GPI-anchored proteins to the Golgi and suggest that coatomer might function in the retrieval of specific factors from the Golgi which are required for the transport of GPI-anchored proteins from the ER to the Golgi.

MATERIALS AND METHODS

Strains and growth conditions

Strains of *Saccharomyces cerevisiae* used for this work are listed in Table 1. Precultures were grown to saturation in complete medium (1% yeast extract, 2% peptone, 2% glucose, 40 μg/ml each of adenine and uracil) and used to inoculate overnight cultures. For metabolic labeling experiments, overnight cultures were grown at the permissive temperature in SDYE (0.2% yeast extract, 0.67% yeast nitrogen base without amino acids, 2% glucose and the required nutrients) to exponential growth phase (0.5-2×10^7 cells/ml).

Antibodies

Polyclonal antisera directed against Gas1p, invertase, Emp47p and against α-1,3-mannose modifications were described previously (Horvath et al., 1994; Schröder et al., 1995). Polyclonal antisera against Och1p were kindly provided by G. Waters (Princeton University, Princeton, USA) and Y. Jigami (National Institute of Bioscience and Human Technology, Tsukuba, Japan). Polyclonal antiserum against Yap3p was obtained from Y. Bourbonnais (Biotechnology Research Institute, Montreal, Canada) and Wbp1 antiserum was raised in rabbits using an expression construct provided by S. te Heesen (Eidgenössische Technische Hochschule, Zürich, Switzerland).

Table 1. Strains of *Saccharomyces cerevisiae* used in this work

Strain	Genotype	Source
RH448	*Mat**a** his4 leu2 ura3 lys2 bar1*	Lab. strain
RH1433	*Matα sec23-1 leu2 ura3*	Lab. strain
RH1473	*Mat**a** sec21-1 his4 leu lys2 bar1*	Lab. strain
RH1489	*Mat**a** sec13-1 leu2 ura3 lys2 bar1*	Lab. strain
RH1491	*Mat**a** sec12-4 his4 ura3 lys2 bar1*	Lab. strain
RH1737	*Mat**a** sec18-1 his4 ura3 leu2 bar1*	Lab. strain
RH1800	*Mat**a** his4 ura3 leu2 bar1*	Lab. strain
RH2607	*Mat**a** lcb1-100 his4 ura3 leu2 bar1*	Lab. strain
RH2688	*Mat**a** sec27-1 his4 ura3 leu2 bar1*	Lab. strain
RH2856	*Matα gaa1-1 ura3 leu2*	Lab. strain
RH3187	*Mat**a** ret1-1 his4 ura3 leu2 trp1 bar1*	Lab. strain
RH3466	*Matα ade2 his4 ura3 leu2 lys2 suc2Δ9*	Lab. strain
RH3467	*Mat**a** lcb1-100 ade2 his4 ura3 leu2 suc2Δ9*	Lab. strain
EGY101	*Mat**a** ret1-1 ura3 leu2 his3 trp1 suc2Δ9*	P. Cosson
PC130	*Mat**a** ret2-1 his4 ura3 leu2 lys2 suc2Δ9*	P. Cosson
FLY74	*Matα ret3-1 his4 ura3*	F. Letourneur

Radiolabeling and immunoprecipitations

Cells were grown overnight in SDYE, harvested and washed with SD* (2% glucose, 0.67% yeast nitrogen base and the required nutrients). 2.5×10^7 cells per time point were resuspended in 1 ml SD*, preincubated for 1-10 minutes at the indicated temperatures and pulsed with 100 μCi TRANS ^{35}S-label (Dupont de Nemours, Germany) per time point and chased as indicated. The chase was initiated by adding a 1/100 volume of chase cocktail (0.3% methionine, 0.3% cysteine, 0.3 M $(NH_4)_2SO_4$). At the indicated chase times, samples were taken and NaN_3 and NaF were added to 10 mM final concentration each. Radiolabeled cells were collected, resuspended in TEPI (100 mM Tris-HCl, pH 7.5, 10 mM EDTA, proteinase inhibitors: 1 μg/ml pepstatin, 1 μg/ml leupeptin, 1 μg/ml antipain) and lysed by vortexing 4 times for 1 minute with glass beads. The lysates were boiled in the presence of 1% SDS for 5 minutes and centrifuged for 15 minutes in an Eppendorf centrifuge. The supernatants were added to 5 ml TNET (100 mM Tris-HCl, pH 8, 100 mM NaCl, 5 mM EDTA, 1% Triton X-100) and incubated with antiserum and Protein A-Sepharose (Pharmacia, Uppsala, Sweden) for 3 hours at room temperature. The beads were washed 4 times with TNET, once with 20 mM Tris-HCl, pH 7.5, and then resuspended in Laemmli protein sample buffer. The immunoprecipitate was analyzed by SDS-PAGE with subsequent exposure and quantitation of the gel on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). For Gas1p pulse-chase experiments, the percentage of matured Gas1p was determined by taking the ratio of the 125 kDa mature form to the total signal (125 kDa and 105 kDa forms) and multiplying by 100.

For double immunoprecipitations, Gas1p or CPY were eluted from the Protein A-Sepharose beads by resuspension in 100 μl 1% SDS and boiling for 5 minutes. The eluate was split into equal samples which were added to TNET and reprecipitated as above with either the same antiserum or with antiserum directed against α-1,3-mannose modifications. After antibody and Protein A-Sepharose incubation, the sample was treated as described previously.

The invertase secretion experiment was performed as described (Kübler et al., 1994). Analysis of the intracellular transport of Inv-Wbp1 was performed based on the protocol by Gaynor et al. (1994). In brief, cells expressing the Inv-Wbp1 fusion protein were radiolabeled for 5 minutes after a 2-minute preincubation at 37°C and chased for the indicated time. The fusion protein was recovered by immunoprecipitation with antiserum directed against invertase. The immune complexes were resuspended in 32 μl 0.5% SDS, 1% β-mercap-

toethanol, boiled for 5 minutes and the eluates were incubated in 40 μl final volume 50 mM sodium citrate, pH 5.5, and 1 mU Endoglycosidase H (Böhringer Mannheim) overnight at 37°C. The percentage of processed fusion protein was determined as the ratio of the processed 56 kDa to the total signal (70 kDa and 56 kDa forms) times 100. The background band found mostly from wild type, which migrates at approximately the same molecular mass as the processed form, but which was already present at 0 minute chase, was not included in the quantitation.

Immunoblotting of Yap3p

Total protein extracts from the indicated yeast strains were prepared and the proteins were separated by SDS-PAGE. After transfer to nitrocellulose, the filters were blocked with 2% milk in PBS, and during the incubation with the Yap3 antiserum (1/1,000), mannose was added to 100 mM to block unspecific sugar binding. Anti-rabbit IgG coupled to peroxidase (Sigma) was used as secondary antibody and the immune-reactivity was detected using the ECL kit (Amersham, Int., Amersham, UK) and quantified by scanning films with a laser densitometer (Molecular Dynamics, Sunnyvale, CA). The ratio of the 68 kDa core glycosylated ER form (Ash et al., 1995) to the total signal determined the extent of ER to Golgi transport.

Subcellular fractionation by velocity sedimentation on sucrose density gradients

1.5×10^8 cells were radiolabeled for 5 minutes at 37°C as described above and chased for 30 minutes. Cells were spheroplasted in a volume of 500 μl as described previously (Schimmöller and Riezman, 1993) and lysed by the addition of 500 μl H_2O containing protease inhibitors. The lysate was cleared once of unbroken spheroplasts by centrifuging for 5 minutes at 500 ***g*** and then loaded onto an 11 ml sucrose step-gradient (Schröder et al., 1995). The gradient was spun for 2 hours 20 minutes at 4°C in a TST41.14 rotor (Kontron Instruments, Zürich, Switzerland) at 37,000 rpm and fractionated from the top into 1 ml fractions, the pellet being resuspended in the last fraction. SDS was added to a final concentration of 1%. The samples were boiled for 5 minutes and centrifuged for 15 minutes at full speed in a table top centrifuge. The supernatants were centrifuged again and the distributions of Gas1p, Wbp1p and Emp47p were determined by immunoprecipitation, SDS-PAGE and PhosphorImager analysis. Och1p distribution was assessed by parallel analysis of 5×10^8 unlabeled cells. Samples of each fraction were resolved by SDS-PAGE, transferred to nitrocellulose and probed with an antibody against Och1p. Immune-reactivity was detected using the ECL kit (Amersham, Int., Amersham, UK) and the signal was quantitated by scanning films with a laser densitometer (Molecular Dynamics, Sunnyvale, CA).

GPI-anchor attachment and remodeling

The procedure was performed as described (Nuoffer et al., 1991). In brief, cells were radiolabeled as described above, converted into spheroplasts (Schimmöller and Riezman, 1993) and lysed in TEPI. The lysates were incubated with 1% TX-114 for 30 minutes on ice, then spun for 10 minutes in an Eppendorf centrifuge. The supernatant was incubated for 5 minutes at 32°C and then centrifuged briefly for phase separation. Each phase was reextracted twice. After this first cycle of extractions, the detergent phase was diluted tenfold with TEPI and split into two equal samples. To one half, 0.1 unit PI-PLC (Böhringer Mannheim) was added, the other served as a control. After a 3-hour incubation at 30°C with occasional mixing, phase separation was performed with each sample as described above. Phases were reextracted and total protein was TCA-precipitated from both phases. The protein precipitates were resuspended in 1% SDS and the samples were processed for Gas1p immunoprecipitation. Unanchored Gas1p segregated into the primary aqueous phase while anchored Gas1p partitioned into the primary detergent phase and shifted into the secondary aqueous phase after PI-PLC treatment.

[^{3}H]*myo*-inositol labeling and the determination of mild base sensitivity to assay for GPI-anchor remodeling were performed as described earlier (Horvath et al., 1994). In brief, cells were pulse-labeled with 15 μCi [^{3}H]*myo*-inositol for 20 minutes followed by an 80-minute chase. Cells were lysed by vortexing with glass beads and glycoproteins were affinity-purified using Concanavalin A-Sepharose beads (Pharmacia, Uppsala, Sweden). The glycoproteins were separated by SDS-PAGE and the gel was cut into 4 mm slices which were treated with Pronase to generate protein-free GPI-anchors. The radioactivity was extracted and counted. The fractions of the entire gel were pooled and dried. The GPI-anchors were resuspended in $CHCl_3$:CH_3OH:H_2O (10:10:3) and the sample was split into 4 equal parts (1-4). Two parts were treated with mild base, while the other two were mock treated. TX-114 partition allows the separation of base-sensitive anchors (aqueous phase) from base-resistant anchors (detergent phase). The percentage of base sensitivity was determined in the following manner: average of cpm in the aqueous phase of the base-treated samples minus the average of cpm in the aqueous phase of the mock-treated samples determined the specific base-sensitive cpm. Those cpm over total cpm multiplied by 100 determined the percentage of base sensitivity.

In vitro budding

In vitro budding experiments were performed essentially as described by Doering and Schekman (1996). Briefly, *lcb1-100* or wild-type cells (OD_{600} = 5) were preincubated in medium lacking methionine and ammonium sulfate, and then radiolabeled for 5 minutes at 20°C by the addition of 100 μCi [^{35}S]Promix/OD_{600} (1,200 Ci/mmol; Amersham, Arlinghton Heights, IL). A 0.1 ml portion of radiolabeled cells was diluted tenfold in chase medium (without radiolabel, but containing 0.5 mg/ml each of methionine and cysteine) and incubated for 20 minutes, while the remainder was used to generate perforated spheroplasts for budding assays. Samples of total budding reactions and the vesicle-containing supernatant fractions were digested with trypsin to eliminate material not enclosed in membrane compartments or vesicles. These and chased samples were analyzed by immunoprecipitation with polyclonal Gas1p antiserum and SDS-PAGE.

RESULTS

Gas1p transport is blocked in a mutant defective in ceramide synthesis

The transport of the GPI-anchored protein, Gas1p, from the ER to the Golgi has been shown to be dependent upon genes specifically required for vesicular transport (Conzelmann et al., 1988) and upon addition of the GPI anchor (Nuoffer et al., 1991; Doering and Schekman, 1996). In addition, we have shown that incubation with myriocin, an inhibitor of ceramide synthesis, specifically retards the transport of Gas1p and other GPI-anchored proteins to the Golgi (Horvath et al., 1994). In order to investigate this ceramide requirement further we analyzed the transport of Gas1p in a mutant strain showing a temperature-sensitive defect in ceramide synthesis. This mutant allele was isolated in our laboratory as an endocytosis mutant, *end8-1* (Munn and Riezman, 1994). *end8-1* cells were shown to be defective for ceramide synthesis and the *end8* mutation to be allelic to *LCB1* (B. Stevenson, personal communication), a gene required for serine palmitoyltransferase activity (Buede et al., 1991). Transport of Gas1p can be monitored by following the conversion of the 105 kDa core glycosylated ER form to the mature 125 kDa form, which is only formed upon arrival in the medial Golgi compartment. Mutant *lcb1-100* and wild-type cells were grown overnight at the permissive temperature, preincubated at 24°C or shifted to

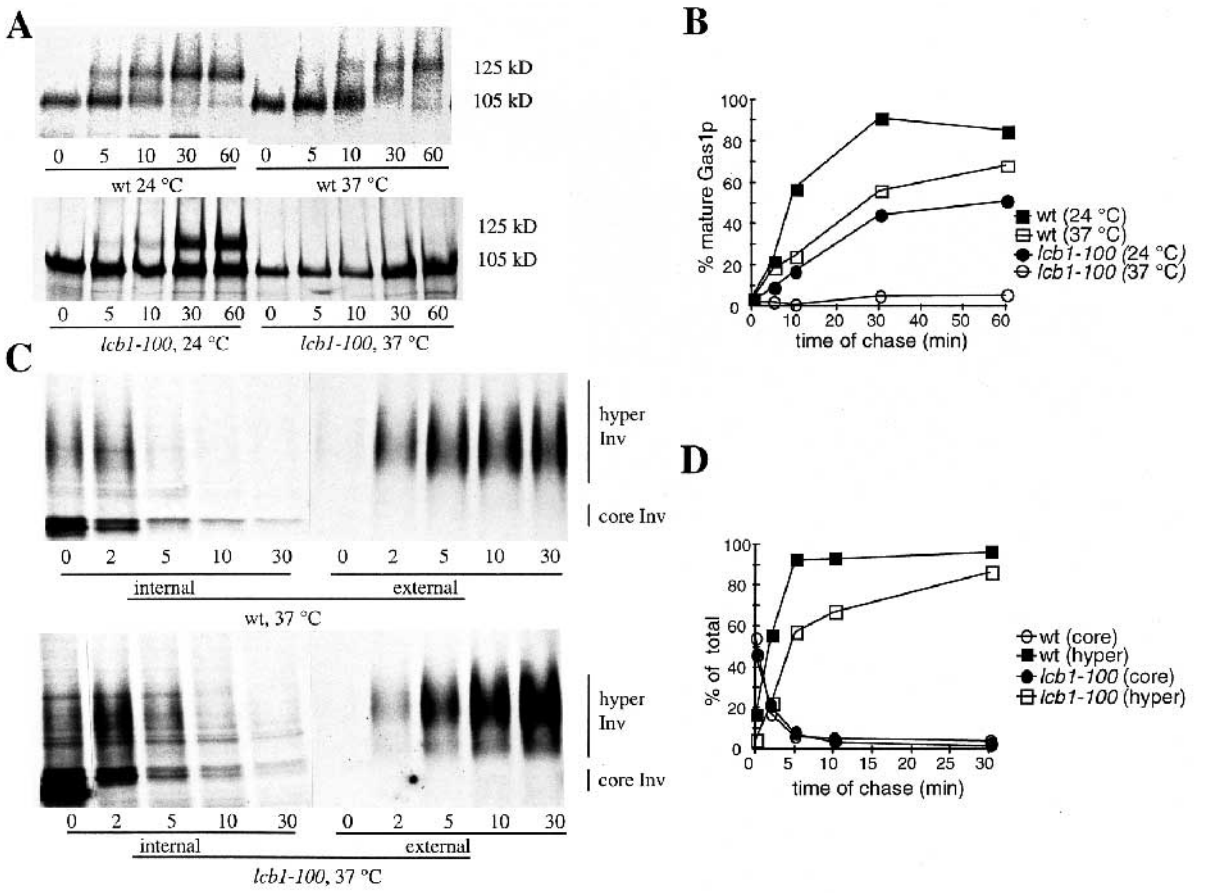

Fig. 1. Protein transport in *lcb1-100* cells. (A) ER to Golgi transport of Gas1p at 24°C and 37°C in wild-type (wt) and *lcb1-100* mutant cells. Wild-type (RH448) and *lcb1-100* (RH2607) cells were preincubated for 2 minutes at 24°C or 37°C, radiolabeled for 5 minutes and chased for the indicated amount of time in minutes. Gas1p was immunoprecipitated from total cell lysate, the immunoprecipitate was separated by SDS-PAGE (7.5% gel) and analyzed by autoradiography. (B) Quantitations of the pulse-chase experiments shown in A. (C) Analysis of invertase secretion in wild-type (RH448) and in *lcb1-100* (RH2607) cells. Spheroplasts derived from wild-type or *lcb1-100* cells were pulse-labeled for 4 minutes after a 2 minute preincubation at 37°C and chased as indicated. After the chase, cells and medium were separated and both were analyzed for invertase by immunoprecipitation. The ER forms can be detected as discrete bands (core Inv) whereas the Golgi modified forms run as a hyperglycosylated smear of 100 to 150 kDa (hyper Inv). (D) Quantitations of the invertase secretion experiment shown in C.

37°C for 2 minutes, pulse-labeled and chased as indicated (Fig. 1A and B). The maturation of Gas1p in wild-type cells at 24°C was rapid with a half time of maturation of 8 minutes (Fig. 1A and B), but was significantly delayed in *lcb1-100* cells at 24°C. At 37°C, maturation of Gas1p in wild-type cells was slower than at 24°C (half time of transport of 25 minutes); however, at this temperature in *lcb1-100*, basically no maturation occurred. We observed that in wild-type cells at 37°C, a portion of the mature form was sensitive to extracellular degradation causing a significant loss of signal (Fig. 1A, unpublished observations). The kinetics of Gas1p transport in wild-type cells at 37°C (Fig. 1B) might therefore represent an underestimate of the actual rate of transport. The results with the *lcb1-100* mutant strengthens our previous argument that ceramide synthesis plays an important role in transport of Gas1p to the Golgi. The partial effect of myriocin on Gas1p transport seen previously was most likely due to an incomplete inhibition of ceramide synthesis by the compound (Horvath et al., 1994).

To investigate whether the mutation in *LCB1* affects the transport of proteins that are not GPI-anchored, we analyzed the transport of the glycoprotein invertase (Schimmöller et al., 1995). The ER-specific core glycosylated invertase gave a 79-83 kDa ladder of distinct bands when resolved by SDS-PAGE. This core glycosylated form was further glycosylated in the Golgi and appeared as a smear of 100-150 kDa. Hyperglycosylated invertase left the Golgi and was secreted into the periplasm (Fig. 1C and D). In *lcb1-100* mutant cells at 37°C, core glycosylated invertase was converted into the hyperglycosylated Golgi form with kinetics very similar to those seen in wild-type cells. Therefore, ER to Golgi transport of invertase was not affected by the *lcb1-100* mutation. A slight delay in secretion of hyperglycosylated invertase into the medium was detected (Fig. 1C and D). We also tested the behavior of a transmembrane protein using a recombinant variant of the Golgi protein Emp47p into which a glycosylation site had been introduced (Schröder et al., 1995), facilitating the analysis of ER to Golgi transport. We found that in *lcb1-100*, the hyperglycosylated form appeared with similar kinetics to those in wild-type cells (data not shown). Also the transport of carboxypeptidase Y (CPY) to the vacuole was shown not to be affected in the *lcb1-100/end8-1* mutant (Munn and Riezman, 1994). We conclude that the block in

ER to Golgi transport caused by the *lcb1-100* mutation is selective for Gas1p.

Coatomer is required for the transport of Gas1p from the ER to the Golgi

Vesicular transport from ER to Golgi has been shown to proceed via COP II-coated vesicles (Pryer et al., 1992; Barlowe et al., 1994). Gas1p transport seems to follow this route because its transport is blocked by sec12 (Schimmöller et al., 1995). Sec12p is required for recruitment of the COP II coat onto ER membranes. In addition, COP II components are the only soluble factors required for the budding of Gas1p-containing vesicles in vitro (Doering and Schekman, 1996). To confirm this finding in vivo, we examined the effects of mutations in the COP II coat components, Sec13p and Sec23p. Pulse-chase analysis showed that mutations in either the *SEC23* and *SEC13* genes abolished transport of Gas1p to the Golgi (Fig. 2).

Coatomer has been proposed to be involved in both anterograde and retrograde transport between the ER and the Golgi (Pepperkok et al., 1993; Rothman, 1994; Bednarek et al., 1995). We addressed the question of whether the COP I coat also plays a role in the transport of Gas1p between the ER and the Golgi by performing pulse-chase experiments in various COP I mutant strains after a 2 minute preincubation at a non-permissive temperature. We found that Gas1p maturation was defective in all of the coatomer mutants tested, but to varying extents (Fig. 2). The strongest effect was seen in *ret1-1* (α-COP), *sec21-1* (γ-COP) and *ret2-1* (δ-COP) mutants. As the effect of the α-COP mutant, *ret1-1,* on Gas1p maturation was very strong and this allele has been reported to have little effect on the anterograde transport of CPY (Letourneur et al., 1994), we decided to investigate Gas1p transport in this mutant more closely. Analysis of *ret1-1* cells that were pulse-labeled after a 2-minute preincubation at the various temperatures demonstrated a temperature-sensitive defect for Gas1p maturation (Fig. 3A).

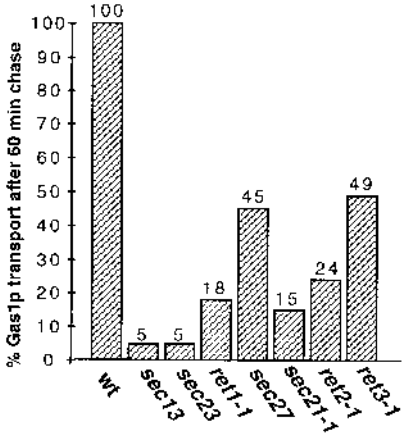

Fig. 2. Gas1p maturation is blocked in COP II and COP I mutants. Wild-type (RH448), *sec13* (RH1489), *sec23* (RH1433), *ret1-1* (RH3187), *sec27* (RH2688), *sec21-1* (RH1473), *ret2-1* (PC130) and *ret3-1* (FLY74) were radiolabeled for 5 minutes after a 2 minute preincubation at the non-permissive temperature and chased for 60 minutes at 37°C. Cells were lysed by the glass bead method and Gas1p was immunoprecipitated from the lysates. The immune complexes were separated by SDS-PAGE and analyzed by autoradiography. The percentage of transported Gas1p was determined by the ratio of the signal in the 125 kDa form to the total signal (105 kDa and 125 kDa) multiplied by 100. The numbers are average values of 3 independent experiments

[^{3}H]*myo*-inositol can be incorporated into the whole spectrum of GPI-anchored proteins. In wild-type cells, most of the radioactivity is incorporated into glycoproteins found as a high molecular mass smear at the top of an SDS-gel. Since these high molecular mass forms are not found in early secretion mutants at non-permissive temperatures (e.g. *sec12*, data not shown), this technique allows the analysis of the transport of other GPI-anchored proteins between the ER and the Golgi. Wild-type and *ret1-1* cells were grown at 24°C overnight, shifted to 37°C for 5 minutes and labeled for 20 minutes with [^{3}H]*myo*-inositol followed by an 80-minute chase. Glycoproteins were affinity-purified and separated by SDS-PAGE. The gel lanes were cut into slices which were treated with Pronase. The radioactivity was extracted and counted. We found that in *ret1-1* cells, [^{3}H]*myo*-inositol was mainly found in proteins of lower apparent molecular mass than in wild-type cells (Fig. 3B). These data suggest that the ER to Golgi transport of other GPI-anchored proteins is also affected in *ret1-1*, as has been previously been shown for myriocin-treated cells (Horvath et al., 1994).

To test if the *ret1-1* mutant displays a more general transport defect, invertase secretion was analyzed. Pulse-chase analysis of spheroplasts after a 2-minute preincubation at 37°C showed that invertase maturation and secretion occurred with very similar kinetics to those in wild-type cells (Fig. 3C). Although a slight reduction in the extent of hyperglycosylation of invertase was seen in *ret1-1* cells, the core glycans of invertase were extensively elongated causing the protein to migrate at a molecular mass well above 100 kDa. This minor defect in glycosylation cannot explain the significant shift in the [^{3}H]*myo*-inositol labeling pattern (Fig. 3B). We conclude that a mutation in the α-subunit of coatomer, Ret1p, inhibits the transport of Gas1p and other GPI-anchored proteins, while the transport of CPY (Letourneur et al., 1994) and invertase occurs with wild-type kinetics.

ER to Golgi transport of Yap3p is defective in *lcb1-100* and *ret1-1*

The aspartyl protease Yap3p represents a further well characterized GPI-anchored protein from yeast (Ash et al., 1995). It occurs as a 68 kDa ER core form whose core glycan chains are extensively elongated upon arrival to the Golgi apparatus, giving rise to a smear of an average molecular mass of about 150 kDa (Ash et al., 1995). To address the transport requirements of Yap3p, wild-type, *lcb1-100*, *ret1-1* and *sec12* cells were grown overnight at the permissive temperature and were either left at 24°C or shifted for 90 minutes to 37°C. Total protein extracts were prepared which were separated by SDS-PAGE and analyzed for Yap3p by western blot analysis. In wild-type cells, the hyperglycosylated form of Yap3p as well as a distinct, but weak band of about 90 kDa were most abundant at both temperatures. In contrast, in *sec12* cells, which are defective in budding from the ER, the 68 kDa core-glycosylated form accumulated and the appearance of the 90 kDa form was enhanced upon shift to the non-permissive temperature. Since the 90 kDa form was also present in wild-type cells, we quantified the amount of 68 kDa form to measure the

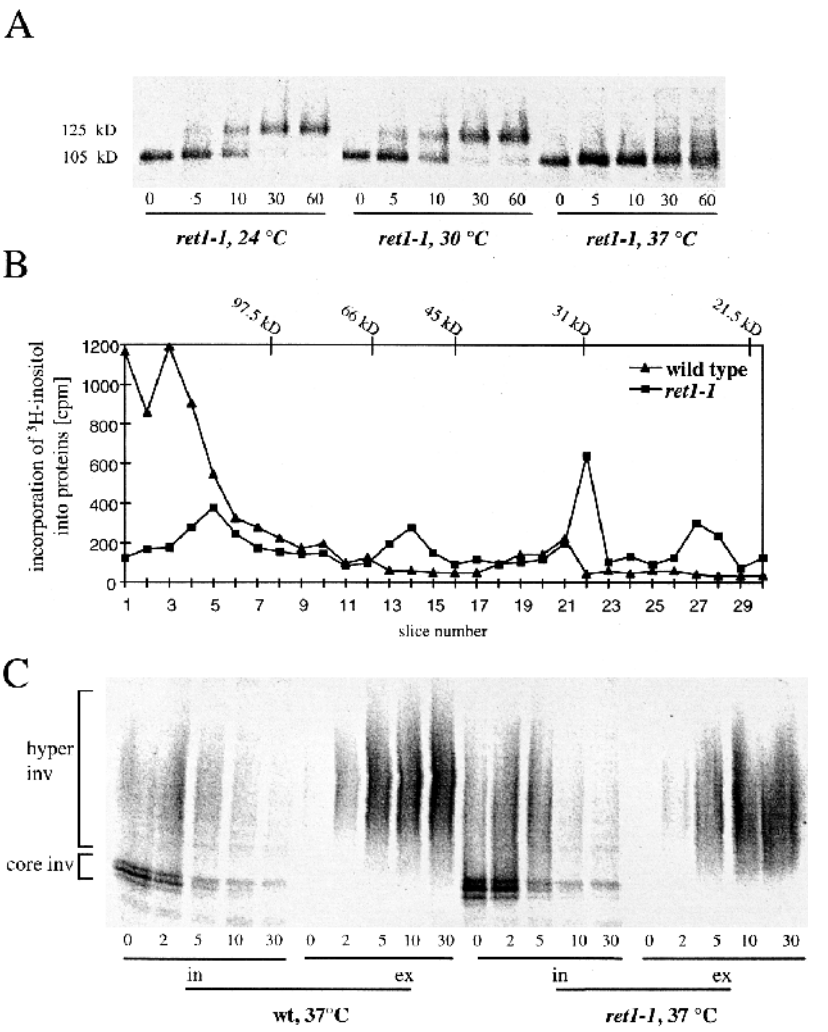

Fig. 3. Gas1p maturation is specifically blocked in *ret1-1* cells in a temperature-dependent manner. Pulse-chase experiments were performed in *ret1-1* (RH3187) to investigate the kinetics of Gas1p transport at different temperatures as described for Fig. 1. (A) Gas1p maturation in *ret1-1* at 24, 30 and 37°C. (B) wild-type (RH448) and *ret1-1* (RH3187) cells were shifted to 37°C for 20 minutes before they were labeled for 20 minutes with [^{3}H]*myo*-inositol with a subsequent chase of 80 minutes. Glycoproteins were isolated, separated by SDS-PAGE (10%) and the lanes were cut into 4 mm slices. The proteins were digested with Pronase and the radioactivity was counted. The migration of molecular size markers is indicated on top. (C) The secretion of invertase was tested at 37°C in wild-type and *ret1-1* cells after a 2 minute preincubation at 37°C. The ER core form is indicated as 'core Inv' and the Golgi modified hyperglycosylated form is indicated as 'hyper Inv'. 'in' denotes internal invertase whereas 'ex denotes the invertase secreted into the medium.

relative block in ER to Golgi transport in various strains (Fig. 4). In *ret1-1* cells, Yap3p transport was blocked to a similar extent as in *sec12* control cells. In *lcb1-100* cells, the 68 kDa form also accumulated significantly, but to a lesser extent than in *ret1-1* or *sec12* cells. We conclude that, as found for Gas1p, Yap3p transport depends on ceramide synthesis; however, the requirement appears to be less stringent than for Gas1p. The *ret1-1* mutation caused a tight block in the transport of Yap3p.

Mutant *lcb1-100* and *ret1-1* cells attach the GPI anchor to Gas1p

GPI-anchor attachment is required for exit of Gas1p from the endoplasmic reticulum (Nuoffer et al., 1993; Doering and Schekman, 1996). One of the proteins that is required for attachment of the GPI anchor, Gaa1p, is an ER protein with a dilysine retrieval motif (Hamburger et al., 1995). *ret1-1* was isolated due to its inability to retrieve dilysine-tagged proteins from the Golgi to the ER. Therefore, it is possible that *ret1-1* and other COP I mutant cells mislocalize essential components of the GPI-anchoring machinery and do not transport Gas1p because they do not attach the GPI-anchor. This is less likely for *lcb1-100* cells because it has been shown previously that inhibition of ceramide synthesis by myriocin does not block anchor addition to Gas1p (Horvath et al., 1994). To test

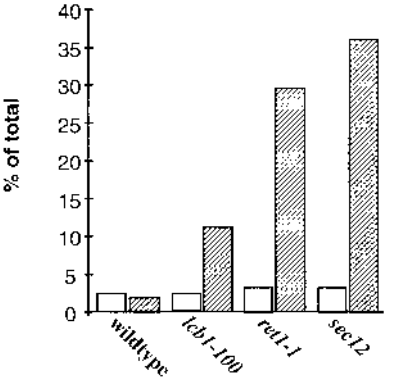

Fig. 4. Yap3p transport in wild-type, *lcb1-100*, *ret1-1* and *sec12* cells. Wild-type (RH448), *lcb1-100* (RH2607), *ret1-1* (RH3187) and *sec12* (RH1491) cells were grown overnight at permissive temperature and left at 24°C (open bars) or shifted to the non-permissive temperature (hatched bars) for 90 minutes. Total protein extracts were separated by SDS-PAGE, transferred to nitrocellulose and decorated with antiserum against Yap3p. The reactivity as revealed by ECL was quantified by densitometric scanning as described. An average value of 3 independent experiments is shown.

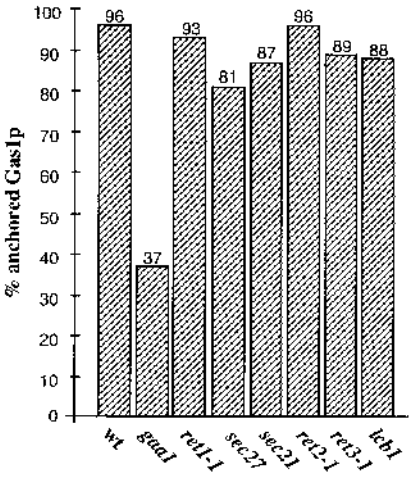

Fig. 5. The GPI-anchor is attached to Gas1p in *lcb1-100* and coatomer mutants. Wild-type (RH448), *gaa1* (RH2856), *ret1-1* (RH3187), *sec27* (RH2688), *sec21-1* (RH1473), *ret2-1* (PC130), *ret3-1* (FLY74) and *lcb1-100* (RH2607) cells were pulse labeled after a 2 minute preincubation at 37°C and chased for 5 minutes. Anchoring efficiency was analyzed by phase-separation into TX-114 with subsequent PI-PLC treatment of the detergent phase. The distribution of Gas1p was determined by immunoprecipitation from each fraction followed by analysis of the precipitates by SDS-PAGE and quantitation on a PhosphorImager. The percentage of anchored Gas1p was determined as described in Materials and Methods. The numbers are average values of 2 independent experiments.

whether the block of Gas1p transport in *ret1-1*, the other coatomer mutants and in *lcb1-100* was due to inefficient anchor attachment we pulse-labeled cells after a 2-minute preincubation at the non-permissive temperature and chased for 5 minutes. Cell lysates were prepared and extracted with Triton X-114. When attached to the membrane via the GPI-moiety, Gas1p partitions into the Triton X-114 detergent phase and can only be shifted into the aqueous phase after enzymatic treatment with PI-specific phospholipase C. Unanchored Gas1p partitions mainly into the aqueous phase and the portion found in the detergent phase is unaffected by phospholipase C treatment (Nuoffer et al., 1991). The phase separation data (Fig. 5) indicate that in wild-type cells, Gas1p was completely anchored, whereas in *gaa1* cells, where anchor attachment is defective (Hamburger et al., 1995), only 37% of Gas1p was anchored. In the *lcb1-100* mutant and all of the COP I mutant strains, including *ret1-1*, Gas1p was efficiently processed with the GPI-anchor, suggesting that the block in Gas1p transport cannot be due to a defect in the attachment of the GPI-anchor.

The *lcb1-100* mutant does not have a Golgi to ER retrieval defect

Since the *ret1-1* mutant is defective in the retrieval of dilysine-tagged proteins from the Golgi to the ER (Letourneur et al., 1994), it is conceivable that *lcb1-100* could also be defective in retrograde transport, thereby causing a block in forward transport. To examine Golgi to ER retrieval in *lcb1-100* mutant cells, we introduced a dilysine-tagged version of invertase (invertase-Wbp1: Gaynor et al., 1994) into *lcb1-100* cells. This fusion protein occurs as a 70 kDa form after treatment with Endoglycosidase H, if localized to the ER as in wild-type cells (Fig. 6). If retrieval to the ER is defective, as seen for *ret1-1* cells, invertase-Wbp1 is transported to the vacuole where it is proteolytically processed and is found as a 56 kDa form after treatment with Endoglycosidase H (Letourneur et al., 1994). In the *lcb1-100* mutant, invertase-Wbp1 remained ER-localized as the amount of processed form found at 37°C after a 45-minute chase was the same in wild-type and *lcb1-100* cells. Similar results were found at 30°C (data not shown). This suggests that *lcb1-100* does not mislocalize dilysine-tagged proteins and that the observed transport block in *lcb1-100* is not due to defective retrograde transport of dilysine-tagged proteins.

The *ret1-1* mutant does not show a defect in remodeling of the GPI-anchor

The lipid portion of most GPI-anchored proteins from yeast is subjected to a lipid remodeling process in which the diacylglycerol moiety of the complete precursor is exchanged for a ceramide moiety (Conzelmann et al., 1992). We have shown previously that upon inhibition of ceramide synthesis by myriocin, remodeling of GPI-anchors is blocked, and we could not exclude that inhibition of anchor remodeling caused the defect in Gas1p secretion (Horvath et al., 1994). We therefore analyzed if the *ret1-1* mutation affects lipid remodeling. Wild-type and *ret1-1* cells were labeled with [^{3}H]*myo*-inositol as described earlier (Fig. 3B). The extracted radioactivity from each gel slice was pooled and half of the sample was treated with mild base, which allows the distinction between base-resistant ceramide anchors and base-sensitive diacylglycerol anchors. Base-resistant anchors were separated from base-sensitive anchors by TX-114 phase partition and the radioactivity of each fraction was counted. In wild-type cells, about 60 to 65% of total GPI-anchors were remodeled. Similar results were found for *ret1-1* cells (Fig. 7). Therefore, remodeling of the GPI-anchor to a ceramide is not defective in *ret1-*

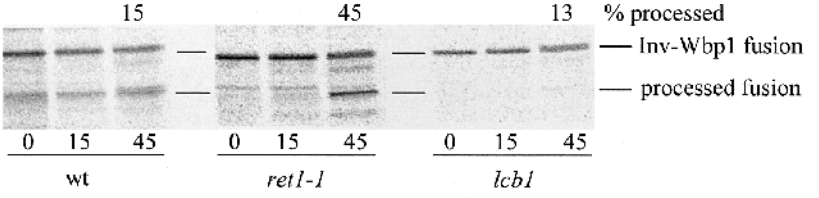

Fig. 6. *lcb1-100* cells are not defective in the recycling of a dilysine-tagged reporter protein. Wild-type (RH3466), *ret1-1* (EGY101) and *lcb1-100* (RH3467) cells harboring the plasmid invertase-Wbp1 (pEG1-KK: Gaynor et al., 1994) were pulse-labeled for 5 minutes after a 2-minute preincubation at 37°C and chased for the indicated amounts of time. The invertase-Wbp1 fusion protein was immunoprecipitated using polyclonal antiserum against invertase. The precipitates were treated with Endoglycosidase H, analyzed by SDS-PAGE and the signal quantified on a PhosphorImager. The percentage of processed fusion protein was determined as described in Materials and Methods.

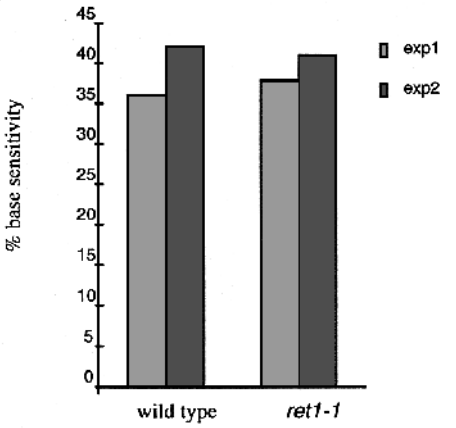

Fig. 7. GPI-anchors are remodeled in *ret1-1* cells. Wild-type (RH448) and *ret1-1* (RH3187) cells were labeled with [^{3}H]*myo*-inositol as described for Fig. 3B. The radioactivity of all gel slices from each strain was pooled and the pooled fractions were split into 4 identical parts. Two parts were subjected to base treatment while the other two were mock treated. The determination of the percentage of base-sensitive anchors is described in Materials and Methods.

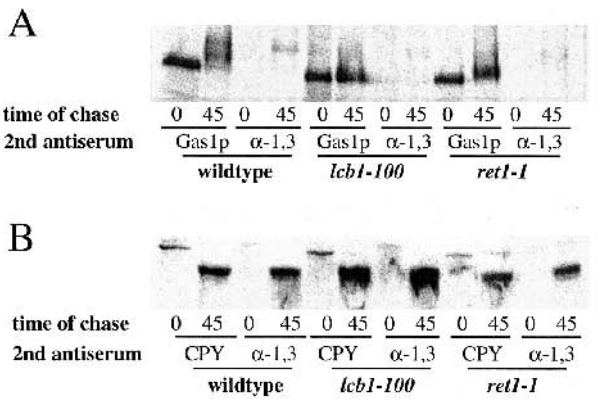

Fig. 8. Gas1p is not modified with mannose in an α-1,3 linkage in *lcb1-100* and *ret1-1* cells. Wild-type (RH448), *lcb1-100* (RH2607) and *ret1-1* (RH3187) cells were radiolabeled for 5 minutes after a 2 minute preincubation at 37°C and chased for 0 or 45 minutes. Cells were lysed and Gas1p or CPY were immunoprecipitated from total lysates. The precipitates were eluted from the Protein A-Sepharose beads. The eluates were split into two equal parts which received the same antiserum or antiserum against mannose in α-1,3 linkage. Reprecipitated immune complexes were analyzed by SDS-PAGE and autoradiography. (A) Double immunoprecipitations using Gas1p (A) or CPY (B) antiserum as primary antiserum.

1 cells and a lack of lipid remodeling cannot explain the transport defect.

Gas1p transport is blocked before arrival to the Golgi in *lcb1-100* and *ret1-1* mutant cells

Previous studies using myriocin showed that ceramide synthesis enhanced the maturation of GPI-anchored proteins and it was implied that the effect was somewhere before the medial Golgi compartment, where the increase in size occurs. We therefore analyzed the site of ceramide requirement further by assaying for the acquisition of modifications of N-linked carbohydrate chains onto the protein. α-1,3-linked mannose is attached to N-linked glycans in a medial Golgi compartment (Herscovics and Orlean, 1993). Wild-type, *ret1-1* and *lcb1-100* cells were grown at the permissive temperature and preincubated at 37°C for 2 minutes. Cells were labeled for 5 minutes and chased for either 0 or 45 minutes. Total lysates were prepared, Gas1p or CPY were immunoprecipitated and reprecipitated with either the same antiserum or with antiserum specific for α-1,3-mannose modifications (Fig. 8A and B). In wild-type cells, only the mature 125 kDa form could be precipitated by both antisera. In *ret1-1* and *lcb1-100* mutant cells, the accumulated from of Gas1p was not recognized by the α-1,3-mannose-specific antiserum, suggesting that in these mutants, transport was blocked before arrival to the medial Golgi compartment. Under the same conditions, the Golgi and vacuolar forms of biosynthetically labeled CPY could be efficiently precipitated by the α-1,3-mannose-specific antiserum (Fig. 8B).

In the *cis*-Golgi compartment, it is believed that one mannose is attached in an α-1,6 linkage by the mannosyltransferase Och1p (Gaynor et al., 1994). Och1p can therefore be used as a marker protein for this compartment. Addition of this single mannose residue would not necessarily have been detected as a change in migration in our gel system. The existing antiserum against this α-1,6-linked mannose residue was not useful in this instance because it recognized unglycosylated, unanchored Gas1p (C. Sütterlin, unpublished results). We therefore investigated the behavior of Gas1p that accumulated in *lcb1-100* and *ret1-1* by velocity gradient fractionation and compared it to the intracellular distribution of Och1p. Our gradient system allows the separation of ER from Golgi membranes (Schröder et al., 1995). The ER comigrates with the plasma membrane at the bottom of the gradient (fractions 8 to 11) whereas the Golgi is enriched in the middle fractions (fractions 5 to 8). Wild-type, *lcb1-100* and *ret1-1* cells were metabolically labeled for 5 minutes after a 2-minute preincubation at the non-permissive temperature, chased for 30 minutes and the lysates were subjected to cell fractionation. The fractions obtained were analyzed for the distribution of Gas1p, Wbp1p (an ER marker), Emp47p (a Golgi marker, data not shown) and Och1p. In wild-type cells (Fig. 9A), Gas1p was completely converted into the mature 125 kDa form and distributed mainly into fractions at the bottom of the gradient (8-11) due to its plasma membrane localization. As expected from known kinetics of maturation, a small amount of mature Gas1p was still found in the Golgi fractions (5-8). In *lcb1-100* and in *ret1-1* cells, only immature Gas1p was found, which colocalized with the ER marker, Wbp1p, at the bottom of the gradient. Almost no Gas1p was found in Och1p-containing fractions (Fig. 9B and C). The distribution of ER and Golgi proteins was the same in wild-type, *lcb1-100* and *ret1-1* cell fractionations indicating that the two mutations affected Gas1p transport without grossly disturbing Golgi and ER structures. These results, together with the lack of α-1,3-mannose modification, strongly suggests that Gas1p transport was specifically blocked in *lcb1-100* and *ret1-1* cells before reaching the *cis*-Golgi.

lcb1-100 membranes are not defective for the budding step

Gas1p can be packaged into ER-derived COP II-coated vesicles in perforated yeast spheroplasts (Doering and

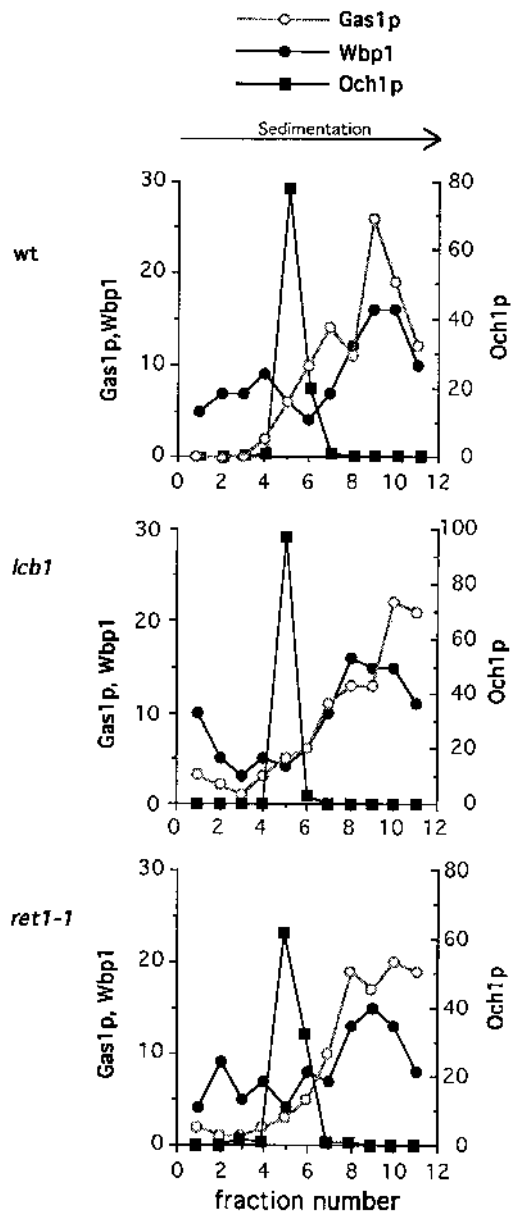

Fig. 9. Newly synthesized Gas1p that accumulates in *lcb1-100* (RH2607) and *ret1-1* (RH3187) cells does not colocalize with the *cis*-Golgi marker Och1p. Wild-type, *lcb1-100* and *ret1-1* cells were pulse-labeled for 5 minutes after a 2-minute preincubation at 37°C and chased for 30 minutes. Cells were lysed and the cleared lysate was loaded onto an 18% to 60% (w/v) sucrose density gradient. The gradients were centrifuged for 2 hours 20 minutes at 4°C at 37,000 rpm. 1 ml fractions were collected from the top (fraction 1) and analyzed for the distribution of Gas1p and Wbp1 by immunoprecipitation followed by SDS-PAGE and autoradiography, and for Och1p by western blotting (see Materials and Methods). In all gradients, fraction 12 was found to contain a large portion of unlysed cells and was omitted from the calculations. The distribution of Wbp1p, Och1p and Gas1p in wild-type (A) in *lcb1-100* (B) or in *ret1-1* cells (C) is shown.

Schekman, 1996). We exploited this in vitro system to investigate the requirements for ceramides in the exit of Gas1p from the ER. Wild-type and *lcb1-100* cells were radiolabeled at 20°C and a portion of each culture was chased at 20°C for 20 minutes to verify the impaired maturation phenotype (Fig. 10, last two lanes). The remaining radiolabeled cells were converted to perforated spheroplasts, washed, and incubated with cytosol from either wild-type or *lcb1-100* cells to induce vesicle release. Parallel control reactions were performed using unlabeled membranes and radiolabeled prepro-α-factor, a well characterized COP II vesicle cargo molecule (Baker et al., 1988). We found that Gas1p, like pro-α-factor, was packaged into vesicles with equal efficiency by membranes from either wild-type or *lcb1-100* cells (Fig. 10). This result was independent of whether cytosol from wild-type or *lcb1-100* cells was used, and of whether radiolabeling was performed at 20°C, 30°C or warmer (data not shown). These results show that Gas1p can exit the ER in vitro under similar conditions to where transport to the Golgi is blocked in vivo. Although it would have been interesting to perform similar experiments with the *ret1-1* mutant, the transport phenotype in this mutant is displayed only at temperatures which are too high for performing in vitro assays (e.g. 37°C).

DISCUSSION

In this study, we show that the transport of GPI-anchored proteins is selectively blocked before the *cis*-Golgi in the two mutant strains *lcb1-100* and *ret1-1*. This demonstrates a strict requirement for ceramide synthesis for Gas1p transport and reveals a novel requirement for the transport of GPI-anchored proteins, namely Ret1p. This is the first time that a forward transport defect of a subset of secretory proteins has been observed in *ret1-1* mutant cells. Furthermore, we provide evidence that remodeling of the GPI-anchor is not sufficient for transport of GPI-anchored proteins to the Golgi. Finally, we present in vitro data suggesting that GPI-anchored proteins can exit the ER in the absence of ongoing ceramide synthesis.

For the sake of discussion, we assume that the well characterized in vitro budding assay faithfully reproduces the in vivo situation. This assumption is supported by the following observations. (1) The budding of Gas1p into COP II-coated vesicles is a highly specific process and a version of Gas1p that is not GPI-anchored due to a mutation in the anchor attachment site (Gas1p-N506Q) has been shown not to enter vesicles (Doering and Schekman, 1996). (2) The process is strongly dependent on cytosol or purified COP II components, with a tenfold increase in signal over background. (3) Even though the efficiency with which Gas1p enters vesicles is low when compared with glyco-pro-α-factor (3% versus approximately 15%), this difference is consistent with the in vivo transport kinetics of the two proteins.

Membranes derived from *lcb1-100* cells were not defective for Gas1p budding from the ER. Under conditions where, in a parallel in vivo experiment, Gas1p transport was blocked to about 80%, no significant difference in Gas1p budding from membranes derived from wild-type or *lcb1-100* cells was observed. These results imply that the Gas1p transport block is between the ER and the Golgi and suggest that a block in ceramide synthesis could therefore affect vesicle fusion. Only the transport of GPI-anchored proteins, and not that of other secretory proteins, was affected by the *lcb1-100* mutation. Our data are consistent with a working hypothesis in which two

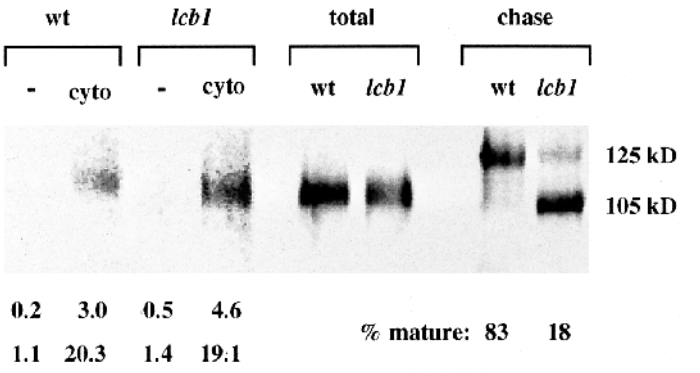

Fig. 10. Gas1p is packaged into transport vesicles from *lcb1-100* membranes. Budding assays were performed (see Materials and Methods) for 30 minutes at 20°C in the absence (–) or presence (cyto) of whole yeast cytosol from wild-type cells. Gas1p was immunoprecipitated from either vesicle containing supernatant fraction (first four lanes), or from one seventh of an unfractionated reaction (*total*, containing all membranes and vesicles). Values for the percentage of total released Gas1p or glycosylated pro-α-factor (gpαf) are indicated below the first four lanes; lanes are averages of values from at least four independent experiments. Gas1p was also immunoprecipitated from chased samples of the radiolabeled cultures (*chase*) and the percentage of Gas1p present in the 125 kDa mature form (assessed by PhosphorImager) is indicated below those lanes.

vesicle populations with specific fusion requirements mediate protein transport between ER and Golgi. It is likely that both vesicles carry a COP II coat because the transport of all secretory proteins that are translocated into the ER is blocked by mutations in COP II coat subunits (Schekman and Orci, 1996). Our suggestion of parallel pathways is not without precedent in the yeast secretory pathway. Parallel pathways have been proposed for Golgi to cell surface (Harsay and Bretscher, 1995) and Golgi to vacuole (Cowles et al., 1997) transport.

The formation of two vesicle populations that carry the same coat, but contain different cargo, can most simplistically be explained by the existence of membrane microdomains within the ER that are enriched in glycosphingolipids (mainly IPC and potentially ceramide as such). The GPI-anchor might allow GPI-anchored proteins to enter such domains, from which other secretory proteins are excluded. Thus, sphingolipid-enriched domains would mediate a protein sorting process simply by separating proteins on the basis of their intrinsic physical properties, and vesicle budding from the ER would produce two distinct COP II-coated vesicles with different content. Some vesicles would contain cargo and lipids that are derived from the glycosphingolipid-enriched membrane microdomains and other vesicles would contain the proteins excluded from such domains as well as phospholipids. Both vesicles would be targeted to the *cis*-Golgi with which they fuse. However, due to the different lipid and cargo composition of the vesicles, fusion requirements might be different for the two vesicle populations and a role for either vesicle specific proteins or lipids to mediate fusion is conceivable. This model could also help to explain the quantitative differences in the effects of the *lcb1-100* mutation on Gas1p and Yap3p transport. One could postulate that Gas1p would partition more efficiently into the sphingolipid-enriched domains than Yap3p and therefore be more dependent on ongoing ceramide synthesis.

Membrane microdomains as a mechanism for protein sorting have been proposed earlier. In the *trans*-Golgi of polarized mammalian cells, membrane subdomains have been described as detergent-insoluble structures which are enriched in glycosphingolipids, sphingomyelin, cholesterol and GPI-anchored proteins (Brown, 1992; Zurzolo et al., 1994). This protein-independent mechanism of protein sorting has been implicated in the segregation of proteins to be transported to the apical surface from basolateral proteins. Polarized sorting depends on ceramide synthesis. Upon its disruption by the inhibitor Fumonisin B1, proteins can exit from the *trans*-Golgi; however, they reach the cell surface in a non-polarized way (Futerman, 1995; Mays et al., 1995). Sphingolipids might therefore play a role in targeting vesicles to the correct surface, but might probably not mediate fusion.

We propose that sphingolipid-enriched microdomains are necessary for sorting and fusion of vesicles that transport GPI-anchored proteins to the Golgi and thereby imply two distinct functions for sphingolipids. Our in vitro data on vesicle budding, however, only suggest a sphingolipid requirement for vesicle fusion, since membranes derived from *lcb1-100* cells were not defective for Gas1p budding from the ER. IPC synthesis is likely to occur in the ER by the transfer of inositolphosphate derived from phosphatidylinositol as donor to ceramide (Becker and Lester, 1980). While IPC turnover in the ER appears to be slow, the level of ceramide itself is more strongly subjected to a block in synthesis because its overall level is low and it is rapidly converted into IPC (Schönbächler et al., 1995). Lipid microdomains enriched in IPC might therefore persist in *lcb1-100*, at least during the time course of our experiment, leading to protein sorting into distinct vesicle populations. A reduced level of ceramide might, however, result in defective fusion of the GPI-containing vesicles.

Previous studies provided a precedent for a role of ceramides in membrane fusion. Fusion of Semliki Forest Virus (SFV) with liposomes is strongly dependent on the lipid composition of the membrane (Nieva et al., 1994; Corver et al., 1995). Cholesterol must be present in the target membrane and is required for the virus to bind to the liposome. Virus entry (membrane fusion), however, requires the presence of ceramides in the target membrane. Ceramides activate membrane fusion at low concentration and in a stereospecific manner (Moesby et al., 1995). In particular, the 3-hydroxyl group and double bond in the sphingolipid backbone, which are also present in yeast sphingolipids, are required for fusion (Corver et al., 1995). The low concentration of sphingolipids needed suggests that they do not play a structural role in the target membranes. In a similar scenario, ceramide could mediate vesicle fusion in a catalytic rather than structural manner. We cannot, however, exclude a role for IPC in the fusion process.

A mutation in the α-subunit of coatomer also specifically blocks the transport of GPI-anchored proteins to the Golgi. Coatomer might be required to recycle a transport factor (GPI-

TF) to the ER that is required for sorting, budding or fusion of GPI-containing vesicles and that might be rapidly depleted from the ER. Such a protein could function as a receptor that recruits GPI-anchored proteins into vesicles. Shr3p has been shown to be specifically required to recruit amino acid permeases into vesicles (Ljundahl et al., 1992; Kuehn et al., 1996). Moreover, cargo receptors have been hypothesized in the ER which, when not retrieved, cause a delay in the transport of their cargo molecules (Gaynor and Emr, 1997). A GPI-transport factor could also be required to mediate fusion of GPI-containing vesicles with the Golgi, e.g. a specific v- or t-SNARE (Bennet, 1995). If such proteins are not recycled efficiently to the ER, one might expect a block of anterograde transport of GPI-anchored proteins but not of other secretory proteins. We have tested whether any of the described ER-to-Golgi v-SNAREs Bet1p, Bos1p and Sec22p specifically mediate transport of Gas1p or CPY. We found, however, that mutations in these v-SNAREs abolished Gas1p and CPY transport to the Golgi to about the same extent (data not shown). This finding suggests that the transport of GPI-anchored proteins depends on the normal transport machinery, but on GPI-specific factors in addition. Furthermore, in contrast to COP II mutants, most mutations in the COP I coat show only partial defects in anterograde transport of Gas1p (Fig. 2). Our model proposes a cotransport of GPI-anchored proteins with glycosphingolipids to the Golgi. Upon arrival in the Golgi, IPC is further modified to give MIPC, whose formation depends on vesicular transport (Puoti et al., 1991). While COP II mutants and *sec18* block MIPC and $M(IP)_2C$ synthesis, this study shows that mutations in the subunits of coatomer affect MIPC synthesis only after a 90-minute preincubation at the non-permissive temperature. This data is consistent with a role for coatomer in retrograde, but not forward, transport.

The inhibition of ceramide synthesis either by myriocin or the *lcb1-100* mutation blocks the exchange of GPI-diacylglycerol to a GPI-ceramide anchor. Although the GPI-anchor of Gas1p is not remodeled to a ceramide, we could not exclude that the lack of remodeling of other GPI-anchored proteins leads to an indirect block in Gas1p transport. At least the *ret1-1* mutant now allows us to uncouple remodeling from transport. Gas1p transport is blocked in this mutant while GPI-anchors are remodeled as efficiently as in wild-type cells. The data show that remodeling of the GPI-anchor is not sufficient for transport and that other specific requirements must exist.

It is of course conceivable that the in vitro system does not reflect the in vivo situation. While in vitro most likely only one round of budding is reproduced, ceramides might be required in vivo at the ER to mediate several rounds of transport thereby producing a ceramide requirement that we could not have detected in vitro. If this is the case, our data suggest that GPI-anchored proteins need, apart from anchor attachment and a functional COP II coat, at least a certain level of sphingolipids as well as a highly specific GPI-transport factor to enter into vesicles.

We have used several biochemical approaches to purify ER-derived vesicles generated in vivo following radiolabeled Gas1p as marker. Even in mutant strains in which vesicle accumulation has been described, like in *sec18*, we were unable to separate ER from ER-derived vesicles (Kaiser and Schekman, 1990). Also, no report on purification of in vivo-generated ER-derived vesicles can be found in the literature. The in vitro budding assay therefore appears to the most covenient system with which to study budding and post-budding requirements and we are currently setting up a system to directly address the ceramide requirement for vesicle fusion.

We are grateful to G. Waters, Y. Jigami, S. te Heesen and Y. Bourbonnais for providing antisera and to P. Cosson and F. Letourneur and strains and the plasmid pEG1-KK. Thanks go to the members of the Riezman laboratory. We appreciate helpful discussion with Randy Schekman and members of the Schekman laboratory. We thank Alan Munn, Mark Schönbächler, Johanna Wahlberg, Pascal Mäser and Rainer Duden for critical comments on the manuscript. Thanks also go to Cristina Baschong, Thomas Aust and Nicolas Stern for excellent technical assistance. This work was made possible by the Canton of Basel Stadt and the Swiss National Science Foundation (to H.R.). T.L.D. was supported by the Howard Hughes Medical Institute.

REFERENCES

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

Ash, J., Dominguez, M., Bergeron, J. J. M., Thomas, D. Y. and Bourbonnais, Y. (1995). The yeast proprotein convertase encoded by *YAP3* is a glycophosphatidylinositol-anchored protein that localizes to the plasma membrane. *J. Biol. Chem.* **270**, 20847-20854.

Baker, D., Hicke, L., Rexach, M., Schleyer, M., Schekman, R. (1988). Reconstitution of *SEC* gene product dependent intercompartmental protein transport. *Cell* **54**, 335-344.

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 drives vesicle budding from the endoplasmic reticulum. *Cell* **77**, 895-907.

Becker, G. W. and Lester, R. L. (1980). Biosynthesis of phosphoinositol-containing sphingolipids from phosphatidylinositol by a membrane preparation from *Saccharomyces cerevisiae*. *J. Bacteriol.* **142**, 747-754.

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

Bennet, M. K. (1995). SNAREs and the specificity of transport vesicle targeting. *Curr. Opin. Cell Biol.* **7**, 581-586.

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

Brown, D. A. (1992). Interactions between GPI-anchored proteins and membrane lipids. *Trends Cell Biol.* **2**, 338-343.

Buede, R., Rinker-Schaffer, C., Pinto, W. J., Lester, R. L. and Dickson, R. C. (1991). Cloning and characterization of *LCB1*, a *Saccharomyces* gene required for biosynthesis of the long-chain base component of sphingolipids. *J. Bacteriol.* **173**, 4325-4332.

Conzelmann, A., Riezman, H., Desponds, C. and Bron, C. (1988). A major 125-kd membrane glycoprotein of *Saccharomyces cerevisiae* is attached to the lipid bilayer through an inositol-containing phospholipid. *EMBO J.* **7**, 2233-2240.

Conzelmann, A., Puoti, A., Lester, R. L. and Desponds, C. (1992). Two different types of lipid moieties are present in glycosylphosphoinositol-anchored membrane proteins of *Saccharomyces cerevisiae*. *EMBO J.* **11**, 457-466.

Corver, J., Moesby, L., Erukulla, R. K., Ressy, K. C., Bittman, R. and Wilschut, J. (1995). Sphingolipid-dependent fusion of Semliki forest virus with cholesterol-containing liposomes requires the 3-hydroxyl group and the double bond of the sphingolipid backbone. *J. Virol.* **69**, 3220-3223.

Cowles, C. R., Snyder, W. B., Burd, C. G. and Emr, S. D. (1997). Novel Golgi to vacuole delivery pathway in yeast: identification of a sorting determinant and required transport component. *EMBO J.* **16**, 2769-2782.

Doering, T. L. and Schekman, R. (1996). GPI anchor attachment is required for Gas1p transport from the endoplasmic reticulum in COPII coated vesicles. *EMBO J.* **15**, 182-191.

Fankhauser, C., Homans, S. W., Thomas-Oates, J. E., McConville, M. J.,

Desponds, C., Conzelmann, A. and Ferguson, M. A. J. (1993). Structure of glycosylphosphatidylinositol membrane anchors from *Saccharomyces cerevisiae*. *J. Biol. Chem.* **268**, 26365-26374.

Futerman, A. H. (1995). Inhibition of sphingolipid synthesis: effects on glycosphingolipid-GPI-anchored protein microdomains. *Trends Cell Biol.* **5**, 377-380.

Gaynor, E. C., Te Heesen, S., 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.

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

Hamburger, D., Egerton, M. and Riezman, H. (1995). Yeast Gaa1p is required for attachment of a complete GPI anchor onto proteins. *J. Cell Biol.* **129**, 629-639.

Harsay, E. and Bretscher, A. (1995). Parallel secretory pathways to the cell surface in yeast. *J. Cell Biol.* **131**, 297-310.

Herscovics, A. and Orlean, P. (1993). Glycoprotein biosynthesis in yeast. *FASEB J.* **7**, 540-550.

Horvath, A., Sütterlin, C., Manning-Krieg, U., Movva, N. R. and Riezman, H. (1994). Ceramide synthesis enhances transport of GPI-anchored proteins to the Golgi apparatus in yeast. *EMBO J.* **13**, 3687-3695.

Kaiser, C. A. and Schekman, R. (1990). Distinct sets of SEC genes govern transport vesicle formation and fusion early in the secretory pathway. *Cell* **61**, 723-733.

Klis, F. M. (1994). Review: Cell wall assembly in yeast. *Yeast* **10**, 851-869.

Ktistakis, N. T., Brown, H. A., Waters, M. G., Sternweis, P. C. and Roth, M. G. (1996). Evidence that phospholipase D mediates ADP ribosylation factor-dependent formation of Golgi coated vesicles. *J. Cell Biol.* **134**, 295-306.

Kübler, E., Schimmöller, F. and Riezman, H. (1994). Calcium-independent calmodulin requirement for endocytosis in yeast. *EMBO J.* **13**, 5539-5546.

Kuehn, M. J., Schekman, R. and Ljungdahl, P. O. (1996). Amino acid permeases require COP II components and the ER resident protein Shr3p for packaging into transport vesicles in vitro. *J. Cell Biol.* **135**, 585-595.

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 dilysine-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.

Lisanti, M. P., Rodriguez-Boulan, E. and Saltiel, A. R. (1990). Emerging functional roles for the glycosyl phosphatidylinositol membrane protein anchor. *J. Membr. Biol.* **117**, 1-10.

Ljundahl, P. O., Gimeno, C. J., Styles, C. A. and Fink, G. R. (1992). SHR3: A novel component of the secretory pathway specifically required for localization of amino acid permeases. *Cell* **71**, 463-478.

Mallabiabarrena, A. and Malhotra, V. (1995). Vesicle biogenesis: The coat connection. *Cell* **83**, 667-669.

Maxwell, S. E., Ramalingam, S., Gerber, L. D., Brink, L. and Udenfriend, S. (1995). An active carbonyl formed during glycosylphosphatidylinositol addition to a protein is evidence of catalysis by a transamidase. *J. Biol. Chem.* **270**, 19576-19582.

Mays, R. W., Siemers, K. A., Fritz, B. A., Lowe, A. W., van Meer, G. and Nelson, W. J. (1995). Hierachy of mechanisms involved in generating Na/K-ATPase polarity in MDCK pithelial cells. *J. Cell Biol.* **130**, 1105-1115.

McConville, M. J. and Ferguson, M. A. J. (1993). The structure, biosynthesis and function of glycosylated phosphatidylinositols in the parasitic protozoa and higher eukaryotes. *Biochem. J.* **294**, 305-324.

Moesby, L., Corver, J., Erukulla, R. K., Bittman, R. and Wilschut, J. (1995). Sphingolipids activated membrane fusion of Semliki forest virus in a stereospecific manner. *Biochemistry* **34**, 10319-10324.

Munn, A. L. and Riezman, H. (1994). Endocytosis is required for the growth of vacuolar H^+-ATPase-defective yeast: identification of six new *END* genes. *J. Cell Biol.* **127**, 373-386.

Nieva, J. L., Bron, R., Corver, J. and Wischut, J. (1994). Membrane fusion of Semliki forest virus requires sphingolipids in the target membrane. *EMBO J.* **13**, 2797-2804.

Nuoffer, C., Horvath, A. and Riezman, H. (1993). Analysis of the sequence requirements for glycosylphosphatidylinositol anchoring of *Saccharomyces cerevisiae* Gas1 protein. *J. Biol. Chem.* **268**, 10558-10563.

Nuoffer, C., Jenö, P., Conzelmann, A. and Riezman, H. (1991). Determinants of glycophospholipid anchoring of the *Saccharomyces cerevisiae* Gas1 protein to the plasma membrane. *Mol. Cell. Biol.* **11**, 27-37.

Orci, L., Palmer, D. J., Amherdt, M. and Rothman, J. E. (1993). Coated vesicle assembly in the Golgi requires only coatomer and ARF proteins from the cytosol. *Nature* **364**, 732-734.

Pepperkok, R., Scheel, J., Horstmann, H., Hauri, H. P., Griffith, G. and Kreis, T. E. (1993). β-COP is essential for biosynthetic membrane transport from the endoplasmic reticulum to the Golgi complex in vivo. *Cell* **74**, 71-82.

Peter, F., Plutner, H., Zhu, H., Kreis, T. E. and Balch, W. E. (1993). β-COP is essential for transport of proteins from the endoplasmic reticulum to the Golgi in vitro. *J. Cell Biol.* **122**, 1155-1167.

Popolo, L., Vai, M., Gatti, E., Porello, S., Bonfante, P., Balestrini, R. and Alberghina, L. (1993). Physiological analysis of mutants indicates involvement of the *Saccharomyces cerevisiae* GPI-anchored protein gp115 in morphogenesis and cell separation. *J. Bacteriol.* **175**, 1879-1885.

Pryer, N. K., Wuestehube, L. J. and Schekman, R. (1992). Vesicle-Mediated Protein Sorting. *Annu. Rev. Biochem.* **61**, 471-516.

Puoti, A., Desponds, C. and Conzelmann, A. (1991). Biosynthesis of mannosylinositolphosphoceramide in *Saccharomyces cerevisiae* is dependent on genes controlling the flow of secretory vesicles from the endoplasmic reticulum to the Golgi. *J. Cell Biol.* **113**, 515-525.

Ram, A. J., Brekelmans, S. S. C., Oehlen, L. J. W. M. and Klis, F. M. (1995). Identification of two cell cycle regulated genes affecting the β-1, 3-glycan content of the cell walls in *Saccharomyces cerevisiae*. *FEBS Lett.* **358**, 165-170.

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

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

Schimmöller, F. and Riezman, H. (1993). Involvement of Ypt7p, a small GTPase, in traffic from late endosome to the vacuole in yeast. *J. Cell Sci.* **106**, 823-830.

Schimmöller, F., Singer-Krüger, B., Schröder, S., Krüger, U., Barlowe, C. and Riezman, H. (1995). The absence of Emp24p, a component of ER-derived COPII-coated vesicles, causes a defect in transport of selected proteins to the Golgi. *EMBO J.* **14**, 1329-1339.

Schönbächler, M., Horvath, A., Fassler, J. and Riezman, H. (1995). The yeast *spt14* gene is homologous to the human *PIG-A* and is required for GPI-anchor synthesis. *EMBO J.* **14**, 1637-1645.

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.

Zurzolo, C., van't Hof, W., van Meer, G. and Rodriguez-Boulan, E. (1994). VIP21/caveolin, glycosphingolipid cluster and the sorting of glycosylphosphatidylinositol-anchored proteins in epithelial cells. *EMBO J.* **13**, 42-53.

(Received 12 May 1997 - Accepted 4 September 1997)