Two GTPase isoforms, Ypt31p and Ypt32p, are essential for Golgi function in yeast

Mustafa Benli, Frank Döring1, David G. Robinson2, Xiaoping Yang and Dieter Gallwitz3

Department of Molecular Genetics, Max Planck Institute for Biophysical Chemistry, PO Box 2841, D-37018 Göttingen and 2Plant Physiology Institute, University of Göttingen, Untere Karspüle 2, D-37073 Göttingen, Germany
1Present address: Institute of Nutritional Sciences, Unit of Biochemistry, University of Giessen, D-35392 Giessen, Germany
3Corresponding author

In eukaryotic cells, monomeric GTPases of the Ypt/Rab family function as regulators at defined steps of vesicular transport in exo- and endocytosis. Here we report on the isolation and characterization of two genes (YPT31 and YPT32) of the yeast *Saccharomyces cerevisiae* which encode members of the Ypt family exhibiting >80% sequence identity. Whereas the disruption of one of the two genes was phenotypically neutral, the disruption of both YPT31 and YPT32 led to lethality. Depletion of wild-type Ypt31p or of a short-lived ubiquitin–Ypt31p in a ypt32 null background led to a massive accumulation of Golgi-like membranes, an inhibition of invertase secretion and defects in vacuolar protein maturation. Similar alterations were observed in a conditional-lethal ypt31-1 mutant at 30 min after shift to the non-permissive temperature. According to subcellular fractionation, a significant part of Ypt31p appeared to be located in Golgi-enriched membrane fractions. In accordance with this, indirect immunofluorescence using affinity-purified anti-Ypt31p antibodies gave a punctate staining similar to that observed with Golgi-located proteins. From the phenotypic alterations observed in ypt31 and ypt32 mutants, it seems likely that the two GTPases are involved in intra-Golgi transport or in the formation of transport vesicles at the most distal Golgi compartment.

Keywords: Golgi/Rab GTPase/secretion/yeast/Ypt31,32 proteins

Introduction

Protein transport and membrane flow in eukaryotic cells involve complex mechanisms whose many intricacies are now being studied intensively using genetic and biochemical approaches. According to present knowledge, vesicular protein transport between different membrane-enclosed compartments regularly involves proteins common to several secretory and endocytotic transport steps as well as proteins with compartmental specificity. The latter group includes receptor molecules on transport vesicles and their respective target membranes (for reviews, see Bennet and Scheller, 1994; Rothman, 1994) and small GTPases of the Ypt/Rab family (for reviews, see Novick and Brennwald, 1993; Zerial and Stenmark, 1993; Nuoffer and Balch, 1994; Grabowski et al., 1995). Many of the components involved in the docking and fusion of vesicular intermediates to their specific target membranes are highly conserved from yeast to man. As yeast is easily accessible for genetic studies, significant progress in the understanding of the components and the functioning of vesicular transport can be ascribed to analyses of this unicellular organism.

The existence of ras-like GTPases, Ypt1p and Sec4p (Gallwitz et al., 1983; Salminen and Novick, 1987), and their essential role at defined steps of the secretory pathway (Goud et al., 1988; Segev et al., 1988; Schmitt et al., 1988) was, for example, first realized in yeast. The accumulation of vesicular intermediates originating from either the endoplasmic reticulum (ER) or the most distal Golgi compartment in ypt1 and sec4 mutant cells (Goud et al., 1988; Becker et al., 1991), argues for a defect of carrier vesicle docking to their specific target compartments, respectively the cis-Golgi in Ypt1p- and the plasma membrane in Sec4p-deficient cells. Defects in the consumption of vesicular intermediates could also be shown in an ER–Golgi in *vitro* transport system depleted of Ypt1p (Rexach and Schekman, 1991; Segev, 1991). The presently favoured hypothesis is that Ypt/Rab GTPases are regulators participating in the formation of the multicomponent docking/fusion complexes which operate in diverse vesicular transport steps in exocytosis (Rothman, 1994; Søgaard et al., 1994).

Small GTPases were also discovered recently to operate in the less well characterized endocytotic pathway of yeast. Ypt7p (Wichmann et al., 1992) and three isoforms of Ypt5p, termed Ypt51/Vps21p, Ypt52p and Ypt53p (Horazdovsky et al., 1994; Singer-Krüger et al., 1994), are, in contrast to Ypt1p and Sec4p, not essential for cell viability. Whereas the three isoforms of Ypt5p appear to act at an early step of the endocytotic pathway, presumably between early and late endosomal compartments (Singer-Krüger et al., 1995), Ypt7p has been shown to be involved in transport between late endosomes and the vacuole (Schimmöller and Riezman, 1993) and in homotypic vacuole–vacuole fusion (Haas et al., 1995).

Although it has been suggested that Ypt1p, in addition to its function in ER–Golgi transport, might also act in intra-Golgi membrane traffic (Bacon et al., 1989; Jedd et al., 1995), evidence for a role for Ypt GTPases in protein transport between different Golgi compartments has not been obtained as yet. By contrast, this seems to be the case for the mammalian Ypt homologue, Rab1p (Plutner et al., 1991; Nuoffer et al., 1994). Thus, convincing evidence for a dual function of Ypt1p in yeast is so
far lacking. Here we report on the characterization of two isoforms of a *Saccharomyces cerevisiae* GTP binding protein, designated Ypt31p and Ypt32p, that are prime candidates for GTPases engaged in intra-Golgi protein transport and/or in the budding of vesicular carriers from a late Golgi compartment.

**Results**

**Isolation and characterization of YPT31 and YPT32 genes**

A 4 kb *HindIII* fragment and two *EcoRI* fragments of ~6 and 2.4 kb from total *S.cerevisiae* DNA digests were found to be labelled in Southern blots with 32P-labelled ypt3 cDNA from *Schizosaccharomyces pombe* under moderately stringent hybridization conditions. After mapping the cloned DNA fragments with different restriction endonucleases, a 1265 bp *HindIII*-PstI fragment derived from the 4 kb *HindIII* fragment was shown by sequence analysis to contain an open reading frame for a 223 amino acid long protein having a high degree of sequence identity with the fission yeast GTPase Ypt3p and its mammalian homologue Rab11 (Figures 1 and 2). The gene was designated *YPT31* in order to distinguish it from a second *S.cerevisiae* gene encoding a homologue of the *S.pombe* ypt3 gene which, in a parallel approach, was identified by the polymerase chain reaction (PCR) using total yeast DNA and two degenerate DNA primers. These corresponded to the amino acid sequences EYD-YLFK found in the fission yeast Ypt3p (Miyake and Yamamoto, 1990) and the mammalian Rab11 protein (Chavrier et al., 1990) and to QIWDTAGQ, typically found in Ypt/Rab proteins (Valencia et al., 1991). One of the cloned PCR fragments, 150 bp in length, encoded part of a small GTPase with an effector region identical to that of the *S.pombe* Ypt3p, the mammalian Rab11 protein and the *S.cerevisiae* Ypt31 protein identified in the genomic screen described above. The cloned PCR fragment was then used to isolate the corresponding gene, which was identified on a genomic 1.6 kb *EcoRV* fragment, cloned, sequenced and designated *YPT32*.

In contrast to the fission yeast *ypt3* gene, the baker’s yeast *YPT31* and *YPT32* genes do not contain intervening sequences. Sequence analysis of the 6 kb *EcoRI* fragment, which harbours the 5’ part of the *YPT31* gene (Figure 1), showed that the gene encoding phosphatidylinerine synthase, mapped on chromosome V (Kiyono et al., 1987), is located 5’ to *YPT31*. Using a commercially available chromoblot (Clontech), the *YPT32* gene was mapped to chromosome VII (data not shown), an assignment verified by sequencing this chromosome (H.Tettelin et al., in preparation). During the course of our investigations, *YPT31* was accidently cloned and named *YPT8* (Lai et al.; 1994), and has been identified on chromosome V thanks to the genome sequencing project (F.S.Dietrich et al., in preparation).

The protein sequences deduced from the DNA sequences of the two genes, shown in Figure 2, revealed that the two isoforms of Ypt3p have an overall identity of 83%. Ypt31p and Ypt32p share >60% identical sequence with the *S.pombe* Ypt3p and the canine Rab11 protein (Figure 2). All four proteins share an identical effector region sequence (SKSTIGVEF) which appears to be diagnostic for functionally equivalent Ypt/Rab GTPases (Kibbe et al., 1993). Whereas the yeast Ypt3p proteins have two consecutive cysteine residues at their C-terminal ends, the mammalian Rab11 protein terminates with two cysteines followed by three other amino acid residues.
Single disruptions of YPT31 or YPT32 are phenotypically neutral, a double disruption is lethal

Gene disruption experiments were performed in order to investigate the importance of the Ypt3 proteins for cell viability. A region of YPT31 extending from 19 nucleotides upstream of the translation initiation codon to codon 118 was deleted (TII111II fragment, Figure 1) and replaced by a DNA fragment harbouring the yeast URA3 gene as a selectable marker. The YPT32 gene was disrupted by inserting a DNA fragment containing the yeast HIS3 gene into the BglII site at codon 130 (Figure 1). The disrupted genes on linear fragments were used to replace one chromosomes copy of the respective wild-type gene in either a Ura− or a His− diploid strain. Following sporulation and tetrad dissection, ypt3I− as well as ypt32− haploids were found to form colonies of similar size to wild-type haploids. Likewise, the inactivation of either YPT31 or YPT32 in a haploid strain did not result in any easily scorable phenotype (growth at different temperatures, cellular morphology, protein secretion, maturation of vacuolar enzymes). We conclude, therefore, that neither YPT31 nor YPT32 by itself is an essential gene.

As Ypt31p and Ypt32p are obvious isoforms of one GTPase, we sought to investigate ypt31/ypt32 double disruption mutants. A diploid strain heterozygous for both ypt3I− and ypt32− was sporulated and tetrads were analysed. The pattern of spore viability observed was consistent with the presence of two unlinked mutations that together lead to lethality. Single ypt31 and ypt32 disruptants were recovered with the expected frequency, but no his3/ura3− double mutants were found, strongly suggesting that the simultaneous inactivation of the two Ypt3p-encoding genes is incompatible with normal cell growth.

To gain an insight into the possible function of Ypt31p and Ypt32p, a haploid ypt32− strain carrying the YPT31 gene under transcriptional control of the regulatable GAL10 promoter was constructed and shown to grow on galactose- but not on glucose-containing media. Depletion of Ypt31p at ~12–14 h after a shift of mutant cells to glucose-containing medium resulted in a cessation of cell growth. This could be seen by comparing the optical density and the cell number of the mutant cell culture grown in galactose and glucose (Figure 3A). Beginning roughly at ~20 h after the GAL10 promoter switch off, the number of viable cells decreased slightly and was reduced to 75% after 30 h. The slow cessation of cell growth was most likely due to the high expression of the GAL10 promoter-controlled YPT31 gene prior to its repression (Figure 3B), providing enough protein for several rounds of cell division. Furthermore, cells lacking Ypt31p and Ypt32p became somewhat larger than Ypt31p-producing cells, explaining the slight increase in the optical density at ~14 h after the medium shift without a concomitant change in cell number (Figure 3A).

Since at least 14 h were needed to deplete cells of Ypt31p and to block cell proliferation after repression of GAL10–YPT31 promoter fusion, we tried to circumvent this problem by generating conditional ypt31 mutants. For this purpose, a previously published method was followed which is based on the formation of highly unstable ubiquitin fusion proteins allowing a much faster depletion of the protein under study after promoter switch off (Park et al., 1992). As shown in Figure 4, a genetic element encoding ubiquitin, followed by a lac repressor segment and a haemagglutinin epitope tag, was fused to the YPT31 gene in such a way that the foreign protein sequences were joined to the N-terminus of Ypt31p. In addition, two versions of the ubiquitin cassette allowed the production of Ypt31 proteins with short N-terminal extensions starting with either methionine, a protein-stabilizing amino acid, or with arginine, a severely destabilizing amino acid residue (Park et al., 1992). In the following, these proteins are designated respectively M-Ypt31p and R-Ypt31p.

The fusion genes under transcriptional control of the GAL10 promoter were chromosomally integrated such as to replace the YPT31 gene in a ypt32− haploid strain. On galactose-containing media, strains expressing either of the two chimeric proteins grew perfectly well, showing that the N-terminal extension of 64 amino acids did not interfere severely with the function of the mutant GTPases. The kinetics of cell growth arrest after blocking M-Ypt31p
production compared well with that observed after inhibition of Ypt31 wild-type protein synthesis (Figure 3A, and data not shown). In contrast, shut-down of R-Ypt31p production resulted in a significantly faster cessation of cell proliferation (~4 h after medium shift) accompanied by the disappearance of Ypt31p (Figure 4B and C). Identical results were obtained in a haploid ypt31Δ strain expressing Ypt32 fusion proteins, demonstrating again that Ypt31p and Ypt32p fulfill identical or overlapping functions.

Depletion of Ypt3 proteins results in vacuole fragmentation and the accumulation of Golgi-like structures

Light microscopic inspection of ypt32/GAL10-R-YPT31 cells grown in galactose (conditions which lead to an overproduction of Ypt31p, see Figure 3B) revealed a lack of typical wild-type yeast vacuoles. Examination of these cells with the vacuole-specific vital stain CDCFDA [5(6)-carboxy-2',7'-dichlorofluorescein diacetate] (Pringle et al., 1989) showed instead numerous small vacuolar compartments (data not shown). Following a shift to glucose-containing medium, typical large vacuoles reappeared after 4-6 h when the cellular level of Ypt31p began to approach that of wild-type cells (Figure 3B). After depletion of Ypt31p, at the 14 h time point, vacuoles once again appeared to be highly fragmented. Due to the faster depletion of Ypt31p in ypt32/GAL10-R-YPT32 cells, vacuolar fragmentation was already visible 3-4 h after the shift to glucose-containing media. These results suggest that overproduction as well as the depletion of Ypt3 proteins disturbs vacuolar morphology.

In order to investigate the morphological alterations in Ypt31p-depleted cells in more detail, we have performed electron microscopy of ypt32/GAL10-R-YPT31 haploids. Cells grown in galactose- and shifted to glucose-containing medium for different periods of time were either subjected directly to potassium permanganate fixation or spheroplasted and then fixed with glutaraldehyde/osmium tetroxide. Both methods are recommended as being particularly useful for visualizing membranous structures in yeast (Stevens and White, 1979; McDonald, 1984). No significant abnormalities in intracellular structures were noticed in ypt32 null mutant cells expressing Ypt31 wild-type protein at normal levels (Figure 5A). High expression of Ypt31p from the GAL10 promoter-controlled gene resulted in the disappearance of large vacuoles normally seen in wild-type cells and the simultaneous appearance of many smaller and spherical compartments filled with darkly stained material (Figure 5B and M). This is in agreement with the results obtained by CDCFDA staining, and argues strongly for a fragmentation of vacuoles in such cells. However, fragmentation of vacuoles in haploid cells overexpressing the fusion proteins M-Ypt31p or R-Ypt31p was not observed (data not shown). This might reflect a suboptimal functionality of the N-terminally extended GTPases.

More importantly, numerous cisternae which formed stacks and discontinuous rings accumulated (Figure 5C, E and G) at those times after GAL10 promoter switch off when the Ypt31 GTPase was well below the wild-type level but just detectable with the antibody used (12 h for wild-type Ypt31p and 2 h for R-Ypt31p, Figures 3B and 4C, respectively). Freeze-fracture micrographs (Figure 5M and N) show that the discontinuous nature of these ring-like structures reflects the high degree of fenestration in a cup-shaped cisterna. These cisternae often could be seen to originate from the stacks, thereby resembling the Golgi stacks which are present in higher organisms. These structures were also seen in permanganate-fixed cells. Stacks with up to five cisternae, which are not or only
rarely seen in wild-type cells (Preuss et al., 1992), were found regularly in the mutant cells fixed with permanganate (Figure 51-L). Some membrane structures resembled the cup-shaped ‘Berkeley bodies’, known to represent abnormal Golgi membranes (Novick et al., 1980).

At times when Ypt31p (or R-Ypt31p) was no longer

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**Fig. 5.** Morphological analysis of Ypt31p-depleted cells. Δypt32/YPT31 cells grown in YEPgal (A) and Δypt32/GAL10-YPT31 cells grown in either YEPgal (B) or in YEPD for 12 (C and I) or 16 h (D) as well as Δypt32/GAL10-R-YPT31 cells, shifted from galactose to glucose for either 2 (G and M) or 4 h (H). Cells were spheroplasted prior to osmium tetroxide-ferrocyanide fixation (A–H) or were fixed directly with potassium permanganate (I–L), or were freeze-fractured (M and N) and viewed by transmission electron microscopy. Small arrows point to Golgi-like structures (C, G and I) which are also shown at higher magnification (E, J, K, L and N). Pleomorphic vacuolar structures (D and F) are highlighted by thick arrows. The arrowhead in (H) points to dilated ER, asterisks in (D) and (H) show structures resembling ‘Berkeley bodies’. N, nucleus; V, vacuole. The bar in the bottom panel represents 0.5 μm.
detectable immunologically, the presumptive Golgi stacks were greatly diminished, and pleomorphic, sometimes club-shaped structures predominated (Figure 5D, F and H). These structures appeared to be of vacuolar origin as several of them contained particulate material and in some cases were found to extend from small vacuoles. An accumulation of ER membranes, as judged by their connection to the nuclear envelope, was also observed (Figure 5H).

It seems, therefore, that in yeast cells lacking Ypt3 GTases, alterations of membrane structures are variable, although a massive accumulation of Golgi-like cisternae is one of the earliest observed phenotypes.

*Generation and accumulation of Golgi-like membrane stacks is a very fast response of ypt31-1 mutant cells at non-permissive temperature*

To be able to distinguish between early and late occurring alterations following the loss of Ypt31p function, we created a conditional-lethal ypt31 mutant by inserting hydroxyamine-treated YPT31 gene sequences into the genome by homologous recombination. A temperature-sensitive mutant with a single amino acid substitution (K127N) in Ypt31p was identified. The amino acid substitution, which proved to be responsible for the mutant phenotype, is within the highly conserved NKXD sequence known to be important for guanine nucleotide binding (Schmitt et al., 1986; Valenza et al., 1991).

After transfer of the ypt31-1 mutant cells to 37°C, cell proliferation ceased within 1 h. At 37°C, the cellular level of the mutant Ypt31(K127N) protein appeared to be somewhat higher than that of Ypt31p in Δypt32/YPT31 cells and did not change significantly during the first 2 h after the temperature shift (data not shown). The ypt31-1 mutant cells under restrictive conditions exhibited phenotypic alterations comparable with those observed in Ypt31p/Ypt32p-depleted cells. Most importantly, already 30 min following the shift to the non-permissive temperature, stacked, Golgi-like cisternae and other membrane-enclosed structures resembling the so-called 'Berkeley bodies' developed whereas the vacuoles appeared perfectly normal (Figure 6). Morphological changes of vacuoles were not seen before 2 h after the shift of mutant cells to the restrictive conditions, demonstrating that this is a secondary lesion. It is also noteworthy that in these experiments, the accumulation of membrane-enclosed organelles resembling Golgi structures was observed in cells subjected to freeze substitution.

*Ypt3 protein-depleted cells are defective in intracellular protein transport*

The severe morphological alterations observed in Ypt3p-deficient cells point to a function of these GTases in intracellular membrane transport, as has been well documented for other members of the Ypt protein family (Brennwald and Novick, 1993; Strom and Gallwitz, 1993). We therefore studied the fate of proteins which pass through the compartments of the secretory pathway on their way to either the plasma membrane (invertase) or the vacuole (soluble and membrane-bound hydrolases). These proteins are modified by glycosylation and proteolytic processing as they move from the ER through different Golgi cisternae and from the Golgi to their final destination. Based on their changing electrophoretic mobility, they can be followed conveniently in pulse-chase experiments.

Most of the studies were performed on ypt32/GAL10-R-YPT31 mutant cells because of the fast depletion of Ypt31p after GAL10 promoter switch off (see above). As shown in Figure 7, an ER and a Golgi precursor form (p1 and p2, respectively) of the vacuolar proteinases carboxypeptidase Y (CPY) and proteinase A (PrA) can be distinguished after a short pulse with radioactive amino acids, and these, as well as the precursor of the membrane-integrated alkaline phosphatase (ALP), can be chased completely to the mature form of the enzymes within <20 min at 30°C. The enzyme maturation kinetics in ypt32/YPT31 cells grown in glucose and in ypt32/GAL10-R-YPT31 cells immediately after a shift from galactose- to glucose-containing medium were virtually indistinguishable. The maturation of all three hydrolases was clearly delayed (Figure 7A) 2 h after the start of Ypt31p depletion when the GTase was still detectable but at a significantly reduced level compared with ypt32 null mutant cells (Figure 4C). This was even more pronounced at the 3 h time point and, at 4 h after initiating the R-YPT31 transcription block, precursors with an electrophoretic mobility of the ER forms of the enzymes were detected nearly exclusively at 40 min chase times (Figure 7A). A smear on top of the p1 forms of CPY and PrA also became apparent at this time point. This is more clearly seen for CPY in Figure 7B. We then decided to determine whether the accumulated precursor forms of vacuolar hydrolases had received outer chain α1-6 and α1-3 mannosyl residues which are diagnostic of early and late Golgi glycosylation (Frazuzzo and Schekman, 1989; Graham et al., 1994), respectively. Three hours after initiating the block of GAL10-R-YPT31 gene transcription, newly synthesized CPY was first precipitated with anti-CPY-specific antibodies and then treated with either anti-α1-6 or anti-α1-3 mannose antibodies. As shown in Figure 7B, α1-6 and α1-3 mannosylation could be clearly demonstrated for the p2 Golgi precursor and the mature form but not for the p1 form itself, suggesting that at least a fraction of the soluble vacuolar hydrolase became correctly processed in cells lacking Ypt3 GTases. Missorting and secretion of CPY, typical for vacuolar protein sorting (vps) yeast mutants, was not observed in Ypt31/Ypt32-depleted cells.

In a second set of experiments, the secretion of invertase was followed in Ypt3p-depleted cells. At various times after a shift of ypt32/GAL10-R-YPT31 cells to glucose-containing medium, invertase synthesis was induced, and the secreted (periplasmic) and intracellular (spheroplast-containing) enzyme levels were examined by activity staining in non-denaturing polyacrylamide gels. This experiment is somewhat complicated by the fact that large amounts of invertase were already found in the periplasmic space in ypt32/GAL10-R-YPT31 cells grown in galactose prior the induction of the enzyme in low glucose medium (0 h time point in Figure 8A). Therefore, only a qualitative assessment of intracellularly accumulated invertase and its state of glycosylation was possible, although the efficiency of invertase secretion could not be assessed. From Figure 8A it is evident that highly glycosylated
invertase, seen in gels as a broad smear, already accumulated at ~1 h after initiating Ypt31p depletion. At later time points, an increasing amount of the intracellularly accumulated secreted form of invertase appeared to be underglycosylated. Small amounts of the ER form of invertase were also found to accumulate in the course of Ypt31p depletion.

The secretion of invertase was also examined in ypt32/GAL10-YPT31 cells. Since it takes >10 h of growth in glucose-containing medium to deplete the cells of wild-type Ypt31p, periplasmically located invertase generated during cellular growth in galactose was less abundant than in the mutant cells described above at times when the efficiency of secretion was measured. At 10, 12 and 14 h after blocking GAL10-YPT31 transcription, cells were induced for invertase synthesis, the enzyme activity being measured before and after spheroplast formation, and the amount of intracellular (periplasmic) invertase was calculated. As can be seen in Figure 8B, the ratio of intracellular to periplasmic invertase increased with the progression of Ypt31p depletion, with the majority of the enzyme accumulating inside the cells at times when Ypt31p was no longer detectable on Western blots (see Figure 3B). This shows that the depletion of Ypt3 GTPases results in a severe inhibition of protein secretion.

In a further set of experiments, invertase secretion was studied during the heat inactivation of the mutant Ypt31 GTPase in ypt31-1 cells. Pulse–chase experiments were performed under permissive (25°C) and restrictive conditions (37°C) as shown in Figure 9. Cells labelled between 30 and 60 min following the shift to the non-permissive temperature were highly defective in invertase secretion and accumulated both highly glycosylated and underglycosylated forms of the enzyme.

**A significant fraction of membrane-bound Ypt31p appears to be Golgi associated**

The cellular localization of regulatory proteins can provide valuable hints regarding their functional roles. We therefore attempted to localize Ypt31p by cell fractionation. A
polyclonal antibody specific for Ypt31p, that did not even cross-react with the closely related Ypt32p, was raised in rabbits and used to identify the GTPase in cellular fractions enriched for either ER or Golgi membranes.

Spheroplasts were prepared and carefully lysed by Dounce homogenization. We found that most of the GTPase was bound to membranes (Figure 10A) and could be solubilized with detergents but not with high salt or at alkaline pH (data not shown). A crude fractionation of cellular membranes and organelles involving consecutive sedimentations at 500, 13 000 (P13) and 100 000 g (P100) revealed that the bulk of the GTPase sedimented in fractions P13 and P100. The distribution between these fractions of several marker proteins for different cell organelles was determined by Western blot analysis (Figure 10A) and sucrose gradient centrifugation (Figure 10B–D). Vacuolar membrane proteins (the 100 kDa subunit of the vacuolar ATPase and ALP) as well as the ER marker Sec61p were clearly enriched in the P13 fraction. The Kex2 protease, a late Golgi compartment marker (Redding et al., 1991), and, less pronounced, the cis-Golgi-associated transport vesicle receptor Sed5p (Hardwick and Pelham, 1992) were enriched in the P100 fraction which is known to contain the majority of Golgi membranes (Baker et al., 1988).

To separate Golgi from ER and vacuole compartments more effectively, the cell lysate, after two spins at 500 g, was subjected to velocity centrifugation through sucrose
gradients (Antebi and Fink, 1992; Schröder et al., 1995). As shown in Figure 10B–D, the bulk Ypt31p migrated to a position close to the Golgi markers Kex2p and Sed5p. A fraction of the GTPase, apparently the soluble cytoplasmic form (see also Figure 10A), appeared at the top of the gradient. Most importantly, Ypt31p did not co-migrate with ER and vacuolar compartments which were identified with antibodies directed against the soluble ER-resident Kar2p (Rose et al., 1989) and ALP, respectively. This could best be seen after densitometric scanning of the Western blots (Figure 10C and D). As on nearly identical sucrose gradients, the plasma membrane has been shown to co-migrate with the ER fraction (Antebi and Fink, 1992), it seems likely to us that no appreciable amount of Ypt31p is associated with the plasma membrane.

In addition to the subcellular fractionation, we sought to investigate the cellular localization of Ypt31p by indirect immunofluorescence. Using affinity-purified anti-Ypt31p antibodies, a punctate staining was observed (Figure 11). This is reminiscent of what has been seen previously with antibodies directed against Golgi- and transport vesicle-associated proteins, like Ypt1p (Segev et al., 1988; Ossig et al., 1995), Kex2p (Franzusoff et al., 1991) or Sed5p (Hardwick and Pelham, 1992). As shown in Figure 11, a control experiment with affinity-purified antibodies directed against the Sed5 protein, which is assumed to localize primarily to the cis-Golgi compartment, resulted in a picture similar to that obtained with anti-Ypt31p antibodies. In both cases, neither a perinuclear staining typical for ER localization nor a staining of the vacuole was evident.

From the combined results of subcellular fractionation and indirect immunofluorescence, we assume that at least part of Ypt31p is bound to Golgi membranes.

Discussion

We have isolated a pair of functionally redundant small GTPases from the budding yeast S. cerevisiae which are members of the Ypt family. According to the currently

Fig. 9. Inhibition of invertase secretion in Δypt32/ypt31-1 mutant cells at the non-permissive temperature. Δypt32/ypt31 and Δypt32/ypt31-1 and sec18-1 cells were grown at 25°C. Portions of the cultures were transferred to low glucose (0.1%) medium and grown at either 25 or 37°C for 30 min. Proteins were labelled with Tran 35S-label for 30 min and, after a chase with cold methionine and cysteine, invertase was immunoprecipitated from the periplasmic (E) and intracellular (I) compartments. S, highly glycosylated, secreted form; ER, core-glycosylated ER form of invertase.

Fig. 10. Subcellular fractionation to localize Ypt31p. Spheroplasts of a wild-type strain were lysed gently and, after removing unbroken cells and debris at 500 g, the lysate was either fractionated by differential (A) or sucrose gradient centrifugation (B). After SDS-PAGE of fraction aliquots, proteins were transferred to nitrocellulose filters, and Ypt31p and different organelle marker proteins were identified with specific antibodies using the ECL system. Densitometric scannings of the blot shown in (B) are presented in (C) and (D). The direction of gradient centrifugation is from left to right.
used nomenclature, we have termed these proteins Ypt31p and Ypt32p. Their primary sequences are identical by >80%. Their closest relatives are the Ypt3p from the fission yeast S.pombe (Miyake and Yamamoto, 1990) and the Rab11 proteins from mammals (Chavrier et al., 1990), with which they share identical effector and α2 helix domain sequences. These are regions in Ypt/Rab GTPases which contribute significantly to the functional specificity of different family members (Haubruck et al., 1989, 1990; Hengst et al., 1990; Brennwald and Novick, 1993; Dunn et al., 1993; Stenmark et al., 1994). The Ypt31p/Ypt32p pair is essential for yeast cell viability, and each of the two GTPases allows undisturbed cell growth and proliferation in the absence of the other. It appears, therefore, that the two proteins fulfill identical functions. Interestingly, the fission yeast has only one Ypt3p-encoding gene, the disruption of which also results in lethality. Functional studies of the S.pombe ypt3 gene, however, have not yet been performed.

The functional role of GTPases in the secretory or the endocytic pathway of yeast has been determined by studying mutants defective in various YPT genes. Ypt1p- and Sec4p-defective S.cerevisiae cells accumulate ER–Golgi and Golgi–plasma membrane transport vesicles, respectively (Goud et al., 1988; Becker et al., 1991), suggesting an involvement of these GTPases in an as yet undefined, molecular mechanism in carrier vesicle docking to and/or fusion with the respective target membrane (Brennwald and Novick, 1993; Zerial and Stenmark, 1993; Grabowski et al., 1995). In contrast to the vital functions of Ypt1p and Sec4p in the secretory pathway, small GTPases involved in endocytotic membrane traffic, Ypt7p (Wichmann et al., 1992) and Ypt51p/Ypt52p/Ypt53p (Horazdovsky et al., 1994; Singer-Krüger et al., 1994), are not indispensable for yeast cell viability. Assuming that Ypt GTPases are essential for secretion, one would predict that the Ypt31p/Ypt32p isoforms are regulators of some step in the biosynthetic transport route. The data which we have obtained and describe in this report support this notion. Ypt31p/Ypt32p-depleted cells are unable to secrete invertase, they accumulate Golgi and ER forms of vacuolar hydrolases and they build up numerous stacks of membrane-enclosed compartments which resemble stacked Golgi cisternae found in mammalian and higher plant cells.

Wild-type S.cerevisiae cells, in contrast to higher eukaryotes and to the evolutionarily distantly related yeast S.pombe, do not organize their Golgi apparatus into stacks but contain instead single curved, disc-like structures (Preuss et al., 1992). The exaggerated stacked cisternae seen in permanganate-fixed cells, their sacculotubular appearance in osmium tetroxide/ferrocyanide-fixed spheroplasts and the morphologically identifiable fenestrated cisternae in freeze fractures of Ypt31p/Ypt32p-depleted cells, all argue for Golgi membranes that accumulate in the absence of Ypt3 GTPases. In fact, the stacked, membrane-enclosed, cisternae are almost indistinguishable from those observed in S.cerevisiae sec7 mutants (Novick et al., 1981; Svoboda and Něcaš, 1987; Rambourg et al., 1993) known to be defective in intra-Golgi protein transport (Franzusoff and Schekman, 1989). What is now needed to demonstrate unequivocally that the accumulated organelles in Ypt31p/Ypt32p-deficient cells are indeed exaggerated Golgi membranes is the identification of Golgi-resident proteins in these structures by immunoelectron microscopy.

Nevertheless, Ypt31p/Ypt32p-depleted cells exhibit similar protein transport defects to the two known S.cerevisiae Golgi mutants, sec7 and sec14: (i) intracellular accumulation and secretion block of glycosylated invertase, and (ii) accumulation of ER and partially and fully glycosylated Golgi forms of the vacuolar hydrolase CPY (Stevens et al., 1982; Franzusoff and Schekman, 1989) which traverses the secretory pathway and is sorted for delivery to the vacuole in a late Golgi compartment. In the present study, we observed that the inhibition of transport and maturation of several soluble and membrane-bound vacuolar hydrolases paralleled the disappearance of the Ypt31 GTPase in ypt32 null mutant cells (or the

![Image of Ypt31p and Sed5p](https://example.com/fig11.png)

**Fig. 11.** Indirect immunofluorescence to localize Ypt31p and the cis-Golgi marker Sed5p. Cells of a wild-type strain were fixed with formaldehyde, spheroplasted and then treated with affinity-purified polyclonal antibodies directed against Ypt31p or Sed5p (left). DIC images of the cells are shown on the right.
depletion of Ypt32p in ypt31 disruptants). That this was a direct consequence of the functional loss of Ypt31p/Ypt32p activity follows from the rapid appearance of transport defects for all proteins travelling along the secretory pathway. Invertase secretion, PrA, CPY and ALP glycosylation and maturation were already severely inhibited during the early phase of the disappearance of the short-lived ubiquitin–Ypt31 fusion protein, i.e. at 1–2 h after initiating the transcription block of the fusion protein-encoding gene. Vacular hydrolases accumulated predominantly as ER forms at the final stage of Ypt31 protein loss, similar to what has been seen in sec7 Golgi mutants at the non-permissive temperature (Franzusoff and Schekman, 1989). By contrast, but similarly to the sec14 Golgi mutants under non-permissive conditions, invertase accumulated intracellularly as heterogeneously and highly glycosylated species, although some ER-glycosylated forms did appear also. In the course of Ypt31p depletion, none of the CPY species with an electrophoretic mobility of the p1 ER form possessed α1-6 or α1-3 mannose residues, suggesting that they did not reach the Golgi compartment or, because of their low degree of mannosylation, were not precipitated efficiently by the antiserum used.

At a later stage of Ypt31p/Ypt32p depletion, the accumulation of predominantly ER-glycosylated vacular hydrolases appeared to coincide with an enrichment of dilated ER compartments. This, we believe, is a secondary effect of a protein transport defect between and from the Golgi cisternae which leads to an accumulation of membrane-enclosed compartments proximal to the Golgi. This argument follows from the finding that the formation of exaggerated Golgi-like cisternae clearly preceded the less prominent accumulation of ER membranes. Comparable ER membrane accumulation has been observed in yeast mutants that are defective in a late step of ER–Golgi vesicle traffic (Kaiser and Schekman, 1990), suggesting that the retention of donor membranes occurs under conditions where transport vesicle docking or fusion to the cis-Golgi compartment is blocked. Another striking morphological alteration of mutants lacking Ypt31 and Ypt32 proteins, the fragmentation of vacleules, also appears to be a secondary effect. Vacular aberrations became apparent after the Golgi-like structures had developed, and this was seen in a ypt32 null mutant in the course of either the slow depletion of wild-type Ypt31p or the rather fast disappearance of the ubiquitin–Ypt31 fusion protein. More importantly, in the temperature-sensitive ypt31-1 mutant, Golgi-like cisternal stacks accumulated without a visible change in vacuole morphology already within 30 min after shifting the mutant cells to the non-permissive temperature. Therefore, it might be that in the complete absence of Ypt33 GTAPases, some component(s) required for vacuole–vacuole fusion or vacuole membrane biogenesis become limiting, while under wild-type conditions they are delivered to this organelle in a Ypt31p/Ypt32p-dependent manner. The yeast vacuole is a dynamic structure consisting of several small compartments under normal growth conditions. These fuse together easily in glucose-deficient media or during cell harvest, and there are many, as yet unexplained, mutants which are defective in forming large vacuoles (Raymond et al., 1992).

The morphological alterations of vacuoles observed at later time points following Ypt31p/Ypt32p depletion might also point to a transport defect occurring between endocytic compartments. Such mutants, however, are usually characterized by missorting and secretion of vacuolar enzymes but are not defective in secretion. In contrast to these so-called vps mutants, Ypt31p/Ypt32p-lacking cells or ypt31-1 mutant cells at the non-permissive temperature did not missort vacuolar hydrolases, such as CPY. One vps mutant, Δvps28, has been reported recently to accumulate a multilamellar membrane compartment which superficially resembles some of the membrane stacks we have observed in ypt31l/ypt32 mutants. While the exaggerated membrane structures in our GTAPase mutants appeared in different forms and larger numbers per cell, apparently only one multilamellar structure, believed to be an exaggerated pre-vacular, endosome-like compartment, occurred in vps28 null mutant cells (Rieder et al., 1996). As in other vps mutants, invertase secretion was undisturbed in Δvps28 cells.

Although we cannot at present exclude the possibility that the Ypt3 GTPases have a function in a post-Golgi transport reaction as well, their role in intra-Golgi transport seems most plausible to us as an explanation for the biochemical and morphological alterations which we have observed in cells lacking Ypt31p and Ypt32p and in ypt31-1 mutant cells at the non-permissive temperature. The strongest indication for this assumption is the block in invertase secretion and its intracellular accumulation, predominantly in the highly glycosylated form, in Ypt31p/Ypt32p-depleted cells and in ER core-glycosylated and highly glycosylated form in ypt31-1 mutant cells at 37°C. As we have not observed an excess of secretory vesicles, which, for example, is seen in sec4 mutants defective in vesicle docking to the plasma membrane (Goud et al., 1988), it might well be that the Ypt3 GTAPases participate in transport vesicle formation at the most distal Golgi compartment. Therefore, it seems likely that the highly glycosylated invertase resides in the exaggerated Golgi-like cisternae without being able to leave it in the absence of Ypt3 GTAPases. Although less likely, the defects seen in ypt31l/ypt32 mutant cells could also be reconciled with a role for Ypt3 GTAPases in retrograde Golgi transport. One might assume that the inhibition of retrograde Golgi–ER membrane traffic results in the formation of exaggerated and functionally defective Golgi structures. A changed membrane composition could thus interfere with transport vesicle formation. As other yeast transport GTAPases, like Ypt1p and Sec4p, act in concert with vesicular and target membrane receptors, so-called v- and t-SNARES (Dascher et al., 1991; Hardwick and Pelham, 1992; Aalto et al., 1993; Protopov et al., 1993; Søgaard et al., 1994), it is worth mentioning in this context that such receptor-like proteins with an apparent function in retrograde Golgi transport of yeast have been identified recently (Banfield et al., 1995; Lewis and Pelham, 1996).

Finally, according to our localization experiments, Ypt31p appears to be associated, at least in part, with Golgi membranes. A significant part of the GTAPase was found in the Golgi-enriched 100 000 g pellet fraction and, on sucrose gradients, most of the Ypt31p, except for its soluble form, co-migrated with two different Golgi membrane proteins, Kex2p and Sed5p. It clearly separated from the ER, the vacuole and the plasma membrane. In
support of the results obtained by subcellular fractionation, indirect immunofluorescence with anti-Ypt31p antibodies resulted in a punctate staining resembling localization of other Golgi proteins. It is not clear, however, to what extent the Ypt31p which was pelleted with the 13 000 g fraction might be associated with Golgi or other membrane compartments. If the Ypt31p-Ypt32 proteins were involved in vesicle formation at a late Golgi compartment, one would expect also to find these GTPases on post-Golgi target membranes.

Materials and methods

Materials and general methods

The S.cerevisiae strains used in this study are listed in Table I. Cloning experiments were performed with Escherichia coli strain dh5α, on standard media (Miller, 1972). E.coli transformations were performed according to Hananah (1983). Yeast media as well as standard yeast genetic techniques (crosses, sporation of diploids and tetrad dissections) were performed as previously described by Sherman et al. (1986). Yeast transformations were performed by the alkali cation treatment of Ito et al. (1983).

Cloning of the YPT31 and YPT32 genes

Total DNA from S.cerevisiae strain DUR was digested with the restriction endonucleases EcoRI and HindIII, and the resulting fragments were separated in a 1% agarose gel and transferred to Nylons membranes (Schleicher & Schüll). Hybridization was performed under moderately stringent conditions (6× SSC, 42°C) with 32P-oigolabelled 1.2 kb cDNA coding for the S.pombe Ypt3p (Miyake and Yamamoto, 1990). DNA fragments of the HindIII and EcoRI digest in the region of the gel giving a hybridization signal (HindIII one 4 kb fragment, EcoRI two fragments of 6 and 2.5 kb) were eluted from preparative agarose gels and cloned into the HindIII or EcoRI site of plBluescripit II SK (+) (Stratagene). The recombinant plasmids were transformed into E.coli and colony screening was performed using hybridization filters and conditions as described above. The 4 kb HindIII fragment of the recombinant plasmid pYPT3H3 was shown to contain a 1.265 kb cross-hybridizing HindIII-PstI fragment, which was subcloned into plBluescriptII SK (+), sequenced and found to contain the YPT31 gene. PCR amplification of gene-specific degenerate primers representing the protein sequence motifs YDLFLK [5'-TA(C/T)(A/G)CTTA(C/T)TA(G/A/G)(T/T)AA-3'] found in mammalian Rab1p (Chavrier et al., 1990) and S.pombe Ypt3p (Miyake and Yamamoto 1990) and QIWDTAG [5'-GCCA(A/G)TCG(A/G)GTTCACA(T/C)TG-3'] found in most Rab/Ypt proteins (Valencia et al., 1991) were used for amplification of yeast DNA (Krawetz et al., 1989). Five low stringency amplifications (37°C annealing, 58°C extension) were followed by 30 cycles with moderate stringency (45°C extension). All steps were allowed to proceed for 1 min (Compton, 1990). The reaction products were cloned in pBluescriptII SK (+) for sequence analysis (Sambrook et al., 1989). The isolation of the entire YPT32 gene was achieved by screening a yeast genomic library (Dascher et al., 1991) with a cloned 150 bp PCR fragment of the YPT32 gene as the hybridization probe. The subgenomic EcoRV fragment (1.6 kb) was inserted into the EcoRV site of plBluescriptII SK (+) for further sequence analysis. To create a loss-of-function allele, the 381 bp Thh111 fragment of YPT31 (Figure 1) was replaced by a 1.2 kb HindIII fragment containing the URA3 marker gene. YPT32 was disrupted by inserting a 1.8 kb BamHI fragment containing the HIS3 marker gene into the BgII site of the coding region of YPT32 (Figure 1). For gene disruption, the mutated versions of both genes were excised either as a 2.2 kb BglII-SmaI fragment (YPT31) or a 3.4 kb EcoRV fragment (YPT32) and used for transformation of the yeast strain RH270-2B. The chromosomal YPT31 gene was replaced by the GAL10 fusion in a strain (YTH5) which was disrupted in YPT32 as follows. Yeast plasmid YEp51-SLY1 (Ossig et al., 1991) was digested with SalI-Nde I. The resulting 2.7 kb SalI-NdeI fragment containing the GAL10 promoter and the LEU2 marker gene were then ligated into the Thh111 site 19 bp upstream of the initiation codon of YPT31. The correct integration of all mutated versions of YPT31 and YPT32 into the genome of the strains (see above) was verified by Southern blot analysis (data not shown).

Plasmid pGEM YPT31 URA3 UBI LAcI flu YPT31 was generated as follows. Plasmid pBaBa4 (provided by J.W.Szostak) was digested with SpIl. The resulting 1.7 kb fragment containing the ubiquitin–Lacl–#haemagglutinin–AR1 disruption was cloned to the SpIl site of pGEM7 z (+) to generate plasmid pGEM UBI LAcI flu. A 2.6 kb PvuII–PstI fragment derived from the EcoIl expression vector pet11a–YPT31 containing the entire YPT31 coding sequence was cloned between the PvuII and PstI sites of pGEM3 z (+). This plasmid was then digested with AarIl and NdeI. The resulting 1.0 kb fragment containing the Ypt31–coding sequence was cloned between the AarIl and Asel sites of pGEM UBI–LAcI flu to generate plasmid pGEM UBI LAcI flu YPT31. For chromosomal integration, plasmid pGEM YPT31 URA3 GAL10 UBI LAcI flu YPT31 was generated as follows. Plasmid pBaBa4 was digested with EcoRl and SpIl. The resulting fragment containing URA3 and the GAL10 promoter sequence was cloned between the EcoRl and SpIl site of pGEM7 z (+) to obtain plasmid pGEM URA3 GAL10. A 400 bp HindIII–EcoRl fragment derived from pBluescript (in which YPT31 was cloned as a 1256 bp HindIII–PstI fragment) was then cloned between the HindIII and EcoRl site of pGEM URA3 GAL10 to generate plasmid pGEM YPT31 URA3 GAL10. A 1.4 kb SpIl fragment derived from pGEM UBI LAcI flu YPT31 was then cloned to the SpIl site of pGEM YPT31 URA3 GAL10 to obtain pGEM YPT31 URA3 GAL10 LAcI flu YPT31.

Yeast cell extracts and immunoblotting

Whole cell extracts were prepared by harvesting three optical density units (OD600) of cells. Lysis was achieved by resuspending the cells in 300 μl of 2 M NaOH, 5% β-mercaptoethanol for 10 min on ice. Trichloroacetic acid (TCA) was then added to give a final concentration of 10%. The samples were kept on ice for an additional 10 min and the

<table>
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Ypt31p and Ypt32p in protein transport

precipitates were collected by centrifugation. The resulting pellet was neutralized by washing with 100 μl of 1.5 M Tris–HCl (pH 7.5) and dissolved in 90 μl of 2× Laemmli dissociation buffer (Laemmli, 1970). After heating at 95°C for 2–5 min, cell debris was sedimented and the supernatant was used for Western blotting or for immuno blotting (Burnette, 1981). For detection of immobilized antigens, the ECL Western blotting system (Amersham) was used. For subcellular fractionation, spheroplasts were obtained from logarithmically grown cells according to Vaeusthube and Schekman (1992). Spheroplasts in lysis buffer (0.1 M sorbitol, 20 mM HEPES, pH 7.4, 50 mM potassium acetate, 2 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulphonyl fluoride (PMSF), 0.1 μg/ml pepstatin, 0.5 μg/ml leupeptin, 20 μM E64, 1 mM benzbamin, 20 μg/ml trypsin inhibitor) at 20 OD600/ml were lysed gently on ice in a Dounce homogenizer with 10–15 strokes using a loosely fitting pestle. After two consecutive centrifugations of the lysate at 500 g for 10 min, the cleared lysate was centrifuged at 13 000 g for 10 min to obtain the P13 pellet. The S13 was then centrifuged at 100 000 g for 1 h to obtain the Golgi-enriched P100 pellet and the soluble proteins (S100 fraction). For gradient fractionation of cell organelles, 1 ml of the cleared lysate was loaded onto a manually generated 12-step sucrose gradient (0.5 ml of 60%, 1 ml each of 50, 48, 45, 43, 41, 38, 36, 33 and 29% and 0.5 ml of 21% sucrose in lysis buffer) and centrifuged at 170 000 g for 3 h at 4°C in a Beckman SW40 rotor. Fifteen fractions were collected manually from the top to the bottom and proteins were precipitated with 10% TCA and processed for Western blotting or analyzed by electrophoresis and scanning densitometry. Western blots were done using the Arcus II scanner (AGFA) and Fotolook SA software. Band densities were calculated with NIH Image 1.59 software.

To determine the nature of the association of Ypt31p with the pelletable material, the cleared lysate (500 g supernatant) was either adjusted to 1 M potassium acetate, 0.1 M sodium carbonate (pH 11), 5 M urea, 1% Triton X 100 or washed twice with ice for 60 min prior to centrifugation at 100 000 g for 1 h. The samples were then analysed by Western blotting as described above.

**Generation of antisera**

Polynuclear antibodies were obtained using Ypt31p and Ypt32p proteins, which were expressed in E.coli using the pET expression system (Novagen). For induction onto the expression vector, an NdeI restriction site was introduced at the initiation codon of YPT31 and YPT32 genes by PCR-mediated mutagenetation. The cloned genes were amplified using PCR primers with the desired restriction sites as 5’ extensions (5’-TTCACATATGCGCGGAGACTAGCGG-3’ for YPT31, 5’-GTATTCCACCATATGAGCAGCAAGC-3’ for YPT32). The PCR was performed using the Vent DNA polymerase following the instructions of the manufacturer (New England Biolabs). After generation of blunt ends with T4 DNA polynucerase, the PCR fragments were first inserted into the Smal site of BluescriptII KS+ (+) (Stratagene) for sequence analysis. YPT31 and YPT32 were then digested using BamHI–Ndel (YPT31 was partially digested by Ndel) and cloned into the vector pET11a to yield pET11-a-YPT31 and pET11-a-YPT32, respectively, for protein production in E.coli strain BL21. Bacterially produced Ypt31p and Ypt32p were purified by a three-step procedure and were used to raise polynuclear antibodies in rabbits as described by Wagner et al. (1992). For the generation of antibodies to vacuolar alkaline phosphatase, a trpE-ALP fusion protein plasmid was produced in E.coli strain DH5a following the method of Körner et al. (1991). The fusion protein was purified by preparative SDS-PAGE, electroeluted and injected into rabbits. Antibodies against vacuolar CPY, the gene name in Saccharomyces cerevisiae, CPY (Boehringer) which was treated with EndoH following the instructions of the manufacturer (New England Biolabs). CPY was then purified further by SDS-PAGE and electroelution (as above). Antibodies specific for BiP/Kar2p and Kex2p were also raised in rabbits using the synthetic peptides GADDYNVGTVL (BiP/Kar2p) and IKQKFPDNDASEAKSLQEL (Kex2p), respectively. For the generation of anti-Sed5p antibodies, a His-tagged Sed5p lacking the C-terminal membrane anchor (amino acids 1–320) was produced in E.coli. Polynuclear antibodies were produced in rabbits (Eurogentech, Brussels). They were affinity purified over immobilized, recombinant Sed5p.

**Protein labelling and immunoprecipitation**

Yeast cells were grown in supplemented minimal medium, SM (Reid, 1989) containing either galactose or glucose in order to induce or repress the expression of genes fused to the GAL10 promoter. The growth properties of cells were not altered in SMM. Cells were harvested (3 OD600 units) by centrifugation, resuspended in 250–500 μl of growth medium containing 0.5 mg ovalbumin/ml and pulse-labelled with 250 μCi Tran 35S-label (ICN) for 15 min. After addition of unlabelled methionine and cysteine (to give final concentrations of 0.5 mg/ml each), the cells were chased for the times indicated. The chase was stopped on ice by the addition of sodium dodecyl sulfate (SDS)-PAGE (final concentrations 2 and 0.2 mM, respectively). The cells were lysed using glass beads, and immunoprecipitated with protein A–Sepharose according to Raymond et al. (1992). To detect outer chain manniosyl residues, the radiolabelled CPY was immunoprecipitated and further treated with anti-α-1,6 Man or α-1,3 Man sera according to Franzusoff and Schekman (1989). The samples were separated using 8% SDS–PAGE. The proteins in gels were fixed and stained with Coomassie blue in 10% acetic acid, 10% methanol. After incubation in Amplify (Amersham) for 30–45 min, the gels were dried and exposed to X-Omat AR (Kodak) at –80°C.

To study processing and secretion of invertase in a ypr31/1 mutant strain, cells were induced for synthesis of secreted invertase in 0.1% glucose for 30 min, left at 25°C or shifted to 37°C for 30 min and then pulse-labelled for 30 s and chased for 20 min at the respective temperature with cold methionine and cysteine as described above. Invertase was precipitated with a specific antibody and samples were processed for Western blotting as described above for CPY.

**Assay for internal and periplasmic invertase**

The Δypt32/GAL10-YPT31 strain was grown for different times under repressing conditions. The cells were then washed twice with medium containing 0.1% glucose and grown for 1 h in the low glucose medium (see above). The cells were collected by centrifugation and washed twice with ice-cold sodium azide (10 mM). The cell density was adjusted to 10 OD600/ml in 0.1 M sodium acetate and the samples divided into two portions. The equivalent of 0.01–0.2 OD600 units of cells was assayed for periplasmic invertase and for total invertase activity as described by Johnson et al. (1987) and Goldstein and London (1975). The intracellular invertase activity was determined by subtraction of the periplasmic activity from the total activity. One invertase unit was defined as the amount (in nmol) of glucose produced per min by 1 OD600 unit of cells. The different stages of glycosylation and secretion of active invertase in S.cerevisiae were analysed as follows: cells were grown in YEPD medium to an OD600 of 2, the cells were then transferred to YEPD medium for different time intervals and finally transferred for 1 h to 0.1% glucose-containing medium in order to depress the synthesis of extracellular invertase (Esmun et al., 1981). The preparation of spheroplasts and fractionation were performed according to Schauer et al. (1985). One OD600 of each sample was applied onto a non-denaturing 7.5% polyacrylamide gel. Invertase activity staining was performed as previously described by Grossmann and Zimmermann (1979).

**Indirect immunofluorescence**

Indirect immunofluorescence using affinity-purified rabbit polyclonal anti-Ypt31p and anti-Sed5p antibodies was performed exactly as described previously (Haas et al., 1995). Cy3-labelled goat anti-rabbit IgG (H+L) from Jackson Immuno Research Laboratories, Inc., served as secondary antibody.

**Light and electron microscopy**

To visualize vacuoles, 500 μl samples were taken from yeast cultures and treated for 30 min with CDC54A as described by Robert et al. (1991). The labelled cells were adhered to concanavalin A-treated (1 mg/ml) coverslips and viewed using a Zeiss Axiopt microscope equipped with Normarski optics.

For electron microscopy, the samples were taken from yeast cultures as shown in Figure 5. These were either prepared by the permanganate fixation method according to Ossig et al. (1991), or spheroplasts were fixed using a modified version of the osmium ferrocyanide procedure of McLaughlin (1964). The primary fixative consisted of 2% glutaraldehyde in a 50 mM KPO4 (pH 7.0) buffer containing 1 mM CaCl2 and 1.2 M sorbitol. After 2 h at room temperature, the spheroplasts were pelleted gently. The supernatant was then removed and replaced directly by the second fixative [2% OsO4 + 0.8% (v/v) K6Fe(CN)6 in 50 mM phosphate buffer]. The spheroplasts were resuspended and allowed to stand for 5 min at room temperature before pelleting as described elsewhere. This procedure was repeated after 10, 25 and 90 min. The spheroplasts were then washed in distilled H2O (3×10 min) and transferred to 2% aqueous uranyl acetate for 12 h at 14°C. After rewashing in distilled H2O (2×10 min), the spheroplasts were embedded in 3% low melting point agar. The agar blocks were taken through a graded aceton series and then subjected to en bloc lead staining in an
absolute ethanol–acetone mixture as described by Kushida (1966). Thin sections were prepared from material embedded in Spurr’s resin. These were post-stained in methanolic uranyl acetate and lead citrate (10 min each), and examined in a Philips CM 10 electron microscope operating at 80 kV.

For freeze substitution, cells were pelleted and a portion of the yeast paste was mounted between sandwich sample holders and quick frozen with liquid propane using a JFD 030 Croyjet (Bal-Tec Balzers, Liechtenstein). The specimens were transferred to liquid nitrogen and inserted into the chamber of an AF5 cryosubstitution apparatus (Reichert, Vienna, Austria) containing acetone at −85°C with 4% OsO4. Over a 90-h period, the specimens were slowly brought to −20°C and then, over a further 9 h, to room temperature. They were then taken through a series of acetone–ethanol mixtures to pure ethanol and finally embedded in London Resin White. Sections were double stained with uranyl acetate and lead citrate.

Acknowledgements
We would like to thank A.Franzusoff (Denver) and T.Rapoport (Boston) for providing anti-α,1,3 mannose and anti-α,1,6-GlcNAc antibodies, respectively, and R.Grabowski for anti-Sed5p antibodies. We also thank H.-H.Trepte for some of the electron micrographs, M.I.Eschen for helping to construct the ubiquitous YPT31 fusion gene, P.Msenkus for technical help, and P.Schleissmman for expert secretarial assistance. This work was supported in part by grants to D.G. from the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie.

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Received on June 8, 1996; revised on August 22, 1996.