

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

Mustafa Benli, Frank Döring<sup>1</sup>,  
David G. Robinson<sup>2</sup>, Xiaoping Yang and  
Dieter Gallwitz<sup>3</sup>

Department of Molecular Genetics, Max Planck Institute for Biophysical Chemistry, PO Box 2841, D-37018 Göttingen and  
<sup>2</sup>Plant Physiology Institute, University of Göttingen, Untere Karspüle 2, D-37073 Göttingen, Germany

<sup>1</sup>Present address: Institute of Nutritional Sciences, Unit of Biochemistry, University of Giessen, D-35392 Giessen, Germany

<sup>3</sup>Corresponding 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

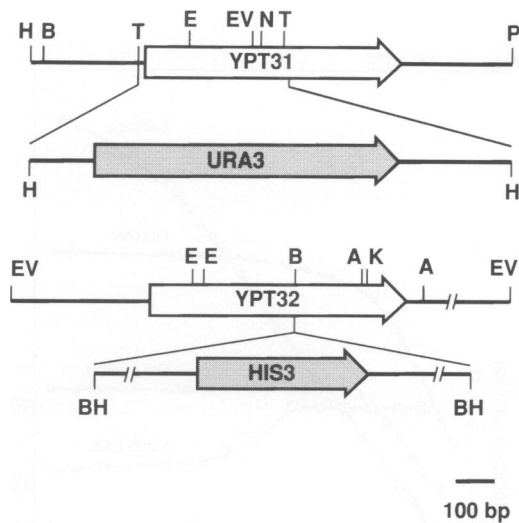
Protein transport and membrane flow in eukaryotic cells involve complex mechanisms whose many intricacies are now being studied intensively using genetic and biochemical approaches. According to present knowledge, vesicular protein transport between different membrane-enclosed compartments regularly 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 multiprotein docking/fusion complexes which operate in diverse vesicular transport steps in exocytosis (Rothman, 1994; Søggaard *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 Ypt1p homologue, Rab1p (Plutner *et al.*, 1991; Nuoffer *et al.*, 1994). Thus, convincing evidence for a dual function of Ypt1p in yeast is so



**Fig. 1.** Schematic representation of the *YPT31* and *YPT32* gene loci with relevant restriction sites: *Acc* I (A), *Bgl* II (B), *Eco*RV (EV), *Eco*RI (E), *Eco*NI (EN), *Hind*III (H), *Kpn*I (K), *Nde*I (N), *Pst*I (P), *Tth*111I (T). The protein-coding regions are shown by open arrows. Also shown are the disruptions of *YPT31* and *YPT32* with the *URA3* and *HIS3* gene, respectively.

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 *Hind*III fragment and two *Eco*RI fragments of ~6 and 2.4 kb from total *S.cerevisiae* DNA digests were found to be labelled in Southern blots with <sup>32</sup>P-labelled *ypt3* cDNA from *Schizosaccharomyces pombe* under moderately stringent hybridization conditions. After mapping the cloned DNA fragments with different restriction endonucleases, a 1265 bp *Hind*III–*Pst*I fragment derived from the 4 kb *Hind*III 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

S.c.Ypt31	1	MSS	EDYGYDYD	L	LFK	I	VL	I	GD	SG	V	G	K	S	N	L	S	R	F	T	K																
S.c.Ypt32	1	MS	NEDYGYDYD	L	LFK	I	VL	I	GD	SG	V	G	K	S	N	L	S	R	F	T	K																
S.p.Ypt3	1	M	COEDEVDY	--	L	FK	I	VL	I	GD	SG	V	G	K	S	N	L	S	R	F	T	R															
C.f.Rab11	1	M	GTRDDEVDY	--	L	FK	V	VL	I	GD	SG	V	G	K	S	N	L	S	R	F	T	R															
S.c.Ypt31	36	NE	F	N	M	D	S	K	S	T	I	G	V	E	F	A	T	R	T	L	E	I	D	C	K	R	I	K	A	O	I	W	D	T	A		
S.c.Ypt32	36	DE	F	N	I	E	S	K	S	T	I	G	V	E	F	A	T	R	T	I	E	V	E	N	K	K	I	K	A	O	I	W	D	T	A		
S.p.Ypt3	33	NE	F	N	I	E	S	K	S	T	I	G	V	E	F	A	T	R	N	I	N	L	D	N	K	K	I	K	A	O	I	W	D	T	A		
C.f.Rab11	34	NE	F	N	L	E	S	K	S	T	I	G	V	E	F	A	T	R	S	I	Q	V	D	C	K	T	I	K	A	O	I	W	D	T	A		
S.c.Ypt31	71	G	O	E	R	Y	R	A	I	T	S	A	Y	Y	R	G	A	V	G	A	L	I	V	Y	D	I	S	K	S	S	S	Y	E	N	C	N	
S.c.Ypt32	71	G	O	E	R	Y	R	A	I	T	S	A	Y	Y	R	G	A	V	G	A	L	I	V	Y	D	I	S	K	S	S	S	Y	E	N	C	N	
S.p.Ypt3	68	G	O	E	R	Y	R	A	I	T	S	A	Y	Y	R	G	A	V	G	A	L	I	V	Y	D	I	I	T	K	S	S	F	D	N	V	G	
C.f.Rab11	69	G	O	E	R	Y	R	A	I	T	S	A	Y	Y	R	G	A	V	G	A	L	I	V	Y	D	I	I	T	K	H	L	T	Y	E	N	V	E
S.c.Ypt31	106	H	W	L	S	E	L	R	E	N	A	D	D	N	V	A	V	G	L	I	G	N	K	S	D	L	A	H	L	R	A	V	P	T	E	E	
S.c.Ypt32	106	H	W	L	T	E	L	R	E	N	A	D	D	N	V	A	V	G	L	I	G	N	K	S	D	L	A	H	L	R	A	V	P	T	D	E	
S.p.Ypt3	103	R	W	L	K	E	L	R	E	H	A	D	S	N	I	V	I	L	V	G	N	K	I	D	L	L	H	L	R	A	V	S	T	E	E		
C.f.Rab11	104	R	W	L	K	E	L	R	H	A	D	S	N	I	V	I	L	V	G	N	K	S	D	L	R	H	L	R	A	V	P	T	D	E			
S.c.Ypt31	141	S	K	T	F	A	C	E	N	Q	L	L	F	T	E	S	A	L	N	S	E	N	V	D	K	A	F	E	L	I	N	T	I	Y			
S.c.Ypt32	141	A	K	N	F	A	V	E	N	Q	L	L	F	T	E	S	A	L	N	S	D	N	V	D	K	A	F	R	E	L	I	V	A	I	F		
S.p.Ypt3	138	A	Q	A	F	A	E	N	N	L	S	F	T	E	S	A	M	D	A	S	N	V	E	E	A	F	O	T	V	L	T	E	I	F			
C.f.Rab11	139	A	R	A	F	A	E	K	N	G	L	S	F	T	E	S	A	L	D	S	T	N	V	E	A	A	F	O	T	I	L	T	E	Y			
S.c.Ypt31	176	Q	K	V	S	K	H	O	M	D	L	G	D	S	S	A	N	G	N	A	N	G	A	S	A	P	N	G	P	T	I	S	L	T	P		
S.c.Ypt32	176	Q	M	V	S	K	H	O	V	D	L	S	G	S	G	T	N	M	G	S	N	G	A	P	K	G	P	T	I	S	L	T	P				
S.p.Ypt3	173	R	I	V	S	N	R	S	L	---	---	E	A	G	D	D	G	V	H	P	T	A	G	T	-	L	N	I	A	P	T						
C.f.Rab11	174	R	I	V	S	Q	K	C	M	---	---	S	D	R	R	E	N	D	M	S	P	S	N	-	N	V	V	P	I	H	V	P					
S.c.Ypt31	211	P	N	E	-	N	K	K	A	N	G	N	C	C																							
S.c.Ypt32	210	P	K	E	-	D	K	K	K	S	S	N	C																								
S.p.Ypt3	201	M	N	D	L	N	K	K	K	S	S	O	C																								
C.f.Rab11	203	T	T	E	N	---	K	P	K	V	Q	C	Q	N	I																						

**Fig. 2.** Amino acid comparison of *S.cerevisiae* (S.c.) Ypt31p and Ypt32p, *S.pombe* (s.p.) Ypt3p and canine (C.f.) Rab11p. Identical amino acids in Ypt31p and Ypt32p or in more than two of the proteins are highlighted on a black background. The sequence data are available from EMBL/GenBank under accession Nos X72833 (*YPT31*) and X72834 (*XPT32*).

then used to isolate the corresponding gene, which was identified on a genomic 1.6 kb *Eco*RV 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 *Eco*RI fragment, which harbours the 5' part of the *YPT31* gene (Figure 1), showed that the gene encoding phosphatidylserine 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 accidentally 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 Ypt3 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.

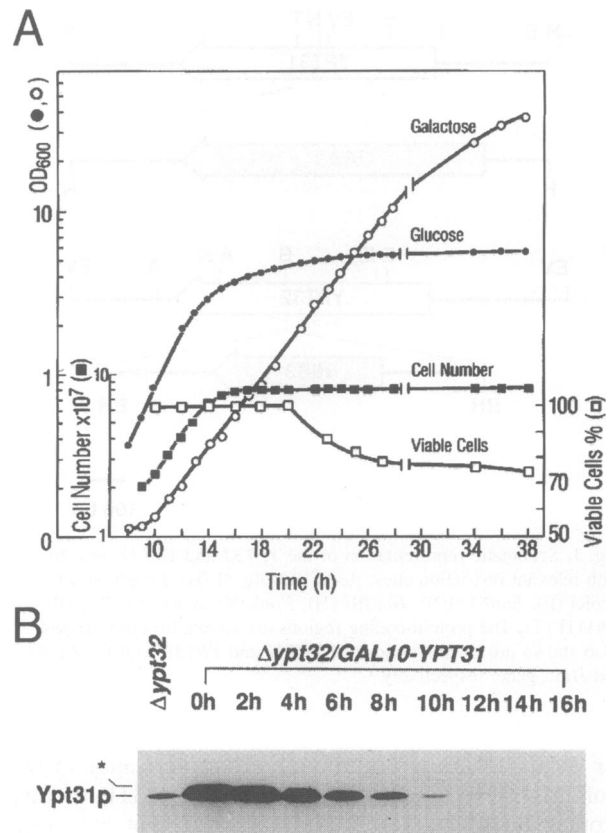
### Single disruptions of *YPT31* or *YPT32* are phenotypically neutral, a double disruption is lethal

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 (*Tth111III* 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 *Bgl*III site at codon 130 (Figure 1). The disrupted genes on linear fragments were used to replace one chromosomal copy of the respective wild-type gene in either a *Ura*<sup>-</sup> or a *His*<sup>-</sup> diploid strain. Following sporulation and tetrad dissection, *ypt31*<sup>-</sup> as well as *ypt32*<sup>-</sup> 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 *ypt31*<sup>-</sup> and *ypt32*<sup>-</sup> 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*<sup>-</sup>/*ura3*<sup>-</sup> 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*<sup>-</sup> 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).

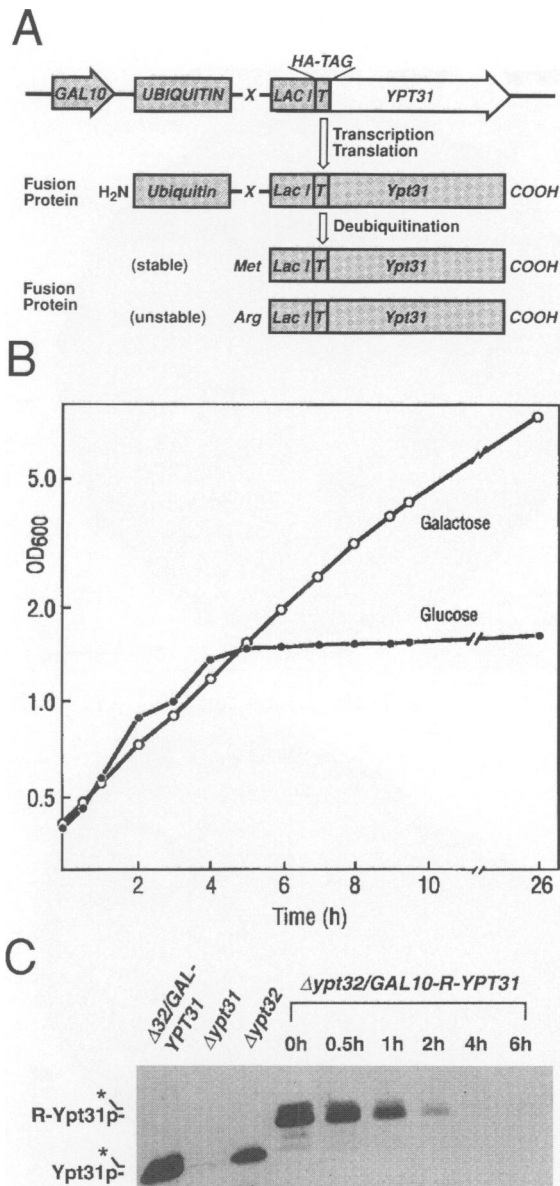
Since at least 14 h were needed to deplete cells of Ypt31p and to block cell proliferation after repression of *GAL10*-*YPT31* promoter fusion, we tried to circumvent this problem by generating conditional *ypt31* mutants. For this purpose, a previously published method was followed which is based on the formation of highly unstable ubiquitin fusion proteins allowing a much faster depletion



**Fig. 3.** Growth properties and Ypt31p expression of a  $\Delta ypt32/GAL10-YPT31$  strain. (A) Cells were grown in YEPgal medium, washed once in water and transferred either to YEPgal medium (○) or to YEPD medium (●). The cell growth was followed by optical density measurement at 600 nm or by haemocytometer counting (■). At the times indicated, aliquots of cells were plated on galactose-containing medium to allow a calculation of viable cells (□). (B) Depletion of Ypt31p after *GAL10* promoter switch off. At 2 h intervals following the transfer of cells to glucose-containing medium, total cellular protein was subjected to SDS-PAGE and Ypt31p was identified on Western blots with a polyclonal anti-Ypt31p antibody. On high expression in galactose-containing medium, a non-prenylated Ypt31p species (\*) can be seen on top of the isoprenylated form. Protein extracts from the isogenic strain with a disrupted *YPT32* gene ( $\Delta ypt32$ ) were included in this analysis.

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*<sup>-</sup> 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



**Fig. 4.** (A) Schematic representation of fusion genes harbouring elements encoding ubiquitin, a *lacI* repressor segment (*LacI*), the haemagglutinin epitope (T) and *YPT31* under the galactose-inducible *GAL10* promoter. After deubiquitination of the fusion proteins, either methionine or arginine is the N-terminal amino acid residue which then determines the stability of the GTPase. (B) Growth properties of  $\Delta ypt32/GAL10-R-YPT31$  cells (with arginine at the N-terminus of Ypt31p) in YEPgal (○) and at different times after shift to YEPD (●) were determined as described in the legend of Figure 3A. (C) Fast depletion of mutant Ypt31p (R-Ypt31p with arginine at the N-terminus) after shift (indicated in hours) from galactose- to glucose-containing medium. Western blots were performed as described in Figure 3. Note that the anti-Ypt31p antibody did not cross-react with Ypt32p (in the  $\Delta ypt31$  deletion strain).

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 *ypt31*<sup>-</sup> strain expressing Ypt32 fusion proteins, demonstrating again

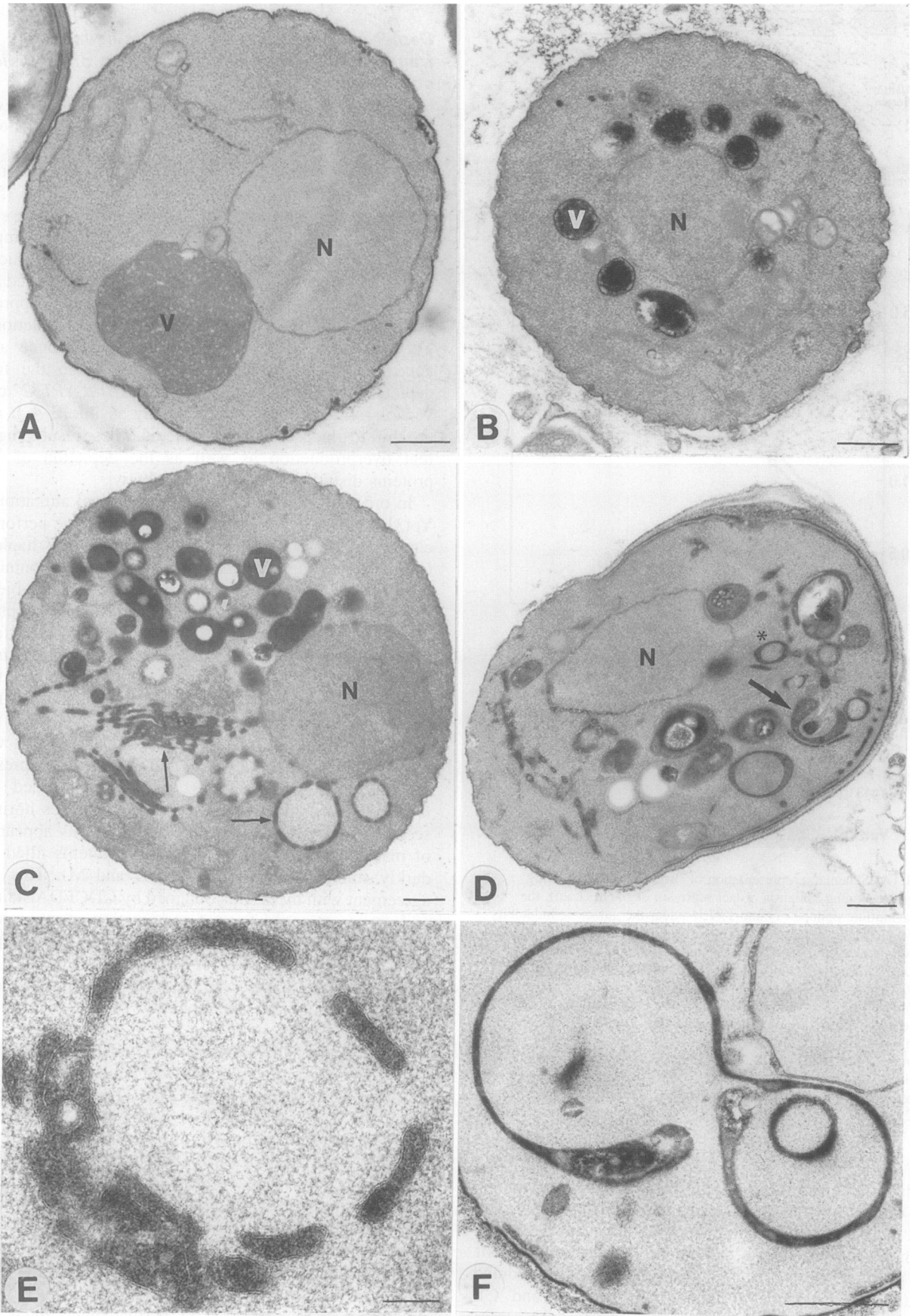
that Ypt31p and Ypt32p fulfill identical or overlapping functions.

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

Light microscopic inspection of *ypt32/GAL10-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 ferrocyanide. 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

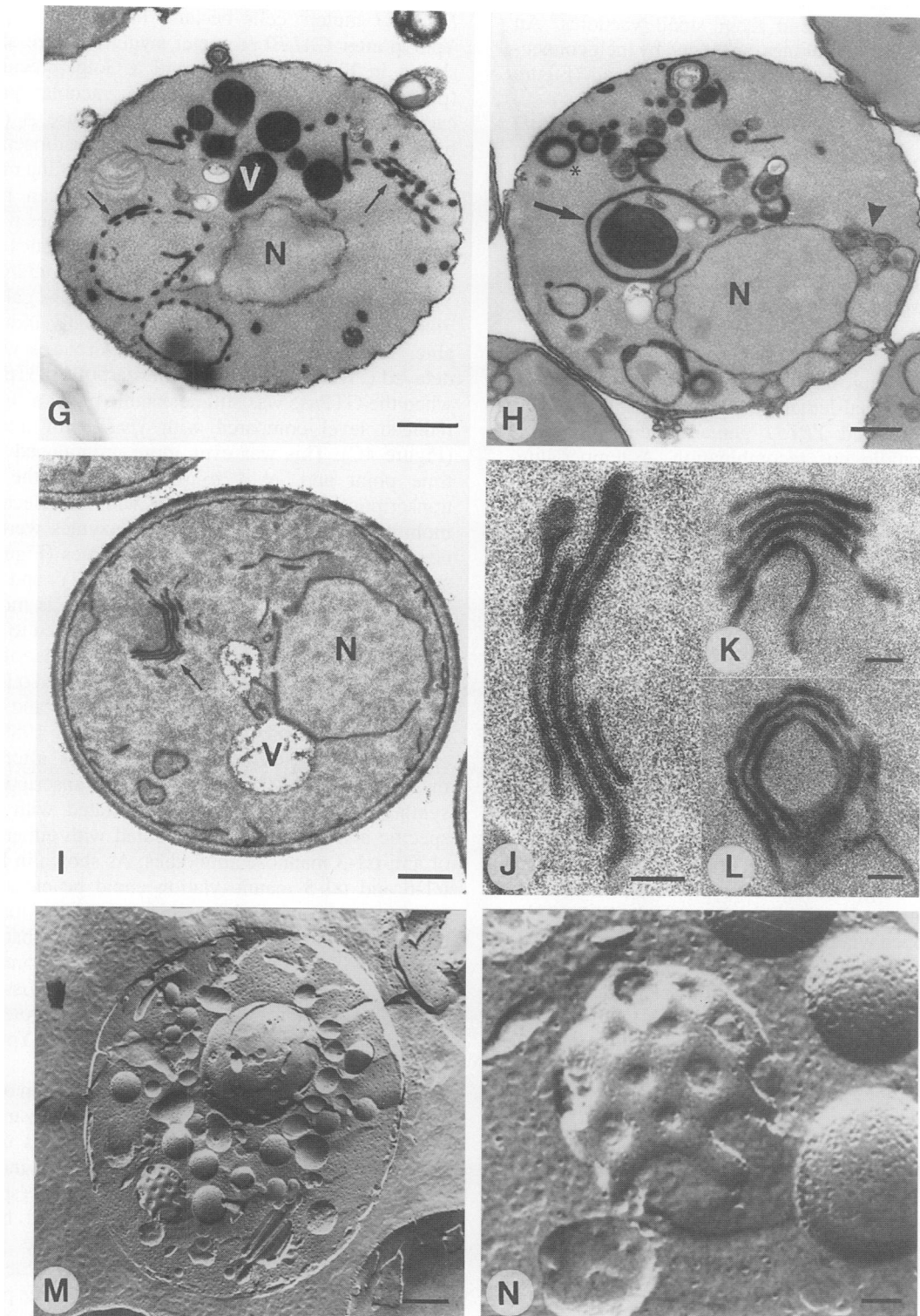




rarely seen in wild-type cells (Preuss *et al.*, 1992), were found regularly in the mutant cells fixed with permanganate (Figure 5I–L). Some membrane structures resembled the

cup-shaped ‘Berkeley bodies’, known to represent abnormal Golgi membranes (Novick *et al.*, 1980).

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



**Fig. 5.** Morphological analysis of Ypt31p-depleted cells.  $\Delta ypt32/YPT31$  cells grown in YEPgal (A) and  $\Delta ypt32/GAL10-YPT31$  cells grown in either YEPgal (B) or in YEPD for 12 (C and I) or 16 h (D) as well as  $\Delta ypt32/GAL10-R-YPT31$  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). Pleiomorphic 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  $\mu\text{m}$ .

detectable immunologically, the presumptive Golgi stacks were greatly diminished, and pleiomorphic, 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 GTPases, 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 hydroxylamine-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; Valencia *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  $\Delta ypt32/YPT31$  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 phenotypical 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.

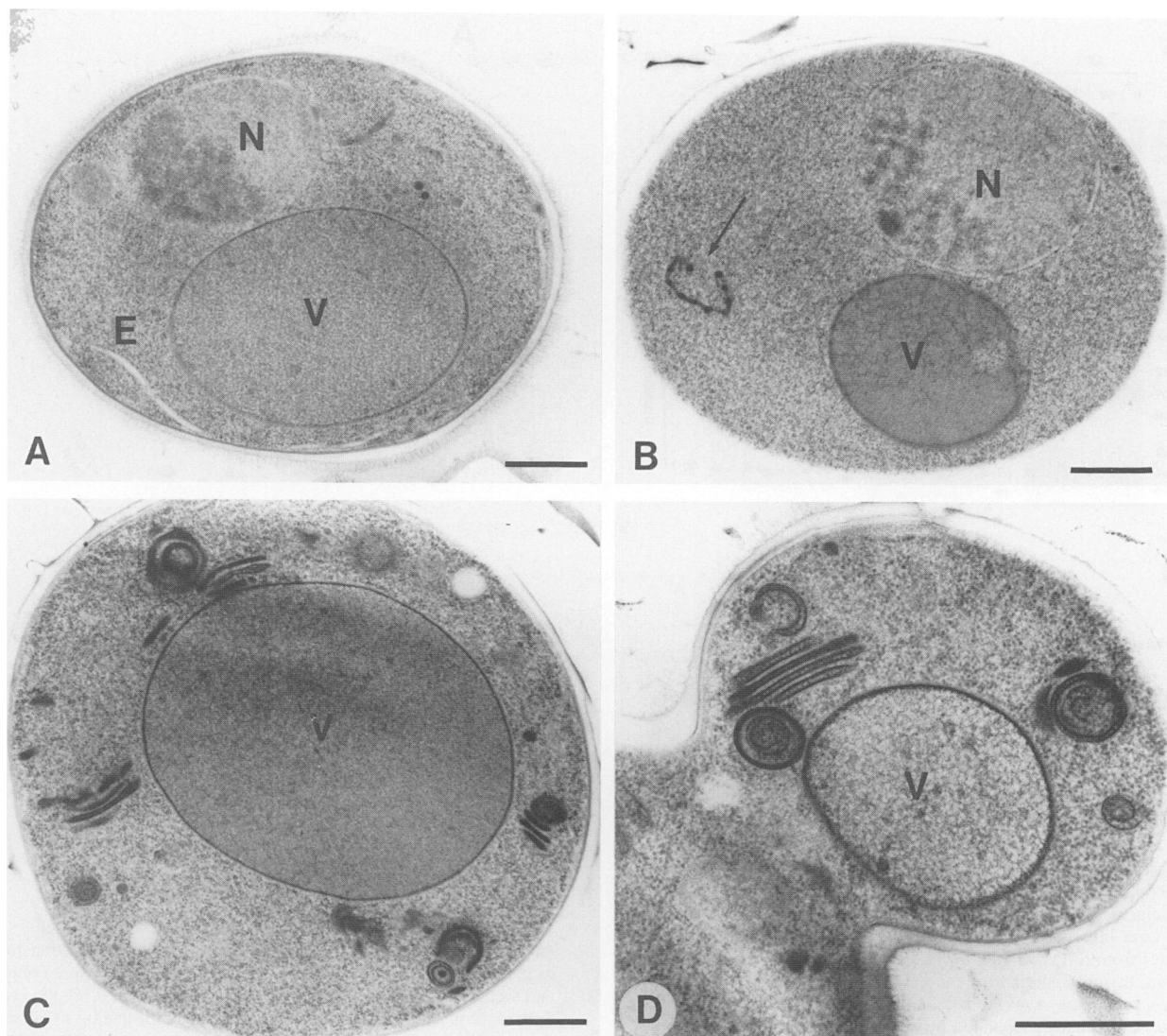
#### **Ypt3 protein-depleted cells are defective in intracellular protein transport**

The severe morphological alterations observed in Ypt3p-deficient cells point to a function of these GTPases 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 GTPase 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  $\alpha$ 1-6 and  $\alpha$ 1-3 mannosyl residues which are diagnostic of early and late Golgi glycosylation (Franzusoff 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- $\alpha$ 1-6 or anti- $\alpha$ 1-3 mannosyl antibodies. As shown in Figure 7B,  $\alpha$ 1-6 and  $\alpha$ 1-3 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 GTPases. 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



**Fig. 6.** Ultrastructural analysis of mutant *ypt31-1* cells. Cells of a  $\Delta ypt32/YPT31$  strain (A) grown at 30°C and cells of a  $\Delta ypt32/ypt31-1$  strain grown either at 25°C (B) or at 37°C for 30 min (C and D) were subjected to freeze substitution as described in Materials and methods. Note the intact vacuoles and the fast appearance of Golgi-like membrane stacks and abnormal membrane structures resembling 'Berkeley bodies' in mutant cells at the non-permissive temperature. In (B), an arrow points to a flat membrane-enclosed structure, presumably Golgi, occasionally visible in *ypt31-1* mutant cells under permissive conditions. N, nucleus; V, vacuole; E, endoplasmic reticulum. The bar represents 0.5  $\mu\text{m}$ .

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 intra- and extracellular (periplasmic) invertase was calculated. As can be seen in Figure 8B, the ratio of intracellular to periplasmic invertase increased with the

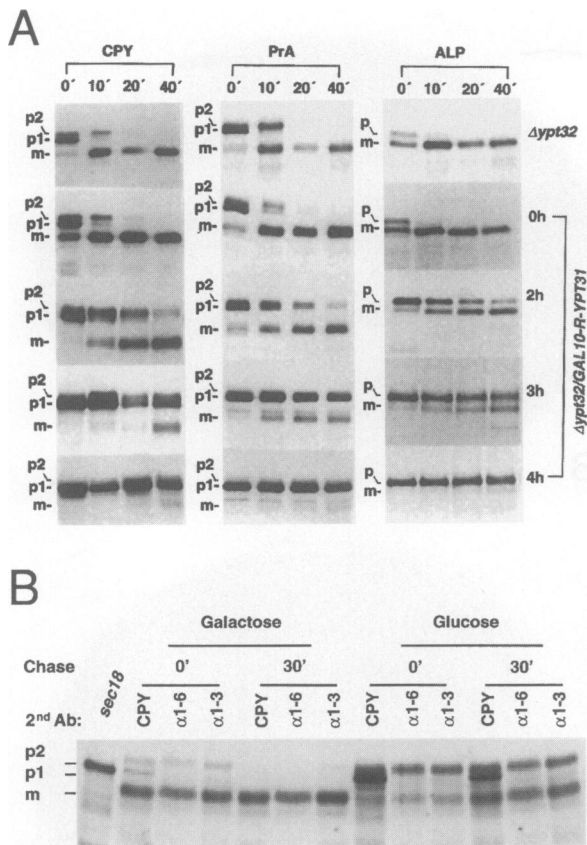
progression of Ypt31p depletion, with the majority of the enzyme accumulating inside the cells at times when Ypt31p was no longer detectable on Western blots (see Figure 3B). This shows that the depletion of Ypt3 GTPases results in a severe inhibition of protein secretion.

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

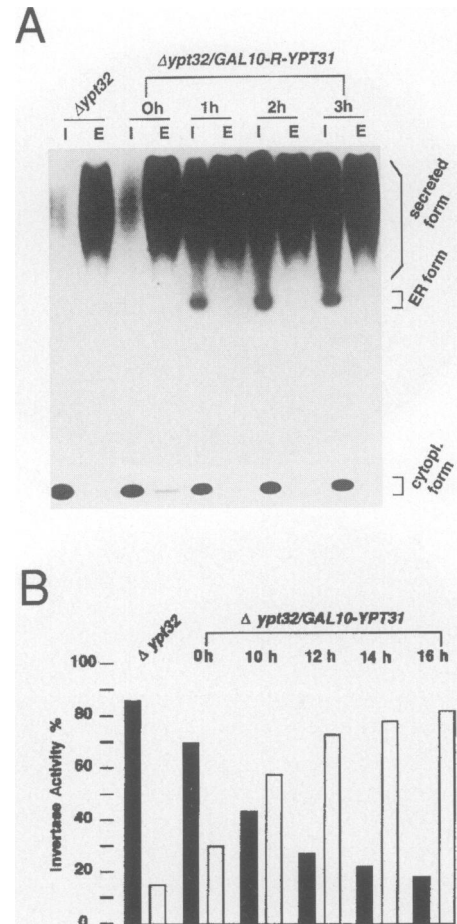




**Fig. 7.** Dependence of vacuolar hydrolase processing on Ypt3 GTPases. (A) For pulse-chase experiments, control cells of a  $\Delta ypt32/YPT31$  strain ( $\Delta ypt32$ ) were grown in semi-minimal medium supplemented with glucose (SMMG) whereas  $\Delta ypt32/GAL10-R-YPT31$  cells were investigated at different times (indicated at the right) after transfer from SMMgal to SMMG. Pulses with Tran  $^{35}\text{S}$ -label were for 15 min, the chase period for the times indicated. Carboxypeptidase Y (CPY), proteinase A (PrA) and alkaline phosphatase (ALP) were immunoprecipitated, separated by SDS-PAGE and identified by autoradiography. (B)  $\Delta ypt32/GAL10-R-YPT31$  cells grown either in SMMgal or 3 h after the transfer from galactose- to glucose-containing SMM were pulse-labelled for 20 min and chased for 30 min. CPY was immunoprecipitated, and one-third each of this immunoprecipitate was treated further with antibodies specific for CPY and  $\alpha 1-6$  or  $\alpha 1-3$  mannosyl residues. The ER form (p1) of CPY was identified with the help of a *sec18* mutant which was pulse-labelled at the non-permissive temperature. (p) proform; (m) mature form of the enzymes.

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.

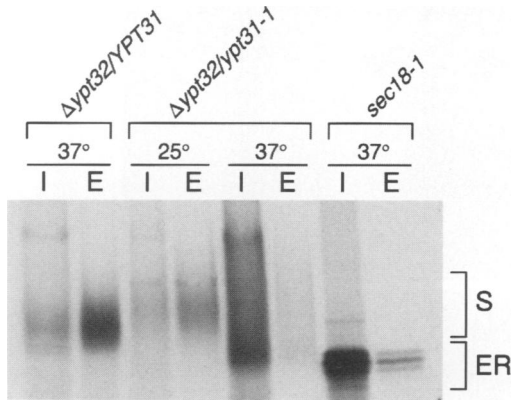
Spheroplasts were prepared and carefully lysed by Dounce homogenization. We found that most of the GTPase was bound to membranes (Figure 10A) and could be solubilized with detergents but not with high salt or at alkaline pH (data not shown). A crude fractionation of cellular membranes and organelles 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



**Fig. 8.** Inhibition of invertase secretion in Ypt31p-depleted cells. (A)  $\Delta ypt32/GAL10-R-YPT31$  cells grown in galactose were transferred to glucose-containing YEP. After 1, 2 and 3 h, samples were collected and were then grown to derepress for a further 1 h in 0.1% glucose. Spheroplasts were prepared and pelleted by centrifugation to obtain the periplasmic (E) and the cytoplasmic (I) fraction. Proteins of both fractions were separated on a 7% non-denaturing polyacrylamide gel followed by activity staining of the invertase. As a control, extracts from  $\Delta ypt32$  and  $\Delta ypt32/GAL10-R-YPT31$  cells (0 h time point) grown in galactose were included in this analysis. Note that significant amounts of highly glycosylated invertase were secreted in galactose-grown cells without treatment of cells in 0.1% glucose. The migration positions of core-glycosylated ER forms, the outer chain glycosylated secreted invertase and the constitutively synthesized cytoplasmic form of the enzyme are indicated. (B)  $\Delta ypt32$  and  $\Delta ypt32/GAL10-R-YPT31$  cells, grown either in YEPgal or in YEPD for 10, 12, 14 and 16 h, were collected by centrifugation. The pellets were resuspended in YEP containing 0.1% glucose and were divided into two. One portion subsequently was grown for a further 1 h to derepress the synthesis of invertase, the other portion was resedimented. Intracellular (open bars) and extracellular (closed bars) invertase activities were then measured as described in Materials and methods and represented as the percentage of the total activity.

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



**Fig. 9.** Inhibition of invertase secretion in  $\Delta ypt32/ypt31-1$  mutant cells at the non-permissive temperature.  $\Delta ypt32/YPT31$ ,  $\Delta ypt32/ypt31-1$  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  $^{35}\text{S}$ -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.

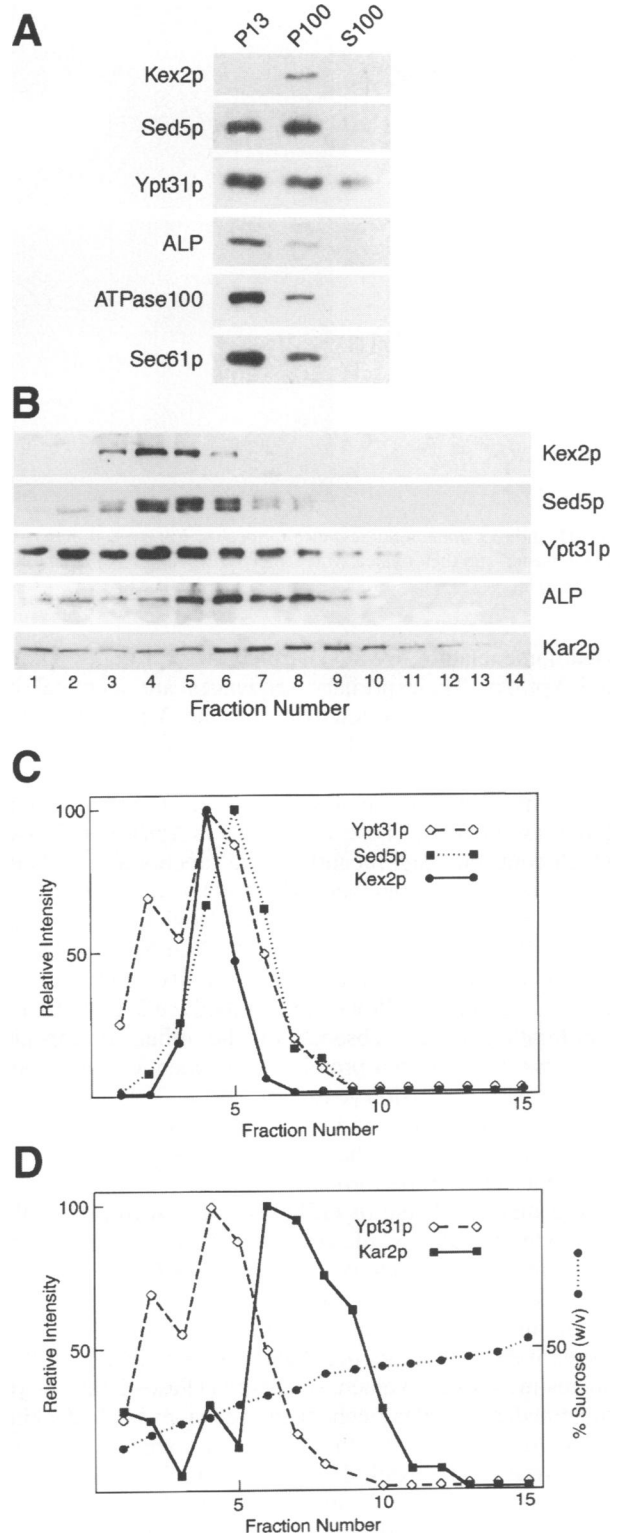
gradients (Antebi and Fink, 1992; Schröder *et al.*, 1995). As shown in Figure 10B–D, the bulk Ypt31p migrated to a position close to the Golgi markers Kex2p and Sed5p. A fraction of the GTPase, apparently the soluble cytoplasmic form (see also Figure 10A), appeared at the top of the gradient. Most importantly, 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 (Franzoso *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.

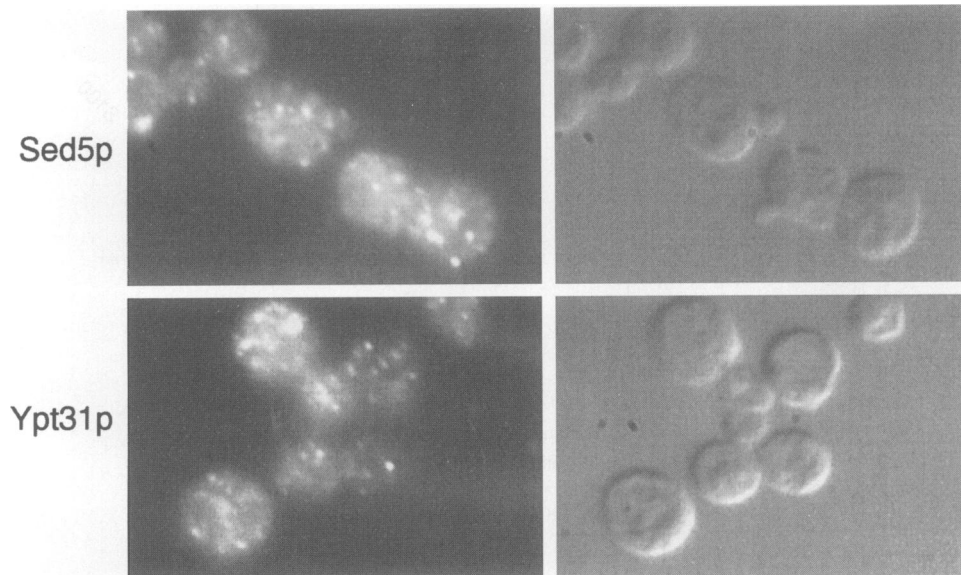
From the combined results of subcellular fractionation and indirect immunofluorescence, we assume that at least part of Ypt31p is bound to Golgi membranes.

## 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. 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 scan of the blot shown in (B) are presented in (C) and (D). The direction of gradient centrifugation is from left to right.



**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.

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  $\alpha 2$  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 endocytotic 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 Ypt31/Ypt32 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 distant 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 Necas, 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

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. Vacuolar 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  $\alpha$ 1-6 or  $\alpha$ 1-3 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 vacuolar 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 vacuoles, also appears to be a secondary effect. Vacuolar 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 Ypt3 GTPases, 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,  $\Delta vps28$ , has been reported recently to accumulate a multilamellar membrane compartment which superficially resembles some of the membrane stacks we have observed in *ypt31/ypt32* mutants. While the exaggerated membrane structures in our GTPase mutants appeared in different forms and larger numbers per cell, apparently only one multilamellar structure, believed to be an exaggerated pre-vacuolar, endosome-like compartment, occurred in *vps28* null mutant cells (Rieder *et al.*, 1996). As in other *vps* mutants, invertase secretion was undisturbed in  $\Delta vps28$  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 on 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 GTPases 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 GTPases. Although less likely, the defects seen in *ypt31/ypt32* mutant cells could also be reconciled with a role for Ypt3 GTPases 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 GTPases, 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; Søggaard *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 GTPase 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



Table I. Strains used in this study

Strain	Genotype	Source
RH270-2B	<i>MATa leu2 ura3 his4 lys2 bar1-1</i>	H.Riezman, Basel
MSUC-3D	<i>MATα leu2 ura3 his3 trp1 lys2</i>	H.D.Schmitt, Göttingen
MSUC 1A	<i>MATa leu2 ura3 his3 trp1 ade</i>	H.D.Schmitt, Göttingen
DUR	<i>MATa/α URA3/ura3 HIS3/his3 TRP1/trp1</i>	Dascher et al. (1991)
YTH1	<i>MATα his3 ura3 ypt31::HIS3 trp1</i>	this study
YTH2	<i>MATa leu2 ura3 ypt32::URA3</i>	this study
YTH3	<i>MATa/α LEU2/leu2 ura3/ura3 ypt32::URA3 HIS3/his3 ypt31::HIS3 TRP1/trp1</i>	this study
YTH4	<i>MATa leu2 GAL10-YPT31-LEU2 ura3 ypt32::URA3</i>	this study
YTH5	<i>MATa leu2 ura3 ypt32::URA3 his4 lys2 bar1-1</i>	this study
YTH11	<i>MATa leu2 ura3 his4 ypt32::HIS4 lys2 bar1-1</i>	this study
YTH7	<i>MATa leu2 GAL10-YPT31-LEU2 ura3 his4 ypt31::HIS4 lys2 bar1-1</i>	this study
YTH8	<i>MATa leu2 ura3 his3 ypt32::HIS3 trp1 ade</i>	this study
YTH9	<i>MATα leu2 ura3 his3 ypt32::HIS3 trp1 GAL10-YPT31-TRP1 lys2</i>	this study
YTH20	<i>MATa leu2 ura3 GAL10-R-YPT31-URA3 his4 ypt32::HIS4 lys2</i>	this study
YTH21	<i>MATα leu2 ura3 GAL10-R-YPT31-URA3 his4 ypt32::HIS4 lys2</i>	this study
YTH22	<i>MATa leu2 ura3 GAL10-M-YPT31-URA3 his4 ypt32::HIS4 lys2</i>	this study
YTH31	<i>MATa leu2 ura3 his3 ypt32::HIS3 ypt31-1-LEU2 trp1 ade</i>	this study
MB18	<i>MATα sec18-1 leu2 ura3 his4 suc2-Δ9</i>	M.Bielefeld, Düsseldorf

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 Ypt31/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 Hanahan (1983). Yeast media as well as standard yeast genetic techniques (crosses, sporulation 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 Nylon membranes (Schleicher & Schüll). Hybridization was performed under moderately stringent conditions (6× SSC, 42°C) with <sup>32</sup>P-oligo-labelled 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 pBluescriptII SK(+) (Stratagene). The recombinant plasmids were transformed into *E.coli* and colony screening was performed using hybridization fragments 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 pBluescriptII SK(+), sequenced and found to contain the *YPT31* gene. PCR amplification of gene-specific degenerate primers representing the protein sequence motifs YDYLFK [5'-TA(C/T)GA(C/T)TA(C/T)TT(A/G)TT(C/T)AA-3'] found in mammalian Rab11p (Chavrier et al., 1990) and *S.pombe* Ypt3p (Miyake and Yamamoto 1990) and QIWDTAG [5'-GCC(A/G/T)GC(A/G/T)GT(A/G)TCCCA(G/T)AT(C/T)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 pBluescriptII SK(+) for further sequence analysis. To create a loss-of-function allele, the 381 bp *Tth1111* 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 *BglIII* 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 *BglIII*-*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 *SaII*-*NdeI*. The resulting 2.7 kb *SaII*-*NdeI* fragment containing the *GAL10* promoter and the *LEU2* marker gene were then ligated into the *Tth1111* 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 *SphI*. The resulting 1.7 kb fragment containing the ubiquitin-LacI-haemagglutinin-ARD1 fusion was cloned to the *SphI* site of pGEM7 z(+) to generate plasmid pGEM *UBI LacI flu*. A 2.6 kb *PvuII*-*PstI* fragment derived from the *E.coli* expression vector pET111-a-*YPT31* containing the entire *YPT31* coding sequence was cloned between the *PvuII* and *PstI* sites of pGEM7 z(+). This plasmid was then digested with *AatII* and *NdeI*. The resulting 1.0 kb fragment containing the Ypt31-encoding sequence was cloned between the *AatII* and *AseI* 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 *EcoRI* and *SphI*. The resulting fragment containing *URA3* and the *GAL10* promoter sequence was cloned between the *EcoRI* and *SphI* site of pGEM7 z(+) to obtain pGEM *URA3 GAL10*. A 400 bp *HindIII*-*EcoRI* fragment derived from pBluescript (in which *YPT31* was cloned as a 1265 bp *HindIII*-*PstI* fragment) was then cloned between the *HindIII* and *EcoRI* site of pGEM *URA3 GAL10* to generate plasmid pGEM *YPT31 URA3 GAL10*. A 1.4 kb *SphI* fragment derived from pGEM *UBI LacI flu YPT31* was then cloned to the *SphI* 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 (OD<sub>600</sub>) 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

precipitates were collected by centrifugation. The resulting pellet was neutralized by washing with 100  $\mu$ l of 1.5 M Tris-HCl (pH 7.5) and dissolved in 90  $\mu$ l of 2 $\times$  Laemmli dissociation buffer (Laemmli, 1970). After heating at 95°C for 2–5 min, cell debris was sedimented and the samples (0.5–1 OD<sub>600</sub>) were separated by SDS-PAGE followed by immunoblotting (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 Wuestehube 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 phenylmethylsulfonyl fluoride (PMSF), 0.7  $\mu$ g/ml pepstatin, 0.5  $\mu$ g/ml leupeptin, 20  $\mu$ M E64, 1 mM benzamidin, 20  $\mu$ g/ml trypsin inhibitor] at 20 OD<sub>600</sub>/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 blot analysis as described above. Densitometric scanning of the Western blots was 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 was left untreated on 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

Polyclonal antibodies were obtained using Ypt31p and Ypt32p proteins, which were expressed in *E. coli* using the pET11-a expression system (Novagen). For insertion into the expression vector, an *NdeI* restriction site was introduced at the initiation codon of *YPT31* and *YPT32* genes by PCR-mediated mutagenesis. The cloned genes were amplified using PCR primers with the desired restriction sites as 5' extensions (5'-TTCACATATGAGCGCGGAGGACTACGGG-3' for *YPT31*, 5'-GTTTCCACATATGAGCAACG-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 polymerase, the PCR fragments were first inserted into the *SmaI* site of BluescriptII KS(+) (Stratagene) for sequence analysis. *YPT31* and *YPT32* were then digested using *BamHI-NdeI* (*YPT31* was partially digested by *NdeI*) and cloned into the vector pET11-a 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 polyclonal 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 dh5 $\alpha$  following the method of Kömer *et al.* (1991). The fusion protein was purified by preparative SDS-PAGE, electroeluted and injected into rabbits. Antibodies against vacuolar CPY were generated from commercially available 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 GADDVENYGTVI (BiP/Kar2p) and IKQKFPNDANAESASNLQEL (Kex2p), respectively. For the generation of anti-Sed5p antibodies, a His<sub>6</sub>-tagged Sed5p lacking the C-terminal membrane anchor (amino acids 1–320) was produced in *E. coli*. Polyclonal 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, SMM (Reid, 1983), 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 OD<sub>600</sub> units) by centrifugation, resuspended in 250–500  $\mu$ l of growth

medium containing 0.5 mg ovalbumin/ml and pulse-labelled with 250  $\mu$ Ci Tran <sup>35</sup>S-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 azide and PMSF (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 mannosyl residues, the radiolabelled CPY was immunoprecipitated and further treated with anti- $\alpha$ 1-6 Man or  $\alpha$ 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 *ypt31-1* 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 min 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  $\Delta$ *ypt32/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 OD<sub>600</sub>/ml in 0.1 M sodium acetate and the samples divided into two portions. The equivalent of 0.01–0.2 OD<sub>600</sub> units of cells was assayed for periplasmic invertase and for total invertase activity as described by Johnson *et al.* (1987) and Goldstein and Lampen (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 OD<sub>600</sub> unit of cells.

The different stages of glycosylation and secretion of active invertase in *S. cerevisiae* were analysed as follows: cells were grown in YEPDgal medium to an OD<sub>600</sub> of 2. The cells were then transferred to YEPD medium for different times and finally transferred for 1 h to 0.1% glucose-containing medium in order to derepress the synthesis of extracellular invertase (Esmon *et al.*, 1981). The preparation of spheroplasts and fractionation were performed according to Schauer *et al.* (1985). One OD<sub>600</sub> 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<sup>TM</sup>-conjugated goat anti-rabbit IgG (H+L) from Jackson Immuno Research Laboratories, Inc., served as secondary antibody.

#### Light and electron microscopy

To visualize vacuoles, 500  $\mu$ l samples were taken from yeast cultures and treated for 30 min with CDCFDA as described by Roberts *et al.* (1991). The labelled cells were adhered to concanavalin A-treated (1 mg/ml) coverslips and viewed using a Zeiss Axiophot 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 McDonald (1984). The primary fixative consisted of 2% glutaraldehyde in a 50 mM KPO<sub>4</sub> (pH 7.0) buffer containing 1 mM CaCl<sub>2</sub> 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% OsO<sub>4</sub> + 0.8% (w:v) K<sub>4</sub>Fe(CN)<sub>6</sub> in 50 mM phosphate buffer]. The spheroplasts were resuspended and allowed to stand for 5 min at room temperature, before pelleting and renewed replacement of the second fixative. This procedure was repeated after 10, 25 and 90 min. The spheroplasts were then washed in distilled H<sub>2</sub>O (3 $\times$ 10 min) and transferred to 2% aqueous uranyl acetate for 12 h at 14°C. After rewashing in distilled H<sub>2</sub>O (2 $\times$ 10 min), the spheroplasts were embedded in 3% low melting point agar. The agar blocks were taken through a graded acetone 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 AFS cryosubstitution apparatus (Reichert, Vienna, Austria) containing acetone at  $-85^{\circ}\text{C}$  with 4% OsO<sub>4</sub>. Over a 90 h period, the specimens were slowly brought to  $-20^{\circ}\text{C}$  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.

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