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Specific binding to a novel and essential Golgi membrane protein (Yip1p) functionally links the transport GTPases Ypt1p and Ypt31p

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The regulation of vesicular transport in eukaryotic cells involves Ras-like GTPases of the Ypt/Rab family. Studies in yeast and mammalian cells indicate that individual family members act in vesicle docking/fusion to specific target membranes. Using the two-hybrid system, we have now identified a 248 amino acid, integral membrane protein, termed Yip1, that specifically binds to the transport GTPases Ypt1p and Ypt31p. Evidence for physical interaction of these GTPases with Yip1p was also demonstrated by affinity chromatography and/or co-immunoprecipitation. Like the two GTPases, Yip1p is essential for yeast cell viability and, according to subcellular fractionation and indirect immunofluorescence, is located to Golgi membranes at steady state. Mutant cells depleted of Yip1p and conditionally lethal *yip1* mutants at the non-permissive temperature massively accumulate endoplasmic reticulum membranes and display aberrations in protein secretion and glycosylation of secreted invertase. The results suggests for a role for Yip1p in recruiting the two GTPases to Golgi target membranes in preparation for fusion.

Keywords: Golgi/secretion/two-hybrid system/vesicular protein transport/Ypt/Rab GTPase

Introduction

Both protein and membrane traffic between the organelles of the secretory and endocytic pathways involve complex regulatory mechanisms. They ensure specificity and directionality of vesicular protein flow as well as a dynamic balance of membrane material between the organelles involved. Genetic and biochemical studies with unicellular yeast and with many specialized mammalian cells revealed that a multitude of proteins, either specific for a particular transport step or with similar function in different stages of transport, participate in vesicular trafficking (Rothman and Wieland, 1996). Several of these proteins are evolutionarily highly conserved (Bennett and Scheller, 1994). Among them are the monomeric GTPases of the Ypt/Rab family that play a decisive role in transport vesicle docking and/or membrane fusion (Lazar *et al.*, 1997; Novick and Zerial, 1997). Their critical function is clearly demonstrated in yeast, as cells depleted of GTPases that act at different stages of the biosynthetic pathway lose viability (Schmitt *et al.*, 1986; Salminen and Novick, 1987; Benli *et al.*, 1996). Although there is evidence for a role of Ypt/Rab GTPases in the priming and pairing of vesicular and target membrane receptors, SNAREs (Lian *et al.*, 1994; Søgaard *et al.*, 1994; Lupashin and Waters, 1997; Mayer and Wickner, 1997), GTPases have not been found in docking/fusion complexes isolated from detergent-lysed cells (Søgaard *et al.*, 1994). This suggests that interactions of transport GTPases with components of the vesicle docking/fusion machinery are short-lived and difficult to detect by biochemical means.

Another technique for detecting specific protein–protein interactions, the two-hybrid system, has also been applied to discover proteins that bind to Ypt/Rab GTPases. Activated, i.e. primarily GTP-bound, forms of several Rab proteins have thus been found to bind to putative effectors, Rab5p to Rabaptin-5 (Stenmark *et al.*, 1995), Rab8p to a Golgi-localized protein kinase (Ren *et al.*, 1996), Rab9p to an endosome-associated 40 kDa protein (Diaz *et al.*, 1997) or Rab6p to a kinesin-related, Golgi-associated protein (Echard *et al.*, 1998). The activated GTPases appear to recruit all of these proteins to the correct membrane, be it a vesicular, donor or acceptor membrane. As transport GTPases, apparently complexed with GDI (GDP dissociation inhibitor) (Soldati *et al.*, 1994; Ullrich *et al.*, 1994), bind to specific membranes on exocytic or endocytic organelles, it seems most likely, but has not been proven, that organelle-specific GTPase-binding proteins exist. The two-hybrid system could also be of value in identifying such putative receptors.

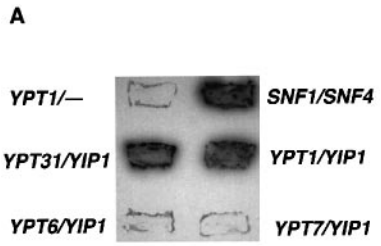
With this in mind, we initiated a two-hybrid screen with the yeast GTPases Ypt1p and Ypt31p which are essential for endoplasmic reticulum (ER) to Golgi and intra-Golgi transport (Lazar *et al.*, 1997). An integral membrane protein of 27 kDa, Yip1p, was discovered that specifically binds the two wild-type GTPases, but not Ypt6p or Ypt7p. The functional properties of this essential protein suggest its involvement in specific membrane binding of two Ypt GTPases that act in consecutive stages of the biosynthetic pathway.

Results

Identification of a novel protein that specifically interacts with Ypt1 and Ypt31 GTPases

Previous attempts in our laboratory to identify, by affinity chromatography, proteins that physically interact with yeast transport GTPases of the Ypt family failed. Likewise, Ypt1p could not be detected in docking complexes of ER-derived vesicles at their target Golgi compartment (Søgaard *et al.*, 1994).

We therefore searched for Ypt1- and Ypt31-interacting proteins using the two-hybrid system (Fields and Song, 1989). In separate experiments, fusions of the Gal4 DNA-



B

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  1 MSFYNTSNNA NNGGGFYQPS AQFAVPQGSM
 31 SFQNTVGSSN TGNDNNLGVA PDPLPVGILH
 61 ALSTKGYPHE PPLLEGIGIN FDHIITKTKM
 91 VLIPIRVGSG VPQEILNDSD LAGPLIFFLL
121 FGLFLLMAGK VHFGYIYGVA LFGTISLHNL
151 SKLMSNNDTS TQTNLQFFNT ASILGYCFLP
181 LCFLSLLGIF HGLNNTTGYV VSVLFVIWST
211 WTSSGFLNSL LQLQNARLLI AYPLLIFYSV
241 FALMVIFV
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Fig. 1. Identification and interaction specificity of Yip1p in the two-hybrid system. (**A**) Wild-type GTPases Ypt1, Ypt31, Ypt6 and Ypt7 fused to the Gal4 DNA-binding domain were used as bait, and Yip1p lacking the N-terminal 11 amino acids and fused to the Gal4 transcription activating domain was used as prey in the yeast two-hybrid analysis to detect β-galactosidase activity. Fusions of the Gal4 domains to the protein kinase Snf1 and its activating subunit Snf4 were used as positive control. The lack of transcription activation by Ypt GTPases alone is shown for Ypt1p. (**B**) Primary sequence of Yip1p. The putative membrane-spanning sequences are underlined. Amino acid substitutions of the temperature-sensitive mutants *yip1-1* (P114L, G129E) and *yip1-2* (G175E) are shown.

binding domain to either Ypt1 or Ypt31 wild-type protein were screened for binding partners expressed from yeast cDNAs fused to the transcription activation domain-encoding *GAL4* gene fragment (gift of S.J.Elledge). In the case of Ypt1p, eight individual recombinant plasmids recovered from ~2.5×10^6 original transformants survived several verification tests for apparently true positives. Of these, one plasmid was recovered twice and, as shown by DNA sequence analysis, expressed a fusion with a 237 amino acid protein fragment. This protein was termed Yip1 (Ypt-interacting protein). Surprisingly, Yip1p was also found 20 times among 38 positive clones in a parallel screen with the Ypt31 GTPase as a bait. The *YIP1* gene was isolated from a genomic library, sequenced and shown to encode a 248 amino acid protein (DDBJ/EMBL/GenBank accession No. X97342). Yip1p has a molecular mass of 27.07 kDa and contains three putative membrane-spanning domains (Figure 1B). It is not significantly related to any other *Saccharomyces cerevisiae* protein. In an assessment of the specificity of the protein interactions observed, two other Ypt GTPases, Ypt6p (Li and Warner, 1996; Tsukada and Gallwitz, 1996) and Ypt7p (Wichmann *et al*., 1992), were found not to interact with Yip1p in the two-hybrid system (Figure 1A).

Yip1p is an essential protein and involved in vesicular transport

Two strategies were followed to assess the function of *YIP1*: gene disruption and the analysis of conditional mutants. First, the gene on one chromosome VII was knocked out in a diploid strain by deleting a 575 bp fragment including codons 1–190 and replacing it by the *URA3* marker gene (Figure 9A). Cells of a *yip1* deletion strain were sporulated and subjected to tetrad analysis. It was found that all four spores were able to germinate, but only two formed colonies and these were Ura$^-$, showing that *YIP1* is essential for cell growth and proliferation. We then created conditional lethal mutants (i) by PCR mutagenesis and (ii) by placing *YIP1* under transcriptional control of the regulatable *GAL10* promoter (Figure 9B and C), allowing us to deplete cells of Yip1p in glucose-containing medium.

Cells containing the *GAL10* promoter-regulated *YIP1* grew normally in galactose-containing medium but ceased proliferation 10–12 h after shift to glucose (data not shown). As shown in Figure 2A, cells depleted of Yip1p accumulated the unprocessed proforms of several vacuolar hydrolases that pass through the ER and the Golgi compartments on the way to their final destination. The transport inhibition resembled that of a *sec18* mutant which, at the non-permissive temperature, completely abolishes ER-to-Golgi vesicular traffic (Novick *et al.*, 1981; Graham and Emr, 1991). A severe inhibition of vacuolar enzyme maturation was also seen in pulse–chase experiments performed with two temperature-sensitive *yip1* mutants having different Yip1 amino acid substitutions (Figure 1B). As shown in Figure 2B and C, after a 15 min pulse of wild-type cells with ^{35}S-labelled amino acids, two proforms of vacuolar carboxypeptidase Y (CPY), the core-glycosylated ER form (p1) and the Golgi-modified form (p2), could be distinguished easily from the mature form (m) which is generated by proteolytic cleavage upon arrival of the p2 form in the vacuole. After a 30 min chase, the proforms were completely matured at 25 and 36°C. In *yip1-1* mutant cells (Figure 2B), the maturation of CPY and of the vacuolar alkaline phosphatase (ALP) was severely impaired already at the permissive and, to a comparable extent, at non-permissive temperature. In contrast, in *yip1-2* mutant cells (Figure 2C), the maturation of CPY at the permissive temperature (25°C) was only slightly disturbed, but was completely inhibited at 36°C. As shown by electrophoretic mobility and the lack of Golgi-acquired α1-6- and α1-3-linked mannosyl residues, it was the ER form of CPY that was accumulated at 36°C. These results suggested that the loss of Yip1p function results in a protein transport defect at an early stage(s) in the biosynthetic pathway.

In following the processing and secretion of invertase by activity staining in non-denaturing gels, we observed that in both conditional lethal *yip1* mutants, especially in *yip1-2*, part of the enzyme accumulated inside the cell in its ER core-glycosylated form. However, the bulk of invertase was severely underglycosylated and transported efficiently to the periplasmic space (Figure 3). This somewhat surprising feature is shared by *yip1* and a previously isolated *ypt1* mutant (Becker *et al*., 1991).

In line with the processing and transport defects of proteins passing along the biosynthetic route, cells depleted of Yip1p (Figure 4) and *yip1* mutant cells at the non-permissive temperature (not shown) massively accumulated ER membranes. That the augmented membranes were part of the ER can be seen by their characteristic connections with the nuclear membrane (Figure 4E and F).

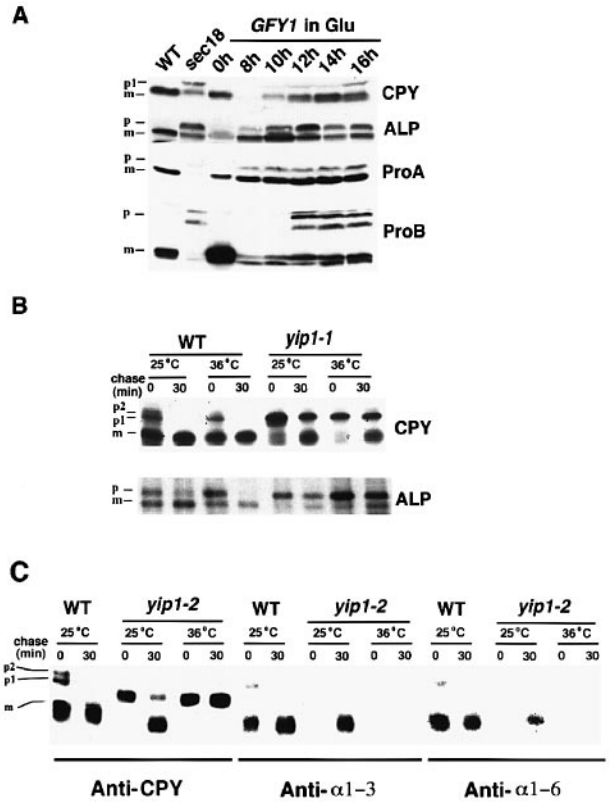

Fig. 2. Inhibition of protein transport in conditional *yip1* mutants. (**A**) Western blot analysis with total cellular proteins of vacuolar soluble proteins (carboxypeptidase Y, CPY, proteinases A and B, ProA/B) and integral membrane hydrolases (alkaline phosphatase, ALP) in the yeast strain GFY1 at different times (h) after shift from galactose- to glucose-containing growth medium which resulted in transcriptional silencing of the *GAL10* promoter-controlled *YIP1* gene. p1, ER core-glycosylated CPY; p, ER- and Golgi-modified proforms; m, mature form of enzymes generated after arrival in the vacuole. Proteins of wild-type (WT) and *sec18* heat-sensitive cells (1 h after shift to non-permissive conditions) were used as controls for normal and inhibited protein transport through the secretory pathway. (**B** and **C**) Pulse–chase experiments with wild-type (WT) and *yip1-1* and *yip1-2* temperature-sensitive mutants at the designated temperatures. Cells were labelled with [^{35}S]amino acids for 15 min and chased for 30 min with cold methionine and cysteine. CPY and ALP were immunoprecipitated, resolved by SDS–PAGE and identified by fluorography. In (C), anti-CPY immunoprecipitates were divided into three equal portions and immunoprecipitation was performed again with antibodies against CPY, α1-3- and α1-6-linked mannosyl residues, respectively. ER core-glycosylated (p1), Golgi-glycosylated (p2) and mature (m) CPY as well as unprocessed (p) and mature (m) ALP could be resolved electrophoretically due to their different molecular masses.

Increased ER membrane proliferation in temperature-sensitive *yip1* mutants was observed as early as 30 min after shift to restrictive conditions. In Yip1p-depleted cells, ER membranes frequently formed multi-layered aggregates (Figure 4D).

Yip1 is an integral membrane protein

A polyclonal antibody was generated against the His_6-tagged N-terminal 106 amino acid-comprising Yip1p fragment to investigate the intracellular localization of the protein. As suggested by its primary structure (Figure 1B), Yip1p has all the properties of an integral membrane protein, with the predicted membrane-spanning domains residing in the C-terminal half of the molecule. To prove

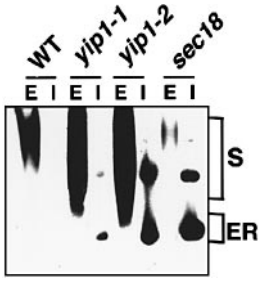

Fig. 3. Fate of secreted invertase in conditionally lethal *yip1* mutants. Activity staining of invertase after separation of periplasmic (E) and intracellular (I) protein fractions in non-denaturing gels. Highly glycosylated invertase (S) is secreted from wild-type cells (WT) and hypoglycosylated invertase from *yip1-1* and *yip1-2* mutant cells (shifted to non-permissive temperature for 1 h in 0.1% glucose medium). Accumulation of intracellular, ER core-glycosylated invertase (ER) in *sec18* mutant cells (1 h at 37°C) served as a control for transport inhibition.

this, cell lysates were incubated on ice in the presence of 5 M urea or 1% Triton X-100, and in high salt or at alkaline pH. After centrifugation at 100 000 *g*, a significant part of Yip1p was detected in the soluble fraction after detergent treatment only (Figure 5A), indicating that this protein is indeed inserted into membranes. It can also be seen from Figure 5A that solubilization with detergent resulted in partial degradation of Yip1p.

The availability of an anti-Yip1p antibody directed against the hydrophilic N-terminal half of Yip1p allowed us to determine the membrane topology of the protein. After careful cell lysis, and the removal of unbroken cells and cell debris, the cellular membranes and organelles precipitating at 100 000 *g* were treated with proteinase K in the presence and absence of detergent. Proteins were then precipitated with trichloroacetic acid (TCA) and subjected to Western blot analysis using anti-Yip1p antibodies or antibodies directed against the Golgi protein Emp47p (Schröder *et al.*, 1995). Emp47p is a type-I integral membrane protein with a C-terminally located membrane-spanning domain. The lumenally oriented N-terminal portion of the protein should therefore be protected against protease digestion. As predicted, Emp47p was digested by proteinase K only after detergent treatment. In contrast, the N-terminal region of Yip1p was digested regardless of whether the P100 fraction was treated with detergent or not (Figure 5B). This shows that the N-terminus of Yip1p faces the cytoplasm.

Yip1p is localized to the Golgi apparatus

To investigate the intracellular localization of Yip1p, subcellular fractionations and indirect immunofluorescence were performed. On differential fractionation of cell lysates, Yip1p was found exclusively in fractions pelleted at either 10 000 or 100 000 *g*, but most of Yip1p was precipitable at 100 000 *g*, like the late Golgi protease Kex2p (Graham and Emr, 1991). Interestingly, high levels of expression of Yip1p from a multicopy plasmid led to the appearance of a sizeable proportion of this protein in the subcellular fraction sedimenting at 10 000 *g*, which is enriched for ER harbouring the Kar2 protein and for vacuolar membranes containing ALP (Figure 6A). Sucrose gradient centrifugation of cell lysates revealed that most of the Yip1 protein in wild-type cells co-sedimented with

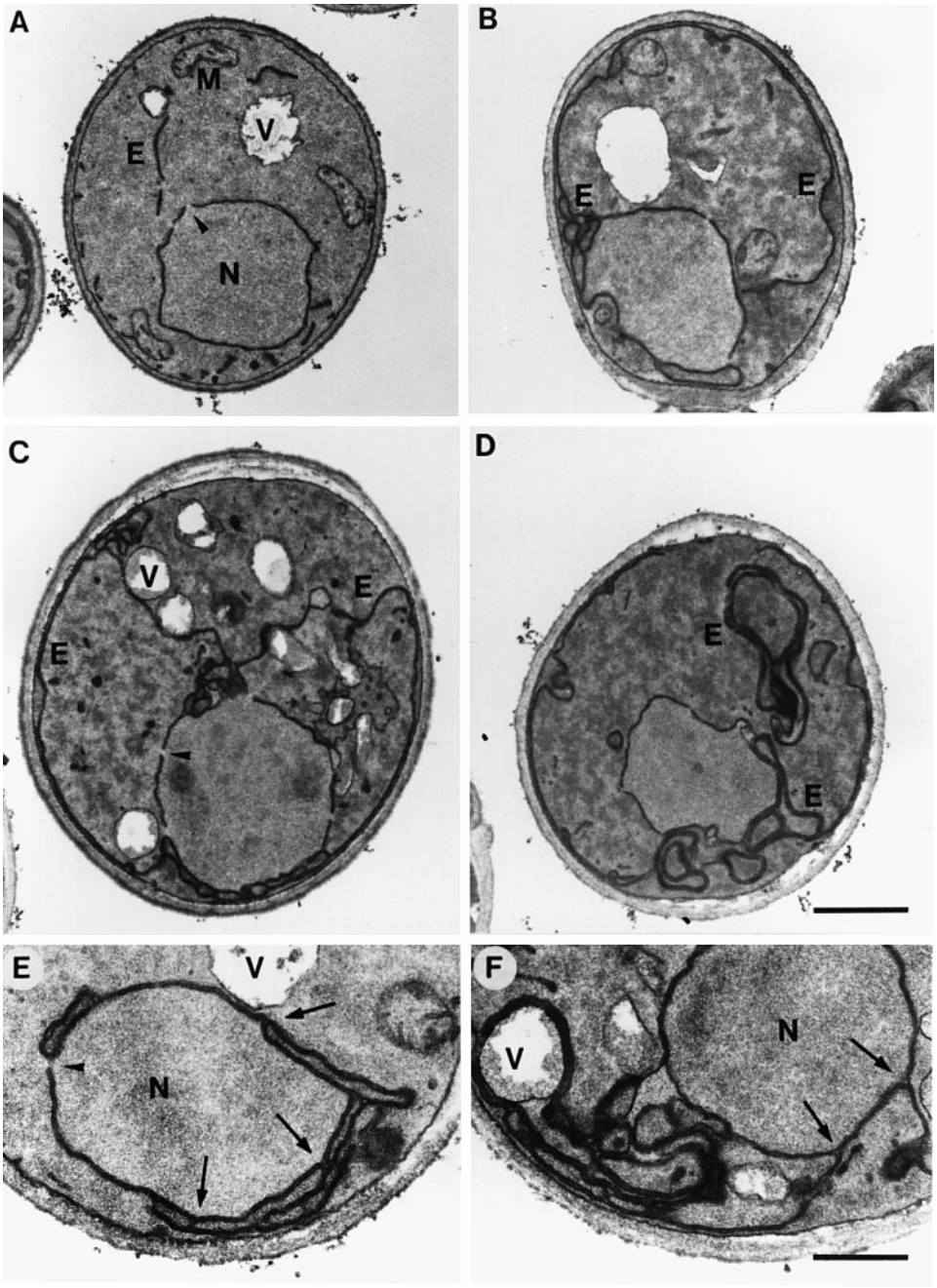

Fig. 4. Accumulation of ER in cells depleted of Yip1 protein. A yeast strain carrying the *YIP1* gene under transcriptional control of the *GAL10* promoter was shifted from galactose- (**A**) to glucose-containing medium for 10 h (**B** and **E**) or 16 h (**C**, **D** and **F**), and cells were fixed with potassium permanganate and subjected to electron microscopic analysis. Arrows point to connections between the ER and nuclear membranes, arrowheads to nuclear pores. N, nucleus; V, vacuole; M, mitochondrion; E, endoplasmic reticulum. The bars in (A–D) and in (E and F) represent 1 and 0.5 μm, respectively.

the Kex2 protease and, in part, with the *cis*-Golgi transport vesicle receptor Sed5p (Hardwick and Pelham, 1992), but not with ER and vacuole membrane markers (Figure 6B).

As shown by indirect immunofluorescence (Figure 7), Yip1p in wild-type cells exhibited a punctate staining pattern typical for Golgi-localized proteins. However, on high expression, Yip1p staining was seen primarily as a perinuclear ring, suggesting ER localization. The phenomenon whereby high levels of expression of Golgi proteins can lead to their accumulation in the ER has been observed previously (Munro, 1991; Machamer *et al.*, 1993). Most importantly, by double immunofluorescence using polyclonal anti-Yip1p antibodies and monoclonal anti-Myc epitope antibodies to identify C-terminally Myc-tagged Emp47p, an almost perfect co-localization of Yip1p and Emp47p was observed (Figure 7E and F). Emp47p was shown previously to be associated primarily with medial-Golgi membranes in logarithmically growing cells (Schröder *et al.*, 1995).

Taken together, these results show that Yip1p, at steady state, is an integral Golgi membrane protein and on high expression can be enriched in the ER.

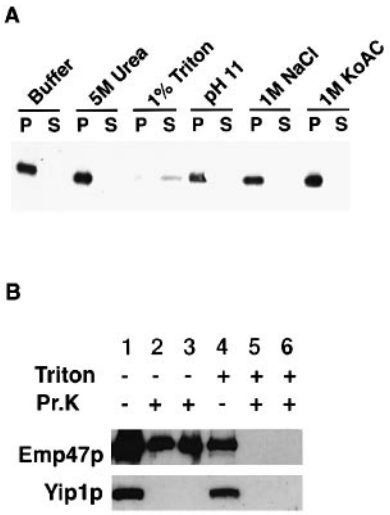

Fig. 5. Membrane localization and topology of Yip1p. (**A**) Logarithmically grown cells were disrupted with glass beads, and the cell lysate (500 *g* supernantant) was treated for 30 min on ice as indicated. After centrifugation at 100 000 *g* for 1 h, soluble and pelleted proteins were separated by SDS–PAGE and subjected to immunoblot analysis using anti-Yip1p antibody. (**B**) Cells were lysed carefully in a Dounce homogenizer, and cellular membranes were precipitated at 100 000 *g*, resuspended in buffer A in the absence or presence of 1% Triton X-100, and incubated without (lanes 1 and 4) or with proteinase K for 30 min (lanes 2 and 5) or 60 min (lanes 3 and 6). Proteins were TCA precipitated, resolved by SDS–PAGE and probed with anti-Emp47p or anti-Yip1p antibodies.

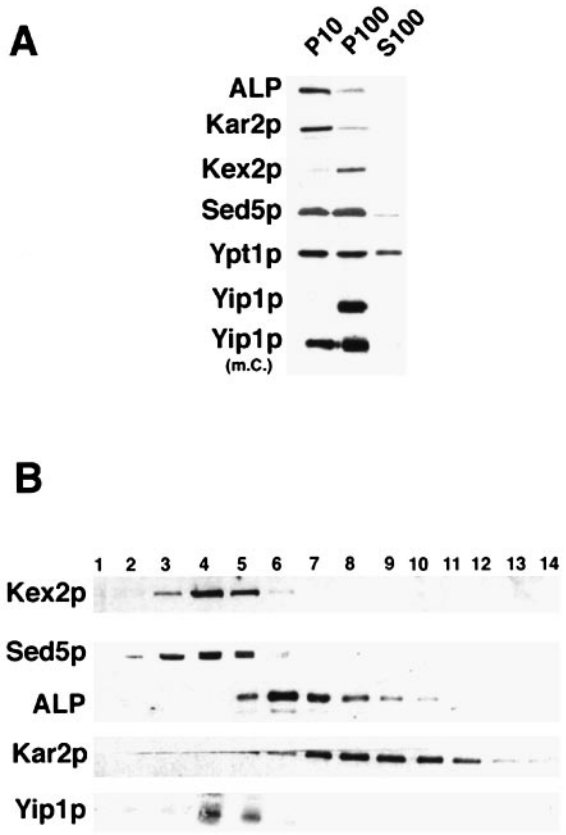

Fig. 6. Intracellular location of Yip1p. Wild-type cells were disrupted with glass beads and subjected to centrifugation at 500 *g* to remove unbroken cells and cell debris. The supernatant was fractionated by differential centrifugation at 10 000 and 100 000 *g* (**A**) or by sucrose gradient centrifugation (**B**). Aliquots of fractions were subjected to SDS–PAGE and Western blot analysis with antibodies against the marker proteins shown to the left. The gradient fractions in (B) are numbered from the lowest (fraction 1) to highest (fraction 14) sucrose density.

Physical interactions of Yip1p with Ypt1 and Ypt31 GTPases

Having shown that protein transport is defective in *yip1* mutants and that these defects were clearly associated with an early step(s) of the secretory pathway, attempts were made to help elucidate the functional relationship of the essential Yip1 protein and the transport GTPases Ypt1 and Ypt31. As the combination of conditional mutations in two separate but functionally related genes is often lethal, we searched for synthetic lethality after crossing the *yip1-1* and the *yip1-2* mutant with either of the heat-sensitive $ypt1^{A136D}$ (Jedd *et al.*, 1995) and $ypt31^{K127N}$ mutant strains (Benli *et al.*, 1996). No haploid *yip1* mutant cell was viable at the otherwise permissive temperature of 25°C when it also carried the *ypt1* or *ypt31* mutant allele, providing further evidence for the functional interplay in protein transport of Yip1p and the two GTPases.

To corroborate the results of the two-hybrid analyses which suggested specific physical interaction of Yip1p with Ypt1p and Ypt31p, we prepared a soluble GST fusion protein that contained the hydrophilic part of Yip1p, termed $Yip1^{N}p$ (amino acid residues 1–99; Figure 1B). The GST–$Yip1^{N}$ fusion protein bound to glutathione–Sepharose-4B (Figure 8B) was used as the affinity matrix in binding experiments with total protein of detergent-lysed yeast cells. As can be seen in Figure 8C, Ypt31p was bound efficiently to the N-terminal hydrophilic domain of Yip1p. Ypt1p could also be detected, but only as a faint band. In this experiment, Ypt7p was not found among the proteins bound to the affinity matrix, and neither of the GTPases was retained by GST alone. These results perfectly mirrored those obtained by a two-hybrid analysis which showed that Ypt31p but not Ypt1p bound efficiently to the N-terminal domain of Yip1p. Both GTPases, however, interacted similarly well with the complete Yip1p (Figure 8A).

To ascertain the interaction of Ypt1p and Yip1p, an affinity-purified anti-Ypt1p antibody was covalently bound to protein A–Sepharose beads and used to co-immunoprecipitate Yip1p from a cleared detergent lysate. Yip1p was in fact found in the immunoprecipitate (Figure 8D). Importantly, Ypt31p was also found in the immunoprecipitate obtained with anti-Ypt1p antibodies (Figure 8D), indicating that Yip1p might be able to bind the two GTPases at the same time.

These results suggest that although Ypt31p and Ypt1p bind to the integral membrane protein Yip1p, both GTPases appear to have other sequence requirements for efficient Yip1p binding.

Discussion

According to present knowledge, Ypt/Rab GTPases are essential regulators of membrane transport at defined stages of secretory and endocytic transport routes (Pfeffer, 1996; Lazar *et al.*, 1997; Novick and Zerial, 1997). It therefore came as a surprise when we discovered Yip1p to be an integral membrane protein that specifically binds two different transport GTPases, Ypt1p and Ypt31p. As

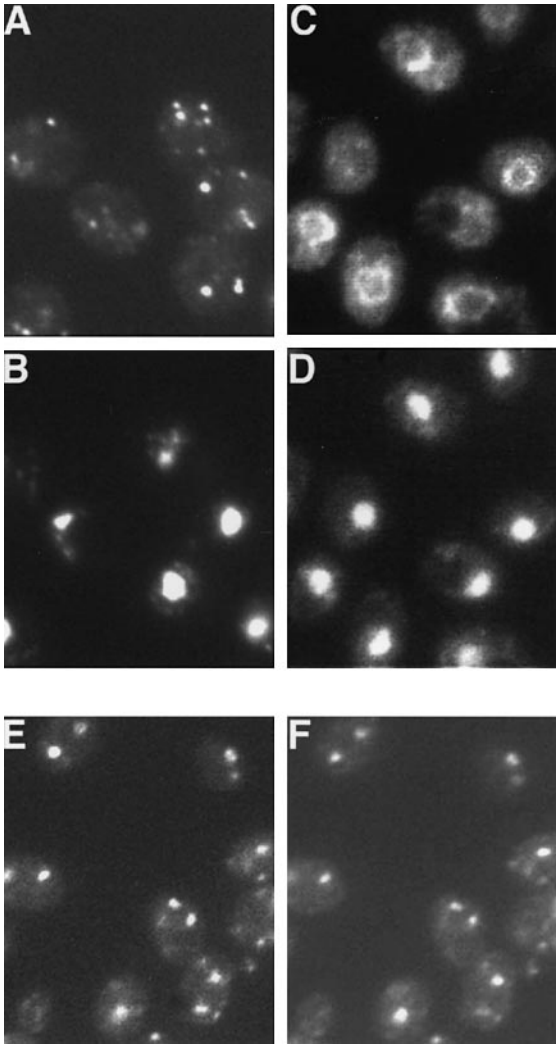

Fig. 7. Localization of Yip1p by indirect immunofluorescence microscopy. Paraformaldehyde-fixed spheroplasts from wild-type cells (**A** and **B**) and from cells of the same strain expressing *YIP1* from a 2 μ-based multicopy vector (**C** and **D**) were treated with affinity-purified anti-Yip1p antibody (A and C). DAPI staining was performed to identify the nuclear region (B and D). Spheroplasts of yeast cells expressing the C-terminally Myc-tagged Golgi protein Emp47p (strain RH3047) were challenged with polyclonal anti-Yip1p antibody and a monoclonal anti-Myc epitope antibody, and then treated with Cy3™- and Cy2™-conjugated second antibodies. Almost perfect co-localization of Yip1p (**E**) and Emp47p (**F**) is observed.

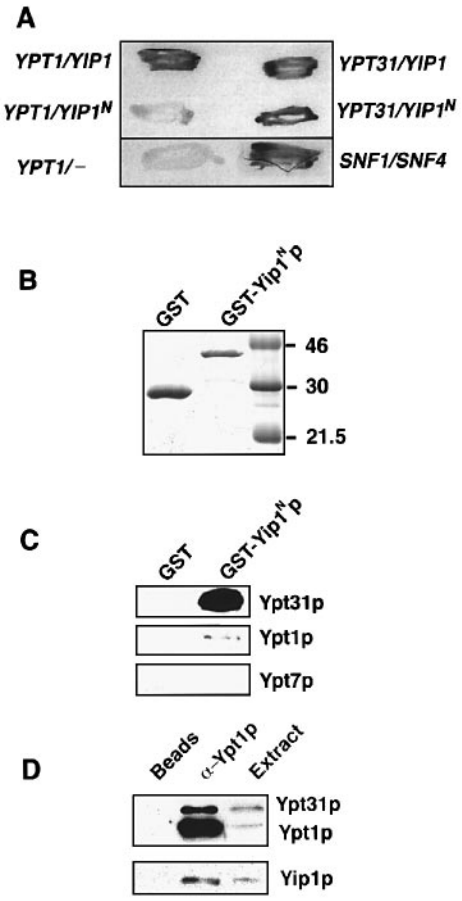

Fig. 8. Specific binding of Yip1p with Ypt31p and Ypt1p. (**A**) Two-hybrid analysis with wild-type Ypt1p and Ypt31p as bait, and with either Yip1p (lacking the N-terminal 11 amino acids) or Yip1^{N}p (amino acids 1–99) as prey. The controls were as in Figure 1. (**B**) GST or a GST–Yip1^{N} fusion protein was expressed in yeast and purified on glutathione–Sepharose-4B beads. Proteins bound to the beads were stained with Coomassie Blue. Molecular mass markers are shown to the right. (**C**) After incubating the beads with proteins of detergent-lysed cells and extensive washing, proteins bound to the beads were separated by SDS–PAGE and subjected to Western blot analysis with antibodies specific for either Ypt31p, Ypt1p or Ypt7p. (**D**) Beads without or with covalently attached anti-Ypt1p antibodies were incubated with a cleared detergent lysate and washed extensively. Proteins bound were separated by SDS–PAGE and searched for Ypt1p, Ypt31p and Yip1p using specific antibodies. Total proteins of alkali-lysed cells (extract) were separated electrophoretically in the same gel to identify the positions of Ypt1p, Ypt31p and Yip1p.

Ypt1p (Segev *et al.*, 1988) and Ypt31p (Benli *et al.*, 1996; Jedd *et al.*, 1997) are bound primarily to Golgi organelles at steady state, we considered the possibility that Yip1p would also be a Golgi-bound protein. According to sub-cellular fractionations and indirect immunofluorescence, this appears to be the case. It is therefore tempting to speculate that Yip1p acts to recruit specifically Ypt1p and Ypt31p to Golgi membranes, a function expected for the often discussed GTPase receptors (Lazar *et al.*, 1997). Studies in mammalian cells have shown that the GDP-bound forms of Rab5p and Rab9p, complexed with GDI, are directed to their target membranes before the GTPases are activated by GDP to GTP exchange (Soldati *et al.*, 1994; Ullrich *et al.*, 1994). As there are a multitude of Ypt/Rab proteins but only a limited number of GDIs (in fact there is only one GDI in yeast; Garrett *et al.*, 1994), specific membrane binding of the transport GTPases cannot be mediated by GDI. Instead, one of the binding determinants appears to be the highly variable region of the C-terminal 40 amino acids of Ypt/Rab proteins (Chavrier *et al.*, 1991). In addition to recognizing certain structural features of the transport GTPase to be bound, a receptor would also be expected to associate preferentially with the GDP-bound form of the GTPase and to have GDI-displacing activity. Although we have not yet analysed in detail whether Yip1p has such properties, we have noted that mutant versions of Ypt1p and Ypt31p deficient in GTP hydrolytic activity (Q to L substitution in the nucleotide-binding domain G3, WDTAGQE) do not interact with Yip1p in the two-hybrid system. This preliminary result suggests a preference of Yip1p for binding Ypt1p and Ypt31p in their GDP-bound conformation. If this were the

case, the Yip1p–GTPase complex could furnish the binding site for a specific guanine nucleotide exchange factor (GEF) like Sec2p, the GEF for the Ypt GTPase Sec4p. Sec2p is a cytosolic protein that stimulates GDP/GTP exchange on membrane-associated Sec4p (Walch-Solimena *et al.*, 1997). Using a cell-free fusion assay, it has been shown that Ypt7p, a GTPase required for vacuole–vacuole fusion (Haas *et al.*, 1995), is involved directly in docking and after-priming of v- and t-SNAREs for interaction on the membrane-enclosed compartments to be fused (Ungermann *et al.*, 1998). It is possible that the activated forms of Ypt1p and Ypt31p recruit additional components and promote the local assembly of protein complexes needed for successful docking and membrane fusion.

Regardless of whether Yip1p acts as a receptor in the sense discussed above, this protein is essential for cell viability, as are the GTPases it binds. Although studies *in vivo* and *in vitro* have shown that Ypt1p is required for docking of ER-derived transport vesicles to an early Golgi compartment (Schmitt *et al.*, 1988; Segev *et al.*, 1988; Becker *et al.*, 1991; Rexach and Schekman, 1991; Søgaard *et al.*, 1994), the analysis of certain *ypt1* mutants has provided evidence for an additional role of Ypt1p in transport between early Golgi compartments (Bacon *et al.*, 1989; Jedd *et al.*, 1995). The function of Ypt31p (and its homologue Ypt32p) is less clear, but investigations with cells depleted of these essential GTPases and with conditionally lethal *ypt31* (or *ypt32*) mutants point to a role in transport between Golgi organelles or even in the generation of transport vesicles at the most distal Golgi compartment (Benli *et al.*, 1996; Jedd *et al.*, 1997). As we previously pointed out, the biochemical and morphological alterations seen in *ypt31* mutants would also be compatible with a function of the Ypt31/32 GTPases in retrograde Golgi transport (Benli *et al.*, 1996; Lazar *et al.*, 1997). Importantly, however, there appears to be spatial overlap of transport steps in which Ypt1p and Ypt31p are involved. This, in fact, is supported by our finding reported here that the combination of *ypt1* and *ypt31* mutant alleles results in synthetic lethality. The striking co-localization of Yip1p and Emp47 that we observed in a double immunofluorescence analysis appears to indicate that Yip1p is concentrated on medial-Golgi membranes under steady-state conditions. This would follow from previous localization studies of the type I transmembrane protein Emp47p which, although cycling between the Golgi apparatus and the ER, was found to reside primarily on a Golgi compartment harbouring α1,3-mannose-modified oligosaccharides (Schröder *et al.*, 1995). Although vesicular and *cis*-Golgi localization of Yip1p cannot be excluded, medial-Golgi attachment would be compatible with the proposed functioning of Ypt31/32p and Ypt1p. Therefore, the Yip1 protein, by being able to recruit Ypt1p and Ypt31p to Golgi membranes, possibly even at the same time, links the two GTPases in regulating transport to and between Golgi organelles. In line with the proposed role of Yip1p in recruiting Ypt1p and Ypt31p to specific membrane compartments, our preliminary data show that in cells depleted of Yip1p the soluble pool of the two GTPases increases in proportion to the membrane-associated pool.

As expected, cells depleted of Yip1p and *yip1* mutants at the non-permissive temperature are defective in protein transport. ER to Golgi transport was significantly delayed in both heat-sensitive mutants, although to a different extent. ER core-glycosylated forms of CPY and invertase accumulated in the *yip1-2* mutant in particular. It might be that the mutant Yip1 proteins, each carrying amino acid substitutions in a separate putative membrane-spanning domain (Figure 1B), are still partially active but that the additive lesions of Ypt1p- and Ypt31p-requiring functions lead to growth arrest at elevated temperature. A striking feature is the hypoglycosylation of the invertase secreted from the two *yip1* mutants. This could result from a general disturbance of Golgi function, perhaps caused by a failure to deliver or distribute properly enzymes such as the glycosyltransferases to or between different Golgi compartments. Interestingly, we previously observed that a conditionally lethal *ypt1* mutant with an amino acid substitution in the effector loop region (Becker *et al.*, 1991) also secreted underglycosylated invertase efficiently. As our studies indicate that Ypt1p and Ypt31p might bind to different regions of Yip1p, it would be of interest to generate and characterize additional *yip1* mutants. For example, mutants in the N-terminal, hydrophilic half of Yip1p which, as expected, faces the cytoplasm and appears to constitute the principle binding region of Ypt31p, could help to elucidate the function of the Ypt31/32 GTPases further.

Materials and methods

Yeast and bacterial strains, growth media

Saccharomyces cerevisiae strains used in this study are listed in Table I. All the mutants used were derived from the wild-type strains MSUC-1A and MSUC-3D which have been described previously (Benli *et al.*, 1996). These strains were used for crossing, transformation, isolating the haploids carrying desired markers or mutations, sporulation of diploids and tetrad analysis experiments. Cells were grown either in YPD medium (1% yeast extract, 2% peptone and 2% dextrose) or in SD medium containing nutritional supplements (Sherman *et al.*, 1986). *Escherichia coli* strains used were DH5α and XL1 blue (Stratagene).

Two-hybrid analysis

An *Nde*I site was created at the ATG initiation codon of *YPT1* (Wagner *et al.*, 1987) by Kunkel mutagenesis (Kunkel *et al.*, 1987) in pLN-YPT1 to facilitate cloning of the *Nde*I–*Bam*HI fragment of *YPT1* into the DNA-binding domain vector pAS1-CYH2 (a gift from S.J.Elledge). An *Nde*I–*Sal*I fragment of *YPT31* (Benli *et al.*, 1996) was also cloned into the pAS1-CYH2 vector after an internal *Nde*I site had been deleted by silent mutagenesis. The pAS-YPT1 and pAS-YPT31 vectors were transformed separately into the yeast reporter strain Y190 (Harper *et al.*, 1993). The strains containing either pAS-YPT1 or pAS-YPT31 were subsequently transformed with an *S.cerevisiae* cDNA library made in the lambda activation domain vector pACT (a gift from S.J.Elledge) and selected as described (Durfee *et al.*, 1993). The candidates turning blue in the X-Gal filter assay were examined for the loss of pAS-YPT1 and pAS-YPT31 by streaking them on SD (lacking leucine, 2.5 μg/ml cycloheximide) plates, and plasmid loss was verified by replica plating onto SD plates lacking tryptophan and leucine. To verify positive clones further, they were mated with Y187 (Harper *et al.*, 1993) containing pAS derivatives expressing a Gal4(1–147) fusion p53 protein which is considered unrelated to Ypt1p and Ypt31p. Then β-galactosidase activity was tested by the X-Gal filter assay. Clones specific for *YPT1* and *YPT31* were taken up for recovering the plasmids, which were then amplified in *E.coli* strain DH5α.

Construction of recombinant plasmids and mutants

All constructs were made in pBS (KS+) (Stratagene) and amplified in *E.coli*. For gene disruption, the *Xho*I–*Nco*I fragment of *YIP1* was replaced by the *URA3* marker gene (Figure 9A). The recombinant

Table I. Strains used in this study

Strain	Genotype	Source
MSUC-1A	MAT**a** *ura3 leu2 trp1 his3 ade2*	this laboratory
MSUC-3D	MATα *ura3 leu2 trp1 his3 lys2*	this laboratory
MB18	MATα *sec18-1 ura3 leu2 his3*	M.Bielefeld
GFY1	MAT**a** *ura3 leu2 trp1 his3 ade2 LEU2–GAL10→YIP1*	this study
YXY10	MAT**a**/MATα *ura3/ura3 trp1/trp1 his3/his3 leu2/leu2 ade2/ADE2lys2/LYS2 yip1::URA3/YIP1*	this study
YXY11a	*MAT***a** *ura3 leu2 trp1 his3 ade2 yip1-1-TRP1*	this study
YXY11α	MATα *ura3 leu2 trp1 his3 lys2 yip1-1-TRP1*	this study
YXY12a	MAT**a** *ura3 leu2 trp1 his3 ade2 yip1-2-TRP1*	this study
YXY12α	MATα *ura3 leu2 trp1 his3 lys2 yip1-2-TRP1*	this study
YXY20	MAT**a**/MATα *ura3/ura3 trp1/trp1 his3/his3 leu2/leu2 ade2/ADE2lys2/LYS2*	this study
YXY136	*MATα ura3 leu2 trp1 his3 lys2 ypt1^{A136D}-LEU2*	this study
YLX7	MAT**a** *ura3 leu2 trp1 his3 ade2 ypt31^{K127N}-LEU2 ypt32::HIS3*	this laboratory
Y190	MAT**a** *gal4 gal80 his3 trp1 ade2 ura3 leu2 URA3::GAL→lacZ LYS2::GAL→HIS cyh^{r}*	S.J.Elledge
Y187	*MATα gal4 gal80 his3 trp1 ade2 ura3 leu2 URA3::GAL→ lacZ*	S.J.Elledge
BJ5457	MAT**a** *ura3 trp1 lys2 leu2 his3 can1 prb1 pep4::HIS3 GAL*	Yeast Genetic Stock Center
RH3047	MAT**a** *his4 leu2 ura3 lys2 bar1-1 emp47::LYS2 myc-EMP47::LEU2*	Schröder *et al.* (1995)

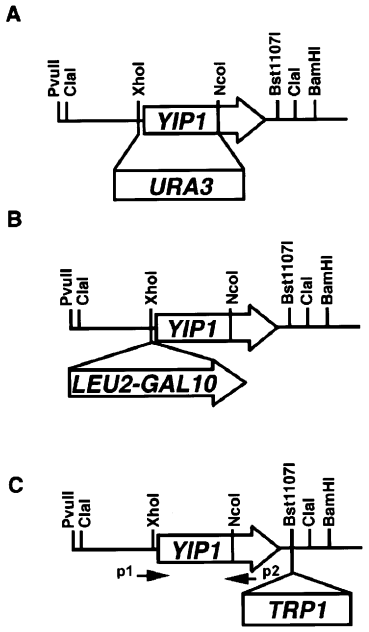

Fig. 9. Schematic representation of *YIP1* gene constructs used. (**A**) Disruption of *YIP1* was achieved by replacing an *Xho*I–*Nco*I fragment with the *URA3* marker gene on a 1.1 kb *Hin*dIII fragment. (**B**) A *LEU2–GAL10* fusion fragment was inserted into the *Xho*I restriction site 6 bp 5′ of the ATG initiation codon to bring *YIP1* under transcriptional control of the *GAL10* promoter. (**C**) The heat-sensitive *yip1-1* mutant was created by PCR mutagenesis using primers p1 and p2. The *yip1-2* mutant (G175E substitution) was created by site-directed mutagenesis. For chromosomal integration of the mutant alleles, the *TRP1* gene was inserted into the *Bst*1107I restriction site 3′ of the stop codon.

plasmid harbouring the disrupted *YIP1* gene was linearized with *Cla*I and transformed into the wild-type diploid strain MSUC-1A/3D to disrupt one chromosomal copy of *YIP1* by homologous recombination. GFY1 (*GAL10* promoter fused to *YIP1*) was constructed by inserting a 2.8 kb *LEU2–GAL10* fragment from the YEp51 vector into the *Xho*I site of *YIP1* (Figure 9B). This construct was digested with *Pvu*II–*Bam*HI and transformed into one of the wild-type MSUC strains. A conditionally lethal mutant, *yip1-1* (YXY11), was created by random PCR mutagenesis as described by Fromant *et al.* (1995). The PCR was carried out in 4 μM dTTP, 0.2 μM dATP, dGTP and dCTP, 10 mM $MgCl_2$ and 0.5 mM $MnCl_2$ at 94°C for 1 min, 55°C for 1.5 min and 72°C for 2 min for 30 cycles using the primers p1, 5′-GACGGGG-AGTACTGCAAGACAC-3′; and p2, 5′-CCAGACGAGGTCCAA-GTACTC-3′. PCR products were digested overnight with *Xho*I–*Nco*I, purified from agarose gels and used to replace the wild-type *YIP1* gene in pBS-YIP1. The plasmids were pooled and linearized with *Cla*I for integration into the genome. The transformants carrying *yip1* mutations were selected by the *TRP1* marker gene which had been inserted into the *Bst*1107I site located 67 bp downstream of the stop codon (Figure 9C). Temperature-sensitive colonies were selected by replica plating and growth at different temperatures. Another conditionally lethal mutant, *yip1-2* (YXY12), was created by site-directed mutagenesis (R.Sternglanz and E.Andrulis, personal communication) in pBS-YIP1. The *ypt1^{A136D}* (YXY136) temperature-sensitive mutant was created as described (Jedd *et al.*, 1995).

Cloning of YIP1 and generation of antibodies

The cDNA clones of *YIP1* identified in the two-hybrid screens with *YPT1* and *YPT31* lacked the codons for the first 11 amino acids. The *YIP1* gene was cloned from a yeast genome library made in YEp13 (Dascher *et al.*, 1991) by colony hybridization. Polyclonal antibodies against a His_6-tagged Yip1p (amino acids 1–106) were raised in rabbits as described (Wagner *et al.*, 1992). Antibodies were purified with the antigen produced in *E.coli* using the AminoLink plus affinity purification system (Pierce).

Subcellular and sucrose gradient fractionation and immunoblot analyses

Yeast cells were harvested at mid-logarithmic phase, the cell pellet was washed with 10 mM cold NaN_3 and resuspended in 3 vols of buffer A [50 mM Tris pH 7.5, 100 mM KCl, 1 mM EDTA, 1 mM dithiothreitol (DTT), 1 mM Pefabloc and proteinase inhibitor mix]. Cells were disrupted with 1 vol. of glass beads and by vortexing six times for 1 min at 4°C. The cell lysate was centrifuged twice at 500 *g* for 5 min to remove cell debris, and the cleared lysate was centrifuged at 10 000 *g* for 15 min to obtain the P10 pellet. The S10 fraction was then subjected to centrifugation at 100 000 *g* at 4°C for 1 h to obtain P100 and S100. The 500 *g* lysate was also subjected to sucrose density gradient centrifugation as previously described (Benli *et al.*, 1996). To investigate membrane localization of Yip1p, the supernatant of the cell lysate after a 500 *g* centrifugation was divided into different portions that were treated for 30 min on ice with either 1% Triton X-100, 5 M urea, 0.1 M sodium carbonate pH 11, 1 M NaCl or 1 M KOAc and then centrifuged at 100 000 *g* to obtain soluble and precipitated proteins. Proteins in different fractions were separated by sodium dodecyl sulfate–gel electrophoresis (SDS–PAGE), and immunoblot analyses were performed using the ECL system (Amersham) and specific antibodies as described (Benli *et al.*, 1996).

Protein labelling, immunoprecipitation and invertase assay

Cells were pulse-labelled for 15 min with *Trans*^{35}S-label (ICN) and chased for 30 min. The labelled proteins were immunoprecipitated using specific antibodies and separated by SDS–PAGE (Benli *et al.*, 1996). After incubating the gel with Amplify (Amersham) for 30–45 min, the proteins were detected by exposing the gels to X-Omat AR (Kodak) at –80°C. Invertase activity staining was carried out as described (Benli *et al.*, 1996).

Indirect immunofluorescence and electron microscopy

Indirect immunofluorescence using rabbit polyclonal anti-Yip1p and monoclonal mouse c-Myc epitope antibodies was performed as described by Schröder *et al.* (1995). Cy3™-conjugated goat anti-rabbit and Cy2™-conjugated goat anti-mouse F(ab′) 2 fragment (Jackson Immuno Research Laboratory Inc.) served as secondary antibody. To study co-localization, a yeast strain expressing C-terminally Myc-tagged Emp47p from the chromosomally integrated mutant gene was used (a gift of S.Schröder). Anti-Myc epitope antibodies were from Santa Cruz Biotechnology. Double immunofluorescence was observed using a Zeiss Axiophot equipped with the appropriate filters. The electron microscopy of potassium permanganate-fixed cells was done as described previously (Benli *et al.*, 1996).

In vitro interaction of GTPases Ypt1p and Ypt31p with Yip1p

Co-affinity purification and immunoprecipitation were carried out to verify the interaction between GTPases Ypt1p and Ypt31p with Yip1p. A DNA fragment encoding the N-terminal 99 amino acids of Yip1p ($Yip1^N$) was cloned in vector NEG-KT, a derivative of pEG-KT (Mitchell *et al.*, 1993), to fuse $Yip1^N$ to the N-terminus of GST. Protein expression and purification were performed as previously described (Grabowski and Gallwitz, 1997). The buffer conditions were (i) disruption buffer: 50 mM Tris–HCl pH 7.5, 100 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, 2% Triton X-100, proteinase inhibitor mix; and (ii) washing buffer: 50 mM Tris-HCl pH 7.5, 1 M KCl, 5 mM $MgCl_2$ and 1 mM DTT. Affinity-purified, polyclonal anti-Ypt1p antibody was coupled to protein A–Sepharose CL-4B (Pharmacia) and cross-linked via the bifunctional coupling reagent, dimethylepimelimidate (DMP), as described by Harlow and Lane (1988). The yeast detergent extract was prepared as described (Søgaard, 1994) and the extract was adjusted to a detergent concentration of 0.5%. It was incubated with GST–$Yip1^N$ or anti-Ypt1p beads at 4°C with end-over-end rotation for 1 h, followed by three washes with phosphate-buffered saline (PBS) buffer with proteinase inhibitor mix. About 50–100 μg of GST–$Yip1^N$, 25–50 μg of antibody and 100 OD_{600} of yeast extract were used in each reaction. A 50 μl aliquot of 2× SDS loading buffer was added to the beads after washing, and the samples were heated for 5 min at 95°C before SDS–PAGE and immunoblot analysis.

Proteinase protection

Spheroplasts from logarithmically grown cells were prepared by digestion with Zymolase 100 T (Seikagaku Corporation). They were suspended in buffer A without proteinase inhibitor mix and lysed with 20 strokes in a Dounce homogenizer. After centrifuging twice at 500 *g*, the supernatant fraction was centrifuged at 100 000 *g* for 1 h, and the pelleted membranes were resuspended in the buffer described above and treated on ice with proteinase K (50 μg/ml) as previously described (Haucke and Schatz, 1997).

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References

Bacon,R.A., Salminen,A., Ruohola,H., Novick,P. and Ferro-Novick,S. (1989) The GTP-binding protein Ypt1 is required for transport *in vitro*—the Golgi apparatus is defective in *ypt1* mutants. *J. Cell Biol.*, **109**, 1015–1022.

Becker,J., Tan,T.J., Trepte,H.H. and Gallwitz,D. (1991) Mutational analysis of the putative effector domain of the GTP-binding Ypt1 protein in yeast suggests specific regulation by a novel GAP activity. *EMBO J.*, **10**, 785–792.

Benli,M., Döring,F., Robinson,D.G., Yang,X. and Gallwitz,D. (1996) Two GTPase isoforms, Ypt31p and Ypt32p, are essential for Golgi function in yeast. *EMBO J.*, **15**, 6460–6475.

Bennett,M.K. and Scheller,R.H. (1994) A molecular description of synaptic vesicle membrane trafficking. *Annu. Rev. Biochem.*, **63**, 63–100.

Chavrier,P., Gorvel,J.P., Stelzer,E., Simons,K., Gruenberg,J. and Zerial,M. (1991) Hypervariable C-terminal domain of rab proteins acts as a targeting signal. *Nature*, **353**, 769–772.

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

Diaz,E., Schimmoller,F. and Pfeffer,S.R. (1997) A novel Rab9 effector required for endosome-to-TGN transport. *J. Cell Biol.*, **138**, 283–290.

Durfee,T., Becherer,K., Chen,P.L., Yeh,H., Yang,Y., Kilburn,A.E., Lee,W.H. and Elledge,S.J. (1993) The retinoblastoma protein associates with the protein phosphatase type 1 catalytic subunit. *Genes Dev.*, **7**, 555–569.

Echard,A., Jollivet,F., Martinez,O., Lacapere,J.J. and Goud,B. (1998) Interaction of a Golgi-associated kinesin-like protein with Rab6. *Science*, **279**, 580–585.

Fields,S. and Song,O. (1989) A novel genetic system to detect protein–protein interactions. *Nature*, **340**, 245–246.

Fromant,M., Blanquet,S. and Plateau,P. (1995) Direct random mutagenesis of gene-sized DNA fragments using polymerase chain reaction. *Anal. Biochem.*, **224**, 347–53.

Garrett,M.D., Zahner,J.E., Cheney,C.M. and Novick,P.J. (1994) *GDI1* encodes a GDP dissociation inhibitor that plays an essential role in the yeast secretory pathway. *EMBO J.*, **13**, 1718–1728.

Grabowski,R. and Gallwitz,D. (1997) High-affinity binding of the yeast *cis*-Golgi t-SNARE, Sed5p, to wild-type and mutant Sly1p, a modulator of transport vesicle docking. *FEBS Lett.*, **411**, 169–72.

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

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

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

Harlow,E. and Lane,O. (1988) *Antibodies: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Harper,J.W., Adami,G.R., Wei,N., Keyomarsi,K. and Elledge,S.J. (1993) The p21 Cdk-interacting protein Cip1 is a potential inhibitor of G1 cyclin-dependent kinases. *Cell*, **75**, 805–816.

Haucke,V. and Schatz,G. (1997) Reconstitution of the protein insertion machinery of the mitochondrial inner membrane. *EMBO J.*, **16**, 4560–4567.

Jedd,G., Richardson,C., Litt,R. and Segev,N. (1995) The Ypt1 GTPase is essential for the first two steps of the yeast secretory pathway. *J. Cell Biol.*, **131**, 583–590.

Jedd,G., Mulholland,J. and Segev,N. (1997) Two new Ypt GTPases are required for exit from the yeast *trans*-Golgi compartment. *J. Cell Biol.*, **137**, 563–580.

Kunkel,T.A., Roberts,J.D. and Zakour,R.A. (1987) Rapid and efficient site-specific mutagenesis without phenotypic selection. *Methods Enzymol.*, **154**, 367–382.

Lazar,T., Goette,M. and Gallwitz,D. (1997) Vesicular transport: how many Ypt/Rab-GTPases make an eukaryotic cell? *Trends Biochem. Sci.*, **22**, 468–472.

Lian,J.P., Stone,S., Jiang,Y., Lyons,P. and Ferro-Novick,S. (1994) Ypt1p implicated in v-SNARE activation. *Nature*, **372**, 698–701.

Li,B. and Warner,J.R. (1996) Mutation of the Rab6 homologue of *Sacharomyces cerevisiae*, *YPT6*, inhibits both early Golgi function and ribosome biosynthesis. *J. Biol. Chem.*, **271**, 16813–16819.

Lupashin,V.V. and Waters,M.G. (1997) t-SNARE activation through transient interaction with a Rab-like guanosine triphosphate. *Science*, **276**, 1255–1258.

Machamer,C.E., Grim,M.G., Esquela,A., Chung,S.W., Rolls,M., Ryan,K. and Swift,A.M. (1993) Retention of a *cis* Golgi protein requires polar residues on one face of a predicted α-helix in the transmembrane domain. *Mol. Biol. Cell*, **4**, 695–704.

Mayer,A. and Wickner,W. (1997) Docking of yeast vacuoles is catalyzed by the Ras-like GTPase Ypt7p after symmetric priming by Sec18p (*NSF*). *J. Cell Biol.*, **136**, 307–318.

Mitchell,D., Marshall,T.A. and Deschenes,R.J. (1993) Vectors for the inducible overexpression of gluthathione *S*-transferase fusion proteins in yeast. *Yeast*, **9**, 715–723.

Munro, S. (1991) Sequences within and adjacent to the transmembrane segment of the α-2,6-sialyltransferase specify Folgi retention. *EMBO J.*, **10**, 3577–3588.

Novick,P. and Zerial,M. (1997) The diversity of Rab proteins in vesicle transport. *Curr. Opin. Cell Biol.*, **9**, 496–504.
Novick,P., Ferro,S. and Schekman,R. (1981) Order of events in the yeast secretory pathway. *Cell*, **25**, 461–469.
Pfeffer,S.R. (1996) Transport vesicle docking: SNAREs and associates. *Annu. Rev. Cell Dev. Biol.*, **12**, 441–461.
Ren,M., Zeng,J., De Lemos-Chiarandini,C., Rosenfeld,M., Adesnik,M. and Sabatini,D.D. (1996) In its active form, the GTP-binding protein Rab8 interacts with a stress-activated protein kinase. *Proc. Natl Acad. Sci. USA*, **93**, 5151–5155.
Rexach,M.F. and Schekman,R.W. (1991) Distinct biochemical requirements for the budding, targeting, and fusion of ER-derived transport vesicles. *J. Cell Biol.*, **114**, 219–229.
Rothman,J.E. and Wieland,F.T. (1996) Protein sorting by transport vesicles. *Science*, **272**, 227–234.
Salminen,A. and Novick,P. (1987) DNA sequencing with chain terminating inhibitors. *Proc. Natl Acad. Sci. USA*, **74**, 5463–5467.
Schmitt,H.D., Wagner,P., Pfaff,E. and Gallwitz,D. (1986) The ras-related *YPT1* gene product in yeast: a GTP-binding protein that might be involved in microtubule organization. *Cell*, **47**, 401–412.
Schmitt,H.D., Puzicha,M. and Gallwitz,D. (1988) Study of a temperature-sensitive mutant of the ras-related *YPT1* gene product in yeast suggests a role in the regulation of intracellular calcium. *Cell*, **53**, 635–647.
Schröder,S., Schimmöller,F., Singer-Krüger,B. and Riezman,H. (1995) The Golgi-localization of yeast Emp47p depends on its di-lysine motif but is not affected by the *ret1-1* mutation in alpha-COP. *J. Cell Biol.*, **131**, 895–912.
Segev,N., Mulholland,J. and Botstein,D. (1988) The yeast GTP-binding Ypt1 protein and a mammalian counterpart are associated with the secretion machinery. *Cell*, **52**, 915–924.
Sherman,F., Fink,G.R. and Hicks,J.B. (1986) *Methods in Yeast Genetics*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Søgaard,M., Ye,K., Tani,R.R., Geromanos,S., Tempst,P., Kirchhausen,T., Rothman,J.E. and Söllner,T. (1994) A rab protein is required for the assembly of SNARE complexes in the docking of transport vesicles. *Cell*, **78**, 937–948.
Soldati,T., Shapiro,A.D., Svejstrup,A.B.D. and Pfeffer,S.R. (1994) Membrane targeting of the small GTPase rab9 is accompanied by nucleotide exchange. *Nature*, **369**, 76–78.
Stenmark,H., Vitale,G., Ullrich,O. and Zerial,M. (1995) Rabaptin-5 is a direct effector of the small GTPase Rab5 in endocytic membrane fusion. *Cell*, **83**, 423–432.
Tsukada,M. and Gallwitz,D. (1996) Isolation and characterization of *SYS* genes from yeast, multicopy suppressors of the functional loss of the transport GTPase Ypt6p. *J. Cell Sci.*, **109**, 2471–2481.
Ullrich,O., Horiuchi,H., Bucci,C. and Zerial,M. (1994) Membrane association of rab5 mediated by GDP-dissociation inhibitor and accompanied by GDP/GTP exchange. *Nature*, **368**, 157–160.
Ungermann,C., Nichols,B.J., Pelham,H.R.B. and Wickner,W. (1998) A vacuolar v/t-SNARE complex, the predominant form *in vivo* and on isolated vacuoles, is disassembled and activated for docking and fusion. *J. Cell Biol.*, **140**, 61–69.
Wagner,P., Molenaar,C.M.T., Rauh,A.J.G., Brökel,R., Schmitt,H.D. and Gallwitz,D. (1987) Biochemical properties of the ras-related *YPT* protein in yeast: a mutational analysis. *EMBO J.*, **6**, 2373–2379.
Wagner,P., Hengst,L. and Gallwitz,D. (1992) Ypt-proteins in yeast. *Methods Enzymol.*, **219**, 369–387.
Walch-Solimena,C., Collins,R.N. and Novick,P.J. (1997) Sec2p mediates nucleotide exchange on sec4p and is involved in polarized delivery of post-Golgi vesicles. *J. Cell Biol.*, **137**, 1495–1509.
Wichmann,H., Hengst,L. and Gallwitz,D. (1992) Endocytosis in yeast: evidence for the involvement of a small GTP-binding protein (Ypt7p). *Cell*, **71**, 1131–1142.

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