Significance of GTP Hydrolysis in Ypt1p-regulated Endoplasmic Reticulum to Golgi Transport Revealed by the Analysis of Two Novel Ypt1-GAPs*

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We here report on the identification and detailed biochemical characterization of two novel GTPase-activating proteins, Gyp5p and Gyp8p, whose efficient substrate is Ypt1p, a Ypt/Rab-GTPase essential for endoplasmic reticulum-to-Golgi trafficking in yeast. Gyp5p accelerated the intrinsic GTPase activity of Ypt1p 4.2×10^4-fold and, surprisingly, the 40-fold reduced GTP hydrolysis rate of Ypt1(Q67L)p 1.5×10^4-fold. At steady state, the two newly discovered GTPase-activating proteins (GAPs) as well as the previously described Gyp1p, which also uses Ypt1p as the preferred substrate, display different subcellular localization. To add to an understanding of the significance of Ypt1p-bound GTP hydrolysis *in vivo*, yeast strains expressing the GTPase-deficient Ypt1(Q67L)p and having different Ypt1-GAP genes deleted were created. Depending on the genetic background, different mutants exhibited growth defects at low temperature and, already at permissive temperature, various morphological alterations resembling autophagy. Transport of proteins was not significantly impaired. Growth defects of Ypt1(Q67L)-expressing cells could be suppressed on high expression of all three Ypt1-GAPs. We propose that permanently active Ypt1p leads to increased vesicle fusion, which might induce previously unnoticed autophagic degradation of exaggerated membrane-enclosed structures. The data indicate that hydrolysis of Ypt1p-bound GTP is a prerequisite for a balanced vesicle flow between endoplasmic reticulum and Golgi compartments.

In eukaryotic cells, a large number of newly synthesized proteins and of proteins internalized from the plasma membrane pass through membrane-enclosed compartments to reach their final destination. Transport between organelles of the biosynthetic and the endocytic pathway involves vesicular intermediates that fuse with specific target compartments to deliver their cargo (1). Transport vesicle formation and targeting require complex molecular machines, and different Ras-like GTPases act as key regulators in assembling specific protein complexes at different donor and target membranes (2–4). Genetic and *in vitro* transport studies in yeast indicate that Ypt/Rab-GTPases act in tethering transport vesicles to their cognate acceptor membranes before SNARE[1] pairing (5, 6), but the molecular details of GTPase function are still unresolved. Of the 11 yeast Ypt/Rab family members, only the ones involved in the secretory pathway are essential for cell viability (7). Among them is Ypt1p, the regulator of forward transport between the ER and the Golgi. Ypt1p appears to exert its vesicle tethering function when bound to the target membrane of early Golgi compartment(s) (8).

Since Ypt/Rab-GTPases, like Ras, cycle between an active, GTP-bound and an inactive, GDP-bound conformation, GDP-GTP exchange by specific exchange factors (guanine nucleotide exchange factors) and hydrolysis of the bound GTP catalyzed by GTPase-activating proteins (GAPs) are expected to play important roles in the functioning of these proteins. The hydrolysis of GTP has been associated with either the membrane fusion process itself, with the recycling of the GTPases, or with the role of the GTPase as a timer for membrane fusion (9, 10). In a perhaps extreme view, it has even been argued that, in the case of Ypt1p, GTP hydrolysis is not important for the protein's function in vesicular transport (11). Although Ypt/Rab-GTPases possess a slow intrinsic GTPase activity, which in most cases is significantly slower than 0.01 min^{-1} at 30 °C (12), effective down-regulation of the regulators requires specific GTPase-activating proteins.

The first Ypt/Rab-specific GAPs, termed Gyp (GAP for Ypt proteins), were isolated from yeast by high expression cloning (13–15). It was subsequently noted that these GAPs contained regions of sequence similarity with many proteins of unknown function from yeast and higher eukaryotes (16). This was also helpful for the identification and cloning of other Ypt/Rab-GAPs from yeast (17–20). So far, six related GAPs from *Saccharomyces cerevisiae* constitute a protein family that we have termed GYP. Most of the yeast Ypt/Rab-GAPs have a rather broad substrate specificity, but all of them are highly active, accelerating the intrinsic GTP hydrolysis rate of their preferred substrates 10^4- to 10^6-fold. Among the six known Gyp proteins, only Gyp1p efficiently uses Ypt1p as substrate *in vitro*, but this GAP accelerates the intrinsic GTPase activities of Ypt51p, Sec4p, and Ypt7p with similar efficiency (18, 20). The overlap-

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[1] The abbreviations used are: SNARE, soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor; GAP, GTPase-activating protein; ER, endoplasmic reticulum; HPLC, high performance liquid chromatography; GFP, green fluorescent protein; CFP, cyan fluorescent protein; YFP, yellow fluorescent protein; P10, pellet after 10,000 × *g* centrifugation; P100, pellet after 100,000 × *g* centrifugation (high speed pellet); VSV, vesicular stomatitis virus.

ping substrate specificity of GYP family members might explain why the chromosomal deletion of individual *GYP* genes is phenotypically neutral. Concerning ER-to-Golgi transport, it was also possible that additional Ypt1-GAPs might participate in the regulation of Ypt1p activity.

We have now isolated two novel GYP genes whose protein products, Gyp5p and Gyp8p, are potent Ypt1-GAPs. Whereas Gyp5p has a remarkable specificity for Ypt1p *in vitro*, Ypt6p, which appears to be functionally linked to Ypt1p (21),[2] is the preferred substrate for Gyp8p. We now find that Ypt1(Q67L)p, unlike the equivalent mammalian Ras(Q61L) mutant protein, served as efficient substrate for Gyp5p, explaining, at least in part, why previous studies using the *ypt1^Q67L* mutant allele did not cause major phenotypic alterations in some yeast strains (11). Synthetic growth defects and morphologically prominent alterations in mutants with a combination of the *ypt1^Q67L* mutant allele and Ypt1-GAP gene deletions, which we describe here, demonstrate that hydrolysis of Ypt1p-bound GTP is in fact required for effective functioning of this regulatory GTPase.

<div align="center">EXPERIMENTAL PROCEDURES</div>

Yeast Strains, Cell Growth, and Genetic Techniques—*S. cerevisiae* strains cl3-ABYS-86 (*MATα ura3-5 leu2-3 112 his3 pra1-1 prb1-1 prc1-1 cps1-3 can*^R) from D. H. Wolf (University of Stuttgart, Germany) and MSUC-3D (*MATα ura3 trp1 leu2 his3 lys2*) (this laboratory) were used for further genetic manipulations. For expression and purification of potential GAPs, the protease-deficient yeast strain BJ5459 (*MAT**a** ura3 leu2 his3 lys2 pep4::HIS3 prb1D.16R kan1*), originally obtained from the Yeast Genetic Stock Center, University of California, Berkeley, was employed. To analyze growth of different strains at various temperatures, logarithmically growing cultures were diluted into fresh YPD medium (1% yeast extract, 2% peptone, 2% dextrose) to an optical density of A_{600} ~0.01. From serial 10-fold dilutions, 4 μl each were spotted onto YPD-agar plates, and cells were grown at the appropriate temperatures. Cell growth in liquid cultures was followed by measuring the optical density at 600 nm, and the duplication time was calculated from the exponential parts of the growth curves.

Gene deletions were performed by replacement with *loxP-KanMX-loxP* cassettes (22); carboxyl-terminal epitope tagging was as described by De Antoni and Gallwitz (23). Replacement of the *YPT1* gene on chromosome VI with the mutant *ypt1^Q67L* gene was achieved by transforming relevant yeast strains with the linearized vector pREypt1^Q67L as previously described (24).

Cloning of GYP Genes—Open reading frames YPL249c (*GYP5*) and YFL027c (*GYP8*) were amplified by high stringency PCRs using proof-reading DNA polymerases (12). Forward primers started with the translation initiation codon (boldface type) and had a 5′ overhang with restriction enzyme recognition sequences (underlined). Reverse primers contained six consecutive histidine codons in front of the translational stop codon: GYP5-forward, 5′-CAT<u>GGATTCC</u>AT**ATG**TCTTCATCAGACACAAATCTATTG-3′; GYP5-reverse, 5′-ACG<u>GCTAGC</u>GTCGAC**TTA**ATGGTGATGGTGATGGTGTTTAAAAACTTTTTTAAAACCAGTC-3′; GYP8-forward, 5′-ATC<u>AGATCT</u>CAT**ATG**CCATTAAGGTCATTATTTC-3′; GYP8-reverse, 5′-GTA<u>CTCGAG</u>AAGC**TTA**GTGATGGTGATGGTGATGTCTAGTTGGATGCCCCAG-3′.

GYP5 PCR products were cleaved with *Bam*HI and *Nhe*I and ligated into *Bam*HI-*Xba*I-cleaved pYES2 plasmid (Invitrogen). GYP8 PCR products were cleaved with *Nde*I and *Xho*I and ligated into pET22a (Novagen) cleaved with the same enzymes. Truncated genes were created by cloning the appropriate PCR products as described above and ligation into pET30a (Novagen). Suppression analysis of yeast mutants was done with *GYP* genes cloned under TPI promoter control in the 2μ-based vector pYX212 (R & D Systems).

Subcellular Fractionations, Protein Blots, and Fluorescence Microscopy—Subcellular fractionation by differential centrifugation and the preparation of yeast cell extracts for immunological protein detection were as described previously (26). Proteins in SDS-PAGE-fractionated extracts were detected immunologically after electrotransfer onto nitrocellulose membranes (Schleicher & Schuell) and incubation with appropriately diluted anti-VSV epitope antibodies (Roche Molecular Biochemicals). Gyp-GFP fusions were prepared by cloning of *Bam*HI- plus

*Sal*I-cleaved *GYP* genes (recognition sequences were included in the PCR primers) into pUG23 vector (27). For YFP-CFP double fluorescence studies, pUG23 was modified by replacing the GFP gene with YFP or CFP. YFP and CFP genes were obtained by PCR from vectors pEYFP and pECFP (CLONTECH), respectively. For coexpression of GYP1-YFP and GYP8-CFP, the GYP8-CFP expression cassette was transplanted from pUG23-GYP8-CFP (*HIS3* marker) into *URA3*-marked vector pUG36 (27). Double transformants were grown in selective medium lacking both histidine and uracil and analyzed by confocal fluorescence microscopy using Leica TCSSP2. Fluorescent data were recorded at CFP- and YFP-specific excitation and emission wavelengths. Records at different wavelengths were merged using Photoshop 5.5 software.

Expression and Purification of Recombinant Proteins—Ypt-GTPases were expressed from pET vectors in *E. coli* BL21(DE3) (Novagen) and purified by anion exchange chromatography and gel filtration (28). Bacterially expressed, catalytically active Gyp5–58p and Gyp8–49p, His₆-tagged at their C termini, were affinity-purified on Ni²⁺-nitrilotriacetic acid-agarose (Qiagen) and further purified by ion exchange chromatography on MonoQ HR10/10 (Amersham Biosciences) and by gel filtration using Sephacryl S-200 HR 16/60. The purification method described (12, 18) was modified in that 1% of Triton X-100 (Baker) was included in the cell lysis buffer to facilitate protein solubility. If required, purified proteins were concentrated by ultrafiltration using Microsep (Pall Filtron Corp.).

Biochemical Analysis of GAPs—Filter GAP assays using [γ-³²P]GTP (PerkinElmer Life Sciences) were performed as described (14). HPLC-based GAP assays were performed exactly as previously described (12). For determining the extremely low intrinsic hydrolysis rates of Ypt1(Q67L)p mutant protein, the incubation time at 30 °C was extended over 8 h. The biochemical properties of Gyp5p with Ypt1p and Ypt1(Q67L)p as substrates were determined using an integrated Michaelis-Menten procedure (29) as described (18). Due to the extremely slow intrinsic GTP hydrolysis rate of Ypt1(Q67L)p, rates of the GAP-accelerated reactions were followed using fixed concentrations of the mutant GTPase (10 and 20 μM, respectively, for the Gyp5p- and Gyp8p-catalyzed reaction) and excessive amounts of the two GAPs.

Electron Microscopy—To augment intracellular membranes, yeast cells were fixed with potassium permanganate and processed for electron microscopy as described previously (26).

<div align="center">RESULTS</div>

Identification of Two Novel Ypt/Rab-specific GAPs—Six moderately conserved sequence segments constitute the GYP domain of the catalytically active region of Ypt/Rab-specific GAPs. Within this region of the biochemically identified GAPs, six from yeast and two from mammals, there are three absolutely conserved "GYP fingerprint" sequences, R*XXX*W, I*XX*-D*XX*R, and Y*X*Q (Fig. 1*A*), which are helpful to identify members of this class of proteins. Using this criterion, two other yeast proteins, encoded by the reading frames YPL249c and YFL027c, were predicted to be members of the GYP family. The genes were amplified by PCR from total *S. cerevisiae* DNA and expressed from a multicopy vector as C-terminally His₆-tagged proteins in yeast. Proteins adsorbed to Ni²⁺-agarose were first tested for GAP activity with different GTP-loaded Ypt-GTPases in a standard assay (12). We found that the protein product of YPL249c, hereinafter termed Gyp5p, accelerated the intrinsic GTP hydrolysis rate of Ypt1p significantly and that of Sec4p marginally. High expression and purification of full-length YFL027c protein was not successful either in yeast or in *Escherichia coli*. Because our previous studies (18) had shown that deletion of sequences outside the catalytic domain can result in improved yield and solubility of bacterially produced Ypt-GAPs, short C-terminal truncations of 33 and 77 amino acids, respectively, were made of the YFL027 protein. The larger truncation was found to allow easy production in *E. coli* of soluble protein with GAP activity toward Ypt1p and Ypt6p. The gene comprising the reading frame YFL027c was therefore designated *GYP8*.

As shown in Fig. 1, the GYP domain of the 101.6-kDa Gyp5p is localized in the C-terminal half of the protein (amino acids 451–624), whereas in the 57.6-kDa Gyp8p it occupies most of
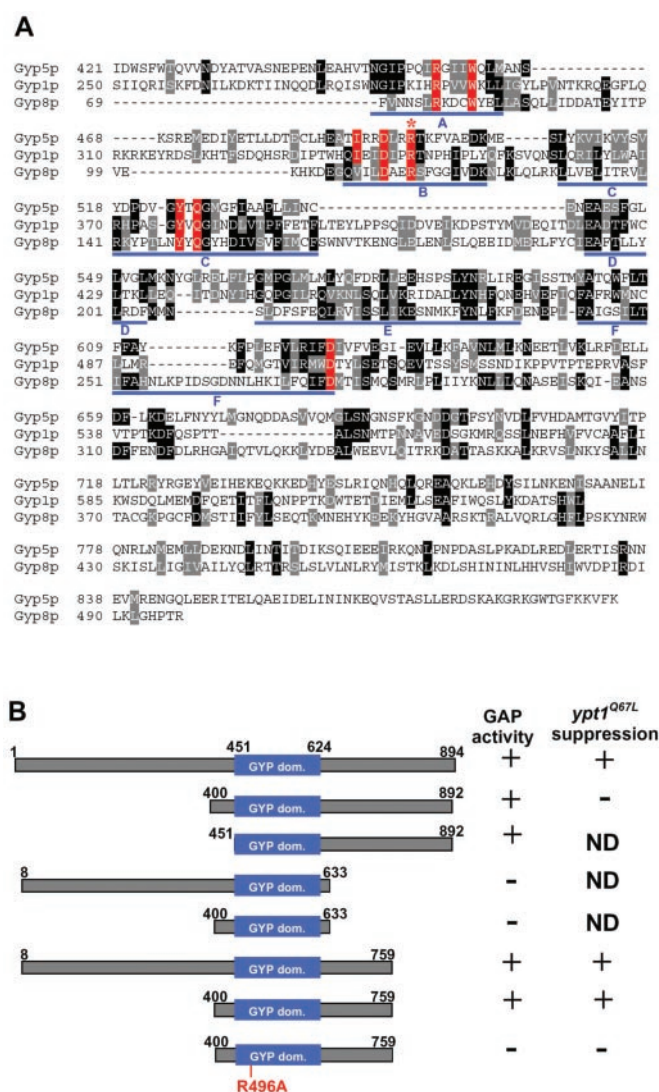

A

B

FIG. 1. **Sequence comparison of the Ypt1-GAPs Gyp1p, Gyp5p, and Gyp8p and truncations of Gyp5p to identify its catalytic domain and suppressor activity.** *A*, protein sequence alignments were done with ClustalW and edited manually. The six conserved motifs (16), which form the core of the Gyp1 catalytic domain (32), are indicated by blue bars. The conserved amino acids of the GYP "fingerprint" sequences are highlighted by a *red background*, and the invariant arginine residue required for catalysis is indicated by a *star*. *B*, schematic representation of Gyp5p and its different truncations. The GYP domain is indicated by a *blue box*. Proteins were tested for GAP activity with GTP-loaded Ypt1p using an HPLC-based assay and for suppressing activity of a cold-sensitive *ypt1*^{Q67L} strain. *ND*, not determined.

the N-terminal half (amino acids 68–275). Gyp5p harbors a coiled-coil region of about 140 amino acid residues at its C terminus and a serine- and proline-rich domain located N-terminally of the GYP domain. In correspondence with mutational studies of Gyp1p and Gyp7p (18), we observed that deletion of the large N-terminal region preceding the GYP domain of Gyp5p did not affect its GAP activity and that a larger sequence segment following the GYP domain was required for the protein's catalytic activity. However, at least 135 amino acids could be removed from the C-terminal end of Gyp5p without interfering with its GAP activity (Fig. 1*B*).

Gyp5p and Gyp8p Are Potent GAPs for Ypt1p—For the biochemical characterization, a 493-amino acid-long fragment of Gyp5p (Gyp5–58p, residues 400–892) and a 420-amino acid-

long Gyp8 protein lacking only the C-terminal 77 residues (Gyp8–49p) were produced in *E. coli* and purified by affinity and ion exchange chromatography and by gel filtration. Our previous studies had shown that various biochemical parameters, like substrate specificity and activation rates, did not change significantly when full-length and truncated active Gyp proteins were compared (18). We first inquired into the substrate specificity of the novel GAPs employing a quantitative HPLC-based method to compare the GAP activity with different GTP-loaded GTPases (12). As shown in Table I, Gyp5–58p exhibited a clear preference for Ypt1p over Sec4p, the only other GTPase whose intrinsic GTPase activity was accelerated by this GAP. For Gyp8–49p, which had a higher specific activity than Gyp5–58p (Fig. 2*A*), Ypt1p and Ypt6p were the most efficient substrates (Table I). Although in the standard assay, the acceleration of the intrinsic GTP hydrolysis rate was higher for Ypt6p due to its extremely slow intrinsic GTPase activity, in absolute terms the reaction rate with Ypt1p ($k = 1.144$ min^{-1}) was higher than that with Ypt6p ($k = 0.385$ min^{-1}). In addition, Sec4p and, less pronounced, the redundant GTPases Ypt31p and Ypt32p served as substrate for Gyp8–49p *in vitro*.

In further experiments, we studied the interaction between Gyp5–58p and its preferred substrate Ypt1p and determined the GAP catalytic activity and the substrate affinity from single time curves by means of an integrated Michaelis-Menten equation (18, 29, 30) (Fig. 2*B*). In a reaction with a 200-fold excess of Ypt1p·GTP over Gyp5–58p, we determined k_{cat} to be 106 min^{-1}, which corresponds to a 4.24×10^{4}-fold acceleration of the intrinsic hydrolysis rate of this GTPase. With a K_m of 74.5 μM, Gyp5–58p, like other Ypt-GAPs (18, 19, 31), has a low affinity for its preferred substrate GTPase Ypt1p. So far, k_{cat} and K_m for Gyp8–49p could not be determined with certainty, since under the experimental conditions of the HPLC-based GAP assay, increasing substrate concentrations tended to inhibit GAP activity.

One of the structural hallmarks of Ypt-GAPs is a conserved arginine within the I*XX*D*XX*R sequence motif (Fig. 1*A*). This residue is critical for GAP catalytic function (18, 31), and the three-dimensional structure of Gyp1p (32) indicates that it might act in catalysis like the "finger arginine" of Ras-GAP (33). We have substituted this arginine with either alanine or lysine in both Gyp5p (Arg^{496}) and Gyp8p (Arg^{114}) and found that each of the four mutant proteins almost completely lost GAP activity (shown for Gyp5p in Fig. 2*A*).

Intracellular Distribution of Gyp5p and Gyp8p—Of the eight Gyp family members in yeast, three (Gyp1p (18, 34), Gyp5p, and Gyp8p (this report)) are now known to be potent GAPs *in vitro* for Ypt1p, the GTPase with an essential function in ER-to-Golgi transport. In a recent report (34), fluorescence micro-
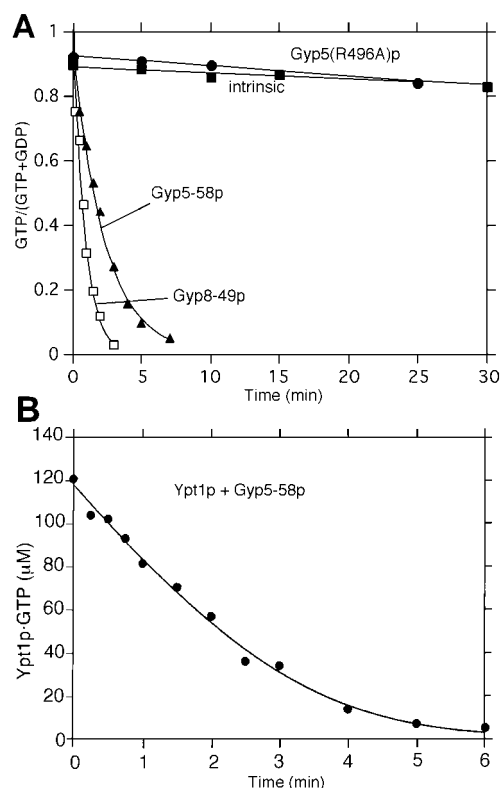
TABLE I
Substrate specificities of Gyp5p and Gyp8p in vitro

GTPase	Intrinsic GTP hydrolysis rate[a]	Gyp5–58p-stimulated GTP hydrolysis acceleration[b]	Gyp8–49p-stimulated GTP hydrolysis acceleration[c]
	min^{-1}	*-fold*	*-fold*
Ypt1p	0.0025	150.0	457.8
Sec4p	0.0016	23.6	175.2
Ypt31p	0.0064	1.2	30.9
Ypt32p	0.0083	1.0	43.0
Ypt51p	0.0052	1.6	6.8
Ypt52p	0.0862	1.4	1.5
Ypt53p	0.0102	1.0	7.2
Ypt6p	0.0002	2.2	1923.0
Ypt7p	0.0023	1.0	6.6

[a] All experiments were performed at 30°C, data from Albert and Gallwitz (17).
[b] 20 μM Ypt-GTP complexes were incubated with 100 nM Gyp5–58p.
[c] 20 μM Ypt-GTP complexes were incubated with 20 nM Gyp8–49p.

Functional Analysis of Novel Ypt1-GAPs

FIG. 2. **Ypt1p is substrate for both Gyp5–58p and Gyp8–49p.** *A*, Ypt1p·GTP complex (20 μM) was incubated with buffer (intrinsic), 0.2 μM Gyp5–58p, 0.2 μM Gyp5–58(R496A)p, or 0.02 μM Gyp8–49p. Ypt1p-bound GTP and GDP were determined by HPLC analysis. Whereas wild type Gyp proteins accelerate GTP hydrolysis efficiently, Gyp5(R496A)p is almost completely inactive. *B*, Ypt1p·GTP complex (120 μM) was incubated at 30 °C with 0.6 μM Gyp5–58p, and the extent of GTP hydrolysis was determined by HPLC. The concentration of Ypt1p·GTP was plotted as a function of time and fitted by an integrated Michaelis-Menten equation.

FIG. 3. **Relative abundance of Ypt1-GAPs.** The reading frames of *GYP1*, *GYP5*, and *GYP8* in their normal chromosomal environments were extended with three consecutive VSV epitope tags. Logarithmically growing cells expressing the three modified GAPs were subjected to alkaline lysis and immunoblotting. Two preparations were probed with anti-VSV antibody for each protein. Signal intensities were quantified with a Lumi-Imager.

scopic data were presented showing that on high expression, red fluorescent protein-tagged Gyp1p partially colocalized in punctate structures with a GFP fusion of the Golgi protein Bet3p, suggesting Golgi association of Gyp1p. In a first attempt to assign Gyp5p and Gyp8p to their main cellular compartment(s) and to determine the relative abundance of the three Ypt1-GAPs, Gyp5p, Gyp8p, and Gyp1p were C-terminally tagged with VSV, MYC, or hemagglutinin epitopes and expressed from the modified genes that were integrated into the genome of the protease-deficient strain Cl3-ABYS-86 such that they replaced the respective chromosomal wild type copies. This experimental procedure was chosen to also avoid possible artifacts resulting from unphysiologically high intracellular concentration of the GAPs. The relative abundance of the three GAPs was calculated from immunoblots of electrophoretically separated total cellular proteins performed with antibodies specific for the protein tags. Fig. 3 shows an immunoblot with VSV-tagged Ypt-GAPs, which was subjected to quantification of the reactive protein bands using a Lumi-Imager. According to these analyses, the intracellular levels of Gyp5p and Gyp1p were about 12.5 and 6 times higher, respectively, than that of Gyp8p. In subcellular fractionation experiments, Gyp1p was distributed almost equally between the P10, P100, and soluble fractions. In contrast, Gyp5p was in the soluble fraction nearly exclusively. Gyp8p was enriched in the P10 fraction, suggesting that a significant portion was either part of large protein complexes or associated preferentially with the ER and/or the plasma membrane (data not shown).
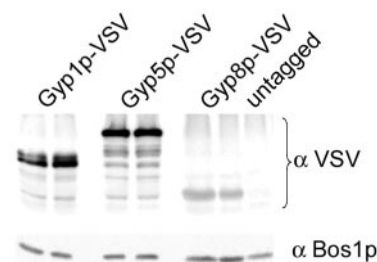
The intracellular distribution of the three Ypt1-GAPs was also studied by fluorescence microscopy using C-terminal GFP fusions. In agreement with the cell fractionation data, the Gyp5-GFP was predominantly cytosolic, whereas Gyp8-GFP localized to few and bright punctate structures (Fig. 4*A*). Gyp1-GFP that, according to a recent study (34), might be confined to Golgi compartments labeled smaller and more frequent punctate structures than Gyp8-GFP. To ascertain this observation, we co-expressed Gyp1-YFP with Gyp8-CFP and examined the cells by confocal microscopy. The fluorescence of YFP and CFP was recorded separately. As shown in Fig. 4*B*, the structures labeled by Gyp1-YFP and Gyp8-CFP are obviously not identical, indicating a concentration of at least part of the two GAPs in different cellular compartments.

These analyses show that despite the different abundance of the three GAPs, each has a typical intracellular localization profile at steady-state.

Synthetic Growth Inhibition in gyp5 Knockout Strains Expressing a GTPase-deficient Ypt1 Mutant Protein—Previous studies have shown that single deletions of either of the known Ypt-GAP-encoding genes did not result in clearly observable phenotypes when cells were grown in rich media. Since Gyp1p, Gyp5p, and Gyp8p are potent Ypt1-GAPs, we deleted the three chromosomal genes singly and in different combination in two strains of different genetic background. All mutants were viable and grew like "wild type"; only the triple $\Delta gyp1$/$\Delta gyp5$/$\Delta gyp8$ deletion in the protease-deficient strain cl3ABYS-86 had a somewhat retarded growth at low temperature (15 °C) compared with single and double deletion mutants. We reasoned that if the three GAPs accepted Ypt1p as substrate also *in vivo*, specific defects might become apparent when the *GYP* genes were deleted in cells that expressed the GTPase-deficient Ypt1(Q67L) mutant protein. Substitution with leucine of this highly conserved glutamine, which in Ras is essential for intrinsic and GAP-accelerated GTP hydrolysis (35), has been reported to be phenotypically silent in yeast, and this was taken as evidence that GTP hydrolysis might not be important for Ypt1p function (11). We first replaced the chromosomal *YPT1* gene with the mutant gene expressing Ypt1(Q67L)p in the haploid strains MSUC-3D and cl3ABYS-86 and found that cell growth of the MSUC-3D(*ypt1^{Q67L}*) strain was unaffected at temperatures ranging from 15 to 37 °C, whereas the protease-deficient strain cl3ABYS-86 did not grow at 15 °C (Fig. 5, *A* and *B*). We then attempted to introduce the *ypt1^{Q67L}* mutant allele into haploid strains lacking one or more of the Ypt1-GAP genes. As can be seen from Table II, the triple deletion of *GYP1*, *GYP5*, and *GYP8* precluded chromosomal exchange of the *YPT1* gene by the *ypt1^{Q67L}* allele in MSUC-3D, indicating that such cells are inviable. In cl3-ABYS-86 background, the replacement of *YPT1* by the *ypt1^{Q67L}* mutant gene was inefficient when one of
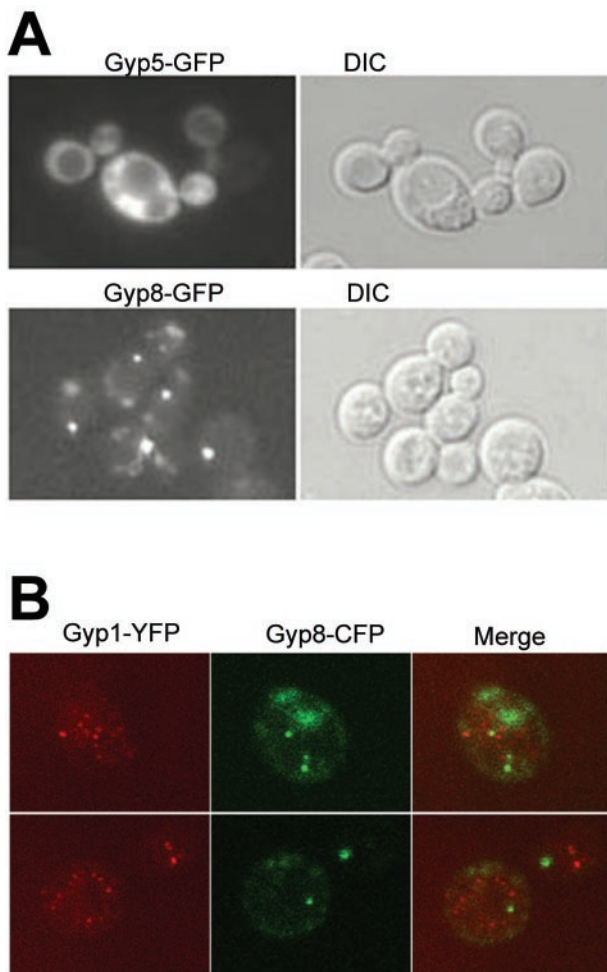
FIG. 4. **Localization of Gyp-GFP fusion proteins.** *A*, Gyp-GFP proteins were expressed from pUG23 vector and visualized by fluorescence microscopy. Nonfluorescent, round areas represent vacuoles (compare with DIC images). *B*, Gyp1-YFP and Gyp8-CFP were coexpressed (see "Experimental Procedures"), and cells were examined by confocal microscopy. YFP and CFP fluorescence was recorded separately and merged. Note that Gyp1-YFP (*red*) and Gyp8-CFP (*green*) decorate different structures.

FIG. 5. **Synthetic growth defects and suppression analysis of mutant yeast strains.** *A*, serial dilutions of wild type and different MSUC-3D mutants (genotypes shown to the *right*) were grown at different temperatures. *B*, the growth of protease-deficient strain cI3-ABYS-86 and the mutant expressing the *ypt1*^{Q67L} allele at 15 and 30 °C. *C*, high expression of Gyp1p, Gyp5p, and Gyp8p, but not of Gyp6p or Gyp7p, suppresses the cold-sensitive phenotype of MSUC-3D(*ypt1*^{Q67L}/Δ*gyp5*/Δ*gyp8*).

TABLE II
Efficiency of YPT1 gene replacement by ypt1^{Q67L} *allele in different strains with Ypt1-GAP gene deletions*

Replacement of *YPT1* by *ypt1*^{Q67L} was verified by PCR analysis followed by digestion with *Xho*I, the restriction site being present only in the mutant allele.

Yeast strains transformed with pRE-*ypt1*^{Q67L}	Leu$^+$ colonies checked	Strains harbouring *ypt1*^{Q67L}
MSUC-3D (WT)	10	10
ADY40 (Δ*gyp1*)	10	10
ADY41 (Δ*gyp5*)	10	9
ADY43 (Δ*gyp8*)	10	10
ADY44 (Δ*gyp5*/Δ*gyp1*)	10	1
ADY45 (Δ*gyp5*/Δ*gyp8*)	10	5
ADY46 (Δ*gyp1*/Δ*gyp8*)	10	7
ADY48 (Δ*gyp5*/Δ*gyp1*/Δ*gyp8*)	20	0
cl3-ABYS-86 (WT)	10	9
ADY20 (Δ*gyp1*)	10	5
ADY21 (Δ*gyp5*)	10	6
ADY23 (Δ*gyp8*)	10	8
ADY24 (Δ*gyp5*/Δ*gyp1*)	10	0
ADY25 (Δ*gyp5*/Δ*gyp8*)	10	0
ADY26 (Δ*gyp1*/Δ*gyp8*)	10	0
ADY27 (Δ*gyp5*/Δ*gyp1*/Δ*gyp8*)	20	0

the three Ypt1-GAP genes was deleted, and it was unsuccessful when two of them were deleted. Importantly, MSUC-3D(*ypt1*^{Q67L}) cells lacking either the *GYP5* gene alone or *GYP5* in combination with *GYP1* or *GYP8* displayed a somewhat reduced growth at suboptimal temperatures (25 or 20 °C) and complete growth inhibition at 15 °C. For example, the growth rates of the *ypt1*^{Q67L} strain and the *ypt1*^{Q67L}/Δ*gyp5* strain at 30 °C were 92 and 101 min, and the rates at 20 °C were 195 and 230 min, respectively. Single deletions of *GYP1*, *GYP8*, or the Ypt7-GAP-encoding *GYP7* gene did not affect the growth properties of the MSUC-3D(*ypt1*^{Q67L}) mutant strain (Fig. 5*A*). The consequences of Ypt1-GAP gene deletions were even more severe in the cl3ABYS-86(*ypt1*^{Q67L}) strain; already single knockouts of either *GYP5* or *GYP8* led to reduced growth at 20 °C. However, regardless of the growth temperature, none of the mutants described above exhibited significant alterations of transport or maturation of newly synthesized secreted invertase or vacuolar carboxypeptidase Y (data not shown).

Collectively, these synthetic negative effects suggest a functional link between the three GAPs and Ypt1p, and they indicate that Gyp5p may be the most effective GAP for Ypt1p *in vivo*.

High Intracellular Levels of Ypt1-GAPs Rescue Ypt1(Q67L)-expressing Mutant Cells from Growth Defects—As an explana-
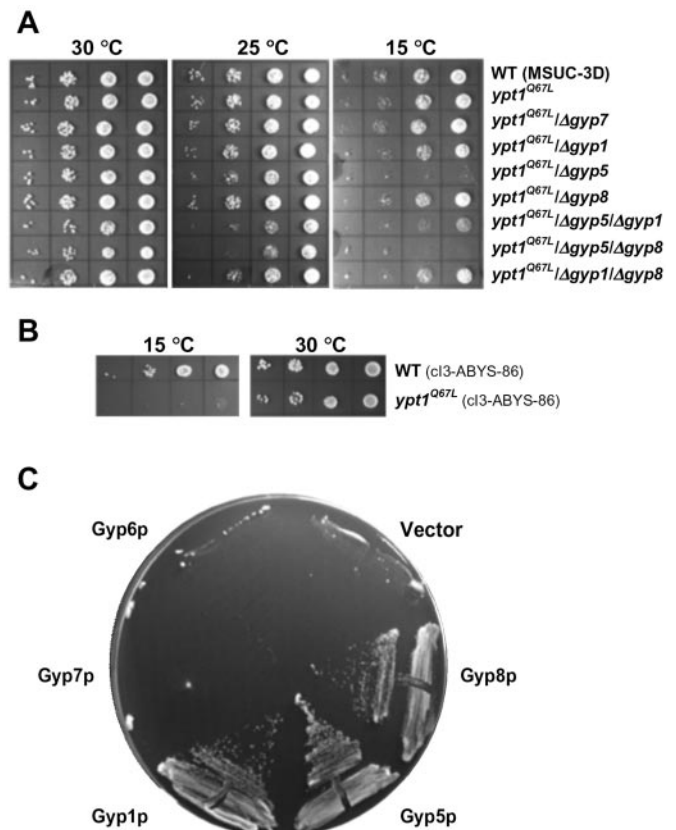
tion for the synthetic growth defects of *ypt1*^{Q67L} mutant cells lacking Ypt1-GAP genes, we envisaged the possibility that the intrinsic GTPase activity of Ypt1(Q67L)p, in contrast to that of the corresponding Ras mutant protein, was still activable by its cognate GAPs. In this case, we anticipated that high expression of the Ypt1-GAPs might rescue *ypt1*^{Q67L} mutant strains from

Functional Analysis of Novel Ypt1-GAPs

growth inhibition at nonpermissive temperature. MSUC-3D(*ypt1^{Q67L}*/Δ*gyp5*/Δ*gyp8*), which does not grow at 15 °C, was transformed with vector pYX212 harboring the Ypt1-GAP gene *GYP1*, *GYP5*, or *GYP8* under transcriptional control of the strong TPI promoter. As controls for Ypt-GAPs with different substrate specificity, recombinant vectors with either *GYP6* (13, 31) or GYP7 (14, 15) were also used to transform the same mutant strain. As shown in Fig. 5*C*, the cold sensitivity of the *ypt1^{Q67L}* strain could be suppressed on high expression of either Gyp1p, Gyp5p, or Gyp8p but not with Gyp6p or Gyp7p.

The results of the suppression analyses suggested that Ypt1(Q67L)p is in fact a substrate for Ypt1-GAPs. We therefore put the mutant GTPase to the test for intrinsic and GAP-accelerated GTPase activity. The intrinsic GTP hydrolysis rate of Ypt1(Q67L)p, followed over a time course of several hours at 30 °C, was determined to be 6×10^{-5} min^{-1}, which is about 40-fold slower than that of the wild type protein. Surprisingly, this very slow intrinsic GTPase activity of Ypt1(Q67L)p could be significantly accelerated by Gyp5p. Following Michaelis-Menten kinetics, the k_{cat} for Gyp5–58p was determined to be 0.89 min^{-1}, which corresponds to a 1.5×10^{4}-fold acceleration of the intrinsic GTP hydrolysis rate of Ypt1(Q67L)p.

Since the N- and C-terminal regions flanking the GYP domain of Gyp5p appear to contain several structural domains, we addressed the question whether these sequence segments are of significance for *in vivo* function of this Ypt1-GAP. Various truncation mutants were tested for their ability to suppress growth inhibition of *ypt1^{Q67L}* mutant cells (Fig. 1*B*). It was found that high expression of the catalytically active fragment lacking the N-terminal 399 amino acids did not overcome the growth inhibition at nonpermissive temperature. We then made the observation that on high expression, this fragment itself was growth-inhibitory unless the C-terminal end including most of the potential coiled-coil domain (which comprises amino acids 720–860) was also deleted. Somewhat surprisingly, this truncation mutant lacking amino acids 2–399 and 760–894 was again active as suppressor. The suppressor activity depended on the GAP activity of the Gyp5p fragment, since it was lost when the presumptive "finger arginine" in position 496 was substituted for alanine or lysine (Fig. 1*B*).

These results suggest that the Ypt1(Q67L) mutant GTPase is a substrate for Gyp1p, Gyp5p, and Gyp8p *in vivo* and that sequences within the N-terminal half of Gyp5p might interact with and neutralize the cell growth-inhibitory effect of the C-terminal 133-amino acid region.

Accumulation of Membrane Material and Alterations Resembling Autophagic Processes in Mutants Defective in Hydrolysis of Ypt1p-bound GTP—Ypt1p is known to have an essential function in ER-to-Golgi trafficking (7). The most surprising discovery that Ypt1(Q67L)p is a very efficient substrate at least for one of the Ypt1-GAPs *in vitro* is likely to explain the lack of or the very mild growth defects of cells expressing the mutant instead of the wild type Ypt1-GTPase. Because a severe impairment of the hydrolysis of Ypt1p-bound GTP is supposed to shift the GTPase to a permanently active state, we searched for possible protein transport and morphological alterations in relevant mutant cells. Glycosylation and secretion of invertase induced in low glucose medium were not significantly affected either in *ypt1^{Q67L}* or in *ypt1^{Q67L}*/Δ*gyp5* mutant cells. Likewise, the processing and sorting of vacuolar CPY was not different in mutant and wild type cells (data not shown).

We next examined, by electron microscopy, cells of strain MSUC-3D and of its mutant derivatives ADY41(Δ*gyp5*), ADY49(*ypt1^{Q67L}*), and ADY51(*ypt1^{Q67L}*/Δ*gyp5*). Cells were grown at the permissive temperature of 30 °C and fixed with potassium permanganate to augment membrane structures. The *gyp5* deletion strain was not significantly different from wild type. The *ypt1^{Q67L}* strain characteristically displayed an accumulation of vesicular structures about 50–150 nm in size. Cells of the double mutant showed in addition some proliferation of ER membranes and, importantly, various alterations of vacuole morphology. In many cells, vacuoles were fragmented or showed long invaginations resembling microautophagic tubes (36); others displayed engulfed cytoplasm and vesicular structures (Fig. 6, *B* and *D*). A number of vacuoles contained round, membrane-bound organelles of about 350 nm that were filled with cytoplasm and membrane material (Fig. 6*C*). These structures resembled autophagic bodies, the outer membranes of which were often partially degraded. Vesicles about 100 nm in size were also frequently observed in vacuoles (Fig. 6*C*). The morphological alterations were even more striking in the proteinase-deficient strain cl3-ABYS-86. Whereas at the permissive temperature of 30 °C, *gyp5* deletion did not result in a clear phenotype, cells with the *ypt1^{Q67L}* mutation (strain ADY29) and especially the *ypt1^{Q67L}*/Δ*gyp5* double mutant cells (strain ADY31) accumulated ER membranes and vesicles of different size that sometimes formed larger clusters. Typical of the mutant cells were pleiomorphic vacuole structures; they sometimes had a club-shaped appearance (Fig. 6*E*). Vacuoles were mostly filled with particulate material; some were packed with vesicles apparently surrounded by two unit membranes. Structures resembling multivesicular bodies that contained vesicles of 40–50 nm were also observed (Fig. 6, *F–H*). Vacuole membranes exhibited several indentations, and in some cases there were clear indications for direct uptake of cytoplasmic and membrane material (Fig. 6, *F* and *H*).

In a first attempt to examine whether an autophagic process caused the morphological alterations in Ypt1(Q67L)p-expressing cells, we tried to additionally delete either *AUT1* or *AUT7*, two nonessential genes involved in autophagy (37, 38). Whereas the knockouts were easily done in MSUC-3D, we were unable to delete either of the two genes in haploid strains MSUC-3D(*ypt1^{Q67L}*/Δ*gyp5*) or in cl3-ABYS-86(*ypt1^{Q67L}*). Although further investigations would be required, these results argue for synthetic negative effects or synthetic lethality. The observed morphological alterations therefore suggest that the effective blocking of the hydrolysis of Ypt1p-bound GTP induces previously unnoticed autophagic processes, most likely to get rid of accumulated membrane material.

DISCUSSION

In this report, we describe and characterize two novel GAPs that act on Ypt/Rab GTPases involved in vesicular protein transport. This brings the GYP family of GTPase-activating proteins in yeast to eight members. According to our computer search, there appears to be only one other uncharacterized protein in budding yeast, the open reading frame of YGL036w, that would satisfy the primary structure criteria (conserved residues of the GYP domain) for a GAP of this family, but until now, we have no indication for it having such a biochemical property. Whereas one of the newly identified GAPs, Gyp5p, has a remarkable specificity for Ypt1p *in vitro*, the second, Gyp8p, exhibits a rather broad substrate specificity like several of the other yeast Ypt-GAPs that we have studied previously. However, Ypt1p is also one of the preferred substrate GTPases for Gyp8p *in vitro*. The two novel GAPs and the previously identified Gyp1p (18, 34) are the three GYP family members that very efficiently accelerate the low intrinsic GTP hydrolysis rate of Ypt1p.

One of the puzzling questions is why the deletion of individual GYP genes, including the newly identified *GYP5* and *GYP8*, allows yeast cells to survive under normal growth conditions
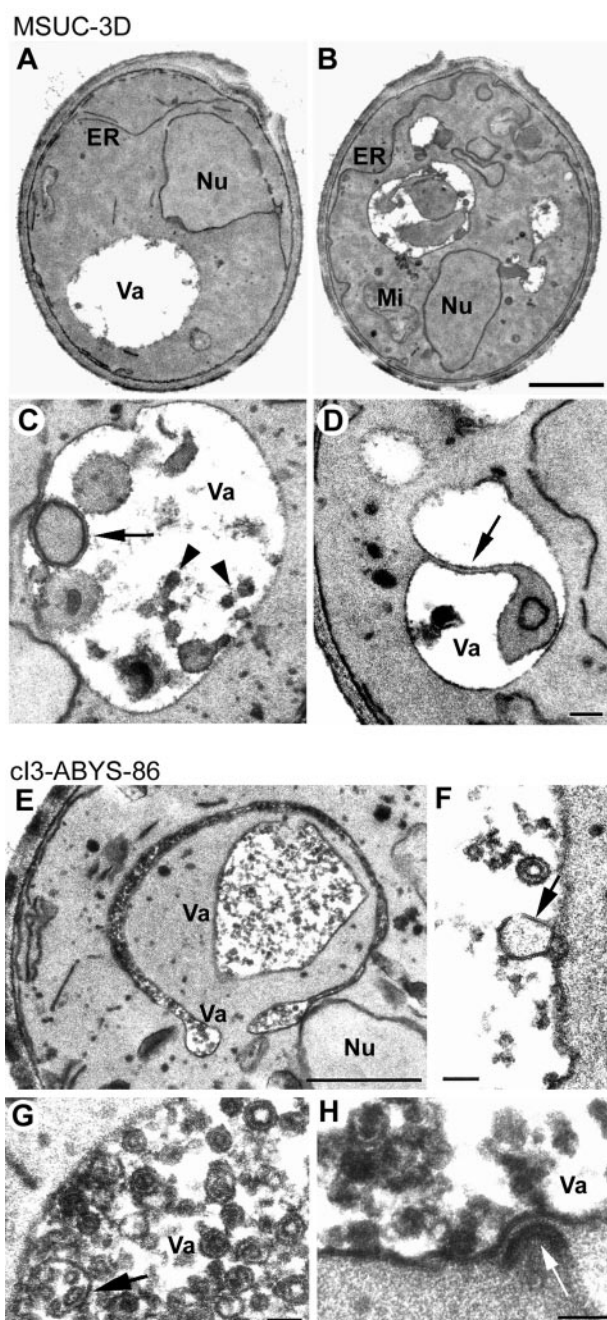
FIG. 6. **Morphological alterations of *gyp5* deletion strains expressing the *ypt1^Q67L^* mutant allele.** Thin section electron microscopy of potassium permanganate-fixed wild type (*A*) and mutant MSUC-3D(*ypt1^Q67L^*/Δ*gyp5*) cells (*B–D*). Accumulation of vesicular structures of different size and of ER membranes as well as vacuolar inclusions are seen (*A*). *C*, the *arrow* points to a structure resembling an autophagosome, and *arrowheads* point to vesicles of about 50 nm. *D*, the *arrow* points to a thin vacuolar invagination. *E–H*, electron micrographs of protease-deficient cI3-ABYS-86(*ypt1^Q67L^*/Δ*gyp5*) mutant cells. Note the pleiomorphic vacuoles (*E*) filled with "vesicles in vesicles" about 100 nm in size (*G*). *G*, the *arrow* points to an intravacuolar organelle containing small vesicles and resembling a multivesicular body. The *arrows* point to a vesicular structure apparently budding from the vacuole membrane into the vacuole lumen (*F*) and to a tubular appearing organelle apparently in preparation for uptake by the vacuole (*H*). *Mi*, mitochondria; *Nu*, nucleus; *Va*, vacuole. *Bars*, 1 μM (*B*, *E*); 200 nm (*D*); 100 nm (*F–H*).

without any obvious defect in growth or in intracellular protein transport. One explanation would be that the functional loss of one GAP can be substituted for by another. This in fact seems to be the case, although, as shown here, it became apparent

only in cells living with the GTPase-deficient Ypt1(Q67L) mutant protein and at less than optimal growth temperatures. The synthetic negative growth defects of various mutant combinations in rich media indicates that all three GAPs, Gyp1p, Gyp5p, and Gyp8p, most likely act on Ypt1p also *in vivo*. Evidence for Gyp1p being a Ypt1-GAP has recently been obtained by Du and Novick (34) who observed various genetic interactions between this GAP and Ypt1p in synthetic growth media. Our experimental strategy, combining various GYP gene deletions with the GTPase-deficient Ypt1(Q67L) GTPase uncovered that it is Gyp5p which apparently is the most effective Ypt1-GAP *in vivo*.

Additional evidence for Gyp1p, Gyp5p, and Gyp8p acting as Ypt1-GAPs *in vivo* comes from our finding that the growth inhibition at 15 °C of a *ypt1^Q67L^*/Δ*gyp5*/Δ*gyp8* strain (MSUC-3D background) can be overcome specifically by high intracellular levels of Gyp1p, Gyp5p, and Gyp8p. Interestingly, this is so although the major fraction of the different GAPs appears to be located in different cellular compartments. Whereas a recent study (20) and our own results show that Gyp1p is distributed between the cytoplasm and punctate structures, apparently representing Golgi membranes (20), Gyp5p is almost completely cytosolic as demonstrated by subcellular fractionation and microscopic inspection of cells expressing a GFP fusion. By confocal microscopy, Gyp8p was primarily found in a few large structures that did not match the apparent Golgi compartments with which part of Gyp1p was associated. Since Gyp8p in high concentration in solution tends to aggregate, it is possible that the intracellular structures seen with the GFP-Gyp8 fusions represent aggregates as well. This then might explain why in protease-deficient cells, high expression of Gyp8p is growth-inhibitory. On the other hand, Gyp1p and Gyp8p are potent GAPs *in vitro* not only for Ypt1p but also for several other Ypt-GTPases. If this were true also for the situation in living cells, different intracellular localization of fractions of the two GAPs at steady-state could be the consequence of their acting at different transport steps.

Since the GTP-loaded Ypt/Rab GTPases are membrane-bound, there should be a mechanism to recruit the GAPs to those membranes where they are needed for inactivation of specific substrates. From the fact that a large part of at least some Ypt/Rab-GAPs can be deleted without affecting their catalytic potency (18), we previously argued that such sequences could be important for membrane association of the GAPs. It was found initially that Gyp5p loses the capability to rescue the GTPase-deficient *ypt1^Q67L^* mutant from growth defect when it lacks an N-terminal fragment of 400 amino acid residues, which otherwise is dispensable for catalytic activity and substrate specificity of this GAP. Although this suggested that the N-terminal half of Gyp5p is likely to contain sequences required for specific membrane association, we subsequently discovered that the ability of the N-terminally truncated Gyp5p to act as suppressor is restored after further deletion of the C-terminal 135 amino acids including most of the coiled-coil region. This indicates that the C-terminal sequences with the potential to form coiled-coils might fulfill a noncatalytic function in concert with sequences of the N-terminal half and affect proper localization and/or catalytic activity. Further functional analysis of the sequences outside the catalytic domain of Gyp5p would also be of interest, since previous studies suggested that the protein product of YPL249c (that we here identify to be the Ypt1-GAP Gyp5p) appears to interact with Rvs167p, a protein apparently involved in actin cytoskeleton function (39, 40). In the case of Gyp1p of which a significant fraction is already bound to membranes, supposedly including the Golgi, the region N-terminal of the catalytic domain of Gyp1p is not re-

quired to rescue, in synthetic media, a Δ*gyp1* deletion strain from growth inhibition at 37 °C (34). This might mean that other sequences, perhaps contained in the C terminus, are used to direct Gyp1p to membranes where it has to act. However, high expression of the GAPs and their fragments was performed in the suppression analyses by Du and Novick (34) and in those reported here. Under these conditions, the level of Ypt1-GAPs at membranes with the GTP-loaded Ypt1p might be sufficiently high to allow down-regulation of the GTPase's activity.

The deletion of one or of several of the Ypt1-GAP genes was phenotypically neutral. We nevertheless assume that the hydrolysis of Ypt1p-bound GTP is severely inhibited in the Δ*gyp1*/Δ*gyp5*/Δ*gyp8* triple deletion mutant, but this has not been investigated. Unless additional GAPs can act on Ypt1p *in vivo*, the very mild growth retardation of these mutant cells seems to suggest that the slow inbuilt GTP hydrolytic activity of the essential Ypt1p suffices to allow ER-to-Golgi traffic to keep cells going under optimal growth conditions. It is important to note here that in the GTP-bound conformation, small GTPases are generally active. Therefore, under conditions where the hydrolysis of Ypt1p-bound GTP is severely impaired, as for instance in the triple *gyp* deletion mutant, one would not necessarily expect deficiencies in ER-to-Golgi traffic, unless GTP hydrolysis would be essential for membrane fusion *per se*. According to the results of our study, a direct involvement of Ypt1p in membrane fusion seems unlikely. This is in accord with the conclusion of a previous analysis in which mutant Rab5-dependent early endosome fusion occurred in the absence of hydrolysis of GTPase-bound xanthosine triphosphate (10). There is ample evidence instead that Ypt/Rab-GTPases could act in the preparation of membrane fusion by recruiting, to defined membranes, components required for transport vesicle docking and/or fusion (3, 4, 41).

An unexpected finding of our investigation is that the Ypt1(Q67L) mutant protein serves as a very efficient substrate for its cognate GAP Gyp5p. As will be reported elsewhere, this appears not to represent a special case but rather a general property of Ypt/Rab-GTPases. Marginal acceleration of the intrinsic GTPase activity of Rab11(Q70L) and Sec4(Q79L) mutant proteins was also noted previously, although these investigations had been performed with crude cell extracts as source for GTPase-activating proteins (42, 43). Our results obtained with purified Ypt/Rab-GAPs and mutant Ypt/GTPases are in sharp contrast to mammalian H-Ras protein carrying the equivalent substitution. Ras(Q61L) is known to be oncogenic (44) because of its significantly reduced intrinsic GTPase activity, which is not accelerated or is only marginally accelerated by its cognate GAPs (35, 45). The substitution with leucine of the conserved Gln[67] in Ypt1p results in a 40-fold decrease of its inbuilt GTPase activity. This drop of activity is even more pronounced than that of the corresponding Ras(Q61L) mutant whose intrinsic GTP hydrolysis rate has been reported to be about 22-fold lower than the hydrolysis rate of wild type H-Ras (46). Importantly, Gyp5p accelerated the intrinsic GTP hydrolysis rate of Ypt1(Q67L)p by more than 10^4-fold, which is even 1000-fold higher than the intrinsic GTPase activity of wild type Ypt1p. Therefore, one can assume that there are mechanistic differences in the mode of activation of GTP hydrolysis by GAPs that are specific for different GTPase subtypes within the Ras superfamily. The acceptance as efficient substrate of Ypt1(Q67L)p by its cognate GAP Gyp5p could explain why cells expressing the GTPase-defective instead of the Ypt1 wild type protein exhibit, depending on the cell's genetic background, no growth or very minor growth or protein transport alterations. The same has been reported for Sec4p, the essential GTPase

involved in exocytosis. Sec4(Q79L)p, analogous to Ypt1(Q67L)p, caused cold sensitivity of mutant cells but only minor lesions of the secretory activity (43). It is likely, therefore, that in cells expressing Ypt1(Q67L)p as the only source for Ypt1p there is sufficient hydrolysis of GTPase-bound GTP to allow cycling and proper functioning of the essential GTPase. In this context, we feel it to be misleading to argue (11) that GTP hydrolysis is not important for Ypt1-GTPase function in vesicular transport.

The prominent phenotypes of mutant cells with severely perturbed hydrolysis of Ypt1p-bound GTP are growth defects at low temperature and various morphological alterations. The latter were mostly independent of the growth temperature and included moderately increased proliferation of ER membranes, pleiomorphic vacuoles, and an accumulation of vesicular structures of different size, most frequently ranging from about 50 to 200 nm. Although we have not attempted to isolate and further examine these membrane-enclosed structures, we feel it to be likely that they could represent ER-derived vesicles and their homotypic fusion products. In yeast, COPII-coated transport vesicles originating from the ER have, on average, a diameter of 50 nm (2). Several early secretory mutants accumulate this type of vesicle (47), among them conditional *ypt1* mutants at the nonpermissive temperature (48). In the mutant cells described here, which lack Ypt1-GAPs and express the GTPase-deficient Ypt1(Q67L) protein as the only source for Ypt1p, the vast majority of the essential GTPase is expected to be in its GTP-bound (*i.e.* active) conformation. This might lead to increased fusion activity similar to that seen in mammalian cells that overexpress the corresponding Rab5(Q79L) mutant GTPase and, as a consequence, form significantly enlarged early endosomes (49). Increased fusion, therefore, might not only occur between ER-derived vesicles and Golgi compartments but also between vesicles themselves. Homotypic COPII vesicle fusion seems to be a regular event in mammalian cells, giving rise to the formation of so-called vesicular-tubular clusters (VTCs) (50, 51).

Accelerated and malcontrolled fusion processes that we assume to take place in mutants with permanently active Ypt1-GTPase obviously do not impair protein transport from the ER to and through the Golgi but rather the equilibrium between membrane-enclosed organelles of the early secretory pathway. The morphological alterations seen in these cells suggest to us that an autophagic response might be induced, presumably to destroy transport vesicles and organelles derived from increased vesicle fusion processes. Vacuolar uptake of vesicular structures was documented most dramatically in protease-deficient cells that lack the Ypt1-GAP Gyp5p and express the Ypt1(Q67L) GTPase. This would resemble pexophagy and mitophagy, the selective degradation in vacuoles of peroxisomes or mitochondria in response to changed nutrient and growth conditions (52). The long tubular invaginations of vacuoles and the apparently direct engulfment and uptake of vesicular and short tubular organelles from the vacuole surface seen in the mutants studied here conspicuously resemble the microautophagic sequestration of peroxisomes, for example (53).

Selective vacuolar sequestration of transport intermediates has not been observed previously. It is possible, therefore, that induction of autophagy is a way to not only selectively destroy unused peroxisomes or mitochondria but also a surplus of organelles of the biosynthetic pathway to keep the dynamic balance of membranes making up its different compartments. However, definite proof for a selective degradation of vesicular transport intermediates via an autophagic pathway has to await further studies involving different autophagic mutants, but preliminary results already suggest that haploid cells ex-

pressing the Ypt1(Q67L) mutant GTPase do not allow the deletion of the otherwise nonessential *AUT1* (37) or *AUT7* (38) genes.

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