Opposite Roles of the F-Box Protein Rcy1p and the GTPase-Activating Protein Gyp2p During Recycling of Internalized Proteins in Yeast

Céline Lafourcade,* Jean-Marc Galan† and Matthias Peter*,[1]

**Swiss Federal Institute of Technology Zurich (ETH), Institute of Biochemistry, 8093 Zurich, Switzerland and* †*Institut Jacques Monod-CNRS, 75251 Paris Cedex 05, France*

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ABSTRACT

The F-box protein Rcy1p is part of a non-SCF (Skp1p-cullin-F-box protein) complex involved in recycling of internalized material. Like *rcy1*Δ, cells lacking the Rab-GTPase Ypt6p or its heterodimeric GEFs Rgp1p and Ric1p are unable to recycle the v-SNARE Snc1p. Here we provide genetic evidence suggesting that Rcy1p is a positive regulator of Ypt6p. Deletion of the GAP Gyp2p restores recycling in *rcy1*Δ, while overexpression of an active form of Ypt6p partially suppresses the recycling defect of *rcy1*Δ cells. Conversely, overexpression of Gyp2p in wild-type cells interferes with recycling of GFP-Snc1p, and the cells accumulate membrane structures as evidenced by electron microscopy. Gyp2p-GFP is distributed throughout the cytoplasm and accumulates in punctate structures, which concentrate in an actin-dependent manner at sites of polarized growth. Taken together, our results suggest that the F-box protein Rcy1p may activate the Ypt6p GTPase module during recycling.

TRAFFICKING of vesicles from and to the plasma membrane and between different cellular compartments is fundamental for the organization and functioning of eukaryotic cells. The Rab/Ypt-GTPases are key regulators of membrane trafficking and together with SNARE proteins mediate selective fusion of vesicles with target compartments (Segev 2001). They cycle between GDP- and GTP-bound forms, and accessory proteins that regulate this cycling are thought to be critical for Ypt/Rab function. Guanine-nucleotide exchange factors (GEFs) stimulate both GDP loss and GTP uptake, while GTPase activating proteins (GAPs) catalyze GTP hydrolysis. A family of related proteins termed Gyp's has been discovered and shown to exhibit GAP activity toward several Ypt/Rab GTPases *in vitro* (Strom *et al.* 1993; Albert and Gallwitz 1999). However, little is known about the role of GEFs and GAPs for Ypt/Rab proteins *in vivo*.

In eukaryotes, most plasma membrane proteins are internalized by endocytosis and either recycle back to the plasma membrane or are degraded in the lysosomal/vacuolar compartment. Internalized proteins travel through two morphologically and biochemically distinct organelles called early and late endosomes, from where they are sorted in the two pathways. Recycling of many signaling receptors back to the plasma membrane allows multiple rounds of ligand binding and internalization. In some specialized cell types the recycling pathway is also used for antigen presentation and transcytosis, as well as for recycling of synaptic vesicle components (von Bartheld *et al.* 2001). However, the machinery and molecular mechanisms controlling recycling of plasma membrane proteins are poorly understood.

In the yeast *Saccharomyces cerevisiae*, several proteins are transported back to the plasma membrane and are thus used as markers to study recycling pathways *in vivo*. For example, the pheromone-induced endocytosis of Ste3p results in its recycling back to the plasma membrane (Chen and Davis 2000; Chen and Davis 2002), while the chitin synthase Chs3p translocates between sites of chitin deposition on the cell surface and an internal structure called the chitosome (Ziman *et al.* 1998; Valdivia *et al.* 2002). Moreover, recycling of the exocytic v-SNARE Snc1p, which is involved in fusion of Golgi-derived secretory vesicles with the plasma membrane, allows reutilization of Snc1p for several rounds of secretion (Lewis *et al.* 2000). The localization and phosphorylation state of green fluorescent protein (GFP)-Snc1p provides a convenient marker of recycling. In wild-type cells, GFP-Snc1p is localized at the plasma membrane with some punctate staining of internal structures, while it accumulates in intracellular structures in recycling mutants (Lewis *et al.* 2000; Galan *et al.* 2001). Recycling can also be quantified by measuring resecretion of previously internalized fluorescent dye FM4-64 (Wiederkehr *et al.* 2000). Using these recycling assays, several components involved in recycling have recently been identified. For example, the Rab-GTPase Ypt6p and its heterodimeric GEF Ric1p/Rgp1p is required for recycling of Snc1p, presumably by mediating fusion of endosomal vesicles with the Golgi compart-

[1]*Corresponding author:* Swiss Federal Institute of Technology Zurich (ETH), Institute of Biochemistry, ETH Hoenggerberg HPM G 6.2, 8093 Zurich, Switzerland. E-mail: matthias.peter@bc.biol.ethz.ch

TABLE 1

Yeast strains

Strain name	Relevant genotype	Background	Source
K699	Wild type	W303	K. Nasmyth
YJMG 263	*MAT***a** *rcy1::KanMX4*	W303	S. Alvaro
YCL 01	*MAT***a** *rcy1::KanMX4 gyp2::lacz cl101*	W303	This study
YCL 03	*MAT***a** *rcy1::KanMX4 gyp2::lacz cl103*	W303	This study
YCL 04	*MAT***a** *rcy1::KanMX4 rdn37-1::lacz cl104*	W303	This study
BY4741	Wild type	BY4741	Euroscarf
YCL 245	*MAT*α *rcy1::KanMX4*	BY4741	Euroscarf
YCL 260	*MAT***a** *rcy1::KanMX4*	BY4741	Euroscarf
YCL 80	*MAT***a** *gyp2::KanMX4*	BY4741	Euroscarf
YCL 79	*MAT***a** *gyp2::KanMX4 rcy1::KanMX4*	BY4741	This study
YCL 152	*MAT***a** *gyp6::KanMX4*	BY4741	Euroscarf
YCL 259	*MAT***a** *gyp6::KanMX4 rcy1::HIS3*	BY4741	This study
YCL 94	*MAT***a** *gyp3::KanMX4*	BY4741	Euroscarf
YCL 92	*MAT***a** *gyp3::KanMX4 rcy1::KanMX4*	BY4741	This study
YCL 172	*MAT***a** *gyp2::HIS3*	W303	This study
YCL 170	*MAT***a** *gyp2::HIS3 rcy1::KanMX4*	W303	This study
YCL 154	*MAT***a** *ypt6::KanMX4*	BY4741	Euroscarf
YCL 257	*MAT***a** *ypt6::KanMX4 gyp2::HIS3*	BY4741	This study
YCL 156	*MAT***a** *ric1::KanMX4*	BY4741	Euroscarf
YCL 258	*MAT***a** *ric1::KanMX4 gyp2::HIS3*	BY4741	This study
YCL 158	*MAT***a** *rgp1::KanMX4*	BY4741	Euroscarf
YCL 261	*MAT***a** *rgp1::KanMX4 gyp2::HIS3*	BY4741	This study
YCL 174	*MAT***a** *gyp2::GYP2-GFP-HIS3*	W303	This study
YCL 211	*MAT***a** *gyp2::GYP2-13Myc-HIS3*	W303	This study
YCL 212	*MAT***a** *rcy1::KanMX4 gyp2::GYP2-13Myc-HIS3*	W303	This study

ment (Siniossoglou *et al.* 2000; Siniossoglou and Pelham 2001). Likewise, Chs3p and Snc1p accumulate in intracellular compartments in cells defective for the SNAREs Tlg1p and Tlg2p (Holthuis *et al.* 1998; Lewis *et al.* 2000). Snc1p colocalizes with Tlg2p, suggesting that Tlg2p may play a direct role in recycling (Lewis *et al.* 2000). Finally, Rcy1p and its binding partner Skp1p are required for recycling of GFP-Snc1p at a postinternalization step of endocytosis (Wiederkehr *et al.* 2000; Galan *et al.* 2001). Rcy1p contains an amino-terminal F-box, which interacts with Skp1p (Bai *et al.* 1996), and a CAAX-box motif at its carboxyl terminus, which mediates its interaction with membranes (Zhang and Casey 1996; Galan *et al.* 2001). Moreover, the carboxy-terminal domain of Rcy1p shows homology to hSec10, a protein involved in membrane trafficking (Wysocki *et al.* 1999). However, the molecular role of Rcy1p in membrane trafficking remains elusive. Despite the functional interaction with Skp1p, the function of Rcy1p may not be linked to ubiquitin-dependent degradation (Galan *et al.* 2001).

Here we have investigated the role of Rcy1p in recycling. We found that deletion of the GAP Gyp2p suppresses the recycling defect of *rcy1*Δ cells. Our results implicate Gyp2p in recycling and suggest that Rcy1p functions as a positive regulator of the Ypt6p module *in vivo*.

MATERIALS AND METHODS

Yeast strains: Yeast strains are described in Table 1. Strains are derived from K699: *MAT***a** *ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3 GAL+ psi+ ssd1-d2* (W303 background) or BY4741: *MAT***a** *his3-*Δ*1 leu2-*Δ*0 met15-*Δ*0 ura3-*Δ*0* (Euroscarf strains). Standard yeast growth conditions and genetic manipulations were used as described (Guthrie and Fink 1991). Strains expressing GFP-, myc-, or hemagglutinin-tagged versions of *GYP2* were constructed as described (Longtine *et al.* 1998).

DNA manipulations: Plasmids are listed in Table 2. Standard procedures were used for recombinant DNA manipulations (Ausubel *et al.* 1991). PCR reactions were performed with the Expand polymerase kit as recommended by the manufacturer (Boehringer Mannheim, Indianapolis). Oligonucleotides were synthesized by Genset (Paris), and the sequences are available upon request.

TABLE 2

Plasmids

Plasmid name	Relevant characteristics	Source
pJMG 118	GFP-SNC1 URA3 CEN	M. Lewis
pCL 145	GFP-SNC1 LEU2 CEN	This study
pCL 46	GAL-GFP-GYP2 URA3 CEN	This study
pCL 66	GAL-GYP2 URA3 CEN	This study
pCL 69	GAL-YPT6Q69L URA3 CEN	This study

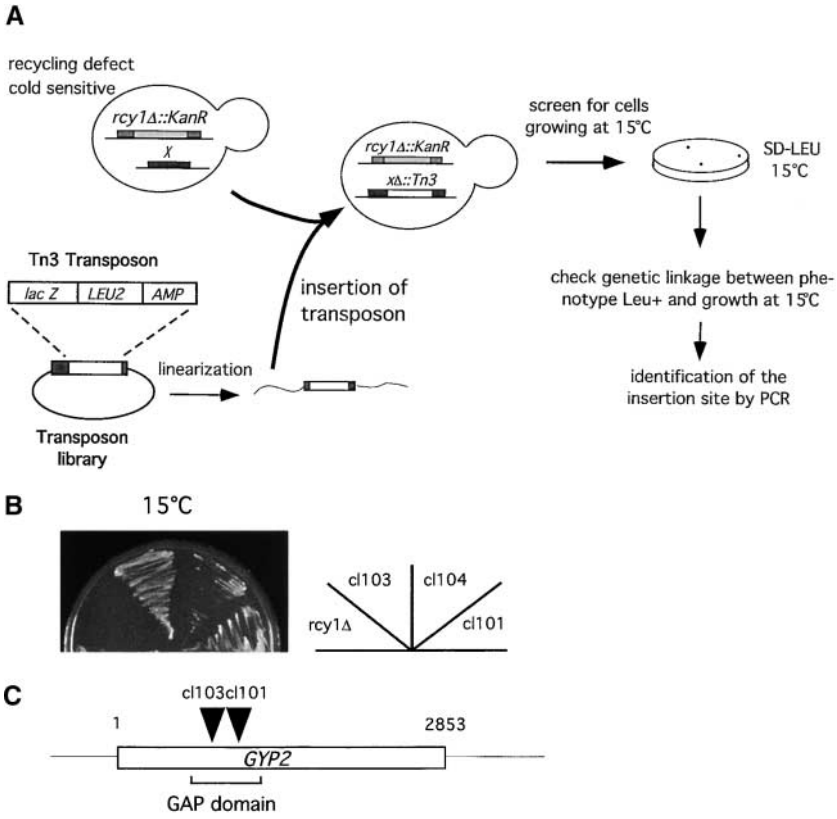

FIGURE 1.—A genetic screen to identify suppressors of the growth defect of *rcy1*Δ cells at 15°. (A) The experimental strategy to identify suppressor of *rcy1*Δ cells. *rcy1*Δ cells (YJMG263) were mutagenized with the transposon library as described previously (BURNS *et al.* 1994), and transformants able to grow at 15° were isolated (B). The genetic linkage of the integrated transposon (marked by *LEU2*) with the suppression phenotype was confirmed by tetrad analysis. The insertion site of the transposon was determined using a degenerate PCR primer, and the flanking region was sequenced. (C) Two independent transposons inserted into the 5′ region of the open reading frame of *GYP2*.

Antibodies, Western blots, phosphatase assays, and microscopy: Standard procedures were used for yeast cell extract preparation and immunoblotting (GALAN and PETER 1999). A polyclonal antibody against Sui2p (eIF2α) was used for loading controls (gift of A. Hinnenbusch). 9E10 antibodies were produced by the ISREC antibody facility.

For microscopy, cells were grown to early log phase and photographed on a Zeiss axiophot fluorescence microscope with a Photometrics CCD camera. GFP-tagged proteins were visualized using a Chroma GFPII filter (excitation 440–470 nm) and analyzed with Photoshop 4.0 software. Cells expressing GFP-Gyp2p from the inducible *GAL* promoter were grown to early log phase at 30° in selective media containing raffinose (2% final concentration), at which time galactose was added (2% final concentration) for 4 hr. Where indicated, the actin polymerization inhibitor latrunculin-A (200 μM final concentration in DMSO), or for control DMSO, was added. Where indicated, α-factor was added to a final concentration of 50 μg/ml.

Electron microscopy: Wild-type cells (K699) overexpressing either no protein (vector) or Gyp2p (Gyp2p) from the inducible *GAL* promoter were grown at 30° in selective medium containing 2% raffinose and induced by adding galactose to 2% final concentration. After 4 hr, yeast cells were fixed by adding 200 μl of 50% aqueous glutaraldehyde to 10 ml of growth medium for 10 min and then centrifuged at 5000 × *g* for 10 min at 4°. After fixation with fresh fixatives for 2 hr at 4°, cells were washed in 0.1 M cacodylate buffer (pH 7.4) and in water. Subsequently, cells were treated with 1% $KMnO_4$ for 2 hr on ice, washed in water, and resuspended in 2% aqueous uranyl acetate for 1 hr at 4°. Cells were dehydrated in a graded series of ethanol, infiltrated in a mixture of ethanol and Spurr's resin, and embedded in Spurr's low viscosity media. Thin sections were cut, stained with lead citrate, and examined in a Tecnai 12 electron microscope.

Determination of half-life: Cultures were grown to early log phase in rich medium at 30°, at which time cycloheximide (CHX; Sigma, St. Louis) was added to a final concentration of 50 μg/ml (stock solution: 10 mg/ml). Aliquots were collected at the times indicated, and protein levels were analyzed by immunoblotting with specific antibodies.

FM4-64 recycling assay: FM4-64 recycling assays were performed as described (WIEDERKEHR *et al.* 2000). Yeast cells were allowed to internalize FM4-64 for 12 min at 24° and washed three times with ice-cold SD medium. After the last wash the cells were resuspended in 10 μl of SD medium and kept on ice. Prewarmed SD medium at 24° was added to the cells and the fluorescence was recorded during 600 sec.

RESULTS

Deletion of GYP2 specifically suppresses the growth defect of *rcy1*Δ cells at 15°: To investigate the role of Rcy1p in recycling, we designed a genetic screen to identify second-site suppressors of the cold-sensitive phenotype of *rcy1*Δ cells (WIEDERKEHR *et al.* 2000). As schematically depicted in Figure 1A, *rcy1*Δ cells (YJMG263) were mutagenized with a transposon library (BURNS *et al.* 1994), and the transformants (18,400 colonies total) were selected for their ability to grow at 15°. All selected mutants were backcrossed with a wild-type strain, and only single gene traits linked to the *LEU2* marker were followed further (Figure 1B). To determine the gene responsible for the observed suppression, we isolated genomic DNA of the mutants and amplified the flanking sequence of the inserted transposon by PCR (DI RIENZO *et al.* 1996). The cl104-transposon inac-

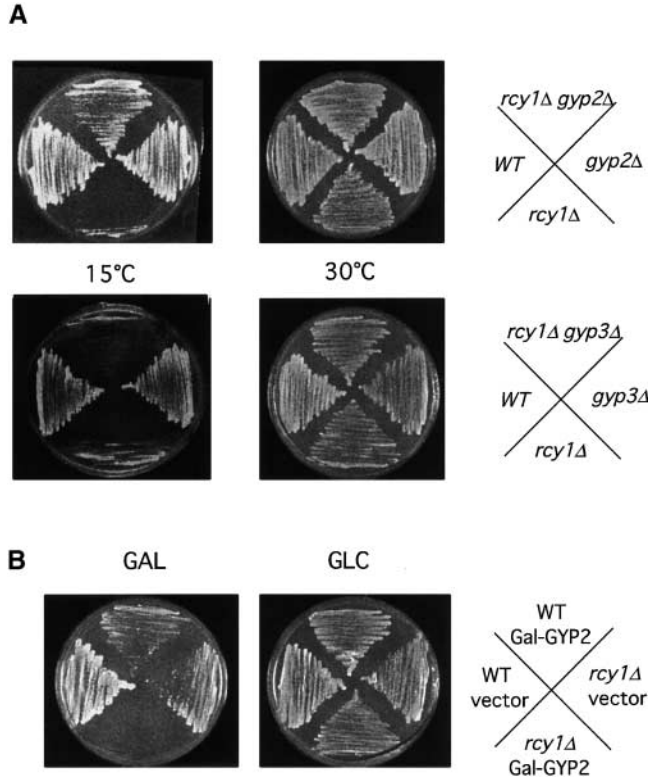

FIGURE 2.—Genetic interactions between *RCY1* and *GYP2*. (A) *rcy1*Δ cells (YCL245) were crossed with cells deleted for *GYP2* (YCL80) or *GYP3* (YCL94), and growth of the resulting double mutants (YCL79 and YCL92, respectively) was compared to wild-type (BY4741) and *rcy1*Δ cells (YCL245) after 7 days at 15° on YPD medium. (B) Overexpression of Gyp2p is toxic in *rcy1*Δ cells. Wild-type (BY4741) and *rcy1*Δ cells (YCL260) were transformed with an empty control plasmid (vector) or a plasmid overexpressing Gyp2p from the inducible *GAL1* promoter (pCL66) and plated on medium containing galactose (left, *GAL* promoter on) or glucose (right, *GAL* promoter off) at 30°. The plates were photographed after 3 days.

tivated the RDN37-1 gene, which codes for a RNA implicated in ribosome biogenesis. However, although deletion of RDN37-1 rescued the cold sensitivity of *rcy1*Δ cells, it did not restore its associated recycling defect (see below; data not shown) and thus was not further characterized. Interestingly, two independent transposons (cl101 and cl103) inserted into the *GYP2* locus and judged by the insertion site are likely to inactivate the gene (Figure 1C). Mdr1p/Gyp2p is a member of the GAPs for the Ypt/Rab family of proteins and was shown to hydrolyze GTP from Ypt6p and Sec4p *in vitro* (ALBERT and GALLWITZ 1999). To ensure that deletion of *GYP2* specifically restores growth to *rcy1*Δ cells at 15°, we compared *rcy1*Δ *gyp2*Δ and *rcy1*Δ *gyp3*Δ double mutants (Figure 2A). Interestingly, only deletion of *GYP2* but not *GYP3* corrected the cold-sensitive phenotype, suggesting that Rcy1p does not generally affect GAPs of the Gyp family. Moreover, we found that overexpression of Gyp2p was toxic in *rcy1*Δ cells at 30° (Figure 2B), while wild-type cells were only weakly affected. Taken together, these genetic experiments suggest that Rcy1p may inhibit the function of Gyp2p *in vivo*.

Deletion of GYP2 suppresses the recycling defect of *rcy1*Δ cells: We next determined whether deletion of Gyp2p suppresses the recycling defect of *rcy1*Δ cells. Two assays have been used as readouts for a functional recycling pathway: first, resecretion of previously internalized FM4-64 (WIEDERKEHR *et al.* 2000) and second, changes in the subcellular localization and phosphorylation state of GFP-Snc1p (LEWIS *et al.* 2000; GALAN *et al.* 2001). As expected, GFP-Snc1p was predominantly intracellular and accumulated in its underphosphorylated form in *rcy1*Δ cells (Figure 3, A and B). In addition, GFP-Snc1p levels are significantly lower in recycling mutants, most likely because GFP-Snc1p is degraded in the vacuole in recycling-deficient mutant cells (C. LAFOURCADE and M. PETER, unpublished results). Interestingly, plasma membrane localization and hyperphosphorylation of GFP-Snc1p was restored in *rcy1*Δ *gyp2*Δ but not in *rcy1*Δ *gyp6*Δ cells (Figure 3, A and B), implying that loss of Gyp2p indeed suppresses the recycling defect of *rcy1*Δ cells. Cells deleted for *GYP2* exhibit no obvious defect of GFP-Snc1p localization or recycling of FM4-64, implying that Gyp2p is not essential for recycling. Consistent with these results, *rcy1*Δ cells exhibited a recycling defect of the fluorescent dye FM4-64, which was suppressed by simultaneous deletion of *GYP2* (Figure 3C). On the basis of these results, we conclude that deletion of *GYP2* restores recycling in *rcy1*Δ cells.

Gyp2p is a component of the Ypt6p GTPase module involved in recycling: Gyp2p has been shown previously to function as a GAP for Ypt6p and Sec4p *in vitro* (ALBERT and GALLWITZ 1999). Moreover, activation of Ypt6p *in vivo* is mediated by the heterodimeric GEF Ric1p/Rgp1p (SINIOSSOGLOU *et al.* 2000). To further characterize the involvement of the Ypt6p module in recycling, we determined the localization and phosphorylation state of GFP-Snc1p in *ypt6*Δ, *ric1*Δ, and *rgp1*Δ cells (Figure 4). As previously reported, GFP-Snc1p accumulated in the cytoplasm in these mutants (Figure 4A; SINIOSSOGLOU *et al.* 2000) and was found predominantly in the unphosphorylated form (Figure 4B), indicative of a recycling defect. The recycling defects of *ypt6*Δ, *ric1*Δ, and *rgp1*Δ cells were not restored by deletion of *GYP2* (Figure 4). Taken together, these results support the notion that Gyp2p and Rgp1p/Ric1p regulate Ypt6p during recycling of GFP-Snc1p *in vivo*. Importantly, overexpression of a mutationally activated form of Ypt6p (Ypt6Q69L) was able to partially restore recycling of GFP-Snc1p in *rcy1*Δ cells (Figure 4C), suggesting that Rcy1p directly or indirectly activates the Ypt6p GTPase module.

Overexpression of Gyp2p inhibits recycling of GFP-Snc1p: To investigate whether wild-type cells overexpressing Gyp2p exhibit a recycling defect, we expressed Gyp2p from the inducible *GAL* promoter. Cells overex-

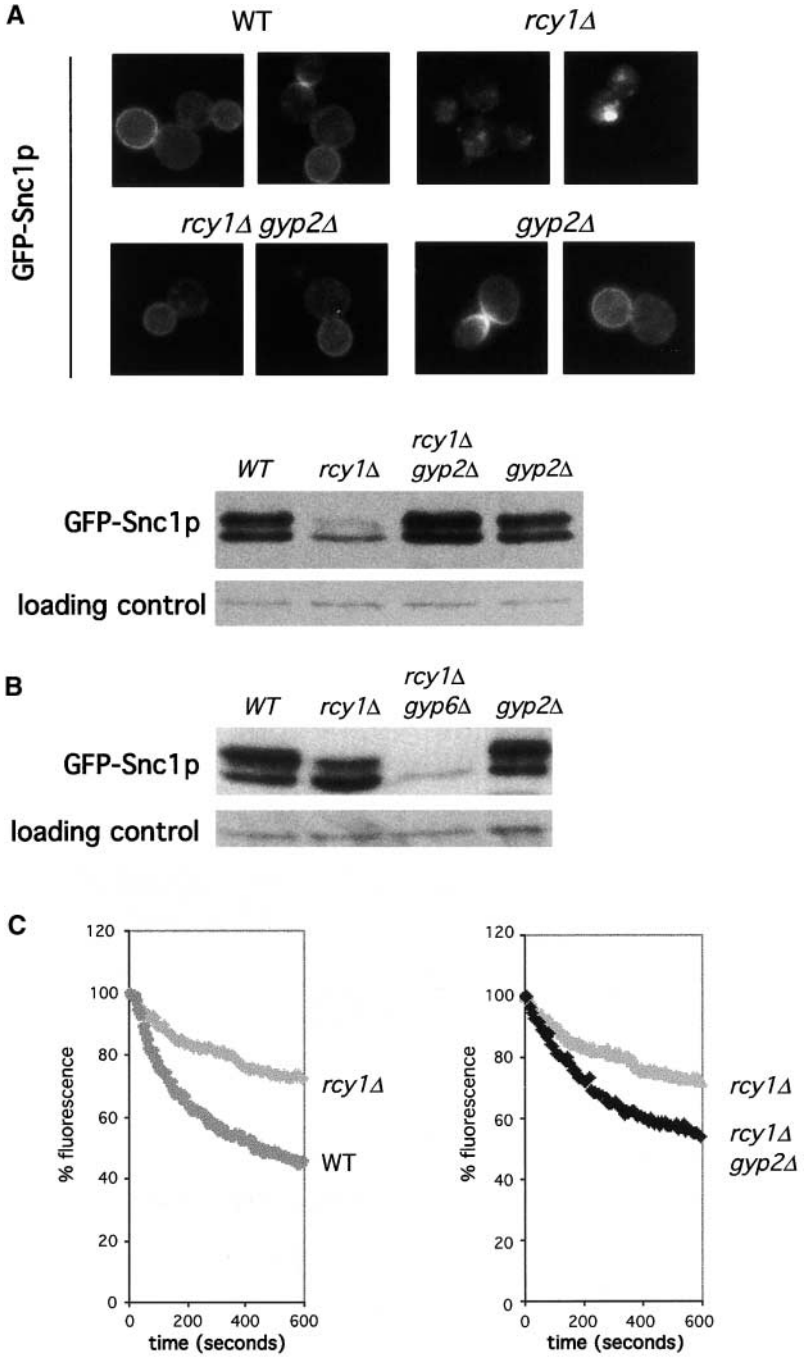

FIGURE 3.—Deletion of *GYP2* restores the recycling defect of *rcy1*Δ cells. (A) Wild-type (K699), *rcy1*Δ (YJMG263), *gyp2*Δ (YCL172), and *rcy1*Δ *gyp2*Δ (YCL170) were transformed with a plasmid expressing the recycling v-SNARE GFP-Snc1p (JMG118), grown in selective SD medium to early log phase, and analyzed by GFP fluorescence microscopy. The phosphorylation state of GFP-Snc1p was examined by Western blot using an antibody raised against GFP. Sui2p was used as a loading control. Note that the phosphorylation state of GFP-Snc1p correlates with its subcellular localization. (B) The phosphorylation state of GFP-Snc1p was also examined by Western blot in wild-type (BY4741), *rcy1*Δ (YCL260), *gyp6*Δ (YCL152), and *rcy1*Δ *gyp6*Δ (YCL259) cells. (C) Wild-type (K699) or the indicated mutant cells were analyzed for their ability to recycle the membrane dye FM4-64 as described in MATERIALS AND METHODS. Note that deletion of *GYP2* restores recycling of GFP-Snc1p in *rcy1*Δ cells.

pressing Gyp2p exhibited a cold- and temperature-sensitive growth defect (data not shown), indicative of a recycling defect. Indeed, GFP-Snc1p accumulated intracellularly in cells overexpressing Gyp2p for 4 hr (Figure 5A), but not in control cells either harboring an empty plasmid (vector) or grown under noninducing conditions (GLC). Likewise, the hyperphosphorylated form of GFP-Snc1p was significantly reduced in a time-dependent manner after inducing Gyp2p by the addition of galactose (Figure 5B). In contrast, overexpression of Gyp2p did not significantly impair targeting of Fur4p to the plasma membrane (data not shown), implying that the secretion pathway is not affected. We conclude that overexpression of Gyp2p specifically triggers a recycling defect. To examine whether a recycling compartment may accumulate in cells overexpressing Gyp2p, we performed electron microscopy on cells grown in galactose for 4 hr. As shown in Figure 5C, many electron-dense membrane structures became apparent (arrowheads), which may resemble the structures accumulating in *rcy1*Δ cells (WIEDERKEHR *et al.* 2000). Taken together, the biochemical and electron microscopy data suggest that overexpression of Gyp2p causes a recycling phenotype, reminiscent of cells lacking Rcy1p.

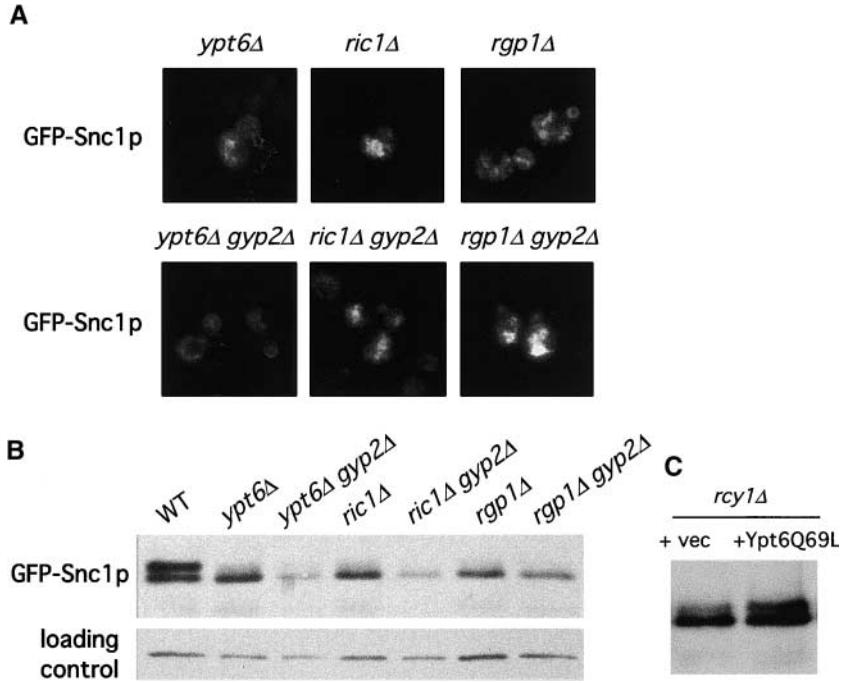

FIGURE 4.—The Ypt6p module is involved in recycling of GFP-Snc1p. (A and B) YCL154 (*ypt6::KanMX4*), YCL156 (*ric1::KanMX4*), YCL158 (*rgp1::KanMX4*), YCL257 (*ypt6::KanMX4 gyp2::HIS3*), YCL258 (*ric1::KanMX4 gyp2::HIS3*), and YCL261 (*rgp1::KanMX4 gyp2::HIS3*) cells transformed with the plasmid JMG118 (GFP-Snc1p) were grown at 30° to early log phase, and the subcellular localization (A) and phosphorylation state (B) of GFP-Snc1p was analyzed as described in the legend to Figure 3. (C) The phosphorylation state of GFP-Snc1p (pCL145) was analyzed in *rcy1*Δ cells (JMG263) overexpressing either no protein (vector) or Ypt6-Q69L (pCL69) from the inducible *GAL* promoter. Note that overexpression of Ypt6Q69L partially restores recycling of GFP-Snc1p in *rcy1*Δ cells.

Gyp2p-GFP localizes to the cytoplasm and specific structures, which accumulate at sites of polarized growth: To localize Gyp2p, we replaced its coding sequence at the endogenous locus with *GYP2-GFP* (Figure 6). In addition, we expressed GFP-Gyp2p from the inducible *GAL* promoter. When overexpressed, GFP-Gyp2p was toxic in *rcy1*Δ cells like untagged Gyp2p (data not shown), suggesting that the fusion protein is functional. Both endogenous and overexpressed GFP-tagged Gyp2p (Figure 6A) were predominantly cytoplasmic and localized to dot-like structures, which accumulated in small buds or at tips of mating projections in cells treated with α-factor (Figure 6B). Interestingly, this polarized localization was dependent on an intact actin cytoskeleton, as the polarized localization of Gyp2p-GFP was disturbed after treating α-factor-arrested cells with the actin-depolymerizing drug latrunculin-A. Thus, the localization of Gyp2p-GFP resembles the subcellular localization of Rcy1p, supporting the notion that Rcy1p may regulate Gyp2p *in vivo*. However, colocalization studies will be required to confirm that Rcy1p and Gyp2p indeed accumulate in the same structure.

The stability of Gyp2p is not regulated by Rcy1p: Several F-box proteins are subunits of SCF E3-ligase complexes, which are involved in degradation of target proteins. Because the phenotype of cells overexpressing Gyp2p resembles the phenotype of *rcy1*Δ cells, we tested whether Rcy1p controls the stability of Gyp2p. To detect Gyp2p, we tagged Gyp2p at its carboxy terminus with 13 copies of the 9E10 epitope (Gyp2p-myc). The half-life of Gyp2p-myc was determined by *GAL* shutoff (data not shown) or cycloheximide chase experiments in either wild-type or *rcy1*Δ cells (Figure 7). Gyp2p-myc was a stable protein and its half-life was not affected by Rcy1p. Several slower migrating forms of Gyp2p-myc were apparent, but the formation of these modified forms was not dependent on the presence of Rcy1p. These results suggest that Rcy1p may not regulate the stability of the bulk part of Gyp2p. Consistent with these observations, we did not detect an interaction between Gyp2p and Rcy1p by two-hybrid assays (data not shown).

DISCUSSION

Here we investigated the role of the F-box protein Rcy1p in recycling of membrane proteins. We identify Gyp2p as a suppressor of the recycling defect of *rcy1*Δ cells and provide *in vivo* evidence that Gyp2p functions as a GAP for Ypt6p during recycling between the endosome and the Golgi compartments. On the basis of genetic evidence, we propose that Rcy1p is a positive regulator of the Ypt6 module *in vivo*, which does not regulate the stability of Gyp2p.

Gyp2p is part of the Ypt6p GTPase module that regulates recycling: Rab/Ypt proteins are key regulators of all steps of vesicular trafficking and primary determinants of compartment specificity. Our results suggest that Gyp2p inactivates Ypt6p during recycling *in vivo*, thereby antagonizing the GEF complex Ric1p/Rgp1p. Consistent with these findings, Gyp2p was recently copurified in a complex containing Ypt6p (SINIOSSOGLOU and PELHAM 2001) and functions as a GAP for both Ypt6p and Sec4p *in vitro* (ALBERT and GALLWITZ 1999). *gyp2*Δ cells do not exhibit an obvious recycling defect, implying that rapid cycling between the GTP- and GDP-bound state of Ypt6p may not be required for its function *in vivo*. It is possible that the intrinsic GAP activity of Ypt6p is sufficient to allow its inactivation. Alternatively, some GAPs of the Gyp family may function in a redundant manner. Indeed, besides Gyp2p, Gyp3p, Gyp4p,

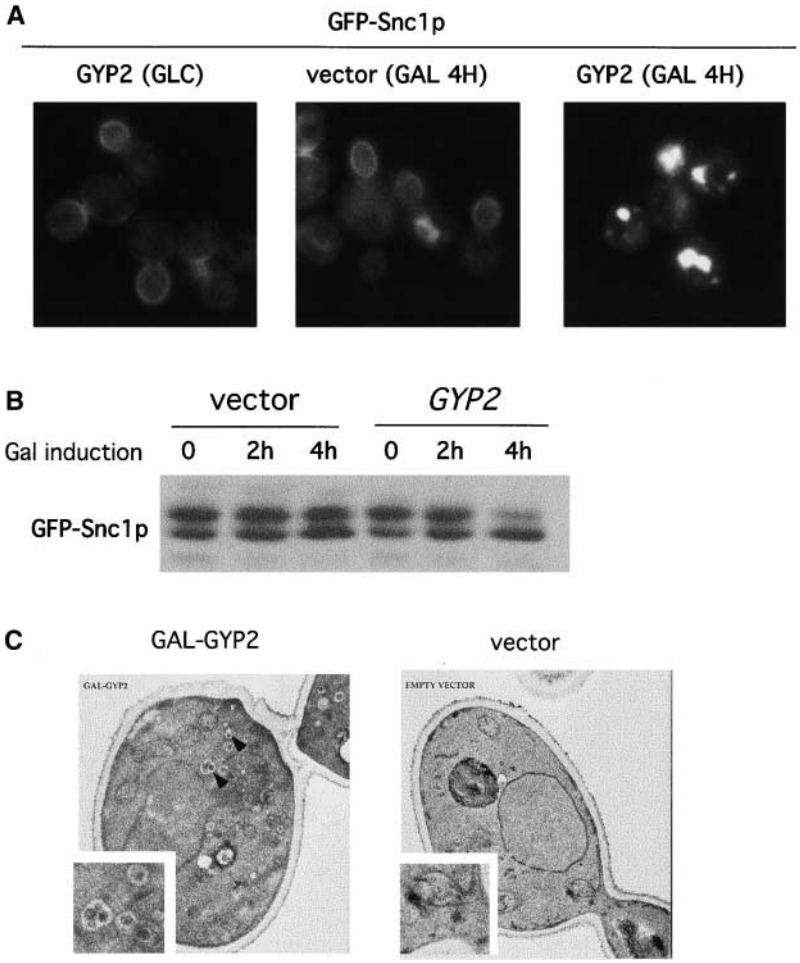

FIGURE 5.—Overexpression of Gyp2p interferes with recycling of GFP-Snc1p. (A and B) The subcellular localization and phosphorylation state of GFP-Snc1p was analyzed in wild-type cells (K699) overexpressing either no protein (vector) or Gyp2p (Gyp2p) from the inducible *GAL* promoter. For control, cells grown under noninducing conditions were included (GLC). Cells were analyzed at the times indicated (in hours) after addition of galactose (GAL) by either GFP fluorescence (A) or immunoblotting with GFP antibodies (B). Note that cells overexpressing Gyp2p are unable to efficiently recycle GFP-Snc1p. (C) Wild-type cells (K699) overexpressing either no protein (vector) or Gyp2p (Gyp2p) from the inducible *GAL* promoter were analyzed by electron microscopy 4 hr after addition of 2% galactose. Cells were fixed, dehydrated, and embedded for electron microscopy as described in MATERIALS AND METHODS. Note that cells overexpressing Gyp2p accumulate membranous structures in the cytoplasm (inset).

and Gyp6p are also able to stimulate hydrolysis of Ypt6p-GTP *in vitro* (ALBERT and GALLWITZ 1999). However, unlike *GYP2*, deletion of *GYP6* was unable to restore recycling of GFP-Snc1p in *rcy1*Δ cells, suggesting that at least these GAPs do not function in a redundant manner during recycling *in vivo*.

Rcy1p may activate Ypt6p *in vivo*: Several lines of evidence suggest that Rcy1p may function as a positive regulator of the Ypt6p module *in vivo*. First, cells lacking *YPT6* or its activators *RGP1/RIC1* exhibit a recycling defect, which is similar to *rcy1*Δ cells. Second, both loss of Gyp2p function and overexpression of the active form of Ypt6p restored recycling of GFP-Snc1p in *rcy1*Δ cells. Finally, overexpression of Gyp2p interfered with recycling of GFP-Snc1p in otherwise wild-type cells. On the basis of these results we suggest that the recycling defect of *rcy1*Δ cells may be due to their inability to produce Ypt6p-GTP. At present we do not know the molecular mechanism for how Rcy1p regulates the Ypt6p module. Given that Rcy1p interacts with Skp1p through its F-box, we tested whether Rcy1p may be involved in ubiquitin-dependent degradation of Gyp2p. However, we were unable to detect a difference in the half-life of Gyp2p in the presence or absence of Rcy1p, consistent with the previous finding that Rcy1p may not be part of a conventional E3 ligase of the SCF family (GALAN *et al.* 2001). Although ubiquitination of several membrane proteins is important for their internalization, currently no evidence suggests that Rcy1p may function at subsequent steps, which involve ubiquitin. Moreover, recent evidence suggests that ligand-induced endocytosis and recycling of Ste3p does not require ubiquitination (CHEN and DAVIS 2002). Besides the F-box, the sequence of Rcy1p does not provide clues to its function. It is possible that Rcy1p inhibits the GAP activity of Gyp2p, although by two-hybrid assay we were unable to detect a physical interaction between Gyp2p and Rcy1p. Alternatively, Rcy1p may function as a GEF for Ypt6p or may activate the GEF Ric1p/Rgp1p. It is worthwhile to note that the known GEFs for Ypt-GTPases share no obvious sequence homology, and thus *in vitro* GAP and GEF assays are needed to test these possibilities. However, we cannot exclude that Rcy1p regulates the Ypt6p module indirectly and thus may not physically interact with its components. For example, Rcy1p may regulate the localization or expression of Ypt6p or its activators Ric1p/Rgp1p, or it may function in a pathway unrelated to Ypt6p, which may nonspecifically interfere with Ypt6p function in the absence of Rcy1p.

Role of Yptp-GAPs in trafficking: While the function of Ypt/Rab proteins in specific steps of vesicular trafficking is rapidly emerging, little is known about the

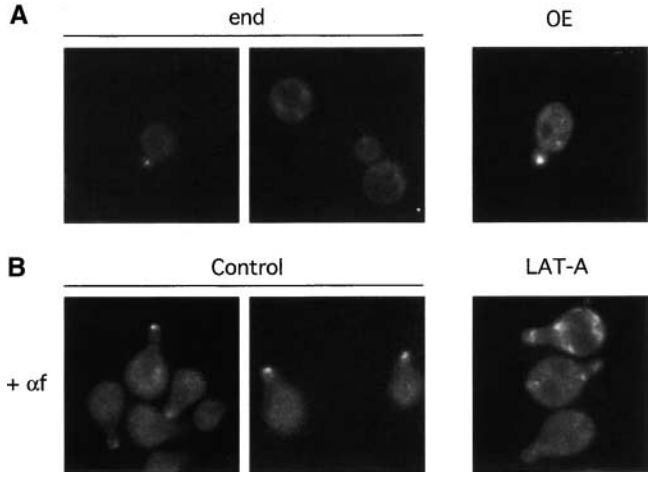

FIGURE 6.—Localization of Gyp2p. (A) Wild-type cells (K699) harboring *GYP2-GFP* integrated at its endogenous locus (end, YCL174) or a plasmid overexpressing GFP-Gyp2p from the *GAL* promoter (OE, right) were analyzed by GFP microscopy. (B) YCL174 cells were treated with α-factor for 2 hr (+ αf), and Gyp2p-GFP was analyzed by GFP microscopy. Where indicated, the cells were treated with latrunculin-A for 5 min (right, + LAT-A). Note that Gyp2p-GFP is concentrated at growth sites in an actin-dependent manner.

physiological role of their GAPs *in vivo*. This is particularly challenging, as the GAPs of the Gyp family exhibit a surprisingly broad substrate specificity when assayed on different Ypt/Rab proteins *in vitro*. For example, Gyp1p hydrolyzes GTP *in vitro* on Sec4p, Ypt1p, Ypt7p, and Ypt51p (Du *et al.* 1998). However, Gyp1p is partly localized to the Golgi apparatus, and genetic evidence suggests that it acts specifically on Ypt1p during secretion (Du and Novick 2001). Msb3p/Gyp3p and Msb4p/Gyp4p were identified as suppressors of particular *cdc24-ts* mutants, and genetic evidence suggests that they may contribute to the polarized assembly of the actin cytoskeleton downstream of Cdc42p (Bi *et al.* 2000). Consistent with these observations, Msb3p and Msb4p localize to the incipient bud site (Bi *et al.* 2000) and are proposed

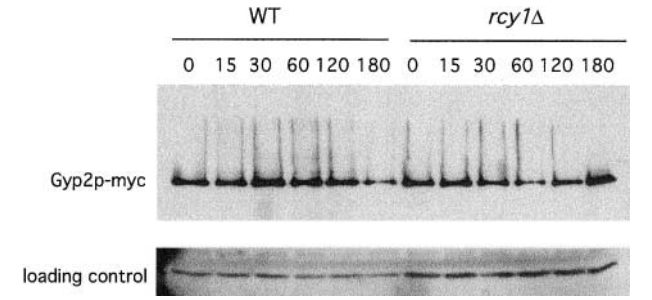

FIGURE 7.—The stability of Gyp2p-myc is not affected by Rcy1p. The half-life of Gyp2p-myc was determined by CHX chase in either wild-type (YCL211) or *rcy1*Δ (YCL212) cells. Cells were grown in rich medium to mid-log phase at which time CHX was added (time 0) to 50 μg/ml final concentration. Aliquots were removed at the times indicated (in minutes) and analyzed by immunoblotting with 9E10-antibodies.

to act primarily on Sec4p, Ypt6p, and Ypt7p during polarized secretion (Albert and Gallwitz 2000). In this study, we identified Gyp2p as a likely regulator of Ypt6p during recycling of membrane proteins. Gyp2p is distributed throughout the cytoplasm and accumulates in specific structures, while Ypt6p and the GEF Ric1p/Rgp1p localize to the late Golgi (Siniossoglou *et al.* 2000). It thus appears that individual Gyp-GAPs may function like their Ypt/Rab substrates at discrete steps of vesicle trafficking *in vivo*. An important challenge for the future is to decipher the physiological role of the unclassified GAPs of the Gyp family for their specific role during intracellular trafficking.

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