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Ric1p and the Ypt6p GTPase Function in a Common Pathway Required for Localization of *Trans*-Golgi Network Membrane Proteins

Eric S. Bensen,* Bonny G. Yeung, and Gregory S. Payne†

Department of Biological Chemistry, University of California, Los Angeles, Los Angeles, California 90095-3717

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In *Saccharomyces cerevisiae*, clathrin is necessary for localization of *trans*-Golgi network (TGN) membrane proteins, a process that involves cycling of TGN proteins between the TGN and endosomes. To characterize further TGN protein localization, we applied a screen for mutations that cause severe growth defects in combination with a temperature-sensitive clathrin heavy chain. This screen yielded a mutant allele of *RIC1*. Cells carrying a deletion of *RIC1* (*ric1*Δ) mislocalize TGN membrane proteins Kex2p and Vps10p to the vacuole. Delivery to the vacuole occurs in *ric1*Δ cells also harboring *end3*Δ to block endocytosis, indicative of a defect in retrieval to the TGN rather than sorting to endosomes. *SYS1*, originally discovered as a multicopy suppressor of defects caused by the absence of the Rab GTPase *YPT6*, was identified as a multicopy suppressor of *ric1*Δ. Further comparison of *ric1*Δ and *ypt6*Δ cells demonstrated identical phenotypes. Multicopy plasmids expressing v-SNAREs Gos1p or Ykt6p, but not other v- and t-SNAREs, partially suppressed phenotypes of *ric1*Δ and *ypt6*Δ cells. *SLY1–20*, a dominant activator of the *cis*-Golgi network t-SNARE Sed5p, also functioned as a multicopy suppressor. Because Gos1p and Ykt6p interact with Sed5p, these results raise the possibility that TGN membrane protein localization requires Ric1p- and Ypt6p-dependent retrieval to the *cis*-Golgi network.

INTRODUCTION

Localization of proteins to appropriate membrane organelles is crucial for the functional compartmentalization of eukaryotic cells. For proteins that function in organelles of the secretory and endocytic pathways, localization requires not only targeting to the proper destination but mechanisms to maintain residence despite extensive membrane and protein flux through each organelle. Continued residence can be achieved through retention mechanisms that restrict incorporation into transport vesicles departing from an organelle and/or retrieval mechanisms that carry out vesicle-mediated return from distal sites in the pathway (Pelham and Munro, 1993; Rothman and Wieland, 1996).

The Golgi apparatus in the yeast *Saccharomyces cerevisiae*, like its mammalian counterpart, is organized into dynamic, functionally distinct subcompartments that pose additional challenges for protein localization. Though not arranged into the cisternal stacks characteristic of the mammalian cell Golgi apparatus, yeast Golgi subcompartments can be considered functionally analogous to the mammalian *cis*-Golgi network (CGN), *medial* Golgi, and *trans*-Golgi (TGN) network (Graham and Emr, 1991; Preuss *et al.*, 1992). The CGN serves as the site where endoplasmic reticulum–derived transport carriers dock and fuse and where mannose residues are first added to the core oligosaccharides of glycoproteins (Gaynor *et al.*, 1994; Graham and Emr, 1991). Accordingly, this compartment is enriched for the t-SNARE Sed5p involved in the fusion of ER transport carriers and in the α-1,6 mannosyltransferase Och1p (Gaynor *et al.*, 1994; Hardwick and Pelham, 1992). The medial Golgi compartment carries out elaboration of glycoprotein carbohydrate side chains and contains a collection of different glycosyltransferases (Herscovics and Orlean, 1993). The TGN is the compartment where proteolytic maturation of the α-factor mating pheromone is initiated by the furin-related endoprotease Kex2p, dipeptidyl aminopeptidase A (DPAP A) and the carboxypeptidase Kex1p. The TGN is also a major sorting station, giving rise to vesicles targeted to the plasma membrane, endosomes, vacuoles, and probably to earlier Golgi compartments (Conibear and Stevens, 1998).

Studies of membrane protein localization suggest that both retention and retrieval play important roles at multiple

* Present address: Department of Genetics, Cell Biology and Development, University of Minnesota, Twin Cities, St. Paul, MN 55108.
† Corresponding author. E-mail address: gpayne@mednet.ucla.edu.

levels of the yeast Golgi apparatus. The molecular basis for localization has been most clearly established for TGN membrane proteins. Sequences have been defined in the cytoplasmic domains of Kex2p and DPAP A that delay egress from the TGN, implying that retention contributes to the localization of these proteins (Brickner and Fuller, 1997; Bryant and Stevens, 1997). In addition, aromatic amino acid retrieval signals are present in the cytoplasmic domains of these proteins as well as Vps10p and probably Kex1p (Cereghino *et al.*, 1995; Cooper and Bussey, 1992; Cooper and Stevens, 1996; Nothwehr *et al.*, 1993; Wilcox *et al.*, 1992). Vps10p is the sorting receptor for the vacuolar hydrolase carboxypeptidase Y (CPY) and acts in the TGN to divert CPY from the secretory pathway into vesicles targeted to endosomes (Cooper and Stevens, 1996; Marcusson *et al.*, 1994). Characterization of mutant cells with defects in sorting of CPY to the vacuole (primarily *vps* mutants) has revealed that TGN-localized α-factor maturation enzymes and Vps10p follow the same basic itinerary, cycling between the TGN and a prevacuolar endosomal compartment (PVC) (Conibear and Stevens, 1998). Significant advances have been achieved in defining the molecular components of this cycling pathway. Sorting into the endosome-targeted pathway depends on clathrin and the dynamin-like GTPase Vps1p, suggesting that clathrin-coated vesicles mediate transport from the TGN (Nothwehr *et al.*, 1995; Seeger and Payne, 1992; Wilsbach and Payne, 1993). A number of proteins have been identified that function in targeting and fusion of TGN-derived vesicles to the PVC (Conibear and Stevens, 1998). These include members of the vesicle (v-) and target membrane (t-) SNARE protein family involved in vesicle fusion (Sollner *et al.*, 1993; Weber *et al.*, 1998), the Sec1p family of t-SNARE–interacting proteins (Halachmi and Lev, 1996), and the rab family of GTPases thought to recruit vesicle-target membrane tethering factors, which facilitate interaction between v- and t-SNAREs (Gonzalez and Scheller, 1999; Martinez and Goud, 1998; Waters and Pfeffer, 1999). Retrieval from the PVC requires, in addition to a retrieval signal on the cargo protein, components of a multimeric complex proposed to act as a coat for retrograde vesicles targeted to the Golgi apparatus (Horazdovsky *et al.*, 1997; Nothwehr and Hindes, 1997; Nothwehr *et al.*, 1999; Seaman *et al.*, 1997, 1998). In contrast to the aforementioned steps, less is known about proteins involved in targeting and fusion of retrograde vesicles.

As an approach to identify additional factors involved in TGN protein localization we previously carried out a screen for mutations (*tcs*) that cause synthetic growth defects when combined with a temperature-sensitive form of clathrin heavy chain (Bensen *et al.*, 2000). A subset of *tcs* mutations by themselves caused defects in α-factor maturation and missorting of CPY, suggestive of Kex2p and Vps10p localization defects. In agreement with this interpretation, *tcs* mutations were identified in *VPS* genes whose products are known to act in TGN protein localization. In addition, one *tcs* mutant was found to contain a mutation in the *RIC1* gene. *RIC1* was initially identified in a screen for mutations that reduce ribosome synthesis (Mizuta *et al.*, 1997). However, this screen yielded at least six mutations that impaired the secretory pathway (Li and Warner, 1996; Mizuta and Warner, 1994), making it likely that the decrease in ribosome synthesis is a secondary consequence of membrane trafficking defects (Bensen *et al.*, 2000). Here we present biochemical and genetic characterization of cells lacking *RIC1*. Our results suggest Ric1p is necessary for efficient TGN protein localization, acting together with the Rab family GTPase Ypt6 and v-SNAREs Gos1p and Ykt6p in a retrograde pathway targeted to the CGN.

MATERIALS AND METHODS

Plasmids and Nucleic Acid Techniques

Plasmid constructions were performed using standard molecular biology techniques. A *Sal*I-*Not*I fragment containing *RIC1* was subcloned from p426-RIC1 (Bensen *et al.*, 2000) into pRS316 (Sikorski and Hieter, 1989) to form p316-RIC1. pric1-Δ2 was constructed by replacing the *TRP1* gene in pric1-Δ1 with the *URA3* gene. pric12μ1A is from a genomic library containing a fragment from chromosome X from approximately bp 429,661 to ~ 437,299. This region contains six open reading frames (ORFs) including *SYS1*. pARL1–1 resulted from the ligation of a (1.2 kb) *Sac*I-*Bam*HI genomic fragment isolated from a multicopy suppressing library clone into pRS315 (Sikorski and Hieter, 1989). The *Sac*I-*Bam*HI fragment from pARL1–1 was subcloned into pRS426 (Christianson *et al.*, 1992) to form p426-ARL1. *YPT6* was cloned by suppression of a *ypt6*Δ strain with a single copy genomic library (ATCC number 77162). DNA was isolated from clones that were able to grow at 37° and was electroporated into *Escherichia coli*. The presence of *YPT6* was verified by sequencing. A 1.45-kb *Xba*I fragment containing *YPT6* was subcloned into pRS316 to generate p316-YPT6. A 1.45-kb *Sac*I-*Bam*HI fragment from p316-YPT6 was subcloned into pRS426 to form p426-YPT6. A 1.6-kb fragment containing *GOS1* was amplified from genomic DNA by the PCR using the following primers: 5′-CCGG-GAATTCACCAAGAAAAGGCATATGGA-3′ and 5′-CCGGG-GATCCAATGCATCTGGATGAGGTCGT-3′ and cloned into pBluescript KS(+) (Stratagene, La Jolla, CA) to form pBKS-GOS1.1. The integrity of the amplified fragment was assessed by sequencing. An *Eco*RI-*Bam*HI fragment from pBKS-GOS1.1 was subcloned into pRS426 to form p426-GOS1. *TLG1* was cloned by screening a genomic library (see above) for clones that suppressed the temperature-sensitive growth defect of *tlg1*Δ cells. DNA was isolated from colonies that were able to grow at 37°C, as described previously, and electroporated into *E. coli*. DNA was isolated from bacteria, and the presence of *TLG1* in the genomic insert was confirmed by sequencing. A 2.55-kb *Hin*dIII-*Eco*RI fragment containing the *TLG1* ORF was subcloned into pRS426 to generate p426-TLG1. pSLY1–20, pSED5, pBET1, pSEC22, pYKT6, pVTI1 and pSFT1-URA3 were all generously provided by M.G. Waters (Princeton University, Princeton, NJ). All constructs are 2-μm–based multicopy plasmids containing the *URA3* gene (VanRheenen *et al.*, 1998). pAN109, a 2-μm–based multicopy plasmid containing *BOS1* and *URA3* (VanRheenen *et al.*, 1998), was a gift of S. Ferro-Novick (Yale University School of Medicine, New Haven, CT). pHPD1–2, a 2-μm–based multicopy plasmid containing *TLG2* and *URA3*, was kindly provided by L. Robinson (Louisiana State University Medical Center, Shreveport, LA).

Strains, Media, and Genetic Techniques

Strains used in this study are shown in Table 1. To generate a disruption in the *YPT6* gene with *HIS3*, the primer pairs 5′-GAT-TCTGAACAGTAAAAGATAAACAAAGAAGAGATTAACAATG GATTGTACTGAGAGTGCACC-3′, 5′-GGCGCAAATCCTGATC-CAAAC-3′ and 5′-GTTCTCCTTATGCCCTATAGAACTGAAAT-ATTAGGTGCTACATCTGTGCGGTATTTCACACCG-3′, 5′-CGGCT-GGTCGCTAATCGTTG-3′ were used to generate two overlapping PCR products using pRS303 (Sikorski and Hieter, 1989) as the template. These PCR products were transformed into SEY6210 to generate GPY1700. *RIC1* was disrupted with *URA3* to generate GPY1701 by transforming SEY6210 with pric1-Δ2 that had been

Table 1. Strains used in this study

Strains	Genotype	Reference
SEY6210	*MATα ura3-52 leu2-3, 112 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9*	Robinson *et al.* 1988
TVY1	*MATα ura3-52 leu2-3, 112 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 pep4::LEU2*	S. Emr UCSD
GPY1437	*MATα ura3-52 leu2-3, 112 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 tcs1-1(ric1)*	This study
GPY1480	*MATα ura3-52 leu2-3, 112 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 ric1::TRP1*	This study
GPY1608	*MATα ura3-52 leu2-3, 112 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 pep4::LEU2 ric1::TRP1*	This study
GPY1609	*MATα ura3-52 leu2-3, 112 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 end3::LEU2 ric1::TRP1*	This study
GPY1700	*MATα ura3-52 leu2-3, 112 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 ypt6::HIS3*	This study
GPY1701	*MATα ura3-52 leu2-3, 112 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 ric1::URA3*	This study
GPY1708	*MATα ura3-52 leu2-3, 112 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 ypt6::HIS3 ric1::TRP1*	This study
GPY2108	*MATα ura3-52 leu2-3, 112 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 gos1::HIS3*	This study

linearized with *Xho*I. To construct *ypt6*Δ *ric1*Δ and *pep4*Δ *ric1*Δ strains, GPY1700 and TVY1 were transformed with pric1-Δ1 that had been linearized with *Xho*I to generate GPY1708 and GPY1608, respectively. To generate GPY1609, a *ric1*Δ *end3*Δ double mutant, GPY1480 was transformed with an *Apa*I to *Xba*I fragment of pMP16, which contains *LEU2* inserted into *Bam*HI and *Xho*I sites in *END3* (gift of L. Hicke, Northwestern University, Evanston, IL). To generate a disruption in the *GOS1* gene with *HIS3*, the primer pairs 5′-ACACAGGGAAAAGCCCAATTCCAGACAAGCAACCACAC-CACGATTGTACTGAGAGTGCACC-3′, 5′-GGCGCAAATCCTGA-TCCAAAC-3′ and 5′-AGTATACAAAGGGTGGTTATCGTGG-CCAATACAAACGCGTTCATCTGTGCGGTATTTCACACCG-3′, 5′-CGGCTGGTCGCTAATCGTTG-3′ were used to generate two overlapping PCR products using pRS303 as the template. These PCR products were transformed into SEY6210 to generate GPY2108.

YPD medium is 1% Bacto-yeast extract (Difco, Detroit, MI), 2% Bactopeptone (Difco), and 2% dextrose. SD is 0.67% yeast nitrogen base without amino acids and 2% dextrose. Supplemented SD is SD with 40 μg/ml adenine, 30 μg/ml leucine, 30 μg/ml lysine, 20 μg/ml histidine, 20 μg/ml uracil, and 20 μg/ml tryptophan. SD CAA medium is supplemented SD with 5 mg/ml vitamin assay casamino acid mix (Difco). SD CAA -ura is SD CAA without uracil. SDYE is supplemented SD with 0.2% yeast extract. Cell densities in liquid culture were measured in a 1-cm plastic cuvette using a Beckman Instruments DU-62 spectrophotometer (Palo Alto, CA). One A_{500} unit is equivalent to 2.3×10^7 cells/ml.

Standard techniques for yeast mating, sporulation, and tetrad analysis were used (Guthrie and Fink, 1991). DNA transformations were performed as previously described (Gietz and Schiestl, 1995).

Metabolic Labeling and Immunoprecipitation

For metabolic labeling experiments, cells were grown to midlogarithmic phase in SDYE at 30° or in SD CAA -ura for the experiment in Figure 7. Labeling and immunoprecipitation of α-factor was performed as described by Seeger and Payne (1992b) except that labeling time was as indicated in figure legends. Labeling and immunoprecipitation of CPY was as described by Seeger and Payne (1992a) except for the changes mentioned below. Cells were labeled in supplemented SD, pH 5.7, with 1 mg/ml BSA and 10 μg/ml α_2-macroglobulin for 10 min. Aliquots were removed at 0, 10, and 30 min after the addition of unlabeled amino acids. Kex2p was metabolically labeled and immunoprecipitated as described by Chu *et al.* (1999). Vps10p and alkaline phosphatase were labeled and immunoprecipitated using the same protocol as Kex2p except samples were removed at chase times indicated in the figure legend. For invertase analysis strains carrying invertase on a multicopy plasmid (pRB58; ref) were used to facilitate detection of invertase. Cells grown to midlogarithmic phase were transferred to SD -ura medium containing 0.1% instead of 2% dextrose. After a 30-min incubation to derepress expression of secreted invertase, cells were labeled and processed as described for CPY except that only the periplasmic fraction was analyzed for external invertase.

Differential Centrifugation

For fractionation by differential centrifugation, cells were grown to midlogarithmic phase in YPD at 30°C. Cells were converted to spheroplasts, resuspended at 20 OD_{500}/ml in lysis buffer (0.2 M sorbitol, 50 mM potassium acetate, 2 mM EDTA, 20 mM HEPES, pH 6.8, 1 mM DTT, and 1XPIC), and lysed by 20 strokes in a 7-ml Dounce homogenizer with the B pestle. The lysate was subjected to centrifugation at $300 \times g$ for 5 min at 4°C, and the supernatant (S1) was subjected to centrifugation at $10,000 \times g$ in a HB4 rotor at 4°C for 15 min. An aliquot of the supernatant (S2) was reserved and the pellet (P2) was resuspended in volume of lysis buffer equivalent to the volume of S1. The supernatant (S2) was subjected to centrifugation at $200,000 \times g$ for 17 min in TLA 100.2 rotor (Beckman Instruments). The supernatant (S3) was reserved, and the pellet (P3) was resuspended in a volume of lysis buffer equivalent to the volume of S2. Equal volumes of each fraction were analyzed by SDS-PAGE and immunoblotting. Antibodies were visualized using color development for alkaline phosphatase (Bio-Rad, Richmond, CA).

FM4–64 Labeling

Vacuolar membrane staining with the vital dye FM4–64 was performed as described previously (Vida and Emr, 1995)

Screen for Multicopy Suppressors of **ric1**

A strain carrying a mutant allele of *RIC1* (GPY1437) was transformed with a 2 micron *URA3* based genomic library (Carlson and Botstein, 1982). After an overnight incubation at 30°, the plates were transferred to 37° to screen for suppressors of the *ric1* growth phenotype. Approximately 800 colonies from an estimated total of 34,000 transformants grew at 37°. To determine plasmid dependence, 84 colonies that grew at 37° were assayed for their ability to grow on 5-fluoro-orotic acid (5-FOA), a drug that selects against the *URA3* gene product, at 30 and 37°C. Of the 84 colonies tested, only 7 were temperature sensitive on 5-FOA medium, suggesting that the remaining colonies were revertants or contained extragenic suppressors unlinked to the plasmid. DNA was isolated from the seven strains and electroporated into *E. coli* (see above). Restriction map analysis revealed DNA isolated from five of the seven strains to contain the *RIC1* gene. The genomic fragments from the remaining two genomic clones were identified by DNA sequence analysis. Single ORFs from these clones were subcloned and retransformed into the *ric1* strain to identify the complementing genes.

RESULTS

Mislocalization of Kex2p in ric1Δ *Cells*

The mutant allele of *RIC1* identified in the *tcs* screen, *tcs1*, was generated by UV irradiation (Bensen *et al.*, 2000). Because the nature of the mutation was not characterized, we generated a new strain carrying a deletion of *RIC1* in order to determine the consequences of a complete deficiency of Ric1p (see Materials and Methods). Like the *ric1* strain isolated in the *tcs* screen, *ric1*Δ cells display a growth defect at 37°C but grow at nearly wild-type rates at 24 and 30°C (Bensen, unpublished results). As an initial assay for TGN protein localization, α-factor maturation was monitored. The 13 amino acid, α-factor peptide is synthesized as part of a larger precursor polypeptide that receives core oligosaccharides upon translocation into the ER and extensive further glycosylation in the Golgi apparatus. In the TGN, the highly glycosylated precursor is subjected to proteolytic maturation initiated by Kex2p (Fuller *et al.*, 1988). Defects in Kex2p localization lowers the efficiency of α-factor precursor maturation, leading to secretion of the highly glycosylated precursor (Payne and Schekman, 1989; Wilsbach and Payne, 1993). To assess the form of α-factor secreted by *ric1*Δ cells, mutant and congenic wild-type cells were labeled with [^{35}S]methionine and cysteine, and then α-factor was immunoprecipitated from the culture media and analyzed by SDS-PAGE and autoradiography. Although wild-type cells secreted only mature pheromone, *ric1*Δ cells secreted substantial levels of highly glycosylated precursor (67% by phosphorimage analysis) (Figure 1A, lanes 1 and 2). This defect was somewhat more severe than that of *tcs1* cells, which secreted 50% of α-factor as precursor (Bensen *et al.*, 2000), suggesting that the *tcs1* mutation does not completely eliminate Ric1p activity. Other strains analyzed in this and subsequent figures will be discussed in later sections.

As a more direct measure of Kex2p localization, Kex2p stability was monitored. In wild-type cells, efficient cycling of Kex2p between the TGN and endosomes confers stability to the protein. If this cycling is impaired, either by mutations in the Kex2p retrieval signal or by mutations in components of the TGN–endosome trafficking pathways, Kex2p transits to the vacuole where it is degraded (Brickner and Fuller, 1997; Seaman *et al.*, 1997; Voos and Stevens, 1998; Wilcox *et al.*, 1992; Wilsbach and Payne, 1993). Kex2p stability was compared in *ric1*Δ and wild-type cells using a pulse-chase protocol and immunoprecipitation. Over a 90-min chase period, a conspicuous decrease in the stability of Kex2p was evident in the *ric1*Δ cells (Figure 1B, lanes 1–3 compared with lanes 4–6). Kex2p stability was restored in *ric1*Δ cells also carrying *pep4*Δ, which eliminates most vacuolar proteolytic activity (Jones, 1991) (Figure 1B, lanes 7–9). This result argues that Kex2p is mislocalized to the vacuole in *ric1*Δ cells.

Defects in different stages of the cycling pathway between TGN and endosomes can be distinguished by establishing the route followed by mislocalized TGN proteins. Mutation of clathrin heavy chain or Vps1p, both required for sorting from the TGN, results in transport of TGN proteins to the plasma membrane. An endocytic defect in clathrin mutants causes accumulation of the mislocalized TGN proteins at the cell surface (Seeger and Payne, 1992). However, Vps1p mutations do not affect endocytosis, and the TGN proteins are rapidly internalized, delivered to the vacuole, and degraded (Nothwehr *et al.*, 1995). TGN protein degradation in *vps1* cells can be prevented by introducing a mutation that blocks endocytosis (Nothwehr *et al.*, 1995). In contrast, in cells with a defect in retrieval from endosomes, TGN proteins are directly transported from the PVC to the vacuole so that inhibiting endocytosis does not have an effect on turnover (Nothwehr *et al.*, 1999). Therefore, to determine whether *ric1*Δ preferentially affects sorting from the TGN or retrieval from the PVC, we introduced the *end3*Δ mutation, which blocks endocytosis (Benedetti *et al.*, 1994). Kex2p stability in *ric1*Δ *end3*Δ cells was indisinguishable from that observed in *ric1*Δ cells (Figure 1B, lanes 10–12 compared with lanes 4–6). These data argue that Kex2p is mislocalized to the

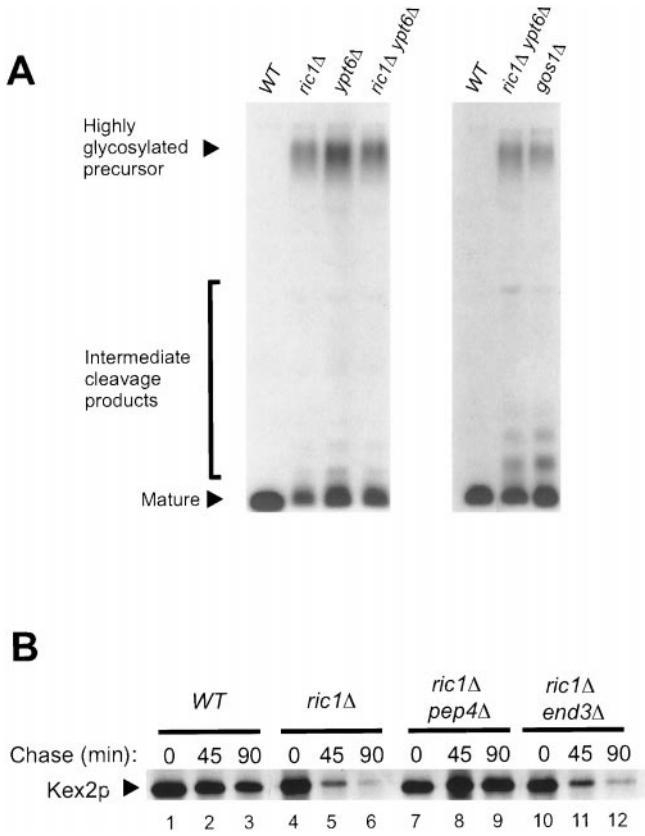

Figure 1. Pheromone α-factor maturation and Kex2p stability are defective in *ric1*Δ, *ypt6*Δ, and *gos1*Δ cells. (A) Maturation of α-factor. Wild-type (*WT*, SEY6210), *ric1*Δ (GPY1480), *ypt6*Δ (GPY1700), *ric1*Δ *ypt6*Δ (GPY1708) and *gos1*Δ (GPY2108) strains were grown in SDYE media overnight at 30°C to midlogarithmic phase. Cells were labeled for 45 min at 30°C with [^{35}S]methionine/cysteine. α-Factor was immunoprecipitated from the culture supernatant and subjected to SDS-PAGE and autoradiography. B) Kex2p stability. Wild-type (*WT*, SEY6210), *ric1*Δ (GPY1480), *ric1*Δ *pep4*Δ (GPY1608), and *ric1*Δ *end3*Δ (GPY1609) strains were grown overnight in SDYE media at 30°C to midlogarithmic phase. Cells were labeled with [^{35}S]methionine/cysteine at 30°C for 15 min followed by the addition of excess nonlabeled amino acids (chase). Samples were removed at 0, 45, and 90 min after the addition of chase, and Kex2p was immunoprecipitated from cell lysates. Kex2p was visualized by SDS-PAGE followed by autoradiography.

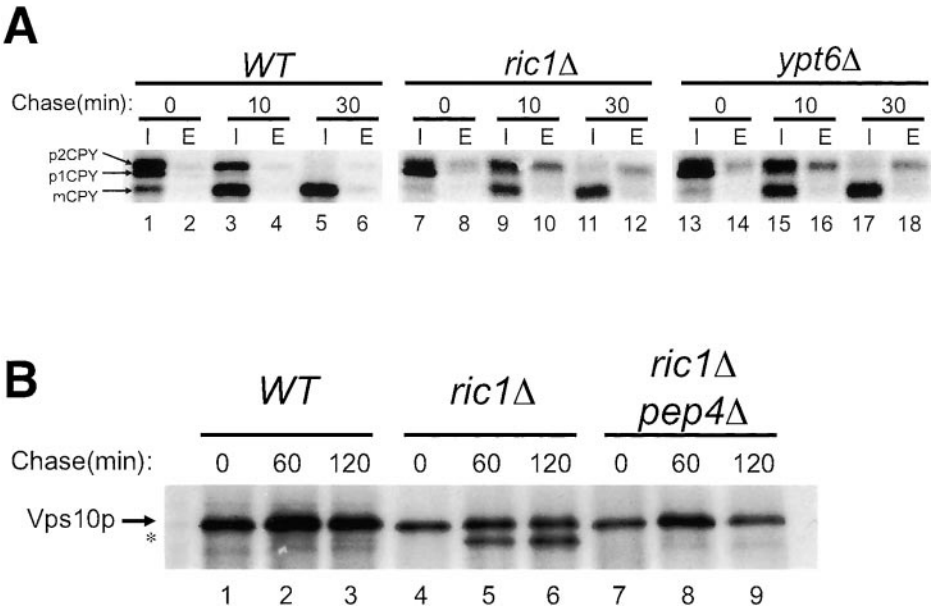

Figure 2. CPY sorting and Vps10p stability are defective in *ric1*Δ and *ypt6*Δ strains. (A) CPY sorting. Wild-type (*WT*, SEY6210), *ric1*Δ (GPY1701) and *ypt6*Δ (GPY1700) strains were grown and subjected to pulse-chase analysis as described in the legend to Figure 1B. After a 15-min labeling period, samples were removed after 0, 10, and 30 min of chase and CPY was immunoprecipitated from internal (I) and external (E) fractions. CPY was resolved by SDS-PAGE and subjected to phosphorimage analysis. (B) Vps10p stability. Wild-type (*WT*, SEY6210), *ric1*Δ (GPY1480), and *ric1*Δ *pep4*Δ (GPY1492) strains were grown and subjected to pulse-chase analysis as described in the legend to Figure 1B. Following a 10-min labeling period, cells were removed after 0, 60, and 120 min of chase and Vps10p was immunoprecipitated from cell lysates. Vps10p was visualized by SDS-PAGE followed by autoradiography. The 170-kDa Vps10p proteolytic cleavage product is denoted by an asterisk.

vacuole in *ric1*Δ cells without traveling to the plasma membrane, suggesting that Ric1p is required for TGN protein retrieval.

Missorting of the Vacuolar Hydrolase CPY in ric1Δ *Cells*

To characterize the effects of *ric1*Δ on TGN localization of another protein, we examined Vps10p, the sorting receptor responsible for directing newly synthesized CPY from the TGN into endosome-targeted transport vesicles (Cooper and Stevens, 1996; Marcusson *et al.*, 1994). Vps10p-mediated sorting was assessed by analyzing the biosynthesis of CPY (Stevens *et al.*, 1982). CPY is synthesized as an inactive precursor that is core glycosylated in the ER to produce 67-kDa p1CPY. Limited additional glycosylation in the Golgi apparatus yields the 69-kDa p2 form. Vps10p binds p2CPY in the TGN and carries its cargo to the PVC. It is thought that Vps10p releases p2CPY in the PVC and then is recycled to the TGN while p2CPY proceeds to the vacuole where proteolytic maturation generates 61-kDa mCPY. Sorting defects are manifested as the secretion of the Golgi-modified p2CPY (Bankaitis *et al.*, 1986; Rothman and Stevens, 1986; Stevens *et al.*, 1986). An initial qualitative indication that *ric1*Δ cells missort CPY was obtained using a nitrocellulose overlay assay and a CPY precursor-specific antibody (Bensen *et al.*, 2000). We applied pulse-chase immunoprecipitation to provide a quantitative measure of missorting. Wild-type and *ric1*Δ cells were metabolically labeled with [^{35}S]methionine and cysteine and harvested at designated intervals after initiation of the chase. Samples were separated into internal and external fractions, and CPY was immunoprecipitated and analyzed by SDS-PAGE, autoradiography, and phosphorimaging. All three CPY forms were detected immediately after the labeling period in the internal fraction from wild-type cells; 16% of the total CPY was mature (Figure 2A, lanes 1 and 2). At the 10-min chase period, 65% of CPY was mature, and the remainder was in the p2 form (Figure 2A, lanes 3 and 4). By 30 min of chase, 96% of CPY was mature (Figure 2A, lane 5). Sorting was essentially complete: <5% of the total CPY was detected in the external fraction at 30 min (Figure 2A, lane 6). In contrast, at the 0-min chase point, *ric1*Δ cells contained primarily ER-modified p1CPY, and mature CPY constitituted 10% of the total (Figure 2A, lanes 7 and 8). At the 10-min chase point, 45% of the internal CPY was mature with the remainder mostly in the p2 form, and by 30-min maturation was essentially complete (86% of internal CPY) (Figure 2A, lanes 9 and 11). The slight kinetic delay in CPY maturation, attributable to slowed conversion of p1 to p2CPY, is indicative of a delay in CPY transport between the ER to the Golgi apparatus or through early Golgi compartments. In addition to the change in maturation kinetics, 20% of the total CPY was secreted from *ric1*Δ cells (Figure 2A, lane 12), revealing a mild sorting defect.

Vps10p stability was compared in wild-type, *ric1*Δ, and *ric1*Δ *pep4*Δ cells using pulse-chase immunoprecipitation. Like Kex2p, defects in Vps10p localization result in transport to the vacuole (Brickner and Fuller, 1997; Cereghino *et al.*, 1995; Cooper and Stevens, 1996; Seaman *et al.*, 1997; Voos and Stevens, 1998). Vacuolar degradation of the 190-kDa Vps10p gives rise to a relatively stable 170-kDa product (Cereghino *et al.*, 1995). Whereas intact Vps10p was stable over a 120-min chase period in wild-type cells, degradation to an 170-kDa fragment was readily apparent at the 60- and 120-min chase points in *ric1*Δ cells (Figure 2B, lanes 1–6). Degradation was dependent on vacuolar protease activity since Vps10 was stable in *ric1*Δ *pep4*Δ cells (Figure 2B, lanes 7–9). These results argue that missorting of CPY in *ric1*Δ cells is due to a defect in localization of Vps10p. Similarly to Kex2p, Vps10p degradation still occurred in *ric1*Δ cells carrying *end3*Δ, supporting the interpretation that *ric1*Δ causes a defect in retrieval of TGN residents (Bensen, unpublished results).

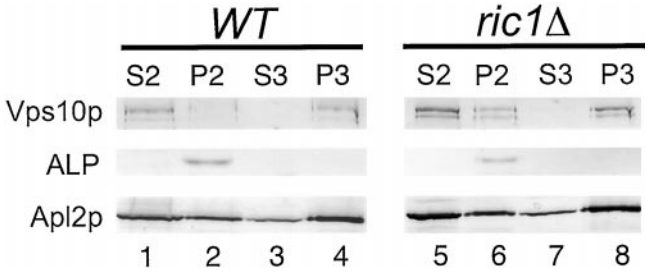

Figure 3. Vps10p is mislocalized in *ric1*Δ cells. Wild-type (*WT*, SEY6210) and *ric1*Δ (GPY1408) cells were grown at 30°C, converted to spheroplasts, and subjected to differential centrifugation. S2 and P2 are, respectively, the supernatant and pellet fractions from centrifugation at 10,000 × *g* for 15 min.; S3 and P3 are, respectively, the supernatant and pellet fractions from centrifugation at 200,00 × *g* for 17 min. Pellets were resuspended in volumes equal to the supernatants, and equal volumes were analyzed by SDS-PAGE and immunoblotting.

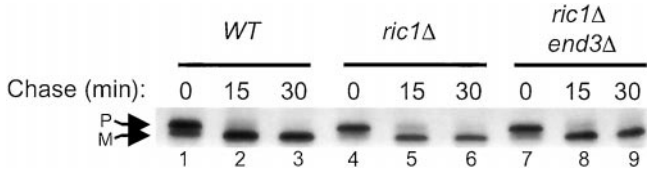

Figure 4. Efficient transport of ALP to the vacuole in *ric1*Δ and *ric1*Δ *end3*Δ cells. Wild-type (*WT*, SEY6210), *ric1*Δ (GPY1480), and *ric1*Δ *end3*Δ (GPY1609) strains were grown and subjected to pulse-chase analysis as described in the legend to Figure 1B. After a 10-min labeling period, cells were removed at 0, 15, and 30 min after the addition of chase, and ALP was immunoprecipitated from cell lysates. ALP was visualized by SDS-PAGE followed by autoradiography. P and M, precursor and mature forms of ALP, respectively.

As an additional assay for Vps10p localization, the subcellular distribution of Vps10p was assessed by differential centrifugation. Extracts of wild-type and *ric1*Δ cells were cleared of unbroken cells and large structures by low-speed centrifugation and then were subjected to sequential centrifugation steps to generate a medium-speed supernatant and pellet (S2, P2) and a high-speed supernatant and pellet (S3, P3). In wild-type cells (Figure 3, lanes 1–4), Vps10p fractionated almost completely in the P3 fraction. Vacuoles, as detected by immunoblotting for vacuolar membrane protein alkaline phosphatase (ALP), sedimented in the P2 fraction (Figure 3, lanes 1–4). In *ric1*Δ cells, a conspicuous shift of Vps10p into the P2 vacuole-containing fraction was evident (Figure 3, lanes 5–8). As a control for nonspecific effects of *ric1*Δ on membrane fractionation properties, we also examined the distribution of the clathrin adaptor AP-1 β subunit, Apl2p. This protein distributes between P2, P3, and S3 fractions in wild-type cells, and *ric1*Δ did not change this pattern. These results provide further evidence that Ric1p is necessary for the proper localization of Vps10p.

Efficient ALP Transport to the Vacuole in ric1Δ *Cells*

We examined whether *ric1*Δ affects a recently discovered pathway from the Golgi apparatus to the vacuole that bypasses endosomes (Cowles *et al.*, 1997b; Piper *et al.*, 1997). Cargo that follow this pathway include the vacuolar membrane protein ALP and the vacuolar t-SNARE Vam3p (Cowles *et al.*, 1997a,b; Piper *et al.*, 1997). The pathway is independent of clathrin but requires the clathrin adaptor-related complex AP-3 (Cowles *et al.*, 1997a; Stepp *et al.*, 1997; Vowels and Payne, 1998). Vacuolar delivery of ALP results in proteolytic activation of a precursor form, which allows the AP-3–dependent pathway to be assessed by pulse-chase immunoprecipitation (Klionsky and Emr, 1989). ALP biosynthesis was investigated in wild-type, *ric1*Δ, and *ric1*Δ *end3*Δ strains. The *ric1*Δ *end3*Δ strain was included to investigate the possibility that in *ric1*Δ cells, ALP is rerouted from the TGN to the vacuole by way of the plasma membrane. ALP maturation occurred at nearly the same rates in all three strains (Figure 4). Thus, Ric1p is not necessary for sorting into or transport through the AP-3–dependent pathway.

Vacuole Fragmentation in ric1Δ *Cells*

Vacuole morphology in *ric1*Δ cells was visualized with vital dyes FM4–64 and 5-(and -6)-carboxy-2′,7′-dichlorofluorescein diacetate (CDCFDA). Both dyes revealed fragmentation of vacuoles (Figure 5; Bensen, unpublished results). Cells cultured in defined synthetic medium (SDYE and SD CAA; see Materials and Methods) appeared to display more severe fragmentation than cells grown in more complex medium (YPD; see Materials and Methods). The basis for this difference has not been addressed. The aberrant vacuolar morphology in *ric1*Δ cells is consistent with a role in vacuole biogenesis.

Multicopy Suppressors of ric1Δ

Cells carrying *ric1*Δ grow at near wild-type rates at 24°C but very poorly at 37°C (Figure 6A; Mizuta *et al.*, 1997). As an approach to gain insights into Ric1p function, suppressors of the temperature-sensitive growth defect of *ric1*Δ cells were sought. For this purpose, a yeast genomic DNA library carried in a multicopy vector was introduced into *ric1*Δ cells, and the resulting transformants were screened for the ability to grow at 37°C. Of seven plasmids capable of restoring growth at 37°C, five contained *RIC1*. The other two plasmids carried regions from chromosome X and chromosome II. Subcloning of these regions revealed the suppressing genes to be the Arf-like GTPase *ARL1* from chromosome X (Lee *et al.*, 1997) and the *SYS1* gene from chromosome II (Tsukada and Gallwitz, 1996) (Figure 6A).

Phenotypic Similarities in ric1Δ *and* ypt6Δ *Cells*

SYS1 was originally identified as a multicopy suppressor of the temperature-sensitive growth defects of cells lacking the Rab family GTPase Ypt6p (Tsukada and Gallwitz, 1996). The common suppression of both *ric1*Δ and *ypt6*Δ cell growth defects prompted a comparison of the phenotypes caused by mutation of the two genes. Published reports suggested that, like disruption of *RIC1*, disruption of *YPT6* results in temperature-sensitive growth, α-factor maturation defects, missorting of CPY, and fragmented vacuoles (Li and Warner, 1996; Tsukada and Gallwitz, 1996). We directly compared the effects of *ric1*Δ and *ypt6*Δ in our strain background and

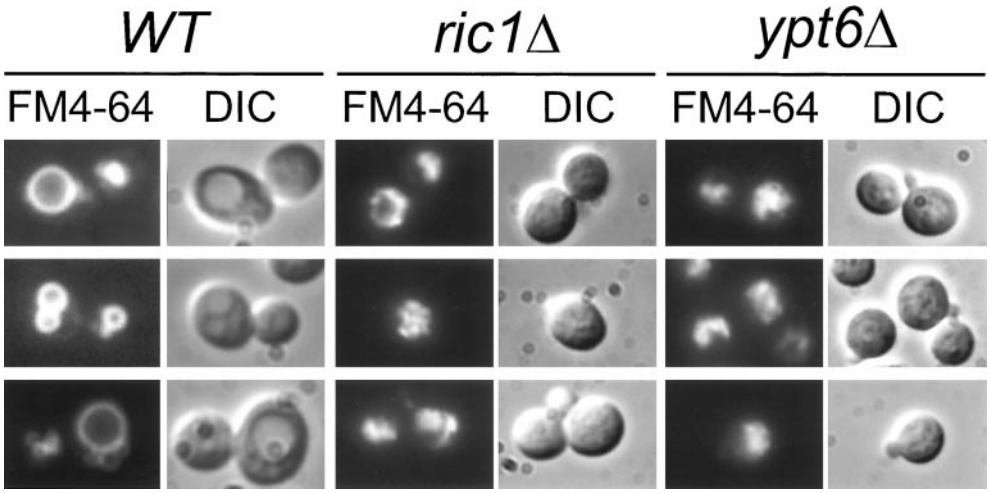

Figure 5. ric1Δ and *ypt6*Δ cells display fragmented vacuoles. Wild-type (*WT*, SEY6210), *ric1*Δ (GPY1480), and *ypt6*Δ (GPY1700) strains were grown overnight in SDYE media, at 30°C, to midlogarithmic phase. Cells were incubated with FM4–64 for 15 min, resuspended in fresh media devoid of dye, and allowed to internalize the dye for 45 min, at 30°C, before viewing. FM4–64, fluorescence (left); DIC, differential interference contrast optics (right).

observed that the two strains displayed equivalent defects in α-factor maturation (Figure 1A), maturation and sorting of CPY (Figure 2, lanes 7–18), vacuole fragmentation (Figure 4), and growth at elevated temperatures (Figure 6, A and B). Also, the temperature-sensitive growth defect of each strain was suppressed to the same degree by multiple copies of *SYS1* and *ARL1* (Figure 6, A and B). The extensive phenotypic similarities caused by disruption of *RIC1* and *YPT6* suggest that the gene products function in the same pathway.

Additional genetic analyses support the hypothesis that Ric1p and Ypt6p act in the same pathway. Combining *ric1*Δ and *ypt6*Δ did not exacerbate the growth (Bensen, unpublished results) or α-factor maturation defects (Figure 1A) caused by either mutation alone. This finding contrasts with the severe accentuation of growth defects that ensued when either *ric1*Δ or *ypt*Δ was combined with a temperature-sensitive allele of *CHC1* or *vps1*, *vps5*, *vps16*, *vps18*, *vps21*, and *vps35* (Bensen and Costaguta, unpublished results).

In the case that two gene products act in the same pathway, reciprocal tests of overexpression suppression can provide an indication of the order of action. The results in Figure 6, A and B, demonstrate that *YPT6* carried on a multicopy plasmid suppresses the 37°C growth defect of *ric1*Δ cells, but multicopy *RIC1* does not suppress the growth defect of *ypt6*Δ cells. Immunoblots confirm that the *RIC1* multicopy plasmid leads to more than 10-fold overexpression of Ric1p (Bensen, unpublished results). We also investigated whether multicopy *YPT6* could suppress the α-factor maturation defect in *ric1*Δ cells grown at the permissive temperature. Multicopy *YPT6* reduced the level of precursor α-factor secreted by *ric1*Δ cells by ~50% (Figure 7A). Consistent with the effects on growth, multicopy *RIC1* had no effect on the α-factor maturation defect of *ypt6*Δ cells (Figure 7B). These results suggest that high levels of Ypt6p can overcome the absence of Ric1p, providing a genetic argument that Ric1p acts upstream of Ypt6p.

Efficient Secretion of Invertase by ypt6Δ *and* ric1Δ *Cells*

Previous studies have implicated Ypt6p in transport through early stages of the secretory pathway, based on analysis of CPY and the secreted protein invertase in *ypt6*Δ cells shifted to 37°C, a nonpermissive growth temperature (Li and Warner, 1996). In contrast, our characterization of CPY and ALP transport in *ypt6*Δ and *ric1*Δ cells at permissive growth temperatures did not reveal significant transport delays at early stages of the secretory pathway. To further address whether Ypt6p is required for secretory pathway function, invertase secretion was monitored in wild-type and *ypt6*Δ cells by pulse-chase analysis. Invertase is expressed in two forms: a constitutive cytoplasmic form and a glucose-repressed secreted form (Carlson and Botstein, 1982). To analyze invertase secretion, cells were first incubated in low-glucose medium to induce expression of the secreted form of invertase and then were subjected to a pulse-chase regimen (Esmon *et al.*, 1981). At designated intervals cells were harvested and separated into internal and external fractions, and invertase was imunoprecipitated. Upon translocation, invertase is core-glycosylated, yielding a form that migrates more slowly (Figure 8, core) than unglycosylated cytoplasmic invertase (Figure 8, cyto). Upon reaching the Golgi apparatus, invertase carbohydrate moieties are extensively and heterogeneously elaborated to yield a highly glycosylated species (Figure 8, highly-glycosylated) that is secreted from the cells. At the permissive growth temperature of 30°C, invertase was efficiently glycosylated and secreted from both wild-type (Figure 8, lanes 1–6) and *ypt6*Δ cells (Figure 8, lanes 7–12). As with the analysis of CPY, a slight kinetic delay was apparent in conversion of the ER form of invertase to the highly glycosylated Golgi form (Figure 8, lanes 1 and 2 compared with lanes 7 and 8). Similar results were obtained with *ric1*Δ cells. These analyses provide additional evidence that Ypt6p (and Ric1p) do not play significant roles in transport through the secretory pathway.

Multicopy Suppression of ric1Δ *and* ypt6Δ *by Specific SNARE Proteins*

While the precise molecular function of Rab GTPases has yet to be elucidated, current models implicate Rabs as key regulators of the molecular interactions leading to vesicle docking and ultimately to fusion between the vesicle and the target organelle membranes (Gonzalez and Scheller, 1999;

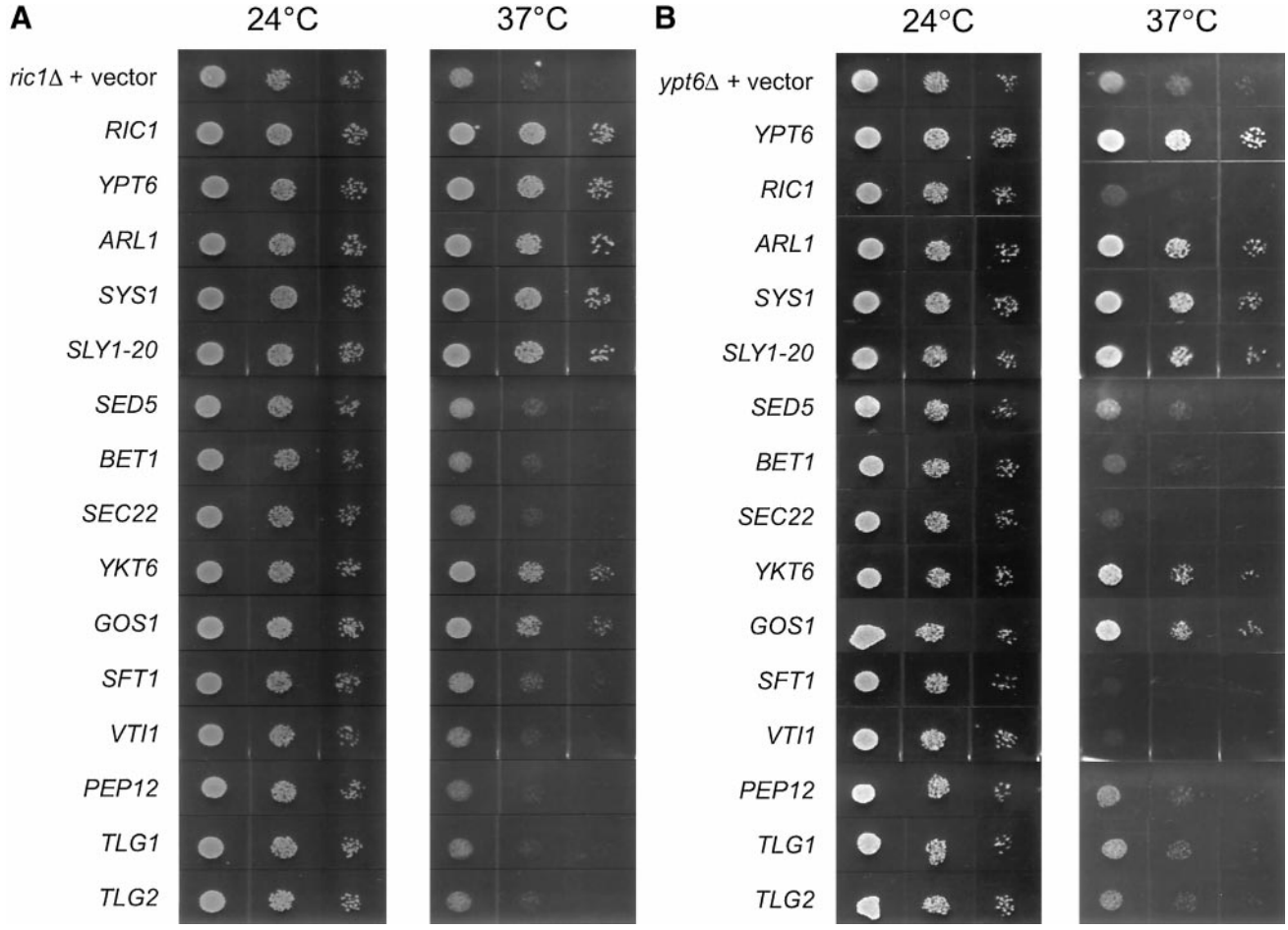

Figure 6. Suppression of the growth defect of *ypt6*Δ and *ric1*Δ cells. (A) GPY1480 (*ric1*Δ) was transformed with a vector control (pRS426), a *RIC1* centromeric plasmid (p316-RIC1), and multicopy plasmids carrying the following genes: *YPT6* (p426-YPT6), *ARL1* (p426-ARL1), *SYS1* (pric12u1A), *SLY1–20* (pSLY1–20), *SED5* (pSED5), *BET1* (pBET1), *SEC22* (pSEC22), *YKT6* (pYKT6), *GOS1* (p426-GOS1), *SFT1* (pSFT1-URA3), *VTI1* (pVTI1), *PEP12* (pCB31), *TLG1* (p426-TLG1), and *TLG2* (pHPD1–2). Strains were grown to saturation at 24°C in SD CAA-ura and serial dilutions of the cultures were spotted onto supplemented SD plates at 24 and 37°C. (B) GPY1700 (*ypt6*Δ) was transformed with a vector control (pRS426), a *YPT6* centromeric plasmid (*YPT6*), and the same plasmids as in Figure 6A, except the multicopy *YPT6* plasmid was replaced with a multicopy *RIC1* plasmid (p426-RIC1).

Martinez and Goud, 1998; Waters and Pfeffer, 1999). Fusion appears to be driven by formation of parallel coiled-coil complexes consisting of v-SNAREs on the vesicle membrane and t-SNAREs on the target membrane (Weber *et al.*, 1998). Consistent with the key function of SNARE proteins at the last step of the vesicle docking and fusion process, studies in yeast have indicated that overexpression of appropriate SNARE proteins can suppress defects caused by mutations in specific Rab proteins (Brennwald *et al.*, 1994; Dascher *et al.*, 1991; Stone *et al.*, 1997). On the basis of these precedents, we tested a set of multicopy plasmids expressing different SNAREs for the ability to rescue defects in *ypt6*Δ and *ric1*Δ cells. These included: v-SNAREs Bet1p and Sec22p, involved in transport between the ER and Golgi apparatus; Sft1p, Gos1p, and Ykt6p, thought to play roles in intra-Golgi transport; and Vti1p, which participates in several steps including transport from the TGN to endosomes (Nichols and Pelham, 1998). Also tested were the CGN t-SNARE Sed5p, which acts in ER-to-Golgi transport and retrograde transport to the CGN, the Golgi/endosome t-SNAREs Tlg1p and Tlg2p, which are proposed to function in traffic between endosomes and the TGN, and the endosomal t-SNARE Pep12p, which is required for transport from the TGN to the PVC (Nichols and Pelham, 1998). Only multicopy *YKT6* and *GOS1* allowed growth of *ric1*Δ or *ypt6*Δ cells at 37°C (Figure 6, A and B). The α-factor maturation defects were also partially suppressed by multicopy plasmids carrying *YKT6* and *GOS1* but not other SNAREs tested in Figure 6 (Figure 7, A and B; Bensen, unpublished results).

Gos1p and Ykt6p interact with the t-SNARE Sed5p, as do Bet1p, Sec22p, Sft1p, and Vti1p (Banfield *et al.*, 1995; Lupashin *et al.*, 1997; McNew *et al.*, 1997; Sogaard *et al.*, 1994; von Mollard *et al.*, 1997). We did not detect suppression of *ric1*Δ or *ypt6*Δ by multicopy *SED5*; however, this result could be complicated by the detrimental effects of Sed5p overexpression on growth (Wooding and Pelham, 1998). As an alter-

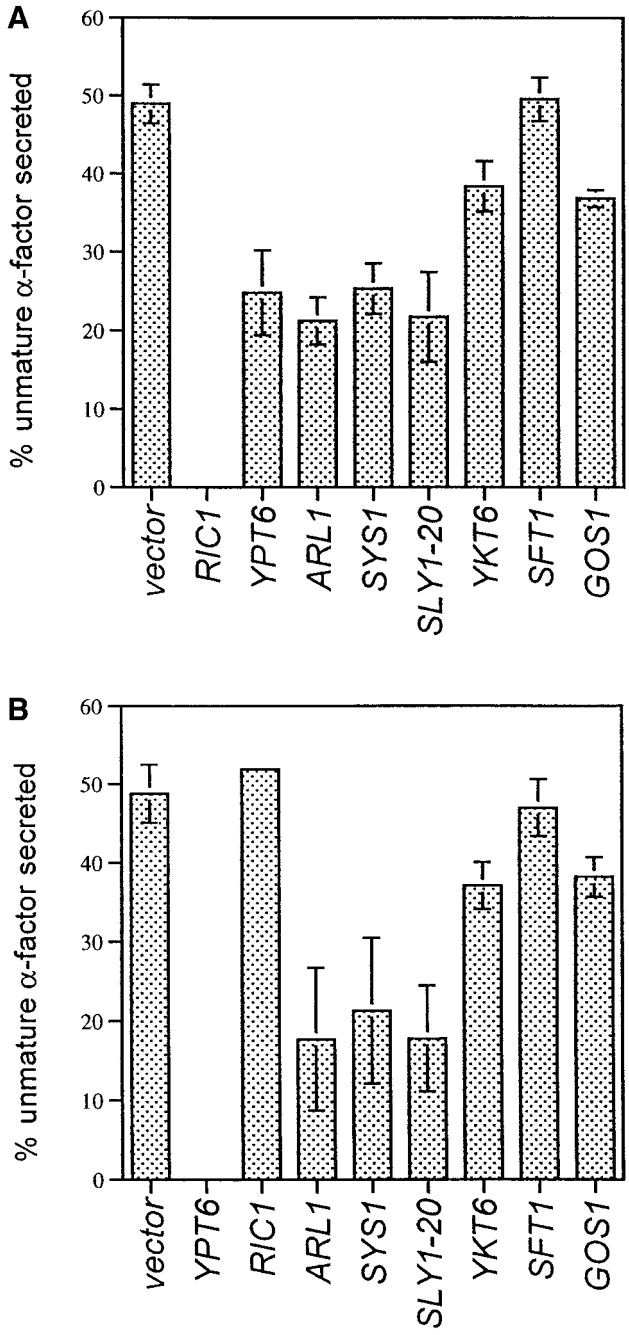

Figure 7. Multicopy suppression of the *ric1*Δ and *ypt6*Δ α-factor defects. *ric1*Δ strains (A) carrying plasmids described in the legend to Figure 6A or *ypt6*Δ strains carrying plasmids described in the legend to Figure 6B were grown overnight at 30°C in SD CAA-ura to midlogarithmic phase. α-Factor was metabolically labeled and immunoprecipitated as described in Figure 1A followed by phosporimage analysis using a Molecular Dynamics PhosporImager and ImageQuaNT software. Percent of unmature α-factor was calculated by dividing the amount of highly glycosylated precursor plus intermediate cleavage products by the total amount of α-factor. Values were normalized to either the *RIC1*-carrying strain (A) or *YPT6*-carrying strain (B). Error bars were calculated on the basis of three experiments except for *GOS1* suppression, which was based on two experiments.

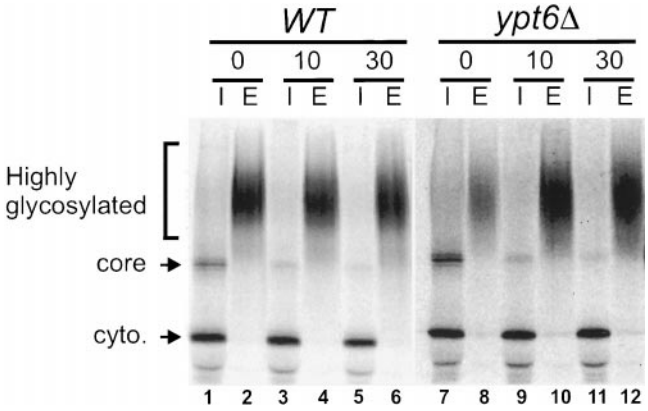

Figure 8. Invertase is efficiently secreted by *ypt6*Δ cells. Wild-type (*WT*, SEY6210) and *ypt6*Δ (GPY1700) strains expressing *SUC2* from a multicopy plasmid (pRB58) were grown overnight at 30°C to midlogarithmic phase. After a 30-min incubation in low glucose media (0.1%) to induce invertase expression, cells were metabolically labeled with [^{35}S]methionine/cysteine followed by the addition of excess nonlabeled amino acids (chase). Samples were removed after 0, 10, and 30 min of chase, and invertase was immunoprecipitated from internal (I) and external (E) fractions. Invertase was resolved by SDS-PAGE and subjected to phosphorimage analysis. Cytoplasmic (cyto), core, and highly glycosylated mature forms of invertase are shown.

native approach to assess the involvement of Sed5p, a plasmid expressing the *SLY1–20* allele was tested. Sly1p interacts with Sed5p and is a member of the Sec1 family of SNARE regulators (Grabowski and Gallwitz, 1997; Sogaard *et al.*, 1994). The dominant *SLY1–20* allele allows suppression of defects caused by deletion of Ypt1p, a Rab GTPase necessary for ER-to-Golgi transport (Dascher *et al.*, 1991; Ossig *et al.*, 1991). *SLY1–20* suppressed both the growth (Figure 6, A and B) and α-factor maturation defects (Figure 7, A and B) of *ric1*Δ and *ypt6*Δ cells. These suppression results implicate Ric1p and Ypt6p in events leading to formation of SNARE complexes containing Gos1p, Ykt6p, and Sed5p.

Defective α-factor Maturation in gos1Δ *Cells*

Cells deficient in Gos1p have been reported to exhibit a kinetic delay in CPY maturation and missorting of p2CPY, similar to the defects in *ric1*Δ and *ypt6*Δ cells (McNew *et al.*, 1998). To extend comparison between *gos1*Δ, *ric1*Δ, and *ypt6*Δ cells, α-factor maturation was analyzed in a congenic set of strains. Maturation of α-factor was incomplete in *gos1*Δ cells (31% precursor), suggesting that localization of Kex2p is defective in the absence of Gos1p (Figure 1A).

DISCUSSION

A mutant allele of the *RIC1* gene was identified through a screen for mutations that cause synthetic growth defects in cells expressing a temperature-sensitive clathrin heavy chain (Bensen *et al.*, 2000). Our results argue that Ric1p acts upstream of Ypt6p in a pathway that is necessary for retrieval of TGN proteins from the PVC. Very recently, Siniossoglou and colleagues reported that a complex of Ric1p and Rgp1p

functions as a nucleotide exchange factor for Ypt6p (Siniossoglou *et al.*, 2000). Our results are entirely consistent with this function. Ypt6p has been suggested to act in retrieval of proteins from endosomes to the TGN (Siniossoglou *et al.*, 2000; Tsukada and Gallwitz, 1996; Tsukada *et al.*, 1999). However, defects caused by the absence of either Ric1p or Ypt6p can be suppressed by multicopy plasmids expressing the v-SNAREs Ykt6p and Gos1p, as well as *SLY1–20*, which is an activator of the CGN t-SNARE Sed5p. On the basis of these results we suggest that localization of TGN proteins may involve Ypt6p- and Ric1p-mediated targeting and fusion of retrograde vesicles with the CGN.

Two prior models for the role of Ypt6p have been presented. The first proposes that Ypt6p acts in a retrograde pathway from endosomes to the TGN (Tsukada and Gallwitz, 1996; Tsukada *et al.*, 1999). The second places Ypt6p at an early secretory pathway step, either ER to CGN or CGN to *medial* Golgi compartments (Li and Warner, 1996, 1998). Our results are more concordant with the first model, which was primarily based on vacuolar protease-dependent instability of Kex2p, missorting of CPY, and accumulation of 40- to 50-nm vesicles in *ypt6*Δ cells (Tsukada and Gallwitz, 1996; Tsukada *et al.*, 1999). Our analyses of *ric1*Δ cells revealed many of the same phenotypes and also show instability and mislocalization of Vps10p. Importantly, our studies offer evidence that Ric1p and, by extension Ypt6p, are necessary for optimal retrieval from the PVC rather than transport from the TGN. First, despite the presence of *end3*Δ to block the endocytic pathway, Kex2p and Vps10p were unstable, arguing that the TGN proteins were still mislocalized to the vacuole in *ric1*Δ *end3*Δ double mutants. This property distinguishes *ric1*Δ cells from *vps1* mutants, in which a block in TGN to endosome traffic results in missorting to the plasma membrane and subsequent endocytosis-dependent transport to the vacuole (Nothwehr and Stevens, 1994). Second, the rapid kinetics of CPY maturation in *ric1*Δ and *ypt6*Δ cells indicate that transport from the TGN to endosomes to vacuoles is unaffected by loss of Ric1p or Ypt6p. By these properties, *ric1*Δ cells resemble other *vps* mutants with defects in retrograde traffic from endosomes.

Although the data presented here support the hypothesis that Ypt6p acts in a retrograde pathway from endosomes, the results suggest that the pathway leads to the CGN rather than the TGN (Figure 9, pathway 1). This conclusion is based on suppression of growth and α-factor maturation defects in *ric1*Δ or *ypt6*Δ cells by multicopy plasmids expressing v-SNAREs Ykt6p or Gos1p, or *SLY1–20*, observations that implicate the CGN t-SNARE Sed5p in the Ypt6p-dependent pathway. Studies of other yeast Rabs indicate that multicopy suppression of Rab-deficient phenotypes is a reliable approach to identify SNAREs that are regulated by a particular Rab protein. For example, the v-SNARE Bet1p involved in ER-to-CGN transport was identified in a screen for multicopy suppressors that could restore viability and ER-to-CGN traffic in cells lacking Ypt1p (Dascher *et al.*, 1991; Ossig *et al.*, 1991), and the t-SNARE Sec9p was identified as a multicopy suppressor of viability and exocytosis in cells expressing a mutant form of Sec4p (Brennwald *et al.*, 1994). In the case of *ric1*Δ and *ypt6*Δ cells, of the 10 genes encoding v- and t-SNAREs that we tested, only multicopy *GOS1* and *YKT6* functioned as suppressors. Both of these SNAREs form complexes with Sed5p, the CGN t-SNARE (McNew *et al.*, 1997; Sogaard *et al.*, 1994). Suppression was specific to Gos1p and Ykt6p because other SNAREs that interact with Sed5p—Bet1p, Sec22p, Bos1p (Bensen, unpublished results), Sft1p, and Vti1p—did not suppress *ric1*Δ or *ypt6*Δ defects when expressed from multicopy plasmids. Thus, Gos1p and Ykt6p represent a functionally distinct subset of Sed5p-interacting SNAREs, defined by the ability to suppress the loss of Ypt6p. In addition to the multicopy suppression results, the similar effects of *gos1*Δ and *ypt6*Δ on α-factor maturation, CPY sorting, and vacuole morphology also support a role for Gos1p in a Ypt6p-dependent pathway (Figure 1A; McNew *et al.*, 1998). The phenotypes of *ykt6*Δ cells are less informative because the lethal consequences of gene disruption, multiple anomalies in CPY maturation upon Ykt6p depletion, and interactions with multiple t-SNAREs suggest that Ykt6p acts in more than one transport step (McNew *et al.*, 1997; Ungermann *et al.*, 1999).

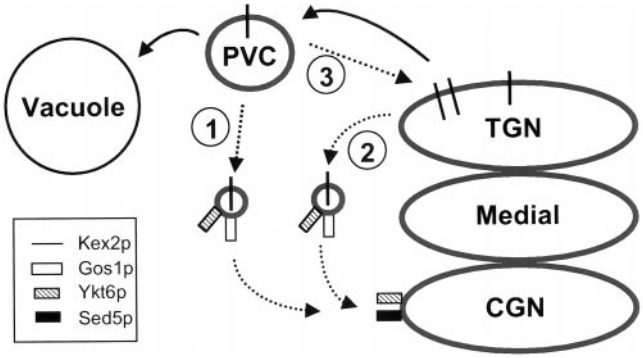

Figure 9. Models for Ypt6p/Ric1p-mediated retrieval of TGN membrane proteins. Dotted arrows indicate possible Ypt6-dependent pathways. CGN-targeted vesicles containing Gos1p and possibly Ykt6p may originate from the prevacuolar endosome compartment (PVC; pathway 1) or from the TGN (pathway 2). Ykt6p may act as part of a Sed5p t-SNARE rather than as a vesicle-associated v-SNARE. See text for details.

Suppression of *ypt6*Δ defects by *SLY1–20* offers further support for the participation of Sed5p in the Ypt6p-mediated pathway (Figures 7 and 8; Mizuta and Warner, 1994). Sly1p is a member of the Sec1 family of proteins, which interact with t-SNAREs and are thought to regulate SNARE complex formation (Halachmi and Lev, 1996). Sly1p binds to Sed5p and is necessary for ER-to-CGN transport (Grabowski and Gallwitz, 1997; Lupashin *et al.*, 1996; Ossig *et al.*, 1991; Sogaard *et al.*, 1994). *SLY1–20* bypasses the need for Ypt1p in ER-to-CGN traffic (Dascher *et al.*, 1991; Ossig *et al.*, 1991), presumably by alleviating the need for Ypt1p in formation of Sed5p-containing SNARE complexes (Lupashin and Waters, 1997). By analogy, it is likely that the effects of *SLY1–20* on Sed5p also bypass the need for Ypt6p in formation of Sed5p-containing SNARE complexes mediating fusion of retrograde vesicles to the CGN. Because there are fewer identifiable Sec1-like proteins than t-SNAREs encoded in the yeast genome, it could be argued that *SLY1–20* suppresses *ypt6*Δ through effects on another t-SNARE. The most logical alternative t-SNAREs for a retrieval pathway are the TGN/endosomal t-SNAREs Tlg1p and Tlg2p, particularly because loss of either protein leads to TGN protein mislocalization to the vacuole (Holthuis *et al.*, 1998). However, Tlg1p and

Tlg2p associate with another Sec1-like protein Vps45p (Nichols *et al.*, 1998), and interactions between Tlg1p/2p and Gos1p have not been detected (Nichols and Pelham, 1998). Thus, it is unlikely that these t-SNAREs function in the Ypt6p-dependent pathway. Instead, the similar effects on TGN protein localization caused by *ypt6* and either *tlg1*Δ or *tlg2*Δ suggest that both pathways are required for optimal TGN protein localization.

We cannot discount the possibility that SNARE overexpression rescues mutant phenotypes by increasing traffic through a Ypt6p-independent pathway because suppression occurs in cells carrying a deletion of *YPT6*. In this scenario, Ypt6p and Ric1p could act in a pathway from endosomes to the TGN (Figure 9, pathway 3), perhaps in concert with Tlg1p, Tlg2p, and the putative tethering complex containing Vps52p, Vps53p, and Vps54p (Conibear and Stevens, 2000). Defective retrieval to the TGN could then be balanced by enhanced transport to the CGN caused by overexpression of *GOS1*, *YKT6*, or *SLY1–20* and mediated by another Ypt such as Ypt1p.

In its simplest form, our model predicts that Ypt6p is necessary for formation of a SNARE complex involving Gos1p and Ykt6p on retrograde vesicles and Sed5p at the CGN (Figure 9). However, a recent study of SNARE specificity suggested that Ykt6p may serve as a t-SNARE light chain for Sed5p (McNew *et al.*, 2000). Regardless of the precise t-SNARE architecture, we did not detect a decrease in the levels of Ykt6p or Gos1p associated with immunprecipitated Sed5p in *ypt6*Δ cells compared with wild-type cells (Bensen, unpublished results). One explanation for this observation is functional redundancy between Ypt6p and Ypt1p. Overexpression of Ypt1p can suppress *ypt6*Δ defects in vivo, suggesting that Ypt1p is capable of acting in place of Ypt6p (Li and Warner, 1998). It is possible that, in *ypt6*Δ cells, normal levels of Ypt1p provide sufficient Ypt6p-redundant function to obscure effects on SNARE complex formation as assayed by coimmunoprecipitation of SNAREs. Alternatively, in addition to the Ypt6p-dependent SNARE complex, Gos1p and Ykt6p could be partnered with Sed5p and other v-SNAREs in functionally distinct Ypt6p-independent complexes (Nichols and Pelham, 1998). Such complexes would complicate detection of specific effects on Ypt6p-dependent interactions. Finally, as mentioned above, Gos1p, Ykt6p, and Sed5p may function in a Ypt6p-independent pathway. Additional experiments are required to evaluate these possibilities.

A role for Ypt6p in delivery of retrograde vesicles to the CGN can accommodate the results that prompted the model that Ypt6p acts in anterograde transport to and through the Golgi apparatus. An anterograde role for Ypt6p was proposed on the basis of accumulation of p1CPY, a decrease in external invertase in *ypt6*Δ cells incubated at the nonpermissive growth temperature, and genetic interactions between *YPT6* and *YPT1* (Li and Warner, 1996, 1998). In general, our results are inconsistent with significant roles for Ric1p and Ypt6p in anterograde transport. Cells grown at permissive growth temperatures displayed only a slight delay in conversion of p1 to p2CPY and in conversion of core invertase to highly glycosylated invertase. Furthermore, there was little or no impairment of ALP or Kex2p transport through the early stages of the secretory pathway. These results are in accord with those of Gallwitz and colleagues (Tsukada and Gallwitz, 1996; Tsukada *et al.*, 1999) and strengthen the contention Ypt6p does not play a significant role in anterograde traffic. The genetic interactions between *YPT1* and *YPT6* are not at odds with a role for Ypt6p in retrograde transport because regulation of the same t-SNARE (Sed5p) by both Ypt proteins would provide a molecular basis for the observed genetic effects. In the context of the Ypt6p retrograde model it is possible to envision effects of *ypt6*Δ on anterograde transport. For example, if the CGN-directed retrograde pathway plays some role in retrieval of Golgi proteins necessary for anterograde transport, then blocking the retrograde pathway would have an effect on anterograde traffic that could be exacerbated by a shift to elevated temperatures. Wild-type cells shifted to 37°C show a conspicuous increase in vacuolar-protease–dependent turnover of Kex2p (Wilcox *et al.*, 1992) and accumulate endosomal compartments (Mulholland *et al.*, 1999), raising the possibility that temperature stress inhibits retrieval from endosomes. When combined with the effects of *ypt6*Δ, such a temperature-sensitive inhibition of retrograde transport could further compromise the Ypt6-dependent pathway and/or an alternative pathway, thereby severely depleting necessary anterograde transport factors from early Golgi compartments.

The compartment from which Ypt6-dependent retrograde vesicles emanate is not entirely clear. The PVC is one likely possibility (Figure 9, pathway 1). If Ypt6p-dependent vesicles bud from the PVC and deliver TGN protein cargo to the CGN or TGN, then the defect in retrieval of Kex2p and Vps10p from the PVC in *ypt6*Δ cells would be explained by a block in vesicle fusion leading to sequestration of proteins important in formation of retrieval vesicles. Alternatively, Ypt6-dependent vesicles could originate from the TGN (Figure 9, pathway 2). In this case, localization of TGN proteins would involve contributions from this TGN-to-CGN pathway together with other retrograde routes such as PVC to TGN. In *ypt6*Δ mutants, sequestration of factors important in budding of the TGN-derived retrograde vesicles would increase levels of TGN proteins entering vesicles targeted to the PVC. Because retrieval of TGN proteins from the PVC is saturable, the ensuing increase in TGN proteins reaching the PVC could overwhelm the retrieval process, leading to default delivery to the vacuole (Cereghino *et al.*, 1995; Cooper and Bussey, 1992; Nothwehr *et al.*, 1993; Roberts *et al.*, 1992; Wilcox *et al.*, 1992).

Given prevailing evidence that retrograde traffic plays an important role throughout the Golgi apparatus (Allan and Balch, 1999; Nichols and Pelham, 1998), defects in Ypt6-mediated retrograde transport to the CGN might be expected to affect localization of proteins resident in earlier Golgi compartments. However, at permissive growth temperatures the absence of glycosylation defects in *ric1*Δ and *ypt6*Δ cells suggests that localization of glycosyltransferases in early Golgi compartments is not grossly affected. Furthermore, the CGN α-1,6 mannosyltransferase Och1p and the *medial* Golgi α-1,6 mannosyltransferase Mnn1p exhibited no changes in stability in *ric1*Δ or *ypt6*Δ cells, indicating that these proteins are not mislocalized to the vacuole to any appreciable extent (Bensen, unpublished results). Thus, the Ypt6p-dependent pathway appears to be selectively involved in TGN protein localization. Two v-SNAREs, Sft1p and Vti1p, which have been implicated in retrograde traffic

within or to the Golgi apparatus (Banfield *et al.*, 1995; Lupashin *et al.*, 1997; von Mollard *et al.*, 1997), did not suppress *ric1*Δ or *ypt6*Δ defects when expressed from multicopy plasmids. These SNAREs therefore are likely to define additional retrograde pathways that act in conjunction with the Ypt6p-dependent pathway to maintain the overall organization of the Golgi apparatus.

Our studies add to an increasingly complex picture of traffic pathways involved in organization of the Golgi complex. First, TGN protein localization may depend on both retrieval to the TGN and retrieval to the CGN. Second, only a subset of the many Sed5p-interacting v-SNAREs can suppress *ypt6*Δ, thereby distinguishing the transport pathway responsible for suppression from other pathways targeted to Sed5p at the CGN. At least one of the Ypt6p-dependent pathway v-SNARES, Ykt6p, displays genetic and physical interactions with v- and t-SNAREs in other pathways, consistent with emerging evidence that combinatorial arrangements of SNAREs mediate distinct fusion steps. Deciphering the mechanisms that govern the combinatorial distribution of SNAREs in different vesicle populations represents a future challenge for understanding the specificity of vesicle targeting and fusion.

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