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Analysis of subcellular localization and function of the yeast Rab6 homologue, Ypt6p, using a novel amino-terminal tagging strategy

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ABSTRACT

Ypt6p, the yeast homologue of mammalian Rab6, is involved in the multiple processes regulated by membrane trafficking such as vacuole maturation and membrane protein recycling. Although several lines of evidence suggest that Ypt6p is possibly localized to multiple membrane compartments, the precise localization of endogenous Ypt6p remains to be elucidated. In this study, we developed a novel method for N-terminal tagging of endogenous protein based on homologous recombination and investigated the subcellular localization and function of Ypt6p. Ypt6p and its GTP-bound form were predominantly localized to the *cis*- to *medial*-Golgi compartments whereas the GDP-bound form of Ypt6p was localized to the cytosol. Ric1p, a component of the specific GEF complex for Ypt6p, largely colocalized with Ypt6p in the early Golgi, and localization of Ypt6p changed to the cytosol in *ric1*Δ cells. On the other hand, Gyp6p, a putative GAP for Ypt6p, was localized to the *trans*-Golgi compartment and deletion of *GYP6* increased the localization of Ypt6p at the *trans*-Golgi, suggesting that Gyp6p promotes the dissociation of Ypt6p from the Golgi when arriving at the *trans*-Golgi compartment. Additionally, we demonstrated that overexpression of the GDP-bound form of Ypt6p caused defective vacuole formation and recycling of Snc1p to the plasma membrane. These results suggest that the GTP-binding activity of Ypt6p is necessary for *intra*-Golgi trafficking and protein recycling in the early Golgi compartment.

1. Introduction

Rab/Ypt GTPases are well-characterized regulators of intracellular membrane trafficking, functioning as molecular switches that alternate between GTP- and GDP-bound forms. The biological activity and localization of Rab/Ypt proteins are controlled by multiple factors, including guanine nucleotide dissociation inhibitor (GDI), guanine nucleotide exchange factor (GEF), and GTPase-activating protein (GAP) [1]. Covalent isoprenylation of the C-terminal cysteine residues is also required for proper localization and activity of Rab/Ypt proteins [2]. GDP-bound prenylated Rab/Ypt proteins bind to GDI, which masks their isoprenyl anchor and thereby maintains them in soluble cytosolic form [1]. Conversion of the GDP-bound Rab/Ypt into the GTP-bound form is catalyzed by a specific GEF, which promotes the exchange of GDP with GTP, resulting in binding of Rab/Ypt proteins to their effector proteins [1]. GTP-bound Rab/Ypt no longer associates with GDI and therefore can stably reside on membrane compartments via the prenylated C terminus. After arriving at the target membrane, Rab/Ypt proteins are inactivated by hydrolysis of the bound GTP to GDP, extracted from the membrane by GDI, and recycled back to the cytosol [1].

The mechanisms of trafficking along the secretory pathway are well conserved from yeast to mammalian cells [3]. In yeast, the secretory pathway is known to be regulated by sequential activation and inactivation of three types of Rab protein – Ypt1p, Ypt31p/32p and Sec4p – by their GEFs and GAPs [4,5]. A recent study has demonstrated a novel Rab-GAP cascade in the secretory pathway that regulates the conversion of Ypt6p to Ypt32p during *intra*-Golgi trafficking; Ypt6p was shown to reside at the *medial*-Golgi and to dissociate from the membrane upon arrival of Ypt31p/32p that recruit Gyp6p, a putative GAP for Ypt6p, to the Golgi [6]. Previous studies have reported that Ypt6p, together with

Abbreviations: GEF, guanine nucleotide exchange factor; GAP, GTPase-activating protein; VPS, vacuolar protein sorting; GFP, green fluorescent protein.

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its GEF Ric1p/Rgp1p complex, mediates the fusion of vesicles from endosomes to the *trans*-Golgi by recruiting the Golgi-associated retrograde protein (GARP) complex [7,8]. These observations suggest roles of Ypt6p at the *medial*- or *trans*-Golgi, but the precise localization of endogenous Ypt6p has yet to be determined, and therefore the precise step(s) controlled by Ypt6p remains unclear.

Chromosomal GFP tagging of endogenous protein at the C terminus has become a standard technique for analyzing the localization of protein in yeast [9]. However, C-terminal tagging of Ypt6p, which contains conserved C-terminal cysteine residues that are required for prenylation, renders Ypt6p non-functional because it interferes with the latter's membrane anchoring. Thus, a novel strategy for tagging GFP at the N terminus is necessary for analyzing the localization and function of endogenous Ypt6p. We recently developed a novel method for N-terminal tagging of endogenous protein based on homologous recombination [10]. In the present study, using this novel method, we generated cells chromosomally expressing GFP-Ypt6p and determined the localization of Ypt6p. Additionally, based on the principles of this method, we developed new techniques for one-step introduction of specific point mutations into a gene and replacement of the promoter region of the gene at the chromosomal locus. Using these techniques, we demonstrate that Ypt6p and its GTP-bound form were predominantly localized to the *cis*- to *medial*-Golgi compartments whereas the GDP-bound form of Ypt6p was localized to the cytosol. We also demonstrate that overexpression of the GDP-bound form of Ypt6p caused defective vacuole formation and recycling of Snc1p to the plasma membrane.

2. Material and methods

2.1. Yeast strains, growth conditions, and plasmids

The yeast strains used in this study are listed in Supplementary Table 1. All strains were grown in standard rich medium (YPD) or synthetic medium (SM) supplemented with 2% glucose and appropriate amino acids. The N-terminal GFP tag was integrated at the endogenous locus of the *YPT6* gene as follows: 333-bp 5′ UTR of *YPT6* gene and the N-terminal fragment of the *YPT6* ORF (nt 1–210) were generated by PCR and cloned into the BamHI or BglII site of pBS-GFP-HIS3 vector [10] (pBS-P_{YPT6}-GFP-*YPT6*(1–210)-HIS3). To integrate GFP at the N terminus of the *YPT6* gene, the integration plasmid was linearized by XcmI and transformed into yeast. The extra region generated by insertion of the integration plasmid was removed by PCR-based homologous recombination as shown in Fig. 1A. GFP-tagged Ypt6(Q69L)p and Ypt6(T24N)p were generated as follows: The N-terminal fragment of the *YPT6* ORF (nt 1–636) was subcloned into BamHI-digested pBS and mutagenized using a PCR-based mutagenesis protocol. To create integration plasmids, these mutagenized fragments were replaced with the N-terminal fragment of the *YPT6* of pBS-P_{YPT6}-GFP-*YPT6*(1–210)-HIS3, and the integration plasmid was linearized by HindIII and transformed into yeast. The integration plasmid for Ypt6p overexpression was generated by replacing the *YPT6* promoter of pBS-P_{YPT6}-GFP-*YPT6*(1–210)-HIS3 with the *TPI1* promoter (418-bp 5′ UTR of *TPI1* gene). The C-terminal GFP or mCherry tagging of proteins was performed by PCR-based homologous recombination using pFA6a-GFP(S65T) or pFA6a-mCherry, respectively, as a template [9].

2.2. Fluorescence microscopy

Fluorescence microscopy was performed using an Olympus IX81 microscope equipped with a x100/NA 1.40 (Olympus) objective and Orca-AG cooled CCD camera (Hamamatsu), using Metamorph software (Universal Imaging). Simultaneous imaging of red and green fluorescence was performed using an Olympus IX81 microscope and an image splitter (Dual-View; Optical Insights) that divided the red and green components of the images with a 565-nm dichroic mirror and passed the red component through a 630/50 nm filter and the green component through a 530/30 nm filter. Fluorescence labeling of α-factor was performed as described previously [11].

2.3. Cell extract preparation and immunoblotting

Yeast cells were grown in YPD medium at 25 °C for 16–20 h. The cells collected from 50 mL of the cultures were washed twice with distilled water, frozen in liquid nitrogen, and powdered using mortar and pestle. After suspended the cell powder in lysis buffer (20 mM Tris–HCl, pH 8.0, 300 mM NaCl, 2 mM EDTA, 10 mM EGTA, 1% Triton X-100, 15% glycerol, 1 mM PMSF and protease inhibitor cocktail), cell lysates were prepared using ultrasonication. After being centrifuged, the supernatants were separated on SDS–PAGE and transferred onto PVDF membranes (Bio-Rad). The membrane was blocked overnight with 5% nonfat dry milk in phosphate buffer saline containing 0.05% Tween 20 (PBS-T) and incubated for 2 h at room temperature with anti-GFP antibody (Life Technologies) diluted in PBS-T containing 1% nonfat dry milk. After washing in PBS-T, the membrane was incubated with horseradish peroxidase-conjugated donkey anti-rabbit IgG (GE Healthcare). Immunoreactive protein bands were visualized by exposing the membrane for 1 min to an ECL Plus chemiluminescence reagent (GE Healthcare).

3. Results and discussion

3.1. Endogenous Ypt6p is predominantly localized to the cis- to medial-Golgi compartments

In a recent study, we developed a novel method for N-terminal GFP tagging of endogenous protein [10]. Using this method, a GFP-tag was added to the N terminus of Ypt6p (Fig. 1A) (see Methods for details). To test the functionality of GFP-tagged Ypt6p, we examined its ability to complement the phenotype of *ypt6*Δ cells. A previous study had shown that *ypt6*Δ cells exhibit a temperature-sensitive growth defect [12]. As shown in Fig. 1B, we found that expression of GFP-Ypt6p was able to restore the growth defect of *ypt6*Δ cells at 39 °C. GFP-Ypt6p was clearly detected as numerous small puncta throughout the cytoplasm (Fig. 1C). The puncta of GFP-Ypt6p were highly colocalized with those of Vrg4p (~80.0%), a *cis*-Golgi marker, and Mnn5p (~70.6%), a *medial*-Golgi marker (Fig. 1D and E), but only partly colocalized with Sec7p (~28.8%), a *trans*-Golgi marker, or Vps10p (~27.5%), a marker for the *trans*-Golgi and late endosomal compartments [13,14] (Fig. 1D and E). Interestingly, Ypt6p was highly localized to COPI-coated vesicles labeled by Sec21p (~65.5%), whereas it was rarely localized in COPII-coated vesicles labeled by Sec13p (~8.0%) (Fig. 1D and E), suggesting the localization of Ypt6p at the early Golgi compartments. Such localization differs somewhat from previous observations showing that Ypt6p resides at the boundary between the *cis*- and *trans*-Golgi, 54% and 58% of GFP-Ypt6p being colocalized with *cis*- and *trans*-Golgi markers, respectively [6]. We also examined the localization of Ypt6p in endocytic compartments by colabeling GFP-Ypt6p with Alexa Fluor 594-α-factor (A594-α-factor) [11], and found that Ypt6p little colocalizes with A594-α-factor at any of the time points examined. From these results we concluded that endogenous Ypt6p is localized to the Golgi apparatus, particularly the *cis*- to *medial*-Golgi compartments.