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Involvement of Golgi-associated retrograde protein complex in the recycling of the putative Dnf aminophospholipid flippases in yeast

Keiko Takagi, Kunihiko Iwamoto [1], Shingo Kobayashi, Hiroyuki Horiuchi, Ryouichi Fukuda, Akinori Ohta *

Department of Biotechnology, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan

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ABSTRACT

It is widely accepted that phosphatidylethanolamine (PE) is enriched in the cytosolic leaflet of the eukaryotic plasma membranes. To identify genes involved in the establishment and regulation of the asymmetric distribution of PE on the plasma membrane, we screened the deletion strain collection of the yeast *Saccharomyces cerevisiae* for hypersensitive mutants to the lantibiotic peptide Ro09-0198 (Ro) that specifically binds to PE on the cell surface and inhibits cellular growth. Deletion mutants of *VPS51*, *VPS52*, *VPS53*, and *VPS54* encoding the components of Golgi-associated retrograde protein (GARP) complex, *YPT6* encoding a Rab family small GTPase that functions with GARP complex, *RIC1* and *RGP1* encoding its guanine nucleotide exchange factor (GEF), and *TLG2* encoding t-SNARE exhibited hypersensitivity to Ro. The mutants deleted for *VPS51*, *VPS52*, *VPS53*, and *VPS54* were impaired in the uptake of fluorescently labeled PE. In addition, aberrant intracellular localization of the EGFP-tagged Dnf2p, the putative inward-directed phospholipid translocase (flippase) of the plasma membrane, was observed in the mutant defective in the GARP complex, Ypt6p, its GEF proteins, or Tlg2p. Our results suggest that the GARP complex is involved in the recycling of Dnf flippases.

1. Introduction

In biological membranes, phospholipids are considered to distribute asymmetrically between the two leaflets of the bilayer. In eukaryotic plasma membrane, it is widely accepted that phosphatidylethanolamine (PE) and phosphatidylserine (PS) reside predominantly in the inner leaflet, whereas phosphatidylcholine (PC) and sphingomyelin are enriched in the outer leaflet [1]. The asymmetric distribution of phospholipids is believed to be generated and maintained by transporter proteins that facilitate inward or outward transport (flip-flop) of lipids across cellular membranes [1,2]. The type 4 P-type ATPases (P4-ATPases) are primary candidates for inward-directed phospholipid transporter (also called 'flippase') [3–7]. In budding yeast *Saccharomyces cerevisiae*, five P-type ATPases, Drs2p, Dnf1p, Dnf2p, Dnf3p, and Neo1p, are classified in the P4-ATPase subfamily [8,9]. Among P4-ATPases in yeast, Dnf1p and Dnf2p localize in the plasma membrane [9–12]. Deletion of *DNF1* and *DNF2* deteriorates the uptake of fluorescently labeled PE, PS, and PC, probably due to the defects in the ATP-dependent transport of these phospholipid analogs from the outer to the inner leaflet of the plasma membrane [10,13]. In contrast, involvement of Drs2p and/or Dnf3p in the phospholipid translocation in *trans*-Golgi network (TGN) or post-Golgi secretory vesicles was reported [9,14,15]. Quadruple deletion of *DNF1, DNF2, DNF3*, and *DRS2* was lethal, suggesting that these genes share essential function(s) in yeast [9]. These P4-ATPases interact with the conserved membrane proteins, which constitute Cdc50 family including Cdc50p, Lem3p/Ros3p, and Crf1p [11,16,17]. Dnf1p and Dnf2p interact with Lem3p, Dnf2p also interacts with Cdc50p, and Dnf3p binds to Crf1p [11,12]. Cdc50 family proteins are required for the exit of P4-ATPases from the ER [11,12], but it remains plausible that they might play additional regulatory roles in flippase activities of P4-ATPases. Triple deletions of *CDC50*, *LEM3*, and *CRF1* resulted in lethality [16] and hence Cdc50 family proteins appear to share essential function(s) in yeast.

Ro09-0198 (Ro) is a lantibiotic peptide that specifically binds to PE on the surface of liposome or mammalian cells, and causes leakage of liposome contents or induces cytolysis of mammalian cells [18–20]. The biotinylated Ro (Bio-Ro) conjugated with

Abbreviations: DIC, differential interference phase contrast microscopy; DOPC, dioleoylphosphatidylcholine; EGFP, enhanced green fluorescent protein; GARP, Golgi-associated retrograde protein; GEF, guanine nucleotide exchange factor; GFP, green fluorescent protein; M-C_6-NBD-PE, 1-myristoyl-2-[6-{(7-nitro-2-1,3-benzoxadiazol-4-yl)amino}hexanoyl]-*sn*-glycero-3-phosphoethanolamine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PMME, phosphatidylmonomethylethanolamine; PS, phosphatidylserine; Ro, Ro09-0198; TLC, thin layer chromatography; TGN, *trans*-Golgi network; VPS, vacuolar protein sorting; VFT, Vps fifty-three.

* Corresponding author. Fax: +81 3 5841 8015.
E-mail address: aaohta@mail.ecc.u-tokyo.ac.jp (A. Ohta).
[1] Present address: Molecular Function and Pharmacology Laboratories, Taisho Pharmaceutical Co. Ltd., 1-403 Yoshino-cho, Kita-ku, Saitama-shi, Saitama 331-9530 Japan.

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fluorescein-labeled streptavidin was used to analyze the distribution of PE on the plasma membrane of Chinese hamster ovary cells [21,22]. Ro also inhibited growth of yeast, and Bio-Ro was used as a specific probe to evaluate localization of PE in the outer leaflet of the yeast plasma membrane [13,17]. *LEM3*/*ROS3* (Ro-sensitive 3) gene was identified by the analysis of Ro-hypersensitive mutants [17]. Single or double deletion mutants of *DNF1* and *DNF2* exhibited hypersensitivity to Ro [10,13]. Ro-sensitivity of these mutants is probably due to the increase of PE in the outer leaflet of the plasma membrane, which was likely resulted from the defects in the inward transport of exposed PE. In this study, we extended genetic screen for new factors involved in the asymmetric distribution of PE in the plasma membrane and surveyed the yeast deletion mutant collection for the Ro-sensitive phenotype. We found that the deletion mutants of *VPS51*, *VPS52*, *VPS53*, and *VPS54*, which are encoding components of the Golgi-associated retrograde protein (GARP) complex, exhibited hypersensitivity to Ro. They were also defective in the uptake of fluorescence-labeled-PE and accumulated enhanced green fluorescent protein (EGFP)-tagged Dnf2p in the intracellular compartment, suggesting that the GARP complex is involved in the recycling of the putative phospholipid flippases.

2. Materials and methods

2.1. Strains, materials, and media

Yeast strains used in this study are listed in Table S1. Ro was kindly provided by M. Umeda (Kyoto University). Ro was also purified from the culture supernatant of *Streptoverticillium griseoverticillatum* NAR164C-MY6 strain (ATCC) as described by Takemoto et al. [23]. Unless otherwise indicated, yeast strains were grown at 30 °C in YPD medium composed of 1% yeast extract (Difco), 2% Polypeptone (Nihon Seiyaku), and 2% glucose. Strains carrying plasmids were grown in SD medium {0.17% yeast nitrogen base without ammonium sulfate and amino acids (Difco), 0.5% ammonium sulfate, 2% glucose} containing 2% casamino acids (Difco).

2.2. Plasmids

Plasmids and primers used in this study are listed in Tables S2 and S3, respectively. To construct YCpDNF1-GFP and YCpDNF2-GFP, *DNF1* and *DNF2* ORFs with their 5′-non-coding regions were amplified by PCR from W303-1A genomic DNA using primers DNF1-GFP-I-f and DNF1-GFP-III-r, and DNF2-U-Xba and DNF2-L-Sph, respectively. Amplified fragments were digested with *Sac*I and *Bam*HI or *Xba*I and *Sph*I. The GFP coding region was amplified from pEGFP (CLONTECH, Palo Alto, CA) by PCR using primers EGFP-BamHI or EGFP-U-EcoRI-SphI and GFP-L-SalI. The amplified fragment was digested with *Bam*HI and *Sal*I or *Sph*I and *Sal*I. The terminator region of GAPDH gene of *S. cerevisiae* was excised from pYPR2831 with *Sal*I and *Hin*dIII [24]. These fragments were inserted into YCplac33 [25].

2.3. Ro sensitivity assay

Yeast cells were grown at 30 °C to log phase, and diluted to 3 OD_{660} units/ml. Five microliters of every 10-fold diluted cultures was spotted onto YPD plate with or without 50 μM Ro. Cells were incubated at 30 °C for 48 h.

2.4. Phospholipid analysis

Cells cultivated to log phase in YPD medium were washed and resuspended in 0.15 M KCl. Phospholipids were extracted by Bligh–Dyer's method [26], and separated by two-dimensional thin layer chromatography (TLC) on silica gel plate (TLC Glass Plates, Si 60, Merck). Solvent systems used for chromatography are as follows: chloroform/methanol/acetic acid (65:25:10, v/v/v) for the first dimension and chloroform/methanol/formic acid, (65:25:10, v/v/v) for the second dimension. Lipid spots were scraped and amounts of phospholipids were measured by phosphorus assay [27].

2.5. Internalization of fluorescently labeled PE into cells

Fluorescently labeled PE, 1-myristoyl-2-[6-{(7-nitro-2-1,3-benzoxadiazol-4-yl)amino}hexanoyl]-*sn*-glycero-3-phosphoethanolamine (M-C_6-NBD-PE) (Avanti Polar Lipids Inc.) was purified by TLC. Purified M-C_6-NBD-PE and dioleoylphosphatidylcholine (DOPC) (Avanti Polar Lipids Inc.) were used for vesicle preparation. Measurement of M-C_6-NBD-PE internalization was performed as previously described [13,17].

2.6. Fluorescence microscopy

Microscopic images were acquired a BX52 microscope (Olympus, Tokyo, Japan) equipped with ORCA-ER (Hamamatsu Photonics, Hamamatsu, Japan).

To visualize GFP-tagged proteins, cells were grown to log phase in SD medium containing 2% casamino acids. Aliquot were mounted on glass slides and immediately observed. For FM4-64 uptake assay, cells were grown to log phase in YPD medium at 30 °C, resuspended to 1×10^7 cells/200 μl in YPD medium, and incubated with 32 μM FM4-64 (Molecular Probes) for 15 min at 30 °C. Cells were washed three times with ice-cold SD medium, resuspended in 200 μl of YPD medium, and incubated for at least 90 min at 30 °C before microscopic observation.

3. Results

3.1. Screening of deletion mutants hypersensitive to Ro

Hypersensitivity of the *ROS3*-, *DNF1*-, or *DNF2*-deleted mutant to the PE-binding lantibiotic peptide Ro led us to an assumption that Ro-sensitive mutants are high in PE content in the outer leaflet of the plasma membrane and possibly defective in the mechanism for establishment of the asymmetric distribution of PE in the plasma membrane [10,13,17]. To reveal how the asymmetric distribution of PE is established, we screened the EUROSCARF yeast deletion mutant collection for Ro-sensitive mutants and found that mutants in genes *VPS51*, *VPS52*, *VPS53*, and *VPS54* were similarly sensitive to 50 μM Ro (Fig. 1A).

These genes are encoding components of GARP complex, which are also known as VFT (Vps fifty-three) complex. The GARP complex is a member of the quatrefoil family of multi-subunit tethering complexes that functions in tethering of vesicles derived from early and late endosomes to the TGN through binding to t-SNARE component Tlg1p [28–31]. The mutants of the genes encoding the Rab family small GTPase Ypt6p, which functions together with GARP complex [30], and its guanine nucleotide exchange factors (GEF) Ric1p and Rgp1p [32] showed hypersensitivity to Ro (Fig. 1A). Its GTPase-activating protein Mdr1p, however, did not show hypersensitivity [33]. Although we could not test Ro-sensitivity of the deletion mutant of *TLG1* because it was not included in the viable deletion mutant collection, the deletion mutant of *TLG2* encoding an SNARE protein probably forming t-SNARE complex with Tlg1p exhibited hypersensitivity to Ro (Fig. 1A). We focused on the involvement of the GARP complex in the dynamics of PE in the plasma membrane.

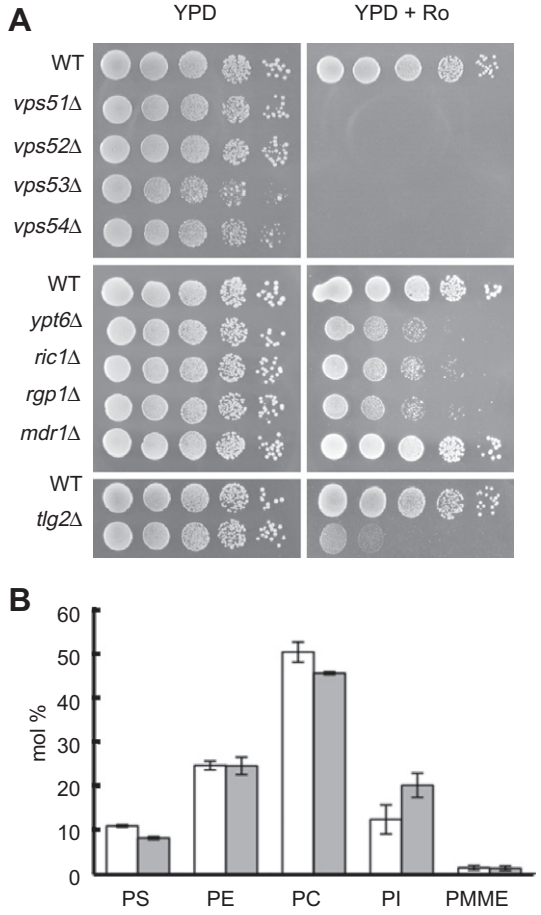

Fig. 1. Deletion mutants of the genes encoding the components of GARP complex show the sensitivity to Ro. (A) Growth of the deletion mutants on YPD medium with or without 50 μM Ro was analyzed as described in Section 2. (B) Phospholipid composition of the *vps51*Δ (gray bars) cells. Phospholipids were extracted from the wild-type (white bars) or *vps51*Δ cells, separated, and quantified as described in Section 2. Values are means expressed as the percentage of total major phospholipid ± S.E. from three independent experiments.

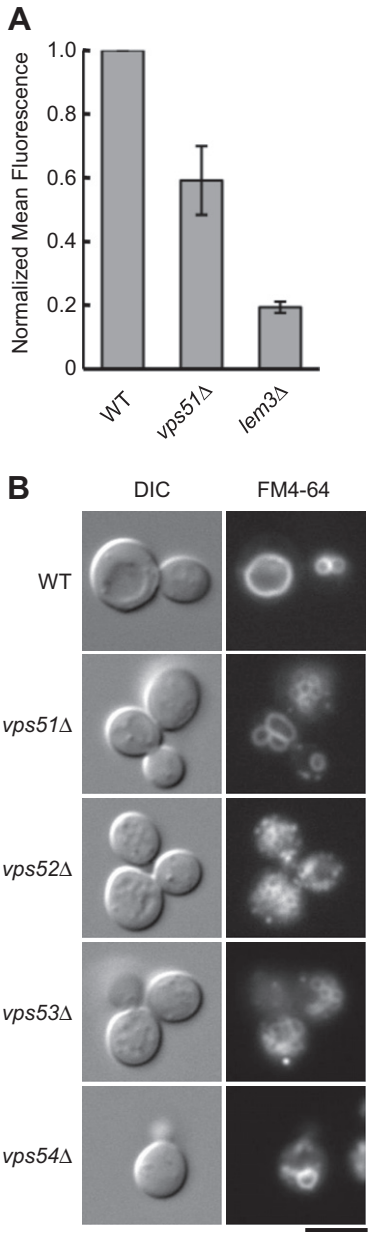

Fig. 2. Uptakeof M-C_6-NBD-PE or an endocytic maker in the deletion mutants of the genes encoding the components of GARP complex. (A) Internalization of M-C_6-NBD-PE by the deletion mutants. Yeast cells were grown to log phase and incubated with vesicles containing M-C_6-NBD-PE, and the M-C_6-NBD-PE fluorescence was measured by the flow cytometry. Data represent the normalized mean fluorescence intensity to that of wild-type cells. Values are average of means ± S.E. from four independent experiments. (B) Uptake of endocytic markers in deletion mutants. Yeast cells were grown to log phase and incubated with FM4-64, as described in Section 2. Images of differential interference phase contrast microscopy (DIC) (left) and FM4-64 fluorescence (right) are shown. Bar, 5 μm.

3.2. Defects of deletion mutants of GARP complex genes in the internalization of PE

We first analyzed the composition of major phospholipids in *vps51*Δ cells. The relative amount of PE in *vps51*Δ cells was not significantly altered from that in the wild-type cells (Fig. 1B), suggesting that Ro sensitivity of *vps51*Δ cells is not due to increase in the amount of PE.

Next, we investigated the dynamics of PE on the plasma membrane in *vps51*Δ cells by analyzing the internalization of M-C_6-NBD-PE into the cells. M-C_6-NBD-PE added in the culture medium is considered to be internalized by transbilayer transport across the plasma membrane in yeast [34,35]. We incubated yeast cells with liposomes containing M-C_6-NBD-PE for 30 min, and measured fluorescence intensity of NBD in the cells with flow cytometry. As reported previously, significant decrease in the uptake of M-C_6-NBD-PE was observed in the deletion mutant of *LEM3* [17]. The deletion mutant of *VPS51* exhibited approximately 40% lower fluorescence intensity than the wild-type cells (Fig. 2A). Decrease in the uptake of NBD-labeled PE was also observed in *vps52*Δ, *vps53*Δ, and *vps54*Δ cells (data not shown). In the fluorescence microscopic analysis, similar localization pattern of NBD fluorescence was observed in the deletion mutants of these *VPS* genes, compared to the wild-type cells, although the overall intensity of intracellular fluorescence in the deletion mutants was lower (data not shown).

The endocytic activities of these deletion mutants were analyzed by evaluating the incorporation of endocytic marker, FM4-64. As is known that the deletion mutants of the genes encoding the components of GARP complex exhibit the aberrant vacuolar morphology [28,29], the FM4-64-stained vacuolar patterns of the mutants were different from that in the wild-type cells, but it is evident that FM4-64 was incorporated in deletion mutants of

VPS51, *VPS52*, *VPS53*, and *VPS54*, implying that these mutants are not defective in endocytic incorporation of FM4-64 (Fig. 2B). In addition, no significant defect was observed in the incorporation of another endocytic marker, Lucifer Yellow, in these *vps* mutants (data not shown).

Taken together, these results suggest that GARP complex are implicated in the translocation step of PE from the outer to the inner leaflet of the plasma membrane.

3.3. Requirement of the GARP complex for the localization of putative phospholipid flippase to the plasma membrane

To elucidate how GARP complex is engaged in the translocation of PE in the plasma membrane, we looked at the behavior of the putative phospholipid flippase, Dnf2p, in the deletion mutant of the genes encoding the components of GARP complex. Dnf2p fused with EGFP at its C terminus was expressed under its native promoter on a low copy plasmid YCplac33. Functionality of this fusion protein was confirmed by the complementation of the hypersensitivity of *dnf2*Δ cells to Ro by expression of this fusion protein (data not shown). The amounts of Dnf2p-EGFP in the deletion mutants, estimated by using anti-EGFP antibody, are not significantly altered from that in the wild-type strain (data not shown).

We examined localization of Dnf2p-EGFP in *vps51*Δ, *vps52*Δ, *vps53*Δ, and *vps54*Δ mutants (Fig. 3 and Table 1). In the wild-type cells, Dnf2p-EGFP localized at the cell surface, particularly at the small bud cortex and the bud neck of mitotic large-budded cells, in agreement with previous reports [10,12]. In contrast, the EGFP fluorescence was observed in the intracellular structures in the deletion mutants of the genes encoding the components of GARP complex. Similar aberrant intracellular localization of another putative flippase, Dfn1p, tagged with EGFP was observed in these deletion mutants, although its fluorescent intensity was lower than that of Dnf2p-EGFP (data not shown). In the deletion mutants of *YPT6*, *RIC1*, and *RGP1*, around 40% mutant cells were aberrant in the localization of Dnf2p-EGFP and in the mutant of *TLG2*, higher population (77%) of cells were recognized as aberrant, whereas *mdr1*Δ cells were 80% normal in the Dnf2p-EGFP localization (Table 1). These are consistent with their less severe sensitivity to Ro. These results suggest that the GARP complex and retrograde traffic from early (and possibly late) endosomes to TGN are required for the proper localization of Dnf2p and Dnf1p at the plasma membrane, and sensitivity to Ro and the deficiency in the internalization of fluorescently-labeled PE in the deletion mutants of the genes encoding the components of GARP complex and Rab family GTPase are due to the aberrant localization of Dnf proteins.

Table 1
Localization of Dnf2p-EGFP in the deletion mutants.

Strain	Localization (%)		*n*
	Cell surface	Intracellular	
WT	97.0	3.0	164
*vps51*Δ	5.6	94.4	124
*vps52*Δ	4.2	95.8	119
*vps53*Δ	0.0	100.0	108
*vps54*Δ	1.0	99.0	104
*ypt6*Δ	63.6	36.4	143
*ric1*Δ	55.7	44.3	140
*rgp1*Δ	58.5	41.5	118
*mdr1*Δ	80.0	20.0	115
*tlg2*Δ	22.8	77.2	114

Cells were categorized by pattern of the observed EGFP fluorescence and expressed as the percentages of the cell population. 'Cell surface', Dnf2p-EGFP fluorescence localized near the cell surface; 'Intracellular', Dnf2p-EGFP fluorescence was observed at intracellular structures or was diffused in the cytosol; '*n*', the number of the cells categorized.

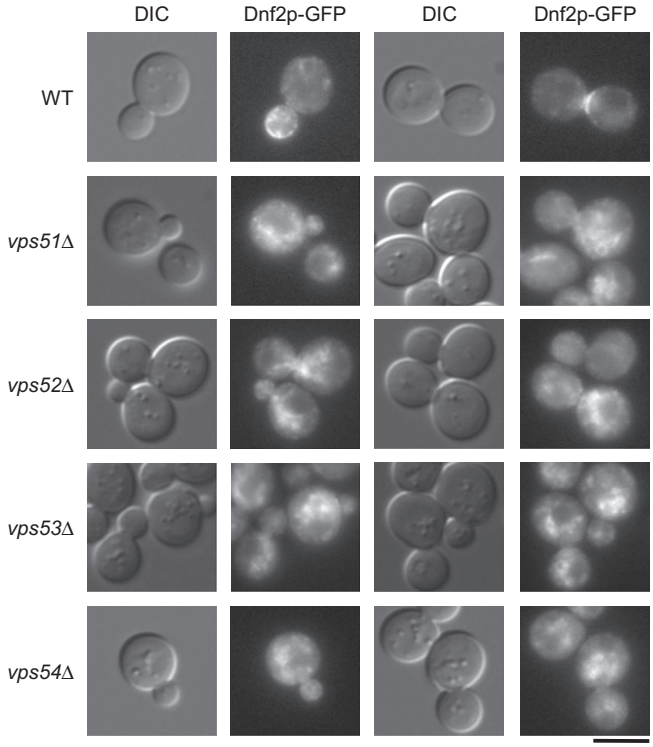

Fig. 3. Localization of Dnf2p-EGFP in the deletion mutants of the genes encoding the components of GARP complex. The wild-type, *vps51*Δ, *vps52*Δ, *vps53*Δ, and *vps54*Δ cells harboring YCpDNF2-GFP were grown to log phase, followed by microscopic observation, as described in Section 2. Images of DIC and GFP fluorescence are shown. Bar, 5 μm.

4. Discussion

The deletion mutants of the genes encoding the components of GARP complex exhibited hypersensitivity to the PE-binding lantibiotic peptide Ro09-0198, deficiency of the uptake of M-C_6-NBD-PE, and altered localization of the putative aminophospholipid flippases, Dnf1p and Dnf2p. GARP complex is considered to be a tethering complex for SNARE dependent vesicle fusion, required for retrograde traffic from both early and late endosomes to the TGN [29,31]. Deletion of *VPS51* or *VPS52* impaired the recycling of the secretory vesicle v-SNARE Snc1p to the plasma membrane and caused intracellular accumulation of Snc1p tagged with GFP due to the defect in the membrane traffic from the early endosome to the TGN [29,31]. Dnf1p localizes to both the plasma membrane and the TGN/endosome. It was suggested that Dnf1p recycles between these membranes since it was accumulated on the plasma membrane in the mutant defective in endocytosis [11,36]. Dnf2p appears to localize predominantly to the plasma membrane and partly to the internal punctate structures, and its cycling between the endocytic and exocytic pathways was predicted by Hua et al. [9,10,37]. Thus, the defects in the recycling route from early or late endosome to the TGN in the deletion mutants of the genes encoding the components of GARP complex, Ypt6p, its GEF regulators, or Tlg2p could result in the intracellular mislocalization of Dnf1p and Dnf2p, leading to the hypersensitivity to Ro and the defect in the uptake of M-C_6-NBD-PE. Although the organelles, in which Dnf proteins mislocalize in these mutants, are not determined yet, they might accumulate in the vacuole or transport vesicles as proposed for Snc1p [29].

The results in this study raise the possibility of the regulation of membrane integrity by the endocytic recycling of phospholipid flippases. Dnf1p and Dnf2p are enriched at the small bud cortex and at the bud neck of mitotic large-budded cells [10–12]. Recycling of Dnf proteins could contribute to establish or regulate their localization on the plasma membrane in cell cycle progression. It was reported that Dnf1p might function on the TGN/endosome [9]. Thus, the recycling of Dnf proteins could also engage in the control of the spatial distribution of Dnf proteins between the plasma membrane and the TGN/endosome.

It has been reported that asymmetric distribution of PE between inner and outer leaflets of biological membranes is involved in the polarized cell growth and membrane traffic [3,13,38]. In addition, regulation of Dnf proteins by the protein kinase network was proposed [37,39]. However, the significance of the asymmetric distribution of PE and its regulation still remain to be established. Isolation and characterization of hypersensitive yeast mutants to Ro as described here will contribute to address these unsolved issues common to and probably critical for eukaryotic cells.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.11.147.

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