Role of Phosphatidylserine in Phospholipid Flippase-Mediated Vesicle Transport in Saccharomyces cerevisiae

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Phospholipid flippases translocate phospholipids from the exoplasmic to the cytoplasmic leaflet of cell membranes to generate and maintain phospholipid asymmetry. The genome of budding yeast encodes four heteromeric flippases (Drs2p, Dnf1p, Dnf2p, and Dnf3p), which associate with the Cdc50 family noncatalytic subunit, and one monomeric flippase Neo1p. Flippases have been implicated in the formation of transport vesicles, but the underlying mechanisms are largely unknown. We show here that overexpression of the phosphatidylserine synthase gene CHO1 suppresses defects in the endocytic recycling pathway in flippase mutants. This suppression seems to be mediated by increased cellular phosphatidylserine. Two models can be envisioned for the suppression mechanism: (i) phosphatidylserine in the cytoplasmic leaflet recruits proteins for vesicle formation with its negative charge, and (ii) phosphatidylserine flipping to the cytoplasmic leaflet induces membrane curvature that supports vesicle formation. In a mutant depleted for flippases, a phosphatidylserine probe GFP-Lact-C2 was still localized to endosomal membranes, suggesting that the mere presence of phosphatidylserine in the cytoplasmic leaflet is not enough for vesicle formation. The CHO1 overexpression did not suppress the growth defect in a mutant depleted or mutated for all flippases, suggesting that the suppression was dependent on flippase-mediated phospholipid flipping. Endocytic recycling was not blocked in a mutant lacking phosphatidylserine or depleted in phosphatidylethanolamine, suggesting that a specific phospholipid is not required for vesicle formation. These results suggest that flippase-dependent vesicle formation is mediated by phospholipid flipping, not by flipped phospholipids.

Type 4 P-type ATPases (P4-ATPases), highly conserved membrane proteins among eukaryotic cells, are phospholipid flippases that selectively transport phosphatidylserine (PS) and phosphatidylethanolamine (PE) from the exoplasmic to the cytoplasmic leaflet of the plasma membrane and internal membranes to generate and maintain asymmetrical distribution of phospholipids (1–4). P4-ATPase deficiencies are associated with human diseases (e.g., intrahepatic cholestasis type 1) (5). However, much remains to be learned about the functions and physiological and pathological importance of P4-ATPases.

In the budding yeast Saccharomyces cerevisiae, there are five P4-ATPases: Drs2p, Dnf1p, Dnf2p, Dnf3p, and Neo1p. Of these, Drs2p, Dnf1p/Dnf2p, and Dnf3p form complexes with the Cdc50 family noncatalytic subunits Cdc50p, Lem3p, and Crl1p, respectively, for their exit out of the endoplasmic reticulum (ER) and proper localization (6, 7). These four flippases are collectively essential for viability and have redundant roles for vesicle transport in various transport pathways (8, 9), including the early endosome–trans-Golgi network (TGN) retrieval pathway or the endocytic recycling pathway (7). Neo1p does not associate with Cdc50 family members and thus might function without a noncatalytic subunit. Neo1p is an essential protein by itself, possibly because it is involved in various cell functions other than the endocytic recycling pathway, including retrograde transport from Golgi bodies to the ER (10) and membrane trafficking within endosomal/Golgi system (11).

Amino phospholipids, PS and PE seem to be preferred substrates of flippases. In vitro studies using a fluorescent analog (NBD-PS and NBD-PE) suggested that Cdc50p-Drs2p prefers PS with a minor activity toward PE (12–14). In contrast, Lem3p-Dnf1p and -Dnf2p translocate NBD-PE and NBD-PC when they are localized at the plasma membrane (9, 15). However, since all of these studies essentially used a phospholipid analog or a lipid-binding peptide, it needs to be further demonstrated that phospholipids are involved in flippase functions in vivo.

Cdc50p-Drs2p, which is mainly localized to early endosome/TGN membranes, is implicated in the formation of clathrin-coated vesicles from these membranes (16–19). Because clathrin has no intrinsic lipid-binding ability, it is linked to membranes by adaptors, which bind to lipids and/or the cytoplasmic domains of cargo proteins (20). The AP-1 clathrin adaptor has been suggested to function downstream of Cdc50p-Drs2p (18, 19), but the underlying mechanisms are unknown.

One important question is how flippase activity is harnessed to form a transport vesicle. Flippases would influence physicochemical properties of membranes by an asymmetric membrane structure with a high concentration of substrate lipids, such as PS and PE, in the cytoplasmic leaflet. These phospholipids could recruit adaptor proteins for vesicle formation by their specific properties (e.g., a negative charge of PS). A second important consequence of a flippase activity is an increase in phospholipid number within the cytoplasmic leaflet relative to the luminal leaflet. This could induce bending in the membrane toward the cytosol, a process that is essential to vesicle budding (21).

We show here that overexpression of the PS synthase gene CHO1, which resulted in an increase in PS, suppressed defects in the endocytic recycling of flippase mutants. Our results suggest that PS in the cytoplasmic leaflet is not sufficient for vesicle for-
### TABLE 1 Yeast strains used in this study

| Strain* | Relevant genotype | Source or reference |
|---------|-------------------|--------------------|
| YEF473  | MATα a lys2-801/lys2-801 ura3-52/ura3-52 his3Δ-200/none3-200 trplΔ-63/trplΔ-63 leu2Δ-1/leu2Δ-1 | 26 |
| YKT1066 | MATα lys2-801 ura3-52 his3Δ-200 leu2Δ-1 TRP1 | This study |
| YKT1651 | MATα neo1-101 | This study |
| YKT1781 | MATα neo1-101::LEU2 TRP1 | This study |
| YKT1782 | MATα KanMX6::P_GAL1-3HA-DC50 TRP1 | This study |
| YKT1783 | MATα HplMX4::P_GAL1-3HA-DC50 neo1-101::LEU2 TRP1 | This study |
| YKT1629 | MATα TRP1::P_GAL1-3HA-DC50 | This study |
| YKT1784 | MATα TRP1::P_GAL1-3HA-DC50 neo1-101 | This study |
| YKT1785 | MATα DNF2-EGFP::natMX TRP1 | This study |
| YKT1786 | MATα neo1-101::LEU2 DNF2-EGFP::natMX TRP1 | This study |
| YKT1787 | MATα KanMX6::P_GAL1-3HA-DC50 DNF2-EGFP::KanMX6 TRP1 | This study |
| YKT1788 | MATα HplMX4::P_GAL1-3HA-DC50 neo1-101::LEU2 DNF2-EGFP::natMX TRP1 | This study |
| YKT1777 | MATα LEU2::mRFP::SNC7 TRP1 | This study |
| YKT1789 | MATα HplMX4::P_GAL1-3HA-DC50 neo1-101::LEU2 LEU2::mRFP::SNC7 TRP1 | This study |
| YKT1790 | MATα HplMX4::P_GAL1-3HA-DC50 neo1-101::LEU2 TRP1 | This study |
| YKT1650 | MATα HIS3MX6::P_GAL1-3HA-DC50 neo1-101 | This study |
| YKT1529 | MATα KanMX6::P_GAL1-3HA-DC50 | This study |
| YKT1791 | MATα KanMX6::P_GAL1-3HA-DC50 | This study |
| YKT1792 | MATα KanMX6::P_GAL1-3HA-DC50 | This study |
| YKT1793 | MATα TRP1::P_GAL1-3HA-DC50 | This study |
| YKT1513 | MATα KanMX6::P_GAL1-3HA-DC50 leu2Δ::HIS3MX6 crf1Δ::HplMX3 LEU2::GFP::SNC1 TRP1 | This study |
| YKT1660 | MATα KanMX6::P_GAL1-3HA-NEO1 | This study |
| YKT1796 | MATα HplMX4::P_GAL1-3HA-DC50 neo1-101::LEU2 leu2Δ::HIS3MX6 crf1Δ::HplMX4 TRP1 | This study |
| YKT1797 | MATα DNF1-EGFP::KanMX6 TRP1 | This study |
| YKT1798 | MATα HplMX4::P_GAL1-3HA-DC50 neo1-101::LEU2 DNF1-EGFP::KanMX6 TRP1 | This study |
| YKT1799 | MATα LEU2::GFP::Lact-C2 TRP1 | This study |
| YKT1800 | MATα KanMX6::P_GAL1-3HA-DC50 | This study |
| YKT1801 | MATα KanMX6::P_GAL1-3HA-DC50 | This study |
| YKT1428 | MATα cho1Δ::KanMX4 TRP1 | This study |
| YKT1642 | MATα APL2-EGFP::KanMX6 TRP1 | This study |
| YKT1803 | MATα cho1Δ::natMX APL2-EGFP::KanMX6 TRP1 | This study |
| YKT1507 | MATα cdc50Δ::HIS3MX6 TRP1 | This study |
| YKT1804 | MATα cdc50Δ::HplMX4 cho1Δ::KanMX4 TRP1 | This study |
| YKT1805 | MATα DNF2-EGFP::HplMX4 TRP1 | This study |

* YKT strains are isogenic derivatives of YEF473.

### TABLE 1 (Continued)

| Strain* | Relevant genotype | Source or reference |
|---------|-------------------|--------------------|
| YKT1806 | MATα cho1Δ::natMX DNF2-EGFP::KanMX6 TRP1 | This study |
| YKT1807 | MATα KanMX6::P_GAL1-3HA-DC50 cho1Δ::natMX DNF2-EGFP::KanMX6 TRP1 | This study |
| YKT1808 | MATα KanMX6::P_GAL1-3HA-DC50 cho1Δ::natMX SEC7-EGFP::KanMX6 DNF2-EGFP::HplMX4 TRP1 | This study |
| YKT1809 | MATα psd2Δ::HIS3MX6 TRP1 | This study |
| YKT1812 | MATα URA3::GFP::SNC1 TRP1 | 64 |
| YKT1810 | MATα DNF2-EGFP::natMX TRP1 | This study |
| YKT1871 | MATα DNF2-EGFP::natMX TRP1 | This study |
| YKT1872 | MATα HplMX4::P_GAL1-3HA-DC50neo1-101::LEU2 SEC63-mRFP::KanMX6 TRP1 | This study |

**MATERIALS AND METHODS**

**Media and genetic methods.** Unless otherwise specified, strains were grown in YPD medium (1% Bacto yeast extract [Difco Laboratories, Detroit, MI], 2% Bacto peptone [Difco], 2% glucose, and 0.01% adenine). Strains carrying plasmids were selected in synthetic medium (SD) containing the required nutritional supplements (22). Synthetic medium (SC) was SD medium containing all required nutritional supplements. When appropriate, 0.5% Casamino Acids were added to SD medium without uracil (SDA-U). For induction of the GAL1 promoter, 3% galactose, and 0.2% sucrose were used as carbon sources instead of glucose (YPGA, SG-U, and SGA-U). When required, 2 mM ethanolamine and 2 mM choline were supplemented to medium to support growth of the cho1Δ and psd2Δ mutants, respectively. Standard genetic manipulations of yeast were performed as described previously (23). The lithium acetate method was used for introduction of plasmids into yeast cells (24, 25).

**Escherichia coli** strains DH5α and XL1-Blue were used for construction and amplification of plasmids.

**Strains and plasmids.** Yeast strains constructed in the YEF473 background (26) are listed in Table 1. Because flippase and cho1Δ mutants exhibit defects in tryptophan uptake (27), a strain in which trp1Δ-63 was replaced with TRP1 was constructed (YKT1066), and most strains used in the present study were derived from this strain. PCR-based procedures were used to construct gene deletions and gene fusions with the GAL1 promoter, green fluorescent protein (GFP), and mRFP (26). Some gene deletions (psd2Δ) and psd2ΔA were constructed by transformation with PCR-products from knockout strains. The psd2ΔA mutant was kindly provided by S. Maye-Royall. All constructs produced by the PCR-based procedure were verified by colony-PCR amplification to confirm the replacement occurred at the expected locus. When required, selection markers of mutant alleles were changed appropriately by cassette exchange (28).

The GFP-tagged Lact-C2 plasmid (pRS416-GFP-Lact-C2) (29) was purchased from Hematologic Technologies, Inc. (Essex Junction, VT). The URA3::GFP::Lact-C2 strain was constructed by integrating the linearized pRS306-GFP-Lact-C2 into the URA3 locus, and the URA3::GFP::Lact-C2AAA (Lact-C2-W26A, W33A, F34A) strain was similarly constructed. Lact-C2AAA (29), CHO1(D148A), and CH01(D152A) mutations were constructed by site-directed mutagenesis as described previously (30). The LEU2::mRFP::SNC1 strain was constructed by integrating the linearized pRS305-mRFP::SNC1 into the LEU2 locus, and the LEU2::mRFP::SNC1(pm) strain was similarly constructed. The neo1-101::LEU2
strain was constructed by integrating the linearized pRS305-neo1-101-C, which contained only the carboxyl-terminal neo1-101 mutation site fragment, into the NEO1 locus. The plasmids used in the present study are listed in Table 2. Schemes detailing the construction of plasmids and DNA sequences of nucleotide primers are available upon request.

**Determination of the neo1-101 mutation site.** The neo1-101 mutant gene was cloned by the gap repair method (31). The pRS316-NEO1ΔbgII plasmid was linearized with HindIII and Hpal, followed by transformation into the neo1-101 strain (YKT1651). Plasmids were recovered from several independent Ura+ transformants and sequenced. One mutation, which changed GTG (Val) of the codon 1145 to ATG (Met) in the C-terminal cytosolic region of Neo1p, was identified.

**Isolation of multiplicity suppressors of the Pgal1p-Cdc50p neo1-101 mutant.** The Pgal1p-Cdc50p neo1-101 strain was transformed with a yeast genomic DNA library constructed in the multiplicity plasmid YEp24 (32). Transformants were selected on SGA-U plate at 30°C and then replica plated onto YPDA plates. Plasmids were recovered from the transformants that grew on YPDA, and those containing CDC50 or NEO1 were identified by PCR and eliminated. Restriction enzyme digestion of the remaining plasmids indicated that 20 different clones were isolated. Nine of these plasmids reproducibly conferred growth on YPDA. Three clones that exhibited clearer suppression were chosen, and the genes responsible for the suppression were determined to be CHO1, YCK1, and ART5 by fragment subcloning and DNA sequencing.

**Microscopic observations.** Cells were observed using a Nikon Eclipse E800 microscope (Nikon, Inc., Tokyo, Japan) equipped with an HB-10103AF super high-pressure mercury lamp and a 1.4 numerical aperture 100 x Plan Apo oil immersion objective lens with appropriate fluorescence filter sets or differential interference contrast optics. Images were acquired using a digital cooled charge-coupled device camera (C7429-95-12NR; Hamamatsu Photonics, Hamamatsu, Japan) using AQUACOSMOS software (Hamamatsu Photonics). Observations are compiled from the examination of at least 100 cells. To visualize GFP- or mRFP-tagged proteins, cells were grown to early to mid-logarithmic phase, harvested, and resuspended in SDA- or SD-based medium. Cells were mounted on microscope glass and immediately observed using a GFP band-pass or G-2A (for mRFP) filter set.

**Phospholipid analysis.** Cells were grown in 12 ml of appropriate medium containing 1 μCi of [38P]orthophosphoric acid (Perkin-Elmer-Cetus, Norwalk, CT) to an optical density at 600 nm of 0.5 for 12 h at 30°C to achieve steady-state labeling. The cells were harvested by centrifugation, washed with sterile water, and transferred to screw-cap glass tubes. The cells were treated with 5% trichloroacetic acid for 1 h and then washed with cold water three times. Phospholipids were extracted basically by the Bligh and Dyer method (33), using 0.1N HCl as the aqueous phase as described previously (34). The cells were resuspended in 1.2 ml of CHCl3-MeOH (2:1) and an aliquot of the sample was spotted onto silica gel plates, and the plate was exposed to an imaging plate for 2 days, and the signal was detected and quantitated by using a FLA 3000 phosphor imager. The phospholipids were identified by comparison to commercial standards (Avanti Polar Lipids, Alabaster, AL).

**RESULTS**

**Combination of neo1-101 and cdc50Δ mutations result in synthetic defects in endocytic recycling.** We previously screened for mutations that were synthetically lethal with cdc50Δ and obtained an allele of NEO1, neo1-101 (36). Neo1-101p had a Valine 1145 to-methionine substitution in the C-terminal cytoplasmic region. NEO1 is an essential gene, but the neo1-101 mutation did not affect the growth rate (Fig. 1A). The neo1-101 mutation was combined with conditional alleles of flippase genes, Pgal1p-CDC50 and Pgal1p-Drs2p alleles, whose expression is repressed by glucose. Both Cdc50p- and Drs2p-depleted neo1-101 cells exhibited severe growth defects (Fig. 1A). The neo1-101 mutation did not exhibit synthetic growth defects with lem3Δ or dnf1Δ dnf2Δ mutations (data not shown).

Because a previous study showed that Cdc50p-Drs2p, Lem3p-
Dnf1/2p, and Crf1p-Dnf3p had redundant roles in the early endosome to TGN recycling pathway (7), we examined whether the neo1-101 mutation aggravated the recycling defect in Cdc50p-depleted cells. Dnf2p, which is mainly localized to polarized growth sites of the plasma membrane (8, 9, 36), is recycled from the plasma membrane via the early endosome to the TGN (37). The neo1-101 mutant cells exhibited normal polarized localization of Dnf2p-GFP at a bud or a cytokinesis site (99%, n/H11005 = 143 budded cells) like wild-type cells (99%, n/H11005 = 145) (Fig. 1B). In the Cdc50p-depleted cells in which Cdc50p was partially depleted for 12 h in the presence of glucose, Dnf2p-GFP was internally accumulated in some cells, but most of the cells still exhibited polarized Dnf2p-GFP (90%, n/H11005 = 122), as reported previously for GFP-Snc1p (18). In contrast, in the Cdc50p-depleted neo1-101 mutant, only 8% (n = 127) of the cells exhibited polarized Dnf2p-GFP, and the remaining 92% accumulated Dnf2p-GFP in internal structures that seemed to be early endosome-derived abnormal membranes.

To confirm these results, we examined other cargos transported through this pathway. A v-SNARE Snc1p, involved in the fusion of Golgi-derived secretory vesicles with the plasma membrane, is recycled through the endocytic recycling pathway (38). Tlg1p, an essential t-SNARE mediating fusion of endosome-derived vesicles with the TGN, is recycled between the early endosome and the TGN (39, 40). In wild-type cells, mRFP-Snc1p was localized to polarized plasma membrane sites such as Dnf2p-GFP, whereas GFP-Tlg1p was observed as internal punctate structures reminiscent of endosomal/TGN membranes (Fig. 1C). In the Cdc50p-depleted neo1-101 mutant, both mRFP-Snc1p and GFP-Tlg1p were localized to internal enlarged compartments (82%, n = 124). These compartments were independent of a TGN marker Sec7p-mRFP (Fig. 1D), suggesting that they were early endosome-derived membranes. These results suggest that combination of cdc50 and neo1-101 mutations caused the synthetic defect in the retrieval pathway from the early endosome to the TGN.

Overexpression of the phosphatidylserine synthase gene CHO1 suppresses the endocytic recycling defects in the Cdc50p-depleted neo1-101 mutant. To obtain a clue to flippase functions, we isolated multicopy suppressors of the synthetic growth defect.
FIG 2 Overexpression of CHO1 suppresses defects in growth and endocytic recycling of Cdc50p-depleted neo1-101 cells. (A) Suppression of the growth defects. Cells were grown to early log phase in SGA-U medium, and cell growth was examined at 30°C for 1 day as in Fig. 1A with an initial cell concentration of 1.0 × 10^7 cells/ml. The strains were as follows (abbreviations used in the figure are indicated in brackets): YKT1760 (PGAL1-CDC50 neo1-101) carrying YEp24 [empty], YEp24-CHO1 (pKT1753) [pCHO1], or YEplac195-CDC50 (pKT1263) [pCDC50]. (B) Localization of the overproduced Cho1p-GFP to the ER membrane. Cells harboring YEplac195-CHO1-GFP (pKT2112) were grown in SDA-U medium at 30°C for 12 h. The strains were YKT1871 (SEC63-mRFP) and YKT1872 (PGAL1-CDC50 neo1-101 SEC63-mRFP). Bar, 5 μm. (C) Suppression of the defects in endocytic recycling of Dnf2p-GFP. Localization of Dnf2p-GFP was examined in the cells grown in SDA-U medium at 30°C for 12 h to deplete Cdc50p. The strains were YKT1878 (PGAL1-CDC50 neo1-101 DNF2-GFP) carrying the same plasmids as in panel A. Arrows indicate that Dnf2p-GFP is localized to polarized plasma membrane sites, including the bud or the cytokinesis site. Bar, 5 μm. (D) PS is increased by overexpression of CHO1. The cells described in panel A carrying YEp24 (empty) or YEp24-CHO1 (pCHO1) were labeled with 32P during Cdc50p depletion in SDA-U medium at 30°C for 12 h. Wild-type cells (YKT1066) carrying YEp24 were similarly cultured and 32P labeled as a control. Phospholipids were extracted, separated, and quantified as described in Materials and Methods. The data represent percentages of total phospholipids with means ± the standard deviations of three independent experiments. Asterisks indicate a significant difference in the Student t test (*, P < 0.05). PC, phosphatidylcholine; PI, phosphatidylinositol. (E) Failure of catalytically inactive cho1 mutant genes to suppress the growth defect. Cells were grown to early log phase in SGA-U medium, and cell growth was examined at 30°C for 1 day as in Fig. 1A with an initial cell concentration of 2.0 × 10^6 cells/ml. The strains were YKT1872 (PGAL1-CDC50 neo1-101) carrying YEplac195 (empty), YEplac195-CHO1 (pKT2111, pCHO1), YEplac195-cho1(D148A) [pKT2114, pcho1(D148A)], YEplac195-cho1(D152A) [pKT2115, pcho1(D152A)], or YEplac195-CDC50 (pKT1263, pCDC50).

of the Cdc50p-depleted neo1-101 mutant. CHO1, encoding the unique phosphatidylserine synthase in yeast, was isolated (Fig. 2A). Cho1p catalyzes the synthesis of PS from CDP-DAG and serine in the ER (41). To confirm that the overexpressed Cho1p was normally localized to the ER membrane, we constructed the CHO1-GFP allele. CHO1-GFP was functional, because it complemented the choline auxotrophy and the cold-sensitive growth of the cho1Δ mutant (data not shown). The Cho1p-GFP overexpression from a multicopy plasmid was colocalized with an ER marker Sec63p-mRFP (42) in the Cdc50p-depleted neo1-101 mutant, as well as in the wild type (Fig. 2B), indicating that the overexpressed Cho1p-GFP was normally localized to the ER. We confirmed that Cho1p-GFP was overexpressed in these strains, because the Cho1p-GFP signal was undetectable under the same condition in a strain expressing Cho1p-GFP from its genomic locus (data not shown).

We examined whether the CHO1 overexpression also suppressed the recycling defect of Dnf2p-GFP in the Cdc50p-depleted neo1-101 mutant. When Cdc50p in the neo1-101 mutant was depleted for 12 h in the glucose-containing synthetic medium (SDA-U), Dnf2p-GFP was polarized in 13% (n = 142 budded cells) of the cells, which was slightly higher compared to cells grown in YPDA rich medium (8%, Fig. 1B). The CHO1 overexpression increased the cells with polarized Dnf2p-GFP to 36% (n = 140) (Fig. 2C). This partial suppression was consistent with the partial suppression of the growth defect. Because it was previously shown that CHO1 overexpression increased cellular PS levels (43), these results suggest that the endocytic recycling of Dnf2p-GFP was restored by increased PS.

To confirm that PS content was increased in the Cdc50p-depleted neo1-101 mutant by CHO1 overexpression, we analyzed phospholipid composition in the cells grown under the same condition as in Fig. 2C. Interestingly, we noticed that PS content was decreased from 17.0% ± 0.9% of the wild type to 11.1% ± 1.9% in
FIG 3 Overexpression of CHO1 suppresses defects in growth and endocytic recycling of the Cdc50p-depleted dnf1Δ crf1Δ mutant. (A) Suppression of the growth defects. Cells were grown to early log phase in SGA-U medium, and growth was examined at 25°C for 2 days as in Fig. 1A with an initial cell concentration of 2.0 × 10⁶ cells/ml. The strains were as follows (abbreviations used in the figure are indicated in brackets): YKT1529 (PGAL1-CDC50 dnf1Δ crf1Δ), YKT173 (PGAL1-CDC50 dnf1Δ crf1Δ DNF2-GFP), YKT1791 (PGAL1-CDC50 dnf1Δ crf1Δ DNF2-GFP) carrying pRS416-mRFP-SNC1, pKT1785 (DNF2-GFP), and YKT1792 (PGAL1-CDC50 dnf1Δ crf1Δ DNF2-GFP) carrying pRS416-mRFP-SNC1. Bar, 5 μm. (B) Suppression of the defects in endocytic recycling of Dnf2p-GFP. Localization of Dnf2p-GFP was examined in the wild type and the strains described in panel A carrying YEp24 [empty] or YEp24-CHO1 (pKT1753) [pCHO1]. (C) Suppression of the defects in endocytic recycling of Dnf2p-GFP. Localization of Dnf2p-GFP was examined in the cells grown as in panel B. The strains were YKT1791 (PGAL1-CDC50 dnf1Δ crf1Δ DNF2-GFP) carrying the same plasmids as in panel A. Arrows indicate that Dnf2p-GFP is localized to the polarized plasma membrane sites. Bar, 5 μm. (D) Suppression of the defects in endocytic recycling of Dnf2p-GFP. Localization of Dnf2p-GFP was examined in the cells grown as in panel B. The strains were YKT1791 (PGAL1-CDC50 dnf1Δ crf1Δ DNF2-GFP) carrying the same plasmids as in panel A. Arrows indicate that Dnf2p-GFP is localized to the polarized plasma membrane sites. Bar, 5 μm. (E) GFP tagging of Snc1p, but not of Snc1p(pm), inhibits the suppression of growth defects by CHO1 overexpression in the Cdc50p-depleted dnf1Δ crf1Δ mutant. Cell growth was examined as in panel A at 25°C for 2 days with initial cell concentration of 8.0 × 10⁶ cells/ml. The strains were YKT1792 (PGAL1-CDC50 dnf1Δ crf1Δ DNF2-GFP) carrying pRS416-mRFP-SNC1 and YKT1793 (PGAL1-CDC50 dnf1Δ crf1Δ DNF2-GFP) carrying the same plasmids as in panel A. (E) Increase in PS by overexpression of CHO1. The phospholipid content in the wild type and the strains described in panel A carrying YEp24 [empty] or YEp24-CHO1 (pKT1753) [pCHO1] was analyzed as in Fig. 2D, including statistical analysis.

The Cdc50p-depleted neo1-101 mutant, whereas PI content was increased from 16.2% ± 2.4% to 22.4% ± 0.7% (Fig. 2D). The molecular basis for these phospholipid changes is currently unclear, but this seems to be specific to the Cdc50p-depleted neo1-101 mutant, because these changes were subtle (PS) or not observed (PI) in the Cdc50p-depleted dnf1Δ crf1Δ mutant (Fig. 3E). Overexpressed CHO1 resulted in 1.7-fold increase of PS content (from 11.1% ± 1.9% to 18.6% ± 0.6%) in the Cdc50p-depleted neo1-101 mutant (Fig. 2D). In contrast, the phosphatidylinositol (PI) level was decreased from 22.4% ± 0.7% to 14.9% ± 0.8% by CHO1 overexpression, probably because CDP-DAG is also a precursor for PI synthesis (44). Because the suppression was dependent on a remaining flippase (see below), increase of PS, a known substrate of flippases, seems to be responsible for the suppression.

To confirm that the suppression is dependent on the enzymatic activity of Cho1p, we examined catalytically inactive mutants of Cho1p. The CDP-alcohol phosphotransferase motif was previously suggested as a catalytic site of the enzymes, including Cho1p that catalyze the synthesis of a phospholipid by the displacement of CMP from a CDP-alcohol by a second alcohol to form a phosphoester bond. Two aspartic acid residues in this motif, Asp131 and Asp135, were shown to be essential for the catalytic activity of yeast cholinephosphotransferase (Cpt1p) (45). The corresponding residues in Cho1p, Asp148 and Asp152, were replaced with alanine to form Cho1p(D148A) and Cho1p(D152A), respectively. As expected, neither cho1p(D148A) nor cho1p(D152A) complemented the choline auxotrophy and the cold-sensitive growth of the cho1Δ mutant (data not shown). As shown in Fig. 2E, overexpression of these cho1 mutant genes did not suppress the growth defect of the Cdc50p-depleted neo1-101 mutant. We confirmed that both the overexpressed Cho1p(D148A)-GFP and Cho1p(D152A)-GFP were normally localized to the ER as Cho1p-GFP was (data not shown).

The CHO1 overexpression also suppresses the endocytic recycling defects in the Cdc50p-depleted dnf1Δ crf1Δ mutant. To examine whether the suppression was specific to neo1-101, we
overexpressed CHO1 in the Cdc50p-depleted dnf1Δ crf1Δ mutant in which Dnf2p–GFP could be also used as a marker for endocytic recycling. As shown in Fig. 3A, the CHO1 overexpression suppressed the growth defect in the Cdc50p-depleted dnf1Δ crf1Δ mutant. In this mutant in which Cdc50p was depleted for 12 h, Dnf2p–GFP was accumulated in intracellular membranes, whereas mRFP-Snc1p(pm), a mutant of Snc1p, was localized to the plasma membrane (Fig. 3B). mRFP-Snc1p(pm) is normally transported to the plasma membrane by the exocytosis pathway but is not endocytosed due to its defects in endocytosis (38). Thus, these results suggest that the Cdc50p-depleted dnf1Δ crf1Δ mutant is defective in the endocytic recycling pathway, but not in the exocytosis pathway, and that Dnf2p–GFP was accumulated in early endosome-derived membranes. In the Cdc50p-depleted dnf1Δ crf1Δ cells, Dnf2p–GFP was localized to the plasma membrane only in 9% of the cells (n = 107 budded cells), whereas it increased to 59% when CHO1 was overexpressed (n = 137) (Fig. 3C). These results suggest that the CHO1 overexpression partially restored endocytic recycling of Dnf2p–GFP in the Cdc50p-depleted dnf1Δ crf1Δ mutant.

We also examined whether the CHO1 overexpression restored endocytic recycling of GFP-Snc1p in the Cdc50p-depleted dnf1Δ crf1Δ cells. However, interestingly, the expression of GFP-Snc1p inhibited the suppression of growth defects by the CHO1 overexpression (Fig. 3D). Consistently, endocytic recycling of GFP-Snc1p was not restored by the CHO1 overexpression either (data not shown). This inhibitory effect was not observed with GFP-Snc1p(pm) (Fig. 3D). Since Snc1p is a cargo of a vesicle formed from early endosomes, these results may suggest that GFP-tagging of Snc1p interferes with some step in vesicle formation, which is promoted by PS increase.

We confirmed that PS content was increased from 14.2% ± 1.6% to 20.4% ± 0.2% in the Cdc50p-depleted dnf1Δ crf1Δ mutant by CHO1 overexpression, as in the Cdc50p-depleted neo1-101 mutant (Fig. 3E). Unexpectedly, a slight decrease in the PC level was observed from 43.0% ± 1.1% to 38.4% ± 1.5% for an unknown reason. A previous study showed that depletion of PEM2 involved in PC synthesis decreased PC content from 41 to 37% (46). We examined whether the pem2Δ mutation suppressed the growth defect in the Cdc50p-depleted dnf1Δ crf1Δ mutant, but it did not (data not shown), suggesting that the PC decrease was not responsible for the suppression. Taken together, these results suggest that the defects of growth and endocytic recycling in flipase mutants could be suppressed by PS increase.

**Suppression by PS increase seems to be dependent on a remaining flipase.** Our results suggest that increase of PS promotes vesicle formation from early endosomes in the flipase mutants. One possible mechanism is that increased PS is used by a remaining flipase to increase efficiency of flipase-mediated vesicle formation, whereas the other possibility is that PS in the outer leaflet of endosomal membranes is sufficient for vesicle formation by itself (e.g., PS recruits vesicle coat proteins). If the suppression is dependent on a flipase, it would not occur in the absence of flipases. We overexpressed CHO1 in the Cdc50p-depleted lem3Δ crf1Δ mutant in which Drs2p, Dnf1p, Dnf2p, and Dnf3p are not functional, but CHO1 weakly suppressed the growth defect (Fig. 4A). We reasoned that Neo1p might promote vesicle formation with increased PS in this mutant because Neo1p functioned with Drs2p in the endocytic recycling pathway as shown in Fig. 1. In fact, overexpression of NEO1 suppressed the growth defect of the Cdc50p-depleted lem3Δ crf1Δ mutant, and co-overexpression of CHO1 and NEO1 enhanced this suppression (Fig. 4A). Then, we wanted to examine whether CHO1 overexpression would suppress the growth defect in the Cdc50p- and Neo1p-depleted lem3Δ crf1Δ mutant. However, CHO1 overexpression did not suppress the growth defect of even the Neo1p-depleted single mutant (Fig. 4B). This may be because Neo1p is involved in various cell functions other than the endocytic recycling pathway, including retrograde transport from the Golgi bodies to the ER (10) and membrane trafficking within the endosomal/Golgi system (11). Consistently, the growth defect of Neo1p-depleted cells was not suppressed by the overexpression of CDC50/DRS2, CDC50/DRS2 and CHO1, or LEM3/DFN1 (Fig. 4B).

We next examined the neo1-101 allele, which seems to be specifically defective in the endocytic recycling pathway. The growth defect of the Cdc50p-depleted neo1-101 mutant was suppressed by overexpression of LEM3/DFN1, as well as CHO1 (Fig. 4C). In addition, the suppression was enhanced by co-overexpression of LEM3/DFN1 and CHO1. Dnf1p–GFP was normally localized to polarized plasma membrane sites as Dnf2p–GFP was, but in the Cdc50p-deleted neo1-101 mutant, Dnf1p–GFP was localized to endosomal membranes in which mRFP–Snc1p was accumulated (Fig. 4D). These results suggest that overexpressed Lem3p–Dnf1p supported vesicle formation from early endosomes in the Cdc50p-depleted neo1-101 mutant and that this vesicle formation was enhanced by PS increase.

Finally, the growth defect in the Cdc50p-depleted neo1-101 lem3Δ crf1Δ mutant was not suppressed by overexpression of CHO1 (Fig. 4E). Thus, we concluded that the suppression of flipase mutations by increased PS was mediated by a remaining flipase.

**PS is present in the cytoplasmic leaflet of early endosome membranes even in the absence of flipases.** If PS in the cytoplasmic leaflet of early endosome membranes plays a direct role in vesicle formation, PS may not be found in the cytoplasmic leaflet of endosomal membranes that are accumulated in the flipase mutants. Distribution of PS in the cytoplasmic leaflet of the plasma membrane and internal membranes could be monitored with GFP-Lact-C2, the GFP-fused C2 domain of lactadherin, which specifically binds to PS (29).

In wild-type cells, GFP-Lact-C2 was exclusively localized to the plasma membrane, and no intracellular localization was observed, as reported previously (Fig. 5A) (29, 47). In contrast, in the Cdc50p-depleted dnf1Δ crf1Δ mutant, GFP-Lact-C2 was also localized to endosomal membranes merged with mRFP–Snc1p (94%, n = 100). This GFP-Lact-C2 signal was not observed with a mutant version of GFP-Lact-C2, GFP-Lact-C2-AAA, which does not bind to PS (29). We confirmed that expression of GFP-Lact-C2 did not affect the PS content in the Cdc50p-depleted dnf1Δ crf1Δ cells (Fig. 5B). Because it was possible that the PS in the cytoplasmic leaflet resulted from PS flipping by remaining flipases, including Lem3p–Dnf2p and Neo1p, we examined the localization of GFP-Lact-C2 in the mutant in which all known flipases are not functional, that is, the Cdc50p- and Neo1p-depleted lem3Δ crf1Δ mutant. GFP-Lact-C2 was again localized to the mRFP–Snc1p–containing membranes (98%, n = 122) (Fig. 5C), although this mutant seems to accumulate TGN membranes in addition to endosomal membranes due to possible inhibition of the exocytosis pathway (our unpublished results).

These results seem to be consistent with the expected transbi...
FIG 4 Suppression by the CHO1 overexpression is dependent on a remaining flippase. (A) Suppression of the growth defect in the Cdc50p-depleted lem3Δ crf1Δ mutant by CHO1 and/or NEO1 overexpression. Cells were grown to early log phase in SG-LU medium, and cell growth was examined at 30°C for 1 day as in Fig. 1A with an initial cell concentration of 4.0 × 10^6 cells/mL. The strains were as follows (abbreviations used in the figure are indicated in brackets): YKT1513 (P_{GAL1-CDC50 lem3Δ crf1Δ} carrying pRS425 and YEp24 [empty]), pRS425 and YEp24-CHO1 (pKT1753) [pCHO1], pRS425-NEO1 (pKT1788) and YEp24 [pNEO1], pRS425-NEO1 and YEp24-CHO1 [pNEO1 pCHO1], and pRS425 and YEp195-CDC50 (pKT1263) [pCDC50]. (B) Growth defects in the Neo1p-depleted mutant are not suppressed by overexpression of either CHO1 or other flippases. Cells were grown to early log phase in SGA-UW medium, and cell growth was examined at 30°C for 1 day as in Fig. 1A with an initial cell concentration of 8.0 × 10^6 cells/mL. The strains were as follows (abbreviations used in the figure are indicated in brackets): YKT1650 (P_{GAL1-CDC50 neo1-101} [empty]), pRS425 and YEp24-CHO1 (pKT1753) [pCHO1], pRS425-NEO1 (pKT1788) and YEp24 [pNEO1], YEp195-CDC50 (pKT2097) [pCDC50], YEp195-LM3 (pLM3 dDNF1), YEp195-LM3 (pLM3 dDNF1 pCHO1), and YEp195-CDC50 (pKT1264) [pCDC50]. (C) Colocalization of Dnf1p-GFP with mRFP-Snc1p in early endosomal membranes in the Cdc50p-depleted neo1-101 mutant. Localization of Dnf1p-GFP and mRFP-Snc1p was examined in the cells grown in SDA-U medium at 30°C for 12 h to deplete Cdc50p. The strains were as follows (abbreviations used in the figure are indicated in brackets): YKT1797 (DNP1-GFP) and YKT1798 (DNP1-GFP) carrying pRS416-Neo1. 5 μM. (E) Growth defects in the Cdc50p-depleted neo1-101 lem3Δ crf1Δ mutant are not suppressed by CHO1 overexpression. Cells were grown to early log phase in SGA-U medium, and cell growth was examined at 30°C for 1 day as in Fig. 1A with an initial cell concentration of 8.0 × 10^6 cells/mL. The strains were as follows (abbreviations used in the figure are indicated in brackets): YKT1796 (P_{GAL1-CDC50 neo1-101 lem3Δ crf1Δ} carrying YEp24 [empty], YEp24-CHO1 (pKT1753) [pCHO1], and YEp195-CDC50 (pKT1263) [pCDC50].
cho1Δ mutant exhibited a synthetic growth defect (Fig. 6B). This growth defect paralleled the defect in endocytic recycling of Dnf2p-GFP: Dnf2p-GFP was normally polarized in cho1Δ cells (83%, n = 117 budded cells) and was significantly polarized in Cdc50p-depleted cells (34%, n = 119) but not in Cdc50p-depleted cho1Δ cells (5%, n = 104) (Fig. 6C). In the Cdc50p-depleted cho1Δ cells, Dnf2p-GFP was localized to membrane structures that were not colocalized with Sec7p-mRFP (Fig. 6D), suggesting that Cdc50p-Drs2p and Cho1p redundantly function in the endocytic recycling pathway. We concluded that, although PS increase alleviates growth and endocytic recycling defects in flippase mutants and PS is involved in the flippase function, PS is not an essential phospholipid for the flippase-mediated vesicle formation from early endosomes.

Increased PE also alleviates defects in the flippase mutants. The results described above suggest that, in the cho1Δ mutant, phospholipids other than PS are utilized by flippases to promote vesicle formation from early endosomes. Because PE is also a potential substrate of flippases (6, 9, 13), we next examined whether increased PE would suppress the defects in the flippase mutants. PSD1 and PSD2 encode phosphatidylserine decarboxylases that catalyze formation of PE from PS. Psd1p is engaged in mitochondrial PE biosynthesis (51, 52), whereas Psd2p is implicated in PE synthesis in endosomal/TGN membranes (53, 54). As shown in Fig. 7A, overexpression of PSD2 weakly suppressed the growth defect of Cdc50p-depleted dnf1Δ crf1Δ cells, as well as Cdc50p-depleted neo1-101 cells, although this suppression was observed in synthetic (SDA) medium, but not in rich (YPD) medium (data not shown). We confirmed that the total cellular PE content was increased from 18.3% ± 1.6% to 22.4% ± 1.5% and from 19.9% ± 2.1% to 23.5% ± 1.9% by PSD2 overexpression in the Cdc50p-depleted neo1-101 and Cdc50p-depleted dnf1Δ crf1Δ cells, respectively (Fig. 7B). Consistent with the weak suppression of growth defects, cells with polarized Dnf2p-GFP were increased from 11.5% ± 3.0% to 23.0% ± 3.2% by PSD2 overexpression in the Cdc50p-depleted dnf1Δ crf1Δ cells (Student t test, *P < 0.05, four independent experiments) (data not shown).

We next examined whether PE is required for endocytic recycling of GFP-Snc1p. Although complete depletion of PE results in lethality, the psd1Δ psd2Δ mutant is viable, because Dpl1p coding for dihydrophosphoginosine-1-phosphate lyase permits low levels of PE synthesis (55, 56). In the psd1Δ psd2Δ mutant, PE content was markedly decreased to 2.2% ± 0.2% from 16.6% ± 1.4% in the wild type (Fig. 7C). However, GFP-Snc1p was normally localized to polarized sites in the psd1Δ psd2Δ mutant (Fig. 7D). In addition, the psd1Δ psd2Δ cdc50Δ mutant did not exhibit synthetic defects in growth and endocytic recycling of GFP-Snc1p (data not shown). These results suggest that the low level of PE does not cause a defect in the flippase-mediated vesicle formation from early endosomes.

Taken together, our results raise the possibility that, in addition to PS, PE could be also utilized by flippases to promote vesicle formation from early endosomes in the early endosome-to-TGN pathway.

DISCUSSION

In this study we demonstrate that an increase in PS caused by the overexpression of CHO1 alleviates defects in the growth and endocytic recycling of flippase mutants. Many studies using a fluorescence-labeled phospholipid analogue or a lipid-binding peptide have suggested that flippases translocate phospholipids, but it
needs to be demonstrated that flippases act on endogenous phospholipids. Our results provide genetic evidence for the functional relevance between flippases and endogenous phospholipids.

Neo1p is distinct from other flippases in that it is an essential protein and does not associate with a Cdc50p family member. Isolation and characterization of the neo1-101 mutant suggested that Neo1p is involved in the endocytic recycling pathway. We previously isolated NEO1 as a multicopy suppressor of the cdc50-11 lem3A crf1Δ mutant (7). Although Neo1p has not been demonstrated to possess a flippase activity, these results imply that Neo1p functions as a flippase like other flippases.

Overexpression of CHO1 caused two opposite effects in the phospholipid content: an increase in PS and a decrease in PI. Although we cannot exclude a possibility that a decrease in PI is involved in the suppression, the increase in PS, a known substrate of flippases, seems to be responsible, because the suppression was dependent on remaining flippases. Thus, it was suggested that increased PS was flipped by the flippases to promote vesicle formation from early endosomes. The cdc50Δ mutation exhibited synthetic defects with cho1Δ, but not with psd1Δ psd2Δ, suggesting that PS is functionally more relevant to flippases than PE. Since PS has been suggested to be a preferable substrate of Drs2p in vitro (12, 14), PS seems to be more effective in Drs2p-mediated vesicle formation than other phospholipids. In contrast, it has not been clearly demonstrated that Lem3p-Dnf1p/2p flips PS: NBD-labeled PS was still flipped in the lem3Δ mutant probably due to an unidentified protein on the plasma membrane (57), and NBD-PS was a less preferred substrate of Dnf1p compared to NBD-PC and NBD-PE (58). However, growth of the lem3Δ mutant was clearly sensitive to papuamide B, a cyclic lipopeptide that shows cytotoxicity by binding to PS in biological membranes (59), and this sensitivity was suppressed by the cho1Δ mutation (our unpublished results), indicating that PS is exposed on the cell surface in this mutant. These results may suggest that Lem3p-Dnf1p/2p flips PS more efficiently than NBD-PS.

Two possible mechanisms could be envisioned regarding how increased PS enhances vesicle formation. One is that flipped PS in the cytoplasmic leaflet recruits adaptor or coat proteins for vesicle formation with its negative charge. PS has been suggested to be an important factor for directing endocytic proteins to the plasma membrane (60). In the cdc50Δ and crf1Δ mutants, in which early endosomal membranes are intracellularly accumulated, endocytic proteins were assembled on those membranes, probably in a PS-dependent manner (60, 61). In mammalian cells, PS in recycling endosomes recruited evectin-2 via interaction with the pleckstrin homology (PH) domain (62). However, GFP-Lact-C2 detected PS in the cytoplasmic leaflet of early endosome membranes in flippase mutants. This PS seems to be transported from the plasma membrane through the endocytosis-recycling route (60). These results suggest that the presence of PS in the cytoplasmic leaflet is not sufficient for vesicle formation.

Thus, we favor the other mechanism: PS flipping by a flippase induces a local membrane curvature that assists in vesicle formation (21). Elucidation of how this membrane curvature is harnessed to form a vesicle is the next challenge. Proteins containing an amphipathic lipid packing sensor (ALPS) motif (63) are candidates that recognize the flippase-induced membrane curvature. We previously showed that the Arf1p GTPase activating protein gene GCS1 genetically interacts with CDC50 (18) and have recently shown that its ALPS motif is involved in this functional interaction (64). More recently, Gcs1p has been proposed to be an effector that recognizes the membrane curvature induced by Cdc50p-Drs2p through its ALPS motif (65). However, the endocytotic recycling defects in the gcs1Δ mutant are negligible compared to the cdc50Δ/drs2Δ mutant (our unpublished results), indicating that there should be another protein that recognizes the membrane curvature formed by Cdc50p-Drs2p.

Although PS has been suggested to be a preferable substrate of Drs2p in vitro (12, 14), the endocytic recycling pathway was not totally dependent on PS. Endocytic recycling was not significantly affected in the cho1Δ mutant but severely impaired in the cho1Δ cdc50A mutant, indicating that Cdc50p-Drs2p has a function(s) in the endocytic recycling pathway in the absence of PS. Cdc50p-Drs2p might perform a flippase-independent function as suggested for mammalian ATP8B1 flippase (66), but another interesting possibility is that Cdc50p-Drs2p flips PE, as suggested previously (6, 13), to form a vesicle in the absence of PS.

Similar to PS, increased PE alleviated the growth defect of the
Cdc50p-depleted neo1-101 and Cdc50p-depleted dnf1Δ crf1Δ mutants, which contain Lem3p-Dnf1/2p and Lem3p-Dnf2p, respectively. These flipases may utilize increased PE to form a vesicle because Lem3p-Dnf1/2p has been suggested to translocate a fluorescently labeled PE and PC (9, 15). Interestingly, simultaneous depletion of PS and PE (cho1Δ cells grown in SD medium supplemented with 2 mM choline) did not cause an obvious recycling defect (our unpublished results). It is possible that Lem3p-Dnf1/2p also flips PC in the absence of both PS and PE to form a vesicle from early endosomes.

Because both Cdc50p-Drs2p and Lem3p-Dnf1/2p are involved in the endocytic recycling pathway, it seems likely that PE/PC flipping contributes to flipase-mediated vesicle formation, albeit with reduced efficiency compared to PS. This is also consistent with our notion that flipase-mediated vesicle formation is promoted by membrane curvature rather than the chemical or physical properties of a specific phospholipid.

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