Fluorescent, Acyl Chain-labeled Phosphatidylcholine Analogs Reveal Novel Transport Pathways across the Plasma Membrane of Yeast*

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Acyl chain-labeled NBD-phosphatidylcholine (NBD-PC) has been used to identify three gene products (Lem3p, Dnf1p, and Dnf2p) that are required for normal levels of inward-directed phospholipid transport (flip) across the plasma membrane of yeast. Although the head group structure of acyl chain-labeled NBD phospholipids has been shown to influence the mechanism of flip across the plasma membrane, the extent to which the acyl chain region and the associated fluorophore affect flip has not been assessed. Given the identification of these proteins required for NBD-PC flip, it is now possible to determine whether the fluorophore attached to a phospholipid acyl chain influences the mechanism of flip. Thus, flip of phosphatidylcholine molecules with three different Bodipy fluorophores (Bodipy FL, Bodipy 530, and Bodipy 581) was tested and compared with that of NBD-PC in strains carrying deletions in LEM3, DNF1, and DNF2. Deletion of these genes significantly reduced the flip of NBD-PC and Bodipy FL-PC but had no effect on that of Bodipy 581-PC and Bodipy 530-PC. These data, in combination with comparisons of the effect of ATP depletion, collapse of the proton electrochemical gradient across the plasma membrane, and culture density led to the conclusion that at least three different flip pathways exist in yeast that are selective for the structure of the fluorophore attached to the acyl chain of phosphatidylcholine molecules.

Fluorescent acyl chain-labeled phospholipids have been used extensively as reporters to study the transport and intracellular trafficking of phospholipids in a wide range of cells (for recent reviews see Refs. 1–3). Attachment of the fluorophore to the terminal end of one acyl chain permits the synthesis of glycerophospholipids and sphingolipids with a wide range of different head groups. The fluorophore is generally attached to a shortened acyl chain in the sn-2 position to facilitate the introduction of these reporter phospholipids into the outer leaflet of the plasma membrane. This approach has demonstrated that the nature of the head group is a major determinant of the pathway of internalization and subsequent intracellular trafficking and localization of these reporter phospholipids (4).

Application of genetic and genomic approaches available for Saccharomyces cerevisiae has led to the identification of three gene products that are required for the normal uptake of a phosphatidylcholine reporter labeled in the sn-2 acyl chain with NBD4 (7-nitrobenz-2-oxa-1,3-diazol-4-yl). NBD-PC uptake is greater than 80% inhibited in strains in which the LEM3/ROS3 gene is deleted (5, 6). A similar inhibition of NBD-PC internalization is observed in strains deleted in both DNF1 and DNF2 (7). Dnf1p and Dnf2p are members of the P-type ATPase family that couple ATP hydrolysis to the transmembrane transport of a wide spectrum of ions. They belong to a subfamily of P-type ATPases that has been reported to actively transport phospholipids (8, 9). Lem3p encodes an integral membrane protein that is necessary for exit of Dnf1p from the endoplasmic reticulum, suggesting a role as a chaperone but not ruling out a role as a subunit of a functional transporter complex at the plasma membrane (10).

The identification of three proteins that define an internalization pathway for NBD-PC provides the opportunity for the first time to determine whether the structure of the fluorescent reporter group in the acyl chain region affects the mechanism of internalization. With this goal in mind, the plasma membrane flip of NBD-PC was compared with that of phosphatidylcholine molecules differing only in the structure of the Bodipy probe attached to the sn-2 acyl chain in strains deleted for LEM3, DNF1, and DNF2. The results indicated that, dependent on the structure of the fluorescent probe in the acyl chain, phosphatidylcholine molecules were flipped across the plasma membrane by at least two different mechanisms.

NBD-PC flip across the plasma membrane is also inhibited by ATP depletion, nutrient depletion, and collapse of the proton electrochemical gradient across the plasma membrane. Comparison of the plasma membrane flip of the four phosphatidylcholine reporter molecules in response to these perturbations led to the identification of a third flip mechanism dependent on the fluorophore structure. Thus, at least three different mechanisms for phosphatidylcholine flip across the plasma membrane of yeast can be distinguished by altering the structure of the fluorescent moiety in the acyl chain region.

EXPERIMENTAL PROCEDURES

Materials—Yeast medium was obtained from Difco. All other reagents, unless otherwise noted, were purchased from Sigma. NBD-PC was purchased from Avanti Polar Lipids. All other lipids, including Bodipy FL-PC, Bodipy 530-PC, and Bodipy 581-PC, were purchased from Molecular Probes. Stocks of the four fluorescent phospholipids (5 mM) were prepared by solubilization of the dried phospholipids in

* This work was supported by National Institutes of Health Grant GM064770 (to J. W. N.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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4 The abbreviations used are: NBD, 7-nitrobenz-2-oxa-1,3-diazol-4-yl; NBD-PC, 1-myristoyl-2-(4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-pentanoyl)-1-hexadecanoyl-sn-glycero-3-phosphocholine; Bodipy FL-PC, 2-(4,4 difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-pentanoyl)-1-hexadecanoyl-sn-glycero-3-phosphocholine; Bodipy 530-PC, 2-(4,4 difluoro-5-(5,7-diphenyl-4-bora-3a,4a-di aza-s-indacene-3-pentanoyl)-1-hexadecanoyl-sn-glycero-3-phosphocholine; Bodipy 581-PC, 2-(4,4 difluoro-5-(4-phenyl-1,3-buta dienyl)-4-bora-3a,4a-di aza-s-indacene-3-pentanoyl)-1-hexadecanoyl-sn-glycerol-3-phosphocholine; CCP, carbonyl cyanide m-chlorophenylhydrazide; SDC, synthetic complete medium: 0.67% yeast ni trogen base, 2% glucose, complete amino acid supplements; SC-NaNO3, SDC lacking glucose but containing 2% sorbitol and 20 mM NaNO3.
TABLE ONE

| Yeast Strains used in this study | Genotype | Source |
|----------------------------------|----------|--------|
| LMY94                            | MATα leu2 MET15 lys2 ura3 | Research Genetics* |
| LMY102                           | MATα Δhel3 leu2 met15 LYS2 ura3 | Research Genetics* |
| LMY137                           | MATα ENDM4 his4 leu2 ura3 lys2 bar1 | Howard Riezman, Univ. of Basel |
| LMY138                           | MATα end4::LEU2 his4 leu2 ura3 lys2 bar1 | Howard Riezman, Univ. of Basel |
| LMY161                           | MATα DNE1 DNF2 his3 leu2 ura3 lys2 | Todd Graham, Vanderbilt Univ. |
| LMY165                           | MATα Δdnf1 his3 leu2 ura3 lys2 | Todd Graham, Vanderbilt Univ. |
| LMY166                           | MATα Δdnf2 his3 leu2 ura3 lys2 | Todd Graham, Vanderbilt Univ. |
| LMY167                           | MATα Δdnf1Δdnf2 his3 leu2 ura3 lys2 | Todd Graham, Vanderbilt Univ. |

* Haploid strains were produced by sporulation of homozygous diploid strains obtained from Research Genetics, courtesy of Scott Devine.

Dimethyl sulfoxide. Phospholipids were stored at −20 °C and periodically monitored for purity by thin-layer chromatography. Phospholipid concentrations were determined by absorbance in methanol at 466 nm for NBD-PC (ε = 22,000), 503 nm for Bodipy FL-PC (ε = 80,000), 534 nm for Bodipy 530-PC (ε = 64,000), and 582 nm for Bodipy 581-PC (ε = 124,000). Extinction coefficients were provided by Molecular Probes.

Yeast Strains and Culture—The *S. cerevisiae* strains used in this study are shown in TABLE ONE. Unless otherwise noted, cultures were grown in SDC to early log phase (A600 0.2–0.4) from overnight cultures as described (11). Strains used in the examination of growth phase were cultured as described above to varying A600.

Internalization of Phospholipids into Yeast—For analysis of wild-type and deletion strains at physiological temperature, overnight cultures were diluted to 0.1 A600 and grown for several hours to early log phase (A600 0.2–0.4) in SDC at 30 °C. Dimethyl sulfoxide-solubilized lipid was added to a final concentration of 5 μM and vortex mixed. After a 30-min incubation with the phospholipid at 30 °C or a 1-h incubation with the phospholipid at 2 °C, cells were harvested and washed three times with ice-cold SC-NaNO3. Cells were analyzed using fluorescence microscopy and/or flow cytometry.

For analysis of ATP-depleted parent strains, cultures were grown as described above. Immediately prior to labeling, cells were harvested, resuspended in SC-NaNO3, and incubated for 10 min at 30 °C. Labeling was then performed at 2 °C as described above. Collapse of the plasma membrane proton electrochemical gradient was accomplished by incubation of the cells in 0, 1, 5, 20, or 50 μM CCCP in SDC for 10 min at 30 °C preceding labeling at 2 °C. After labeling with the fluorescent phospholipids, washed cells were incubated for 15 min with 1 μg/ml 4’,6-diamidino-2-phenylindole to label mitochondrial and nuclear DNA prior to imaging by fluorescence microscopy.

The percentage of lipid remaining intact after internalization was determined for each strain and each fluorescent phospholipid used in this study via analysis of TLC-separated cell extracts following labeling. Quantification of fluorescent products present in the extracts revealed greater than 80% of fluorescence was from intact lipid for all fluorescently labeled phosphatidylcholines (PCs) in all strains.

Flow Cytometry—Flow cytometric analysis of the phospholipid-labeled cells was performed with a FACSCalibur cytometer (BD Biosciences). For all analyses, 10 μl of a 75-μM stock solution of propidium iodide was added to ~4 × 106 cells in 1 ml of SC-NaNO3 immediately prior to dilution (~3-fold) and flow cytometric analysis on a cytometer equipped with an argon laser operating at 488 nm. Ten thousand cells were analyzed without gating during the acquisition. Analysis was performed with CellQuest (BD Biosciences) software. A dot plot of forward scatter versus the red fluorescence channel (propidium iodide) was used to set a gate that excluded dead cells from the analysis. The percentage of excluded dead cells was <5% during analysis of untreated mutant and parent strains. This percentage increased to a maximum of 15% in those experiments involving ATP depletion, CCCP treatment, and high density. Channels for analysis of each fluorophore-conjugated lipid were chosen based on the maximum wavelengths of emission for each fluorophore.

Fluorescence Microscopy—Fluorescence microscopy was performed on a Zeiss Axiovert 135 microscope equipped with barrier filters allowing for detection of each probe. Filter sets were obtained from Chroma Technology Corp. A multiband dichroic mirror (86000 BS) was used with the filter combinations exciter S490/20 and emitter S528/38 for NBD and Bodipy FL and exciter S555/28 and emitter S617/73 for Bodipy 530 and Bodipy 581. The fluorescence images were captured with a Qimaging Retiga Exi CCD camera and analyzed using Meta morph software (Universal Imaging). The mean pixel intensity for each cell region was calculated and corrected for background fluorescence of a region containing no cells. Data from each independent experiment represent the average pixel intensity of no less than 30 cells.

Although analysis by flow cytometry is quantitatively more accurate, microscopic pixel analysis was necessary to quantify the internalization of Bodipy 581-PC because of the lack of a suitable laser for its excitation. The remaining three fluorescent PC molecules were analyzed by both methods to demonstrate the degree of reproducibility between the
methods. In all cases, the qualitative differences in cell-associated fluorescence observed between strains and treatment conditions were maintained.

RESULTS

The Bodipy and NBD Phosphatidylcholine Analogs Are Distributed to Different Intracellular Organelles—In previous work, we demonstrated that at 30 °C NBD-PC is internalized primarily by flip and trafficked to the lumen of the vacuole via the prevacuolar compartment (12) where it is degraded by vacuolar hydrolases (13). To determine whether phosphatidylcholine molecules with different fluorophores attached to the acyl chain were trafficked to the vacuole in a similar manner, we labeled cells at 30 °C with three different Bodipy fluorophores (Bodipy 581-PC, Bodipy FL-PC, and Bodipy 530-PC; see Fig. 1 for structures) and observed their distribution within the cells by fluorescence microscopy (Fig. 2). NBD-PC was the only fluorescent-tagged phosphatidylcholine molecule that was trafficked to the vacuole. Bodipy 581-PC localized primarily to the mitochondria, as shown by co-localization with the 4′,6-diamidino-2-phenylindole stained mitochondrial DNA. Bodipy FL-PC- and Bodipy 530-PC-labeled membranes surrounding 4′,6-diamidino-2-phenylindole stained nuclear DNA, indicative of nuclear/endoplasmic reticulum membranes, as well as additional unidentified membranous organelles. The various distribution patterns observed for the Bodipy-labeled phosphatidylcholine reporter molecules are unlikely to be caused by differences in degradation because >85% remained intact as determined by quantification of TLC-separated cell extracts following labeling (data not shown).

At 30 °C, NBD-PC is internalized primarily by flip (inward-directed transport) across the plasma membrane based on the observation that its internalization is unaffected in strains in which endocytosis was blocked by the deletion of END4 (14). Similar results were observed for the three Bodipy-labeled phosphatidylcholine analogs. Deletion of END4 had little or no effect on the 30 °C internalization and localization of each of the three Bodipy-labeled phosphatidylcholine molecules (data not shown), indicating that, as with NBD-PC, internalization of the Bodipy-labeled phosphatidylcholine molecules is not dependent on endocytosis and thus is internalized primarily by flip across the plasma membrane. These data indicate that all four of the phosphatidylcholine reporter molecules are internalized predominantly by flip across the plasma membrane and that the structure of the fluorescence moiety attached to the acyl chain is sufficient to alter their partitioning and/or trafficking from the inner leaflet of the plasma membrane to the other membranous organelles of the cell. The work reported below will focus on the mechanism of flip and address specifically whether the Bodipy-labeled phosphatidylcholine molecules are flipped by the same mechanism as identified previously for NBD-PC. The molecular details underlying the observed differential localization patterns for the fluorescent reporter molecules will be addressed in future investigations.

LEM3 Is Required for Flip of NBD-PC and Bodipy FL-PC, but Not for Bodipy 581-PC and Bodipy 530-PC—In our previous work, we demonstrated that the net accumulation of NBD-PC in cells labeled at 30 °C is determined by the rates of both inward-directed (flip) and outward-directed (flop) transport across the plasma membrane (15, 16). To determine the effect of gene deletions on the rate of flip in the absence of flop, the net accumulation of each of the fluorescent phosphatidylcholine molecules was measured at 2 °C (Figs. 3A and 4A, left panels). At

![FIGURE 2. Fluorescently labeled phosphatidylcholine analogs localize to different intracellular regions at 30 °C. Differential interference contrast and fluorescence microscopy images of parent S. cerevisiae strain LMY94 labeled with NBD-PC, Bodipy FL-PC, Bodipy 530-PC, and Bodipy 581-PC at 30 °C as described under “Experimental Procedures.” Bar in bottom right frame represents 5 microns.

![FIGURE 3. Comparison of the effect of LEM3 deletion on low temperature flip of the four fluorescently labeled phosphatidylcholine analogs. Fluorescence microscopy images (A) and quantification of fluorescence by mean pixel intensity analysis (B) and flow cytometry (C) of LEM3 parent strain LMY94 and Δlem3 strain LMY102 as described under “Experimental Procedures.” Data are shown as a percentage of fluorescence in the parent strain. Data presented in graphs represent the average of three independent experiments. Error bars represent the S.D. between experiments. Bar in bottom right frame of panel A represents 5 microns.](image-url)
Phosphatidylcholine Label Determines Flip Pathway

FIGURE 4. Comparison of the effect of DNF1 and DNF2 deletion on low temperature flip of the four fluorescently labeled phosphatidylcholine analogs. Fluorescence microscopy images (A) and quantification of fluorescence by mean pixel intensity analysis (B) and flow cytometry (C) of DNF1 DNF2 parent strain LMY 161, ∆dnf1 strain LMY165, ∆dnf2 strain LMY166, and ∆dnf1Δdnf2 strain LMY167 as described under "Experimental Procedures." Data are shown as a percentage of fluorescence in the parent strain. Data presented in graphs represent the average of three independent experiments. Error bars represent the S.D. between experiments. Bar in bottom right frame of panel A represents 5 microns.

this temperature, the rate of flip for NBD-PC (16) and each of the Bodipy-labeled phosphatidylcholine molecules (data not shown) is reduced to negligible levels. Furthermore, because endocytosis and vesicle budding and fusion are also blocked at 2 °C (14, 17–19), the net accumulation of each of the fluorescent phosphatidylcholine molecules at this temperature reflects the rate of flip in the absence of significant contributions from flip or endocytosis.

Based on the previous observation that deletion of LEM3 inhibits flip of NBD-PC by >80%, we tested whether deletion of LEM3 had a similar effect on flip of the three Bodipy-labeled phosphatidylcholine molecules. Thus, a ∆lem3 strain (LMY102) and its isogenic LEM3 parent strain (LMY94) were labeled with each of the fluorescent phosphatidylcholine molecules at 2 °C. Visualization of the fluorescence images and quantification by image analysis and flow cytometry (Fig. 3) confirmed the expected reduction in NBD-PC flip in the ∆lem3 strain (6). Deletion of LEM3 decreased flip of Bodipy FL-PC in a manner similar to NBD-PC, whereas flip of Bodipy 581-PC and Bodipy 530-PC was actually slightly increased. These data indicate that Bodipy 581-PC and Bodipy 530-PC are internalized by a flip mechanism that is independent of the expression of the LEm3 protein. Because each of these fluorescent phospholipids has the identical glycerophosphorylcholine backbone and head group, these data demonstrate that the selectivity for the flip mechanism is determined by the structure of the acyl chain and the attached fluorophore in addition to that of the head group.

DNF1 and DNF2 Are Required for Flip of NBD-PC, but Not Bodipy 581-PC or Bodipy 530-PC—Because deletion of DNF1 and DNF2, alone and in combination, has been shown to reduce the low temperature flip of NBD-PC (7), it was of interest to determine whether flip of the three Bodipy-labeled phosphatidylcholine molecules was similarly dependent on their expression. We measured the low temperature flip of the four fluorescent phosphatidylcholine reporters in strains deleted in DNF1 (LMY165), DNF2 (LMY166), and the two combined (LMY167). As expected from the results of Pomorski et al. (7), flip of NBD-PC was slightly reduced, if at all, in the ∆dnf1 strain, reduced ~50% in the ∆dnf2 strain, and reduced ~80% in the double deletion ∆dnf1Δdnf2 strain (Fig. 4). On the other hand, flip of Bodipy 530-PC is slightly inhibited in both ∆dnf1 and ∆dnf2 single deletion strains but significantly increased in the double deletion ∆dnf1Δdnf2 strain. A similar increase in Bodipy 581-PC flip was observed in the ∆dnf1Δdnf2 strain, although little or no difference was observed between the parent and single deletion strains. These data indicate that contrary to flip of NBD-PC, flip of Bodipy 530-PC and Bodipy 581-PC does not require the expression of Dnf1p and Dnf2p. In fact, it is actually increased in their absence. This finding suggests that the deletion of DNF1 and DNF2 results in the up-regulation of an alternative mechanism(s) that is/are responsible for flip of Bodipy 581-PC and Bodipy 530-PC.

Quantitative data in Fig. 4, B and C, indicate that the amount of Bodipy FL-PC taken into the cell is only slightly reduced in the ∆dnf1Δdnf2 strain. However, microscopic images shown in Fig. 4A illustrate that the cell-associated fluorescence is accumulated at the plasma membrane as opposed to internal membranes seen in the parent and single deletion mutant strains. Based on the observed partial inhibition of Bodipy FL-PC flip in both of the single dnf null mutants, the appearance of plasma membrane labeling in the ∆dnf1Δdnf2 strain suggests a complete block of flip resulting in accumulation in the outer leaflet of the plasma membrane. However, one cannot rule out the possibility that in the double deletion strain, the Bodipy FL-PC is flipped to the inner leaflet of the plasma membrane but its transfer to internal membranes is blocked. Further experiments will be required to distinguish between these two possibilities.

Fluorescently Labeled Phosphatidylcholine Analogs Differ in Their Response to ATP Depletion—Depletion of cellular ATP stores has been shown previously to inhibit the low temperature flip of NBD-PC by ~80% (14). In an effort to characterize further the putative alternative flip pathways identified for the Bodipy-labeled phosphatidylcholine analogs, we tested the effect of ATP depletion on their low temperature flip. Cells were labeled with each of the fluorescent phosphatidylcholine analogs following ATP depletion by incubation in glucose-deficient medium containing 10 mM NaN3 (see "Experimental Procedures" for
Phosphatidylcholine Label Determines Flip Pathway

FIGURE 5. The effect of ATP depletion on low temperature flip of phosphatidylcholine depends on the fluorophore. Fluorescence microscopy images (A) and quantification of fluorescence by mean pixel intensity analysis (B) and flow cytometry (C) of parent strain LMY94 with and without ATP depletion by azide treatment as described under "Experimental Procedures." Data are shown as a percentage of fluorescence of cells with no azide treatment. Data presented in graphs represent the average of three independent experiments. Error bars represent the S.D. between experiments. Bar in bottom right frame of panel A represents 5 microns.

FIGURE 6. Collapse of the proton electrochemical gradient by CCCP treatment inhibits low temperature flip. Parent strain LMY94 was treated with various concentrations of CCCP before labeling at 2 °C as described under "Experimental Procedures." Data are shown as a percentage of fluorescence relative to no CCCP treatment. Fluorescence measurements were acquired using flow cytometry for NBD-PC, Bodipy FL-PC, and Bodipy 530-PC and mean pixel intensity as determined by microscopy for Bodipy 581-PC. Data presented in graphs represent the average of three independent experiments. Error bars represent the S.D. between experiments.

Culture Growth Past Early Log Phase Reduces Flip of All Lipids Except Bodipy 581-PC—Flip of NBD-PC is at its most active in early log phase cultures, and the rate steadily declines as the culture density increases and the culture medium is depleted of nutrients (16). To determine whether low temperature flips of the Bodipy-labeled phosphatidylcholine analogs were affected similarly, we measured their flip in cells at various times following inoculation into fresh SDC medium (see “Experimental Procedures” for details). Plots of low temperature flip versus growth phase represented by A600 of the cultures (Fig. 7) revealed three different patterns of regulation depending on the structure of the fluorophore. Flip of NBD-PC and Bodipy FL-PC declined steadily with increasing culture density, whereas Bodipy 581-PC flip was independent of culture density over the range studied. Flip of Bodipy 530-PC, however, exhibited a biphasic response. Its flip was initially reduced, followed by a recovery to maximum rates at higher culture density. These data provide additional support for the identification of three different mechanisms for low temperature flip depending on the structure of the fluorophore.

7 H. C. Stevens and J. W. Nichols, unpublished observations.

details). This treatment reduces ATP levels by 80% (13). Both the fluorescent images and quantitative data concur that ATP depletion substantially reduces the rate of NBD-PC and Bodipy FL-PC flip to <40% of normal (Fig. 5). The low temperature flip of Bodipy 581-PC is slightly inhibited by ATP depletion, whereas that of Bodipy 530-PC is surprisingly increased. The increase in low temperature flip of Bodipy 530-PC following ATP depletion suggests yet a third mechanism of transport that is specific for Bodipy 530-PC relative to the other phosphatidylcholine analogs and is inhibited or down-regulated in the presence of normal levels of ATP.

The Plasma Membrane Proton Electrochemical Gradient Is Required for Normal Flip for Each of the Four Phosphatidylcholine Analogs—Incubation of yeast cells with the protonophore CCCP in a concentration sufficient to collapse the proton electrochemical gradient across the plasma membrane almost completely blocks the low temperature flip of NBD-PC (16). Because yeasts grown in glucose do not depend on oxidative phosphorylation to produce ATP, it is possible to collapse the proton electrochemical gradient across the plasma membrane without substantially depleting cellular ATP levels (20). Thus, the proton electrochemical gradient is required for NBD-PC flip even in the presence of ATP. Given this requirement for NBD-PC flip, it was of interest to determine whether the Bodipy-labeled phosphatidylcholine analogs were similarly dependent on the plasma membrane proton electrochemical gradient. The effect of increasing concentrations of CCCP on the low temperature flip of each of the fluorescent phosphatidylcholine analogs was measured (Fig. 6). CCCP treatment reduced flip of all four of the fluorescent phosphatidylcholine analogs without affecting the pattern of intracellular localization (data not shown). NBD-PC and Bodipy 581-PC flip was inhibited by ~90% at 50 μM CCCP, whereas that of Bodipy FL-PC and Bodipy 530-PC was affected to a lesser extent (40–50%). These data indicate that flip of all four of the fluorescent phosphatidylcholine analogs is at least partially dependent on the proton electrochemical gradient across the plasma membrane. The partial inhibition observed for Bodipy FL-PC and Bodipy 530-PC suggests an alternative pathway that is independent of the proton electrochemical driving force.
Phospholipid Label Determines Flip Pathway

We have shown that at the physiological temperature of 30 °C only NBD-PC is transported to the vacuole for degradation, whereas each of the Bodipy-labeled phosphatidylcholine analogs is internalized to a different extent. Interestingly, flip of the other two Bodipy analogs was actually reduced, although not to the same extent. Deletion of the other two Bodipy analogs was actually increased relative to the isogenic parent strain. Thus, Bodipy 530-PC and Bodipy 581-PC are not dependent on the expression of Lem3p to localize to the plasma membrane, suggesting Bodipy FL-PC flips across the plasma membrane. In addition, the observed increase in flip in the Δlem3 strain suggests the up-regulation of an alternative flip pathway that selectively transports Bodipy 530-PC and Bodipy 581-PC, but not Bodipy FL-PC or NBD-PC.

Comparison of the low temperature flip of the four phosphatidylcholine analogs in strains deleted in both DNF1 and DNF2 provides further support for a second selective flip pathway. Deletion of DNF1 and DNF2 dramatically inhibited the low temperature flip of NBD-PC as shown previously (7). However, flip of Bodipy 530-PC and Bodipy 581-PC was increased in this strain. As a result of the accumulation of Bodipy FL-PC at the plasma membrane in the Δdnf1Δdnf2 strain, the cell-associated fluorescence measured by flow cytometry and pixel analysis does not appear to be significantly reduced. However, the fluorescence is primarily localized to the plasma membrane, suggesting Bodipy FL-PC flips across the plasma membrane or its subsequent delivery to intracellular membranes is blocked in the absence of Dnf1p and Dnf2p expression.

Summarizing the genetic perturbation experiments, the data demonstrated the presence of at least two pathways for low temperature flip that almost exclusively distinguish NBD-PC as substrate from Bodipy 530-PC and Bodipy 581-PC. Bodipy FL-PC appears to be recognized to some extent by both pathways based on the partial inhibition in the Δlem3 strain and the appearance of plasma membrane staining in the Δdnf1Δdnf2 strain.

Distinction of a third putative selective flip pathway was identified from the effect of biochemical perturbations on the low temperature flip of the four phosphatidylcholine analogs. Separate mechanisms for the low temperature flip of Bodipy 530-PC and Bodipy 581-PC can be distinguished by the effect of ATP depletion and culture density (Figs. 5 and 7). Depletion of cellular ATP by treatment with NaN3 modestly reduced the low temperature flip of Bodipy 581-PC, whereas that of Bodipy 530-PC actually increased. In addition, flip of Bodipy 581-PC was unaffected by culture density, whereas that of Bodipy 530-PC was regulated in a biphasic pattern, early inhibition followed by a return to starting values. Bodipy FL-PC flip decreased steadily with increasing culture density in a manner observed previously for NBD-PC (16).

Flip of all four phosphatidylcholine analogs was shown to be partially inhibited by collapse of the proton electrochemical gradient across the plasma membrane with CCCP.

**TABLE TWO**

Summary of effectors of fluorescent phosphatidylcholine flip

| Effectors | PMF Depletion | ATP Depletion | Growth Past Early Log Phase |
|-----------|--------------|--------------|-----------------------------|
| Δlem3     | Δdnf1/2      | Δdnf1/2      | Δdnf1/2                     |
| NBD-PC    | □            | □            | □                           |
| Bodipy FL-PC | □        | □            | □                           |
| Bodipy 530-PC | □       | □            | □                           |
| Bodipy 581-PC | □       | □            | □                           |

* Size and direction of arrows reflect the relative effect of perturbation on fluorescent-labeled phosphatidylcholine internalization at 2 °C.

DISCUSSION

Data presented here provide the first demonstration that the acyl chain moiety of phosphatidylcholine molecules can determine the pathway by which a phospholipid molecule is transported in the cell. We have shown that at the physiological temperature of 30 °C only NBD-PC is transported to the vacuole for degradation, whereas each of the Bodipy-labeled phosphatidylcholine analogs is internalized to a different combination of intracellular membranes (Fig. 2). The observation that the extent of internalization and intracellular distribution of the four fluorescent phosphatidylcholine analogs is unaffected in strains in which endocytosis is blocked by the deletion of END4 (data not shown) suggests that each of the phosphatidylcholine analogs is internalized by flip across the plasma membrane. These data demonstrate that the structure of the fluorophore attached to the acyl chain of a phosphatidylcholine molecule is sufficient to produce the differential distribution between the intracellular membranes observed for the four labeled phosphatidylcholine molecules. The fluorophore likely alters some combination of the following: the pathway for flip, the pathway for flop, the mechanism of intracellular trafficking, or the spontaneous transport and partitioning between membranes.

To focus on the mechanism of flip across the plasma membrane, internalization of the four phosphatidylcholine analogs was studied at 2 °C. At this temperature, not only is endocytosis blocked but efflux (flop) across the plasma membrane is also blocked (see Ref. 16 for NBD-PC; data not shown for Bodipy analogs). Thus, the effect of genetic and biochemical perturbations on the mechanism of flip could be studied in isolation. At this temperature, each of the phosphatidylcholine analogs was flipped across the plasma membrane and differentially distributed among the intracellular membranes (Figs. 3A and 4A). The relative effects of each of the experimental parameters on the low temperature flip of the four phosphatidylcholine analogs are summarized in TABLE TWO.

As shown previously (5, 6), deletion of LEM3 significantly inhibited the low temperature flip of NBD-PC (Fig. 3). Low temperature flip of Bodipy FL-PC was also significantly inhibited, although not to the same extent. Interestingly, flip of the other two Bodipy analogs was actually increased relative to the isogenic parent strain. Thus, Bodipy 530-PC and Bodipy 581-PC are not dependent on the expression of Lem3p to flip across the plasma membrane. In addition, the observed increase in flip in the Δlem3 strain suggests the up-regulation of an alternative flip pathway that selectively transports Bodipy 530-PC and Bodipy 581-PC, but not Bodipy FL-PC or NBD-PC.

Comparison of the low temperature flip of the four phosphatidylcholine analogs in strains deleted in both DNF1 and DNF2 provides further support for a second selective flip pathway. Deletion of DNF1 and DNF2 dramatically inhibited the low temperature flip of NBD-PC as shown previously (7). However, flip of Bodipy 530-PC and Bodipy 581-PC was increased in this strain. As a result of the accumulation of Bodipy FL-PC at the plasma membrane in the Δdnf1Δdnf2 strain, the cell-associated fluorescence measured by flow cytometry and pixel analysis does not appear to be significantly reduced. However, the fluorescence is primarily localized to the plasma membrane, suggesting Bodipy FL-PC flips across the plasma membrane or its subsequent delivery to intracellular membranes is blocked in the absence of Dnf1p and Dnf2p expression.

Summarizing the genetic perturbation experiments, the data demonstrated the presence of at least two pathways for low temperature flip that almost exclusively distinguish NBD-PC as substrate from Bodipy 530-PC and Bodipy 581-PC. Bodipy FL-PC appears to be recognized to some extent by both pathways based on the partial inhibition in the Δlem3 strain and the appearance of plasma membrane staining in the Δdnf1Δdnf2 strain.

Distinction of a third putative selective flip pathway was identified from the effect of biochemical perturbations on the low temperature flip of the four phosphatidylcholine analogs. Separate mechanisms for the low temperature flip of Bodipy 530-PC and Bodipy 581-PC can be distinguished by the effect of ATP depletion and culture density (Figs. 5 and 7). Depletion of cellular ATP by treatment with NaN3 modestly reduced the low temperature flip of Bodipy 581-PC, whereas that of Bodipy 530-PC actually increased. In addition, flip of Bodipy 581-PC was unaffected by culture density, whereas that of Bodipy 530-PC was regulated in a biphasic pattern, early inhibition followed by a return to starting values. Bodipy FL-PC flip decreased steadily with increasing culture density in a manner observed previously for NBD-PC (16).

Flip of all four phosphatidylcholine analogs was shown to be partially inhibited by collapse of the proton electrochemical gradient across the plasma membrane with CCCP. At the highest concentration of CCCP...
tested (50 μM), NBD-PC and Bodipy 581-PC were inhibited from 80 to 90% whereas Bodipy FL-PC and Bodipy 530-PC were inhibited 40–50%. The difference in the effect of Bodipy 530-PC and Bodipy 581-PC provides further support for a third flip pathway that distinguishes between these two analogs.

The observations presented and discussed above raise several interesting questions worthy of further investigation. Although the data indicate that more than one pathway for flip of phosphatidylcholine analogs exists, the nature of these pathways has yet to be determined. It is possible that the flip pathways that are independent of the expression of LEM3, DNF1, and DNF2 are the result of membrane perturbations, either the loss of transbilayer asymmetry or changes in membrane composition, that allow for the spontaneous, protein-independent movement across the plasma membrane. Although this possibility cannot be ruled out, observations that Bodipy 530-PC and Bodipy 581-PC respond differently to the depletion of cellular ATP and culture density are not easily reconciled with a spontaneous leak pathway. A more likely interpretation is that the flip pathways that are independent of LEM3, DNF1, and DNF2 result from the regulated expression of additional protein-dependent flip mechanisms. In this case, the expected membrane perturbations resulting from the loss of expression of LEM3, DNF1, and DNF2 may be the signal for the increased expression of genes encoding additional flippases with different specificities for the acyl chain region.

This type of regulation has already been suggested for Rsb1p, a protein first identified as being involved in the translocation of sphingoid long-chain bases (21). Overexpression of Rsb1p has also recently been shown to increase flip and decrease flop of NBD-PC and NBD-labeled phosphatidylethanolamine in yeast (21). In addition, expression of Rsb1p was increased in both Δlem3 and Δdnf1Δdnf2 strains (21). These data demonstrate that expression of proteins involved in lipid translocation can be altered by the absence of other lipid translocation proteins. Further experimentation is needed to determine whether Rsb1p is acting in a lipid translocation pathway independent of Lem3p and Dnf1p/Dnf2p, as well as whether the Rsb1p pathway contributes to flip of Bodipy 581-PC and/or Bodipy 530-PC.

These data raise the question of what structural and/or physical-chemical properties of the phosphatidylcholine analogs determine the different mechanisms of low temperature flip. It is possible that the three different flip mechanisms reflect the presence of three different transporters that recognize the fluorescent groups attached to the phosphatidylcholine molecules. In this case, the transporter would recognize the fluorophore and the remainder of the phospholipid molecule would play a minor role in the recognition and/or transport process. This possibility can be ruled out for NBD- and Bodipy FL-labeled phospholipids and sphingolipids because the head group has been shown to be a major determinant of the mechanism of internalization regardless of the structure of the fluorophore (1, 3, 4). Similar experiments determining the head group dependence of phospholipids labeled in the acyl chain with Bodipy 530 and Bodipy 581 have not been performed, and therefore primary recognition of the structure of Bodipy 530 and Bodipy 581 by different transporters cannot be excluded. However, based on the NBD and Bodipy FL results, it seems more likely that either the recognition by and/or accessibility to the appropriate flipase is determined by the physical-chemical properties of the fluorescent groups (e.g., hydrophobicity and size). Different flippases may be required to transport phosphatidylcholine molecules that differ in the hydrophobicity of the acyl chains. For example, phosphatidylcholine molecules with shorter, more unsaturated acyl chains may be recognized by a different flipase than those with long saturated chains. On the other hand, the physical-chemical properties may determine accessibility to different flippases by favoring partitioning into different microdomains within the plasma membrane. Flippases may differentially partition into these microdomains such that they only flip phosphatidylcholine molecules with access to the same microdomain. For example, if certain fluorescently labeled phosphatidylcholine analogs preferentially partition into the more ordered “lipid raft” type domains, they would have access to lipid transporters present in these domains. In this case, these lipids may be more accurately representing what occurs with endogenous long chain, di-saturated species of phosphatidylcholine. Likewise, fluorescent phosphatidylcholine analogs that partition into the unordered phase would have access to another set of transporters not present in the ordered phase of the membrane. These probes may therefore be representing endogenous species of lipids that are more likely to partition into the unordered phase, such as di-unsaturated species.

The differential partitioning of NBD-PC and Bodipy FL-PC has previously been examined and supports the hypothesis that the fluorophore can affect partitioning into membrane phases (22). In these experiments it was shown that NBD-PC has a 2-fold higher affinity for vesicles containing 1-palmitoyl-2-oleoyl-phosphatidylcholine/cholesterol over vesicles containing sphingomyelin/cholesterol relative to Bodipy FL-PC, although both phospholipids preferred unordered membrane domains. Based on their structures, the differences in relative affinities seen between NBD-PC and Bodipy FL-PC may be even further exaggerated when comparing NBD-PC to the other, more bulky, and hydrophobic Bodipy 530 and Bodipy 581 groups used in this study. Further investigation will be needed to determine whether all the probes used in our study partition differentially into ordered and unordered domains, as well as whether particular proteins involved in lipid translocation are present in these domains.

This study demonstrates that the nature of the fluorescent group attached to a phosphatidylcholine molecule has a profound effect on its intracellular trafficking as well as the mechanism by which it is flipped across the plasma membrane. The putative identification of two additional mechanisms for phosphatidylcholine flip that are independent of LEM3, DNF1, and DNF2 expression suggests that the regulation of phospholipid transport and asymmetry is more complex than previously appreciated. These studies provide the basis for the identification of additional flip mechanisms as well as the tools required to uncover the physical-chemical properties of the acyl chain region of phosphatidylcholine species that determine their differential trafficking in cells.

Acknowledgment—We thank Lynn Malone for expert technical assistance.

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