Lem3p Is Essential for the Uptake and Potency of Alkylphosphocholine Drugs, Edelfosine and Miltefosine*

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The alkylphosphocholine class of drugs, including edelfosine and miltefosine, has recently shown promise in the treatment of protozoal and fungal diseases, most notably, leishmaniasis. One of the major barriers to successful treatment of these infections is the development of drug resistance. To understand better the mechanisms underlying the development of drug resistance, we performed a combined mutant selection and screen in Saccharomyces cerevisiae, designed to identify genes that confer resistance to the alkylphosphocholine drugs by inhibiting their transport across the plasma membrane. Mutagenized cells were first selected for resistance to edelfosine, and the initial collection of mutants was screened a second time for defects in internalization of a short chain, fluorescent (7-nitrobenz-2-oxa-1,3-diazol-4-yl (NBD))-labeled phosphatidylcholine reporter. This approach identified mutations in a single gene, YNL323W/LEM3, that conferred resistance to alkylphosphocholine drugs and inhibited internalization of NBD-labeled phosphatidylcholine. Loss of YNL323W/LEM3 does not confer resistance to N-nitroquinoline N-oxide or ketoconazol and actually increases sensitivity to cycloheximide. The defect in internalization is specific to NBD-labeled phosphatidylcholine and phosphatidylethanolamine. Labeled phosphatidylserine is internalized at normal levels in lem3 strains. LEM3 is a member of an evolutionarily conserved family and has two homologs in S. cerevisiae. Single point mutations that produce resistance to alkylphosphocholine drugs and inhibition of NBD-labeled phosphatidylcholine internalization were identified in several highly conserved domains. These data demonstrate a requirement for Lem3p expression for normal phosphatidylcholine and alkylphosphocholine drug transport across the plasma membrane of yeast.

The alkylphosphocholine class of drugs, originally developed for their anti-cancer activities, has shown great promise in the treatment of parasitic infections including those caused by Leishmania spp., Trypanosoma cruzi, and Trypanosoma brucei (1, 2). In particular, in vitro and in vivo studies in animals have shown miltefosine (hexadecylphosphocholine) and edelfosine, also known as rac-1-octadecyl-2-methoxy-glycerol-3-phosphocholine, to be effective killers of Leishmania in both promastigote and amastigote morphological stages (3, 4). Miltefosine was recently approved as the first orally administered drug for the treatment of leishmaniasis based on several highly successful clinical trials (5–9). Although no consensus has emerged regarding the mode of action of these drugs in cancer cells or in parasites, several likely intracellular targets related to signal transduction and lipid biosynthesis have been identified. In cancer cells, edelfosine is a potent inducer of apoptosis (10, 11). Although the alkyl-specific acyl-CoA acyltransferase enzyme required for remodeling of acyl- and alkylglycerophospholipids has been identified as a potential target for edelfosine and miltefosine in Leishmania (12), a consensus for the primary mode of action has not emerged.

Regardless of whether one or numerous targets are responsible for the efficacy of this class of drugs, all of the known targets appear to require its internalization. Thus, differences in the net drug accumulation resulting from regulation of the influx and/or efflux pathways may account for the differential sensitivity observed among cancer cells as well as strains of Leishmania (13). For example, overexpression of a P-glycoprotein-like transporter in Leishmania tropica confers resistance to miltefosine and edelfosine as well as other drugs (14). Alternatively, increased or decreased drug influx may determine the sensitivity of a particular cell type or strain to the alkylphosphocholine drugs and may provide a mechanism for the development of resistance. Apart from the evidence presented herein, the influx mechanism for either edelfosine or miltefosine is not known for any organism.

Although it is well established that the aminophospholipids phosphatidylserine and phosphatidylethanolamine are transported from the outer to inner leaflet of the plasma membrane in a wide range of cells (15, 16), similar transport of phosphocholine lipid analogues is limited to a few cell types. Initial studies of the internalization of short chain reporter phosphatidylcholine analogues led to the understanding that the inward-directed transport of phosphatidylcholine across the plasma membrane was insignificant relative to its internalization by endocytosis. However, more recent data imply that inward-directed, transbilayer transport of short chain reporter phosphatidylcholine analogues is a feature of particular cell types. For example, transformation of human lung fibroblasts (WI-38 cells) with the SV40 virus (VA-2 cells) was shown to substantially increase the fraction of fluorescent-labeled phosphatidylcholine (7-nitrobenz-2-oxa-1,3-diazol-4-yl (NBD)-PC)1 transported from the outer to inner leaflet of the plasma membrane.

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1 The abbreviations used are: NBD, 7-nitrobenz-2-oxa-1,3-diazol-4-yl; PC, phosphatidylcholine; M-C16-NBD-PC, 1-myristoyl-2(6-NBD-amino-caproyl)-phosphatidylcholine; M-C16-NBD-PE, 1-myristoyl-2(6-NBD-amino-caproyl)-phosphatidylethanolamine; P-C16-NBD-PS, 1-palmitoyl-2(6-NBD-aminocaproyl)-phosphatidylserine; GFP, green fluorescence protein; ER, endoplasmic reticulum; PDR, pleiotropic drug resistance; 4-NQO, N-nitroquinoline N-oxide.
layer transport relative to endocytosis (17). More recently, inward-directed phosphatidylcholine transbilayer transport, monitored with a spin-labeled analogue, was observed in both the apical and basolateral membranes of the dog kidney epithelial cell line, MDCK II (18). However, this is not characteristic of all epithelial cell lines since in the same study, phosphatidylcholine transbilayer transport was not observed in the human colon epithelial cell line, Caco-2. Inward-directed transbilayer transport of fluorescent-labeled phosphatidylcholine has also been demonstrated in the yeast, Saccharomyces cerevisiae (19–21). In this case, phosphatidylcholine transport is dependent on the proton electrochemical gradient and is downregulated by nutrient starvation (21). Gain-of-function mutations in the pleiotropic drug resistance transcription factors, regulated by nutrient starvation (21). Gain-of-function mutations in the phosphatidylcholine transport is inhibited when mutated, confer resistance to alkylphosphocholine drugs by inhibiting their transport across the plasma membrane (21). These studies suggest that the transbilayer transport of phosphatidylcholine and its analogues is associated with the regulation of cell growth and drug resistance.

In this manuscript we present the results of a combined mutant selection and screen designed to identify genes that, when mutated, confer resistance to alkylphosphocholine drugs by inhibiting their transport across the plasma membrane. Mutagenized cells were first selected for resistance to edelfosine, and the initial collection of mutants was screened a second time for defects in internalization of the short chain, fluorescent labeled phosphatidylcholine reporter (1-myristoyl-2-(6-NBD-aminocaproyl))-phosphatidylcholine (M-C₆-NBD-PC). This approach identified mutations in a single gene, YNL323W/LEM3/BRE3/ROS3, that conferred resistance to both ether lipids and inhibited internalization of NB-D-PC. This gene was initially identified as a ligand-effect modulator (LEM3) in a screen for mutants that increased the dexamethasone-dependent activation of a glucocorticoid response element fused to a reporter gene (22). The alias BRE3 was identified in a screen of deletion strains that have increased sensitivity to brefeldin A, a blocker of intracellular transport and membrane biogenesis (23). More recently, this gene was identified in a screen for mutations that confer resistance to the drug Ro09-0198. Ro09-0198 is a tricyclic peptide antibiotic that causes cell lysis upon binding to phosphatidylethanolamine in the outer leaflet of the plasma membrane. Deletion and truncation mutants of LEM3 produced increased sensitivity to Ro09-0198 presumably by increasing the amount of phosphatidylethanolamine exposed to the exoplasmic surface. This conclusion was based in part on the observation that lem3 strains were significantly impaired in their ability to internalize NB-D-PE by flip. These data combined infer a significant role for Lem3p in the transbilayer translocation of phosphatidylethanolamine and phosphatidylethanolamolamine as well as the alkylphosphocholine drugs across the plasma membrane.

**EXPERIMENTAL PROCEDURES**

**Materials**—Yeast media was obtained from Difco. All other reagents, unless otherwise noted, were purchased from Sigma. NB-D-PC labeled phospholipids M-C₆-NBD-PC, 1-myristoyl-2-(6-NBD-aminocaprooyl)-phosphatidylethanolamine (M-C₆-NBD-PE), 1-myristoyl-2-(6-NBD-acyclopropl)-phosphatidic acid, 1-palmitoyl-2-(6-NBD-acyclopropl)-phosphatidylethanolamine (P-C₆-NBD-PS), and edelfosine (also known as 1-O-octadecyl-2-O-methyl-α-glycero-3-phosphocholine) were purchased from Avanti Polar Lipids (Alabaster, AL). Miltefosine (hexadecylphosphocholine) and cycloheximide were purchased from Sigma-Aldrich. Phospholipids were stored at −20 °C and periodically monitored for purity by thin-layer chromatography. Phospholipid concentrations were determined by the lipid phosphorous assay or by absorbance at 466 nm (for NB-D-phospholipids only).

**Yeast Strains and Cultures**—All yeast strains used in the selection and screen were derived from AGY75 (Table I), a yeast strain disrupted in the PDR1 and PDR3 loci. Homozygous diploid deletion strains were derived from the Yeast Genome Deletion Project and were the kind gift of Scott Devine. Strains carrying the three combinations of double deletions of LEM3, CDC50, and YNR048W were created by tetrad analysis of mating of haploid genes from the appropriate homozygous diploid deletion strains. The endocytic mutants were donated by the Reinman laboratory. The temperature sensitivity and endocytosis defects of these strains were confirmed before use in this study. For all experiments, early log-phase cultures (O₅₆₀ = 0.2–0.4) were grown in SDC (0.67% yeast nitrogen base, 2% glucose, and Complete amino acid supplement (Table I)) from overnight cultures. SCN₃₆₅ is SDC media lacking glucose but containing 2% sorbitol and 20 mM sodium azide. Temperature-sensitive strains were grown at a permissive temperature of 23 °C, and edelfosine killing assays were performed at both 23 and 37 °C.

**Mutant Screen for Edelfosine Resistant and M-C₆-NBD-PC Uptake-Deficient Strains**—AGY75 was mutagenized with ethyl methanesulfonate as described by Adams et al. (25). Briefly, cells were washed twice with distilled water and then suspended in 0.1 M sodium phosphate buffer, pH 7.0. Ethyl methanesulfonate was added to a final concentration of 3% and dispersed by vigorous vortex mixing. Cells were incubated for 1 h with shaking at 30 °C. The cells were then pelleted, and the ethyl methanesulfonate-containing buffer was removed. Cells were washed twice with 5% sodium thiosulfate to neutralize any remaining ethyl methanesulfonate. This treatment resulted in 61% killing when compared with the unmutagenized control. Mutagenized cells were diluted into YPAD (1% yeast extract, 2% peptone, 0.004% adenine, 2% glucose) and distributed throughout the wells of microtiter plates, achieving ~5 viable cells per well. After allowing 3–4 days for growth, a prong inoculator was used to transfer mutants to SDC containing 10 µg/ml edelfosine. Cells were harvested from 72 wells where significant growth in the presence of the drug was apparent. After single colony purification, the drug-resistant strains were screened for lack of M-C₆-NBD-PE uptake.
NBD-PC uptake by flow cytometry.

Identification and Generation of Mutant Alleles—The original mutations leading to loss of function in the lem3 strains were identified by sequencing the mutant allele rescued by gap repair as described by Rothstein (26). Additional missense mutations were identified by in vitro mutagenesis of a GFP-LEM3 fusion in the GFP-N-FUS vector described in Niedenthal et al. (27). Briefly, primers specific for GFP and the CYC1 terminator were designed and used to amplify GFP-LEM3 via PCR. MnCl2 was added to this reaction at a final concentration of 100 μM to increase the frequency of errors. This PCR product and linearized GFP-N-FUS were transformed into a lem3Δ strain. Transformants were screened for resistance to edelfosine. Resistant strains were further characterized by Western blot using anti-GFP primary antibodies (Molecular Probes). Plasmids were harvested from the edelfosine-resistant strains that expressed full-length GFP-Lem3p. Sequencing identified the mutations causing loss of LEM3 function (L179P, I234N, and Q60R/Q141R).

Lipid Preparation—Phospholipids dissolved in chloroform were dried under a stream of nitrogen and desiccated under vacuum for 1 h or overnight. Desiccated lipids were resuspended in the appropriate amount of Me2SO or ethanol to obtain a 100% stock concentration of NBD-phospholipids or edelfosine, respectively.

Internalization of Phospholipids into Yeast Cells—Cells were grown to early log phase in SDC at 30 °C. For flow cytometry experiments, cells were chilled on ice for 10 min before the addition of Me2SO or ethanol. The suspension was added to wells containing 50 μl of medium and varying concentrations of drug. Plates were incubated at 37 °C, temperature-sensitive lesions in neither end4Δ nor end4Δ strain, whereas endocytosis from the plasma membrane (33), was not resistant to edelfosine when compared with its wild-type parent (Fig. 2). Furthermore, at 37 °C, temperature-sensitive lesions in neither end3Δ nor end4 improved the survival of edelfosine-treated cells as assessed by methylene blue staining (data not shown). If endocytosis were the primary mechanism of edelfosine uptake, then endocytic mutants would be expected to exhibit increased resistance to edelfosine. Because this was not the case, we concluded that edelfosine was internalized by a non-endocytic mechanism. This result is consistent with previous work demonstrating that the fluorescent-labeled phosphatidylethanolamine, M-ε-NBD-PC, which differs from edelfosine only in the linkage and structure of the hydrophobic chain, is not significantly internalized by endocytosis (20, 34). M-ε-NBD-PC internalization is not inhibited in the end4Δ strain, whereas endocytosis of the fluid phase endocytosis marker, FM4–64, is almost completely blocked (see Fig. 4B in Hanson et al. (34)). This result and the observation that low temperature block of endocytosis (2 °C) inhibited, but did not block M-ε-NBD-PC internalization led to the conclusion that M-ε-NBD-PC is internalized by inward-directed transbilayer transport, or flip, across the plasma membrane (34). The demonstration that edelfosine wild-type strain is sensitive to the ether lipid drug edelfosine at levels similar to those reported previously (28). Because gain-of-function mutations in the pleiotropic drug resistance (PDR) network have been shown to affect intracellular accumulation of a wide range of drugs (29) as well as phospholipid analogs (30), we examined the effects of gain-of-function mutations in PDR1 and PDR3 on edelfosine resistance. As seen in Fig. 1, these mutations resulted in a dramatically resistant strain, which could be due to down-regulated influx (21), up-regulated efflux (29), altered membrane permeability (31), or a combination of these PDR-mediated effects.

Edelfosine Is Not Internalized by Endocytosis—In studies of mammalian cells, endocytosis appears to play a role in the internalization of ether lipid drugs in some of the cell lines tested (32). To determine whether endocytosis is a significant pathway for the internalization of edelfosine in S. cerevisiae, we evaluated the sensitivity of strains defective in the internalization step of endocytosis. An end4 null strain, which is blocked in both fluid-phase and receptor-mediated endocytosis from the plasma membrane (33), was not resistant to edelfosine when compared with its wild-type parent (Fig. 2). Furthermore, at 37 °C, temperature-sensitive lesions in neither end3Δ nor end4 improved the survival of edelfosine-treated cells as assessed by methylene blue staining (data not shown). If endocytosis were the primary mechanism of edelfosine uptake, then endocytic mutants would be expected to exhibit increased resistance to edelfosine. Because this was not the case, we concluded that edelfosine was internalized by a non-endocytic mechanism. This result is consistent with previous work demonstrating that the fluorescent-labeled phosphatidylethanolamine, M-ε-NBD-PC, which differs from edelfosine only in the linkage and structure of the hydrophobic chain, is not significantly internalized by endocytosis (20, 34). M-ε-NBD-PC internalization is not inhibited in the end4Δ strain, whereas endocytosis of the fluid phase endocytosis marker, FM4–64, is almost completely blocked (see Fig. 4B in Hanson et al. (34)). This result and the observation that low temperature block of endocytosis (2 °C) inhibited, but did not block M-ε-NBD-PC internalization led to the conclusion that M-ε-NBD-PC is internalized by inward-directed transbilayer transport, or flip, across the plasma membrane (34). The demonstration that edelfosine

**RESULTS**

Sensitivity of S. cerevisiae to Edelfosine Is Reversed by Mutations in PDR1 and PDR3—Anti-tumor ether lipid drugs have been reported to exhibit cytostatic effects on a wide variety of cell types including yeasts (28). Fig. 1 demonstrates that our

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**FIG. 1.** Gain-of-function mutations in transcription factors PDR1 and PDR3 confer resistance to edelfosine. Pleiotropic drug-resistant strains of S. cerevisiae were grown for 48 h in microtiter plates containing serial dilutions of the alkylphosphocholine drug edelfosine. Growth was measured by the A595 of each well in a microtiter plate reader (see “Experimental Procedures” for details). ●, LKY118 (PDR1PDR3); ○, LKY154 (PDR1Δ1PDR3); ▼, LKY156 (pdr3Δ PDR1Δ1). Data points and error bars represent the means and S.D. of at least three independent experiments.

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toxicity is not reduced in cells in which endocytosis is inhibited suggests it, like M-C<sub>6</sub>-NBD-PC, is internalized by flip across the plasma membrane.

Identification of Mutants Resistant to Edelfosine and Defective in PC Flip—Given that both M-C<sub>6</sub>-NBD-PC (21) and edelfosine (Fig. 2) appear to be internalized by flip across the plasma membrane, we hypothesized that the transport of the two compounds was mediated by the same transporter. To identify this transporter or novel regulators of its activity, we chemically mutagenized a strain carrying disruptions in both PDR1 and PDR3 (see “Experimental Procedures” for details). This strain background was chosen to eliminate the possibility of isolating mutants in these well characterized transcription factors. Of the nearly 10,000 mutants screened, 72 were initially identified as being resistant to edelfosine at a concentration of 10 μg/ml while remaining sensitive to a battery of other drugs including cycloheximide and 4-NQO. To focus our screen on cells defective for drug internalization rather than an intracellular target of edelfosine, we tested the resistant strains for their ability to internalize M-C<sub>α</sub>-NBD-PC. These experiments were performed at low temperature (−2 °C) to block both endocytosis and NBD-phospholipid efflux (21). Twenty-four of the edelfosine-resistant mutants were also dramatically defective for M-C<sub>α</sub>-NBD-PC uptake. Ten of the strains that met both criteria were analyzed and found to carry recessive mutations that fell into one complementation group, indicating that a single gene was likely responsible for the observed phenotype. These mutants were back-crossed at least three times to remove spurious background mutations and tested for the presence of additional phenotypes such as temperature sensitivity of growth, respiration defects, and sensitivity to high salt concentration. No other defects were found.

Identification of YNL323W, a Regulator of Edelfosine and M-C<sub>α</sub>-NBD-PC Flip—To identify the defective gene, we transformed a representative mutant with a CEN-based yeast genomic library, YPH1 (courtesy Philip Hieter), and used fluorescent-activated cell sorting to collect transformants exhibiting rescue of the M-C<sub>α</sub>-NBD-PC internalization defect. Only one rescuing plasmid was identified by this approach, and it contained a 10.5-kilobase region of chromosome XIV. We tested homozygous deletions of each of the full open reading frames in this fragment and found that only one gene affected edelfosine resistance and M-C<sub>α</sub>-NBD-PC uptake. As seen in Fig. 3, deletion of YNL323W causes both resistance to edelfosine and a dramatic defect in NBD-PC internalization. We then subcloned this gene into the centromeric vector pRS416 and transformed it into both the YNL323W deletion strain and our isolated mutants. The transformants were rescued for both phenotypes (Fig. 3, A and B), indicating that YNL323W/LEM3 is required for normal phosphatidylcholine and, presumably, edelfosine transblayer transport. Lem3p is predicted to be a 414-amino acid-long, integral membrane protein containing a predicted transmembrane domain near both the N- (74–95) and C- (376–400) termini (predicted with HMMTOP). The null phenotype is viable (35, 36), and no known functional motifs were identified in a search of the Prosise data base.

Loss of YNL323W/LEM3 Does Not Confer Multidrug Resistance—To determine whether loss-of-function mutations in YNL323W/LEM3 conferred resistance to a wide range of drugs, we tested a lem3 strain for its sensitivity to several commonly used drugs (cycloheximide, 4-NQO, ketoconazole) as well as another member of the alkylphosphocholine class (miltefosine/hexadecylphosphocholine). The lem3 strain exhibited increased resistance to miltefosine (Fig. 4A), whereas its resistance to 4-NQO and ketoconazole was unchanged (data not shown). Interestingly, deletion of LEM3 resulted in increased sensitivity to cycloheximide (Fig. 4B). We concluded that deletion of LEM3 specifically inhibited the internalization by transbilayer transport of the phosphocholine containing drugs but had no effect or perhaps increased the internalization of drugs structurally unrelated to phospholipids.

The Phospholipid Uptake Defect in YNL323W/LEM3 Mutants Is Head Group-specific—To determine whether YNL323W regulated the transblayer transport of phospholipids with other head groups, we examined the uptake of M-C<sub>α</sub>-NBD-PE and P-C<sub>α</sub>-NBD-PS analogs in the homozygous deletion strain at low temperature (2 °C). The 2 °C block of NBD-phospholipid efflux (21) allows the effect of this mutation on unidirectional influx to be tested. As seen in Fig. 5 (leftmost group of bars), deletion of YNL323W/LEM3 reduces the low temperature influx of M-C<sub>α</sub>-NBD-PC by −90% and that of M-C<sub>α</sub>-NBD-PE by −80%. Low temperature uptake of P-C<sub>α</sub>-
LEM3 is required for the normal internalization of NBD-labeled phosphatidylcholine and phosphatidylethanolamine but has little or no effect on the transport of the phosphatidylserine reporters across the plasma membrane. Lem3p Is Predominantly Localized to the Nuclear Envelope/ER—To determine the intracellular location of Lem3p, we first constructed a fusion protein with GFP attached to the C terminus of LEM3 integrated at its gene locus by homologous recombination using PCR-based gene modification (37). Although proper integration was confirmed by PCR, strains expressing the fluorescent fusion protein did not internalize M-C6-NBD-PC detected by flow cytometry and fluorescence microscopy and were resistant to edelfosine and miltefosine, indicating that the C-terminal fusion protein did not functionally complement the endogenous gene. Using the same approach, construction of an N-terminal fusion protein under the control of the GAL1 promoter produced a functional protein, as judged by the same criteria. Upon induction with galactose, the expression of GFP-Lem3p was directly correlated with the low temperature internalization of M-C6-NBD-PC (Fig. 6A). These data indicated that the expression of Lem3p rapidly leads to the production of necessary components of the phosphatidylcholine internalization pathway. No localization pattern was detected during the first 2 h after induction with galactose. After 2 h (Fig. 6B), GFP fluorescence was detected in a pattern that is consistent with localization in perinuclear and cortical ER (38). In a subset of cells a continuous ring of fluorescence at the plasma membrane is consistent with plasma membrane localization. This pattern of fluorescence is very similar to that recently demonstrated for a C-terminal-tagged Lem3p fusion protein expressed on a plasmid behind the endogenous promoter (39). In contrast to our results, Kato et al. (39) observe that the C-terminal-tagged protein could complement another phenotype associated with the deletion of LEM3, that is, sensitivity to lysis by the phosphatidylethanolamine binding protein, Ro09-0198 (39). Thus, it appears that overexpression of Lem3p by the GAL1 promoter does not alter the localization pattern and that Lem3p is predominantly associated with the ER/nuclear envelope with a smaller fraction associated with the plasma membrane. However, the observation that M-C6-NBD-PC flip is induced before the appearance of a detectable GFP-Lem3p pattern of localization precludes a definitive statement regarding the localization of the activity required for M-C6-NBD-PC flip.

LEM3 is a Member of an Evolutionarily Conserved Family—Lem3p has been identified as a member of the LEM3 (ligand-effect modulator 3) family/CDC50 family in the PFAM data base (protein families data base accession number P42838). This family comprises three homologues in yeast and has been evolutionarily conserved among organisms ranging from yeast to humans and plants. Although a role as a plasma membrane phospholipid transporter has been suggested (39) and a role in transcriptional regulation is inferred from other sources (see “Discussion”), the molecular function of these proteins has not been established. The S. cerevisiae homologue, CDC50, is pre-
dicted to encode a protein that is 40% identical and 59% similar to that of \( \text{LEM3} \) at the amino acid level. The \( \text{YNR048W} \) gene encodes a predicted protein 38% identical and 54% similar to \( \text{Lem3p} \). None of these genes is essential under normal growth conditions in \( \text{S. cerevisiae} \), although a deletion of \( \text{CDC50} \) confers a cold-sensitive growth phenotype and increased sensitivity to trifluoperazine, \( \text{ZnCl}_2 \), \( \text{CoCl}_2 \), and \( \text{MnCl}_2 \) (40).

Deletion of either \( \text{CDC50} \) or \( \text{YNR048W} \) alone had little or no effect on sensitivity to miltefosine (Fig. 4A) or \( \text{M-C}_6\text{-NBD-PC} \), \( \text{M-C}_6\text{-NBD-PE} \), and \( \text{P-C}_6\text{-NBD-PS} \) internalization (Fig. 5); however, strains with either \( \text{CDC50} \) or \( \text{YNR048W} \) deleted were slightly more sensitive to cycloheximide than their isogenic parent strain but not as sensitive as the \( \text{lem3} \) strain (Fig. 4B).

The three combinations of double deletions of the \( \text{LEM3} \)/\( \text{CDC50} \) family genes were constructed and tested for synthetic effects on NBD-phospholipid internalization (Fig. 5) and drug resistance (Fig. 7). Because LMY114 \( (\text{lem3} \Delta \text{cdc50} \Delta) \) grew very slowly in SDC, drug sensitivity of the double deletion strains were performed in YPAD. Deletion of all three homologous genes has been reported to produce a slow growth defect (41), precluding the study of strains in which all members of the family were deleted. Several interesting synthetic interactions were observed (Fig. 5). Of particular note is the opposite effect of the deletion of \( \text{YNR048W} \) and \( \text{CDC50} \) in the \( \text{lem3} \) strain. Introduction of a \( \text{ynr048w} \) deletion in the \( \text{lem3} \) strain significantly reduced the low temperature internalization of \( \text{P-C}_6\text{-NBD-PS} \) but had no effect on the internalization of \( \text{M-C}_6\text{-NBD-PC} \) or \( \text{M-C}_6\text{-NBD-PE} \). On the other hand, introduction of a \( \text{cdc50} \) deletion in the \( \text{lem3} \) strain increased the internalization of \( \text{M-C}_6\text{-NBD-PC} \) and \( \text{M-C}_6\text{-NBD-PE} \) but had no significant effect on \( \text{P-C}_6\text{-NBD-PS} \) internalization. The double deletion of \( \text{CDC50} \) and \( \text{YNR048W} \) in the parent \( \text{LEM3} \) strain had

![Fig. 4. Deletion of LEM3 increases resistance to miltefosine but decreases resistance to cycloheximide.](http://www.jbc.org/)

See the legend to Fig. 1 and “Experimental Procedures” for method details.

- ●, LMY94 \( (\text{LEM3}) \);
- ○, LMY102 \( (\text{lem3} \Delta) \);
- ▼, LMY86 \( (\text{cdc50} \Delta) \);
- ●, LMY107 \( (\text{ynr048w} \Delta) \) in both panels A and B. \( \text{wt} \), wild type.
no significant effect on the internalization of any of the NBD-phospholipids tested.

To gain further insight into the function of this gene family, synthetic interactions related to resistance to the alkylphosphocholine drugs and cycloheximide were measured in the double-deletion strains (Fig. 7). With the exception of the cdc50Δynr048Δ strain, the extent of low temperature M-C6-NBD-PC internalization was qualitatively correlated with sensitivity to miltefosine (Fig. 7B). M-C6-NBD-PC internalization in the lem3Δynr048Δ strain (Fig. 5) is inhibited to the same extent as in the lem3Δ strain and exhibits a similar level of resistance to miltefosine (Fig. 7B). M-C6-NBD-PC internalization and sensitivity to miltefosine are also roughly correlated in the lem3Δcdc50Δ strain. This correlation is not as clear for resistance to edelfosine, at least in part due to the decreased difference in sensitivity between the LEM3 and lem3Δ strains when measured in YPAD as opposed to SDC. Although the lem3Δ strain continues to be resistant to edelfosine when grown in YPAD, none of the double deletion strains appear to differ significantly from their isogenic parent. The increased sensitivity to cycloheximide is similar between the lem3Δ and lem3Δcdc50Δ strains. Cycloheximide sensitivity of the other two double-deletion strains is intermediate between that of lem3Δ and LEM3 strains.

These data indicate that all three members of this gene family genetically interact to regulate the internalization of NBD phospholipids and drug resistance. Deletion of YNR048W in combination with LEM3 decreases the internalization of P-C6-NBD-PS (p < 0.05; Student’s t test), whereas deletion of CDC50 and LEM3 increases the M-C6-NBD-PC and M-C6-NBD-PE internalization (p < 0.05; Student’s t test). Single and double deletions cause effects ranging from increased alkylphosphocholine drug resistance or increased cycloheximide sensitivity to no effect at all.

Mutations in Conserved Domains of LEM3 Lead to Loss of Function—The original mutation leading to loss of function in the lem3–1 strain was identified by sequencing the mutant allele rescued by gap repair. The nucleotide change was G517A, which translates into the amino acid change V173I. Interestingly, isoleucine in place of valine at this location, which resides just upstream of a highly conserved segment, occurs in the S. cerevisiae CDC50 gene as well as the Schizosaccharomyces pombe CDC50 homologue, suggesting that this amino acid is important in distinguishing the regulation of M-C6-NBD-PC internalization and alkylphosphocholine drug resistance between the two gene products. Three other mutant alleles isolated in the original screen were characterized by this method, and each was found to contain a nonsense mutation. Additional nonsense mutations were created by in vitro mutagenesis of a GFP-LEM3 fusion in the GFP-N-FUS vector (27). Transfomants in the lem3Δ strain were screened for a lack of complementation of the edelfosine resistance phenotype and the ability to produce a full-length GFP-LEM3 fusion protein. This approach identified two additional loss-of-function point mutations (L179P and I234N) and another resulting from the alteration of two amino acids Q60R/Q141R. All four of the mutant strains had mutations that resulted in amino acid alterations within or close to domains that have been evolutionarily conserved. None of these domains has been identified with specific functions according to the Prosite data base. Future studies are required to reveal the significance of these conserved domains in the function of this protein family.

**DISCUSSION**

We have shown here that deletion of the LEM3 gene in *S. cerevisiae* confers resistance to the alkylphosphocholine drugs edelfosine and miltefosine (Figs. 1 and 7) and confers sensitivity to cycloheximide without affecting resistance to other drugs, including 4-NQO and ketoconazole. The LEM3 gene was identified in a combined screen in which mutants were first selected for their ability to grow in concentrations of edelfosine that are toxic to wild-type laboratory strains. These edelfosine-resistant strains were then screened for defective internalization of a fluorometric, structural analog, M-C6-NBD-PC. M-C6-NBD-PC shares the glycerophosphorylcholine structure with edelfosine and has a similar degree of hydrophobicity although it differs in the carbon chain length and linkage. This second screen served to identify mutations leading to a defect in drug internalization as
opposed to numerous other intracellular targets that may confer resistance. This approach led to the identification a single gene, LEM3, whose expression is required for normal sensitivity to edelfosine and normal internalization of M-C6-NBD-PC.

M-C6-NBD-PC has been shown previously to be predominantly internalized by inward-directed, transbilayer transport across the plasma membrane referred to as flip (20, 21). This conclusion is based in part on the demonstration that M-C6-NBD-PC internalization is not affected by the deletion of the END4 gene that is required for both receptor-mediated and fluid phase endocytosis (33). To rule out endocytosis as the mechanism for edelfosine internalization, the sensitivity of an end4/H9004 strain to edelfosine was compared with that of its isogenic parent. No difference was detected (Fig. 2), leading to the conclusion that endocytosis is not required for edelfosine toxicity and that edelfosine, like M-C6-NBD-PC, is likely internalized by flip. The published observation that deletion of LEM3 does not inhibit the internalization of endocytosis markers FM4–64 and lucifer yellow (39) further supports the interpretation that LEM3 expression is required for flip, but not endocytosis, of edelfosine and M-C6-NBD-PC.

Deletion of LEM3 does not confer multidrug resistance. The lem3Δ strain is resistant to the alkylphosphocholine drugs edelfosine and miltefosine (Fig. 1 and 7) but not to the structurally unrelated fungal drugs cycloheximide (Fig. 7), ketoconazole, and 4-NQO (data not shown). In fact, sensitivity to cycloheximide was increased in the lem3Δ strain. Deletion of LEM3 has also been reported to increase the sensitivity to brefeldin A, a drug that disrupts Golgi organization, but has no effect on sensitivity to monensin (23). Thus, the deletion of LEM3 confers either resistance, sensitivity, or no effect, depending on the drug. At this time the causal relationship of LEM3 deletion and these various drug resistance and sensitivity phenotypes is not clear.

To gain additional insight into the structural features recognized by the LEM3-dependent transport mechanism, internal-
ization of several different NBD-labeled phospholipid head groups were tested. The lem3Δ strain was greater than 90% inhibited in the internalization of M-C6-NBD-PC and ∼80% inhibited in the internalization of M-C6-NBD-PE, whereas the internalization of the phosphoserine (Fig. 5) and phosphatidate analogs (data not shown) was not significantly affected. Similar results were obtained by Kato et al. (39). These data imply that the majority of M-C6-NBD-PC and the alkylphosphocholine drugs are internalized predominantly by a Lem3p-dependent pathway. The fact that internalization of the remaining three NBD-labeled phospholipid analogues are at most partially inhibited implies that at least one Lem3p-independent uptake pathway is yet to be identified for these different phospholipids.

By expressing an N-terminal GFP-LEM3 fusion under the control of the GAL1 promoter, the uptake of M-C6-NBD-PC was shown to be correlated with the expression of GFP-LEM3 (Fig. 6). Although the functional expression could be detected after only 10 min by flow cytometry, the appearance of localized GFP fluorescence in the fluorescence microscope was only detectable after approximately 2 h. The fluorescence pattern was consistent with a primary localization in the perinuclear and cortical ER, with a smaller amount in the plasma membrane. A similar pattern was also observed by Kato et al. (39) or a C-terminal GFP-LEM3 fusion protein expressed on a centromeric plasmid behind its own promoter in a lem3 strain. Thus, overexpression of N-GFP-Lem3p does not appear to alter its localization pattern during the first few hours of induction. Kato et al. (39) also show by Western analysis using an anti-Lem3p antibody that a fraction of Lem3p co-fractionated with the plasma membrane protein Pma1p and was associated with detergent-insoluble glycolipid-enriched complexes or lipid rafts. Demonstration of plasma membrane localization of Lem3p provides a necessary requirement for a direct role in the transport of phosphatidylcholine, phosphatidylethanolamine, and the alkylphosphocholine drugs across the plasma membrane.

However, data from other sources suggest that Lem3p may regulate translocation in addition to or instead of directly transporting phospholipids. The function of Lem3p was first suggested by its identification in a genetic screen to identify proteins that modulate the cellular response to the glucocorticoid, dexamethasone (22). In this study, a yeast strain was transfected with a glucocorticoid receptor expression vector and two reporter genes (HIS3 and lacZ) expressed behind the glucocorticoid response element. A mutation in Lem3 was found to confer increased expression of the reporter genes in response to dexamethasone. Thus it was considered a ligand effect modulator. lem3 strains displayed a marked increase in both the sensitivity to and maximum activation by dexamethasone. Increased reporter gene activation of a lem3 strain was maintained even in strains expressing a truncated glucocorticoid receptor lacking the hormone binding domain but was lost by truncation of the N-terminal regulatory domain that harbors activation, repression, and synergy functions. These data suggest that Lem3p likely affects a downstream step in the glucocorticoid receptor pathway such as nuclear localization, DNA binding, or transcriptional activation as opposed to regulating intracellular availability or binding of dexamethasone to the glucocorticoid receptor (22). The observation that Lem3p has been shown by high throughput mass spectrometric protein complex identification (42) to interact with two proteins involved in nuclear import (YLR347C/KAP95 and YJR132W/NMD5) is consistent with a nuclear function.

Additional insight into the function of Lem3p can be gained from studies of its two yeast homologues, CDC50 and YNR048W. cdc50-1 was initially identified by its cold-sensitive arrest in G1 (43). The cold-sensitive growth arrest of a cdc50Δ strain is suppressed by overexpression of CDC39/NOT1, suggesting that Cdc50p may interact or act in parallel with Cdc39p. Cdc39p is a transcriptional regulator involved in gene expression essential for cell cycle progression at G1 (44, 45). It associates with a multiple protein complex to produce both negative and positive transcriptional regulation (46–48). In particular, it negatively regulates CYC1 expression. Based on the genetic interaction between CDC50 and CDC39, the effect of CDC50 deletion on CYC1 expression was tested (41). Deletion of CDC50 increased the expression of a lacZ reporter fused to the CYC1 promoter at the non-permissive temperature, suggesting that Cdc50p plays a similar role to Cdc39p as a negative regulator of CYC1 expression. Deletion of CDC50 in combination with either LEM3 or YNR048W further increased lacZ expression, suggesting similar negative regulatory roles for the two paralogues (41). CDC50 has also been implicated in the regulation of vesicular trafficking steps required for polarized cell growth (49).

Our studies demonstrate that deletion of CDC50 or YNR048W in combination with LEM3 has complex effects on drug sensitivity and NBD-labeled phospholipid internalization that are not easily reconciled with a direct role in phospholipid transport for all three members of this gene family. Although the reduction of P-C6-NBD-PS internalization by the deletion of YNR048W in the lem3 strain is consistent with this possibility, it is difficult to explain how the deletion of a second phospholipid transporter can increase the internalization of M-C6-NBD-PC and M-C6-NBD-PE as observed in the lem3cdc50Δ strain. Furthermore, single or double deletions of members of this gene family tend to decrease sensitivity to the alkylphosphocholine drugs while increasing their sensitivity to cycloheximide. These opposite effects can be explained if the phospholipid transport across the plasma membrane required for alkylphosphocholine drug sensitivity disturbs the membrane structure, thus increasing passive leakage as suggested previously for PDR16 (31). However, if this was the case, one would expect a similar increase in sensitivity to ketoconazole and 4-NQO, but this was not observed.

At this time no single hypothesis has emerged to explain the multiple phenotypes associated with loss-of-function and null mutations in LEM3 and its yeast homologues CDC50 and YNR048W. These family members may function independently or as part of a multisubunit complex to transport phospholipids across cellular membranes, or alternatively, they may function as transcriptional regulators that are released from the membrane by proteolysis similar to that observed for the membrane-associated SREBP (50). Given the latter case, the LEM3/CDC50 family would function as positive regulators of the expression of yet to be identified phospholipid and alkylphosphocholine drug transporters, perhaps members of the DR52/NEO1 subfamily of P-type ATPases (51–53). On the other hand, the LEM3/CDC50 family may encode essential membrane components of a transporter complex or be required for chaperoning components of that complex to the proper membrane. In this case, the observed negative effects on transcription (22, 41) would be a downstream consequence of altered membrane phospholipid topology. Future studies will be aimed at distinguishing these possibilities to determine the molecular mechanism underlying the requirement for Lem3p expression for normal phosphatidylcholine and alkylphosphocholine drug transport across the plasma membrane of yeast.

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