ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, May 2004, p. 1778–1787 Vol. 48, No. 5
0066-4804/04/$08.00+0 DOI: 10.1128/AAC.48.5.1778–1787.2004

Membrane Sphingolipid-Ergosterol Interactions Are Important Determinants of Multidrug Resistance in *Candida albicans*

Kasturi Mukhopadhyay,[1]† Tulika Prasad,[1]† Preeti Saini,[1] Thomas J. Pucadyil,[2] Amitabha Chattopadhyay,[2] and Rajendra Prasad[1]*

Membrane Biology Laboratory, School of Life Sciences, Jawaharlal Nehru University, New Delhi 110067,[1] and Centre for Cellular and Molecular Biology, Hyderabad 500007,[2] India

Received 19 December 2003/Accepted 5 January 2004

In this study, we examined the importance of membrane ergosterol and sphingolipids in the drug susceptibilities of *Candida albicans*. We used three independent methods to test the drug susceptibilities of *erg* mutant cells, which were defective in ergosterol biosynthesis. While spot and filter disk assays revealed that *erg2* and *erg16* mutant cells of *C. albicans* became hypersensitive to almost all of the drugs tested (i.e., 4-nitroquinoline oxide, terbinafine, *o*-phenanthroline, itraconazole, and ketoconazole), determination of the MIC at which 80% of the cells were inhibited revealed more than fourfold increase in susceptibility to ketoconazole and terbinafine. Treatment of wild-type *C. albicans* cells with fumonisin B1 resulted in 45% inhibition of sphingolipid biosynthesis and caused cells to become hypersensitive to the above drugs. Although *erg* mutants displayed enhanced membrane fluidity and passive diffusion, these changes alone were not sufficient to elicit the observed hypersusceptibility phenotype of *erg* mutants. For example, the induction in vitro of a 12% change in the membrane fluidity of *C. albicans* cells by a membrane fluidizer, benzyl alcohol, did not affect the drug susceptibilities of *Candida* cells. Additionally, the surface localization of green fluorescent protein-tagged Cdr1p, a major drug efflux pump protein of *C. albicans*, revealed that any disruption in ergosterol and sphingolipid interactions also interfered with its proper surface localization and functioning. A 50% reduction in the efflux of the Cdr1p substrate, rhodamine 6G, in *erg* mutant cells or in cells with a reduced sphingolipid content suggested a strong correlation between these membrane lipid components and this major efflux pump protein. Taken together, the results of our study demonstrate for the first time that there is an interaction between membrane ergosterol and sphingolipids, that a reduction in the content of either of these two components results in a disruption of this interaction, and that this disruption has deleterious effects on the drug susceptibilities of *C. albicans* cells.

Candida albicans is an opportunistic diploid fungus that causes infection in immunocompromised and debilitated patients (30). The widespread and prolonged usage of azoles in recent years has led to the rapid development of the phenomenon of azole resistance, which poses a major threat to antifungal therapy (27, 46). Various mechanisms which contribute to the development of azole resistance in *Candida* have been implicated; these include the overexpression of or mutations in the target enzyme of the azoles, lanosterol 14α-demethylase, as well as the overexpression of genes encoding drug efflux pumps belonging to the ATP-binding cassette (ABC) superfamily, namely, *CDR1* and *CDR2*, and transporters belonging to the major facilitator superfamily (MFS), namely, *MDR1* (1, 10, 11, 16, 30, 31, 45).

It has been shown by various investigators that the action of antifungal agents is modulated by subtle modification of the membrane lipid composition (14, 16, 22, 28). Of note is that clinical as well as adapted azole-resistant isolates of *C. albicans* exhibit altered membrane phospholipid and sterol compositions (16, 22). Among various classes of yeast lipids, membrane sterol, which is also the target of azoles, is one of the important constituents; it is responsible mainly for rigidity, stability, and resistance to physical stresses (32). Therefore, a loss of sterol generally results in destabilization of the membrane, leading to increased membrane permeability and altered drug susceptibilities of yeast cells (28, 32). The sphingolipids of *C. albicans* are another class of important membrane lipid components; they differ from those of mammalian cells in that they are structurally less complex and contain phosphatidylinositol as part of their polar head groups (6, 20). These observations acquire significance when one considers recent reports which show the existence of discrete membrane microdomains, known as lipid rafts and composed predominantly of sphingolipid and sterol, within lipid bilayers (4, 5, 19, 26, 37, 38, 48). Interestingly, the up-regulation of lipids and proteins that constitute lipid rafts and the caveolar membrane has been observed in drug-resistant mammalian cells (18, 19). Additionally, it was recently reported that human P glycoprotein/multidrug resistance protein (Pgp/MDR1), which is a homologue of ABC drug transporter Cdr1p of *C. albicans*, is localized predominantly in cholesterol-enriched membrane domains, and that the depletion of cholesterol impairs human Pgp/MDR1-mediated drug transport (5, 23). Taken together, these findings suggest that sphingolipids and sterol as individual components as well as their mutual interactions play an important role in the functioning of the ABC drug efflux pump proteins (9, 13, 19, 21, 35).

* Corresponding author. Mailing address: Membrane Biology Laboratory, School of Life Sciences, Jawaharlal Nehru University, New Delhi 110067, India. Phone: 91-11-2670-4509. Fax: 91-11-2618-7338. E-mail: address: rp47@mail.jnu.ac.in.

† Kasturi Mukhopadhyay and Tulika Prasad contributed equally to this work.

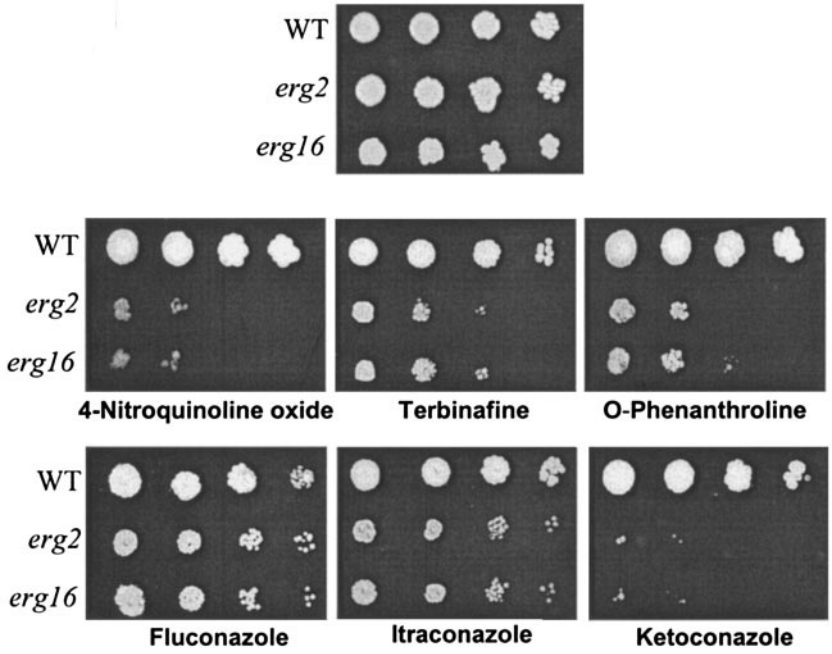


FIG. 1. Drug resistance profiles of *C. albicans* wild-type (WT) and *erg* mutant cells determined by a spot assay as described earlier (28). For this assay, 5-μl samples of fivefold serial dilutions of each yeast culture (each with cells suspended in normal saline to an OD_{600} of 0.1) were spotted on YEPD plates in the absence (control) or in the presence of 4-nitroquinoline oxide (0.1 μg/ml), terbinafine (0.4 μg/ml), *o*-phenanthroline (4 μg/ml), fluconazole (0.4 μg/ml), itraconazole (0.1 μg/ml), and ketoconazole (0.02 μg/ml).

In order to explore the interactions between sphingolipids and ergosterol (a substitute for mammalian sterol) and their involvement in the drug resistance of *C. albicans*, we exploited two sets of conditions in this study. We used *erg* mutants of *C. albicans*, which were deficient in ergosterol content, and we also used *C. albicans* cells with reduced sphingolipid content resulting from a selective blockage of sphingolipid synthesis. We observed that a reduction in either of these two major membrane lipid constituents had deleterious effects on drug resistance, in that *Candida* cells became hypersensitive to most of the drugs tested. Our results further suggest that ergosterol-sphingolipid interactions are important determinants of the surface localization of the major drug extrusion pump protein Cdr1p, which in turn affects the drug susceptibilities of *C. albicans* cells.

MATERIALS AND METHODS

Materials. Media chemicals were obtained from Difco (Detroit, Mich.) and HiMedia (Mumbai, India), and lipid *N*-rhodamine-dioleoyl-phosphatidylethanolamine was purchased from Avanti Polar Lipids, Inc. (Albaster, Ala). L-Dipalmitoyl-phosphatidylcholine, poly-D-lysine, rhodamine 6G (R6G), the fluorescent probe 1,6-diphenyl-1,3,5-hexatriene (DPH), and the drugs *o*-phenanthroline, 4-nitroquinoline oxide, terbinafine, and filipin were obtained from Sigma Chemical Co. (St. Louis, Mo.). Fluconazole was provided by Ranbaxy Laboratories (New Delhi, India), and ketoconazole and itraconazole were provided by Dupont (Wilmington, Del.). ^{3}H-labeled fluconazole was custom prepared by Amersham Biosciences, Little Chalfont, United Kingdom. 1,1′-dilinoleyl-3,3,3′,3′-tetramethylindocarbocyanine, 4-chlorobenzenesulfonate (*FAST*-DiI), and labeled 6-[(7-nitrobenz-2-oxa-1,3-diazol-4-yl)aminohexanoyl] spingosylphosphocholine (NBD-sphingomyelin [NBD-SM]) were purchased from Molecular Probes (Eugene, Oreg.).

Yeast strains and growth media. *C. albicans* strains ATCC 44829 (wild type, 33 ERG^+ ade^-), ATCC 44830 (*erg16* ade^-), and ATCC 44831 (*erg2* ade^-) were procured from the American Type Culture Collection. The strains were maintained as described previously (2). The *Saccharomyces cerevisiae* strain used was PSCDR1-GFP (an AD1-8u$^-$ derivative expressing Cdr1p-green fluorescent protein [GFP]) (36). The yeast strain was cultured in YEPD broth (Bio 101, Vista, Calif.) or SD-URA$^-$ medium (Bio 101).

Drug susceptibility testing of *C. albicans* strains. Drug susceptibilities were measured by using microtiter plate, spot, and filter disk assays. The MICs for the strains were determined with a broth microdilution method as described previously (28). For the spot assay, 5-μl samples of fivefold serial dilutions of each yeast culture (each with cells suspended in normal saline to an optical density at 600 nm [OD_{600}] of 0.1) were spotted onto YEPD plates in the absence (control) or in the presence of the following drugs: 4-nitroquinoline oxide (0.1 μg/ml), terbinafine (0.4 μg/ml), *o*-phenanthroline (4 μg/ml), fluconazole (0.4 μg/ml), itraconazole (0.1 μg/ml), and ketoconazole (0.02 μg/ml). Growth differences were recorded following incubation of the plates for 48 h at 30°C. The filter disk assay was done as described earlier (28); the following drugs were spotted in a volume of 5 to 10 μl: 4-nitroquinoline oxide (3 μg), terbinafine (16 μg), *o*-phenanthroline (64 μg), fluconazole (32 μg), itraconazole (20 μg), and ketoconazole (2 μg).

Labeling of spheroplasts for experiments involving fluorescence recovery after photobleaching (FRAP). Spheroplasts were suspended at a density of 10^8 cells/ml in 1 M sorbitol–0.1 M EDTA buffer and labeled with *FAST*-DiI. Labeling was carried out by using a final concentration of 10 μM *FAST*-DiI with 2% residual ethanol in glass tubes in the dark for 30 min at 25°C with mild shaking. The final pellet of spheroplasts was resuspended in a small volume of 1 M sorbitol–0.1 M EDTA buffer. One drop of this suspension was mounted between a glass slide and a coverslip which had been previously coated with 0.1 mg of poly-D-lysine/ml. The coverslip was sealed with nail enamel and placed inverted on the microscope stage for FRAP experiments.

Fluorescence imaging of *C. albicans* spheroplasts. Spheroplasts labeled with *FAST*-DiI were imaged at a magnification of ×100 with a 1.35 NA oil immersion objective under the fluorescein filter set on an Olympus fluorescence microscope equipped with a charge-coupled device camera (Cool SNAP-Pro) driven by Image-Pro PLUS software. The FRAP experiments were performed in the Gaussian spot-photobleaching and line-scanning mode on a Meridian Ultima 570 confocal laser scanning microscope. Recovery plots were analyzed by nonlinear regression and data were represented graphically by using Meridian Ultima software version 4.15. Fluorescence recovery profiles and diffusion coefficients were analyzed as described by Koppel et al. (17).

FIG. 2. (A) Fluorescence imaging of *C. albicans* spheroplasts labeled with *FAST*-DiI. (a) Chemical structure of *FAST*-DiI. (b and c) *C. albicans* wild-type spheroplasts were labeled with 10 μM *FAST*-DiI (see Materials and Methods for details). Phase-contrast and fluorescence images of the same field of spheroplasts labeled in this manner are shown in panels b and c, respectively. (d to f) Confocal images of wild-type, *erg2*, and *erg16* cells, respectively, labeled with 10 μM *FAST*-DiI. Confocal imaging was carried out by using an open pinhole under conditions described in Materials and Methods, and images were recorded on a 12-bit scale. Bars, 5 μm. (B) Fluorescence recovery plots after photobleaching of spheroplasts of *C. albicans* wild-type, *erg2*, and *erg16* cells labeled with *FAST*-DiI. The difference in normalized fluorescence intensities between the prebleached cells and cells during recovery is plotted as μ over time. Fluorescence recovery profiles were analyzed as described earlier (17). Recovery was monitored for 120 s. The regression line drawn across the data points represents the rate of fluorescence recovery. A qualitative estimate of the diffusion coefficient can be obtained by comparing the slopes of the recovery plots. The plots indicate an increase in the diffusion coefficient for *FAST*-DiI in the order *erg16* > *erg2* > wild type. (C and D) Diffusion coefficients (C) and steady-state fluorescence polarization measurements (D) for *C. albicans* wild-type and *erg* mutant cells. Measurements were determined for spheroplasts by using DPH as the fluorescent probe and excitation and emission wavelengths of 360 and 426 nm, respectively, as described previously (28). The values are means and standard deviations (indicated by bars) of three independent experiments.

TABLE 1. Drug resistance profiles for *C. albicans* strains, as determined by a microtiter assay

Drug	MIC_{80} (μg ml^{-1}) for the following strain:		
	Wild type	*erg2*	*erg16*
4-Nitroquinoline oxide	0.5	0.25	0.25
Terbinafine	2	0.25	0.25
o-Phenanthroline	16	8	8
Fluconazole	0.5	0.5	0.25
Itraconazole	0.4	0.4	0.2
Ketoconazole	0.4	0.008	0.008

Fluorescence polarization studies. Steady-state fluorescence polarization studies of *C. albicans* cells with the fluorescent probe DPH were carried out essentially as described earlier (28).

Drug diffusion and efflux assays. (i) Passive diffusion. Passive diffusion of the fluorescent compound R6G and ^{3}H-labeled fluconazole was determined by using a previously described protocol (28).

(ii) R6G efflux. The functionality of Cdr1p was checked by assaying the energy-dependent efflux of R6G, a known substrate of this drug extrusion pump protein. The protocol for the efflux assay was described previously (16).

Labeling of cells with NBD-SM. Labeling of cells with NBD-SM was carried out essentially as described earlier (28).

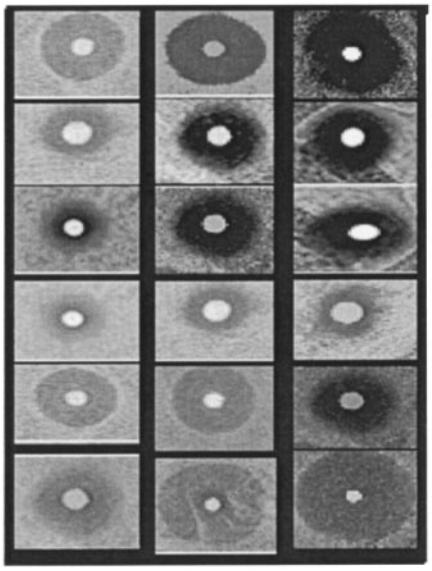
Estimation of sphingolipid synthesis. The assay for sphingolipid synthesis was carried out as described by Mandala et al. (25).

RESULTS

***erg* mutants of *C. albicans* are susceptible to various drugs.** *C. albicans* mutants defective in ergosterol biosynthesis were isolated earlier by Pesti et al. (33). These mutants were exploited in this study to examine whether the reduction in ergosterol levels in any way affects the drug susceptibilities of *C. albicans* cells. As a first step, the lack of ergosterol in *erg* mutant cells was confirmed by reverse-phase high-pressure liquid chromatography (HPLC) and spectroscopic methods (data not shown). We used three independent methods (spot, microtiter plate, and filter disk assays) to examine the drug susceptibilities of *erg* mutant cells (Fig. 1 and Tables 1 and 2).

We observed that in spot assays, the mutants showed increased susceptibilities to 4-nitroquinoline oxide, terbinafine, *o*-phenanthroline, itraconazole, and ketoconazole; however, data on the MICs at which 80% of the cells were inhibited (MIC_{80}) (Table 1) showed that the *erg* mutants were hypersensitive only to terbinafine and ketoconazole (more than twofold). In order to further confirm the susceptibilities of the *erg* mutants, we measured the zones of inhibition obtained in filter disk assays. As shown in Table 2, filter disk assays confirmed the finding of the spot assays (Fig. 1) that *erg* mutants of *C. albicans* were hypersensitive to all of the drugs tested, with the exception of fluconazole. These results emphasize that variations between drug susceptibility assays can exist (8) and that multiple drug susceptibility assays therefore should be used for exact assessment. Of note is that when equal number of cells were spotted on YEPD plates in the absence of drugs, *erg* mutant cells were found to grow relatively slowly, but this minor difference in growth rates did not affect the conclusions with regard to their susceptibilities to drugs. The difference in the rates of growth of *erg* mutant cells with or without drugs was very distinct, clearly indicating that *erg* mutant cells were

TABLE 2. Drug resistance profiles for *C. albicans* strains, as determined by a filter disk assay

Drug (μg)[a]	Wild type	*erg2*	*erg16*	Zone of inhibition (cm) for the following strain:		
				Wild type	*erg2*	*erg16*
4-Nitroquinoline oxide (3)				2.4	2.8	3.4
Terbinafine (16)				1.5	2.1	2.3
o-Phenanthroline (64)				1.5	2.2	2.4
Fluconazole (32)				1.0	1.1	1.2
Itraconazole (20)				2.1	2.4	2.7
Ketoconazole (2)				3.1	4.0	4.6

[a] Drugs were spotted at the indicated concentrations in a volume of 5 to 10 μl.

hypersensitive, a finding which was also supported by the results of filter disk assays.

Membranes of *erg* mutants are more fluid. In order to ascertain the change in membrane fluidity in *erg* mutants, we used the FRAP technique, in which the lateral mobility of a lipid probe (*FAST*-DiI) for *C. albicans* wild-type and *erg* mutant strains was determined. The fluorescence distribution of spheroplasts labeled with *FAST*-DiI is shown in Fig. 2A, panel c; the same field is viewed under phase contrast in Fig. 2A, panel b. There was no observable difference in the surface fluorescence distributions of *FAST*-DiI between wild-type and mutant strains (Fig. 2A, panels d [wild type], e [*erg2*], and f [*erg16*]). Lateral mobility measurements for *FAST*-DiI on spheroplasts labeled predominantly at the plasma membrane (Fig. 2A, panels b and c) were analyzed as described by Koppel et al. (17). Representative fluorescence recovery plots for *C. albicans* wild-type and *erg* mutant strains are shown in Fig. 2B. The absence of ergosterol in the *C. albicans erg* mutants was accompanied by an increase in the diffusion coefficient of *FAST*-DiI (Fig. 2C). The increases in the diffusion coefficient of *FAST*-DiI and fluorescence polarization values (a decrease in fluorescence polarization values implies higher fluidity) demonstrate that *erg* mutants have more fluid membranes (Fig. 2C and D). Thus, the results of two independent fluidity measurements confirm that the membranes of *erg* mutants of *C. albicans* are more fluid.

***erg* mutants demonstrate enhanced passive diffusion of drugs.** The enhanced membrane fluidity of *erg* mutants could affect passive diffusion, which could result in their hypersensitivity to the drugs tested. This possibility was examined by investigating passive drug diffusion into deenergized *erg* mutant cells in which active transport was blocked. We used fluorescent R6G and ^{3}H-labeled fluconazole as described previously (28) as probes to monitor passive diffusion. The R6G distribution was determined by measuring the change in the absorbance values for supernatants withdrawn at various times. Figure 3A shows that both *erg* mutants had reduced concentrations of extracellular R6G in the supernatants over time. These results imply that the passive diffusion of R6G is faster in *erg* mutants than in the wild type. Similar results were obtained when we used ^{3}H-labeled fluconazole to monitor fluconazole accumulation in energy-depleted cells. It is apparent from Fig. 3B that due to enhanced diffusion, the cells of both *erg* mutants accumulated larger amounts of fluconazole than did wild-type cells. The enhanced passive diffusion of R6G and fluconazole by *erg* mutants indicates that the mutants are more permeable, a property that could explain their hypersensitivity to drugs.

In vitro-induced changes in membrane fluidity do not affect drug resistance. In order to determine whether increased membrane fluidity and permeability alone was responsible for the altered drug susceptibilities observed in *Candida erg* mutants, we manipulated the fluidity of membranes of wild-type *Candida* cells in vitro by using a known membrane fluidizer, benzyl alcohol (BA) (28, 34, 39). The addition of 25 mM BA led to a 12% increase in the membrane fluidity of *Candida* wild-type cells (Fig. 3C). However, this BA-induced change in membrane fluidity did not alter susceptibilities to the drugs tested (Fig. 3D). Note that the cause of fluidity changes induced by BA is different from that observed in *erg* mutant cells. Nonetheless, the two experiments suggest that changes in flu-

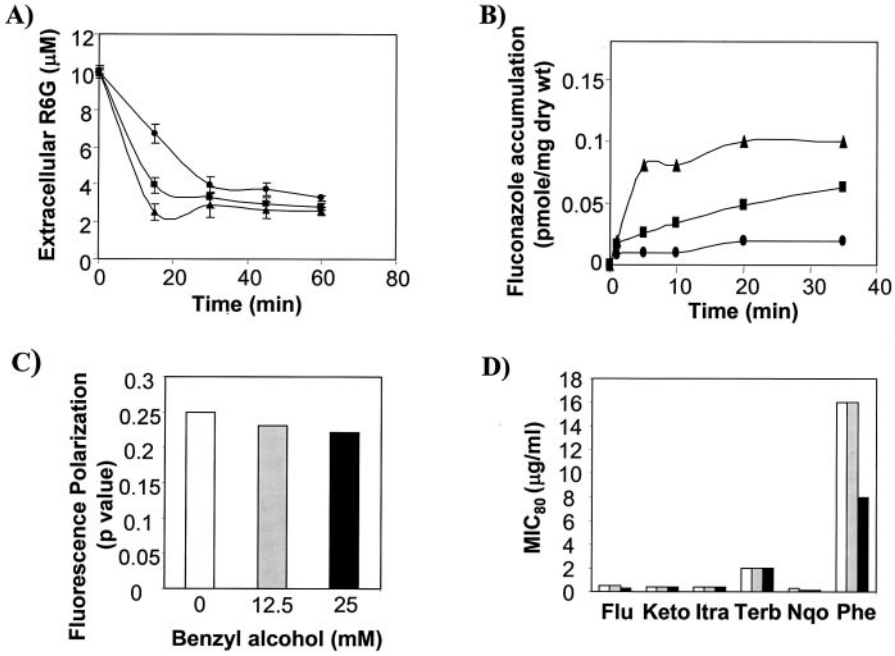


FIG. 3. (A) Extracellular R6G concentrations in *C. albicans* wild-type (●), *erg2* (▲), and *erg16* (■) cells at various time intervals. Deenergized *C. albicans* cells were incubated with R6G at 30°C. At various time points, cells were rapidly centrifuged, and the extracellular concentrations of R6G in the supernatants were determined spectrophotometrically at 527 nm. The values are the means and standard deviations (indicated by bars) of three independent experiments. (B) Accumulation of ^{3}H-labeled fluconazole in deenergized *C. albicans* wild-type (●), *erg2* (▲), and *erg16* (■) cells at various time intervals. (C) Measurements of steady-state fluorescence polarization of *C. albicans* wild-type cells in the absence (white bar) or in the presence of 12.5 mM (gray bar) and 25 mM (black bar) concentrations of BA. Steady-state fluorescence polarization was determined by using DPH as the probe as described in the legend to Fig. 2D. (D) Drug resistance profiles (microtiter assay) of *C. albicans* wild-type cells in the absence (white bar) or in the presence of 12.5 mM (gray bar) and 25 mM (black bar) concentrations of BA. The MIC_{80} was determined as described earlier (28). Abbreviations: Flu, fluconazole; Keto, ketoconazole; Itra, itraconazole; Terb, terbinafine; Nqo, 4-nitroquinoline oxide; and Phe, *o*-phenanthroline.

idity alone were not sufficient to cause the changes in drug susceptibilities that we observed.

NBD-SM is readily exchangeable from *erg* mutants. In view of the interactions of membrane sterol and sphingolipids, observed particularly in membrane microdomains or rafts in other organisms (3, 4), we attempted to explore the occurrence of such interactions in the membrane of *Candida* cells. We labeled *Candida* wild-type and *erg* mutant cells with fluorescent NBD-SM as described earlier (28, 40). The main advantage of this fluorescent lipid analogue is that it can be readily inserted into biological membranes by spontaneous lipid exchange from exogenous carriers (15, 40). Thus, at 90 min postlabeling, when NBD-SM incorporation into the *Candida* membrane was found to be at the maximum (data not shown), the cells were washed and the NBD-SM of labeled cells was back extracted with fatty acid-free bovine serum albumin (BSA). Figure 4 depicts the exchangeable NBD-SM from *Candida* wild-type and mutant cells. It was apparent that the amount of NBD-SM back extracted with BSA from *erg* mutant cells was much higher than that from wild-type cells (Fig. 4). This finding would mean that ergosterol depletion in *erg* mutant cells results in disruption of the interactions between ergosterol and sphingomyelin and thereby leads to enhanced exchangeability of sphinogomyelin. Interestingly, the exchange of NBD-SM was considerably reduced (restored to the level in wild-type cells) when *erg* mutant cells were grown in ergosterol-supplemented media (Fig. 4).

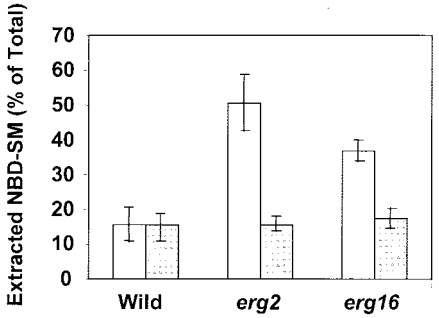
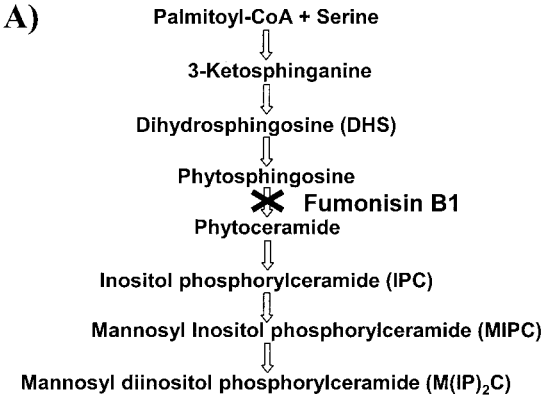


FIG. 4. Postlabeling transbilayer exchange of NBD-SM in *C. albicans* wild-type, *erg2*, and *erg16* cells. Cells were grown in the absence (white bars) or in the presence (gray bars) of medium supplemented with 20 μg of ergosterol/ml. Cells were labeled with NBD-SM and then back extracted with 2% BSA as described in Results. The graph presents data for the 90-min time point, at which the maximum back-extracted fluorescence in the supernatant was observed. The values are the means and standard deviations (indicated by bars) of three independent experiments.

Drug susceptibilities of *C. albicans* cells are affected by fluctuations in sphingolipid content. In order to explore whether the drug susceptibilities of *C. albicans* cells are also affected by membrane sphingolipid levels, we used fumonisin B1, an inhibitor of sphingolipid biosynthesis which blocks the formation

A)



B)

MIC_{80}(μg ml^{-1})

Drugs	WT		*erg2*		*erg16*	
	– FumB1	+FumB1	– FumB1	+FumB1	– FumB1	+FumB1
4-Nitroquinoline oxide	0.5	0.125	0.25	0.25	0.25	0.125
Terbinafine	2	0.125	0.25	0.25	0.25	0.063
o-Phenanthroline	16	0.5	8	8	8	4
Fluconazole	0.5	0.063	0.5	0.25	0.25	0.016
Itraconazole	0.4	0.05	0.4	0.4	0.2	0.05
Ketoconazole	0.4	0.063	0.008	0.008	0.008	0.008

FIG. 5. (A) Schematic representation of the yeast sphingolipid biosynthesis pathway. Fumonisin B1 inhibits the formation of phytoceramide from phytosphingosine. (B) Microtiter assays (MIC_{80}s) of *C. albicans* wild-type (WT) and *erg* mutant cells in the absence or in the presence of 50 μM fumonisin B1 (FumB1) were carried out essentially as described earlier (28).

of phytoceramide from phytosphingosine and thus inhibits the synthesis of the major sphingolipids (Fig. 5A). Wild-type *C. albicans* cells grown in the presence of 50 and 100 μM fumonisin B1 had 45 and 55% reductions in sphingolipid synthesis, respectively (data not shown). Interestingly, the reduction in sphingolipid content due to the blockage of sphingolipid synthesis by fumonisin B1 caused wild-type *C. albicans* cells to become hypersensitive to most of the drugs tested (Fig. 5B). The effect of fumonisin B1 on *erg* mutant drug susceptibilities was variable. While *erg2* mutant cells showed no further significant change in drug susceptibilities in the presence of the inhibitor, *erg16* mutant cells showed even higher susceptibilities to some of the drugs (Fig. 5B). Of note is that the ergosterol content and membrane fluidity of *C. albicans* in the presence of fumonisin B1 did not change significantly (data not shown). Thus, the observed hypersensitivity of fumonisin B1-treated wild-type cells to various drugs could be attributed solely to reduced sphingolipid content. It appears that membrane lipid constituents, particularly ergosterol and sphingolipids, are able to independently affect the drug susceptibilities of *C. albicans* cells. These observations acquire significance when one considers the interactions between membrane sterol and sphingolipids (discussed below).

Depletion of ergosterol or sphingolipids leads to poor surface localization of GFP-tagged Cdr1p. It was observed earlier that Cdr1p functioning was susceptible to the membrane lipid environment and, particularly, that the depletion of ergosterol had deleterious effects on drug susceptibilities (28, 41). The localization of Cdr1p in membrane rafts has not been unequivocally established, but based on the interactions between ergosterol and sphingolipids (membrane raft components) and their effects on drug susceptibilities, it is very likely that Cdr1p, a drug transporter protein, is localized in such domains.

In order to examine whether the depletion of ergosterol or sphingolipids would in any way affect the surface localization of Cdr1p and thus in turn its functioning, we used an *S. cerevisiae* expression system that was developed by Nakamura et al. (29) and that was a generous gift from R. D. Cannon, University of Otago, Dunedin, New Zealand. AD1-8u$^-$, from which seven major ABC transporters have been deleted, was derived from a *pdr1-3* mutant strain that causes hyperinduction of the *PDR5* promoter. We achieved a high level of expression of Cdr1p-GFP by integrating the Cdr1p-GFP open reading frame at the *PDR5* locus downstream from the *PDR5* promoter in strain AD1-8u$^-$; the resulting strain was designated PSCDR1-GFP (36).

Figure 6A shows confocal images of *S. cerevisiae* cells in which Cdr1p is overexpressed as a GFP-tagged protein. It is evident that Cdr1p is localized on the plasma membrane. Upon treatment with 50 μM fumonisin B1 (Fig. 6B), however,

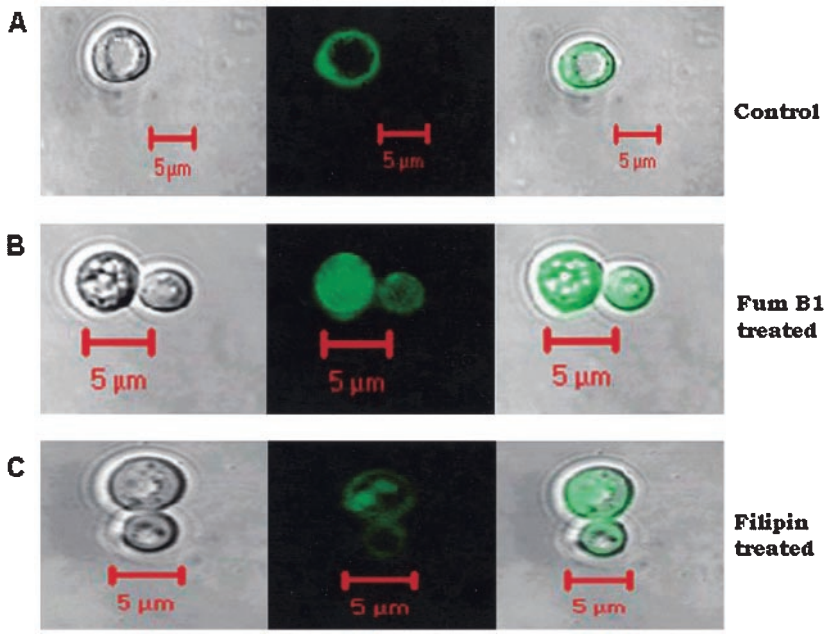


FIG. 6. Phase-contrast imaging (left panels) and fluorescence imaging (middle panels) under a confocal microscope of strain PSCDR1-GFP (A), strain PSCDR1-GFP grown in the presence of 50 μM fumonisin B1 (Fum B1) (B), and strain PSCDR1-GFP incubated with 5 μg of filipin/ml for 2 h (C). Cells were grown overnight and viewed directly for GFP fluorescence on a glass slide under a ×100 oil immersion objective in a Radiance 2100 (AGR3Q/BLD; Bio-Rad) confocal microscope equipped with 488-nm excitation and 500- to 530-nm band-pass emission filters. The fluorescence signal from strain PSCDR1-GFP showed the localization of Cdr1p on the plasma membrane. On treatment with Fum B1 or extended incubation with filipin, the GFP fluorescence from strain PSCDR1-GFP appeared to be concentrated inside the cells, indicating poor surface localization of Cdr1p. The right panels show the merge of the left and middle panels.

the GFP fluorescence appeared to be concentrated inside the cells, implying poor surface localization of Cdr1p. Incubation with the polyene antibiotic filipin for an extended period of time was shown earlier to induce deformations and distortions in sterol-containing membranes (7, 12, 44) because filipin is known to interact with the 3-β-hydroxyl group of sterols. To examine whether filipin-induced distortions would in any way affect Cdr1p localization, we checked the localization of the GFP-tagged protein by confocal microscopy after extended incubation of the cells with filipin. Figure 6C shows that the surface localization of Cdr1p was affected by extended filipin treatment and that this mislocalization was comparable to that seen in fumonisin B1-treated cells (Fig. 6B). It is apparent that the disruption in vitro of interactions between ergosterol and sphingolipids by either filipin or fumonisin B1 results in improper surface localization of Cdr1p.

Sphingolipid and ergosterol depletion results in reduced efflux of R6G. Since the surface localization of Cdr1p appeared to be defective in cells in which interactions between ergosterol and sphingolipids were disrupted, we examined the functionality of this drug extrusion pump by measuring the efflux of R6G. R6G is a known substrate of Cdr1p and was used earlier to monitor the drug efflux activity of this drug transporter (16, 24). R6G was allowed to enter deenergized *Candida* cells by passive diffusion. The energy-dependent extrusion of R6G then was initiated by the addition of glucose. The concentration of extruded dye in the supernatant was measured by monitoring its absorbance at 527 nm. It is apparent from Fig. 7A that the concentration of extruded R6G in the supernatant was lower in both *erg* mutants than in the wild type. Similar results

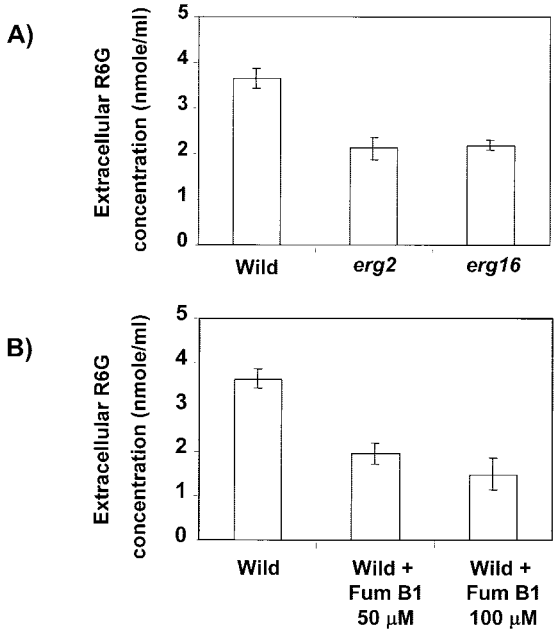


FIG. 7. Glucose-induced R6G efflux from *C. albicans* cells. Deenergized cells were incubated with R6G for 2 h. Efflux was initiated by the addition of 2% glucose, and the concentration of the extruded dye in the supernatant was measured spectrophotometrically at 527 nm. R6G efflux is represented as the extracellular concentration of R6G in the supernatant 60 min after the addition of glucose to wild-type and *erg* mutant cells (A) and to wild-type cells with or without treatment with the indicated concentrations of fumonisin B1 (Fum B1) (B). The values are the means and standard deviations (indicated by bars) of three independent experiments.

were obtained when we examined R6G efflux in wild-type cells treated with various concentrations of fumonisin B1 (Fig. 7B). The inhibitor-treated cells showed much less dye in the supernatant, suggesting reduced Cdr1p-mediated efflux (Fig. 7B). The results indicate that the disruption of interactions between ergosterol and sphingolipids in the membrane results in reduced efflux activity of Cdr1p.

DISCUSSION

Our study demonstrates that *C. albicans erg* mutant cells, which are defective in ergosterol synthesis, are hypersensitive to drugs. The increased drug susceptibilities shown by the *erg* mutant cells were not found to be merely due to an enhancement in membrane fluidity and passive diffusion of drug molecules across the plasma membrane. Rather, the lack of ergosterol in the *erg* mutant cells, which disrupted or destabilized the interactions within ergosterol-sphingolipid-rich microdomains, was the main cause of the observed hypersensitivities of *erg* mutant cells.

In support of the above conclusion, we observed a close relationship between membrane ergosterol-sphingolipid interactions and drug susceptibilities of *C. albicans*. We found that a reduction in sphingolipid levels by the blockage of sphingolipid synthesis also caused *C. albicans* cells to become hypersensitive to various drugs. Further, the depletion of either one of the membrane lipid components appeared to affect the other. For example, we observed that *erg* mutants lacking ergosterol had larger amounts of exchangeable sphingolipids than did the wild type (Fig. 4). Evidence that sphingolipid content can affect the drug susceptibilities of yeast cells was recently reported elsewhere (13). In that study, it was found that *IPT1* gene transcription was responsive to Pdr1p/Pdr3p transcription factors that regulate pleiotropic drug resistance genes of *S. cerevisiae*. The *IPT1* gene encodes the last step of sphingolipid biosynthesis, the loss of which has been shown to have differential effects on drug resistance phenotypes. An interaction between the two membrane lipid components was also evident from several other independent studies in which sphingolipid biosynthesis was shown to be coordinately regulated with cholesterol metabolism in higher eukaryotes (42, 43, 47).

It is important to mention that human Pgp/MDR1 is preferentially localized in microdomains (membrane rafts) rich in cholesterol and sphingolipids in the plasma membrane of mammalian cells and that its function is modulated by cholesterol (23). Our observations that the multidrug ABC transporter Cdr1p was poorly localized and that the efflux of the fluorophore R6G was severely hampered in *Candida* cells when interactions between ergosterol and sphingolipids were disrupted suggest similar possibilities. It is thus tempting to speculate that Cdr1p, a homologue of human Pgp, may also preferentially reside within such membrane rafts, a possibility which needs to be examined. In addition, the disruption of interactions between the membrane lipid components probably results in the mislocalization of Cdr1p, which in turn affects its functioning.

ACKNOWLEDGMENTS

The work presented in this report was supported in part by grants to R.P. from the Department of Biotechnology, New Delhi, India (BT/PR1110/MED/09/186/98), the Volkswagen Foundation, Deutschland, Germany (1/76 798), and the European Commission, Brussels, Belgium (QLK-CT-2001-02377), and by grants to A.C. from the Council of Scientific and Industrial Research, Government of India. T.P., P.S., and T.J.P. thank the Council of Scientific and Industrial Research for the award of a senior research fellowship. K.M. thanks the Council of Scientific and Industrial Research, Government of India, for the award of Pool Officer.

We thank R. D. Cannon for generously providing yeast strains, Nandini Rangaraj for excellent technical assistance during FRAP experiments, and Charu Tanwar for assisting in confocal microscopy. We also thank Shahid Jameel, International Centre for Genetic Engineering and Biotechnology, New Delhi, India, and Amitabha Mukhopadhyay, National Institute of Immunology, New Delhi, India, for providing us with confocal microscopy facilities. Special thanks are extended to Sneha Sudha Komath for valuable suggestions during preparation of the manuscript.

REFERENCES

1. **Albertson, G. D., M. Niimi, R. D. Cannon, and H. F. Jenkinson.** 1996. Multiple efflux mechanisms are involved in *Candida albicans* fluconazole resistance. Antimicrob. Agents Chemother. **40:**2835–2841.
2. **Ansari, S., P. Gupta, S. K. Mahanty, and R. Prasad.** 1993. The uptake of amino acids by *erg* mutants of *Candida albicans*. J. Med. Vet. Mycol. **31:**377–386.
3. **Bagnat, M., A. Chang, and K. Simons.** 2001. Plasma membrane proton ATPase Pma1p requires raft association for surface delivery in yeast. Mol. Biol. Cell **1 2:**4129–4138.
4. **Bagnat, M., S. Keranen, A. Shevchenko, A. Shevchenko, and K. Simons.** 2000. Lipid rafts function in biosynthetic delivery of proteins to the cell surface in yeast. Proc. Natl. Acad. Sci. USA **97:**3254–3259.
5. **Demeule, M., J. Jodoin, D. Gingras, and R. Beliveau.** 2000. P-glycoprotein is localized in caveolae in resistant cells and in brain capillaries. FEBS Lett. **466:**219–224.
6. **Dickson, R. C., and R. L. Lester.** 1999. Metabolism and selected functions of sphingolipids in the yeast *Saccharomyces cerevisiae*. Biochim. Biophys. Acta **1438:**305–321.
7. **Drabikowski, W., E. Lagwinska, and M. G. Sarzala.** 1973. Filipin as a fluorescent probe for the location of cholesterol in the membranes of fragmented sarcoplasmic reticulum. Biochim. Biophys. Acta **291:**61–70.
8. **Espinel-Ingroff, A.** 2001. In vitro fungicidal activities of voriconazole, itraconazole, and amphotericin B against opportunistic moniliaceous and dematiaceous fungi. J. Clin. Microbiol. **39:**954–958.
9. **Ferte, J.** 2000. Analysis of the tangled relationships between P-glycoprotein-mediated multidrug resistance and the lipid phase of the cell membrane. Eur. J. Biochem. **267:**277–294.
10. **Franz, R., S. L. Kelly, D. C. Lamb, D. E. Kelly, M. Ruhnke, and J. Morschhauser.** 1998. Multiple molecular mechanisms contribute to a stepwise development of fluconazole resistance in clinical *Candida albicans* strains. Antimicrob. Agents Chemother. **42:**3065–3072.
11. **Franz, R., M. Ruhnke, and J. Morschhauser.** 1999. Molecular aspects of fluconazole resistance development in *Candida albicans*. Mycoses **42:**453–458.
12. **Friend, D. S., and E. L. Bearer.** 1981. β-Hydroxysterol distribution as determined by freeze-fracture cytochemistry. Histochem. J. **13:**535–546.
13. **Hallstrom, T. C., L. Lambert, S. Schorling, E. Balzi, A. Goffeau, and W. S. Moye-Rowley.** 2001. Coordinate control of sphingolipid biosynthesis and multidrug resistance in *Saccharomyces cerevisiae*. J. Biol. Chem. **276:**23674–23680.
14. **Hitchcock, C. A.** 1993. Resistance of *Candida albicans* to azole antifungal agents. Biochem. Soc. Trans. **21:**1039–1047.
15. **Kean, L. S., A. M. Grant, C. Angeletti, Y. Mahe, K. Kuchler, R. S. Fuller, and J. W. Nichols.** 1997. Plasma membrane translocation of fluorescent-labeled phosphatidylethanolamine is controlled by transcription regulators, *PDR1* and *PDR3*. J. Cell Biol. **138:**255–270.
16. **Kohli, A., Smriti, K. Mukhopadhyay, A. Rattan, and R. Prasad.** 2002. In vitro low-level resistance to azoles in *Candida albicans* is associated with changes in membrane lipid fluidity and asymmetry. Antimicrob. Agents Chemother. **46:**1046–1052.
17. **Koppel, D. E., M. P. Sheetz, and M. Schindler.** 1980. Lateral diffusion in biological membranes: a normal-mode analysis of diffusion on a spherical surface. Biophys. J. **30:**187–192.
18. **Lavie, Y., G. Fiucci, and M. Liscovitch.** 1998. Up-regulation of caveolae and

caveolar constituents in multi-drug-resistant cancer cells. J. Biol. Chem. **273:**32380–32383.
19. **Lavie, Y., and M. Liscovitch.** 2001. Changes in lipid and protein constituents of rafts and caveolae in multidrug resistance cancer cells and their functional consequences. Glycoconjugate J. **17:**253–259.
20. **Lester, R. L., and R. C. Dickson.** 1993. Sphingolipids with inositolphosphate-containing head groups. Adv. Lipid Res. **26:**253–274.
21. **Liscovitch, M., and Y. Lavie.** 2000. Multidrug resistance: a role for cholesterol efflux pathways? Trends Biochem. Sci. **25:**530–537.
22. **Loffler, J., H. Einsele, H. Hebart, U. Schumacher, C. Hrastnik, and G. Daum.** 2000. Phospholipid and sterol analysis of plasma membranes of azole-resistant *Candida albicans* strains. FEMS Microbiol. Lett. **185:**59–63.
23. **Luker, G. D., C. M. Pica, A. S. Kumar, D. F. Covey, and D. Piwnica-Worms.** 2000. Effects of cholesterol and enantiomeric cholesterol on P-glycoprotein localization and function in low-density membrane domains. Biochemistry **39:**7651–7661.
24. **Maesaki, S., P. Marichal, H. V. Bossche, D. Sanglard, and S. Kohno.** 1999. Rhodamine 6G efflux for the detection of *CDR1*-overexpressing azole-resistant *Candida albicans* strains. J. Antimicrob. Chemother. **44:**27–31.
25. **Mandala, S. M., R. A. Thornton, M. Rosenbach, J. Milligan, M. Garcia-Calvo, H. G. Bull, and M. B. Kurtz.** 2002. Khafrefungin, a novel inhibitor of sphingolipid synthesis. J. Biol. Chem. **272:**32709–32714.
26. **Maxfield, F. R.** 2002. Plasma membrane microdomains. Curr. Opin. Cell Biol. **14:**483–487.
27. **Morschhauser, J.** 2002. The genetic basis of fluconazole resistance development in *Candida albicans*. Biochim. Biophys. Acta **1587:**240–248.
28. **Mukhopadhyay, K., A. K. Kohli, and R. Prasad.** 2002. Drug susceptibilities of yeast cells are affected by membrane lipid composition. Antimicrob. Agents Chemother. **46:**3695–3705.
29. **Nakamura, K., M. Niimi, K. Niimi, A. R. Holmes, J. E. Yates, A. Decottignies, B. C. Monk, A. Goffeau, and R. D. Cannon.** 2002. Functional expression of *Candida albicans* drug efflux pump Cdr1p in a *Saccharomyces cerevisiae* strain deficient in membrane transporters. Antimicrob. Agents Chemother. **45:**3366–3374.
30. **Odds, F. C.** 1988. *Candida* and candidosis: a review and bibliography. Balliére Tindall, London, England.
31. **Pao, S. S., I. T. Paulsen, and M. H. Saier, Jr.** 1998. Major facilitator superfamily. Microbiol. Mol. Biol. Rev. **62:**1–34.
32. **Parks, L. W., and W. M. Casey.** 1995. Physiological implications of sterol biosynthesis in yeast. Annu. Rev. Microbiol. **49:**95–116.
33. **Pesti, M., J. M. Campbell, and J. F. Peberdy.** 1981. Alteration of ergosterol content and chitin synthase activity in *Candida albicans*. Curr. Microbiol. **5:**187–190.
34. **Regev, R., Y. G. Assaraf, and G. D. Eytan.** 1999. Membrane fluidization by ether, other anesthetics, and certain agents abolishes P-glycoprotein ATPase activity and modulates efflux from multidrug-resistant cells. Eur. J. Biochem. **259:**18–24.
35. **Seitsma, H., R. J. Veldman, and J. W. Kok.** 2001. The involvement of sphingolipids in multidrug resistance. J. Membr. Biol. **181:**153–162.
36. **Shukla, S., P. Saini, Smriti, S. Jha, S. V. Ambudkar, and R. Prasad.** 2003. Functional characterization of *Candida albicans* ABC drug transporter Cdr1p. Eukaryot. Cell **2:**1361–1375.
37. **Simons, K., and E. Ikonen.** 1997. Functional rafts in cell membranes. Nature **387:**569–572.
38. **Simons, K., and D. Toomre.** 2000. Lipid rafts and signal transduction. Nat. Rev. Mol. Cell. Biol. **1:**31–39.
39. **Sinicrope, F. A., P. K. Dudeja, B. M. Bissonnette, A. R. Safa, and T. A. Brasitus.** 1992. Modulation of P-glycoprotein-mediated drug transport by alterations in lipid fluidity of rat liver canalicular membrane vesicles. J. Biol. Chem. **267:**24995–25002.
40. **Smriti, S. Krishnamurthy, B. L. Dixit, C. M. Gupta, S. Milewski, and R. Prasad.** 2002. ABC transporters Cdr1p, Cdr2p and Cdr3p of a human pathogen *Candida albicans* are general phospholipid translocators. Yeast **19:**303–318.
41. **Smriti, S. S. Krishnamurthy, and R. Prasad.** 1999. Membrane fluidity affects functions of Cdr1p, a multidrug ABC transporter of *Candida albicans*. FEMS Microbiol. Lett. **173:**475–481.
42. **Storey, M. K., D. M. Byers, H. W. Cook, and N. D. Ridgway.** 1998. Cholesterol regulates oxysterol binding protein (OSBP) phosphorylation and Golgi localization in Chinese hamster ovary cells: correlation with stimulation of sphingomyelin synthesis by 25-hydroxycholesterol. Biochem. J. **336:**247–256.
43. **Swain, E., K. Baudry, J. Stukey, V. McDonough, M. Germann, and J. T. Nickels, Jr.** 2002. Sterol-dependent regulation of sphingolipid metabolism in *Saccharomyces cerevisiae*. J. Biol. Chem. **277:**26177–26184.
44. **Wachtler, V., S. Rajagopalan, and M. K. Balasubramanian.** 2003. Sterol-rich plasma membrane domains in the fission yeast *Schizosaccharomyces pombe*. J. Cell Sci. **116:**867–874.
45. **White, T. C.** 1997. Increased mRNA levels of *ERG16*, *CDR*, and *MDR1* correlate with increased azole resistance in *Candida albicans* isolates from a patient infected with human immunodeficiency virus. Antimicrob. Agents Chemother. **41:**1482–1487.
46. **White, T. C., S. Holleman, F. Dy, L. F. Mirels, and D. A. Stevens.** 2002. Resistance mechanisms in clinical isolates of *Candida albicans*. Antimicrob. Agents Chemother. **46:**1704–1713.
47. **Worgall, T. S., R. A. Johnson, T. Seo, H. Gierens, and R. J. Deckelbaum.** 2002. Unsaturated fatty acid-mediated decreases in sterol regulatory element-mediated gene transcription are linked to cellular sphingolipid metabolism. J. Biol. Chem. **277:**3878–3885.
48. **Xu, X., R. Bittman, G. Duportail, D. Heissler, C. Vilcheze, and E. London.** 2001. Effect of the structure of natural sterols and sphingolipids on the formation of ordered sphingolipid/sterol domains (rafts). J. Biol. Chem. **276:**33540–33546.