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Membrane fluidity affects functions of Cdr1p, a multidrug ABC transporter of *Candida albicans*

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Abstract

Earlier, we have shown that the overexpression of an ABC transporter, *CDR1*, is involved in the emergence of multidrug resistance in *Candida albicans*. In this study, we checked its function in vivo by expressing it in different isogenic *Saccharomyces cerevisiae erg* mutants, which accumulated various intermediates of the ergosterol biosynthesis and thus altered the membrane fluidity. Functions like the accumulation of rhodamine 123, β-estradiol, fluconazole and floppase activity associated with Cdr1p were measured to ascertain their responses to an altered membrane phase. The floppase activity appeared to be favoured by an enhanced membrane fluidity, while the effluxing of substrates and Cdr1p's ability to confer multidrug resistance were significantly reduced. We demonstrate that only some of the functions of Cdr1p were affected by an altered lipid environment.

Keywords: Multidrug resistance; Membrane fluidity; *Candida albicans*; *CDR1*

1. Introduction

The dimorphic pathogenic yeast, *Candida albicans* is naturally more resistant to several drugs, e.g. cycloheximide, benomyl, methotrexate, than *Saccharomyces cerevisiae* [1–3]. In addition, the incidence of *C. albicans* cells acquiring resistance to azoles and polyenes has increased considerably in recent years, which has posed serious problems towards a successful chemotherapy [3–6]. Therefore, the cloning and sequencing of a *CDR1* (*Candida* drug resistance gene) and its homologues in *C. albicans* is an important step towards understanding the mechanism of drug resistance [7].

Anti-fungals, e.g. polyenes and azoles, which are widely used to treat *C. albicans* infections, have been shown either to complex with fungal sterols or to affect their conversion to a final product [4,8,9]. However, in recent years, this concept has changed to accommodate new observations. It is now apparent that binding of polyenes to membranes does not solely depend upon sterols but that other membrane components and factors also affect their action [10–12]. Similarly, the reduced permeability of azoles across the cell membrane of *Candida* cells is also an important factor in determining azole sensitivities. Newly discovered pumps belonging to the ABC and MFS super families are being implicated [4,7,8,13–16].

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A possible role of membrane lipids in modulation of the drug binding activity of P-glycoprotein (Pgp) has been suggested. Functions such as drug transport and ATPase activities were shown to be affected by the lipid environment and exogenous lipids [17–19]. The ATP binding folds of Pgp might also interact with membrane phospholipid particularly of the inner leaflet [18]. In vitro experiments involving mammalian Pgp indicated that it required fluid phospholipids in its immediate surroundings for an optimal ATPase activity since the membrane binding domains interact with the lipid bilayer and probably their integrity depends on the presence of certain phospholipids [19,20]. The recent demonstration of phospholipid translocation (flippase and floppase) activity associated with some of the MDRs again emphasises the importance of membrane lipids and its asymmetry [20,21]. As a result, considerable importance is attached to membrane lipids vis-à-vis functioning of multidrug transporters [19,22,23].

The availability of isogenic *erg* mutants of *S. cerevisiae* which are resistant to polyenes and expected to have an altered membrane fluidity, provided an opportunity to test in vivo the functioning of Cdr1p, an ABC transporter of *C. albicans* which we have cloned earlier [14]. We have expressed *CDR1* in *erg* mutants and tested the drug sensitivities, phosphatidylethanolamine (PtdEtn) translocase (floppase) activity, accumulation of rhodamine 123, β-estradiol and fluconazole in the transformants. This report for the first time describes the effect of the lipid environment on a yeast ABC transporter. We demonstrate that only some of the functions of Cdr1p are affected by an altered lipid environment.

2. Materials and methods

2.1. Materials

The chemicals used were of analytical grade and were from Sigma Chemical (USA). All media components were either from Difco (USA) or Himedia (India). Fluconazole was kindly provided by Pfizer (Sandwich, Kent, UK). The [^{3}H]fluconazole and [^{3}H]estradiol were from obtained from Amersham (UK).

2.2. Strains and media

The *S. cerevisiae* strains used in this study, ABC287 (*Mat* a, *ura*3-52, *leu*2Δ1, *lys*2-801, *his*3Δ200, *pep*4Δ::*HIS*3, *prb*1Δ1.6R, *can*1), ABC271 (*Mat* a, *ura*3-52, *leu*2Δ1, *lys*2-801, *his*3Δ200, *pep*4Δ::*HIS*3, *prb*1Δ1.6R, *can*1, *erg*2Δ::*LEU*2), ABC261 (*Mat* a, *ura*3-52, *leu*2Δ1, *lys*2-801, *his*3Δ200, *pep*4Δ::*HIS*3, *prb*1Δ1.6R, *can*1, *erg*3Δ::*LEU*2), ABC283 (*Mat* a, *ura*3-52, *leu*2Δ1, *lys*2-801, *his*3Δ200, *pep*4Δ::*HIS*3, *prb*1Δ1.6R, *can*1, *erg*4Δ) and ABC265 (*Mat* a, *ura*3-52, *leu*2Δ1, *lys*2-801, *his*3Δ200, *pep*4Δ::*HIS*3, *prb*1Δ1.6R, *can*1, *erg*6Δ::*LEU*2), were a kind gift from Dr. A.K. Bachhawat (IMTECH, Chandigarh, India). Yeast strains were grown at 30°C in YNB medium containing 2% glucose with additives (lysine (30 μg ml^{-1}), leucine (40 μg ml^{-1}) and uracil (30 μg ml^{-1})). The wild-type and mutant strains were transformed with the *CDR1* gene by the method described elsewhere [14]. Hereafter, the *CDR1* transformant of the wild-type will be mentioned as WT-T and *erg* mutants as *erg*-T, unless otherwise specified. All the transformants were grown in YNB medium containing 2% glucose with proper additives.

2.3. Accumulation of [^{3}H]-β-estradiol and rhodamine 123

The accumulation of steroid hormone [^{3}H]-β-estradiol and the accumulation of rhodamine 123 was determined by a method as described earlier [24,25].

2.4. Accumulation of fluconazole

The accumulation of [^{3}H]fluconazole in transformants of *erg* mutants of *S. cerevisiae* was determined essentially by the following method. The mid exponential cells were centrifuged at 500×*g* for 3 min and resuspended as a 5% cell suspension in fresh YNB medium containing 2% glucose. An aliquot of cell suspension was incubated in a shaking water bath at 150 rpm at 30°C for 10 min. The reaction was initiated by the addition of 100 nM [^{3}H]fluconazole (0.7 TBq mmol^{-1}). The cells were incubated with [^{3}H]fluconazole for 60 min and an aliquot was taken, filtered rapidly and washed three times with 10 mM PBS, pH 7.4, containing 2% glu-

cose on Millipore manifold filtration assembly using a 0.45 μm cellulose nitrate filter (Millipore, USA). The filter discs were dried and put in cocktail-O for measurement of the radioactivity in a liquid scintillation counter (Beckman, USA). The accumulation was expressed as pmol mg^{-1} dry weight.

2.5. Fluorescamine labelling, lipid extraction and thin layer chromatography

To check the labelling of PtdEtn in *CDR1* transformants of *erg* mutants and their wild-type, cells were harvested in the mid-exponential phase by centrifuging at 3000 rpm for 5 min at 4°C and washed two or three times with buffer A (100 mM potassium phosphate, 5 mM EDTA, pH 7.5). The harvested cells (0.6 g wet weight) were resuspended in 5 ml of buffer B (100 mM potassium phosphate and 600 mM KCl, pH 8.2) and kept at 4°C with gentle swirling. 15.6 mM of fluorescamine in dehydrated DMSO was added dropwise to the cell suspension with constant gentle swirling. After 30 s, the reaction was stopped by adding an equal volume of 1 M ammonia in 600 mM KCl. The cells were centrifuged and washed at 4°C, three or four times till the colour of the dye disappeared from the supernatant. The lipid extraction of yeast cells and separation of derivatised lipids was done by thin layer chromatography as described elsewhere [23].

3. Results and discussion

3.1. Transformation of erg mutants

The isogenic *ERG* disruptants (*erg2*, *erg3*, *erg 4* and *erg 6*) of *S. cerevisiae* accumulate various intermediates of the sterol metabolism and as a result have an altered phase of membrane lipids. Our studies as well as other studies showed that in general the membranes of *erg* mutants become more fluid (Fig. 1). It was interesting to ascertain if various functions attributed to *CDR1* would be affected in such an altered lipid environment. These *erg* mutants as well as its wild-type strain were transformed with a centromeric plasmid pYEURA3 carrying the *CDR1* gene [14]. The transformation of *erg* mutants with *CDR1* was confirmed by plasmid recovery. None of the transformants showed any significant difference in growth rate compared to the recipient strain (data not shown).

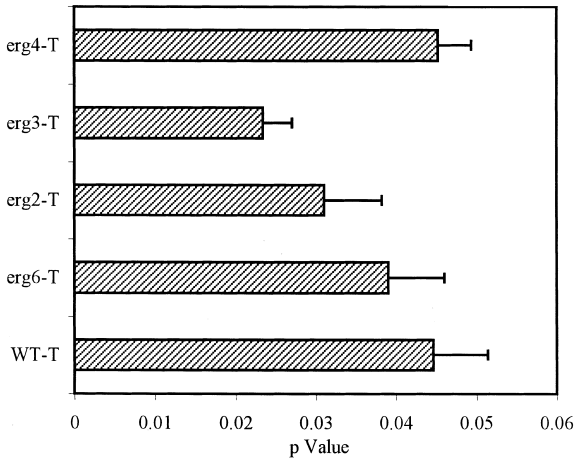

Fig. 1. Fluidity measurements in different *CDR1* transformants. Fluidity measurements in *CDR1* WT-T and different *erg*-T of *S. cerevisiae* were carried out on whole cells at an excitation and emission wavelength of 360 and 450 nm, respectively. The fluorescence anisotropy values were calculated as described earlier [27]. Each experiment was done in triplicate and the values represent the mean of three experiments ± S.D.

3.2. Expression of CDR1 in erg mutants leads to altered drug sensitivities

In order to assess the functional efficiency of Cdr1p, the susceptibility to different drugs to which Cdr1p conferred resistance was assessed. When *CDR1* was expressed in a wild-type strain of *S. cerevisiae*, the WT-T could tolerate up to 0.1 μg ml^{-1} of cycloheximide. However, all the *erg*-T expressing *CDR1* displayed an increased sensitivity to cycloheximide as demonstrated by the increase in the diameter of the growth inhibition zone (Table 1). Similar findings were made for 4-NQO and *o*-phenanthroline for which *erg*-T were also more sensitive. However, they were in general more resistant to fluconazole which could be related to their altered permeability (see below).

3.3. The floppase activity of Cdr1p is enhanced by a change in the membrane fluidity

Previously, we have shown that Cdr1p can trans-

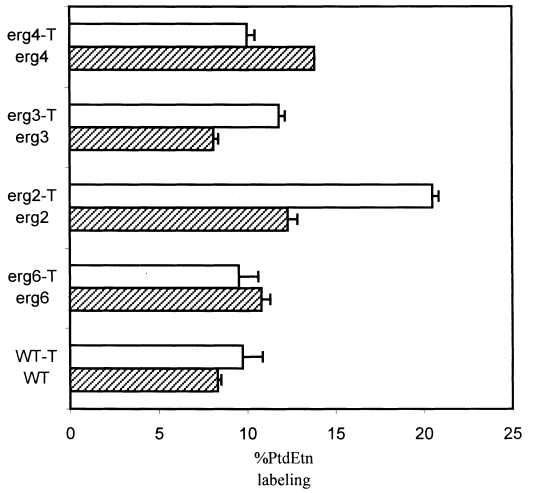

Fig. 2. The percentage labelling of PtdEtn in the plasma membrane of different *CDR1* transformants and their isogenic recipients. The fluorescamine labelling of transformants was done as described in Section 2. Derivatised PtdEtn and other phospholipids were extracted and quantified as described previously [23]. Each experiment was done in triplicate and the values represent the mean of at least three independent experiments ± S.D.

locate phospholipids from the inner monolayer to the exterior monolayer of the plasma membrane of *C. albicans,* which might be its normal physiological function [23]. Our present investigation revealed that the change in membrane fluidity led to an enhanced floppase activity of Cdr1p in some of the *erg*-T cells. As shown in Fig. 2, the percentage labelling of external PtdEtn by fluorescamine was enhanced in transformants expressing Cdr1p as compared to their isogenic recipients. This would imply that the translocation of PtdEtn from the inner to the outer leaflet of the plasma membrane, which is mediated by Cdr1p, is stimulated in response to an increased membrane fluidity (Figs. 1 and 2).

3.4. The accumulation of steroid and rhodamine is affected in transformants

We have previously demonstrated that Cdr1p can mediate the efflux of rhodamine 123, β-estradiol and drugs like fluconazole [24,25]. The WT-T and *erg*-T

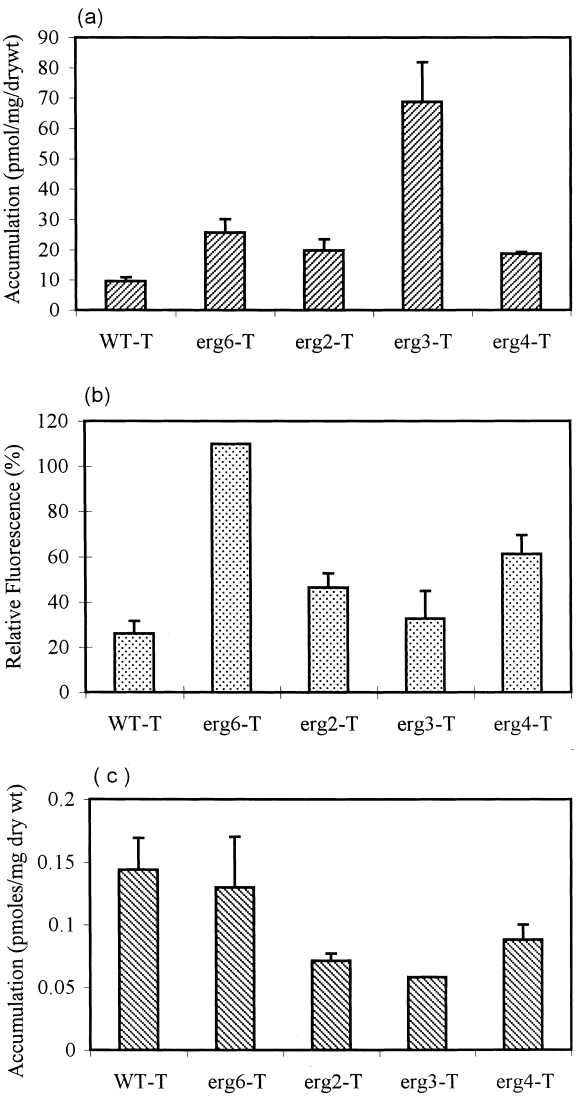

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Fig. 3. Rhodamine 123, [^{3}H]-β-estradiol and fluconazole accumulation in transformants of wild-type and *erg* mutants of *S. cerevisiae*. (a) For rhodamine 123 accumulation, mid-exponential phase cells were harvested and washed twice in PBS and a 2% cell suspension in PBS was used for each experiment as described earlier in Section 2. The values are mean of three independent experiments ± S.D. (b) For the accumulation of [^{3}H]estradiol in transformants, mid-exponential phase cells were harvested and resuspended as 10% suspensions in fresh medium. An aliquot of cells was incubated with 2 nM of β-estradiol (3.37 TBq mmol^{-1}). Aliquots of 0.5 ml were withdrawn after 60 min and filtered rapidly, followed by three washes of buffer (PBS pH 7.4, containing 2% glucose). The radioactivity retained on the filter was measured. The results are the mean of three independent experiments ± S.D. (c) The accumulation of fluconazole was done as described in Section 2. The values are the mean of three independent experiments ± S.D.

Table 1
Drug sensitivity profile of *erg* mutants transformed with *CDR1*

Strains	Cyh (0.1)†	Phe (10)	4-NQO (5)	Flu (100)
WT-T	0.5	N*	1.8	3.9
erg6-T	2.6	1.6	3.4	2.5
erg2-T	2.3	2.8	2.2	3.0
erg3-T	2.45	1.7	2.8	3
erg4-T	1.6	N	2.4	1.3

6×10^5 yeast cells ml^{-1} were mixed with molten YNB agar (~40°C) and poured on a Petri plate. After solidifying, the filter discs were kept on the plate and drugs were spotted on to the discs. The following amounts of drugs (in μg) were applied on a disc in 1–5 μl volumes (solvents used are given in parenthesis): cycloheximide (water), 0.1; *o*-phenanthroline (ethanol), 10; 4-nitrosoquinoline-*N*-oxide (acetone), 5; fluconazole (methanol), 100. The plates were incubated at 30°C and after 2–3 days when clear circular zones appeared, the plates were scored for drug sensitivity by measuring the diameter of the zone of inhibition around the filter discs. Such filter disc assays with each drug were repeated at least four or five times. The solvents used to solublise different drugs were also tested and there was no inhibition of growth due to the solvents used.

†The value given in parenthesis denotes the amount in μg of drugs, added on to the filter disk during the filter disk assay.

*The given values are in cm.

N = no zone.

were tested for the transport of some of the putative substrates of Cdr1p to check its functionality in an altered membrane environment. All the transformants showed varying levels of effect on the steroid transport although all elicited an enhanced accumulation of β-estradiol (Fig. 3a). The increased accumulation indicates a reduced efflux of incoming steroids [25]. Similar to steroid hormone, the rhodamine 123 efflux was also reduced in the transformants as evident from an enhanced accumulation of the dye (Fig. 3a and b). Interestingly, the efflux of fluconazole was increased in all the transformants except erg6-T which does not differ significantly (Fig. 3c). The reduced accumulation of fluconazole in *erg*-T, in contrast to other substrates, could be due to the fact that this drug is a preferred substrate for Cdr1p and has a high affinity for the drug which does not change significantly even when membranes are more fluid.

This study, for the first time, provides an understanding of how a yeast multidrug transporter protein, Cdr1p, changes its behaviour in response to an altered membrane environment. Given the intimate relationship between Pgp, its hydrophobic substrates and the surrounding membrane environment, it would be expected that membrane lipids have a considerable influence over the functioning of Cdr1p. Interestingly, the effect of lipid phase alterations due to the accumulation of various intermediates of the sterol biosynthesis in *erg* mutants had a different effect on various functional aspects of Cdr1p. For example, the floppase activity associated with Cdr1p could sustain the fluctuation in the membrane fluidity of various *erg* mutants. The percentage increase in labelling of PtdEtn by fluorescamine suggested that the floppase activity was enhanced in wild-type and *erg* mutants expressing *CDR1*. It has been shown that classes I and II Pgps of rodents and humans confer MDR and exhibit a broad specificity with respect to the lipid translocase activity while those of class III do not and elicit only a phosphatidylcholine translocase activity [21]. Although several homologues of mammalian Pgps have been detected in yeast including in *C. albicans*, there is no ABC transporter yet identified which, like class III Pgp, would exclusively flip membrane lipids. In this regard, the yeast ABC transporter, which confers multidrug resistance, is similar to its mammalian counterpart since a protein like Cdr1p cannot only confer drug resistance but also can flip membrane phospholipids [23,24]. This inherent duality of the yeast drug transporter is under investigation.

The transformant's ability to efflux drugs was severely hampered since all of them became more sensitive to most of the tested drugs to which their wild-type was resistant. That Pgp prefers a less fluid state of the membrane was also evident from earlier studies on mammalian systems [26]. In this regard, it is interesting to note the conservation of such a property down the evolutionary scale. It is even more

surprising since most of the other reported membrane functions prefer a fluid phase lipid.

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