Asymmetric Distribution of Phosphatidylethanolamine in *C. albicans*: Possible Mediation by *CDR1*, a Multidrug Transporter Belonging to ATP Binding Cassette (ABC) Superfamily

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By using two molecular probes, we demonstrate that only 4% of total phosphatidylethanolamine (PtdEtn) in the plasma membrane (PM) of a human pathogenic yeast, *Candida albicans*, is present in its external half. Evidence is presented to show that the availability of PtdEtn could be related to the expression of a multidrug transporter *CDR1* of *C. albicans*, and the process is energy-dependent. A homozygous *CDR1* disruptant strain of *C. albicans* shows almost 23% reduction in the external labelling of PtdEtn. This report shows that, similar to human MDRs, yeast multidrug transporter could also be involved in aminophospholipid translocation.

KEY WORDS — membrane asymmetry; multidrug resistance; aminophospholipid translocation; ABC transporter; *CDR1*; *C. albicans*

INTRODUCTION

Membrane phospholipids are asymmetrically distributed across the plasma membrane (PM) of a variety of cells (Diaz and Schroit, 1996; Williamson and Schlegel, 1994). Most of the phosphatidylethanolamine (PtdEtn) and phosphatidylserine (PtdSer) are located on the inner leaflet of the lipid bilayer, while phosphatidylcholine (PtdCho) and other lipids are predominantly localized on the outer half of the bilayer (Schroit and Zwaal, 1991). The asymmetrical distribution of membrane lipids is very specific and the loss of this asymmetry has been linked to various physiological processes (Kean *et al.*, 1997; Diaz and Schroit, 1996; Tang *et al.*, 1996; Utsugi *et al.*, 1991; Herrmann and Devaux, 1990).

Overexpression of a 170 kDa glycoprotein (P-glycoprotein) is involved in multidrug resistance (MDR). Such MDR proteins belong to a group of membrane transporters known as ABC (ATP binding cassette) transporters, which are widely distributed throughout the evolutionary scale (Prasad *et al.*, 1996; Hyde *et al.*, 1990; Higgins, 1992; Lewis, 1994). The completion of the *S. cerevisiae* genome project has revealed that there are 29 putative ABC proteins, which could be homologues of MDRs (Decottignies and Goffeau, 1997). The multidrug transporters in yeast not only include members of the ABC family but also a family of membrane facilitators (MFS), which are probably antiporters (Goffeau *et al.*, 1997; Balzi and Goffeau, 1994). In addition, regulatory networks of PDR (pleiotropic drug resistance) genes, backed by transcription regulators, are also well characterized (Balzi and Goffeau, 1994, 1995).

The studies involving MDR in yeast have attracted much attention, since they are good model systems and some of them are also pathogenic. *Candida albicans* is a dimorphic and opportunistic human pathogen (Gow *et al.*, 1994; Prasad, 1991), which is naturally more resistant to several drugs than *S. cerevisiae* (Prasad *et al.*,

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1996). In recent years, the incidence of *C. albicans* cells acquiring resistance to azoles and polyenes has increased alarmingly (Prasad *et al.*, 1996; Van den Bossche, 1995). We had earlier cloned and sequenced a multidrug transporter, *CDR1* (*Candida* drug resistance) of *C. albicans*, which is a homologue of *S. cerevisiae* multidrug efflux pump *PDR5* (Prasad *et al.*, 1995b; Balzi and Goffeau, 1994, 1995). A few more homologues of *CDR1* have been identified recently in *C. albicans* (Walsh *et al.*, 1997; Balan *et al.*, 1997; Sanglard *et al.*, 1997) and many genes specific to fluconazole and benomyl resistance have also been characterized (Alarco *et al.*, 1997; Prasad *et al.*, 1995; Prasad *et al.*, 1996).

Earlier, human Mdr1p and Mdr3p and mouse mdr2p have been shown to have flippase activity, which mediates phospholipid translocation directed towards the exoplasmic leaflet of the lipid bilayer (Ruetz and Gros, 1994; Bosch *et al.*, 1997; Van Helvoort *et al.*, 1996). Recently, Kean *et al.* (1997) have shown that translocation of PtdEtn in *S. cerevisiae* is controlled by *PDR1* and *PDR3* and identified two new alleles, *tpe1-1* (dominant) and *tpe2-1* (semidominant) for *PDR1* and *PDR3*, respectively. Pdr1p and Pdr3p are transcription regulators which control the expression of several genes including ABC transporters like *PDR5* and *SNQ2* (Balzi and Goffeau, 1994, 1995).

Asymmetrical distribution of phospholipids is not restricted to mammalian cells. The PM of *S. cerevisiae* cells is also asymmetric with regard to aminophospholipids and is controlled by an energy-dependent aminophospholipid translocase (Kean *et al.*, 1997; Tang *et al.*, 1996). This communication, for the first time, by using two molecular probes, shows that *C. albicans* PM is asymmetric, where PtdEtn is predominantly confined to the cytoplasmic leaflet of PM. We further show that the expression of *CDR1*, a multidrug resistance ABC transporter gene, affects the availability of PtdEtn in the outer half of the PM in an energy-dependent process, and present evidence that Cdr1p could be a phospholipid translocator.

MATERIALS AND METHODS

Materials

Yeast media were obtained from Difco laboratories (USA). All other chemicals were purchased from Sigma Chemicals Co. (USA). Precoated silica gel G-60 F TLC plates were obtained from E. Merck.

Strains and culture conditions

Candida albicans ATCC 10261 was grown in YEPD medium (2% bactopeptone, 1% bactoyeast extract and 2% glucose) (Jethwaney *et al.*, 1997). *C. albicans* strains CAF2-1 (*Δura3::imm434/URA3*), DSY449 (*Δcdr1::hisG/Δcdr1::hisG*), DSY1025 (*Δcdr1::hisG/Δcdr1::hisG*, *Δcdr2::hisG/Δcdr2::hisG*) were also maintained and grown in YEPD medium, as described earlier (Sanglard *et al.*, 1997). *S. cerevisiae* JG436 (Mat **a**, *PDR5::Tn5*, *leu2*, *met5*, *ura3-52*, *mak71*, *KRB1*) was a kind gift from Dr J. Golin, Catholic University of America, Washington, DC (Prasad *et al.*, 1995b). *S. cerevisiae* AD 1234568 (Mat **a**, *pdr1-3*, *his1*, *ura3*, *pdr5Δ*, *snq2Δ*, *pdr10Δ*, *pdr11Δ*, *pdr15Δ*, *yor1Δ*, *ycf1Δ*), was a kind gift from A. Decottignies, E. Balzi and A. Goffeau, Université Catholique de Louvain, Belgium. Both the strains, which were hypersensitive to cycloheximide, were transformed with the *CDR1* gene by a method described earlier (Prasad *et al.*, 1995b). The resulting transformants S-12 and AD-CDR1 were hyper-resistant to cycloheximide and to many other unrelated drugs and were expressing *CDR1*, as was evident from their Northern analysis (data not shown).

Trinitrobenzenesulphonic acid (TNBS) labelling

TNBS labelling was done essentially by the method described earlier (Balasubramanian and Gupta, 1996). *C. albicans* cells were harvested in mid-log 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. An equal volume of indicated concentration of TNBS (freshly prepared and chilled) in buffer B was added to the cell suspension. After indicated time, the reaction was stopped by adding equal volume of buffer C (200 mM potassium acetate, 600 mM KCl, pH 4·5). The suspension was centrifuged and washed two or three times with buffer C until the colour of free TNBS disappeared from the supernatant.

Table 1. Percentage of total and labelled PtdEtn in different hosts and their transformants, using two different probes, TNBS and fluorescamine[a].

Strains	Genotypes	Percentage PtdEtn of total phospholipids	Percentage labelled PtdEtn of total PtdEtn[b]	
			TNBS	Fluorescamine
S. cerevisiae				
JG436	Mata, *PDR5::Tn5*, *leu2*, *met5*, *ura3-52*, *mak71*, *KRB1*	26·47 ± 0·50	18·7 ± 0·28	18·3 ± 0·4
S-12	JG436 harbouring *CDR1*	29·25 ± 0·71	24·4 ± 1·2	24·1 ± 0·1
NC34[c]	JG436 harbouring *CaMDR1*	22·1 ± 1·28	18·7 ± 0·72	19·1 ± 0·56
AD	Mata, *pdr1-3*, *his1*, *ura3*, *pdr5Δ*, *snq2Δ*, *pdr10Δ*, *pdr11Δ*, *pdr15Δ*, *yor1Δ*, *ycf1Δ*	28·17 ± 1·28	13·9 ± 0·11	12·7 ± 0·60
AD-CDR1	AD harbouring *CDR1*	27·77 ± 1·44	23·56 ± 0·6	23·23 ± 0·38
C. albicans				
ATCC10261		15·16 ± 0·71	4·3 ± 0·14	4·2 ± 0·28
CAF 2-1	*Δura3::imm434/URA3*	22·62 ± 0·05	—	7·33 ± 0·11
DSY 449	*Δcdr1::hisG/Δcdr1::hisG*	22·64 ± 1·33	—	5·68 ± 0·18
DSY 1025	*Δcdr1::hisG/Δcdr1::hisG*, *Δcdr2::hisG/Δcdr2::hisG*	22·90 ± 0·41	—	4·4 ± 0·26

[a]Percentage values represent mean of at least 15–20 determinations.
[b]20 mM TNBS or 15·6 mM fluorescamine was used to label non-permeabilized cells. The incubation time of labelling for TNBS was 15 h while it was a few seconds in the case of fluorescamine. The derivatized PtdEtn was separated and quantitated as described in 'Materials and Methods'.
[c]Transformant carrying *CaMDR1* (MFS type) gene of *C. albicans*.

Fluorescamine labelling

Fluorescamine labelling of *C. albicans* cells was done essentially as described by Balasubramanian and Gupta (1996). Cells were harvested and washed as described above for TNBS labelling. Cells (0·6 g wet weight), were suspended in 3 ml of buffer B. Indicated concentrations of fluorescamine in dehydrated DMSO were added dropwise to the cell suspension with constant gentle swirling. After 30 s the reaction was stopped by adding equal volume of 1 M ammonia in 600 mM KCl. The cells were centrifuged and washed at 4°C three to four times until the colour of the dye disappeared from the supernatant.

Lipid extraction and thin layer chromatography

Lipids were extracted as described earlier (Ibrahim and Ghannoum, 1996; Koul and Prasad, 1996). Solvents used in the first and second dimensions were chloroform:methanol:30% ammonium hydroxide (65:25:6 v/v) and chloroform:water:methanol:glacial acetic acid:acetone (45:4:8:9:16 v/v), respectively. Resolved phospholipids and derivatized phospholipid were scraped off from plates and their phosphate content was estimated.

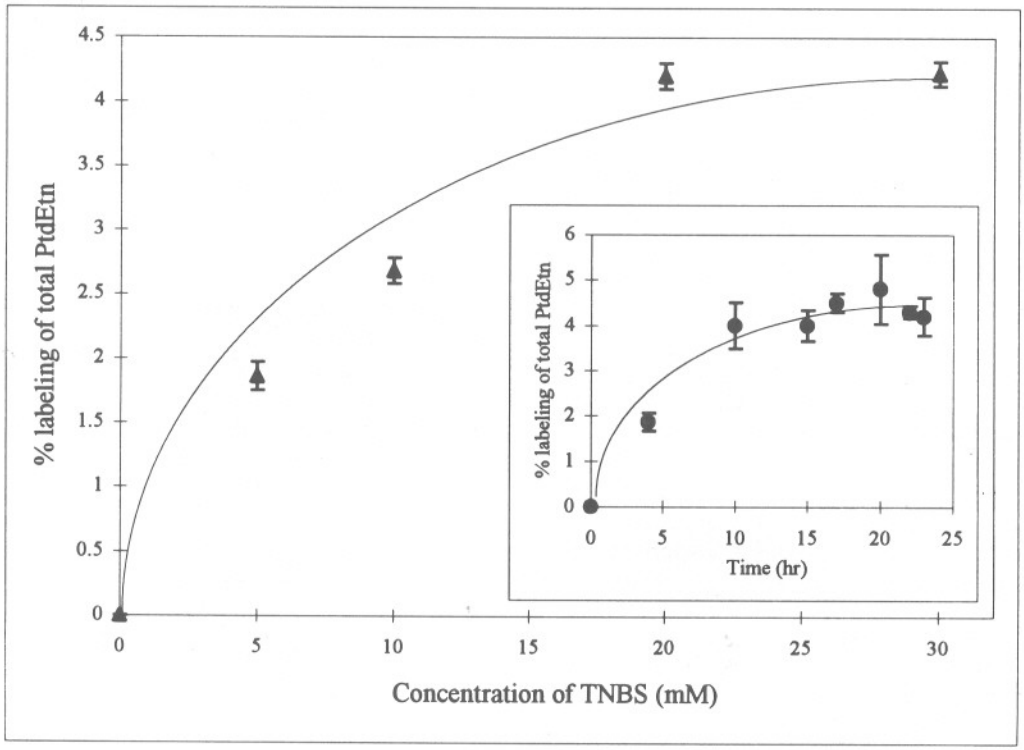
Isolation of total RNA and Northern analysis

Total RNA isolation from *C. albicans* cells and Northern analysis were performed as described elsewhere (Sambrook *et al.*, 1989; Carlson and Botstein, 1992).

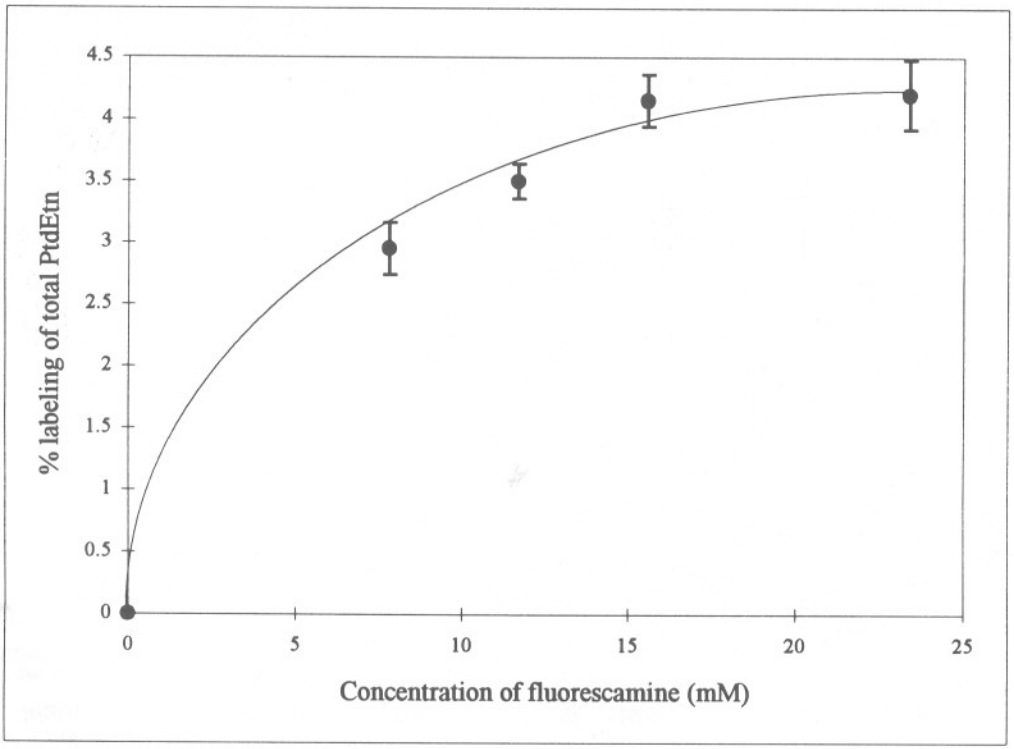
pH-Induced germ tube formation

Bud and mycelia were generated by procedure described elsewhere (Kaur *et al.*, 1988). *C. albicans* 10261 cells were inoculated into 25 ml of amino acid-rich medium (pH 6·8) in plastic Erlenmeyer flasks, and grown at 25°C up to the late log phase of growth (5×10^7 cells/ml). For zinc depletion, cells were inoculated into a fresh flask and maintained at 25°C at stationary phase for 48 h, resulting in a final concentration of $2{\cdot}5 \times 10^8$ cells/ml. Stationary phase G1 singlets ($1{\cdot}5 \times 10^9$ cells/ml) were lightly sonicated at a power of 5 μ peak to peak amplitude, using an MSE Ultrasonic Disintegrater at 2–4°C for 1 min, and incubated in 300 ml nutrient media in 500 ml Erlenmeyer flasks. Cells were grown at 37°C at either pH 4·5 or pH 6·5 to induce bud or hypha formation, respectively. Aliquots were removed at different time

(a)


% labeling of total PtdEtn
Concentration of TNBS (mM)
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
0
5
10
15
20
25
30
% labeling of total PtdEtn
Time (hr)
0
1
2
3
4
5
6
0
5
10
15
20
25

(b)


% labeling of total PtdEtn
Concentration of fluorescamine (mM)
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
0
5
10
15
20
25

intervals, and cell divergence was microscopically examined.

RESULTS

Labelling of plasma membrane PtdEtn of intact C. albicans cells

TNBS has been widely used in determining aminophospholipid distribution in membranes of different cell types. Recently, Balasubramanian and Gupta (1996) have shown the suitability of TNBS and fluorescamine for labelling the external PtdEtn of yeast PM. It is evident from Table 1 that both the probes were equally efficient in labelling the exposed PtdEtn of different yeast strains. The suitability of these probes was further established when we checked their impermeability under our labelling conditions. Under experimental conditions, the probes were impermeable to yeast cells and could only label the PtdEtn of the external leaflet. If cells were permeabilized, the same reagents could label up to 80% of the aminophospholipids, including PtdSer (it was not labelled in normal cells by either of the reagents, since most of PtdSer is localized in the cytoplasmic leaflet). Under the microscope, permeabilized cells labelled with fluorescamine showed dispersed fluorescence, while the latter was localized to the exteriors of non-permeabilized cells (data not shown). The results of the present study thus confirm that TNBS and fluorescamine can be used to label PtdEtn of *C. albicans* and *S. cerevisiae* cells.

As can be seen from Figure 1a, b, PtdEtn could be labelled by both the probes in a concentration-dependent manner. In purified PM of *C. albicans*, of the total phospholipids present, ~15% was PtdEtn (data not shown) and only ~4% of it was labelled with both the aminophospholipid labelling reagents in intact cells. This would mean that, out of total PtdEtn in non-permeabilized *C. albicans* cells, only ~4% is available for external labelling and the rest (~96%) is localized in the inner leaflet of PM. It must be pointed out that about 10–15 h were required for TNBS (20 mM) labelling (Figure 1a, inset), while fluorescamine (15·6 mM) could label the aminophospholipid in 30 s (data not shown). Since the labelling with fluorescamine is very rapid, it is expected to cause much less perturbation in the lipid organization, and therefore was routinely used in the following experiments (Rawyler *et al.*, 1984).

PtdEtn translocation from inner to outer leaflet is energy dependent and protein-mediated

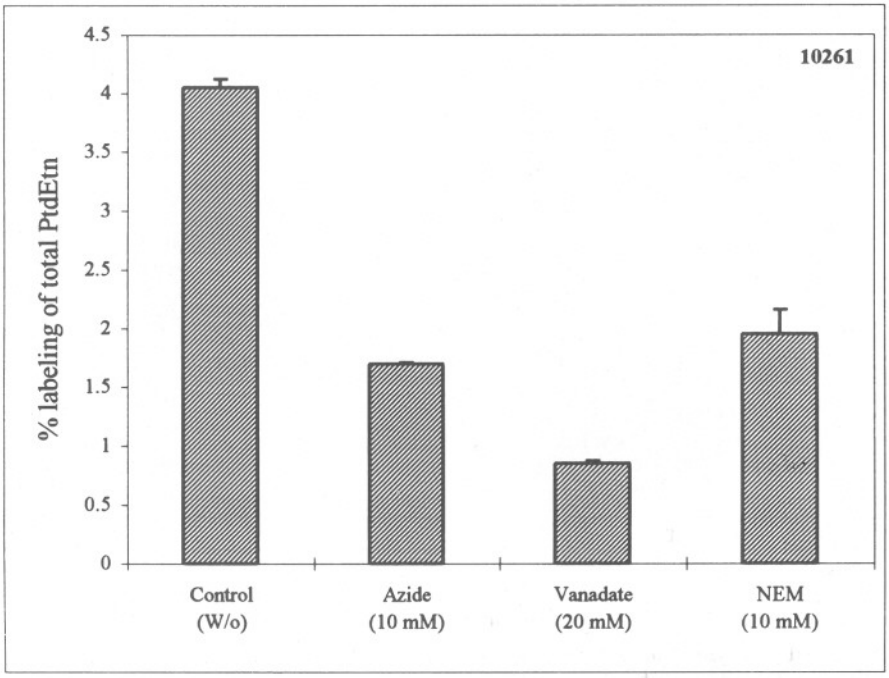
The movement of PtdEtn across PM of *C. albicans* could be prevented if cells were preincubated with energy inhibitors like sodium azide and sodium orthovanadate. Figure 2a, depicts labelling of PtdEtn in the presence of these inhibitors. Sodium azide, which is a mitochondrial ATP synthesis inhibitor, and sodium orthovanadate, which is a typical inhibitor of PM-ATPase, if added prior to labelling of PtdEtn with fluorescamine, resulted in reduced labelling. Almost 58–80% labelling of PtdEtn was inhibited by 10 mM of azide and 20 mM of vanadate. NEM (10 mM), which blocks free thiol groups of proteins and thus prevents protein-mediated processes, when added before labelling, was also able to block PtdEtn labelling with fluorescamine by about 52% (Figure 2a). If cells were starved of glucose for 14 h for the depletion of cellular ATP, the labelling was reduced by 60%. However, if cells were repleted with glucose, the total labelling of PtdEtn was again restored within 6 h. As shown earlier, for *S. cerevisiae* cells (Balasubramanian and Gupta, 1996) there may be an energy dependent aminophospholipid translocase in *C. albicans* which controls the movement of PtdEtn across the two monolayers (Figure 2b). It must be mentioned that the total phospholipid composition of *C. albicans*, determined under various experimental conditions, did not vary significantly (data not shown).

Expression of CDR1 is linked to PtdEtn translocation

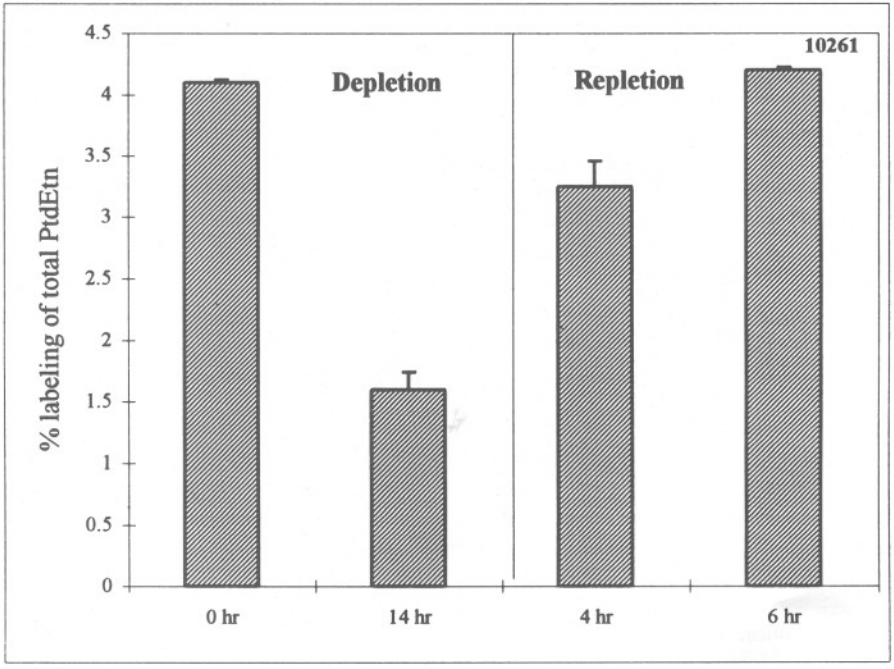
In order to ascertain whether *CDR1* expression could contribute in PtdEtn translocation, *S. cerevisiae* transformant S-12, which was expressing *CDR1*, was used to examine the PtdEtn distribution (Prasad *et al.*, 1995b). Interestingly, in S-12 strain out of total PtdEtn, almost 24% of it was labelled by both fluorescamine and TNBS, as

Figure 1. Percentage labelling of PtdEtn of plasma membrane of *Candida albicans* ATCC 10261 with (a) TNBS and (b) fluorescamine. The inset (a) shows the optimum time required for a 20 mM TNBS labelling. The labelling was done as described in Methods. Derivatized PtdEtn and other phospholipids were extracted and quantified as mentioned in 'Materials and Methods'.

(a)


10261
% labeling of total PtdEtn
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
Control (W/o)
Azide (10 mM)
Vanadate (20 mM)
NEM (10 mM)

(b)


10261
Depletion
Repletion
% labeling of total PtdEtn
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
0 hr
14 hr
4 hr
6 hr

compared to its host strain, JG436 (~19%) lacking *CDR1* and also having disrupted *PDR5*, which is a homologue of *CDR1* (Table 1). That *CDR1* could be involved in PtdEtn translocation was further confirmed when another transformant of *S. cerevisiae* (NC34), which was expressing a non-ABC transporter benomyl resistance (*CaMDR1*) gene, a membrane facilitator (Prasad *et al.*, personal communication) was checked for PtdEtn localization. It was observed that ~19% of PtdEtn was labelled in NC34 as in its host strain JG436 (Table 1). It must be pointed out that only 4% of total PtdEtn could be labelled in *C. albicans* 10 261 cells, while 18–24% of it was labelled in *S. cerevisiae* strains.

The expression of *CDR1* is morphologically regulated. As can be seen from Figure 3a that *CDR1* transcript is higher in mycelial form than in the bud form of *C. albicans* (Figure 3a and 3b). Interestingly, PtdEtn, in the outer leaflet of membrane, was also found to be much higher in the mycelial form as compared to bud form (Figure 3c). It is pertinent to mention that there was no significant change in phospholipid composition including that of PtdEtn between bud or mycelial forms of *C. albicans* cells (data not shown). Thus, the expression of *CDR1* appears to be linked to PtdEtn translocation.

S. cerevisiae strain S-12 having disrupted *PDR5*, is known to harbour several other ABC transporters, which might mask the function of Cdr1p (Decottignies and Goffeau, 1997). We, therefore, transformed *S. cerevisiae* (AD1234568) which was (called as AD hereafter, in this paper) constructed by A. Decottignies (in A. Goffeau's group), wherein seven ABC transporter genes, e.g. *PDR5* (pleiotropic drug resistance gene), *PDR10*, *PDR11*, *PDR15*, *SNQ2* (*Saccharomyces* nitroquinoline-N-oxide resistance gene), *YCF1* (yeast cadmium factor gene) and *YOR1* (yeast oligomycin resistance gene) were disrupted (Decottignies *et al.*, 1998). AD strain was transformed with the *CDR1* gene (Prasad *et al.*, 1995b) and the resulting transformant was designated as AD-CDR1. Both the strains were subjected to fluorescamine labelling. The labelling of PtdEtn was about 24% in AD-CDR1, as compared to its host strain AD where only 13–14% of PtdEtn could be labelled with fluorescamine (Table 1). Similar to *C. albicans* cells (Figure 2a, b), the translocation of PtdEtn mediated by *CDR1* was energy dependent as the labelling with fluorescamine was inhibited by azide and vanadate. NEM could also prevent the translocation of this aminophospholipid in AD-CDR1 strain (Figure 4a). ATP depletion of cells clearly prevented the translocation of PtdEtn which could be restored upon its repletion (Figure 4b). A homozygous disrupted *CDR1* strain of *C. albicans* DSY449 was used to check if the non-availability of functional Cdr1p could affect the availability of external PtdEtn (Sanglard *et al.*, 1997). It is evident from Table 1 that DSY449 has about 23% less of external PtdEtn than its parent strain CAF2-1. Interestingly, a double disruptant of *CDR1* and *CDR2* (a homologue of *CDR1*) DSY1025, showed even less (~40% less as compared to parent strain) of PtdEtn in the external half of PM (Table 1) suggesting the involvement of both the ABC transporters in PtdEtn translocation. It should be mentioned that the difference in external labelling of PtdEtn in strain 10261 and CAF2-1 could be due to their genotypic differences.

DISCUSSION

We have shown that PM of *C. albicans* cells is asymmetric with regard to PtdEtn distribution. Most of the PtdEtn was localized in inner leaflet and only 4% of it was exposed to external leaflet. The distribution of PtdEtn was affected if cells were preincubated with energy inhibitors prior to the labelling with fluorescamine. It was apparent from our results that availability of PtdEtn in outer half was mediated by an energy-dependent process. In this regard it is pertinent to mention about recent reports where aminophospholipid translocase activities in various systems including yeast cells have been shown to control the aminophospholipid movement (Decottignies *et al.*, 1998; Kean *et al.*, 1997; Balasubramanian and Gupta, 1996; Tang *et al.*, 1996). However, the presence of such translocase was not demonstrated in

Figure 2. Effect of (a) different inhibitors, (b) depletion and repletion of cellular ATP on PtdEtn of the plasma membrane of *Candida albicans* ATCC 10261 with fluorescamine (15·6 mM). Cells were incubated with the indicated concentrations of sodium azide (2 h), sodium orthovanadate (30 min) and NEM (10 min). For ATP depletion, cells were starved for 14 h and were repleted with the addition of 2% glucose for 4–6 h. All the treated cells were subjected to fluorescamine labelling, as described in 'Materials and Methods'.

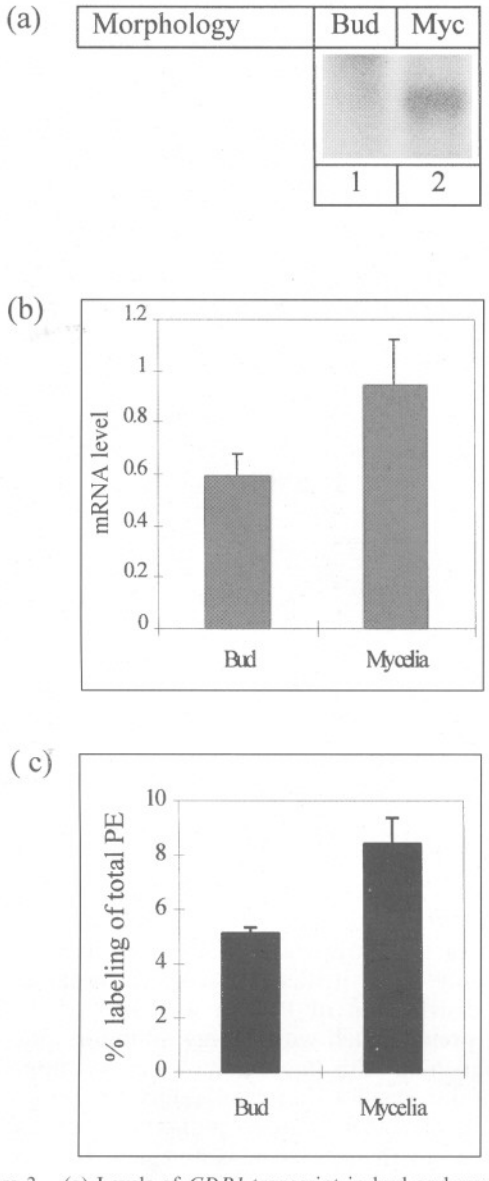


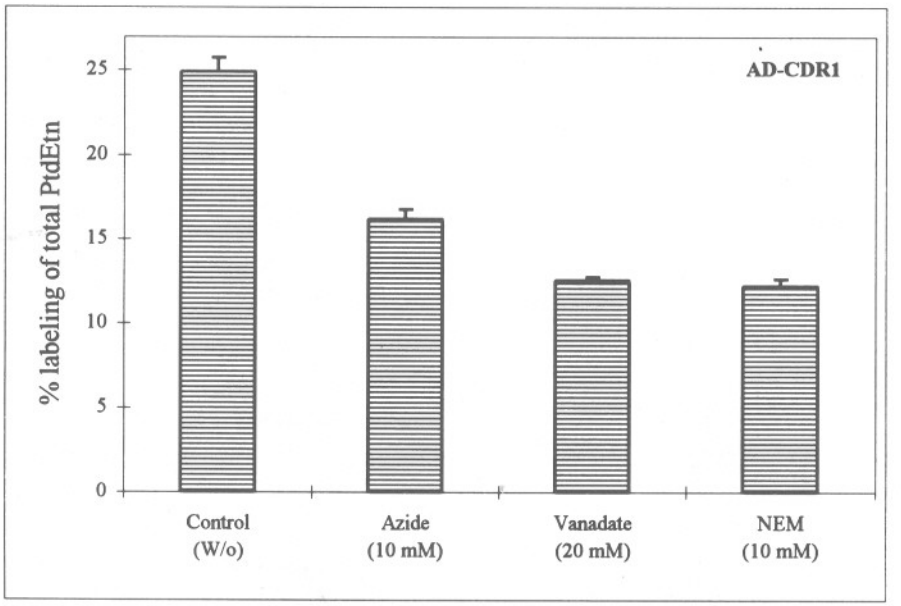
Figure 3. (a) Levels of *CDR1* transcript in bud and mycelial forms. (b) The normalized value of *CDR1* transcript after considering *ACT1* (actin) as internal control. Details of Northern analysis and RNA isolation were similar to those described earlier. (c) Percentage labelling of PtdEtn with fluorescamine (15·6 mM) in bud and mycelial forms. The bud and mycelial forms were formed by pH-regulated dimorphism, as described in 'Materials and Methods'. The fully differentiated population of cells as bud or mycelial forms was taken for Northern and lipid analyses.

C. albicans cells. This report for the first time confirms that flippase activities could be a common phenomenon in lower eukaryotes.

We present evidence to demonstrate that *CDR1*, which is a homologue of *PDR5*, has flippase activity which translocates PtdEtn to the outer leaflet of the membrane. First, there is an energy-dependent movement of PtdEtn in *S. cerevisiae* transformants which express *CDR1* of *C. albicans*. This translocation was even more evident when *CDR1* was expressed in the AD strain of *S. cerevisiae* (AD-CDR1), which was deleted in seven ABC transporters. Interestingly, the host AD strain showed some reduction in fluorescamine labelling after treatment with energy inhibitors and also after depletion of ATP (data not shown). This could be due to the presence of other, as yet unidentified, phospholipid translocators in *S. cerevisiae*, including the known translocator Drs2p which is a P-type transport ATPase involved in PS translocation (Tang *et al.*, 1996; Kean *et al.*, 1997). Second, the involvement of Cdr1p in phospholipid translocation was further evident from the fact that the increased amount of PtdEtn in the outer leaflet of PM of mycelial form correlated well with *CDR1* expression, which was also more pronounced in the mycelial than in the bud form of *C. albicans*. The decrease in the availability of PtdEtn in the outer half of PM of a homozygous *CDR1* disruptant also suggests that it might be involved in PtdEtn translocation. It is interesting to note that, similar to mammalian MDRs (e.g. *MDR1* and *MDR2*), *CDR2*, a homologue of *CDR1*, may also contribute to phospholipid translocation. However, this aspect remains to be investigated. This was further confirmed when *S. cerevisiae* transformant expressing a non-ABC transporter (*CaMDR1*), of *C. albicans* was found to have no affect on the PtdEtn distribution pattern between the two leaflets.

Our results, therefore, suggest that the PtdEtn translocation is linked to an ABC transporter Cdr1p of *C. albicans*. Whether Cdr1p, like human Mdr1p, is a general phospholipid translocator or specific to aminophospholipids remains to be resolved (Ruetz and Gros, 1994; Bosch *et al.*, 1997; Van Helvoort *et al.*, 1996). As mentioned earlier, the contribution of other ABC transporters, like *CDR3* (Balan *et al.*, 1997; White, 1997) in *C. albicans*, and of others as yet uncharacterized transporters which may be involved in phospholipid translocation, can not be excluded from these results. What the mechanism of the movement of phospholipid from the outer to the cytoplasmic leaflet in *C. albicans* cells is, and how many proteins control it, are some of the questions which remain to be resolved.

(a)



(b)

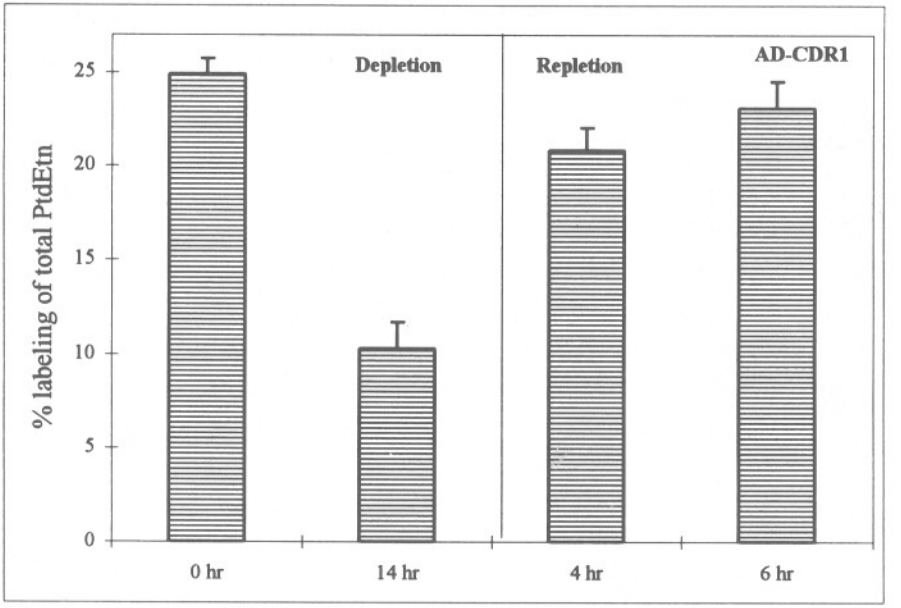


Figure 4. Percentage labelling of PtdEtn with fluorescamine (15·6 mM), (a) in the presence of inhibitors and (b) after depletion and repletion of cellular ATP in AD-CDR1. All the labelling conditions and preincubation with inhibitors, etc., were similar to those described in the caption for Figure 2.

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REFERENCES

Alarco, A. M., Balan, I., Talibi, D., Mainville, N. and Raymond, M. (1997). AP1-mediated multidrug resistance in *Saccharomyces cerevisiae* requires *FLR1* encoding a transporter of the major facilitator superfamily. *J. Biol. Chem.* **272,** 19,304–19,313.

Balan, I., Alarco, A. M. and Raymond, M. (1997). The *Candida albicans CDR3* gene codes for an opaque-phase ABC transporter. *J. Bacteriol.* **179,** 7210–7218.

Balasubramanian, K. and Gupta, C. M. (1996). Transbilayer phosphatidylethanolamine movements in the yeast plasma membrane. Evidence for a protein-mediated, energy-dependent mechanism. *Eur. J. Biochem.* **240,** 798–806.

Balzi, E. and Goffeau, A. (1994). Genetics and biochemistry of yeast multidrug resistance. *Biochim. Biophys. Acta* **1187,** 152–162.

Balzi, E. and Goffeau, A. (1995). Yeast multidrug resistance: the PDR network. *J. Bioenerget. Biomemb.* **27,** 71–76.

Bosch, I., Joannopoulos, K. D., Wu, R.-L., Furlong, S. T. and Croop, J. (1997). Phosphatidylcholine and phosphatidylethanolamine behave as substrates of the human MDR1 P-glycoprotein. *Biochemistry* **36,** 5685–5694.

Carlson, M. and Botstein, D. (1992). Two differentially regulated mRNAs with different 5′ ends encode secreted and intracellular forms of yeast invertase. *Cell* **28,** 145–154.

Decottignies, A. and Goffeau, A. (1997). Complete inventory of the yeast ABC proteins. *Nature Genet.* **15,** 137–145.

Decottignies, A., Grants, A. M., Nicholas, J. W., Wet, H. D., McIntosh, D. B. and Goffeau, A. (1998). ATPase and multidrug transport activities of the overexpressed yeast ABC protein Yor1p. *J. Biol. Chem.* **273,** 12,612–12,622.

Diaz, C. and Schroit, A. J. (1996). Role of translocases in the generation of phosphatidylserine asymmetry. *J. Memb. Biol.* **151,** 1–9.

Goffeau, A., Park, J., Paulsen, I. T., Jonniaux, J.-L., Dinh, T., Mordant, P. and Saier, M. H., Jr (1997). Multidrug-resistant transport proteins in yeast: complete inventory and phylogenetic characterization of the yeast open reading frames within the Major Facilitator Superfamily. *Yeast* **13,** 43–54.

Gow, N. A. R., Hube, B., Bailey, D. A., Schofield, D. A., Munro, C., Swoboda, R. K., Bertram, G., Westwater, C., Broadbent, I., Smith, R. J., Gooday, G. W. and Brown, A. J. P. (1994). Genes associated with dimorphism and virulence of *Candida albicans*. *Can. J. Bot.* **73** (Suppl.), s335–s342.

Herrmann, A. and Devaux, P. F. (1990). Alteration of the aminophospholipid translocase activity during *in vivo* and artificial aging of human erythrocytes. *Biochim. Biophys. Acta* **1027,** 41–46.

Higgins, C. F. (1992). ABC transporters: from microorganisms to man. *Ann. Rev. Cell Biol.* **8,** 67–113.

Hyde, S. C., Emsley, P., Hartshorn, M. J., Mimmack, M. M., Gileadi, U., Pearce, S. R., Gallagher, M. P., Gill, D. R., Hubbard, R. E. and Higgins, C. F. (1990). Structural model of ATP binding proteins associated with cystic fibrosis, multidrug resistance and bacterial transport. *Nature* **346,** 362–365.

Ibrahim, A. S. and Ghannoum, M. A. (1996). Chromatographic analysis of lipids. In Prasad R. (Ed.), *Manual on Membrane Lipids.* Springer-Verlag, Berlin/Heidelberg, pp. 52–79.

Jethwaney, D., Höfer, M., Khaware, R. K. and Prasad, R. (1997). Functional reconstitution of a purified proline permease from *Candida albicans*: interactions with the antifungal cispentacin. *Microbiology* **143,** 397–404.

Kaur, S., Mishra, P. and Prasad, R. (1988). Dimorphism-associated changes in intracellular pH of *Candida albicans*. *Biochim. Biophys. Acta* **972,** 277–282.

Kean, L. S., Grant, A. M., Angeletti, C., Mahe, Y., Kuchler, K., Fuller, R. S. and Nichols, J. W. (1997). Plasma membrane translocation of fluorescent-labeled phosphatidyl ethanolamine is controlled by transcription regulators, *PDR1* and *PDR3*. *J. Cell. Biol.* **138,** 255–270.

Koul, A. and Prasad, R. (1996). Extraction of membrane lipids. In Prasad, R. (Ed.), *Manual on Membrane Lipids.* Springer-Verlag, Berlin, pp. 37–52.

Lewis, K. (1994). Multidrug resistance pumps in bacteria: variation on theme. *Trends Biochem. Sci.* **19,** 119–123.

Prasad, R. (1991). *Candida albicans: Cellular and Molecular Biology.* Springer-Verlag, Berlin.

Prasad, R., Krishnamurthy, S., Prasad, R. and Gupta, V. (1995a). Multiple drug resistance in *Candida albicans*. *Acta Biochem. Polonica* **42,** 497–504.

Prasad, R., Worgifosse, P. D., Goffeau, A. and Balzi, E. (1995b). Molecular cloning and characterisation of a novel gene of C. albicans, *CDR1*, conferring multiple

resistance to drugs and antifungals. *Curr. Genet.* **27,** 320–329.

Prasad, R., Krishnamurthy, S., Prasad, R., Gupta, V. and Lata, S. (1996). Multidrug resistance: an emerging threat. *Curr. Sci.* **71,** 205–213.

Rawyler, A., Roelofsen, B. and Op den Kamp, J. A. F. (1984). The use of fluorescamine as a permeant probe to localize phosphatidylethanolamine in intact Friend erythroleukaemic cells. *Biochim. Biophys. Acta* **769,** 330–336.

Ruetz, S. and Gros, P. (1994). Phosphatidylcholine translocase: a physiological role for the *mdr2* gene. *Cell,* 1071–1081.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor Laboratory Press, New York.

Sanglard, D., Ischer, F., Monod, M. and Bille, J. (1997). Cloning of *Candida albicans* genes conferring resistance to azole antifungal agents: characterisation of *CDR2*, a new multidrug ABC transporter gene. *Microbiology* **143,** 405–416.

Schroit, A. J. and Zwaal, R. F. A. (1991). Transbilayer movement of phospholipids in red cell and platelet membranes. *Biochim. Biophys. Acta* **1071,** 313–329.

Tang, X., Halleck, M. S., Schlegel, R. A. and Williamson, P. (1996). A subfamily of P-type ATPases with aminophospholipid transporting activity. *Science* **272,** 1495–1497.

Utsugi, T., Schroit, A. J., Connor, J., Bucana, C. D. and Fidler, I. J. (1991). Elevated expression of phosphatidylserine in outer membrane leaflet of human tumor cells and recognition by activated human blood monocytes. *Cancer Res.* **51,** 3062–3066.

Van Helvoort, A., Smith, A. J., Sprong, H., Fritzche, I., Schinkel, A. H., Borst, P. and Van Meer, G. (1996). MDR1 P-glycoprotein is a lipid translocase of broad specificity, while MDR3 P-glycoprotein specifically translocates phosphatidylcholine. *Cell,* 87, 507–517.

Van den Bossche, H. (1995). Chemotherapy of human fungal infections. In Lyr, H. (Ed.), *Modern Selective Fungicides. Properties, Applications, Mechanisms of Action.* Gustav Fisher Verlag, Jena, pp. 431–484.

Walsh, T. J., Kasai, M., Francesconi, A., Landsman, D. and Chanock, S. J. (1997). New evidence that *Candida albicans* possesses additional ATP-binding cassette MDR-like genes: implications for antifungal azole resistance. *J. Med. Vet. Mycol.* **35,** 133–137.

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.

Williamson, P. and Schlegel, R. A. (1994). Back and forth: the regulation and function of transbilayer phospholipid movement in eukaryotic cells. *Mol. Memb. Biol.* **11,** 199–216.