Asymmetric Distribution of Aminophospholipids in Plasma membrane of C. albicans
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3.1.1. INTRODUCTION

Membrane phospholipids are asymmetrically distributed across the Plasma membrane (PM) of a variety of cells (Diaz and Schroit, 1996; Ohga et al., 1998; Bevers et al., 1999). Most of the PtdEtn and PtdSer are located on the inner leaflet of lipid bilayer while PtdCho and other lipids are predominantly localized on the outer half of the bilayer (Schroit and Zwaal, 1991; Schroit, 1998; Bevers et al., 1999). The asymmetrical distribution of membrane lipids is very specific and the loss of this asymmetry has been linked to various physiological processes such as procoagulant activity; removal of erythrocytes from blood, which is known to affect the shape of the cell; surface potential of membranes; and their interaction with each other etc. (Herrmann and Devaux, 1990; Utsugi et al., 1991; Fadok et al., 1992; Cerbón and Calderón, 1995; Tang et al., 1996; Kean et al., 1997).

One of the major problems, which arose due to irrational use of drugs in chemotherapy is Multi-Drug Resistance (MDR). Overexpression of a 170 kDa glycoprotein called P-glycoprotein (Pgp) is found to be involved in 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 (Hyde et al., 1990; Higgins, 1992; Prasad et al., 1996; Quentin et al., 1999; Klein et al., 1999; Bauer et al., 1999a). The completion of S. cerevisiae genome project has revealed that there are 33 putative ABC proteins, which are homologues of MDRs (Decottignies and Goffeau, 1997; Bauer et al., 1999a). The multidrug transporters in yeasts not only include members of the ABC family but also a family of Membrane Facilitators (MFS), which are probably antiporters (Balzi and Goffeau, 1994; Goffeau et al., 1997; Pao et al., 1998; Paulsen et al., 1998). In addition, regulatory networks of PDR (Pleiotropic Drug Resistance) genes, backed by transcription regulators, are also well characterized (Balzi and Goffeau, 1994; Balzi and Goffeau, 1995).

The studies involving MDR in yeast have attracted much attention, since they are good model systems and some of them are also pathogenic. C. albicans is a dimorphic and opportunistic human pathogen (Prasad, 1991; Gow et al., 1994; Staib et al., 2000), which is
naturally more resistant, to several drugs, than \textit{S. cerevisiae} (Prasad et al., 1996; White, 1997b). In recent years, the incidence of \textit{C. albicans} cells acquiring resistance to azoles and polyenes has increased alarmingly (Vanden Bossche, 1995; Prasad et al., 1996; White et al., 1998; Ha and White, 1999). Prasad et al cloned and sequenced a multidrug transporter, \textit{CDRI} (\textit{Candida} Drug Resistance), from \textit{C. albicans} which is a homologue of \textit{S. cerevisiae} multidrug efflux pump \textit{PDR5} (Prasad et al., 1995a; Balzi and Goffeau, 1995). A few more ABC transporters and homologues of \textit{CDRI} e.g. \textit{CDR2}, \textit{CDR3} and \textit{CDR4} have been identified recently in \textit{C. albicans} (Prasad et al., 1995a; Sanglard et al., 1997; Balan et al., 1997; Franz et al., 1998b). Many other genes specific to fluconazole and benomyl resistance e.g. \textit{CaMDRI} and \textit{FLU1} have also been characterized (Fling et al., 1991; Ben-Yaacov et al., 1994; Gupta et al., 1998; Calabrese et al., 1999) in \textit{C. albicans}.

Earlier human Mdr1p and Mdr3p and mouse mdr2p have been shown to have flippase activity which mediate phospholipid translocation directed towards exoplasmic leaflet of the lipid bilayer (Ruetz and Gros, 1994c; Van Helvoort et al., 1996; Bosch et al., 1997; Bevers et al., 1999). Among these human Mdr3p and mouse mdr2p are specific translocators of PtdCho as compared to human Mdr1p which has been shown to translocate broad range of short chain fluorescent lipids (Van Helvoort et al., 1996). \textit{MRP1} (Multidrug Resistance Protein), the so called GS-X pump, that transports drugs as glutathione conjugates has also been shown to be involved in translocation of fluorescent analogues of PtdEtn, PtdSer and sphingolipids across the lipid bilayer (Kamp and Haest, 1998; Dekkers et al., 1998a; Raggers et al., 1999b). Purified and reconstituted ABC transporter Lmr of \textit{Lactobacillus lactis} is another transporter, which is shown to mediate specifically the transbilayer movement of fluorescent PtdEtn (Margolles et al., 1999). Three other ABC transporters were recently shown to be involved in lipid transport: \textit{ABCR}, also called Rim protein, was shown to be defective in Stargardt's macular dystrophy; this protein probably transports a complex of retinaldehyde and PtdEtn in the retina of the eye (Borst et al., 2000). \textit{ABCI} was shown to be essential for the exit of cholesterol from cells and is probably a cholesterol transporter (Borst et al., 2000). The \textit{ALDp} family members, peroxisomal ATP-binding cassette transporters Pxa2/Pat1p (Pat) and Ssh2/Pal1/pxa1/pat2p of \textit{S. cerevisiae} which are highly homologous to the gene mutated in X-linked adermoleukodystrophy (X-ALD) are required for the import of activated fatty acids into peroxisomes (Bauer et al., 1999a; Hettema and Tabak, 2000).
Asymmetric distribution of phospholipids

Phospholipid translocase activity in yeast has also been demonstrated by Kean et al. (1997) where they have shown that translocation of PtdEtn in *S. cerevisiae* is controlled by *PDR1* and *PDR3* and identified two new alleles *tpel-1* (dominant) and *tpe2-1* (semidominant) for *PDR1* and *PDR3*, respectively (Kean et al., 1997). Pdr1p and Pdr3p are transcription regulators which control the expression of several genes including ABC transporters like *PDR5*, *YOR1* and *SNQ2* (Balzi et al., 1994; Decottignies et al., 1995; Balzi and Goffeau, 1995; Decottignies et al., 1998).

Asymmetrical distribution of phospholipids is not restricted to mammalian cells. Plasma membrane of *S. cerevisiae* cells are also asymmetric with regard to aminophospholipids and is controlled by an energy dependent aminophospholipid translocase (Balasubramanian and Gupta, 1996; Tang et al., 1996; Kean et al., 1997). The work embodied in this section deals specifically with determining the asymmetric distribution of aminophospholipids particularly PtdEtn in PM of C. albicans by using two molecular probes. It was observed from results that PM is asymmetric with regard to its PtdEtn distribution. Further it was observed that the expression of *CDRI*, a multidrug resistance ABC transporter gene affects the availability of PtdEtn in the outer half of PM, in an energy dependent process and presents evidence that Cdr1p, ABC multidrug transporter of *C. albicans* is a phosphatidylethanolamine translocator.

3.1.2. RESULTS

3.1.2.1. Labeling of plasma membrane PtdEtn of intact *C. albicans* and *S. cerevisiae* cells

To determine the distribution of aminophospholipids in membranes various techniques and probes are known, but among them trinitrobenzenesulfonic acid (TNBS) is the most commonly used one. 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 labeling the external PtdEtn of yeast PM (Balasubramanian and Gupta, 1996). It was evident from Figure 12 & 13, that both the probes were equally efficient in labeling the PtdEtn of outer leaflet of 10261 Candida cells. Under experimental conditions, it was observed that these two probes were impermeable to yeast cells and could only label the external PtdEtn. If cells were
Figure 12. Time and concentration dependent percentage PtdEtn labeling of plasma membrane of *C. albicans* ATCC 10261 using trinitrobenzenesulfonic acid (TNBS). TNBS labeling was done as described in 'Materials and Methods'. Briefly, mid log phase *C. albicans* cells were harvested by centrifuging at 3000 rpm for 5 min at 4° C. The cells were washed two to three times with buffer A (100 mM potassium phosphate 5 mM EDTA, pH 7.5). The harvested cells (0.6 gm wet wt.) 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 different concentrations of TNBS in buffer B was added to the cell suspension. 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 to three times with buffer C till the colour of free TNBS disappeared from the supernatant. Derivatized or labeled PtdEtn and other phospholipids were extracted and quantitated as mentioned in 'Materials and Methods'. The inset Figure depicts the time dependent PtdEtn labeling of ATCC 10261 cells with 20 mM concentration of TNBS. The values shown are the mean of more than three independent experiments and the bar shows the ±SD values.
Figure 13. Concentration dependent percentage PtdEtn labeling of plasma membrane of *C. albicans* ATCC 10261 using fluorescamine. fluorescamine labeling was done as described in ‘Materials and Methods’. Briefly, mid log phase *C. albicans* cells were harvested by centrifuging at 3000 rpm for 5 min at 4°C. Cells (0.6 gm wet wt.), were suspended in 3 ml of buffer B (100 mM potassium phosphate and 600 mM KCl, pH 8.2). Indicated concentrations of fluorescamine in dehydrated DMSO were added drop wise to the cell suspension with constant gentle swirling. After 30 sec 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 as described in ‘Figure1’ three to four times till the colour of the dye disappeared from the supernatant. Derivatized or labeled PtdEtn and other phospholipids were extracted and quantitated as mentioned in ‘Materials and Methods’. The inset Figure depicts the PtdEtn and PtdSer labeling of ATCC 10261 cells at 15.6 mM concentration of fluorescamine under permeabilized conditions. The values shown are the mean of more than three independent experiments and the bar shows the ±SD values.
permeabilized, the same reagents could label up to 85% of the aminophospholipids, including PtdSer (it was not labeled in normal cells by either of the reagents, since most of PtdSer is localized in the cytoplasmic leaflet) (Figure 13, inset). The suitability of these two probes is observed for other yeast strains also (Table 14A & B and Figure 14A & B) and it was observed that similar to C. albicans cells, these two probes could effectively label other yeast cells and their transformants. The results of present study thus confirms that TNBS and fluorescamine can be used to label PtdEtn of C. albicans and S. cerevisiae cells.

3.1.2.1.1. Time and concentration dependent labeling
It was observed from Figure 12 & 13 that PtdEtn could be labeled by both TNBS and fluorescamine probes in a concentration dependent manner. In purified PM of C. albicans, out of total phospholipids present, ~15% was PtdEtn (Table 14A) and only ~4% of it was labeled with both the aminophospholipid labeling reagents in intact cells. It would mean that out of total PtdEtn in non-permeabilized C. albicans cells only ~4% is available for external labeling and the rest (~96%) is localized in the inner leaflet of PM. It must be pointed out that about 10-15 hrs were required for TNBS (20 mM) labeling (Figure 12, inset) while fluorescamine (15.6 mM) could label the aminophospholipid in 30 seconds. Since the labeling 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).

3.1.2.2. PtdEtn translocation between two monolayers of plasma membrane 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 15(A, B & C), depicts labeling of PtdEtn in presence of these inhibitors respectively. Sodium azide, which is a mitochondrial ATP synthesis inhibitor, and sodium orthovanadate, which is a typical inhibitor of PM-ATPase, if added prior to labeling of PtdEtn with fluorescamine, resulted in reduced labeling. The concentrations of sodium azide, sodium orthovanadate and NEM which showed maximum inhibition in PtdEtn labeling with fluorescamine was used for
Table 14. Percentage of total and labeled PtdEtn in cells of host strains and in their transformants of Candida albicans (A) and Saccharomyces cerevisiae (B). Fluorescamine labeling was done as described in ‘Materials and Methods’ and in ‘figure 2’. Mid log phase cells were harvested at 4°C and cells (0.6 gm wet wt.), were suspended in 3 ml of buffer B (100 mM potassium phosphate and 600 mM KCl, pH 8.2). 15.6 mM of fluorescamine in dehydrated DMSO was then added drop wise to the cell suspension with constant gentle swirling. After 30 sec the reaction was stopped and cells were washed till the colour of the dye disappeared from the supernatant. Derivatized or labeled PtdEtn and other phospholipids were extracted and quantitated as mentioned in ‘Materials and Methods’ and in ‘figure 2’. The values shown are the mean of 15-20 independent experiments and the bar shows the ±SD values. NC34°: transformant carrying CaMDRI (MFS type) gene of C. albicans (Gupta et al., 1998).
Figure 14. Percentage labeling of PtdEtn of plasma membrane of *S. cerevisiae* cells using fluorescamine. Fluorescamine labeling in cells of JG436 and its transformants S-12, NC34 (A) and in AD and its transformant AD-CDR1 (B) was done as described in ‘Figure 2’ and in ‘Materials and Methods’ using 15.6 mM concentration of fluorescamine. After labeling extraction of derivatized or labeled PtdEtn and other lipids was done as mentioned in ‘Materials and Methods’. These lipids were then separated on Thin layer chromatography (TLC) plate. lipids were then scrapped off and quantified as described in ‘Materials and Methods’. The values shown are the mean of more than three independent experiments and the bar shows the ±SD values.
Figure 15. Concentration dependent inhibition by various inhibitors on PtdEtn labeling of plasma membrane of *C. albicans* ATCC10261 cells. To study the effect of different concentrations of various inhibitors on PtdEtn labeling of plasma membrane, mid log phase cells of ATCC 10261 were harvested, washed with water and a 5% cell suspension was made in YEPD (Yeast extract peptone dextrose) media. In order to see the effect of various inhibitors, 5% cell suspension was incubated with indicated concentrations of various inhibitors (A) sodium azide for 2 hr, (B) sodium orthovanadate for 30 min and (C) NEM for 10 min. After incubation with inhibitors, cells were subjected to fluorescamine labeling as described in ‘Figure 2’ and in ‘Materials and Methods’. Derivatized or labeled PtdEtn and other phospholipids were extracted and separated on TLC plate as described in ‘Materials and Methods’. These lipids were then scrapped off and quantitated as mentioned in ‘Materials and Methods’. The values shown are the mean of more than three independent experiments and the bar shows the ±SD values.
Asymmetric distribution of aminophospholipids.

subsequent experiments [Figure 15(A, B & C)]. Almost 58-80% labeling of PtdEtn was inhibited by 10 mM of azide and 20 mM of vanadate (Figure 16). NEM (10 mM) which blocks free thiol groups of proteins and thus prevents protein mediated processes, when added before labeling, was also able to block PtdEtn labeling. If cells were starved of glucose, a time dependent effect of starvation was observed in PtdEtn labeling (Figure 17). At 14 hrs of depletion of cellular ATP, the labeling was maximally reduced by 60% (Figure 17). However, if cells were repleted with glucose, the total labeling of PtdEtn was again restored within 6 hrs. These results pointed out that there might be an energy dependent aminophospholipid translocase in C. albicans, which controls the movement of PtdEtn across the two monolayers (Figure 17). It must be mentioned that total phospholipid composition of C. albicans, determined under various experimental conditions did not vary significantly (Table 15).

3.1.2.3. Expression of CDRI is linked to PtdEtn translocation

In order to ascertain if CDRI expression could contribute in PtdEtn translocation, S. cerevisiae transformant S-12 which was expressing CDRI was used to examine the PtdEtn distribution (Prasad et al., 1995a). Interestingly, in S-12 strain out of total PtdEtn, almost 24% of it was labeled by both fluorescamine and TNBS as compared to its host strain JG436 (~19%) lacking CDRI and also having disrupted PDR5, which is a homologue of CDRI (Figure 14A and Table 14B). That CDRI could be involved in PtdEtn translocation was further confirmed when another transformant of S. cerevisiae (NC34) which was expressing a non ABC transporter BENr (benomyl resistance) like gene, a membrane facilitator (Ben-Yaacov et al., 1994; Gupta et al., 1998) was checked for PtdEtn localization. It was observed that ~19% of PtdEtn was labeled in NC34 as in its host strain JG436 (Figure 14A and Table 14B). It must be pointed out that only 4% of total PtdEtn could be labeled in C. albicans 10261 cells, while 18-24 % of it was labeled in S. cerevisiae strains.

S. cerevisiae strain S-12 having disrupted PDR5, is known to harbor several other ABC transporters, which might mask the function of Cdr1p (Decottignies and Goffeau, 1997). S. cerevisiae (AD1234568) which was called as AD 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
Figure 16. Effect of various inhibitors on PtdEtn labeling of plasma membrane of *C. albicans* ATCC10261 cells. To study the effect of various inhibitors on PtdEtn labeling of plasma membrane, mid log phase cells of ATCC 10261 were harvested, washed with water and a 5% cell suspension was made in YEPD (Yeast extract peptone dextrose) media. In order to see the effect of various inhibitors, 5% cell suspension was incubated with indicated concentrations of various inhibitors (A) sodium azide for 2 hr, (B) sodium orthovanadate for 30 min and (C) NEM for 10 min. After incubation with inhibitors cells were subjected to fluorescamine labeling as described in ‘Figure 2’ and in ‘Materials and Methods’. Labeled or derivatized PtdEtn and other phospholipids were then extracted and quantitated as described in ‘Materials and Methods’. The values shown are the mean of more than three independent experiments and the bar shows the ±SD values.
Figure 17. Effect of depletion and repletion of cellular ATP on PtdEtn labeling of plasma membrane of *Candida albicans* ATCC 10261. To study the effect of energy depletion and repletion on PtdEtn labeling of plasma membrane, mid log phase cells of ATCC 10261 were harvested, washed with water and then a 5% cell suspension was made in YEPD (Yeast extract Peptone Dextrose) media without glucose. A portion of glucose starved cells were then subjected to fluorescamine labeling at indicated time intervals. Another portion of glucose starved cells was harvested after 14 hrs and then incubated in YEPD (Yeast extract Peptone Dextrose) media with glucose. These ATP repleted cells were then subjected to fluorescamine labeling. Fluorescamine labeling was done as described in ‘Figure 2’ and in ‘Materials and Methods’. Labeled or derivatized PtdEtn and other phospholipids were then extracted and quantitated as mentioned in ‘Materials and Methods’. The values shown are the mean of more than three independent experiments and the bar shows the ±SD values.
<table>
<thead>
<tr>
<th>Strain</th>
<th>Treatment with various inhibitors</th>
<th>%PtdEtn</th>
<th>%PtdCho</th>
<th>%PG</th>
<th>%PtdSer</th>
<th>%PI</th>
</tr>
</thead>
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<tr>
<td>10261</td>
<td>Control</td>
<td>16.3±0.21</td>
<td>39.6±0.32</td>
<td>10.44±0.66</td>
<td>13.36±0.01</td>
<td>9.0±0.44</td>
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<td>10261</td>
<td>Sodium azide</td>
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<tr>
<td>10261</td>
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<td>12.11±0.45</td>
<td>9.11±0.46</td>
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<tr>
<td>10261</td>
<td>NEM</td>
<td>16.78±0.32</td>
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<td>11.1±0.56</td>
<td>13.1±1.44</td>
<td>9.89±1.0</td>
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</table>

Table 15. Percentage of different phospholipids in 10261 cells of *C. albicans* before and after treatment with various inhibitors. Cells were treated with various inhibitors (Sodium azide, Sodium orthovanadate and NEM as described in ‘Figure 5’) and then labeled with fluorescamine. Labeled or derivatized PtdEtn and other phospholipids were then extracted and the percentage of each phospholipid was determined as described in ‘Materials and Methods’. PtdEtn: Phosphatidylethanolamine, PtdCho: Phosphatidylcholine, PtdSer: Phosphatidylserine, PG: Phosphatidylglycerol, PI: Phosphatidylinositol. The values shown are the mean of more than three independent experiments and the bar shows the ±SD values.
Asymmetric distribution of aminophospholipids.

resistance gene), ycf1 (yeast cadmium factor gene) and yor1 (yeast oligomycin resistance gene) were disrupted (Decottignies et al., 1998) was used under study. AD strain was transformed with the CDR1 gene (Prasad et al., 1995a) and the resulting transformant was designated as AD-CDR1. Both the strains were subjected to fluorescamine labeling. The labeling of PtdEtn was about 24% in AD-CDR1, as compared to its host strain AD where only 13-14% of PtdEtn could be labeled with fluorescamine (Figure 14B and Table 14B).

Similar to C. albicans cells [Figure 15(A, B & C) & Figure 16], the translocation of PtdEtn mediated by CDR1 was energy dependent as the labeling with fluorescamine was inhibited by azide and vanadate [Figure 18(A, B & C) & Figure 19]. NEM could also prevent the translocation of this aminophospholipid in AD-CDR1 strain (Figure 18C & Figure 19). ATP depletion of cells clearly prevented the translocation of PtdEtn, which could be restored upon its repletion (Figure 20).

3.1.2.4. PtdEtn translocation from inner to outer leaflet IN CDR1 and CDR2 disruptants

To further check the involvement of CDR1, PtdEtn labeling was done in disruptants of CDR1 and CDR2. A homozygous disrupted CDR1 strain of C. albicans DSY449 was used to check if the nonavailability of functional Cdr1p could affect the availability of external PtdEtn (Sanglard et al., 1997). It is evident from Figure 21 and Table 16 that DSY449 had 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 (Figure 21 and Table 16) suggesting the involvement of both the ABC transporters in PtdEtn translocation.

3.1.2.5. Morphological regulation of aminophospholipid translocase activity

In order to investigate if the two morphological form of C. albicans which show different level of expression of CDR1, could also show difference in accessibility of PtdEtn, both bud and mycelia cells were labeled with fluorescamine and TNBS. Bud and mycelia were formed by a pH dependent protocol where the two forms were clearly distinguishable from each other (Figure 22). As can be seen from Figure 23A, that CDR1 transcript was higher in mycelial form than in the bud form of C. albicans (Figure 23A & 23B). Interestingly, PtdEtn
Figure 18. Concentration dependent inhibition by various inhibitors on PtdEtn labeling of plasma membrane of *S. cerevisiae* cells of AD and its transformant AD-CDR1. To study the effect of different concentrations of various inhibitors on PtdEtn labeling of plasma membrane, mid log phase cells of AD, AD-CDR1 were harvested, washed with water and a 5% cell suspension was made in YNB (Yeast extract nitrogen base) media. In order to see the effect of various inhibitors, 5% cell suspension was incubated with indicated concentrations of various inhibitors (A) sodium azide for 2 hr, (B) sodium orthovanadate for 30 min and (C) NEM for 10 min. After incubation with inhibitors cells were subjected to fluorescamine labeling as described in ‘Figure 2’ and in ‘Materials and Methods’. Labeled or derivatized PtdEtn and other phospholipids were then extracted and quantitated as described in ‘Materials and Methods’. The values shown are the mean of more than three independent experiments and the bar shows the ±SD values.
Figure 19. Effect of various inhibitors on PtdEtn labeling of plasma membrane of S. cerevisiae AD-CDR1 cells. To study the effect of various inhibitors on PtdEtn labeling of plasma membrane, mid log phase cells of AD and AD-CDR1 were harvested, washed with water and a 5% cell suspension was made in YNB (Yeast nitrogen base) media. In order to see the effect of various inhibitors, 5% cell suspension was incubated with indicated concentrations of various inhibitors (A) sodium azide for 2 hr, (B) sodium orthovanadate for 30 min and (C) NEM for 10 min. After incubation with inhibitors cells were subjected to fluorescent labeling as described in Figure 2' and in 'Materials and Methods'. Labeled or derivatized PtdEtn and other phospholipids were then extracted and quantitated as described in ‘Materials and Methods’. The values shown are the mean of more than three independent experiments and the bar shows the ±SD values.
Figure 20. Effect of depletion and repletion of cellular ATP on PtdEtn labeling of plasma membrane of *S. cerevisiae* AD and AD-CDR1 cells. To study the effect of energy depletion and repletion on PtdEtn labeling of plasma membrane, mid log phase cells of AD and AD-CDR1 were harvested, washed with water and then a 5% cell suspension was made in YNB (Yeast extract nitrogen base) media without glucose. A portion of glucose starved cells were then subjected to fluorescamine labeling at indicated time intervals. Another portion of glucose starved cells was harvested after 14 hrs and then incubated in YNB (Yeast extract nitrogen base) media with glucose. These ATP repleted cells were then subjected to fluorescamine labeling. Fluorescamine labeling was done as described in 'Figure 2' and in 'Materials and Methods'. Labeled or derivatized PtdEtn and other phospholipids were then extracted and quantitated as mentioned in ‘Materials and Methods’. The values shown are the mean of more than three independent experiments and the bar shows the ±SD values.
Figure 21. Percentage labeling of PtdEtn of plasma membrane of *Candida albicans* cells using fluorescamine. Fluorescamine labeling in cells of CAF2-1 and in its disruptants DSY449, DSY1025 was done as described in ‘Figure 2’ and in Materials and Methods’ using 15.6 mM concentration of fluorescamine. After labeling extraction of derivatized or labeled PtdEtn and other lipids was done as mentioned in ‘Materials and Methods’. Extracted lipids were then quantified as described in ‘Materials and Methods’. The values shown are the mean of more than three independent experiments and the bar shows the ±SD values.

<table>
<thead>
<tr>
<th>STRAINS</th>
<th>GENOTYPE</th>
<th>Percentage PtdEtns of total phospholipids</th>
<th>Percentage labeled PtdEtn of total PtdEtn</th>
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<tbody>
<tr>
<td>CAF2-1</td>
<td>Δura3::imm434/URA3</td>
<td>22.62 ± 0.05</td>
<td>Fluorescamine: 7.33 ± 0.11</td>
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<tr>
<td>DSY449</td>
<td>Δcdr1::hisG/Δcdr1::hisG</td>
<td>22.64 ± 1.33</td>
<td>Fluorescamine: 5.68 ± 0.18</td>
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<tr>
<td>DSY1025</td>
<td>Δcdr1::hisG/Δcdr1::hisG, Δcdr2::hisG/Δcdr2::hisG</td>
<td>22.90 ± 0.41</td>
<td>Fluorescamine: 4.4 ± 0.26</td>
</tr>
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</table>

Table 16. Percentage of total and labeled PtdEtn in cells of host strain CAF2-1 and in its disruptants of *Candida albicans*. Labeling of cells with fluorescamine was done as mentioned in ‘Materials and Methods’. Derivatized or labeled PtdEtn and other phospholipids were extracted and were separated on TLC plate using two different solvents as described in ‘Materials and Methods’. These lipids were then scrapped off and quantitated as mentioned in ‘Materials and Methods’. The values shown are the mean of 15-20 independent experiments and the bar shows the ±SD values.
Figure 22. Photographs depicting the bud and mycelia form. Bud and mycelia form were generated by a procedure described in ‘Materials and Methods’. Briefly, *C. albicans* 10261 cells were maintained on a nutrient agar slants at 4 °C. For induction of mycelia cells were inoculated into 25 ml of Lee’s media rich in amino acids (pH 6.8) with 0.2 μM zinc sulfate and were grown at 25 °C for 48 hr to stationary phase of growth. After 48 hrs late log phase cells at a sphere concentration of roughly $5 \times 10^7$ cells/ml were diluted into a new flask at an initial concentration of $2 \times 10^5$ cells/ml in a zinc sulfate-less Lee's media and were incubated up to stationary phase for 48 hr at 25 °C resulting in a final concentration of $2.5 \times 10^8$ cells/ml. One half ml of stationary phase cells were sonicated at a power of 5 microns peak to peak amplitude using a MSE ultrasonicator at 2-4 °C for 1 min and then inoculated into two 500 ml plastic Erlenmeyer flasks each containing 300 ml nutrient media: one at pH 4.5 and another at pH 6.5 at 37 °C in a shaker at 200 rpm. Samples were removed at different time intervals and checked for the cell divergence under microscope. By this method, the cells grown at pH 4.5 and 6.5 were exclusively differentiated as ellipsoidal buds or as elongated hyphae, respectively.
Figure 23. PtdEtn labeling and mRNA expression in bud and mycelial forms. (A) levels of CDRI transcript in bud and mycelial forms. (B) The normalized value of CDRI transcript after considering ACTI (actin) as internal control. Northern analysis and RNA isolation was done as described in ‘Materials and Methods’. (C) Percentage labeling of PtdEtn with fluorescamine (15.6) in bud and mycelial forms. The bud and mycelial forms were formed by pH-regulated dimorphism, as described in ‘Materials and Methods’ and ‘Figure’. The fully differentiated population of cells as bud and mycelial forms were taken for Northern and lipid analysis. Fluorescamine labeling was done as described in ‘Materials and Methods’ and ‘figure 2’. Extraction and quantification of phospholipids were done as mentioned in ‘Materials and Methods’. The values are the mean of three independent readings ± S.D.
in the outer leaflet of membrane, was also found to be much higher in the mycelial form as compared to bud form (Figure 23C). It is pertinent to mention that there was no significant change in phospholipid composition including that of PtdEtn between bud or mycelial forms of \textit{C. albicans} cells. Thus the expression of \textit{CDR}1 appears to be linked to PtdEtn translocation.

3.1.2.6. PtdEtn labeling in azole resistant and sensitive \textit{C. albicans} clinical isolates and its relation with \textit{CDR}1 expression

To further correlate the results of \textit{CDR}1 expression and exposure of PtdEtn on outer leaflet of plasma membrane, a PtdEtn labeling study is done in several azole resistant and sensitive clinical isolates having differential \textit{CDR}1 expression. The azole resistant strains YI.552, YI.547, YI.549, showed high expression of \textit{CDR}1 (Figure 24) and an increased PtdEtn labeling was also observed as compared to azole sensitive isolate YI.344 (Figure 25) having low expression of \textit{CDR}1 (Figure 13). At the same time another azole sensitive isolate which has low level of \textit{CDR}1 expression (YI.456) also showed equal or more PtdEtn labeling as azole resistant strains (Figure 24 & Figure 25). No direct correlation between \textit{CDR}1 expression and PtdEtn labeling could be observed from this set of isolates. It is pertinent to mention here that there are several ABC transporters of \textit{C. albicans} (\textit{Candida} genome project suggests about 29 ABC transporters (http://alces.med.umn.edu/candida.html) which could contribute in maintaining PtdEtn asymmetry in cells. Percentage composition of other lipids was not significantly different between different isolates. (Table 17).

3.1.3. DISCUSSION

Results presented in this chapter demonstrated that PM of \textit{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 labeling 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 (Balasubramanian and Gupta, 1996;
Figure 24. Expression of *CDR1* in different azole resistant and sensitive clinical isolates of *C. albicans*. Northern blot analysis of total RNA from different clinical isolates of *C. albicans* was carried out. Cells were harvested at 10 hr of growth and total RNA was isolated as described in ‘Materials and Methods’. The gel load (25S) rRNA is shown for these clinical isolates. R: azole resistant isolate; S: azole sensitive isolate.

Figure 25. Percentage labeling of plasma membrane PtdEtn of azole resistant and sensitive *C. albicans* clinical isolates using fluorescamine. Fluorescamine labeling was done as described in ‘Material and Methods’ and in ‘Figure 2’. Mid log phase *C. albicans* cells were harvested at 4°C and cells (0.6 gm wet wt.), were suspended in 3 ml of buffer B (described in ‘Figure1’). 15.6 mM of fluorescamine in dehydrated DMSO was then added drop wise to the cell suspension with constant gentle swirling. After 30 sec the reaction was stopped and cells were washed till the colour of the dye disappeared from the supernatant. Derivatized or labeled PtdEtn and other phospholipids were extracted and quantitated as mentioned in ‘Materials and Methods’. The values shown are the mean of more than three independent experiments and the bar shows the ±SD values.
Table 17. Percentage composition of derivatized or labeled PtdEtn and of other phospholipids in azole resistant (R) and sensitive (S) C. albicans clinical isolates. To study the labeled PtdEtn percentage and composition of other lipids, Fluorescamine labeling was done as described in ‘Material and Methods’ and in ‘Figure 2’. Briefly, mid log phase C. albicans cells were harvested at 4°C and cells (0.6 gm wet wt.) were suspended in 3 ml of buffer B (100 mM potassium phosphate and 600 mM KCl, pH 8.2). 15.6 mM of fluorescamine in dehydrated DMSO was then added drop wise to the cell suspension with constant gentle swirling. After 30s the reaction was stopped and cells were washed till the colour of the dye disappeared from the supernatant. Derivatized or labeled PtdEtn and other phospholipids were extracted as mentioned in ‘Materials and Methods’. These lipids were then analyzed on TLC plate and then each lipid was scrapped off and quantitated as described in ‘Materials and Methods’. The values shown are the mean of more than three independent experiments and the bar shows the ±SD values. PG: Phosphatidyl glycerol; PtdEtn: phosphatidylethanolamine; PtdCho: Phosphatidylinositol; PI: Phosphatidylinositol.
Asymmetric distribution of aminophospholipids.

Tang et al., 1996; Kean et al., 1997; Decottignies et al., 1998; Hettema and Tabak, 2000). However, the presence of such translocase was not demonstrated in those reports. Our results for the first time confirm that floppase activities could be a common phenomenon in lower eukaryotes.

Evidence is presented to demonstrate that \textit{CDRI}, which is a homologue of \textit{PDR5}, has floppase activity, which translocates PtdEtn to the outer leaflet of membrane. Firstly, there is an energy dependent movement of PtdEtn in \textit{S. cerevisiae} transformants, which express \textit{CDRI} of \textit{C. albicans}. This translocation was even more evident when \textit{CDRI} was expressed in AD strain of \textit{S. cerevisiae} (AD-\textit{CDR1}) which was deleted in seven ABC transporters. Interestingly, the host AD strain, showed some reduction in fluorescamine labeling after treatment with energy inhibitors and also after depletion of ATP (Figure 18A B & C, Figure 19 & Figure 20). This could be due to the presence of other yet unidentified phospholipid translocators in \textit{S. cerevisiae} including the known translocator Drs2p which is a P-type transport ATPase involved in aminophospholipids translocation (Tang et al., 1996; Marx et al., 1999). Although involvement of \textit{DRS2} in aminophospholipids translocation is reputed (Siegmund et al., 1998; Chen et al., 1999). According to some groups it is involved whereas according to others it is not involved in aminophospholipids translocation. Secondly, the involvement of Cdr1p in phospholipid translocation was further evident from the fact that increased amount of PtdEtn in the outer leaflet of PM of mycelial form correlated well with \textit{CDRI} expression, which was also more pronounced in mycelial form than in bud form of \textit{C. albicans}. The decrease in the availability of PtdEtn in the outer half of PM of a homozygous \textit{CDRI} disruptant also suggests that it might be involved in PtdEtn translocation. It is interesting to note that similar to mammalian MDRs e.g. \textit{MDR1} and \textit{MDR2}, \textit{CDR2}, a homologue of \textit{CDRI} may also contribute to phospholipid translocation. However, this aspect remains to be investigated. It was further confirmed when \textit{S. cerevisiae} transformant expressing a non ABC transporter (\textit{CaMDRI}), of \textit{C. albicans} was found to have no affect on PtdEtn distribution pattern between the two leaflets.

Results therefore suggest that the PtdEtn translocation is linked to an ABC transporter Cdr1p of \textit{C. albicans}. Whether Cdr1p, like human Mdr1p, is a general phospholipid translocator or specific to aminophospholipids remains to be resolved (see Chapter 2) (Ruetz and Gros, 1994c; Van Helvoort et al., 1996; Bosch et al., 1997). As mentioned earlier the contribution
of other ABC transporter like *CDR3* (Balan *et al.*, 1997; White, 1997) in *C. albicans* and of other yet uncharacterized transporters which may be involved in phospholipid translocation, can not be excluded from these results. What is the mechanism of the movement of phospholipid from outer to cytoplasmic leaflet in *C. albicans* cells and how many proteins control it, are some of the questions which remain to be resolved (see Chapter 2).

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