Appendices
A novel catalytic mechanism for ATP hydrolysis employed by the N-terminal nucleotide-binding domain of Cdr1p, a multidrug ABC transporter of Candida albicans

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A R T I C L E  I N F O

Article history:
Received 3 January 2008
Received in revised form 9 April 2008
Accepted 21 April 2008
Available online 2 May 2008

Keywords:
ATP binding cassette
ATP hydrolysis
Fluorescence resonance energy transfer
Multidrug resistance
Nucleotide-binding domain

A B S T R A C T

Although essentially conserved, the N-terminal nucleotide-binding domain (NBD) of Cdr1p and other fungal transporters has some unique substitutions of amino acids which appear to have functional significance for the drug transporters. We have previously shown that the typical Cys193 in Walker A as well as Trp326 and Asp327 in the Walker B of N-terminal NBD (NBD-512) of Cdr1p has acquired unique roles in ATP binding and hydrolysis. In the present study, we show that due to spatial proximity, fluorescence resonance energy transfer (FRET) takes place between Trp326 of Walker B and MIANS (2-(4-maleimidoanilino)naphthalene-6-sulfonic acid) on Cys193 of Walker A motif. By exploiting FRET, we demonstrate how these critical amino acids are positioned within the nucleotide-binding pocket of NBD-512 to bind and hydrolyze ATP. Our results show that both Mg2+ coordination and nucleotide binding contribute to the formation of the active site. The entry of Mg2+ into the active site causes the first large conformational change that brings Trp326 and Cys193 in close proximity to each other. We also show that besides Trp326, typical Glu238 in the Q-loop also participates in coordination of Mg2+ by NBD-512. A second conformational change is induced when ATP, but not ADP, docks into the pocket. Ast328 does sensing of the γ-phosphate of the substrate in the extended Walker B motif, which is essential for the second conformational change that must necessarily precede ATP hydrolysis. Taken together our results imply that the uniquely placed residues in NBD-512 have acquired critical roles in ATP catalysis, which drives drug extrusion.

1. Introduction

In the human pathogenic yeast Candida albicans, overexpression of the drug efflux pump encoding genes CDRI and CDRI2 belonging to the ABC [1–7] and CaM6R1 belonging to MFS family of transporters is one of the principal mechanisms ofazole resistance [8–10]. Among ABC transporters, Cdr1p has emerged as a major drug efflux protein involved in azole resistance [6,11]. Thus, Cdr1p has not only acquired significant clinical importance but is also considered an important target in any design of strategies to combat antifungal resistance [12].

Cdr1p, like other members of the ABC superfamily, has four distinct modules: two TMDs consisting of six transmembrane segments and two NBDs located on the cytosolic side of the membrane. The NBDs, which couple energy of ATP hydrolysis to power drug export are highly conserved throughout the evolutionary scale [2,11]. Each NBD contains three characteristic motifs: Walker A and Walker B motifs, which form the nucleotide-binding site [13], and an ABC signature sequence, or C motif, for which several functions have been proposed, including communication between the TMDs and NBDs during the transport cycle [14].

Currently, there is very limited information regarding the structural architecture of NBDs of ABC proteins. For many ABC transporters there is evidence that the two NBDs, although highly similar in sequence, may adopt different functional roles in the transport cycle [15,16]. A high-resolution crystal structure for the NBD subunit of histidine permease (HisP) has its two catalytic sites facing away from each other in the crystal dimer [17]. Jones and George, on the other hand, proposed a model of interaction between the two NBDs in which the Signature motif of one of the NBDs is directly involved in ATP binding by the other and is important for catalysis [18]. A similar
structure was proposed for the catalytic domain of Rad50 [1]. In human P-gp, a close homologue of Cdr1p, the issue of functional asymmetry of the two NBDs remains contentious with proposals both in favor [19,20] and against [21–23] functional asymmetry.

In contrast to most ABC transporters, the NBDs of fungal transporters, including Cdr1p, have unique positioning of a typical Cys193 in Walker A of N-terminal NBDs as well as Trp326 and Asn328 in and adjacent to Walker B motifs, respectively [24]. Thus the otherwise much conserved N-terminal NBDs of fungal transporters have the distinction of unique positioning of typical amino acid residues. On the other hand, the C-terminal NBD of Cdr1p and other ABC fungal transporters possess perfectly conserved motifs, which are essentially identical to those in non-fungal transporters as well [24].

Based on sequence analyses and these unique substitutions, it was earlier suggested that the N-terminal NBD of Pdr5p from S. cerevisiae is probably unable to perform ATP hydrolysis and that the transporter might function with only one of its two NBDs [24]. Since Pdr5p is a close homologue of Cdr1p, we expected that such a model, by extension, would be applicable to Cdr1p as well. However, we found that the NBDs in Cdr1p were functionally asymmetric and the swapping of NBDs resulted in non-functional Cdr1p chimeras, suggesting that the two NBDs were essential and non-exchangeable [25]. Further, by purifying the N-terminal NBD of Cdr1p in isolation, and generating several mutants, we have recently shown in a series of papers that not only is this domain capable of ATPase activity, but its unique amino acid substitutions also figure in this functioning.

To begin defining the functional significance of the conserved substitutions in N-terminal NBD of Cdr1p, we demonstrated that replacement of the unique Cys193 with Ala gravely impaired ATP hydrolysis without affecting its ability to bind the nucleotide [26,27]. On the other hand, substitution of Trp326 with Ala resulted only in the loss of ATP binding [28]. Substitution of the highly conserved, putative catalytic residue, Asp327 with Asn yielded a mutant variant protein with strongly impaired ATPase activity but comparable nucleotide binding to that of the wild type protein. Thus Asp327 of Cdr1p, unlike in other non-fungal ABC transporters, is a catalytic base and is unlikely to be involved in Mg\textsuperscript{2+} coordination [28,29]. In each case, we backed up results obtained from domain analysis by making the same binding to that of the wild type protein. Thus Asp327 of Cdr1p, unlike in other non-fungal ABC transporters, is a catalytic base and is unlikely to be involved in Mg\textsuperscript{2+} coordination [28,29]. In each case, we backed up results obtained from domain analysis by making the same binding to that of the wild type protein.

The current paper deals with the mechanistic details of how the N-terminal NBD of Cdr1p actually binds and hydrolyzes ATP. Using FRET we show that there are two distinct conformational changes in this domain that correspond to Mg\textsuperscript{2+} coordination and ATP binding, respectively, and which are both important for the subsequent ATP hydrolysis activity.

### Table 1

<table>
<thead>
<tr>
<th>Name</th>
<th>Description</th>
<th>Phenotype</th>
</tr>
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<tbody>
<tr>
<td>SJGN1-8</td>
<td>BL21(DE3)pLysS cells carrying the pSJGN1-8 plasmid</td>
<td>Wild type functional and active</td>
</tr>
<tr>
<td>VRGN W326A</td>
<td>SJG1-8p cells carrying the pVRGN W326A plasmid</td>
<td>Decrease in ATP binding but can hydrolyze ATP with high K_M and V_max values</td>
</tr>
<tr>
<td>VRGN D327A</td>
<td>SJG1-8p cells carrying the pVRGN D327A plasmid</td>
<td>Loss in ATP hydrolysis</td>
</tr>
<tr>
<td>VRGN D327N</td>
<td>SJG1-8p cells carrying the pVRGN D327N plasmid</td>
<td>Loss in ATP hydrolysis</td>
</tr>
<tr>
<td>VRGN CW4</td>
<td>SJG1-8p cells carrying the pVRGN CW4 plasmid</td>
<td>Functional like wild type</td>
</tr>
<tr>
<td>VRGN E328A</td>
<td>SJG1-8p cells carrying the pVRGN E328A plasmid</td>
<td>Decrease in ATP binding but can hydrolyze ATP with high K_M and V_max values</td>
</tr>
<tr>
<td>VRGN E328Q</td>
<td>SJG1-8p cells carrying the pVRGN E328Q plasmid</td>
<td>Functional albeit to a moderate level</td>
</tr>
<tr>
<td>VRGN E328D</td>
<td>SJG1-8p cells carrying the pVRGN E328D plasmid</td>
<td>Functional albeit to a moderate level</td>
</tr>
<tr>
<td>VRGN N328E</td>
<td>SJG1-8p cells carrying the pVRGN N328E plasmid</td>
<td>Loss in ATP hydrolysis</td>
</tr>
<tr>
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<td>SJGN C193A</td>
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</table>

2. Experimental procedures

2.1. Materials

Disodium ATP and ADP, NEM and DTE were purchased from Sigma Chemical Co. MIANS was supplied by Molecular Probes.

2.2. Methods

2.2.1. Construction of mutant variant of NBD-512 by site directed mutagenesis

Site directed mutagenesis was performed using the quick-change site directed mutagenesis kit (Stratagene, La Jolla, CA) as described previously [29]. Sequential Cys and Trp mutations and other mutations were introduced into plasmid pSJGN1-8 according to the manufacturer's instructions, and sequentially mutated plasmid was transformed in E. coli BL21 (DE3) pLysS cells listed in Table 1. Oligonucleotides used in this study, as listed in Supplementary data Table TS1 were commercially procured from Sigma Genosys, Inc. The presence and authenticity of the desired mutations were confirmed by dideoxy sequencing.

2.2.2. NBD-512 (NBD1) and its mutant variant's purification and measurement of ATPase activity

NBD-512 was purified from SJGN1-8 bacterial strain as described previously [26]. Mg\textsuperscript{2+} dependent ATPase activity of the purified NBD-512 was determined by analyzing the release of inorganic phosphate from ATP, as described previously [26] in the presence of 5 mM ATP and 8 mM MgCl\textsubscript{2} at 30 °C.

2.2.3. Labeling of purified NBD-512 and its mutant variants with MIANS

To carry out labeling of purified NBD-512, ~50 μg protein was incubated in ATPase buffer (60 mM Tris-Cl pH 6.5 and 8 mM MgCl\textsubscript{2}) at 22 °C with 50 μM MIANS for 15 min. Unreacted MIANS was quenched with 1 mM DTE. Control unlabelled NBD-512 protein was obtained from the same purified protein preparation. All protein concentrations were adjusted to ~50 μg/ml.

2.2.4. Resonance energy transfer measurements

Fluorescence intensities were measured at 22 °C in a 1 cm-path length cuvette using Cary Eclipse Varro spectrophotometer with slit bandwidths of 5 nm for excitation as well as emission. Measured fluorescence intensities were corrected for dilution, for buffer fluorescence, contribution from free and protein-bound MIANS (this was particularly important for Trp emission spectra and is detailed in the next section) and for inner filter effect wherever required. Unless otherwise specified, 1 ml protein samples (0.4 μM) in 60 mM Tris-HCl pH 6.5 containing 8 mM MgCl\textsubscript{2} were used for the fluorescence experiments.

2.2.5. Determination of parameters for FRET analysis

The efficiency (E) of resonance energy transfer between the donor and acceptor can be written as $E = 1 - R_0^2$, where $R_0$ is the fluorescence intensities of the donor in the presence and absence of the acceptor, respectively. The spatial distance is given by $R = R_0 (E - 1)^{1/2}$, where $R_0$ is the Förster distance corresponding to 50% energy transfer. $R_0$ was calculated from $(R_0)^2 = (10^{2.2} \times 10^{2.2})$ (Å), where $r$ is the spectral overlap integral between donor and acceptor and was determined as in [30,31]. orientation factor $k$ was taken as 2/3; $Q_0$ is the fluorescence quantum yield of the donor, and $q$ is the refractive index of the medium, which was taken as 1.33 for dilute aqueous solution [30]. Since MIANS labeling led to a red shift in the emission maximum of Trp fluorescence, we calculated the quantum yield of Trp in MIANS-labelled NBD-512 CW4 (NBD-512 CW4-MIANS) rather than in the unlabelled protein. For this, we corrected the protein emission spectrum for contribution from protein-bound MIANS by obtaining the spectrum of the MIANS-labelled protein after oxidation of its Trp residue using NBS.
Based on previous results, we hypothesized a model for ATP hydrolysis by the N-terminal NBD (NBD-512) of Cdr1p involving the role of typically placed residues such as Cys193 in Walker A as well as Trp326, Asp327 and Asn328 in the Walker B and extended Walker B motifs [29]. The conserved motifs and location of these typical amino acids within them are shown in Fig. 1. However, so far experimental data was insufficient to suggest how these residues are placed with respect to one another for binding and hydrolysis of ATP. To this end, in the present study, we exploited FRET between critical residues of NBD-512. The assumption was that if Trp326 and Cys193 are in close proximity to bind and hydrolyze ATP, respectively, one should be able to monitor FRET between them by fluorescence labeling Cys193. The role of other conserved residues could also be studied by monitoring the effect of their substitution mutations on this FRET.

Due to the fact that NBD-512 possesses four additional Trp and Cys residues and to avoid complications in interpretation of the FRET data, we generated a mutant variant of NBD-512 (designated as NBD-S12) in which all Cys and Trp residues with the exception of Cys193 and Trp326 were replaced with Ala. Having determined that NBD-512 CW4 bound ATP (Kd 0.072±0.04 mM) and hydrolyzed it (Km 0.185±0.04 mM, Vmax 80±5 mmol/min/mg) in a manner comparable to NBD-512, we used this mutant variant for further studies.

3.1. MIANS can selectively label Cys193 of NBD-512 CW4 mutant variant

MIANS selectively and covalently modifies cysteine side chains under appropriate conditions and becomes highly fluorescent upon binding to proteins [30,31]. The purified N-terminal NBD (NBD-512) of Cdr1p has five Cys residues that could potentially react with MIANS. Expectedly, the addition of MIANS to purified wild type NBD-512 led to a rapid increase in the fluorescence intensity at 450 nm in a concentration dependent manner (Fig. 2A). Pretreatment of NBD-512 with NEM (another Cys modifying agent) caused no further increase in fluorescence upon addition of MIANS, indicating that the two agents compete for the same site(s) (data not shown).

When MIANS labeling was done with NBD-512 CW4, one could specifically label Cys193. Similar to the wild type NBD-512, an increase in the MIANS fluorescence is observed in this mutant variant, albeit to a lesser extent (Fig. 2C).

Since we know from our previous study that Cys193 is critical for ATP hydrolysis we expected that the presence of ATP prior to MIANS labeling will physically block the access of the fluorophore (Supplementary data Fig. S1). A short pre-incubation with ATP resulted in reduction in the extent of modification of NBD-512 with MIANS (Fig. 2B). There was practically no labeling with MIANS if NBD-512 CW4 was pre-equilibrated with ATP (Fig. 2D). The importance of Cys193 in ATP catalysis was further evident when MIANS labeling was done with NBD-512 C193A mutant variant in which Cys193 was mutated to Ala. It was observed that MIANS could label the other cysteines of NBD-512 C193A mutant variant, even after pretreatment with ATP (Fig. 2E and F). These observations confirmed that modification by MIANS at Cys193 was specifically susceptible to the presence of ATP.
3.2. MIANS-labelled Cys193 continues to sense ATP docking in the active site

MIANS labeling results in ~5 nm red shifted emission for Trp326 in NBD-512 CW4-MIANS, suggesting that the probe does perturb the nucleotide-binding pocket of the protein. However, our results show that both NBD-512-MIANS and NBD-512 CW4-MIANS continue to sense Mg–ATP docking, exhibiting a concentration dependent, saturable quenching of MIANS fluorescence upon titration with Mg–ATP (Fig. 3A and B). The $K_d$ for Mg–ATP binding to NBD-512-MIANS as well as NBD-512 CW4-MIANS was effectively unaltered (Fig. 3C) [28]. Very similar $K_d$ values were obtained upon titration with Mg–ADP as well (NBD-512-MIANS, 0.07 ± 0.03 mM; NBD-512 CW4-MIANS, 0.07 ± 0.04 mM). Thus, it was possible to extrapolate the information from ATP binding to NBD-512 CW4-MIANS to understand how NBD-512 might function. Of note, ATP caused no shift in MIANS emission maximum in NBD-512-MIANS or NBD-512 CW4-MIANS, indicating that the polarity of the environment around MIANS in either variants remained effectively unaltered.

3.3. Energy transfer occurs between Trp326 and MIANS-labelled Cys193

The fluorescence emission spectrum of Trp326 has a significant degree of overlap with the absorption spectrum of MIANS ($\lambda_{ex}, \lambda_{em}$ for tryptophan is 295 and 340 nm; $\lambda_{ex}, \lambda_{em}$ for MIANS is 322 and 450 nm).
within close proximity to each other. To test this, purified NBD-512 and NBD-512 CW4, in a buffer containing Mg\(^{2+}\), were separately excited at 295 nm in the presence of increasing concentrations of MIANS, and fluorescence emission recorded between 310–500 nm (Fig. 4A and B, respectively). We observed a sharp increase in fluorescence emission of protein-bound MIANS at 450 nm; the fluorescence emission of Trp at 340 nm was simultaneously quenched. This effect, due to resonance energy transfer, was concentration dependent.

Trp emission was also ~5 nm red shifted, suggesting that Trp326 in NBD-512 CW4 becomes more solvent-exposed upon incorporation of MIANS. Hence, to obtain reliable estimates of Förster's radius and spatial distances between the two fluorophores, it was important to differentiate between quenching as a result of change in polarity of the environment around Trp from that due to FRET [Supplementary data Table TS2]. For this purpose, we subtracted from the emission spectrum of NBD-512 CW4-MIANS a spectrum of the protein after treatment with NBS as described under Methods. The resultant spectrum was taken as that of Trp326 alone in NBD-512 CW4-MIANS.

3.4. FRET monitors conformational changes induced by Mg\(^{2+}\)

We showed previously that Mg-ATP causes a significant conformational change in NBD-512 [28]. However, whether the conformational changes were the result of metal ion coordination or due to nucleotide docking was not apparent from that study [28]. By exploiting FRET and NBD-512 CW4 mutant variant, we re-addressed this issue. Interestingly, in the presence of EDTA and no Mg\(^{2+}\), poor FRET was seen in NBD-512 CW4 upon titration with MIANS (Fig. 5A). MIANS on Cys193 of NBD-512 CW4 was estimated to be at a distance of ~25 Å from Trp326 in the presence of EDTA (Table 2).

![Fig. 3. ATP binding with MIANS-labelled NBD-512 and its mutant variant NBD-512 CW4 monitoring extrinsic MIANS fluorescence. To achieve complete labeling, samples containing 0.4 μM protein in ATPase buffer were incubated with 50 μM MIANS for 15 min. Fluorescence spectra were recorded upon excitation at 322 nm and emission was recorded between 400 and 500 nm. (A) Fluorescence emission spectra of MIANS-labelled NBD-512 in presence of increasing concentrations of ATP (curve 1, no ATP; curve 2, 0.05 mM; curve 3, 0.2 mM; curve 4, 0.5 mM; curve 5, 1.0 mM). (B) Fluorescence emission spectra of MIANS-labelled NBD-512 CW4 mutant variant measured in presence of increasing concentrations of ATP (curve 1, no ATP; curve 2, 0.5 mM; curve 3, 1.0 mM). (C) Concentration dependent binding of ATP to the MIANS-labelled NBD-512 and its mutant variant NBD-512 CW4. Normalized fluorescence intensity (F/F) was plotted as a function of ATP concentration, where F refers to the fluorescence intensity of the sample in absence of ATP and F represents the fluorescence emission intensity at 450 nm upon ATP addition (background corrections as mentioned under Methods). The plot represents averages of three different experiments done in duplicates. The bars represent standard deviations. From the slope and ordinate of the Scatchard plot (plot not shown), dissociation constant (Kd) for ATP binding was determined to be 0.07±0.03 mM for NBD-512 and 0.08±0.04 mM for NBD-512 CW4 assuming a single binding site per molecule.

Hence, it is possible to monitor the spatial proximity between the two using FRET. Since MIANS could label Cys193 of NBD-512 CW4, it could form a donor–acceptor pair with Trp326 provided the fluorophores lay

![Fig. 4. Fluorescence resonance energy transfer between the donor (Trp) and acceptor (MIANS) in NBD-512 and NBD-512 CW4. Purified 0.4 μM NBD-512 (A) or NBD-512 CW4 (B) mutant variant in ATPase buffer was titrated with increasing concentrations of MIANS (0–50 μM), incubated for 2 min after addition of each aliquot and emission spectra recorded between 310 and 500 nm.](image-url)
We had earlier observed that NBD-512 C193A mutant variant binds ATP in a manner similar to wild type and has similar degrees of accessibility to small molecular weight fluorescence quenchers [28]. The $K_d$ for ATP calculated from changes in fluorescence of NBD-512 CW4-MIANS also supports this contention that the protein can bind Mg-ATP even when the Cys193 is labelled by MIANS (Fig. 3C).

It should be pointed out that the probe led to a slight perturbation of the pocket. This, along with the fact that the probe itself is roughly 10 Å in length [31] implies that the distances estimated here should not be taken as the exact spatial distance between Trp326 and Cys193 in the unlabelled protein. Notwithstanding this, the estimated distances between the two fluorophores certainly provide a useful handle for monitoring conformational changes occurring upon ligand binding.

Of note, we observed that another mutant variant, NBD-512 CW4 D327N, crippled in ATP hydrolysis showed conformational change and FRET similar to NBD-512 CW4 when titrated with Mg$^{2+}$ (Fig. 5B). NBD-512 CW4 D327A too behaved very much like NBD-512 CW4 D327N (Supplementary data Fig. S2). This reconfirmed our previous results that the conserved Asp at position 327, unlike its conventional role, does not participate in metal ion coordination (Table 2). Likewise, the catalytically deficient mutant variant NBD-512 CW4 N328A too exhibited FRET comparable to NBD-512 CW4 mutant variant with Mg$^{2+}$ (Fig. 5C; Table 2), suggesting that Asn328 also had no direct role in Mg$^{2+}$ coordination.

3.5. Glu238 also coordinates Mg$^{2+}$

Because of the unique placements of residues such as Cys193, Trp326, Asp327 and Asn328 in N-terminal NBD of Cdr1p, it appears that the conventional residues are not involved in Mg$^{2+}$ coordination. We already knew that the abstraction of metal ion directly affects the fluorescence spectrum of Trp in NBD-512 [28]. Titration with Mg$^{2+}$ results in an enhancement of the Trp fluorescence intensity for NBD-512 [28] as well as for NBD-512 CW4 (data not shown). Thus, Trp326 itself appears to be important for Mg$^{2+}$ coordination and the consequent conformational change in NBD-512. Yet all our previous as well as current FRET results indicate that Trp326 alone cannot be responsible for Mg$^{2+}$ coordination. Results from other NBDs of ABC transporters indicate that the commonly occurring glutamine of Q-loop plays an important role either in contacting the γ-phosphate or in interdomain communication [33,34]. In ButCD the conserved Gln in the Q-loop (Gln80) has also been shown to be involved in metal ion coordination [35].

Addition of Mg$^{2+}$ leads to increased FRET and an enhancement of MIANS fluorescence in NBD-512 CW4 (Fig. 5A; Table 2). This further confirms that Trp326 and MIANS on Cys193 arrive within greater spatial proximity (18.2 Å) once Mg$^{2+}$ is coordinated within the nucleotide-binding pocket. That such a conformational change is essential for ATP hydrolysis is clear since the domain is incapable of catalysis in the absence of the metal ion [26,28]. Further, addition of ATP in the absence of the metal ion could not cause any significant effect on FRET, suggesting that binding of Mg$^{2+}$ was a prerequisite for ATP binding. It may also be noted that Mg$^{2+}$ did not affect the fluorescence of MIANS itself in NBD-512 CW4 (data not shown). Hence, it appears unlikely that Cys193 has a direct role in Mg$^{2+}$ coordination.
The conserved Q-loop of NBD-512 has Glu238 replacing Gin and is a typical substitution for all fungal transporters (Fig. 1). As expected, mutant variants NBD-512 E238A and NBD-512 CW4 E238A were impaired in ATP binding as well as ATPase activity (Fig. 6A and B). Further, FRET analysis showed that this protein underwent a lesser than expected conformational change upon Mg$^{2+}$ addition (Fig. 6C; Table 2). Thus, the mutation of Glu238 specifically impaired the ability of the protein to sense the metal ion. It may be mentioned here that, since Trp326 is intact, metal ion coordination is diminished but not abolished and hence some FRET is expected in this mutant variant.

Replacement with a residue capable of metal ion coordination, as in NBD-512 E238Q and NBD-512 E238D allowed Mg$^{2+}$-ATP binding in a manner comparable to NBD-512 and NBD-512 CW4 proteins [K$_d$ for NBD-512 E238Q was 0.08 ± 0.01 mM and NBD-512 E238D was 0.07 ± 0.01 mM]. Thus, the uncommon Glu238, is also involved in Mg$^{2+}$ coordination.

Of note, the loss of activity in some of the mutant variants of NBD-512 was not due to their inability to form homodimer; our results show that NBD-512 is a monomer in solution (Supplementary data Fig. S3).

3.6 ATP docking subsequent to Mg$^{2+}$ coordination induces further conformational changes

Having shown that Mg$^{2+}$ significantly reduces the distance between Trp326 and Cys319, we checked whether any further enhancement in FRET would occur on addition of ATP. Interestingly, ATP brought the two fluorophores even closer and the R for NBD-512 CW4 was reduced by ~6 Å (Fig. 7A; Table 2). ADP, on the other hand, causes a smaller change in distance (~4 Å) between the two (Fig. 7B; Table 2). A similar effect was observed for the catalysis deficient mutant NBD-512 CW4 D327N (Fig. 7C and D; Table 2), further confirming that unlike other ABC transporters, the conserved Asp327 in NBD 512 is not involved in Mg$^{2+}$ coordination nor important for ATP binding [29]. Expectedly, NBD-512 CW4 E238A, which is affected in Mg$^{2+}$ binding, also shows poor conformational change upon ATP/ADP addition (Fig. 6D).

In comparison to ATP, the inability of ADP to further induce FRET emphasizes the role of γ-phosphate in the docking of the substrate. In other words, the presence of Mg–ADP results in a more open conformation for the binding pocket as compared to when the substrate Mg–ATP docks in. Thus, the formation of a functional nucleotide-binding pocket is dictated both by the metal ion as well as by the nucleotide itself.

3.7. Asn328 is a γ-phosphate sensor

How does this nucleotide-binding domain discriminate between ATP and ADP? For this purpose we examined the role of vicinal residues of Trp326 and Cys319. In particular, we were interested in Asn328 that is again unique to N-terminal NBDs of fungal ABC transporters (Fig. 1) and which we have previously shown to be important for the ability of the protein to impart drug resistance to the cell [29].
Monitoring the quenching of Trp fluorescence upon the addition of Mg-ATP suggested that NBD-512 N328E or NBD-512 N328A bound Mg-ATP with an affinity similar to NBD-512. So did the mutant variant NBD-512 CW4 N328A (K_d for NBD-512 N328E (0.08 ± 0.01 mM); NBD-512 N328A (0.08 ± 0.01 mM)).

That NBD-512 CW4 N328A continues to bind Mg^{2+} can also be deduced from the K value obtained (Table 2). However, unlike NBD-512 CW4, the mutant variant NBD-512 CW4 N328A exhibits a smaller conformation change upon ATP docking (Fig. 7E; Table 2). Indeed, despite proper Mg^{2+} coordination, NBD-512 CW4 N328A does not
differentiate between ATP and ADP [Fig. 7E and F; Table 2]. This would also explain its inability to hydrolyze ATP. Thus, Asn328 appears to be a sensor of the γ-phosphate of ATP and the subsequent conformational change therefore critical for catalysis.

It may also be pointed out here that a great deal of debate has taken place on the role of the equivalent Glu residue at this position in other ABC transporters that has also been shown to be essential for ATPase activity. It had been suggested that the Glu acts as a general or catalytic base [36]. However, Schmitt et al. have pointed out that this may not be the whole story since converting it to Gln in other ABC transporters led to varying degrees of ATPase activity [36]. We have previously shown that the Asp327 adjacent to Asn328 had taken on the catalytic role reserved for the glutamate in other systems. Our results suggest that Asn328 too has acquired a new role, as a sensor of the γ-phosphate. Given that in NBD-512 several residues have taken on new roles, this is perhaps an extension of this trend.

4. Discussion

Besides demonstrating the essentiality of the two NBDs, we have also shown the functional asymmetry of the two NBDs in Cdr1p [25,37], and unequivocally shown that the unique substitutions in the N-terminal domain have significance for ATP hydrolysis by the domain and to the functioning of the full protein in the cell [26–29]. Using isolated N-terminal NBD (NBD-512) of Cdr1p which probably functions as monomer and making the corresponding mutations in the full protein, we showed that Cys193 in Walker A and Asp327 in Walker B motifs of NBD-512 are both essential for ATP hydrolysis [26,28,29]. Further, we showed that Asp327 could act as a catalytic base since replacement by Asn resulted in interesting pH effects on ATP hydrolysis [29]. ATP binding by NBD-512 requires Mg2+ as well as the presence of Trp326 in the Walker B motif of binding pocket [28]. ATP binding to NBD-512 induces a conformation change, which is specific to Mg2+ docking rather than ATP hydrolysis in the wild type NBD-512 [28]. This was supported by the fact that the catalysis deficient mutant variants, NBD-512 C193A and NBD-512 D327N continued to exhibit conformational change upon ATP binding similar to native NBD-512 [28]. Together these results suggested that the typical residues work in conjunction and contribute physically to the active site formation [26,28,29]. Thus the N-terminal NBD of Cdr1p and, by extension those of other fungal transporters like Pdr5p, have evolved so as to use their unique substitutions to perform the task of ATP binding and hydrolysis [26,28].

In the current study we have attempted to obtain greater mechanistic details of the mechanism of ATP hydrolysis owing to the unusual substitutions within this otherwise conserved domain. By employing FRET, we have resolved the issues related to Mg2+ coordination and ATP binding in the N-terminal NBD of Cdr1p. For this purpose, we generated a mutant variant NBD-512 CW4 that had a single Cys (193) and a single Trp (326). We also established that this mutant variant functioned similar to the wild type NBD-512 protein as far as ATP binding and hydrolysis was concerned.

Using this mutant variant, we for the first time confirm that both Mg2+ and nucleotide binding contribute to the formation of the active site in the domain. The entry of Mg2+ into the active site causes a first large conformational change that brings Trp326 and Cys193 into close proximity. Further, Mg2+ has a distinct effect not only on FRET between Trp326 and the MIANS on Cys193, but also causes an enhancement of the intrinsic fluorescence of Trp326 in the mutant variant NBD-512 CW4. Thus Trp326 plays a significant role in Mg2+ coordination, which in turn induces a conformational change within the domain. Our FRET results further show that the typically conserved Glu238 of Q1oop of NBD-512, is another residue involved in coordination of the metal ion since its substitution as in NBD-512 CW4 E238A does not allow Trp326
and Cys193 to move sufficiently close in the nucleotide-binding pocket despite the presence of Mg2+. Thus the uniquely placed Glu238, which replaces Gin in the N-terminal NBD of all fungal ABC transporters, takes over the role of Mg2+ coordination. On the other hand, the uniquely placed Asn328, which is also common to all fungal transporters, acts primarily as a γ-phosphate sensor and is responsible for the second conformational change that occurs upon ATP binding.

Taken together, one can visualize a three-step mechanism of ATP catalysis at NBD-512. The first step occurs when Mg2+ enters the pocket and contacts Trp326 of Walker B and Glu238 of Q-loop (Fig. 8 Step I). This induces a conformational change that drags Cys193 of the Walker A motif towards the catalytic pocket. As a result, Trp326 and Cys193 come within close proximity (R decreases to ~18.2 Å from 24.5 Å). Subsequently, a second conformational change occurs when ATP docks with its phosphates directed towards the pocket. Such a positioning automatically brings the γ-phosphate close to the uniquely placed Asn328 residue in the extended Walker B motif. The sensing of the γ-phosphate by Asn328 is responsible for the second small but significant conformational change in the NBD. As a result Cys193 comes even closer to Trp326 (R ~12 Å, Fig. 8 Step II). This sensing of the γ-phosphate and closing in of the Walker A and Walker B motifs towards the substrate constitutes an important event preceding ATP hydrolysis. The third step is the actual catalysis step in which the catalytic residues, Cys193 of Walker A and Asp327 of Walker B, participate (Fig. 8 Step III). Thereafter, the protein has a more open conformation (R is ~14 Å in the presence of Mg-ADP) that allows ADP to leave.

As mentioned earlier, we have previously shown that Asp327 acts as a catalytic base in ATP hydrolysis [29]. In order to understand the role of Cys193 in the catalysis, we generated the mutant variant NBD-512 C193S. In this mutant, Cys was replaced by Ser whose hydroxyl has a higher pKa than the thiol. At pH 6.5, this mutant variant has showed equal if not better ATPase activity as compared to NBD-512, suggesting that the abstraction of the proton too is an essential step in catalysis at NBD-512. The first step occurs when Mg2+ enters the pocket/hydrolyzed in this second NBD, it allows the domain to swing out, a conformational change that is transduced to the first NBD via the Signature sequence of the second. Such a sequence of events could also be cooperative and require a concerted action of both NBDs without either half being capable of functionally replacing the other.

Like the other motifs in the N-terminal NBDs, the ABC signature sequences of Cdrlp and other fungal transporters too appear to have diverged away from that of other ABC transporters. Whether this became necessary to compensate for the substitutions in their N terminal NBDs or whether there have evolved a new mechanism for coming together for ATP hydrolysis and drug efflux, is a question we are currently examining.

Acknowledgements

The work presented in this paper has been supported in parts by grants from Department of Biotechnology, (DBT/PR3825/ Med/14/48(II)/2003), Council of Scientific and Industrial Research (39/1122/06 EMR-II), Department of Science and Technology (SR/SO/BB-12/2004) and European Commission (QLK2-CT-2001-02377). VR and MG acknowledge the University Grants Commission, India for the support in the form of Senior Research Fellowship.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, if the online version, at doi:10.1016/j.bbamem.2008.04.010.

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Conserved Asp327 of Walker B Motif in the N-Terminal Nucleotide Binding Domain (NBD-1) of Cdrlp of Candida albicans Has Acquired a New Role in ATP Hydrolysis

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Received July 30, 2006; Revised Manuscript Received September 20, 2006

ABSTRACT: The Walker A and B motifs of nucleotide binding domains (NBDs) of Cdrlp though almost identical to all ABC transporters, has unique substitutions. We have shown in the past that Trp326 of Walker B and Cys193 of Walker A motifs of N-terminal NBD of Cdrlp have distinct roles in ATP binding and hydrolysis, respectively. In the present study, we have examined the role of a well conserved Asp327 in the Walker B motif of the N-terminal NBD, which is preceded (Trp326) and followed (Asn328) by atypical amino acid substitutions and compared it with its equivalent well conserved Asp1026 of the C-terminal NBD of Cdrlp. We observed that the removal of the negative charge by D327N, D327A, D1026N, D1026A, and D327N/D1026N substitutions, resulted in Cdrlp mutant variants that were severely impaired in ATPase activity and drug efflux. Importantly, all of the mutant variants showed characteristics similar to those of the wild type with respect to cell surface expression and photoaffinity drug analogue [125I]IAAP and [3H]azidopine labeling. Although the Cdrlp D327N mutant variant showed comparable binding with [γ-32P]8-azido ATP, Cdrlp D1026N and Cdrlp D327N/D1026N mutant variants were crippled in nucleotide binding. That the two conserved carboxylate residues Asp327 and Asp1026 are functionally different was further evident from the pH profile of ATPase activity. The Cdrlp D327N mutant variant showed ~40% enhancement of its residual ATPase activity at acidic pH, whereas no such pH effect was seen with the Cdrlp D1026N mutant variant. Our experimental data suggest that Asp327 of N-terminal NBD has acquired a new role to act as a catalytic base in ATP hydrolysis, a role normally conserved for Glu present adjacent to the conserved Asp in the Walker B motif of all the non-fungal transporters.

One of the most clinically significant mechanisms of azoles resistance in the pathogenic fungi, C. albicans is an over-expression of the drug efflux pumps encoding genes CDR1 and CDR2 belonging to the ATP binding cassette (ABC) (1-7) and CaMdr1 belonging to the major facilitator superfamily (MFS) of transporters (8-10). Among the ABC transporters, the high level of expression of CDR1 invariably contributes to an increased efflux of fluconazole and, thus, corroborates its direct involvement in drug efflux (6, 11, 12). Hence, Cdrlp has not only acquired significant clinical importance but is also considered an important target in any design of strategies to combat antifungal resistance (7, 13, 14).

Typically, like other ABC proteins, the functional form of Cdrlp consists of two hydrophobic nucleotide binding domains (NBDs) located at the cytoplasmic surface of the membrane that harness energy from ATP hydrolysis and two hydrophobic transmembrane domains (TMDs) that are thought to form the translocation pathway for drug efflux. These NBDs of the ABC proteins contain three conserved motifs required for nucleotide binding and hydrolysis: the Walker A and Walker B motifs (15) and the ABC signature

‡ The work presented in this article was supported in part by grants from the Department of Biotechnology (DBT/PR3825/Med/14/488/03), Council of Scientific and Industrial Research (38(1122)(06)EMR-II), Department of Science and Technology (SR/S0/BB-12/2004), and the European Commission, Brussels (QLK2-CT-2001-02377), V.R., M.G., and S.S. acknowledge the University Grants Commission, India for support in the form of Junior and Senior Research Fellowships. S.S. and S.V.A. acknowledge the financial support through the intramural program of the National Institutes of Health, National Cancer Institute, and Center for Cancer Research.

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‖ National Institutes of Health.

1 Abbreviations: ABC, ATP binding cassette; ATP, adenosine-5'-triphosphate; ADP, adenosine-5'-diphosphate; ATPase, adenosine-5'-triphosphatase; PDR, pleiotropic drug resistance; EDTA, ethylenediaminetetraacetic acid; kDa, kilodalton(s); NBD, nucleotide binding domain; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; TMD, trans membrane domain; TMS, trans membrane segment; GFP, green fluorescent protein; ORF, open reading frame; PM, plasma membrane; FACS, fluorescence associated cell sorting; FLU, fluconazole; MIC, miconazole; CYCLO, cycloheximide; ANISO, amastatin; KETO, ketoconazole; ITRA, itraconazole; R6G, rhodamine 6G; [125I]IAAP, [3H]iodoarylazidoprazosin.
sequence (16). ATP hydrolysis and substrate transport is dependent on the cooperativity between NBDs. The Walker A motif (GxG/K/C/T/S) forms the phosphate binding loop, or P-loop, which wraps around the phosphate chain of ATP within this motif and makes extensive hydrogen bonding with β-phosphate (17). The Walker B motif ([I/H]_x[D] provides a carboxylate residue that coordinates and stabilizes the Mg^{2+}, which is a mandatory cofactor in the hydrolysis of ATP (18). A recent crystal structure of the stable MJ0796-E171 Q dimer, an ABC transporter of *Methanococcus jannaschii*, describes the 3D structure of the nucleotide binding pocket wherein it is proposed that NBDs form a symmetrical dimer in which two ATP molecules are sandwiched between the Walker A and Walker B motifs of one NBD and the signature motif of another NBD (19). The deduced 3D structure has essentially identical to non-fungal transporters of the super family (20).

Unlike most other ABC transporters, the NBDs of *C. albicans* and all other fungal transporters of the super family have unique positioning of a typical amino acid Cys193 in the Walker A, Trp326, and Asn328 in and adjacent to the Walker B motifs of N-terminal NBD, respectively (20). Interestingly, the C-terminal NBD of Cdr1p and other ABC fungal transporters possesses conserved motifs that are essentially identical to non-fungal transporters (20). To begin defining the functional significance of the conserved substitutions in the N-terminal NBD of Cdr1p, we have recently demonstrated that the replacement of Cys193 with Ala gravely impaired the ATP hydrolysis without affecting its ability to bind the nucleotide (21, 22). However, substitution of Trp326 with Ala resulted in the loss of ATP binding to purified N-terminal NBD (NBD-512) (23). A mutagenesis screen of another conserved residue underscored the importance of the highly conserved, putative catalytic carboxylate residue Asp327 of the Walker B motif in the purified N-terminal NBD of Cdr1p. The substitution of Asp327 with Asn yielded a mutant variant protein with strongly impaired ATPase activity, although it showed comparable nucleotide-binding to that of wild type protein. In the crystal structure of HisP, the ATPase subunit of an ABC transporter histidine permease from *Salmonella typhimurium*, the negatively charged side chain of this equivalent Walker B Asp residue (Asp178) hydrogen bonds a water molecule in proximity to the γ-phosphate of ATP. This water molecule has been proposed to replace Mg^{2+} in the crystal structure (24), implying that Asp178 and its equivalent in P-gp (Asp555/Asp1200) coordinate Mg^{2+} (25). However, our initial results suggest that an equivalent Asp residue (Asp327) in the Walker B motif of N-terminal NBD of Cdr1p, unlike in other non-fungal ABC transporters, may not be involved in Mg^{2+} coordination (23).

In this study, the precise role of Asp327 of N-terminal NBD in ATP binding and hydrolysis is further explored and is compared with its counterpart Asp1026 of C-terminal NBD. The substitution of both Asp residues resulted in severely impaired ATPase activity and drug transport. Importantly, both mutant variants showed characteristics similar to those of the wild type with respect to photoaffinity drug analogues of prazosin, [3H]IAAP (idoazidoprazosin) and dihydropyridine, and [3H]azido labeling but showed differential labeling with [α-32P]-8-azido-ATP. Although Cdr1p D1026N and Cdr1p D327N/D1026N were severely impaired in nucleotide binding, indicating a direct conventional role of Asp1026 in Mg^{2+} coordination, mutant variants Cdr1p D327N or Cdr1p D327A elicited nucleotide binding comparable to that of wild type Cdr1p. Our results show that because of the replacement of conserved Glu with Asn at position 328 in N-terminal NBD of Cdr1p and as well as other fungal ABC transporters, the conserved Asp327 of the Walker B motif of N-terminal NBD has acquired a new role to act as a catalytic base for ATP hydrolysis.

**EXPERIMENTAL PROCEDURES**

**Materials.** Ribonucleotides (ATP, ADP), protease inhibitors (PMSF, leupeptin, pepstatin A, aprotinin, TLCK, and TPCK) and drugs, miconazole, cycloheximide, anisomycin, ketoconazole, itraconazole, R6G, DTT, oligomycin, and other molecular grade chemicals, were obtained from Sigma Chemical Co. (St. Louis, MO). Oligonucleotides used in this study, as listed in Table 1, were commercially procured from Sigma Genosys, Inc. Fluconazole was kindly provided by Ranbaxy Laboratories (New Delhi, India). Anti-GFP mono-

### Table 1: List of Oligonucleotides Used in This Study

<table>
<thead>
<tr>
<th>name</th>
<th>sequence</th>
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<tbody>
<tr>
<td>D327A/F</td>
<td>5'-CTAAATCCATTTTGGAAGATCCACTAGAGG-3'</td>
</tr>
<tr>
<td>D327A/R</td>
<td>5'-CCCTCTAGTGGCCAAATTTGGGATTATTAG-3'</td>
</tr>
<tr>
<td>D327N/F</td>
<td>5'-CTAATACATTTTGGAAGATCCACTAGAGG-3'</td>
</tr>
<tr>
<td>D327N/R</td>
<td>5'-CCCTCTAGTGGCCAAATTTGGGATTATTAG-3'</td>
</tr>
<tr>
<td>D327E/F</td>
<td>5'-CTAAATCCATTTTGGAAGATCCACTAGAGG-3'</td>
</tr>
<tr>
<td>D327E/R</td>
<td>5'-CCCTCTAGTGGCCAAATTTGGGATTATTAG-3'</td>
</tr>
<tr>
<td>N328E/F</td>
<td>5'-CTAAATCCATTTTGGAAGATCCACTAGAGG-3'</td>
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<tr>
<td>N328E/R</td>
<td>5'-CCCTCTAGTGGCCAAATTTGGGATTATTAG-3'</td>
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<tr>
<td>N328A/F</td>
<td>5'-CTAAATCCATTTTGGAAGATCCACTAGAGG-3'</td>
</tr>
<tr>
<td>N328A/R</td>
<td>5'-CCCTCTAGTGGCCAAATTTGGGATTATTAG-3'</td>
</tr>
<tr>
<td>D1026N/F</td>
<td>5'-CTAAATCCATTTTGGAAGATCCACTAGAGG-3'</td>
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<td>D1026N/R</td>
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<tr>
<td>E1027Q/R</td>
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<tr>
<td>E1027A/F</td>
<td>5'-TAACTCGTGAATCGTTGGGATTATTAGAAGGTTAGA-3'</td>
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<tr>
<td>E1027A/R</td>
<td>5'-TAACTCGTGAATCGTTGGGATTATTAGAAGGTTAGA-3'</td>
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clonal antibody and [α-32P] 8-azido ATP (15–20 Ci/mmol) were purchased from BD Biosciences Clontech (Palo Alto, CA) and Affinity Labeling Technologies, Inc. (Lexington, KY), respectively. [3H] azidopine (60 Ci/mmol) was obtained from Amersham Biosciences (Arlington Heights, III). The radio labeled [32P] IAAP (2,200 Ci/mmol) was procured from Perkin-Elmer Life Sciences (Boston, MA).

Media Chemicals and Strains. Plasmids were maintained in Escherichia coli DH5α. E. coli was cultured in Luria–Bertani medium (Difco, BD Biosciences, NJ) to which ampicillin was added (0.1 mg/mL). The yeast strains were cultured in YEPD broth (Bio101, Vista, CA) or SD-ura broth (Bio101, Vista, CA) as described previously (26).

Methods

Site-Specific Mutagenesis. Site directed mutagenesis was performed using the quick-change mutagenesis system as described previously (26). The mutations were introduced into plasmid pPSCDR1-GFP according to manufacturer’s instructions, and the mutated plasmid pPSCDR1-GFP linearized with XbaI was used to transform AD1-8u− cells by the lithium acetate transformation protocol exploiting uracil prototrophy (26, 27). Table 2 lists all of the strains used in this study.

Confocal Microscopy and Flow Cytometry. Confocal imaging and flow cytometric (FACS) analysis of Cdr1p and its mutant variants carrying S. cerevisiae cells were performed with a Bio-Rad confocal microscope (MRC 1024) with a 100× oil immersion objective and FACSsort flow cytometer (Becton-Dickson Immuno cytometry systems, San Jose, CA) as described previously (26).

Photoaffinity Labeling. Photoaffinity labeling of Cdr1p and its mutant variants with 0.5 μM [3H]-azidopine (specific activity 60 Ci/mmol), 7.5 nM [125I] IAAP (2200 Ci/mmol), and 10 μM [α-32P] 8-azido ATP (7.5 μCi/mmol) was performed with PM proteins (30–50 μg) as described previously (26).

Homology Modeling of NBDs of Cdr1p. Because no structural data is currently available for NBDs of Cdr1p, the model of the CDR1–NBD catalytic dimer along with the ligands (ATP and Mg++) was generated by homology modeling to verify experimental findings. The crystal structure of the dimeric E171Q mutant of MJ0796, an ABC transporter from M. jannaschii (Protein Data Bank (PDB) code 1L2T) (19) was used as a template for modeling the dimer. The target sequence was then aligned using ClustalX version 1.83 (29, 30). The alignment was manually edited so that the loop regions involved with ATP were not influenced by the template structure but by the ATP and metal ligand. The model was built using the program Modeller8 v. 2 (31). An ensemble of 50 structures was generated, and the one with the lowest objective function (energy) was chosen for further analysis. Model evaluation was done using PROCHECK v. 3.5.4 (32).

Routine Procedures. Protein concentrations were determined by a bicinchoninic acid method (33). SDS–PAGE was carried out according to Laemmli (34), using the Mini–PROTEAB II gel and electrophoresis transfer system (Bio-Rad).
RESULTS

Cdr1p Mutant Variants with Conserved Amino Acid Substitutions in the Walker B Motif of NBDs Are Properly Expressed and Surface Localized. Asp327 of N-terminal NBD and Asp1026 of C-terminal NBD in the Walker B motif of Cdr1p or its equivalent in other ABC transporters are well conserved (Figure 1). To probe whether Asp327 has acquired a new role because of its neighborhood conserved substitutions and to compare it is functioning with its counterpart Asp1026 in the Walker B motif of C-terminal NBD, we made point mutations of these residues. Thus, we constructed the mutant variants D327N, D327A, D1026N, D1026A, and D327NI D1026N of Cdr1p. Making the equivalent mutations in both the nucleotide sites of Cdr1p allowed us to examine their roles in ATP catalysis.

The mutant variants, designated as Cdr1p D327N, Cdr1p D327A, Cdr1p D1026N, Cdr1p D1026A, and Cdr1p D327N/D1026N were constructed by site directed mutagenesis and stably overexpressed from the genomic PDR5 locus in a S. cerevisiae mutant AD1-8u" as a heterologous expression system (35). We also tagged the green fluorescent protein (GFP) gene at the C-terminal end of CDR1, which was overexpressed as a fusion protein (26). The host AD1-8u" was derived from a Pdr1-3 mutant strain with a gain-of-function mutation in the transcription factor Pdr1p, resulting in the constitutive hyper-induction of the PDR5 promoter (27). Single copy integration of each transformant at the PDR5 promoter was confirmed by Southern hybridization (data not shown). The wild type Cdr1p and its mutant variants were expressed at equivalent levels as evident from the Western blot analysis of the PM fraction of cells expressing these mutant variant proteins (Figure 2A). All of the mutant variants and wild type Cdr1p were properly cell surface localized as confirmed by confocal microscopy and FACS analysis (Figure 2B).

Mutation of Conserved Carboxylate Residues in the Walker B Motif of NBDs Resulted in Abrogation of Drug Resistance, Drug Transport, and ATPase Activity. We examined the effect of these mutations on drug sensitivities of the cells expressing wild type Cdr1p and its mutant variants by two independent drug susceptibility assays viz., MIC80 (minimum inhibitory concentration for 80% inhibition in growth) and spot assays (26). MIC80 assays revealed that the host strain (AD1-8u") was expectedly hyper-sensitive to all of the tested drugs compared to the growth control (without drug). On the contrary, for the cells expressing wild type Cdr1p, substantial growth in the presence of drugs was observed. Compared to the host strain (AD1-8u"), MIC80 for cells expressing wild type Cdr1p was considerably higher (MIC80 64 μg/mL for fluconazole, 16 μg/mL for anisomycin, 2.0 μg/mL for miconazole, 1.0 μg/mL for ketoconazole, 4.0 μg/mL for Itraconazole, and 0.5 μg/mL for cycloheximide) (Figure 3A). Interestingly, cells expressing Cdr1p mutant variants Cdr1p D327N, Cdr1p D327A, Cdr1p D1026N, Cdr1p D1026A, and Cdr1p D327N/D1026N behaved like the host strain (AD1-8u") and remained hypersensitive to the tested drugs. Accordingly, they displayed extremely low MIC80 values (Figure 3A). The results of spot assays also corroborated MIC80 results (Figure 3B).
FIGURE 2: Membrane localization and expression profile of wild type and carboxylate residue mutant variant Cdr1p. The boxed panel at the top shows the sequence of Walker B and extended Walker B motifs of N-terminal and C-terminal NBDs of Cdr1p. (A) PM of wild type and mutant variant protein-expressing cells were prepared, and their immunodetection was performed as described earlier (26). (B) Fluorescence imaging (upper panel) by a confocal microscope showing membrane localization of Cdr1-GFP (Cdr1p) and its mutant variant protein-expressing cells. Flow cytometry (lower panel) of S. cerevisiae expressing Cdr1p and its mutant variants. The histogram derived from the cell quest program depicts fluorescence intensities for AD1-8u (control) (purple filled area) and PSCdr1-GFP (solid green line) for each panel, and the other extra line represents that for the respective Cdr1p mutant variant-expressing cells.

To check if the observed drug sensitivity by Cdr1p carboxylate mutant variants expressing cells was associated with the impaired extrusion ability of the cell, we performed a rhodamine 6G (R6G) efflux assay (described under Experimental Procedures), which is a good indicator of Cdr1p activity (28). Strikingly, cells expressing these Walker B mutant variants Cdr1p D327N, Cdr1p D327A, Cdr1p D1026N, Cdr1p D1026A, and Cdr1p D327N/D1026N were severely impaired in efflux function (Figure 3C). It is apparent that the disruption of either NBD results in a non-functional protein, and neither the N-terminal nor the C-terminal ATP site can function independently of each other.

The ability of conserved Walker B mutant variants of Cdr1p to hydrolyze ATP was also measured as the oligomycin-sensitive release of Pi from Mg-ATP as described under Experimental Procedures. PM preparations from cells expressing wild type Cdr1p elicited an ATPase activity of ∼50-55 nmol of Pi released per min per mg of protein. None of the mutant variant proteins displayed ATPase activity above the host AD1-8u control (discussed later; Figure 5A). These data suggest that the lack of transport function of mutant variant proteins (Figure 3C) is attributed to the impaired ATPase activity and that both NBDs need to be functional to generate an active transporter.

Drug Binding Remains Unaffected in the Walker B Mutant Variants of NBDs. The drug binding properties of the wild type and mutant variant proteins were assessed by photoaffinity labeling with radio labeled drug substrate analogues of dihydropyridine ([3H]azidopine) and of prazosin ([125I]iodosarylazidoprazosin and [125I]IAAP). These photoaffinity analogues have been successfully used earlier to study drug binding sites on Cdr1p (26). PM preparations derived from yeast cells expressing wild type Cdr1p or its mutant variants Cdr1p D327N, Cdr1p D327A, Cdr1p D1026N, Cdr1p D1026A, and Cdr1p D327N/D1026N were labeled with [3H]azidopine or [125I]IAAP (as described under Experimental Procedures) and subjected to SDS–PAGE and autoradiography. As expected, no binding of these analogues was observed in the host AD1-8u negative control, whereas an efficient labeling of [3H]azidopine (Figure 4A) and [125I]IAAP (Figure 4B) was seen with all of the mutant variant proteins, comparable to that of the wild type protein. Photoaffinity labeling of these drug analogues with the wild type and its mutant variant proteins was specific because [125I]IAAP labeling can be competed out by Cdr1p drug substrates such as nystatin (Figure 4B). These data suggest that mutations made in the Walker B motifs of the N- and C-terminal NBDs do not affect the ability of the transporter to recognize the drug substrates analogues.
Walker B Mutant Variants of NBDs Differ in Their Ability to Bind $[\alpha-3^{2}P]$ 8-Azido ATP. To further examine the cause of impaired drug transport and ATPase activity, we explored the effect of single or double mutations in the Walker B motif on ATP binding in general and particularly exploring whether a mutation in either ATP site would allow nucleotide binding and hydrolysis at the other native site. For this, we examined $[\alpha-3^{2}P]$ 8-azido ATP labeling binding of Cdr1p and its mutant variants, which we had earlier shown to bind efficiently to Cdr1p (26). PM preparations were photoaffinity-labeled with 10 $\mu$M $[\alpha-3^{2}P]$ 8-azido ATP in the presence of 8 mM MgCl$_2$ as described in Experimental Procedures. An equivalent amount of PM derived from the AD1-8u$^-$ host was used as a control, and as expected, no binding of $[\alpha-3^{2}P]$ 8-azido ATP was observed in this case. Membranes expressing wild type Cdr1p and its mutant variants were, however, labeled with $[\alpha-3^{2}P]$ 8-azido ATP, and the labeling of the analogue could be competed out with molar excess of cold ATP (Figure 4C). This labeling was strictly dependent upon Mg$^{2+}$, because the presence of 2 mM EDTA completely eliminated labeling (data not shown). Although the $[\alpha-3^{2}P]$ 8-azido ATP labeling in membranes expressing Cdr1p D1026N and its double mutant variant Cdr1p D327N/D1026N was severely affected, there was no significant difference in labeling between wild type Cdr1p and Cdr1p D327N or Cdr1p D327A. It should imply that the ability to bind ATP to the C-terminal ATP sites is reduced significantly in the protein containing a mutation in the Mg$^{2+}$ binding site (D1026N), whereas binding to the N-terminal ATP site with the D327N mutation is not compromised.

**D327 Is Involved in ATP Catalysis.** The photoaffinity labeling of Cdr1p mutant variants with $[\alpha-3^{2}P]$ 8-azido ATP suggested that Asp1026 of Walker B motif of C-terminal NBD is implicated in ATP binding or Mg$^{2+}$ coordination, similar to its proposed conventional role in other NBDs (18). However, despite being crucial for ATP hydrolysis, Asp327 in the Walker B motif of N-terminal NBD does not appear to be involved in Mg$^{2+}$ coordination (Figure 5A). To further explore the role of Asp327, we examined the pH dependence of ATPase activity of the wild type and its mutant variants to highlight different roles of the two-conserved Asp. As shown in Figure 5B, ATPase activity of Walker B mutant variant Cdr1p D1026N does not show any pH dependence, whereas the pH profile of the enzyme activity of Cdr1p D327N mutant variant (retaining around 10% residual activity) was drastically different. Of note, the Cdr1p D327N mutant variant retains $\sim$40% ATPase activity at acidic pH (4.5–5.5) (Figure 5B), whereas its Ala substitution, that is,

![Figure 3](https://via.placeholder.com/150)

**FIGURE 3:** (A) MIC$_{50}$ values of cells expressing Cdr1p and its mutant variants in the presence of different drugs tested. Drug susceptibility (MIC$_{50}$) of S. cerevisiae cells expressing wild type and mutant variants of Cdr1p MICs were determined by a microdilution method as described previously (26). (B) Drug resistance profiles of the wild type and mutants determined by spot assay. It was done as per the protocol described earlier (26). The concentrations of the drugs used: fluconazole, 5 $\mu$g/mL; anisomycin, 1.0 $\mu$g/mL; miconazole, 0.5 $\mu$g/mL; and ketoconazole, itraconazole, and cycloheximide, 0.15 $\mu$g/mL. (C) Rhodamine 6G efflux by the wild type Cdr1p and its mutant variant protein-expressing cells. The R6G efflux was measured as described previously (28). The values are mean ± SD (error bars) for three independent experiments.
Cdrlp D327A does not show similar enhancement of ATPase activity at low pH. No similar restoration of ATPase activity at acidic pH (4.5–5.5) could also be seen with Cdrlp D1024N or Cdrlp D1026A mutant variants (Figure 5B). Interestingly, the restoration of ATPase activity by cells expressing only the Cdrlp D326N mutant variant could also partially restore resistance to tested drugs when grown at low pH, whereas that was not the case with cells expressing the Cdrlp D326A mutant variant (Figure 5C). Furthermore, as evidenced by the MIC<sub>50</sub> values (Figure 3A) and ATPase activity (Figure 5A), the mutant variant Cdrlp D327E also behaved very much like the wild type protein, confirming the importance of the carboxylate residue at the 327 position.

Isolated N-Terminal Nucleotide Binding Domain D327N (NBD-512 D327N) Confirms pH Dependence. To further confirm that the observed pH dependence was indeed the result of the unique placement of critical residues in the N-terminal NBD of Cdrlp, we used purified N-terminal NBD (NBD-512) protein (27). Indeed, the purified NBD-512 D327N mutant variant protein also showed restoration of ATPase activity at low pH (4.5), which was not the case with the NBD-512 D327A mutant variant protein (data not shown). Interestingly, when purified native NBD-512 and mutant variant NBD-512 D327N were exposed to low pH prior to assessing their ATPase activities at the normal pH of 6.5, the mutant variant protein retained enhanced activity (Figure 5D).
Asp327 Serves as a Catalytic Base

FIGURE 5: (A) ATPase activity of Cdr1p with its mutant variants. ATPase activity of the PM fraction of cells expressing the wild type Cdr1p and its mutant variants were assayed as described previously (26). The values are the mean ± standard deviation of three independent experiments. (B) pH dependence of ATPase activity of Cdr1p and its mutant variants. ATPase activity of PM fractions expressing wild type Cdr1p and its mutant variants were assayed at different pH. The scattered plot represents ADL-8u- (●), PSCdr1-GFP (○), VRGC D327N (▲), VRGC D1026N (▼), VRGC D1026A (□), and VRGC D327N/D1026N (△). (C) Reversal of drug resistance at low pH by the VRGC D327N strain. The reversal of drug resistance by the VRGC D327N strain at different pH was corroborated by a spot assay by growing the cells at different pH in presence of drug; Flu, 0.5 μg/mL; Mico, 0.125 μg/mL; Keto, 0.0156 μg/mL; Itra, 0.125 μg/mL; Cclo, 0.0625 μg/mL; and Aniso, 0.5 μg/mL. (D) ATPase activity of isolated N-terminal NBD (NBD-512) of Cdr1p and its mutant variant proteins. ATPase activity of NBD-512 and its mutant variant proteins at pH 6.5 was assayed after the preincubation of the protein at low pH (4.5).

Substitution of Conserved Asn328 Resulted in Selective Loss of Function, but Unlike Cdr1p D327N, It Does Not Show pH Dependence. In all non-fungal ABC transporters, a very well conserved residue Glu adjacent to conserved Asp (equivalent to Asp327) of the Walker B motif has been implicated to function as a catalytic base in the ATP catalysis cycle (35–39). However, all fungal ABC transporters including Cdr1p lack this conserved Glu residue adjacent to the Walker B core at N-terminal NBD, which is replaced by Asn (Figure 1). Given the importance of the conserved Glu residue in the catalytic cycle of non-fungal ABC transporters, we explored the role of Asn328 in Cdr1p. To investigate this, Asn328 of N-terminal NBD and Glu 1027 of C-terminal NBD at the equivalent position, adjacent to the core Walker B motif of Cdr1p (Figure 6), were substituted. The mutant variants designated Cdr1p N328E, Cdr1p N328A, Cdr1p E1027Q, Cdr1p E1027A, and Cdr1p N328E/E1027Q were constructed and expressed in S. cerevisiae mutant host strain (AD1-8u-). Substitution of these highly conserved residues did not affect cell surface expression of Cdr1p as determined by FACS and confocal analysis (Figure 6A). However, all these mutant variants were abrogated in their ability to efflux R6G (Figure 6B). This loss of efflux was the result of impaired ATPase activity in these mutant variants (Figure 6F). Interestingly, the binding of photoaffinity drug analogues, [3H]-azidopine (Figure 6C) and [125I] IAAP (Figure 6D), and ATP analogue [α-32P] 8-azido ATP to these mutant variants remains unaffected (Figure 6E). Importantly, neither of these mutant variants showed pH dependence of ATPase activity as shown by the Cdr1p D327N mutant variant (Figure 6F, inset).

Homology Modeling of Nucleotide Binding Domains (NBDs) of Cdr1p. In the absence of high-resolution structural data for NBDs of Cdr1p and to validate experimental data, we generated a homology model based on the dimeric E171Q mutant structure of a known homologue MJ0796, an ABC transporter of M. jannaschii. The crystallographic and functional evidence for ABC transporters indicates that residues from both NBDs contribute to the nucleotide binding site. Therefore, it is sensible to refer to ATP binding to sites rather than to individual NBDs (19). By homology modeling, we mapped the sequence of the Cdr1p N- and C-terminal NBDs onto the two subunits of the dimeric E171Q mutant of the MJ0796. Analysis of the ATP-binding pocket shows that ATP is present at the interface of the sandwich dimer formed between the Walker A and B motifs of N-terminal NBD and the signature motif of C-terminal NBD (Figure 7A). In all of the 50 models generated, Asp327 is predicted
Figure 6: (A) Membrane localization and expression profile of wild type Cdr1p and mutant variants. The boxed panel at the top shows the sequence of Walker B and extended Walker B motifs of N-terminal and C-terminal NBDs of Cdr1p. Fluorescence imaging (upper panel) and flow cytometry (lower panel) of S. cerevisiae expressing Cdr1p and its mutant variants has been done as mentioned in the legend for Figure 2B. (B) Rhodamine 6G efflux by the wild type Cdr1p and its mutant variants expressing cells. The inset shows the table of MIC₆₀ values of the wild type Cdr1p and its mutant variant-expressing cells for the drug tested. (C) Photoaffinity labeling of wild type Cdr1p and its mutant variants with [³²P]-azidopine. The PM fraction (30 µg) of cells expressing wild type Cdr1p and its mutant variants were photoaffinity labeled with 0.5 µM [³²P]-azidopine as mentioned in the legend for Figure 4A. The loading pattern is AD1-8u (control; lane 1), Cdr1-GFP (lane 2), Cdr1p N328A (lane 3), Cdr1p N328E (lane 4), Cdr1p E1027Q (lane 5), Cdr1p E1027A (lane 6), and Cdr1p N328E/E1027Q (lane 7). (D) Photoaffinity labeling of wild type Cdr1p and its mutant variants with [¹²⁵I]-IAAP. The PM fraction (30 µg) of cells expressing the wild type Cdr1p and its mutant variants were incubated with 7.5 nM [¹²⁵I]-IAAP at 18°C in the absence or presence of 2 µM Nystatin (+lane) as described in Experimental Procedures. (E) Photoaffinity labeling of wild type Cdr1p and its mutant variants with [α-³²P] 8-azido ATP. The PM fraction (30 µg) of cells expressing the wild type Cdr1p and its mutant variants were incubated with 10 µM [α-³²P] 8-azido ATP at 4°C and competed with 10 mM cold ATP (+ATP lane) as described in Experimental Procedures. In panels C, D, and E, the lower panel shows the immunoblotting using anti-GFP antibody to ensure an equal loading of wild type Cdr1p and its mutant variants in all of the lanes. (F) Comparison of ATPase activity of Cdr1p with its mutant variants. ATPase activity of the PM fraction of cells expressing the wild type Cdr1p and its mutant variants were assayed as described under Experimental Procedures; the values represent the average ± standard deviation of three independent experiments. The inset shows the ATPase activity of Cdr1p and its mutant variants at different pH. The scattered plot represents AD1-8u (○), PSCdr1-GFP (○), VRCG N328E (○), VRCG N328A (●), VRCG E1027Q (▲), VRCG E1027A (△), and VRCG N328E/E1027Q (●).
Asp327 Serves as a Catalytic Base

**DISCUSSION**

Several aspects of the mechanisms of ABC drug transporters remain unresolved. Some questions such as what the various steps in the catalytic cycle are, the role of NBDs with respect to ATP binding and hydrolysis, and the nature and type of signal generated at NBDs by ATP hydrolysis, which is transduced to the drug binding sites in TMDs to mediate drug efflux, are poorly understood (1). Fungal ABC transporters pose additional challenges because they have typical amino acid substitutions in conserved motifs of NBDs, which suggest the possibility of mechanistic differences in the ATP catalysis cycle (20). In an attempt to understand the molecular basis of ATP hydrolysis mediated by the major ABC multidrug transporter of *C. albicans*, Cdr1p, we have demonstrated that the uncommon and atypically conserved Trp326 in the Walker B motif of Cdr1p, we have demonstrated that the uncommon and atypically conserved Trp326 in the Walker B motif of Cdr1p (pink), Asn328 (blue), Trp326 (green), and Cys193 (orange) residue side chains.

transporters is the involvement of the residues equivalent to Asp327 and Asp1026 in Mg2+ coordination (25, 42). We observed that mutations in the Walker B region (D327N, D327A, D1026N, D1026A, and D327N/D1026N) showed equivalent cell surface expression levels of Cdr1p but resulted in abrogated efflux of R6G in all of the Cdr1p mutant variants. Single point mutations introduced in either of the two NBD sites of Cdr1p (D327N and D1026N) impaired the capacity of mutant variant protein to confer resistance to all of the tested drugs. Mutations D327N and D1026N as well as the double mutation D327N/D1026N in the Walker B motif of Cdr1p showed severely decreased ATPase activity, though there was no effect on their ability to recognize the substrate per se as confirmed by photoaffinity drug analogue [3H] azidopine and [125I] IAAP labeling. These results establish that both Asp327 and Asp1026 are critical for ATP hydrolysis at their respective positions, and their substitution results in mutant variants that are unable to power drug efflux without affecting drug binding.

Our photoaffinity labeling experiments with [α-32P] 8-azido ATP illustrated the functional asymmetry of Asp327, Asp1026, and the two NBD sites therein. For example, although Cdr1p D327N showed comparable binding of [α-32P] 8-azido ATP to that of the wild type protein, this was not the case with Cdr1p D1026N and its double mutant Cdr1p D327N/D1026N variant protein, which showed severely impaired labeling. The inability of the Cdr1p D1026N mutant variant to efficiently bind [α-32P] 8-azido ATP even though the N-terminal NBD site was intact highlights the differences in the role of the two residues. The photoaffinity labeling data further imply that unlike at the C-terminal NBD with D1026N, ATP binding per se is not the major cause of dysfunction at the N-terminal NBD site with the D327N mutation, ATP binding per se is not the major cause of dysfunction, but the steps after initial binding are probably affected. In a recent in vivo study, the relative contribution

**FIGURE 7:** Atomic model of the N- and C-terminal NBD dimer of Cdr1p. (A) Homology based model of N- and C-terminal NBD of Cdr1p. The structure of N- and C-terminal NBD was generated by the Modeller8v2 program (19, 31). Structural diagrams were produced using visual molecular dynamic (VMD) software (29), where α-helices are represented as ribbons and strands as arrows. The ATP molecule is shown in licorice format and the atoms, i.e., carbon, nitrogen, oxygen, phosphorus, and sulfur and the magnesium ion are represented in cyan, blue, red, tan, yellow, and green, respectively. Functionally important sequence motifs of NBDs are labeled. (B) Blown-up view of the N-terminal nucleotide binding pocket of the dimeric model of Cdr1p, highlighting the close positioning of Asp327 (pink), Asn328 (blue), Trp326 (green), and Cys193 (orange) residue side chains.
of both the N- and C-terminal NBDs in ATP hydrolysis, drug resistance, and drug transport activity of wild type Cdr1p was examined, wherein the unique Cys193 of Walker A of N-terminal NBD (C193K) and a conserved Lys 901 of Walker A of C-terminal NBD (K901C) were replaced (43). Compared to the Cdr1p C193K, the cells expressing the Cdr1p K901C mutant variant became relatively more susceptible to drugs. This clearly suggested that the two NBDs of Cdr1p are functionally asymmetric (43). On the basis of sequence analyses, it was suggested earlier that the N-terminal NBD of Pdr5p (homologue of Cdr1p) of S. cerevisiae is probably unable to perform ATP hydrolysis and that the transporter might function with only one of its two NBDs (20). Interestingly, in the case of P-gp, which is a close homologue of Cdr1p, the two NBDs are partially interchangeable (25). However, in prokaryotic ABC transporters such as the histidine permease of S. typhimurium, both NBDs are functionally identical and contribute equally to the protein’s activity (44). The functional asymmetry of NBDs in Cdr1p was also illustrated in our recent study, where swapping of NBDs resulted in non-functional Cdr1p chimeras and, thus, suggested that the two NBDs are non-exchangeable (45). Taken together, results confirm that the two potential ATP binding sites of Cdr1p are not identical. These observations are also consistent with a model in which the binding of nucleotide at one site is indispensable for hydrolysis at the other site, originally proposed by Senior and colleagues for P-gp (46).

The photoaffinity labeling data further imply that at the N-terminal NBD site with the D327N mutation in the Walker B motif of Cdr1p, ATP binding per se is not the major cause of dysfunction, but the steps after initial binding are probably affected. That Asp327 and Asp1026 are important for ATP hydrolysis was apparent but that they have acquired different roles became clear when we monitored pH dependence of ATP hydrolysis. Although ATPase activity of the Walker B mutant variant Cdr1p D1026N does not show any pH dependence, the Cdr1p D327N mutant variant showed an enhancement in activity at acidic pH. This partial restoration of ATPase activity at acidic pH by the Cdr1p D327N mutant variant expressing cells was sufficient to partially power drug transport otherwise hypersensitive Cdr1p D327N mutant variant expressing cells could grow in the presence of tested drugs. The restoration of ATPase activity at low pH in the Cdr1p D327N mutant variant and not in the Cdr1p D327A mutant variant suggests that Asp327 probably acts as a catalysis base. At physiological pH 7.5, Asp327 of Cdr1p would exist in its deprotonated form, fully capable of abstracting a proton from a water molecule during ATP hydrolysis (Figure 8A). On the contrary, at the same pH, replaced Asn would be unable to abstract a proton. Our observation that the Cdr1p D327N mutant variant does not show any enhancement in ATPase activity at pH 7.5 supports such an assumption (Figure 8B). However, at acidic pH (4.5), solvent accessible Asn is expected to be hydrolyzed, become Asp, which could easily abstract a proton from a water molecule and, thus, promote ATP catalysis (Figure 8C).

Interestingly, the mutation of Asp327 to Glu was functional and was able to power the drug efflux like the wild type protein, demonstrating an exquisitely sensitive requirement for the carboxyl side chain at the 327 position. Our results with the purified domain mutant variant protein NBD-512 D327N further confirmed this pH dependence of Asp327. Interestingly, the prior exposure of the NBD-512 D327N mutant variant to low pH (4.5) was sufficient to enable it to hydrolyze ATP even at pH 6.5. The exposure of the NBD-512 D327N mutant variant protein to acidic pH would allow Asn to become Asp, which once hydrolyzed could now abstract a proton at pH 6.5. Our results strongly suggest that unlike in other non-fungal ABC transport, the conserved Asp327 in the Walker B motif has acquired a new role, where it is not involved in nucleotide binding per se but rather serves as a catalytic base at the N-terminal ATP site of Cdr1p and is involved in the abstraction of a proton from a water molecule. The conserved Asp1026 of C-terminal NBD, however, continues to perform its traditional established role of Mg2+ coordination in ATP catalysis. Consistent with experimental data, the N- and C-terminal NBD heterodimer model shows that indeed Asp327 forms an integral part of the ATP binding pocket. Thus, in addition to extensive biochemical data, homology modeling of NBDs also demonstrates the critical role of Asp327 in ATP hydrolysis.

Conventionally, the residue equivalent to Asp327 is part of the conserved Walker B motif found in many different non-fungal ATPases such as the ABC proteins, including transporters and DNA repair proteins such as Rad50 and MutS as well as helicase (47). It is known to be essential for chelating the Mg2+ bound to the nucleotide and is shown to be essential for activity (18). The very well conserved residue next to this Asp in non-fungal ABC transporters is Glu, which points directly toward the phosphate of the nucleotide bound in the active site (48). It has been proposed for other proteins that in this position, Glu serves as catalytic base to activate a catalytic water molecule for nucleophilic attack on the γ-phosphate (19, 38, 24). For example, mutation of the Glu residue to Ala or Gln in M. jannaschii ABC transporters, MJ0796 and MJ1267, results in the loss of ATPase activity, supporting the hypothesis that this residue is required for ATP hydrolysis (38). In the case of Bacillus subtilis ABC transporter, BmrA, substitution of this Glu with other amino acids resulted in the entrapment of the nucleotide in triphosphate form in the active site, also indicating the disruption of the hydrolysis reaction (36). In P-gp, the mutation of this Glu, however, indicated occlusion of the nucleotide in the diphosphate form, thus suggesting that the residue plays a role in ADP release and turnover rather than catalysis (49, 18). It would appear that the presence of Asn328 next to the well conserved Asp327 (instead of Glu) in the Walker B motif of the N-terminal NBD of Cdr1p and in other fungal ABC transporters has necessitated Asp327 to acquire the role of a catalytic base in ATP hydrolysis.

On the basis of the role of Glu adjacent to the Walker B motif of NBDs of other non-fungal ABC transporters, we argued that the replacement of Asn with the Glu (N328E) mutation in N-terminal NBD of Cdr1p could improve ATP hydrolysis. Contrary to expectation, this mutation (N328E) exhibited severely impaired ATPase and drug transport activity (Figure 6B). Interestingly, this mutant variant unlike Cdr1p D327N did not exhibit any pH dependence of ATPase activity (Figure 6F). These observations suggest that though Asn328 is important for the functionality of Cdr1p, yet unlike its substituted residues in other ABC transporters, it does not directly participate in ATP hydrolysis.
In conclusion, our study provides an instance of a functional residue migration or hopping during the evolution of NBDs. Active site variation might reflect the evolutionary optimization of the catalytic efficiency of fungal ABC transporters. We show that a highly conserved Asp residue at position 327 in the Walker B motif of N-terminal NBD of a major ABC drug transporter Cdr1p has acquired a new role to act as a catalysis base. Thus, the unique placement of Cys193, Trp326, and Asn328 in the conserved motifs of Cdr1p definitely has functional significance. Because such unique placements are also extended to other fungal ABC transporters, it strongly points toward their evolutionary relevance. However, what additional advantage such changes have brought to this class of proteins is not apparent. One needs to wait for the 3D structure of fungal ABC transporters to highlight the mechanistic differences with other well conserved proteins of this super family. Considering that many fungal ABC transporters belong to human pathogenic fungi, it would be interesting to examine whether these unique changes have provided any extra advantage to their survival in the host environment.

ACKNOWLEDGMENT

We thank R.D. Cannon for the gifts of the plasmid and the strains. We thank Ranbaxy Laboratories Ltd., New Delhi, India for providing fluconazole. We are also grateful to R. Serrano for the kind gift of PM-ATPase antibodies. We thank Dr. Andrew M. Lynn for his help in generating the atomic dimeric model of Cdr1p.

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transmembrane domains and nucleotide binding domains, but transmembrane segment 12 is replaceable with the corresponding homologous region of the non-drug transporter Cdr3p, *Microbiology* 152, 1559–1573.


BI061535T
Efflux Pumps in Drug Resistance of Candida

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Abstract: The incidences of human pathogenic yeast Candida albicans and its related species acquiring resistance to antifungals have increased considerably, which poses serious problems towards its successful chemotherapy. The resistance of these pathogenic fungi is not restricted to the commonly used triazole compounds but is even encountered, though not often, with polyene derivatives as well. The efflux pump proteins belonging to ABC (ATP Binding Cassette) and MFS (Major Facilitators) superfamily are the most prominent contributors of multidrug resistance (MDR) in yeasts. The abundance of the drug transporters and their wider specificity suggest that these transporters may not be exclusively drug exporters in yeasts and may have other cellular functions. In this article we focus on some of the recent advances on the structure and function, evolution and transcriptional control of drug efflux proteins of Candida. A short discussion on the physiological relevance of drug transporters is also included.

Keywords: ABC transporter, Candida albicans, multidrug resistance, efflux pumps, MFS, regulation, drug transport, Cdr1p.

INTRODUCTION

Among the various human fungal pathogens, Candida albicans accounts for the majority of systemic infections. But the infections caused by non-albicans species, such as C. glabrata, C. parapsilosis, C. tropicalis and C. krusei are also common particularly in neutropenic patients and neonates [1-4]. Of note, recently, the incidences of C. albicans cells acquiring resistance to antifungals have increased considerably which has posed serious problems towards its successful chemotherapy [5-7]. Unfortunately, the incidence of antifungal resistance is not restricted to C. albicans alone, non-albicans species such as C. glabrata, C. parapsilosis, C. tropicalis and C. krusei also display this phenomenon [1-4,8]. C. albicans as well as non-albicans species have evolved a variety of mechanisms to develop resistance to common antifungals. Some of the important mechanisms are discussed below.

MECHANISMS OF RESISTANCE TO ANTIFUNGALS

Resistance to antifungals could be visualized as a gradual evolving process wherein different mechanisms may appear during the course of chemotherapy. [3,5,6,7]. The main mechanisms of antifungal resistance include alterations in ergosterol biosynthetic pathway by an overexpression of ERG11 gene that encodes the drug target enzyme 14α-demethylase or by an alteration in target enzymes (point mutations) which leads to reduced affinity to fluconazole [3,9,10]. Reduced intracellular accumulation of drugs (due to rapid efflux) is another prominent mechanism of resistance in Candida cells [11]. Most commonly, genes encoding drug efflux pumps belonging to ABC (ATP binding cassette) and MFS (Major Facilitator) superfamilies of proteins are overexpressed in azole resistant Candida isolates [12-16]. Accordingly, it has been well documented by several groups that clinical azole resistant isolates of C. albicans display transcriptional activation of genes encoding ABC (Cdr1p, Cdr2p) or MFS (CaMdr1p) efflux pump proteins [13,15-18]. Invariably, resistant Candida cells, which show enhanced expression of efflux pumps encoding genes, also show simultaneous increase in the efflux of drugs thus implying a causal relationship between efflux pump encoding gene expression levels and intracellular concentration of the drug [13-15,17]. Interestingly, drug inactivation, which is a very common mechanism in bacteria, has not been observed in Candida cells. Of note recent gene profiling results already suggest even more complexities, which may affect and control the phenomena of antifungal resistance [15,19,20].

EFFLUX PUMPS IN DRUG RESISTANCE

In addition to C. albicans, clinical resistance to fluconazole as a result of reduced intracellular accumulation has also been reported for other pathogenic Candida species including C. tropicalis, C. glabrata, C. krusei and C. dubliniensis. Table 1 lists the known MDR transporters in Candida species. Among non-albicans species, C. glabrata has emerged as an important nosocomial pathogen. Homologues of CDR1, CgCDR1 and CgCDR2 have been isolated by functionally complementing a hypersensitive mutant of S. cerevisiae [21]. Another putative ABC transporter encoded by PDH1 gene has also been implicated in azole resistance of C. glabrata [22]. C. dubliniensis is phylogenetically closely related to C. albicans and is associated with oral candidosis. Fluconazole resistant isolates of C. dubliniensis showed reduced accumulation of the drug as compared to susceptible strains. This led to the identification of two ABC transporters viz., CdCDR1 and CdCDR2, which mediate fluconazole resistance in clinical isolates of C. dubliniensis [23]. A potential role of two putative ABC transporters ABC1 and ABC2 in drug resistance has also been suggested for C. krusei [24].

Since ABC and MFS transporters are among the major players that contribute to azole resistance in clinical isolates
Table 1. Efflux Pumps of Albicans and Non-Albicans Candida Species

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<tr>
<th>Organism</th>
<th>Gene name</th>
<th>Function/Substrates</th>
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<tbody>
<tr>
<td>Candida albicans</td>
<td>CaCDR1</td>
<td>Drug efflux pump, phospholipid translocator&lt;sup&gt;a,b,c&lt;/sup&gt;</td>
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<tr>
<td></td>
<td>CaCDR2</td>
<td>Drug efflux pump, phospholipid translocator&lt;sup&gt;a,c&lt;/sup&gt;</td>
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<td></td>
<td>CgCDR2</td>
<td>Drug efflux pump&lt;sup&gt;9&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>PDH1</td>
<td>Drug efflux pump&lt;sup&gt;9&lt;/sup&gt;</td>
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<tr>
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<td>CjCDR1</td>
<td>Drug efflux pump&lt;sup&gt;9&lt;/sup&gt;</td>
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<td>CjCDR2</td>
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<td></td>
<td>ABC2</td>
<td>Drug efflux pump&lt;sup&gt;9&lt;/sup&gt;</td>
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2. MFS transporters

<table>
<thead>
<tr>
<th>Organism</th>
<th>Gene name</th>
<th>Function/Substrates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Candida albicans</td>
<td>CaMDR1/BEN&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Benomyl, methotrexate, sulfonamethuron methyl, NQO and fluconazole&lt;sup&gt;8&lt;/sup&gt;</td>
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<tr>
<td></td>
<td>FLU1</td>
<td>Mycophenolic acid&lt;sup&gt;8&lt;/sup&gt;</td>
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<tr>
<td>Candida dubliniensis</td>
<td>CdMDR1</td>
<td>Fluconazole, amorolfine, brefeldin A, cerulenin, cycloheximide, fluphenazine and NQO&lt;sup&gt;8&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

References:

<sup>a</sup>Balan, I.; Alvarez, A. M.; Raymond, M. J. Bacteriol., 1997, 179, 7210-7218.
<sup>f</sup>Sanglard, D.; Bille, J.; Calabrese, D. Microbiology, 2000, 146, 2743-2754.

of Candida, there is a spurt in research on all aspects of these genes and their encoded proteins. Therefore, considerable attention is also being paid to the structural and functional aspects of these proteins, which in turn could lead to better strategies for designing modulators/inhibitors of these pumps. As an alternate strategy, an understanding of the regulatory circuits controlling the expression of efflux pump encoding genes is also necessary for the development of drugs that could block the expression of these genes. In this review, we begin with a discussion on the structure and function of ABC proteins followed by a section on the regulation of MDR genes. The evolution and physiological roles of drug efflux proteins, particularly belonging to ABC superfamily are also discussed.

STRUCTURE AND FUNCTION OF EFFLUX PROTEINS

ABC Proteins

ABC proteins are generally made up of two transmembrane domains (TMDs) and two cytoplasmically located nucleotide binding domains (NBDs), although 'half proteins' that probably dimerize for full functionality are known in some prokaryotes Fig. (1A) [25]. Typically, the TMDs comprise of α-helices of 12 transmembrane segments (TMS) while the NBDs have α-helices and β-sheets arranged to form a Rossmann-fold architecture Fig. (1B) [25]. While it appears that several TMSs associate together to form the substrate binding site(s), this alone is probably not sufficient for substrate transport across the membrane bilayer.
Efflux Pumps in Drug Resistance of Candida

Vectorial transport of these substrates requires energy from the hydrolysis of ATP carried out at the NBDs. Given their varied roles and the greatly differing characteristics of substrates that members of this superfamily of proteins seem to efflux, it is hardly surprising that despite the overall conservation of the domain architecture of TMDs, their primary sequences are significantly different. On the other hand, NBDs of ABC transporters are highly conserved both in terms of primary structure and architecture [26].

ABC transporters of Candida and other yeasts like S. cerevisiae possess specific domains for membrane association, ATP binding and hydrolysis. The major drug transporter of C. albicans, Cdr1p, is comprised of two homologous halves, each made up of a hydrophilic, cytoplasmic NBD and TMD represented by six TMS. Although many transporters are predicted to have an (NBD-TMD)$_2$ topology, there are also some putative ORFs that have only (NBD-TMD)$_1$ and thus appear to be half transporters. The structural and functional analysis of human P-gp/MDR1 and its other homologues in mouse have demonstrated the importance of NBDs and TMDs in drug extrusion [27]. In comparison, studies pertaining to the identification of the molecular determinants of yeast ABC drug transporters have only recently been initiated [28-31].

Substrate Binding Sites

The range of Cdr1p substrates varies enormously and includes structurally unrelated compounds such as azoles, lipids and steroids [32,33]. This promiscuity towards substrates is a characteristic feature of most ABC type drug transporters and hence makes their functionality all the more complex to understand [33,34]. As expected, predicting which residues are involved in substrate binding without high-resolution structural data to back these claims is a challenge. Yet using a combination of biochemical and transport assays along with site-directed mutagenesis, it has been possible to map the substrate binding pockets of these proteins with some success [30,31,35].

In a recent study Cdr1p was overexpressed as a GFP-tagged fusion protein in a heterologous hyper-expression system [30] and was characterized for drugs and nucleotide binding [30]. Iodoarylazido prazosin (IAAP, a photoaffinity analogue of P-gp substrate, prazosine) and azidopine (a dihydropyridine photoaffinity analogue of P-gp modulator, verapamil) were shown specifically to bind with Cdr1p-GFP. Interestingly, IAAP binding with Cdr1p-GFP was competed out by nystatin while azidopine binding could only be competed out by miconazole, thus, demonstrating the
possibility of different drug binding sites for the two analogues [30]. For detailed structural and functional analysis, more than 60 point mutations were introduced in Cdr1p (unpublished results). Several point mutations yielded variant proteins exhibiting interesting phenotypes Fig. (2). The studies conducted so far with Cdr1p suggest that in spite of topological differences with human P-gp, there is a conserved functional homology between the two multidrug transporters [30]. Gauthier and coworkers [36] have recently shown that membranes prepared from Cdr1p and Cdr2p expressing cells are capable of binding the photoaffinity analogue of rhodamine 123 (\(^{125}\)I) iodoaryl azido-rhodamine 123 (IAARh123) and that both N-terminal and C-terminal halves of Cdr2p contribute to rhodamine binding [36].

ABC drug transporters of other yeasts are also gradually being put to structural and functional analyses. A close homologue of Cdr1p, Pdr5p of S. cerevisiae, was subjected to random mutational analysis wherein Egner and coworkers [28,29] identified several important amino acid residues critical for drug binding and transport. Recently by using a variety of novel substrates of Pdr5p, Golin and coworkers [37] have reported that this ABC drug transporter has at least three drug binding sites and that some substrates might interact at more than one site. Thus, similar to Cdr1p and its other mammalian homologues, Pdr5p also seems to have multiple drug binding sites throughout the protein.

**Nucleotide Binding Domains (NBDs)**

Another important characteristic feature of ABC drug transporters is that they utilize the energy of ATP hydrolysis to transport variety of substrates across the plasma membrane. The conserved NBDs located at the cytoplasmic periphery are the hub of such an activity. The NBDs of all ABC transporters, irrespective of their origin and nature of transport substrate, share extensive amino acid sequence identity and typical motifs [26]. For example, NBDs of ABC transporters have a β-sheet sub-domain containing the typical Walker A and Walker B motifs, as an essential feature of all ATP requiring enzymes [38], along with an α-helical sub-domain that possesses the conserved ABC signature sequence. NBD protein sequences possess certain conserved amino acid stretches, which are considered to be critical for the domain’s functionality [39]. These include: the Walker A, with a consensus sequence GxxGxGKS/T, where ‘x’ represents any amino acid, the Walker B motif i.e. hhhD, where ‘h’ represents any aliphatic residue, and an ABC signature, LSGGQQ/R/KQR. Structural and biochemical analyses of NBDs show that the lysine residue of Walker A motif binds to the β- and γ-phosphates of ribonucleotides and plays a critical role in ATP hydrolysis [39]. Mutation of this lysine residue has been shown to reduce or abolish the hydrolysis activity and in some cases impair nucleotide binding [39,40]. Interestingly, though N-terminal NBD of Cdr1p contains the conserved Walker A

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**Fig. (2). Mutational analysis of Cdr1p:** Cdr1p possesses two transmembrane domains (TMD1 and TMD2) and two nucleotide binding domains (NBD1 and NBD2). The individual TMSs are marked by numbers indicating the beginning and end of the segments. The point mutations introduced in Cdr1p are marked in boxes and the mutations which showed phenotypes are indicated in red color. The N-terminal and C-terminals of Cdr1 are marked by NH\(_2\) and COOH, respectively.
While the highly conserved Asp327 of NBD1 is known to be the catalytic carboxylate in the context of other ABC transporters, in the NBD1 of Cdr1p it does not appear to mediate catalysis via interaction with Mg\(^{2+}\), as is normally expected for similar transporters [43].

**NBD Structure**

No NBDs from any fungal ABC transporter have yet been crystallized, however, ample biochemical and structural data from other homologous transporters already point to a cooperative role between the TMDs and the NBDs [44]. In general, substrate binding works as the signal for the cytoplasmically located NBDs to bind and hydrolyze ATP as well as to dimerize, which in turn produces a conformational change large enough to power transport [44]. How NBDs dimerize and orient in a catalytic cycle of an ABC transporter, are some of the important issues that remain unresolved. Structural evidence from MdrA, a full length ABC protein of *E. coli*, suggest that in the absence of bound nucleotide, the NBDs of the protein are well-apart in the crystal and do not form a dimeric interface. On the other hand, crystal structure data from BtuCD of *E. coli* suggest that when nucleotide is bound to the NBDs (BtuDs) they dimerize in a head-to-tail arrangement [45]. Whether these differences are purely due to the absence or presence of nucleotide or whether they represent fundamental differences in the way the two proteins are organized remains to be verified. An electron microscopic reconstruction of Pdr5p, one of the best biochemically studied ABC multidrug transporters of the yeast *S. cerevisiae*, was recently published [46]. The model suggests that Pdr5p dimerizes with NBDs oriented perpendicular to each other. The particular preparation of Pdr5p used in the study was inactive but the authors speculate that Pdr5p functions only as a multimeric protein. Their conclusion is based on the assumption that one of the NBDs (NBD1) of Pdr5p that contain a unique cysteine instead of well conserved lysine is inactive and thus, in order to complete the ATP cycle, the proteins forms a multimeric complex [46].

Isolated NBD domains have also been crystallized and a great deal of structural information for NBDs from several ABC transporters, both of bacterial and eukaryotic systems, is available. Thus a gradual picture has begun to emerge on the structure, organization and nucleotide binding sites of the NBDs. Several NBDs, such as HisP [47], MalK [48], MJ0796 [49], Rad50 [50], crystalize as a dimeric unit, raising the probability that dimerization of NBDs is important for their in vivo functionality as well. Further, in some NBDs such as MJ0796, ATP is sandwiched between the Walker A motif of one monomer and the ABC Signature sequence of the other [49]. If indeed this is also true in vivo, it would mean that such dimerization could be the basis for global conformational change and drug transport in ABC transporters. Since the crystal structure of at least one full length ABC transporter (BtuCD) supports such a possibility [45], such functional interaction between the two NBDs of the ABC transporter cannot be ruled out. However, the wide variation observed in possible orientations of monomeric units within the crystal of dimeric NBDs must be kept in mind [51,52].

(Grpgagcst) and B (icqwd) motifs, and an ABC signature sequence (vsggerkrvsia) [14], the commonly occurring lysine residue within the Walker A motif is replaced by a cysteine. This replacement appears to be a unique feature of N-terminal NBDs of almost all the known fungal ABC-type transporters. The significance of this replacement from a charged residue to a sulfhydryl amino acid for the in vivo function of the protein is not apparent. Of note, the Walker A motif in the C-terminal NBD of Cdr1p contains the commonly conserved lysine.

Recently, in an attempt to examine the independent functionality of N-terminal NBD of Cdr1p and to ascertain the role of the uncommon cysteine of the Walker A motif, an active N-terminal nucleotide-binding domain (NBD1) of Cdr1p was purified and characterized. This purified domain elicits a cation dependent general ribonucleotide triphosphatase activity [41]. Jha and coworkers found that an evolutionarily divergent Cys193 of the Walker A motif of NBD1 was critical for ATP hydrolysis. In a recent in vivo study, the relative contribution of both the N- and C-terminal NBDs in ATP binding, hydrolysis and transporter activity of native Cdr1p (full protein) was examined wherein the atypical Cys193 of the Walker A motif of NBD1 (C193K) and conserved Lys901 (K901C) in the Walker A motif of NBD2 were replaced [41]. The drug resistance profile of the Cdr1p mutant variant cells harboring C193K or K901C gave interesting insights into the functioning of the two NBDs. The cells expressing K901C showed enhanced hypersensitivity to drugs as compared to C193K variant. These observations clearly established that the two NBDs respond asymmetrically to the substitution of conserved residues of their respective Walker A motifs. Likewise, Prasad and coworkers have shown that protein chimeras containing either two N-terminal or two C-terminal NBDs in Cdr1p resulted in non-functional protein variants (unpublished data). An in-depth analysis of the catalytic cycle, namely nucleotide binding, hydrolysis and substrate binding/efflux is required to resolve the reasons behind the dichotomy in functioning of the NBDs. Studies to date, however, did demonstrate that a diverse N-terminal NBD (GxxGxGCSrr) of Cdr1p is functional wherein its uncommon Cys193 is critical. Considering that all other fungal ABC transporters including the well studied Pdr5p of *S. cerevisiae* have uncommon cysteine in Walker A of NBD1 (with the exception of Ste6p of *S. cerevisiae*), it is expected that this residue will have indispensable role in the catalytic cycle of these proteins as well [42].

Two other residues of the N-terminal NBD from Cdr1p are also found to be important for domain functioning. The unusual Trp326 in the Walker B motif of NBD1, which is conserved in all fungal transporters, appears to be important for ATP binding and for the accompanying conformational change [43]. Thus, although the mutant with W326A appears capable of ATP hydrolysis, it does so with a much higher K_M value, indicating that the docking of substrate in the binding pocket has been altered by the mutation. However, the protein appears capable of near-normal function in cells expressing the full length protein carrying the W326A mutation, implying that the conformational change that normally occurs upon ATP docking cannot by itself be responsible for the cross-talk by the domain with the TMDs.
Transmembrane Domains (TMDs)

Given that ABC proteins are membrane proteins, it is hardly surprising that very little high-resolution structural information of full-length proteins is available. As mentioned above, relatively high-resolution single crystal X-ray diffraction data are now available for two types of ABC transporters, a bacterial lipid A flippase MsbA [53, 54] and a vitamin B12 importer ButCD from E. coli [45]. The structure of MsbA for the first time provided high-resolution structural proof that the TMDs are indeed likely to span the membrane bilayer. The TMDs in MsbA together form a cone like structure with two openings of different diameters at either face of the bilayer wherein TMS2 and TMS5 participate in the major dimerization contacts. However, the analyzed crystals did not have nucleotide bound to the protein and hence could not provide information of the organization of the TMDs upon ATP binding [53].

The structure of ButCD on the other hand, does contain bound nucleotide. The structural data suggests that the TMDs of each ButC subunit assemble to form a translocation channel through which vitamin B12 is likely to be transported across the bilayer. But the similarities in the TMD organization between MsbA and ButCD cannot be extrapolated much further, not merely because of the absence or presence of bound nucleotide. The nature of the translocation channel in the two proteins could be very different since the two proteins bind substrates that are diametrically opposite in their chemical characteristics. The hydrophobic lipid A is the specific substrate for MsbA while the hydrophilic Vit B12 is the specific substrate for ButCD [45, 53].

Mechanism of Drug Transport

As mentioned earlier, how ABC transporters couple ATP hydrolysis to drug transport, is an unresolved issue. Structural and biochemical evidence has strongly favored the hypothesis that drug binding stimulates the NBDs to bind and hydrolize ATP, which in turn results in large conformational changes both within the NBD as well as in the TMDs, causing the bound drug/substrate to be effluxed by the protein. Such a mechanism has been proposed from the structural data available for ButCD, for example [45]. Biochemically, it was shown that trapping the NBDs in their transition state during an ATP hydrolysis cycle for drug transport resulted in protein with low affinity for drug substrate. Thus during the ATP hydrolysis step, substrate binding site(s) formed by the TMDs of the protein favor dissociation rather than association of the drug substrate [44]. While similar information does not yet exist for fungal ABC transporters, it is possible to speculate that such a mechanism also operates in these proteins. However, exactly how drug binding is transduced to the NBDs as a signal is as yet poorly understood. Of note, the well-documented drug stimulated ATPase activity of mammalian ABC transporters has not been found for fungal transporters [13,55-57]. Nevertheless, it is certain that a very close interaction exists between the TMDs and the NBDs in ABC transporters. For example, it is observed that mutations or deletions in small discrete regions adjacent to the Walker B motif of Pgp, result in loss in this signaling and therefore of substrate transport [58].

Does ATP binding, or its subsequent hydrolysis, induce conformational changes within the NBDs that power drug transport? Interestingly, NBDs from several ABC transporters supposedly undergo significant conformational changes due to ATP binding rather than due to its hydrolysis [43,59]. This has also been shown to be the case for isolated NBD1 from the fungal ABC transporter, Cdr1p. Thus catalytically deficient NBD1 variants (with C193A or D327A/N) continue to exhibit conformational changes similar to wild type NBD1 while the binding deficient mutant (with W326A) appears impaired in its ability to perform a similar conformational transition.

MFS Drug Efflux Proteins

MFS proteins are another class of major drug transporters that are involved in drug efflux and thus, have a role in the multidrug resistance phenomenon displayed by yeasts. The MFS was originally defined as a superfamly of permeases that are involved in drug efflux and thus, have a role in the multidrug resistance phenomenon displayed by yeasts. The MFS was originally defined as a superfamly of permeases that are involved in drug efflux and thus, have a role in the multidrug resistance phenomenon displayed by yeasts. The MFS was originally defined as a superfamly of permeases that are involved in drug efflux and thus, have a role in the multidrug resistance phenomenon displayed by yeasts.
halves, which suggests that C-terminal regions are involved in substrate recognition, and N-terminal regions are involved in proton translocation [65,66].

REGULATION OF MULTI-DRUG RESISTANCE

The transcriptional activation of CDR1 in the development of azole resistance is well known [15-17]. However, the mechanisms by which CDR1 levels are altered in clinical resistant isolates are not fully understood. In comparison, the mechanism underlying the upregulation of MDR genes in the development of multidrug resistance is well described in the baker’s yeast S. cerevisiae, wherein several drug extrusion pumps like PDR5 (Pleiotropic Drug Resistance), SNQ2 (Sensitivity to 4-Nitroquinoline N-oxide) and YOR1 (Yeast Oligomycin Resistance), etc. have been implicated in the development of drug resistance [67,68]. In S. cerevisiae three networks of transacting factors, Pleiotropic drug resistance (PDR), Yeast AP-1 like factor (YAP-1) and Yeast reveromycin resistance (YRR) are mainly involved in controlling the expression of MDR genes. The transcription factors (TFs) PDRI and PDR3 belong to the zinc cluster protein family and regulate the transcription of PDR5 and SNQ2 [68]. On the other hand, YAP (Yeast Activator Protein) belongs to bZip family of TFs and confers resistance to a variety of toxicants, including cycloheximide, cadmium, and tolerance to oxidative stress. YAPI targets include an MFS type drug extrusion pump FLR1 (Fluconazole Resistance) [69] and GLRI encoding glutathione reductase, which are involved in conferring oxidative tolerance to yeast cells [70]. Additionally, YAP1 also activates PDR3 expression under stress conditions such as heat shock [71], and a link between “YAP” and “PDR” networks has also been established in S. cerevisiae [72]. Another zinc finger transcription factor, Yeast reveromycin

A resistance (YRR1) is involved in complex PDR network regulation by directly activating SNQ2 and YOR1 [73], both of which are also common targets of Pdr1p, Pdr3p and Yap1p [74]. In addition to PDR1, PDR3, YAPI and YRR1 a transcriptional repressor of PDR has also been identified recently, which is designated as RDR1 (Repressor of Drug Resistance) [75,76]. RDR1 binds to PDER (Pleiotropic Drug Response Element), a cis-acting regulatory element also shared by the master regulator PDR1 and PDR3 [75]. A recent genome wide approach has revealed that PDR genes of S. cerevisiae are regulated by multiple factors, which involves cross talk between several regulatory networks Fig. (3) [77,78].

REGULATION OF MDR IN CANDIDA ALBICANS

As mentioned above, the upregulation of drug extrusion pump-encoding genes belonging to either ABC (CDR1 and CDR2) or MFS (CaMDRI) proteins represents one of the most prevalent mechanisms of drug resistance in Candida [12,14,18,61,79]. The various possibilities that may affect expression of CDR1 in azole resistant clinical isolates of C. albicans include mutations in the promoter region of the gene, altered regulation by trans-regulatory factors controlling expression or molecular changes taking place during mRNA processing [80-83]. In one study, the molecular changes responsible for the CaMDRI activation in matched fluconazole susceptible and resistant isolates wherein drug resistance coincided with the stable CaMDRI activation, was examined by Wirsching and coworkers [81]. Sequence analysis of the CaMDRI regulatory region did not reveal any promoter mutation in the resistant isolates that might account for the enhanced expression of the GFP reporter gene under the control of the CaMDRI promoter [81]. They concluded that CaMDRI promoter was activated

Fig. (3). Regulatory network of major drug efflux pumps in S. cerevisiae. The boxed square plus indicate the transcriptional activation, the square minus indicates the post transcriptional negative cross regulation between YRR1 and YRRJ where as circle minus indicates the transcriptional repression. The PDR network is interconnected with YRR network as well as with YAP net work indicating the complex regulation of the drug resistance in S. cerevisiae. * indicates ABC transporters, ← indicates MFS transporters.
by trans-regulatory factor(s) that might be mutated in fluconazole resistant isolates [81]. Recently mutation in the trans-regulatory factor has been reported for the activation of PDR16 gene (Phosphatidylinositol Inositol Transfer protein), which is coordinately regulated with CDR1 and CDR2 in clinical isolates of C. albicans [80].

**CIS-REGULATORY ELEMENTS OF THE CDR GENES IN C. ALBICANS**

*In-silico* analysis revealed several putative stress inducible cis-regulatory elements (HSE, MDR-NF1/YB-1, AP-1 etc.) within CDR1 promoter region, which may be responsible for its observed transcriptional activation [84]. Puri and coworkers showed several putative upstream activating (UAS) and upstream-repressing sequences (URS) by employing a *Renuilla* luciferase reporter fusion of CDR1 promoter [84]. Additionally, a steroid responsive region (SRR) in the distal part of the promoter consisting of two progesterone responsive sequence (-628 to -594 and -683 to -648) and one beta-estradiol responsive sequence (-628 to -577) has also been identified [85]. Deletion analysis within the SRR further delimited these steroid responsive sequences into two distinct elements, viz. SRE1 and SRE2. While SRE1 responds only to progesterone, SRE2 responds to both beta-estradiol and progesterone. Both SRE1 and SRE2 were specific for steroids, as they did not respond to drugs [85]. Recent genome profiling revealed that yeast perceives steroids as cellular stress since host of stress responsive genes along with the MDR genes were transcriptionally coregulated [86]. Sanglard and his group have identified a common drug/steroid responsive element (DREa and DREib) in CDR1 and CDR2 promoters which unlike SRE1/2 also respond to drugs Fig. (4) [87].

Recently, a Negative Regulatory Element (NRE) has been identified in proximal region of the CDR1 promoter, which is responsible for its basal expression [88]. *In-silico* analysis revealed that NRE (-ccaaCTGATTGAaact-) of CDR1 harbors ATTGA (inverted CCAAT) and shows significant homology with MDR-NF1/YB-1 binding site (ATTGG) in human MDR1 promoter that is known for its involvement in basal as well as in stress-induced expression [89]. Site directed mutational and deletion analysis of NRE in full-length promoter resulted in the constitutive transcriptional activation of CDR1. A purified 55 KDa nuclear protein (NREBP) was shown to specifically bind with the NRE [88]. Considering the importance of NRE in basal expression of CDR1, recently clinical relevance of NRE was explored in two matched pairs of resistant C. albicans clinical isolates wherein drug resistance coincided with the stable transcriptional activation of CDR1 [15,90]. Integration of the CDR1p-GFP and CDR1p-lacZ promoter reporter fusions at an ectopic locus in the genome of clinical isolates and their activities after mutation or deletion of NRE suggested its involvement in CDR1 expression in resistant isolates. Electrophoretic mobility shift assay (EMSA) and DNaseI foot printing further revealed that NREBP has a definite role in transcriptional regulation of CDR1 (unpublished data). In that study, enhanced binding of NREBP to NRE correlated with down-regulation of CDR1 in sensitive isolates whereas its poor binding or protection to CDR1p in resistant background is directly linked to its constitutive overexpression (unpublished data). Prasad and coworkers for the first time showed that NRE has a key regulatory role in the basal transcriptional activity of CDR1 that is differentially recognized by NREBP in sensitive and resistant clinical isolates of C. albicans.

**TRANS-REGULATORS OF MDR IN C. ALBICANS**

The isolation of the C. albicans CDR1 and CDR2 genes by functional complementation of *S. cerevisiae* lacking ABC transporter gene PDR5 suggested that there could also be a common mechanism of regulation of these homologues between the two yeasts, however, this does not seem to be the case [69,91,92]. For example identification of transcription factors like CAP1 (*Candida* AP-1), and FCRs (Fluconazole resistance), a homologue of transcription factors YAP1 and of PDR1/3 respectively (Table 2), turned out to have different roles as compared to their counterparts in *S. cerevisiae* [69,91,92]. FCR1, 2 and 3 have been identified for their ability to complement the fluconazole

![Fig. (4). Identified cis-acting elements in CDR1 promoter: Numbers indicate the position of the elements relative to the transcription start site (TSP) represented by the arrow. RSRK: repressor of steroid responsive region, SRR: steroid responsive region, SRE1: steroid responsive element 1, SRE2: steroid responsive element 2, NRE: negative regulatory element, W1: window1, W2: window2, W3 window3, W4: window4, DREa: drug responsive element la, DREb: drug responsive element lb, BEE: basal expression element (see text for discussion).](image-url)
Table 2. List of Known Transcription Factors of MDR in Yeast and their Targets

<table>
<thead>
<tr>
<th>TFs</th>
<th>Organism</th>
<th>Description</th>
<th>Target genes</th>
<th>Functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDR1&lt;sup&gt;1&lt;/sup&gt;</td>
<td>S. cerevisiae</td>
<td>Zn(II&lt;sub&gt;2&lt;/sub&gt;Cys&lt;sub&gt;6&lt;/sub&gt;)</td>
<td>PDR3, PDR5, PDR15, PDR16, IPTG1, SNQ2, YOR1, HXT9, HXT11</td>
<td>Activation of PDR genes</td>
</tr>
<tr>
<td>PDR3&lt;sup&gt;1&lt;/sup&gt;</td>
<td>S. cerevisiae</td>
<td>Zn(II&lt;sub&gt;2&lt;/sub&gt;Cys&lt;sub&gt;6&lt;/sub&gt;)</td>
<td>PDR3, PDR5, PDR15, PDR10, SNQ2, YOR1, HXT9, HXT11</td>
<td>Activation of PDR genes</td>
</tr>
<tr>
<td>YAP1&lt;sup&gt;-&lt;/sup&gt;</td>
<td>S. cerevisiae</td>
<td>bZip</td>
<td>PDR5, SNQ2, YOR1, YCF1, TRX2, GSH1, GLR1, FLR1, ATR1</td>
<td>Oxidative stress and heavy metal stress response</td>
</tr>
<tr>
<td>RDR1&lt;sup&gt;1&lt;/sup&gt;</td>
<td>S. cerevisiae</td>
<td>Zn(II&lt;sub&gt;2&lt;/sub&gt;Cys&lt;sub&gt;6&lt;/sub&gt;)</td>
<td>PDR5, PDR15, PDR16, YOR1, HXT9, HXT11</td>
<td>Repressor of drug resistance genes</td>
</tr>
<tr>
<td>YRR1&lt;sup&gt;-&lt;/sup&gt;</td>
<td>S. cerevisiae</td>
<td>Zn2Cys6</td>
<td>SNQ2, YOR1, FLR1, AER1, SNQ1, YLR346C, YRL346C, YRM1</td>
<td>Activation of PDR and repressor of YRR1</td>
</tr>
<tr>
<td>YRM1&lt;sup&gt;1&lt;/sup&gt;</td>
<td>S. cerevisiae</td>
<td>Zn2Cys6</td>
<td>YRR1, SNQ2, YOR1, AER1, SNQ1, PDR16, TPO4</td>
<td>Activation of PDR and repressor of YRM1</td>
</tr>
<tr>
<td>FCRs&lt;sup&gt;-&lt;/sup&gt;</td>
<td>C. albicans</td>
<td>Zn2Cys6</td>
<td>YOR1, SNQ2, YOR1, AER1, SNQ1, PDR16, TPO4</td>
<td>Activation of PDR and repressor of YRM1</td>
</tr>
<tr>
<td>CAP1&lt;sup&gt;1&lt;/sup&gt;</td>
<td>C. albicans</td>
<td>bZip</td>
<td>CoGLR1, CaYCF1, CaMDRI, CaTRR1</td>
<td>Negative regulator of drug resistance</td>
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<td>C. albicans</td>
<td>Zn2Cys6</td>
<td>CDR1, CDR2, TF13, IFS1, HSP12</td>
<td>Transcriptional activator of CDR genes</td>
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<tr>
<td>CaUPC2&lt;sup&gt;1&lt;/sup&gt;</td>
<td>C. albicans</td>
<td>Zn(II&lt;sub&gt;2&lt;/sub&gt;Cys&lt;sub&gt;6&lt;/sub&gt;)</td>
<td>ERG1, ERG2, ERG11</td>
<td>Transcriptional regulation of the ERG genes</td>
</tr>
<tr>
<td>CaNdh80&lt;sup&gt;1&lt;/sup&gt;</td>
<td>C. albicans</td>
<td>Novel DNA binding domain</td>
<td>CDR1, CDR2, TF13, IFS1, HSP12</td>
<td>Transcriptional regulator of CDR1</td>
</tr>
<tr>
<td>EFG1&lt;sup&gt;1&lt;/sup&gt;</td>
<td>C. albicans</td>
<td>bHLH</td>
<td>ERG3</td>
<td>Transcriptional regulator of CDR1</td>
</tr>
<tr>
<td>Pdr1-like&lt;sup&gt;1&lt;/sup&gt;</td>
<td>C. glabrata</td>
<td>Zn(II&lt;sub&gt;2&lt;/sub&gt;Cys&lt;sub&gt;6&lt;/sub&gt;)</td>
<td>CgCDR1, CgPDH1</td>
<td>Transcriptional Regulator of PDR</td>
</tr>
</tbody>
</table>

References:
1. [681], [691], [731], [732], [92], [945], [97], [97], [99], [999], [993]

hypersensitive S. cerevisiae null mutants of Δpdr1 and Δpdr3, suggesting that the FCRs are functional homologues of the master regulators PDR1 and PDR3 of S. cerevisiae. But on the contrary, the FCR1 of C. albicans behaves as a negative regulator of drug resistance [92]. Recently, a homologue of PDR1 was identified in the genetically less susceptible yeast Candida glabrata. In addition, a cis-acting regulatory element (PDRE) recognized by the master regulators PDR1 and PDR3, was also found in the promoter region of the genes involved in drug resistance (CDR1 and PDH1) in C. glabrata [93]. This may suggest some degree of similarity with respect to the regulation of drug resistance genes between the two genetically less susceptible yeasts, S. cerevisiae and C. glabrata.

CAP1, a bZip transcription factor, is involved in multidrug resistance as well as in oxidative stress response in C. albicans. Potential CAP1 targets include MDR genes like CaMDR1 and genes which are involved in oxidative stress, e.g., CaYCF1 (essential for conferring cadmium resistance); CaGLR1 (encoding a Glutathione Reductase) and CaTRR1 (encoding a Thioredoxin Reductase) [91,94,95] (Table 2). Moyer-Ruwey and his group have demonstrated that similar to YAP1 in S. cerevisiae, CAP1 protein also has a conserved cysteine in the C-terminal domain essential for its nuclear localization in response to oxidative stress [95]. No direct link between CAP1, FCRs and drug extrusion pumps (CDR1, CDR2 and CaMDR1) has been established.

CaUPC2 of C. albicans (a homologue of ScUPC2 and ScECM22 of S. cerevisiae), is involved in the development of drug resistance that target the product of ERG11 gene and to those drugs that act on ergosterol biosynthesis [96]. A search of promoter sequences of C. albicans ergosterol biosynthetic genes (ERG1, ERG2 and ERG11) revealed presence of the sequence identical to S. cerevisiae core sterol regulatory element (TCGTA) [96].

In order to search for the regulators of CDR, recently, a systematic search of ORFs encoding proteins with Zn(2)-Cys(6) binuclear cluster domain was undertaken by Sanglard and his group [97]. It was based on the fact that transcription factors (TFs) containing these motifs often recognized the cis-acting regulatory regions containing 5' -CGG- 3' triplets with direct, inverted or everted palindromic repeats with various numbers of nucleotides in the intervening regions and these triplets are also present in the promoter of CDR1 and CDR2 genes [87]. A cluster of ORFs containing this domain was found to be located at chromosome 5 of C. albicans (ZNC1, ZNC2 and ZNC3) along with the mating locus. Disruption of these genes led to the identification of
ZNC2 as the transcriptional activator (TAC1) of CDR genes [97].

Tac1p is a 980 amino-acid protein of the Zn(2)-Cys(6) family, it contains, a DNA binding domain, an activation domain rich in acidic residues at the C-terminus. Tac1p is homologous to Hal1p of S. cerevisiae, a TF that enhances salt tolerance through the increased expression of ENA1 (Na⁺/Li⁺ extrusion pump) [98]. Tac1p constitutively bound to the DRE elements in CDR1 and CDR2 promoter and localizes into the nucleus under normal growth conditions [97]. In a genome wide approach in addition to CDR1 and CDR2, RTA3 and IFU5 has also been identified as the Tac1p targets [97]. However, the pathways leading to the activation of CDR1 and CDR2 and other genes through TAC1 are not known yet. Recently, an activator of CDR, CanMDT80 was identified by functionally complementing ScNd80 of S. cerevisiae, which is known as the meiosis specific transcription factor [100,101]. CanNd80 is involved in drug resistance via regulation of CDR1 in C. albicans [99].

Overexpression of a fusion protein containing potential trans-activation domain of CanNd80 and the DNA binding domain of ScNd80 suggested that they recognize the same DNA sequence [99].

**GENOMIC AND PROTEOMIC ANALYSIS OF DRUG RESISTANCE**

MDR in Candida is a multifactorial phenomenon that may involve convergence of many regulators and regulatory circuits. Recent genome based studies have confirmed this perception [19,20,102-104]. To identify the genes coordinately regulated during the development of drug resistance and their regulatory network in C. albicans, the transcriptome of C. albicans was analyzed in different experimental conditions, such as between two matchedazole resistant and sensitive clinical isolates, between drug induced and un-induced strains, sequential development ofazole resistance etc. [19, 20, 102-104]. The expression profiling of Candida genome under variety of conditions revealed differential expression of several unknown genes belonging to various categories such as amino acid and carbohydrate metabolism; cell stress cell wall maintenance, lipid, fatty acids and sterol metabolism and small molecule transport along with the known MDR genes. The list of commonly activated MDR genes also include CDR1, CDR2, ERG2, RTA3, IFU5, GPX1, CaMDR1, GRP2, IFD5, IFP5987, SNG1 and the down regulated genes mainly include EBP1, FET34, FTR2, OP13 and IFP1222 [19,20,102-104].

Recently, a proteomic approach was employed to the matched set of fluconazole susceptible and resistant isolates wherein drug resistance correlated with stable activation of CaMDR1 or CDR1/CDR2 [64,105]. Proteins encoded by YPR1278 gene and several member of the IFD gene family (YPL088) were overexpressed along with the protein encoded by CaMDR1. These proteins, which were upregulated in CaMDR1 overexpressing fluconazole resistant isolates, were not detected in the fluconazole resistant isolates overexpressing CDR1 and CDR2. Therefore, the expression of CaMDR1 and CDR1/CDR2 seems to be controlled by a different regulatory network in C. albicans [64,105].

**EVOLUTION OF EFFLUX PROTEINS**

**ABC Protein**

While ABC proteins like Cdr1p, Cdr2p, Cdr3p and Cdr4p are well characterized in C. albicans, the exact number of the members of this superfamily is not known. By employing TBLASTN searches together with domain analysis of the recently completed sequencing project of C. albicans ([http://www.ncbi.nlm.nih.gov/](http://www.ncbi.nlm.nih.gov/)), Prasad and coworkers identified 81 nucleotide-binding domains (NBDs), which belong to 51 different putative open reading frames (ORFs) (unpublished data). These searches revealed a rather large number of putative ABC proteins (51) as compared to 31 ABC proteins known in S. cerevisiae genome [106,107]. This is because the C. albicans diploid genome sequence explicitly represents both copies of the genome wherein many of the contigs occur in homologous pairs. The DNA sequences of the paired homologous contigs are usually similar throughout, although in certain regions, such as the mating-type locus, divergence between the homologues is considerable [108]. Considering that each allelic pair represents a single ABC protein of the Candida genome, the total number of putative members of this superfamily is twenty-eight.

The domain organization of Candida ABC proteins, resembles similar proteins from other organisms [25]. By employing neighbor-joining (NJ) tree and the SOM based clustering methods, the twenty-eight putative ABC proteins, which ranged in size from 276 to over 1544 amino acid residues, could be segregated into six major clusters Fig. (5). Among the six clusters, the five corresponded to known subfamilies in yeast S. cerevisiae and the sixth cluster contained ABC proteins that did not belong to any known subfamily and is placed in “others” category. Thus, almost all of the C. albicans ABC proteins identified could be assigned to one of the following subfamilies originally identified for S. cerevisiae, namely: the MDR (Multi Drug Resistance), the PDR (Pleiotropic Drug Resistance), the MRP (Multi Drug Resistance-associated Protein), the ALDp (Adrenaline/dopamine transporter protein), the EF3 (Elongation Factor 3) and the RLIP (RNA Inhibitor). The putative ABC proteins identified in C. albicans follow the expected domain organization for each subfamily Fig. (6). Those in which the TMS is predicted to be N-terminal to the NBD (forward orientation) belong to MDR, MRP and ALDp subfamilies, while those in which the NBD precedes the TMS (reverse orientation) are members of the PDR subfamily. The putative ABC genes that do not encode TMS belong to the EF3 and RLIP subfamilies. Two important points emerged from the sequence analysis of different subfamily members. Firstly, the N- and C-terminal NBDs of Candida ABC proteins within each cluster segregated independently, implying that full transporters arose from duplication and fusion of the half transporters [109,110]. Secondly, N-terminal NBDs of different proteins within a subfamily are more similar to one another as compared to their own corresponding C-terminal NBDs. This suggests that full proteins within the same subfamily share a common full progenitor and also points to the possibility that the two halves are functionally distinct [51]. The inventory of the ABC proteins of Candida is expected to provide new
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Fig. (5). Neighbor-joining (NJ) tree of C. albicans NBDs. In order to indicate the subfamily type, the NBDs of both C. albicans and the closest homologue obtained from BLAST searches [130], were aligned using ClustalW [131]. PROTDIST, from the PHYLIP package [132], was used to determine the distances between the members as shown by the scale. The neighbor-joining (NJ) algorithm [133], also from the PHYLIP package was used to generate unrooted NJ tree. The reliabilities of each branch point were assessed by bootstrap analysis with 1000 replicates and the values are indicated on the branches. For clarity, the NBDs of only the close homologues of Candida ABC proteins are labeled where ‘N’ and ‘C’ represent NBD1 and NBD2, respectively.

insights into their role in antifungal resistance as well as help in functional characterization of this superfamily of proteins.

MFS Proteins

S. cerevisiae genome possess 78 MFS proteins [111,112] wherein some of them are drug transporters. So far, however, the exact number of putative MFS proteins in C. albicans genome is not known. Given the amount of similarities with regard to number and type of ABC proteins between S. cerevisiae and C. albicans genomes, the pathogenic yeast may also have several MFS proteins. As depicted in Table 1, however, only a few have been identified as drug transporters.

DRUG EFFLUX PUMPS ARE MULTIFUNCTIONAL PROTEINS

Considering the abundance of the drug transporters and their wider specificity, it is believed that these drug transporters may not exclusively export drugs. Recent evidences confirm that the drug transporters particularly of the ABC superfamily of S. cerevisiae and C. albicans are multifunctional proteins, which mediate important physiological functions. Some of the well-characterized physiological roles of drug transporters are discussed briefly.
Fig. (6). Predicted domain organization of the different ABC protein subfamily members of \textit{S. cerevisiae} and \textit{C. albicans}. The TM domain consists of six predicted transmembrane spans (TMS) represented by hatched cylinders and the NBDs are shown as ovals. The domain order in various proteins is given, but the intervening sequences are not drawn to scale. 'N' and 'C' represent amino and carboxyl terminals, respectively. The putative \textit{Candida} ABC proteins showing homology to the corresponding \textit{S. cerevisiae} ABC proteins are shown in parentheses. \textit{Candida} proteins marked * and ¨ are homologous to \textit{S. cerevisiae} \textit{Pdr5p} and \textit{Yor1p}.

**As Drug Transporters**

The superfamily of ABC transporters came into prominence because of their role as drug transporters. It is well established that genes encoding drug extrusion ABC proteins like some of the \textit{PDRs} of \textit{S. cerevisiae} and \textit{CDRs} of \textit{C. albicans} mediate ATP-dependent rapid efflux of drugs [113-115]. An enhanced accumulation of fluconazole in the homozygous disruptants \textit{vdadr1 and vdcdr2} of \textit{C. albicans} as compared to its wild type host confirmed a causal relationship between efflux mediated by these two proteins and fluconazole accumulation [18].

**As Human Steroid Transporter**

The ability of \textit{PDR5} and \textit{SNQ2} of \textit{S. cerevisiae} and of \textit{CDR1} of \textit{C. albicans} to efflux steroid hormones, like \(\beta\)-estradiol and corticosterone suggests that human steroid hormones are also substrates of these drug transporters [17,114,116]. As discussed above, these hormones also upregulate the expression of \textit{CDR1} in \textit{C. albicans} [32]. Notably, some of the drugs to which \textit{CDR1} confers resistance, could compete for efflux by \(\beta\)-estradiol and corticosterone, thus implying commonality in binding site(s) between steroids and drugs [17]. Ergosterol of yeast is very similar to mammalian cholesterol and thus it is quite possible that yeast multidrug transporters, similar to human Mdr1p, could be involved in the total sterol homeostasis in the yeast cells.

**As Phospholipid Translocator**

Membrane phospholipids are asymmetrically distributed across the plasma membrane of all cell types in various organisms [117,118]. The asymmetrical distribution of membrane lipids is very specific and its loss has been linked to various physiological consequences [117,118]. The lipid asymmetry is maintained by membrane bound phospholipid translocators divided into three classes a) bi-directional energy dependent scramblase, b) energy dependent translocator that moves lipids towards the cytoplasmic surface of the plasma membrane (flipase) or c) away (flopase) from the cytoplasmic surface of the membrane [118]. Recent reports suggest that some of the ABC drug extrusion pumps down the evolutionary scale can mediate phospholipid translocation. Thus, human Mdr1p, Mdr2p are shown to translocate (flip/flop) membrane phospholipids between the two monolayers of the lipid bilayer [119-121]. \textit{Pdr5p} and \textit{Yor1p} of \textit{S. cerevisiae} and \textit{Cdr1p} and \textit{Cdr2p} of \textit{C. albicans}...
 albicans can also mediate phospholipid translocation and therefore, this property of ABC proteins represents another conserved physiological function of these drug transporters [122, 123]. However, this function does not appear to be a general feature of the proteins involved in drug efflux since Snq2p, the close homologue of Pdr5p, is unable to mediate phospholipid translocation [121].

The flopping or translocation of PtdEtn by Cdr1p is an energy-dependent process [34]. The decrease in the availability of PtdEtn in the outer half of the plasma membrane of a Cdr1p strain further confirmed the involvement of Cdr1p in PtdEtn translocation in C. albicans. Interestingly, Cdr1p and Cdr2p whose overexpression leads to drug resistance elicit outwardly directed phospholipid translocase activity (floppases), while Cdr3p which does not confer drug resistance, is involved in inwardly directed translocation of phospholipids (flipase) [123]. In addition to the differences in the directionality of phospholipid translocation, the floppase activities of Cdr1p and Cdr2p and flipase activity of Cdr3p are further distinguishable. Most importantly, drugs like fluconazole, cycloheximide, and miconazole affect transbilayer movement of phospholipids mediated by Cdr1p, Cdr2p but do not affect the Cdr3p mediated translocase activity. These findings suggest that Cdr1p and Cdr2p presumably have common binding sites for drugs and phospholipids while flipase activity of Cdr3p could be independent of drug binding [123]. The differences in the directionality of phospholipid transfer between Cdrps might be linked to their ability to efflux cytotoxic drugs. It is very likely that phospholipid translocation could represent a normal physiological role of ABC proteins irrespective of their participation in drug effusion. CDR4, a homologue of CDR1, CDR2 and CDR3 of C. albicans has not been known to play a role in drug resistance but it also could be a putative phospholipid translocase. Homologues of CDR1 found in other non-albicans species have not been examined for their ability to translocate phospholipids, but considering their structural and functional homology, it is very likely that some of them will also be functioning as phospholipid transporters in those organisms.

Unlike ABC proteins, MFS proteins were identified as nutrient transporters involved in the accumulation of solutes like sugars, metabolites, amino acids, vitamins, and both inorganic and organic ions (Fig. 1) [124-127]. They can also be involved in expelling drugs is a rather recently recognized property [111, 122, 123]. However, unlike the ABC drug transporters, the MFS superfAMILY transporters have not been identified with any cellular function. In one report, however, CamDR1 has been related to virulence of C. albicans. The mouse model showed that the ΔcamDR1 mutant colonizes but become deficient in causing mortality [62]. The exact mechanism by which the disruption of CamDR1 leads to reduced virulence in C. albicans is not known. These results therefore suggest a role of CamDR1 in a function other than the transport of drugs. The completion of Candida genome project followed by functional genomics would certainly highlight the unexplored physiological role of MFS proteins.

CONCLUDING REMARKS

Among several contributors of antifungal resistance, drug efflux mediated by ABC and MFS proteins, represent a prominent mechanism. In this context, the knowledge of structural and functional aspects of drug transport proteins represents an important aspect of research, which awaits further advances. Although advances made in the past several years have contributed greatly in our understanding of molecular mechanism of transport proteins of mammalian and bacterial origin, these aspects of fungal transporters represent a poorly explored area. As we have discussed, though the studies related to molecular mechanism of drug transport by fungal ABC and MFS transporters were initiated rather recently, it is already emerging that yeast drug efflux pump proteins show extensive functional homology to their mammalian counter parts. There are a few elegant studies which deals with 3D structures of ABC transporters of bacterial and mammalian cells, however, no detailed molecular structure of fungal proteins is available. An electron microscopic reconstruction of Pdr5p of yeast S. cerevisiae was recently published which so far represent a single effort towards an understanding of the structure and functional relationship of yeast drug transporters. Considering the interest in the field, it is expected that such studies will be widely taken up to resolve some of the issues related [49] to drug transport.

ACKNOWLEDGEMENTS

Work cited in this article from the author’s (RP) laboratory is supported in parts by grants from Department of Biotechnology, India (BT/PR3825/MED/14/488/a(2003), (BT/PR4862/BRB/10/360/2004), European Commission, Brussels (QLK-CT-2001-02377), DST-DAAD grant (INT/DAAD/ p-79/2003) and Council of Scientific and Industrial research (CSIR) (37/1132/03/EMR-II).

ABBREVIATIONS

ABC = ATP binding cassette
MFS = Major facilitator superfamily
IAAP = Iodoarylazido prazosin
NBD = Nucleotide binding domain
NJ = Neighbor-joining
SOM = Self-organizing map

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