Divergent signature motifs of nucleotide binding domains of ABC multidrug transporter, CaCdr1p of pathogenic *Candida albicans*, are functionally asymmetric and noninterchangeable

Antresh Kumar [a], Suneet Shukla [b], Ajeet Mandal [a], Sudhanshu Shukla [c], Suresh V. Ambudkar [b], Rajendra Prasad [a,*]

[a] *Membrane Biology Laboratory, School of Life Sciences, Jawaharlal Nehru University, New Delhi 110067, India*
[b] *Laboratory of Cell Biology, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892-4254, USA*
[c] *Laboratory of Immune Cell Biology, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892-4254, USA*

ARTICLE INFO

ABSTRACT

Nucleotide binding domains (NBDs) of the multidrug transporter of *Candida albicans*, CaCdr1p, possess unique divergent amino acids in their conserved motifs. For example, NBD1 (N-terminal-NBD) possesses conserved signature motifs, while the same motif is divergent in NBD2 (C-terminal-NBD). In this study, we have evaluated the contribution of these conserved and divergent signature motifs of CaCdr1p in ATP catalysis and drug transport. By employing site-directed mutagenesis, we made three categories of mutant variants. These included mutants where all the signature motif residues were replaced with either alanines or mutants with exchanged equipositional residues to mimic the conservancy and degeneracy in opposite domain. In addition, a set of mutants where signature motifs were swapped to have variants with either both the conserved or degenerated entire signature motif. We observed that conserved and equipositional residues of NBD1 and NBD2 and swapped signature motif mutants showed high susceptibility to all the tested drugs with simultaneous abrogation in ATPase and R6G efflux activities. However, some of the mutants displayed a selective increase in susceptibility to the drugs. Notably, none of the mutant variants and WT-CaCdr1p showed any difference in drug and nucleotide binding. Our mutational analyses show not only that certain conserved residues of NBD1 signature sequence (S304, G306, and E307) are important in ATP hydrolysis and R6G efflux but also that a few divergent residues (N1002 and E1004) of NBD2 signature motif have evolved to be functionally relevant and are not interchangeable. Taken together, our data suggest that the signature motifs of CaCdr1p, whether it is divergent or conserved, are nonexchangeable and are functionally critical for ATP hydrolysis.

© 2010 Elsevier B.V. All rights reserved.

1. Introduction

It is well established that an overexpression of multidrug transporter protein CaCdr1p, belonging to the ABC superfamily, represents one of the predominant attributes of multidrug resistance (MDR) in clinical isolates of *Candida albicans* [1–9]. The protein CaCdr1p is expressed as a single polypeptide of 1501 amino acids which is comprised of two nucleotide binding domains (NBDs) and two transmembrane domains (TMDs) [10]. Each TMD is made up of six transmembrane segments (TMS) that are involved in the formation of overlapping multiple

substrates binding sites [11]. The substrates of CaCdr1p vary enormously and include structurally unrelated compounds such as azoles, lipids, and steroids [4,12]. Several lines of evidence from mammalian ABC proteins suggest that transport of substrates leads to conformation changes in the TMDs region of the protein that converts to a high-affinity substrate binding site (inward facing) to low-affinity binding site (outward facing) [13]. These conformation changes in the TMDs are triggered by the ATP binding and hydrolysis to the NBDs [14,15]. The NBDs are characterized primarily by the presence of several highly conserved motifs such as Walker A (GxxGxGKS/T, where "x" represents any amino acid), Walker B (hhhhD; where "h" represents any aliphatic residue), and signature C (LSGGQQ/R/KQR) motif which couples the ATP hydrolysis to power drug extrusion [16].

We have shown that unlike other transporters of higher eukaryotes, CaCdr1p of *C. albicans* does not share all the consensus conserved motifs of the ABC transporters. Remarkably, in contrast to the Walker A (GRPGAGCS) and B (IQCWD) motifs of the NBD1 of CaCdr1p, which have substitution of typical critical residues that are unique to the fungal

ABC transporters, it has conserved signature motif (VSGGERKRVSIA). On the other hand, the Walker A (GASGAGKS) and B (LLFLD) motifs of the NBD2 are well conserved [10,17,18]; however, its signature motif (LNVEQRKRLTIGV) is degenerated (Fig. 1). The complexity and diversity of the fungal transporters have been analyzed and reveal that the evolutionary uniqueness present in the PDR family is highly conserved in fungal ABC transporters [19,20]). Our group has extensively examined the functional significance of these unique substitutions present in the NBD domains of CaCdr1p. We have established that the substitution of a typical cysteine with lysine (C193K), present in equivalent position in other ABC transporters, lead to severe abrogation of ATP hydrolysis [21] with no effect on its binding. However, exchange of the unique tryptophan of Walker B region of NBD1 with alanine (W326A) resulted in reduced ATP binding with no effect on its hydrolysis [18]. We also showed that substitution of well-conserved aspartate with asparagine (D327N) strongly impaired the ATPase activity without affecting the ATP binding. Unlike the other nonfungal ABC transporters, aspartate (D327) in the Walker B motif of NBD1 of CaCdr1p is not involved in Mg^{2+} coordination and has a role in ATP catalytic cycle [22]. Thus, the unique evolutionary replacements in CaCdr1p and other yeast ABC transporters are functionally indispensable [21–23].

Similar to the ABC transporters associated with antigen processing (TAP) [24,25], cystic fibrosis transmembrane conductance regulator (CFTR) [26], and multidrug resistance protein 1 (MRP1) of humans [27], CaCdr1p presumably also forms two distinct cytosolic ATPase sites [22]. One noncanonical ATPase site is composed of three unique motifs viz degenerated Walker A and B of NBD1 and degenerated signature motif of the NBD2, and another canonical site is formed by the conserved Walker A and B of NBD2 and conserved signature motif of NBD1. The biochemical studies of TAP revealed that the mutant with two de-generated ATPase sites show drastically reduced transport activity and suggested that the canonical ATP binding site is critical for its function

[24]. Several structural and biochemical studies have shown that the signature motifs are involved in the head to tail ATPase site formation with the Walker A and Walker B motifs of the opposite NBDs, sandwiched with two ATP molecules [26,28–30]. The signature motif physically contributes to the dimerization of NBDs forming two hetero-logous nucleotide binding pockets, interacting via hydrogen bonds with ribose and the γ-phosphate moiety of ATP [29]. A similar structure of NBDs was also deduced for a DNA repair ABC protein Rad50 [28]. Mutation in the signature motif affects the protein function. For example, mutation of the second conserved glycine residue (LSG**G**Q) of the signature motif abolished ATP hydrolysis in human CFTR [26]. Mutation at this position in human Pgp also leads to impaired protein function and abrogated interdomain communication [31]. The analo-gous mutation made in the NBD1 signature motif of both the a-factor transporter ScSte6p and the ScYdf1p transporter of *Saccharomyces cerevisiae* drastically impaired the substrate transport [32,33].

Although the role of signature motifs in ATP catalysis, crosstalk between NBD and TMD, and the ATP-mediated drug transport has been analyzed for the mammalian ABC proteins, the role of evolutionary divergent signature motifs of NBD of yeast ABC drug transporters remains unexplored. This is particularly important since not only yeast ABC drug transporters but also C family of human ABC transporters (ABCC) possess divergent residues in their conserved motifs [1].

This study evaluates the role of signature motifs of the PDR subfamily of the yeast ABC transporter; CaCdr1p where NBD1 has a conserved signature motif (VSGGE) while this motif is degenerated in NBD2 (LNVEQ). For this, we mutagenized signature motif residues by either replacing them with alanines or replacing residues with equiposition residues of another signature motif. We also swapped an entire motif with either a conserved or a degenerated signature motif. By employing drug susceptibility test, efflux of substrate, and ATPase activity measurements, we show that the canonical or noncanonical
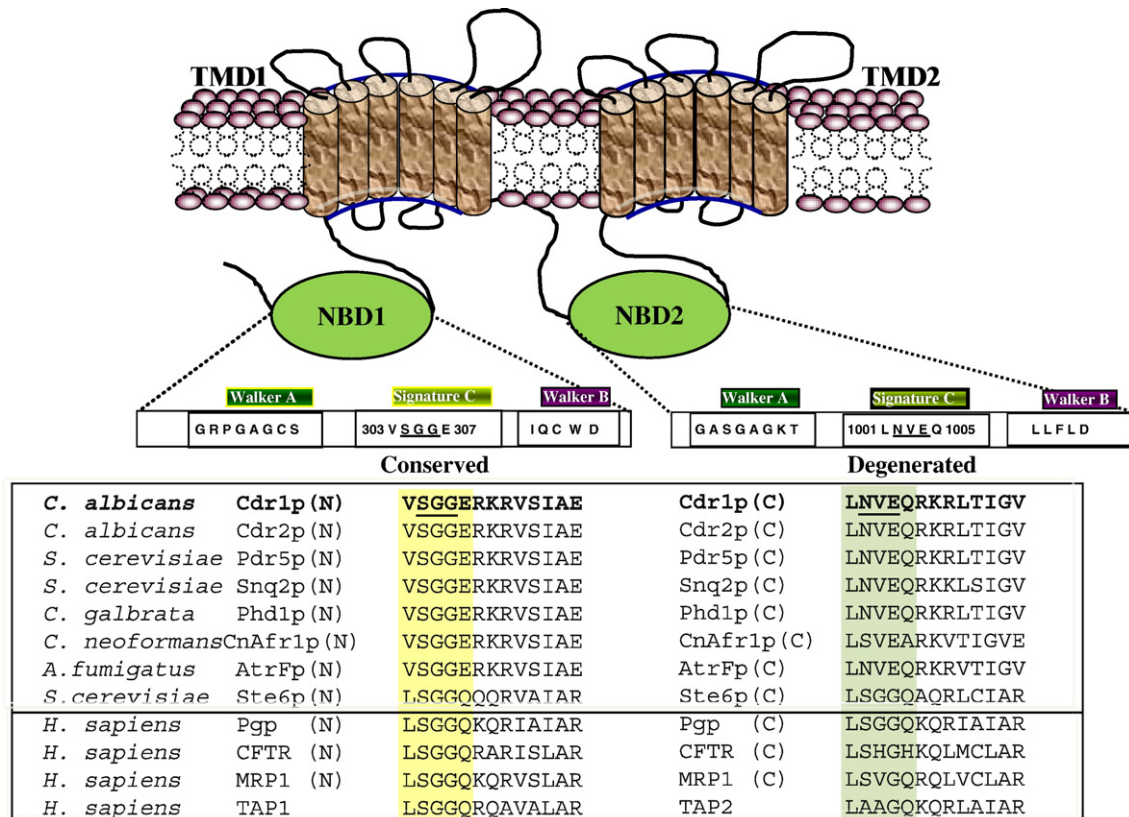

		Walker A	Signature C	Walker B		Walker A	Signature C	Walker B
		G R P G A G C S	303 V S G G E 307	I Q C W D		G A S G A G K T	1001 L N V E Q 1005	L L F L D

		Conserved		Degenerated	
C. albicans	**Cdr1p(N)**	**VSGGERKRVSIAE**	**Cdr1p(C)**	**LNVEQRKRLTIGV**
C. albicans	Cdr2p(N)	VSGGERKRVSIAE	Cdr2p(C)	LNVEQRKRLTIGV
S. cerevisiae	Pdr5p(N)	VSGGERKRVSIAE	Pdr5p(C)	LNVEQRKRLTIGV
S. cerevisiae	Snq2p(N)	VSGGERKRVSIAE	Snq2p(C)	LNVEQRKKLSIGV
C. galbrata	Phd1p(N)	VSGGERKRVSIAE	Phd1p(C)	LNVEQRKRLTIGV
C. neoformans	CnAfr1p(N)	VSGGERKRVSIAE	CnAfr1p(C)	LSVEARKVTIGVE
A. fumigatus	AtrFp(N)	VSGGERKRVSIAE	AtrFp(C)	LNVEQRKRVTIGV
S. cerevisiae	Ste6p(N)	LSGGQQQRVAIAR	Ste6p(C)	LSGGQAQRLCIAR
H. sapiens	Pgp (N)	LSGGQKQRIAIAR	Pgp (C)	LSGGQKQRIAIAR
H. sapiens	CFTR (N)	LSGGQRARISLAR	CFTR (C)	LSHGHKQLMCLAR
H. sapiens	MRP1 (N)	LSGGQKQRVSLAR	MRP1 (C)	LSVGQRQLVCLAR
H. sapiens	TAP1	LSGGQRQAVALAR	TAP2	LAAGQKQRLAIAR

Fig. 1. Topology of CaCdr1p and sequence alignment of signature motifs from various ABC transporters. The sequence alignment of signature motif residues in NBDs with those from other nucleotide binding domains of some known ABC transporters is shown. Signature motifs of CaCdr1p are shown in bold, and the conserved and divergent residues are underlined.

signature motifs in the NBDs of CaCdr1p are functionally asymmetric and critical.

2. Experimental procedures

2.1. Materials

Ribonucleotides (ATP), protease inhibitors (PMSF, leupeptin, pepstatin A, aprotinin, TLCK, and TPCK), and drugs, cycloheximide (CYH), anisomycin (ANI), Rhodamine 6G (R6G), DTT, and 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 (supplementary data), were commercially procured from Sigma Genosys, Inc. Fluconazole (FLC) was kindly provided by Ranbaxy Laboratories (New Delhi, India). Anti-GFP monoclonal antibody and [α-^{32}P] 8-azido ATP (15-20 Ci/mmol) were purchased from BD Biosciences Clontech (Palo Alto, CA) and Affinity Labeling Technologies, Inc. (Lexington, KY), respectively. The radio labeled [^{125}I] IAAP (2300 Ci/mmol) was procured from Perkin-Elmer Life Sciences (Boston, MA).

2.1.1. 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⁻ (Bio101). Table 2 (supplementary data) lists all of the strains used in this study.

2.2. Methods

2.2.1. Site-specific mutagenesis

Site directed mutagenesis was performed using the quick-change mutagenesis system as described previously [34]. The mutations were introduced into plasmid pPSCDR1-GFP according to the manufacturer's instructions, and the mutated plasmid pPSCDR1-GFP linearized with XbaI was used to transform *S. cerevisiae* strain, AD1-8u⁻ cells by the lithium acetate transformation protocol exploiting uracil prototrophy [34,35].

2.2.2. Immunodetection of CaCdr1p

Plasma membrane (PM) was prepared from *S. cerevisiae* cells grown in YEPD to late exponential phase, as described previously [34]. The Western blot analysis was carried out using anti-GFP monoclonal antibody (1:5000 dilution) as described previously [34].

2.2.3. ATPase activity assay

The WT-CaCdr1p associated ATPase activity of the purified PM was measured as an oligomycin-sensitive release of inorganic phosphate as described previously [34]. Briefly, purified PM (10 μg) was incubated at 30 °C in 0.1 ml of reaction mixture containing 8 mM $MgCl_2$ and 60 mM Tris–HCl, pH 7.5 (ATPase reaction buffer), and 20 μM oligomycin where indicated. To eliminate possible contributions from nonspecific vacuolar and mitochondrial ATPases, 50 mM KNO_3 and 10 mM NaN_3, respectively, were included in the reaction mixture. The reaction was started by addition of 5 mM ATP and was stopped by the addition of 0.1 ml of 5% SDS solution. The amount of inorganic phosphate released was determined immediately as described previously [36].

2.2.4. Rhodamine 6G efflux assay

Efflux of Rhodamine 6G (R6G) was determined essentially using a previously described protocol [34,37]. Briefly, approximately 4×10^{-6} yeast cells from an overnight grown culture were transferred into YEPD media and allowed to grow for 5 h. Cells were pelleted and washed and then resuspended in phosphate-buffered saline (PBS) as a 2% cell suspension to which R6G was added at a final concentration of 10 μM and incubated for 1 h at 30 °C. The cells were then washed and resuspended in PBS and reaction was initiated by the addition of 2% glucose. Samples of 1 ml volume were withdrawn at indicated time (30 min) and centrifuged at $9000 \times g$ for 2 min. The supernatant was collected, and absorption was measured at 527 nm.

2.2.5. Drug susceptibility

The susceptibilities of *S. cerevisiae* cells to different antifungal drugs were determined using spot assays. In this assay, 3 μl samples of five-fold serial dilutions of each yeast culture (each with cells suspended in normal saline to an optical density at 600 nm of 0.1) were spotted on to YEPD plates in the absence (control) or in the presence of the drugs [38].

2.2.6. Confocal microscopy and flow cytometry

Confocal imaging and flow cytometric (FACS) analysis of CaCdr1p and its mutant variants carrying *S. cerevisiae* cells were performed with a Bio-Rad confocal microscope (MRC 1024) with a 100-μl oil immersion objective and FACS or flow cytometer (Becton-Dickson Immunocytometry Systems, San Jose, CA) as described previously [34].

2.2.7. Photo affinity labeling with [^{125}I] IAAP

The PM proteins (30 μg) were incubated with the indicated drug for 10 min at 37 °C in 50 mM Tris–HCl, pH 7.5, in the absence of ATP. The samples were brought to room temperature and 7.5 nM [^{125}I] iodoarylazidoprazosin (IAAP) (2300 Ci/mmol) was added and incubated for an additional 5 min under subdued light. The samples were then illuminated with a UV lamp assembly (PGC, Scientifics, Gaithersburg, MD) fitted with two black light (self filtering) UV-A long wavelength F15T8BLB tubes (365 nm) for 10 min at room temperature. Following SDS–polyacrylamide gel electrophoresis (SDS–PAGE) on an 8%Tris–glycine gel at constant voltage, gels were dried and exposed to Bio-MaxMR film (Eastman Kodak, Rochester, NY) at −80 °C for 12 to 24 h. The radioactivity incorporated into the WT-CaCdr1p and its mutant's bands was quantified using a STORM 860 phosphorimager system (Molecular Dynamics, Sunnyvale, CA) and the software Image QuaNTas described previously [34].

2.2.8. Binding of [α-^{32}P] 8-azido-ATP

The PM protein (30 μg) was incubated in the ATPase reaction buffer containing 10 μM [α-^{32}P] 8-azido-ATP (10 μCi/nmol) in the dark at 4 °C (on ice) for 5 min in the presence or absence of 10 mM ATP. The samples were then illuminated with a UV lamp assembly (365-nm wavelength) for 10 min on ice (4 °C) as described previously [39]. Following SDS–PAGE on an 8% Tris–glycine gel at constant voltage, the gels were dried and exposed to Bio-Max MR film at 80 °C for 12–24 h. The radioactivity incorporated into the WT-CaCdr1p and its mutant variants bands was quantified as described above.

3. Results

3.1. Site-directed mutagenesis of signature motif residues

Earlier structural studies have revealed that the first five core residues of signature motif (LSGGQ), which hold the ATP molecule at the catalytic site, are critical [28,29]. To address the role of signature motifs of NBDs of CaCdr1p (Fig. 1), in this study, we have mutagenized the first five residues of both the conserved and the degenerated signature motifs of NBD1 (VSGGE) and NBD2 (LNVEQ), respectively. The first category of mutants included variants where all the residues of both the signature motifs were replaced with alanines such as V303A, S304A, G305A, G306A, and E307A of NBD1 and L1001A, N1002A, V1003A, E1004A, and Q1005A of NBD2. In the second category of mutant variants, we substituted the position of an individual residue of a signature motif into its equipositional residue present in another domain. For example, valine (V303) present in the NBD1 was replaced with leucine which is present at the equivalent position in NBD2. Thus, we constructed V303L, S304N,

G305V, G306E, and E307Q of NBD1 and L1001V, N1002S, V1003G, E1004G and Q1005E of NBD2. Finally, to further evaluate the functional contribution of each signature motif, we swapped the entire signature motif by introducing mutations in a way to produce two consensuses (signature 1-1), two degenerated (signature 2-2) and swapped (signature 2-1) motifs variants which were placed in the third category.

All the mutations were introduced into the C-terminal GFP-tagged CaCdr1p that was stably overexpressed at the *PDR5* locus in a heterologous host *S. cerevisiae* mutant strain, AD1-8u⁻ and expressed as GFP fused CaCdr1p (WT-CaCdr1p) localized in PM [34]. The host AD1-8u⁻ is deleted in seven PM-encoded major ABC transporters and is derived from a *pdr1–3* mutant strain with a gain-of-function mutation in the transcription factor Pdr1p, resulting in a constitutive hyperinduction of the *PDR5* promoter [34]. A single integration copy of *CDR1* ORF at the *PDR5* locus in the host genome of each mutant was confirmed by Southern hybridization (data not shown).

3.2. Substitution of signature motifs residues with alanines abrogates function

Similar to the WT-CaCdr1p, all the alanine scanning mutant variants of signature motif were equally expressed as revealed by Western blot analysis of PM fraction of the cell (Fig. S1, supplementary data) and were properly surface localized as confirmed by confocal microscopy and FACS analysis (Fig. 2A). However, these mutant variants displayed variable drug susceptibilities. For example, cells expressing G306A and E307A of NBD1 and N1002A of NBD2 were hypersensitive to all the tested drugs (Fig. 2B). The enhanced drug sensitivity was corroborated by observed abrogated efflux of a fluorescent substrate R6G [34]. We used intact cells expressing different mutant variants and de-energized them and monitored energy dependent extrusion of R6G, which was initiated by the addition of glucose [37]. As compared to host strain AD1-8u⁻, cells expressing the WT-CaCdr1p were able to extrude higher R6G, which was
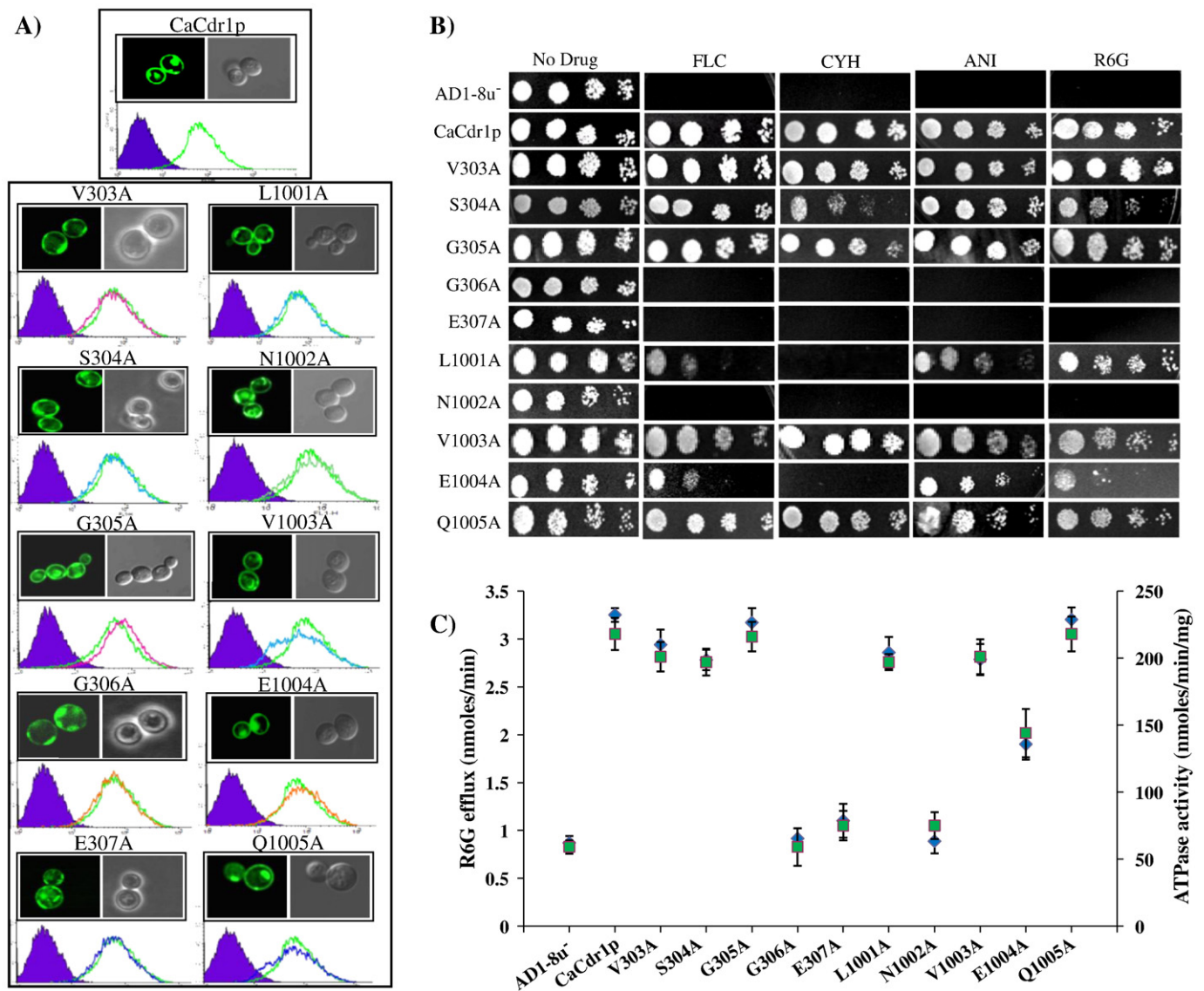

Fig. 2. Alanine scanning of the signature motifs of CaCdr1p. (A) Fluorescence imaging (upper panel) by confocal microscope showing membrane localization of WT-CaCdr1p and its mutant variant protein expressing cells. Flow cytometry (lower panel) of *S. cerevisiae* cells expressing WT-CaCdr1p and its mutant variants. The histogram derived from the cell quest program depicts fluorescence intensities for AD1-8u⁻ (control) (purple filled area) and WT-CaCdr1p (solid orange line) for each panel, and the other extra line represents that for the respective CaCdr1p mutant variant-expressing cells. (B) Drug resistance profile of WT-CaCdr1p and its signature motifs mutant variants is determined by spot assay. It was done as per protocol described earlier [34]. In the spot assays, 5 μl of five-fold serial dilutions of each yeast culture (each with cells suspended in normal saline to an OD of 0.1 (A_{600})) was spotted on YEPD plates in the absence (control) and the presence of the following drugs: FLC, 5 μg/mL; CYH, 0.15 μg/mL; ANI, 1.0 μg/mL; and R6G 5 μg/mL. (C) R6G efflux (left *Y*-axis) indicated by blue diamond (♦) and oligomycin-sensitive ATPase activity (right *Y*-axis) of WT-CaCdr1p with its mutant variants indicated by green square (■) [34,37].

evident from an increase in extracellular concentration of R6G (3 ± 0.5 nmol R6G/ml/30 min). However, drug-hypersensitive mutant variants, G306A and E307A of NBD1 and N1002A of NBD2, displayed severely impaired R6G efflux and behaved like the host strain AD1-8u⁻ (Fig. 2C). The loss of R6G efflux activity in these mutants was associated with a simultaneous impaired ATPase activity (Fig. 2C). Notably, we had earlier observed that unlike mammalian ABC proteins, WT-CaCdr1p does not show dramatic drug stimulated ATPase activity [40]. For this reason, we routinely measure basal (unstimulated) ATPase activity. Mutants such as S304A, L1001A, and E1004A displayed increased sensitivity to only selected drugs. For example, the conserved serine residue of signature motif of NBD1, when replaced with alanine (S304A), was only partially sensitive to cycloheximide (CYH) and R6G while similar to WT-CaCdr1p, remained resistant to other drugs. In contrast, L1001A of NBD2 displayed sensitivity to CYH while it was only partially sensitive to fluconazole (FLC) and anisomycin (ANI). All these mutant variants, which showed partial abrogation in resistance towards selected drugs, did not show any significant difference in their ability to efflux R6G (Fig. 2C). The basal ATPase activity, which drives active extrusion of R6G, was also com-

parable to the WT-CaCdr1p and was not changed. However, mutant variant E1004A was an exception, which was sensitive to CYH and only partly sensitive to FLC, and R6G showed only about 42% decrease in the efflux of R6G (Fig. 2C). Recent studies showed that NBD residues indeed could selectively influence susceptibility towards drugs. H1068A mutation in the H-loop of Pdr5p of S. cerevisiae selectively abrogated resistance towards tested drugs. For example, the mutant variant H1068A remained resistant to ketoconazole (KTC), FLC, and CYH, while it showed dramatic loss of resistance to rhodamine 123 [23].

This decrease in R6G transport was coupled with a simultaneous abrogation of ATPase activity. All the other mutant variants such as V303A, G305A of NBD1, and V1003A and Q1005A of NBD2 had phenotypes, similar to cells expressing WT-CaCdr1p (Fig. 2).

3.3. Restoration of conservancy in signature motifs does not support functionality of NBDs

To further examine the functional compatibility and to underscore significance of the conserved and degenerated signature residues, we
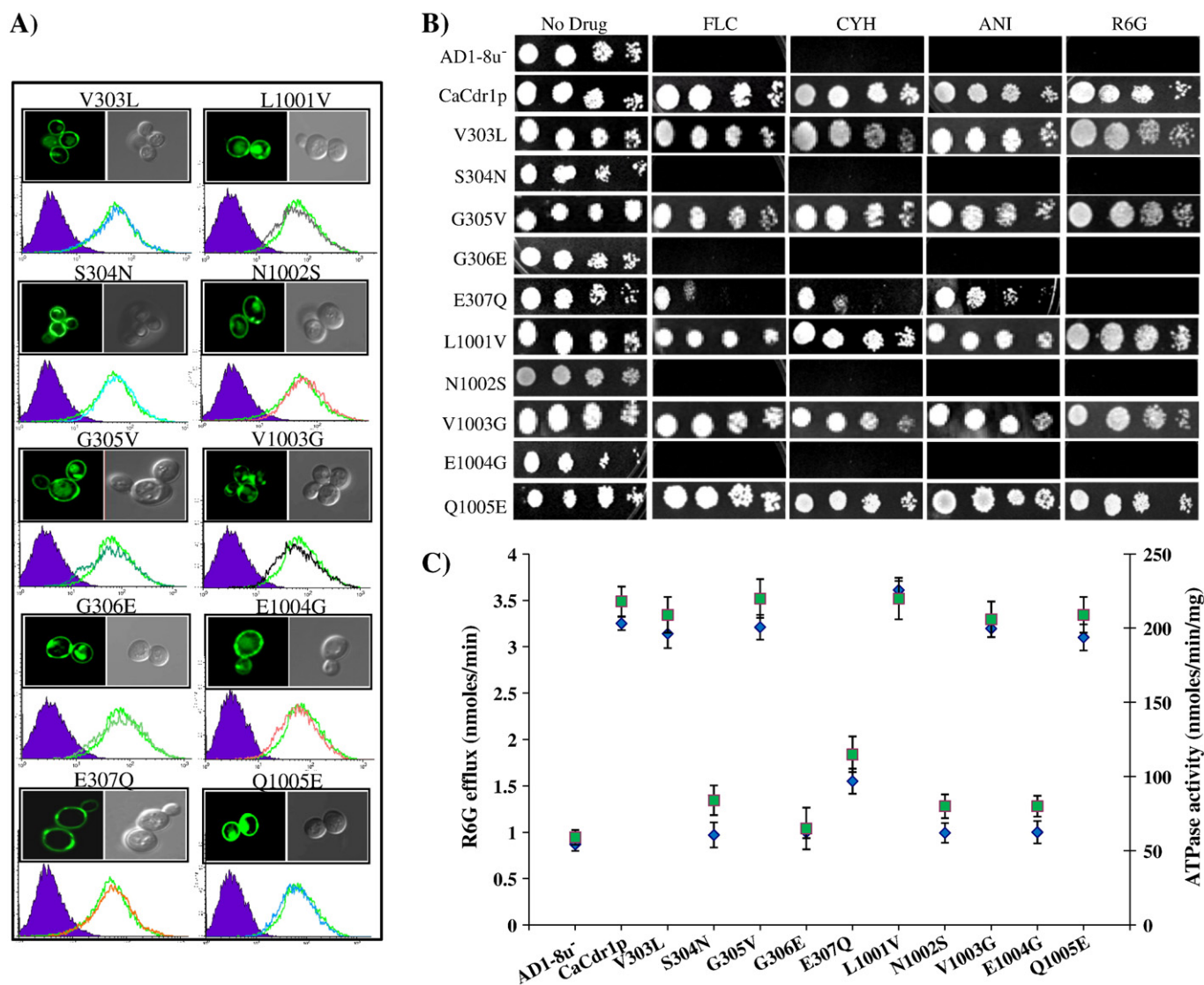

Fig. 3. Equipositional replacements of the signature sequences of CaCdr1p. (A) Fluorescence imaging (upper panel) by confocal microscope showing membrane localization of CaCdr1p and its mutant variant protein expressing cells. Flow cytometry (lower panel) of S. cerevisiae cells expressing WT-CaCdr1p and its mutant variants. The histogram derived from the cell quest program depicts fluorescence intensities for AD1-8u⁻ (control) (purple filled area) and WT-CaCdr1p (solid orange line) for each panel, and the other extra line represents the respective CaCdr1p mutant variant-expressing cells. (B) Drug resistance profile of WT-CaCdr1p and its signature motif mutant variants was determined by spot assays as described for Fig. 2. (C) R6G efflux (left Y-axis) indicated by blue diamond (♦) and oligomycin-sensitive ATPase activity (right Y-axis) of WT-CaCdr1p with its mutant variants indicated by green square (■) [34,37].
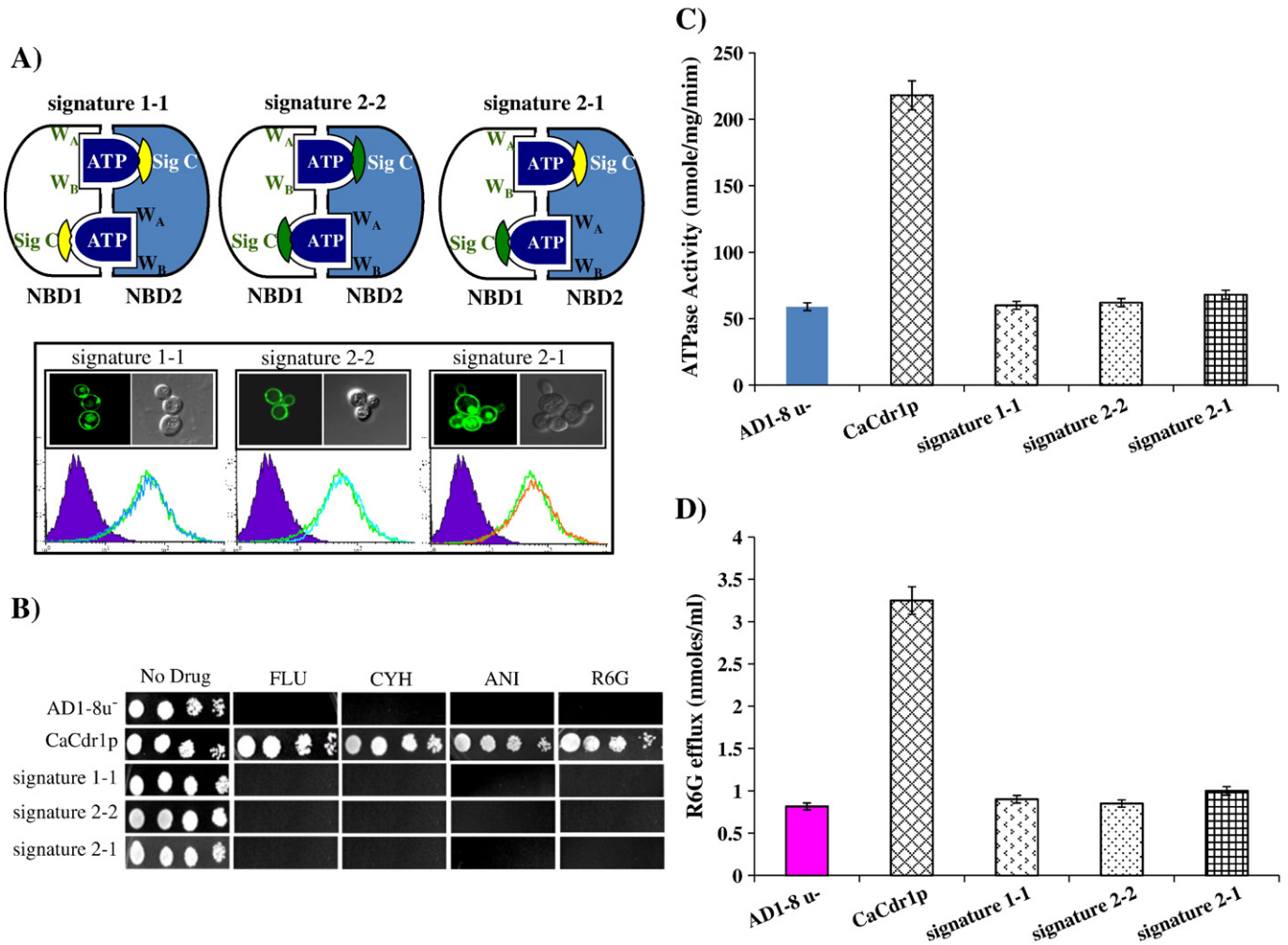
Fig. 4. Swapping of signature motifs of CaCdr1p. (A) Schematic diagrams of different CaCdr1p signature swapped mutants variants. The entire signature motif of NBD1 (white)/NBD2 (purple) was mutated to generate constructs with two conserved (signature 1-1), two degenerated (signature 2 -2) and swapped (signature 2-1) active site (upper panel). Localization and expression profile of WT-CaCdr1p and its swapped signature mutant variants (lower panel). (B) Drug resistance profile of WT-CaCdr1p and its signature motifs swapped mutants was determined by spot assays. (C) Comparison of oligomycin-sensitive ATPase activity of WT-CaCdr1p with its swapped mutant variants. ATPase activity of the PM fraction of cells expressing the WT-CaCdr1p and its mutant variants was assayed as described earlier [34]. (D) R6G efflux by the WT-CaCdr1p and its mutant variant protein-expressing cells. The R6G efflux was measured as described previously [37]. The values are mean SD ($\pm$ error bars) for three independent experiments.

introduced mutations in both the signature motifs by replacing residue with their equivalent positional residue of the other domain. These substitutions did not affect surface expression and localization of WT-CaCdr1p as determined by confocal and FACS analysis (Fig. 3A). Replacement of conserved serine with asparagine (S304N) to mimic degeneracy at this position and asparagine with serine (N1002S) to mimic conservancy in NBD2 resulted in hypersensitivity to all the tested drugs with severely diminished R6G efflux and ATPase activities (Fig. 3C). Similar results were also observed when highly conserved glycine of NBD1 changed into glutamate (G306E) and its equivalent residue glutamate of NBD2 exchanged with glycine (E1004G). Both these mutants were hypersensitive to all the drugs with abrogated R6G efflux and ATPase activity (Fig. 3C). The equipositional mutant E307Q displayed drug selective phenotype, which was hypersensitive to R6G and was only

partially sensitive to other drugs. This mutant variant showed 52% abrogation in R6G efflux with a simultaneous loss of ATPase activity. In contrast, the replacement of equipositional residues, such as V303L, G305V of NBD1, and L1001V, V1003G, and Q1005E of NBD2, had no effect on the drug susceptibility, R6G efflux, and ATPase activity (Fig. 3).

### 3.4. Swapping of the signature motifs results in complete loss of function

The cells expressing the signature swapped mutants, signature1-1, signature 2-2, and signature 2-1, were hypersensitive to all the tested drugs and displayed severely impaired R6G efflux and ATPase activity and behaved more like the host strain, AD1-8u⁻ (Fig. 4). Nonetheless, all the signature swapped variants were properly surface localized and equally well expressed.

Fig. 5. (A). (i) Photoaffinity labeling of WT-CaCdr1p and its mutant variants with [^{125}I]-IAAP. The PM fraction (30 μg protein) of cells expressing WT-CaCdr1p and its mutant variants was incubated with 7.5 nM [^{125}I]-IAAP (2300 Ci/mmol). The samples were UV cross linked and processed as described elsewhere [34]. (ii) Western blot analysis using anti-GFP antibody to ensure equal loading of WT-CaCdr1p and its signature mutant variants. (iii) Normalized incorporated [^{125}I] IAAP labeling with Western blot intensity. The values are shown in percentage. (B) Photoaffinity labeling of WT-CaCdr1p and its mutant variants with [α-^{32}P] 8-azido ATP. The PM fraction (30 μg) of cells expressing the WT-CaCdr1p and its mutant variants was incubated with 10 μM [α-^{32}P] 8-azido ATP 7.5 μCi/nmol at 4 °C and competed with 10 mM cold ATP (+ATP lane) as described in Shukla et al. [34]. (ii) Western blot analysis using anti-GFP antibody to ensure equal loading of WT-CaCdr1p and its signature mutant variants. (iii) Normalized incorporated [α-^{32}P] 8-azido ATP labeling with Western blot intensity. The values are shown in percentage.

A) [^{125}I]-IAAP labeling

i)

ii)

Anti-GFP mAb

iii)

B) [α-^{32}P] 8-azido ATP labeling

i)

ATP (10mM)

ii)

Western blot

Anti-GFP mAb

iii)
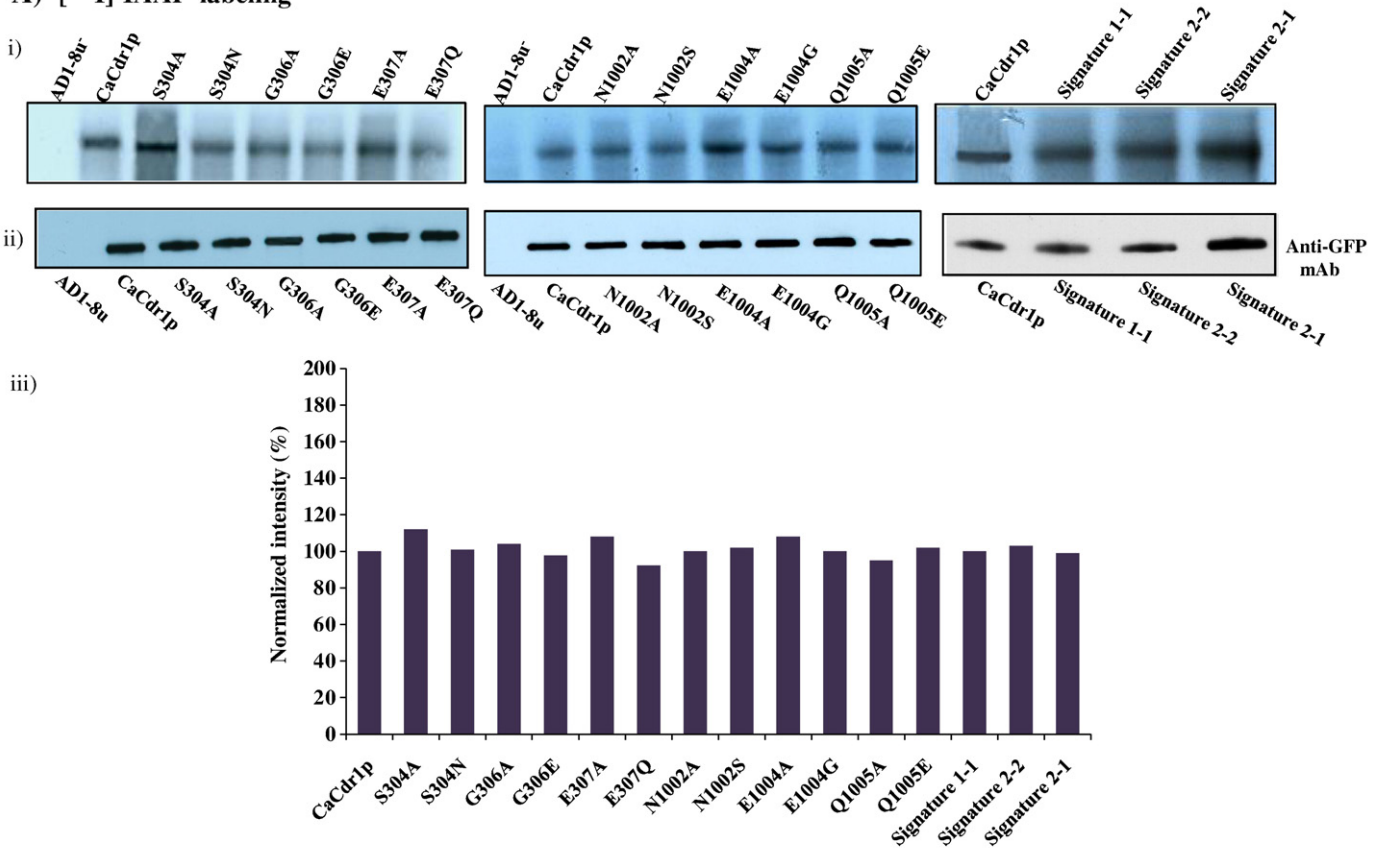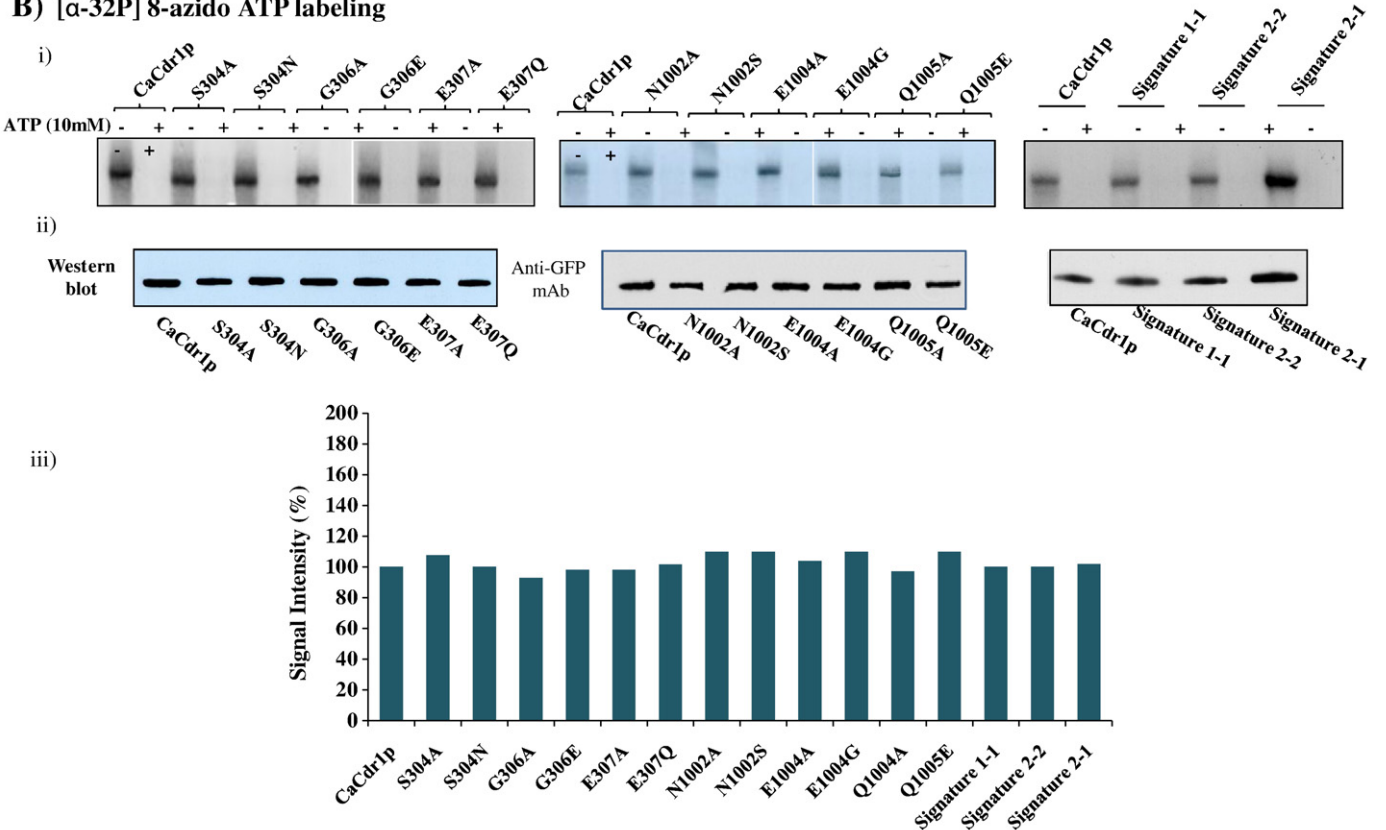
3.5. [^{125}I] IAAP and ATP binding remain unaffected in signature motif mutants

We have examined if the observed impairment of function, i.e., R6G drug transport and ATP hydrolysis of signature mutants was due to their inability to bind to CaCdr1p. We evaluated this aspect by using the [^{125}I] iodoarylazidoprazosin (IAAP) and [α-^{32}P] 8-azido ATP, which are photoaffinity analogues of substrate prazosin and ATP, respectively. We performed the binding experiments with both the signature motifs of NBDs by selecting three signature motif (VS<u>G</u>GE) residues S304, G306, and E307 of NBD1 and equivalent position residues of signature motif (LN<u>VE</u>Q) of NBD2 viz. N1002, E1004, and Q1005. The selection of these residues was based on our data (this study) and from the crystal structure of MJ0796, an ABC protein of *Methanocaldococcus jannaschii*, which showed that these residues are part of ATPase binding pocket and interact with the ATP moiety [29]. All the signature motif mutant variants at a fixed concentration efficiently cross linked to [^{125}I] IAAP comparable to that of WT-CaCdr1p. An equivalent amount of PM derived from the host cells (AD1-8u$^-$) was used as a control, wherein no binding of [^{125}I] IAAP was observed (Fig. 5A). We have earlier shown that [^{125}I] IAAP efficiently cross linked with WT-CaCdr1p and its binding can be competed out with molar excess of nystatin and the binding of [^{3}H] azidopine, a dihydropyridine photoaffinity analogue, is competed out with miconazole [34]. In this study, the competition experiments with drugs were not done since none of the drugs used in this study, which showed selective susceptibilities, compete with [^{125}I] IAAP binding [34]. Thus, [^{125}I] IAAP binding, which did not change in the signature mutant variants, provides limited information and may not be a true indicator of drug binding sites of WT-CaCdr1p.

In addition, all the signature mutant variants were assessed for [α-^{32}P] 8-azido ATP binding, which was normalized by Western blot analysis. No cross linking was observed of this analogue with the host cell (AD1-8u$^-$) membrane, which served as negative control. Notably, binding of 8-azido-[α-^{32}P] ATP to all signature mutant variants was similar to WT-CaCdr1p, which was competed out with a molar excess of the cold ATP (Fig. 5B).

4. Discussion

Signature motifs are the hallmark sequences of NBDs of ABC transporters which display highly conserved sequences across the evolutionary scale; however, there are instances of the appearance of selective divergence within this motif. For example, human ABC transporters such as TAP [24] and CFTR [26] have degenerated signature motifs (Fig 1). In contrast, all the family members of ABC transporters of fungi, particularly of PDR subfamily, display divergence in their signature motifs. Thus, the signature motif of NBD1 of CaCdr1p is well conserved but has NBD2 with a degenerated signature motif (Fig. 1). In the present study, we have explored the significance of degeneration in the signature sequence of NBD2 versus conserved signature motif of NBD1 of CaCdr1p. Our analysis revealed that the conserved and degenerated signature sequences of the CaCdr1p are functionally indispensable and cannot be exchanged. This emphasizes the uncompromised asymmetry that exists between the NBDs of CaCdr1p and in other yeast ABC transporters.

We show that similar to other ABC transporters, the well-conserved serine (S304) and glycine (G306) residues present in conserved signature motif of NBD1 are also critical for the functioning of CaCdr1p. For example, even the substitution at the equivalent position residues of degenerated signature motif of NBD2 with the conserved ones and vice versa does not support the function of the transporter (Figs. 3C and D). In the ABC protein of *Pyrococcus furiosus*, Rad50, replacement of this serine (L<u>S</u>GGQ) to arginine prevents dimerization of NBDs [28], and in human CFTR, polymorphism at this residue with either asparagine, arginine, or isoleucine results in cystic fibrosis [41–43]. Furthermore, if

cysteine was introduced at this position in the CFTR protein, it resulted in total loss of channel activity [44].

The well-conserved glycine present at the fourth position of signature motif (LSG<u>G</u>Q) is involved in the ATP catalysis [25,26,31,32,45,46]. Biochemical analysis revealed that a small change at this position (G→A) results in steric hindrance between methyl group of alanine and γ-phosphate of ATP. If this glycine is exchanged with bulky, charged aspartate or glutamate, it leads to a complete loss of ATPase and protein activity [46,47]. The critical nature of serine and glycine in WT-CaCdr1p can also be compared with similar residue of those proteins whose crystal structures are known. The existing structural information suggests that the signature motifs of ABC proteins; Rad50 of *P. furiosus*, MJ1096 of *M. jannaschii*, GlcV of *Sulfolobus solfataricus*, Sav1866 of *Staphylococcus aureus*, mouse CFTR, HlyB, and MalK of *E. coli*, are involved in the head to tail ATPase site formation with the Walker A and Walker B motifs of the opposite NBDs, sandwiched with ATP molecules wherein the signature motif is a "sensor" for an ATP γ -phosphate in the opposing domain. [28,29,48–52]. Based on the conserved nature of these motifs, it is reasonable to speculate that in CaCdr1p, the conserved S304 and G306 of NBD1 probably fall within close proximity of the ATP binding site. In addition, divergent residues present in NBD2 signature region is also equally important and may be part of the ATPase site as well. However, it still requires experimental validation.

We also provide evidence that in addition to highly conserved and critical S304 and G306 residues, the equipositional residues N1002 and E1004 of degenerated signature motif of NBD2 of WT-CaCdr1p have also evolved to be functionally essential. Notably, pairs of residue like V303 and G305 of NBD1 and L1001, V1003, and Q1005 of NBD2 signature motif, although part of otherwise conserved signature sequences, have apparently no functional relevance. These residues, when replaced with either alanines or with its equipositional substitute, continued to show phenotypes similar to cells expressing WT-CaCdr1p.

Functional nonequivalence in the NBDs of ABC proteins of yeast is the result of variations in the conserved motifs (Walker A, Walker B, H-loop, and signature motif). Our earlier results have shown that the equipositional exchange mutations such as C193K and K901C of Walker A of CaCdr1p yielded selectively impaired functional protein [21]. However, replacement of the entire NBD of CaCdr1p to make a chimera with two NBD1 (NBD1/NBD1) and two NBD2 (NBD2/NBD2) yielded nonfunctional proteins and had severe cellular trafficking problem [53]. These results had established functional asymmetry nature of NBDs in CaCdr1p. These variations in NBD1 may have evolved in response to the degenerated signature motif of NBD2. Thus, in CaCdr1p, both canonical and noncanonical ATP binding sites are formed similar to TAP and CFTR proteins. Recently, Ernst et al. [23,54] hypothesized that in Pdr5p of *S. cerevisiae*, a close homologue of CaCdr1p, one ATP molecule catalyzed at the canonical active site may be sufficient to reset the TMDs, whereas the second noncanonical site (regulatory site) may be engaged to serve as platform for keeping domains in dimeric form (inward facing).

Our equipositional replacements data of signature motifs demonstrate that the mutant variants, S304N and G306E of NBD1 and N1002S and E1004G of NBD2, severely impaired both transport (Fig. 3C) and ATPase activity (Fig. 3C). These results clearly show that both the conserved site and the degenerated site are equally important for ATP catalysis. The signature sequence swapping data highlights the importance of the uniqueness of the signature region of WT-CaCdr1p. Signature swapped mutants with either two conserved (signature 1-1) or two degenerated (signature 2-2) or swapped motif (signature 2-1) rendered nonfunctional protein (Fig. 4) although their surface localization and expression was not affected (Fig. 4A). In this context, it is worth mentioning that TAP protein with two degenerate sites shows dramatically impaired activity and thus demonstrating that consensus site is critical. In contrast, only 10% loss in activity was seen with TAP protein having both consensus sites. In addition, swapping between consensus and degenerative sequences retained

more than 50% of the activity. It would mean that TAP protein can remain partly functional if at least one of the signature motifs retains the consensus site [24]. In contrast, NBDs of Pgp are symmetric in function and possess conserved signature motifs at both positions. The replacement of either serine of both signature motifs (S528/S1173) with alanine resulted in a marginal loss in ATPase activity, whereas double mutation at these positions in the signature motif (S528A/S1173A) alleviates strength of interaction in the transition state, which implies that these serine residues of P-glycoprotein cooperatively accelerate ATP hydrolysis [55]. Taken together, our data clearly establish that the signature motifs of NBDs are functionally nonidentical and that the evolutionary divergence of CaCdr1p and other members of PDR subfamily is critical and noninterchangeable.

Acknowledgements

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbamem.2010.05.017.

References

[1] I. Holland, P. Cole, K. Kuchler, C. Higgins, ABC Proteins from Bacteria to Man, Academic Press, San Diego, CA, 2003.

[2] M.B. Walmsley, K.S. Mckeegan, A.R. Walmsley, Structure and function in efflux pumps that confer resistance to drugs, Biochem. J. 376 (2003) 313–338.

[3] R.A. Calderone, *Candida* and Candidiasis, ASM Press, Washington, DC, 2002.

[4] R. Prasad, S. L. Panwar, Smriti, Drug resistance in yeasts—an emerging scenario, Adv. Microb. Physiol. (Poole, R. K., Ed.) 1st ed., pp 155-201, Academic Press, London, 2002.

[5] T.C. White, Increased mRNA levels of *ERG16, CDR1,* and *MDR1* correlate with increases in azole resistance in *Candida albicans* isolates from a patient infected with human immunodeficiency virus, Antimicrob. Agents Chemother. 41 (1997) 1482–1487.

[6] M.L. Hernaez, C. Gil, J. Pla, C. Nombela, Induced expression of the *Candida albicans* multidrug resistance gene *CDR1* in response to fluconazole and other antifungals, Yeast 14 (1998) 517–526.

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

[8] T.C. White, K.A. Marr, R.A. Bowden, Clinical, cellular, and molecular factors that contribute to antifungal drug resistance, Clin. Microbiol. Rev. 11 (1998) 382–402.

[9] T.C. White, The presence of an R467K amino acid substitution and loss of allelic variation correlate with an azoleresistant lanosterol *14-R demethylase* in *Candida albicans,* Antimicrob. Agents Chemother. 41 (1997) 1488–1494.

[10] R. Prasad, P.D. Wergifosse, A. Goffeau, E. Balzi, Molecular cloning and characterization of a novel gene of *Candida albicans,* conferring multiple resistance to drugs and antifungals, Curr. Genet. 4 (1995) 320–329.

[11] N. Puri, M. Gaur, M. Sharma, S. Shukla, S.V. Ambudkar, R. Prasad, The amino acid residues of transmembrane helix 5 of multidrug resistance protein CaCdr1p of *Candida albicans* are involved in substrate specificity and drug transport, BBA 1788 (2009) 1752–1761.

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

[13] S.V. Ambudkar, In-Wha Kim, Z.E. Sauna, The power of the pump: mechanisms of action of P-glycoprotein (ABCB1), Eur. J. Pharm. Sci. 5 (2006) 392–400.

[14] D. Parcej, R. Tampe, Caught in the act: an ABC transporter on the move, Structure 15 (2007) 1028–1030.

[15] H.W. Pinkett, A.T. Lee, P. Lum, K.P. Locher, D.C. Rees, An inward-facing conformation of a putative metal-chelate-type ABC transporter, Science 315 (2007) 373–377.

[16] E. Schneider, S. Hunke, ATP-binding-cassette (ABC) transport systems: functional and structural aspects of the ATP-hydrolyzing subunits/domains, FEMS Microbiol. Rev. 22 (1998) 1–20.

[17] S. Jha, N. Karnani, S.K. Dhar, K. Mukhopadhayay, S. Shukla, P. Saini, G. Mukhopadhayay, R. Prasad, Purification and characterization of the N-terminal nucleotide binding domain of an ABC drug transporter of *Candida albicans*: uncommon cysteine 193 of Walker A is critical for ATP hydrolysis, Biochemistry 42 (2003) 10822–10832.

[18] V. Rai, S. Shukla, S. Jha, S.S. Komath, R. Prasad, Functional characterization of N-terminal nucleotide binding domain (NBD-1) of a major ABC drug transporter Cdr1p of *Candida albicans*: uncommon but conserved Trp326 of Walker B is important for ATP binding, Biochemistry 44 (2005) 6650–6661.

[19] E. Lamping, P.V. Baret, A.R. Holmes, B.C. Monk, A. Goffeau, R.D. Cannon, Fungal PDR transporters: phylogeny, topology, motifs and function, Fungal Genet. Biol. 47 (2) (2010) 127–142.

[20] M. Gaur, C. Devpriya, R. Prasad, The complete inventory of ABC proteins in human pathogenic yeast, *Candida albicans,* J. Mol. Microbiol. Biotechnol. 9 (2005) 3–15.

[21] S. Jha, N. Dabas, N. Karnani, P. Saini, R. Prasad, ABC multidrug transporter Cdr1p of *Candida albicans* has divergent nucleotide-binding domains which display functional asymmetry, FEMS Yeast Res. 5 (2004) 63–72.

[22] V. Rai, M. Gaur, S. Shukla, S. Shukla, S.V. Ambudkar, S.S. Komath, R. Prasad, Conserved Asp327 of walker B motif in the N-terminal nucleotide binding domain (NBD-1) of Cdr1p of *Candida albicans* has acquired a new role in ATP hydrolysis, Biochemistry 45 (2006) 14726–14739.

[23] R. Ernst, P. Kueppers, C.M. Klein, T. Schwarzmueller, K. Kuchler, L. Schmitt, A mutation of the H-loop selectively affects rhodamine transport by the yeast multidrug ABC transporter Pdr5, PNAS 105 (2008) 5069–5074.

[24] E. Procko, I.F. Connell, Ng. Sze-Ling, R. Gaudet, Distinct structural and functional properties of the ATPase sites in an asymmetric ABC transporter, Mol. Cell 24 (2006) 51–62.

[25] M. Chen, R. Abele, R. Tampe, Functional non-equivalence of ABC signature motifs in the transporter associated with antigen processing (TAP), J. Biol. Chem. 279 (2004) 46073–46081.

[26] P. Melin, V. Thoreau, C. Norez, F. Bilan, A. Kitzis, F. Becq, The cystic fibrosis mutation G1349D within the signature motif LSHGH of NBD2 abolishes the activation of CFTR chloride channels by genistein, Biochem. Pharmcol. 67 (2004) 2187–2196.

[27] X. Ren, T. Furukawa, M. Haraguchi, T. Sumizawa, S. Aoki, M. Kobayashi, S. Akiyama, Function of the ABC signature sequences in the human multidrug resistance protein 1, Mol. Pharmacol. 65 (2004) 1536–1542.

[28] K.P. Hopfner, A. Karcher, D.S. Shin, L. Craig, L.M. Arthur, J.P. Carney, J.A. Tainer, Structural biology of Rad50 ATPase: ATP-driven conformational control in DNA double-strand break repair and the ABC-ATPase superfamily, Cell 101 (2000) 789–800.

[29] P.C. Smith, N. Karpowich, L. Millen, J.E. Moody, J. Rosen, J.P. Thomas, J.F. Hunt, ATP binding to the motor domain from an ABC transporter drives formation of a nucleotide sandwich dimer, Mol. Cell 10 (2002) 139–149.

[30] A.L. Davidson, J. Chen, ATP-binding cassette transporters in bacteria, Annu. Rev. Biochem. 73 (2004) 241–268.

[31] G. Szakacs, C. Ozvegy, E. Bakos, B. Sarkadi, A. Varadi, Role of glycine-534 and glycine-1179 of human multidrug resistance protein (MDR1) in drug-mediated control of ATP hydrolysis, Biochem. J. 356 (2001) 71–75.

[32] B.L. Browne, V. McClendon, D.M. Bedwell, Mutations within the first LSGGQ motif of Ste6p cause defects in a-factor transport and mating in *Saccharomyces cerevisiae,* J. Bacteriol. 178 (1996) 1712–1719.

[33] J.M. Falcon-Perez, M.J. Mazon, J. Molano, P. Eraso, Functional domain analysis of the yeast ABC transporter Ycf1p by site-directed mutagenesis, J. Biol. Chem. 274 (1999) 23584–23590.

[34] S. Shukla, P. Saini, Smriti, S. Jha, S.V. Ambudkar, R. Prasad, Functional characterization of *Candida albicans* ABC transporter Cdr1p, Eukaryot. Cell 2 (2003) 1361–1375.

[35] K. Nakamura, M. Niimi, K. Niimi, A.R. Holmes, J.E. Yates, A. Decottignies, B.C. Monk, A. Goffeau, R.D. Cannon, Functional expression of *Candida albicans* drug efflux pump Cdr1p in a *Saccharomyces cerevisiae* strain deficient in membrane transporters, Antimicrob. Agents Chemother. 45 (2002) 3366–3374.

[36] B. Sarkadi, E.M. Price, R.C. Boucher, U.A. Germann, G.A. Scarborough, M. Niederweis, Expression of the human multidrug resistance cDNA in insect cells generates a high activity drug-stimulated membrane ATPase, J. Biol. Chem. 267 (1992) 4854–4858.

[37] S. Maesaki, P. Marichal, H.V. Bossche, D. Sanglard, S. Kohno, Rhodamine 6G efflux for the detection of CDR1-overexpressing azole-resistant *Candida albicans* strains, J. Antimicrob. Chemother. 44 (1999) 27–31.

[38] K. Mukhopadhyay, A. Kohli, R. Prasad, Drug susceptibilities of yeast cells are affected by membrane lipid composition, Antimicrob. Agents Chemother. 46 (2002) 3695–3705.

[39] Z.E. Sauna, S.V. Ambudkar, Characterization of the catalytic cycle of ATP hydrolysis by human P-glycoprotein, J. Biol. Chem. 276 (2001) 11653–11661.

[40] S. Shukla, V. Rai, D. Banerjee, R. Prasad, Characterization of Cdr1p, a major multidrug efflux protein of *Candida albicans*: purified protein is amenable to intrinsic fluorescence analysis, Biochemistry 45 (2006) 2425–2435.

[41] J. Zielenski, L.C. Tsui, Cystic fibrosis: genotypic and phenotypic variations, Annu. Rev. Genet. 29 (1995) 777–807.

[42] J. Zielenski, P. Patrizio, M. Corey, B. Hanhelin, D. Markiewicz, R. Asch, L.C. Tsui, CFTR gene variant for patients with congenital absence of vas deferens, Am. J. Hum. Genet. 57 (1995) 958–960.

[43] M.P. Anderson, M.J. Welsh, Regulation by ATP and ADP of CFTR chloride channels that contain mutant nucleotide-binding domains, Science 257 (1992) 1701–1704.

[44] J.F. Cotton, M.J. Welsh, Covalent modification of the nucleotide binding domains of cystic fibrosis transmembrane conductance regulator, J. Biol. Chem. 273 (1998) 31873–31879.

[45] Z. Szentpetery, A. Kern, K. Liliom, B. Sarkadi, A. Varadi, E. Bakos, The role of the conserved glycines of ATP-binding cassette signature motifs of MRP1 in the communication between the substrate-binding site and the catalytic centers, J. Biol. Chem. 279 (2004) 41670–41678.

[46] O. Ramaen, C. Sizun, O. Pamlard, E. Jacquet, J.Y. Lallemand, Attempts to characterize the NBD heterodimer of MRP1: transient complex formation involves Gly[771] of the ABC signature sequence but does not enhance the intrinsic ATPase activity, Biochem. J. 391 (2005) 481–490.

[47] G. Schmees, A. Stein, S. Hunke, H. Landmesser, E. Schneider, Functional consequences of mutations in the conserved 'signature sequence' of the ATP binding–cassette protein MalK, Eur. J. Biochem. 266 (1999) 420–430.

[48] G. Verdon, S.V. Albers, N. van Oosterwijk, B.W. Dijkstra, A.J. Driessen, A.M. Thunnissen, Formation of the productive ATP–Mg^{2+}-bound dimer of GlcV, an ABC–ATPase from *Sulfolobus solfataricus*, J. Mol. Biol. 334 (2003) 255–267.

[49] R.J.P. Dawson, K.P. Locher, Structure of a bacterial multidrug ABC transporter, Nature 443 (2006) 180–185.

[50] J. Zaitseva, C. Oswald, T. Jumpertz, S. Jenewein, A. Wiedenmann, I.B. Holland, L. Schmitt, A structural analysis of asymmetry required for catalytic activity of an ABC–ATPase domain dimer, EMBO 25 (2006) 3432–3443.

[51] M.L. Oldham, D. Khare, F.A. Quiocho, A.L. Davidson, J. Chen, Crystal structure of a catalytic intermediate of the maltose transporter, Nature 450 (2007) 515–522.

[52] H.A. Lewis, S.G. Buchanan, S.K. Burley, K. Conners, M. Dickey, M. Dorwart, R. Fowler, X. Gao, W.B. Guggino, W.A. Hendrickson, J.F. Hunt, M.C. Kearins, D. Lorimer, P.C. Maloney, K.W. Post, K.R. Rajashankar, M.E. Rutter, J.M. Sauder, S. Shriver, P.H. Thibodeau, P.J. Thomas, M. Zhang, X. Zhao, S. Emtage, Structure of nucleotide-binding domain 1 of the cystic fibrosis transmembrane conductance regulator, EMBO 23 (2004) 282–293.

[53] P. Saini, N.A. Gaur, R. Prasad, Chimeras of the ABC drug transporter Cdr1p reveal functional indispensability of transmembrane domains and nucleotide-binding domains, but transmembrane segment 12 is replaceable with the corresponding homologous region of the non-drug transporter Cdr3p, Microbiol. 152 (2006) 1559–1573.

[54] R. Ernst, P. Kueppers, J. Stindt, K. Kuchler, L. Schmitt, Multidrug efflux pumps: substrate selection in ATP-binding cassette multidrug efflux pumps—first come, first served, FEBS J. 277 (2010) 540–549.

[55] G. Tombline, L. Bartholomew, K. Gimi, K. Gimi, Grace A. Tyndall, Alan E. Senior, Synergy between conserved ABC signature Ser residues in P-glycoprotein catalysis, J. Biol. Chem. 279 (2004) 5363–5373.