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ABC multidrug transporter Cdr1p of *Candida albicans* has divergent nucleotide-binding domains which display functional asymmetry

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Abstract

In order to ascertain the molecular basis of ATP-mediated drug extrusion by Cdr1p, a multidrug transporter of *Candida albicans*, we recently have reported that the Walker A motif of the N-terminal nucleotide biding domain (NBD) of this protein contains an uncommon cysteine residue (C193; GXXGXGCS/T) which is indispensable for ATP hydrolysis. This residue is exceptionally conserved in N-terminal NBDs of fungal ABC transporters and hence makes these transporters an evolutionarily divergent group. However, the presence of a conventional lysine residue at a similar position in the Walker A motif of the C-terminal NBD warrants the individual contribution of both the NBDs in the ATP-driven efflux function of such transporters. In this study we have investigated the contribution of this divergent Walker A motif in the context of the full Cdr1p protein under in vivo conditions by swapping these two crucial amino acids (C193K in Walker A motif of N-terminal NBD and K901C in Walker A motif of C-terminal NBD) between the two NBDs. Both the native and the mutant variants of Cdr1p were integrated at the *PDR5* locus as GFP-tagged fusion proteins and were hyper-expressed. Our study shows that both C193K- and K901C-expressing cells elicit a severe impairment of Cdr1p's ATPase function. However, both these mutations have distinct phenotypes with respect to other functional parameters such as substrate efflux and drug resistance profiles. In contrast to C193K, K901C mutant cells were substantially hypersensitive to the tested drugs (fluconazole, ansiomycin, miconazole and cycloheximide) and were unable to expel rhodamine 6G. Our results for the first time show that both NBDs influence the Cdr1p function asymmetrically, and that the positioning of the cysteine and lysine residues within the respective Walker A motifs is functionally not interchangeable.
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Keywords: *Candida albicans*; ABC transporter; Multidrug resistance; Cdr1p; Nucleotide-binding domain; ATP hydrolysis; Walker A

1. Introduction

Candida albicans is an opportunistic diploid fungus that causes infections in immunocompromised and debilitated patients [1–3]. Wide-spread and prolonged usage of azoles in recent years has led to the rapid development of the phenomenon of multidrug resistance (MDR) which poses a major hurdle in antifungal therapy. Various mechanisms which contribute towards the development of multidrug resistance have been implicated in *Candida* and some of these include

Abbreviations: ATP, adenosine 5′-triphosphate; ATPase, adenosine-5′-triphosphatase; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethylsulfonylfluoride; kDa, kilodalton(s); ABC, ATP-binding cassette; MDR, multidrug resistance; NBD, nucleotide-binding domain; R6G, rhodamine 6G; FLC, fluconazole; MIC, miconazole; CYH, cycloheximide; ANS, anisomycin.

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overexpression/mutations in the target enzyme of azoles, lanosterol 14α-demethylase [3,4] or overexpression of drug efflux pumps belonging to the ATP-Binding Cassette (ABC) [5] and Major Facilitator Superfamilies of transporters (MFS) [6,7]. Among ABC transporters, Cdr1p has been shown to play a key role in azole resistance in *C. albicans* as deduced from its high level of expression found in several azole-resistant clinical isolates recovered from patients receiving long-term antifungal therapy [8,9]. Additionally, a high level of expression of *CDR1* invariably contributes to an increased efflux of fluconazole, thus corroborating its direct involvement in drug efflux [4,10]. Cdr1p thus has not only acquired significant clinical importance but is considered a critical factor in design of therapeutic strategies to combat antifungal resistance.

Cdr1p is a 1501 amino acid-long polypeptide, organized into two homologous halves. Each half is predicted to include a hydrophilic domain containing a nucleotide-binding domain (NBD), followed by a hydrophobic transmembrane domain (TMD) comprising six transmembrane segments (TMS) (Fig. 1(a)) [11]. The NBDs of ABC-type transporter proteins are the site of ATP hydrolysis and hence the hub of energy generation for drug efflux. Inactivation of these NBDs completely abrogates the functionality of the pump. Studies on human MDR1/P-gp, the mammalian homolog of Cdr1p, suggest that both NBDs are capable of performing an

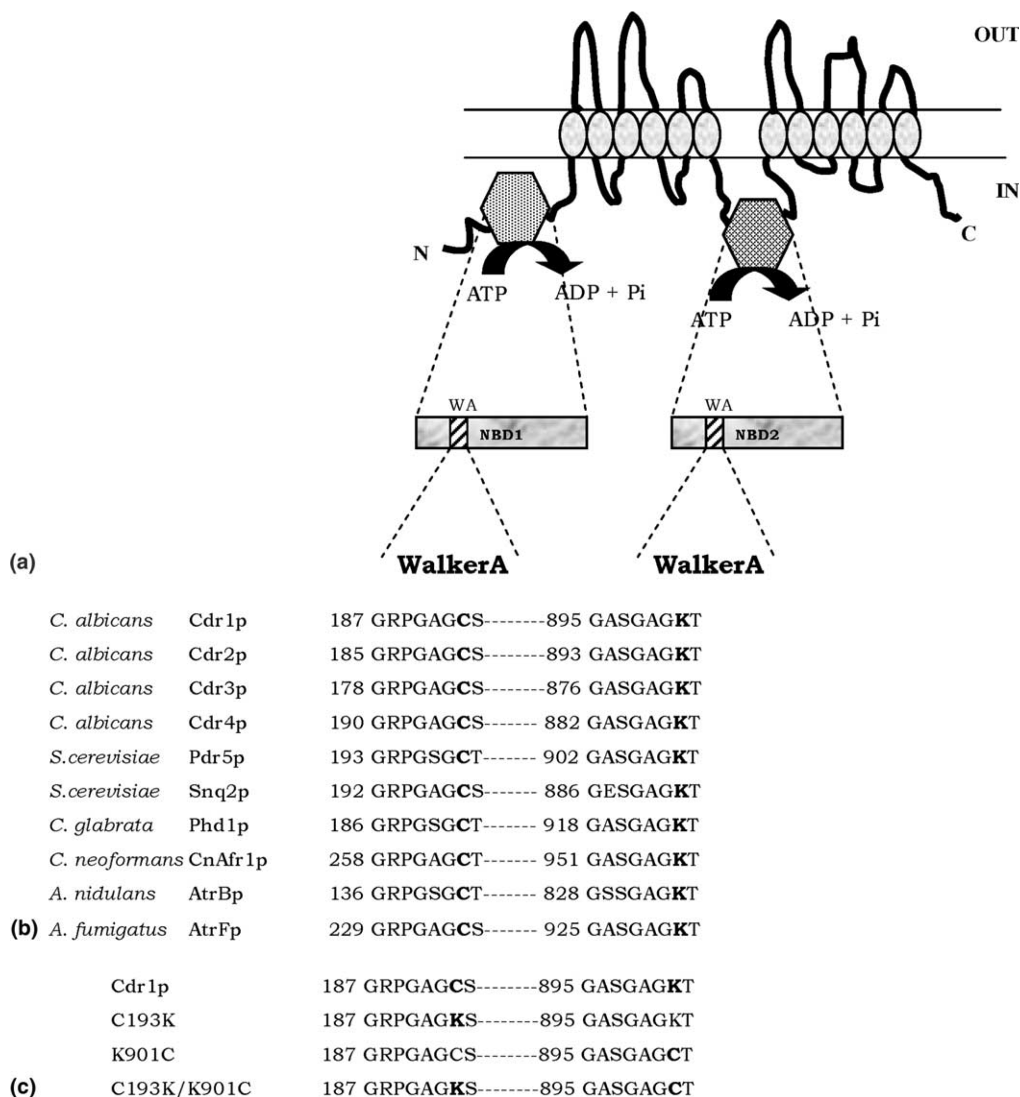

Fig. 1. (a) Topological model of Cdr1p showing two putative transmembrane domains (TMD) and two nucleotide-binding domains (NBD). Each TMD comprises six α-helices spanning the lipid membrane. The cytoplasmic domains of Cdr1p, i.e. NBD1 and NBD2, are located at the N- and C-terminus, respectively. (b) Amino acid sequence alignment of NBD1 and NBD2 of Cdr1p with other fungal ABC type transporters, highlighting (in bold) the conservation of the cysteine and lysine residues within Walker A. (c) Sequence of Walker A motif of Cdr1p, highlighting the amino acid substitution carried out in this study.

ATPase function, and inhibition of hydrolysis at one of the NBDs effectively abolishes hydrolysis at the other [12]. It is also know that hydrolysis at the two NBDs may occur in an alternate fashion. However, the question whether the two NBDs of Cdr1p are functionally identical or asymmetrical still remains unanswered.

The amino acid sequence of NBDs contains certain conserved amino acid stretches, which are critical for this domain's functionality [13]. These include the Walker A motif with a consensus sequence GxxGxG<u>K</u>S/T where 'x' represents any amino acid, the Walker B motif with a hhhhD sequence where 'h' is any aliphatic residue, and an ABC signature consisting of LSGGQQ/R/KQR. Structural and biochemical analyses of NBDs show that the conserved lysine residue within the Walker A motif binds to the β- and γ-phosphates of ribonucleotides and plays a critical role in ATP hydrolysis [14]. It is well-established from studies of other ABC transporters that the mutation of this key lysine residue leads to severe loss in ATPase activity without significantly affecting ATP binding [15].

Studies on various ABC transporters have revealed that ATP hydrolysis and substrate transport are strongly dependent on cooperativity between NBD1 (N-terminal) and NBD2 (C-terminal) [16]. Interestingly, though NBD1 of Cdr1p contains the conserved Walker A (GRPGAG<u>C</u>ST), the commonly conserved lysine residue within the Walker A motif is replaced by a cysteine which appears to be a unique feature of most of the fungal ABC transporters [17]. Conversely, the NBD2 of Cdr1p contains the commonly conserved lysine (GASGAG<u>K</u>T) at equivalent position in its Walker A motif. There is a complete lack of understanding with regard to the functional equivalence of both the NBDs of Cdr1p and the significance of the variation in their Walker A amino acid sequence. We have recently cloned and used purified the soluble domain of NBD1 from the full Cdr1p, to demonstrate in vitro that the uncommon cysteine 193 present within the Walker A motif is critical for ATPase activity [18,19]. Our recent study also has shown that the conserved aspartate 327 of Walker B of NBD1 is critical for ATP binding (unpublished observation).

In the present study we have examined the in vivo significance and contributions of the divergent Walker A motifs of both NBDs of the native Cdr1p with respect to Cdr1p localization, sensitivity to various drugs, ATPase activity, and ability to expel substrates from the cells. Considering all the functional parameters, our results suggest that NBD1 and NBD2 of Cdr1p have asymmetric contributions to the Cdr1p function.

2. Materials and methods

2.1. Strains and media

Plasmids were maintained in *Escherichia coli* DH5α. *E. coli* was cultured in Luria-Bertani medium (Difco), to which ampicillin was added (100 μg ml^{-1}) as required. The bacterial and *Saccharomyces cerevisiae* strains used in this study are listed in Table 1. The yeast strains were cultured in YEPD broth (1% yeast extract, 2% peptone and 2% glucose (HiMedia, Mumbai, India), or in SD ura^{-} drop-out media (0.67% YNB, 0.2% drop-out mix and 2% glucose, Difco). For agar plates, 2% (w/v) Bactoagar (HiMedia) was added to the medium. All chemicals used in this study were of analytical grade.

2.2. Site-directed mutagenesis of CDR1 and generation of transformants

Site-directed mutagenesis was performed by using the QuikChange mutagenesis system from Stratagene (La Jolla, CA, USA). The mutations were introduced into plasmid pPSCDR1-GFP according to the manufacturer's instructions. The primers used for this purpose were complementary oligonucleotides harboring C193K and K901C mutations (Table 2). The desired alterations in nucleotide sequence were confirmed by DNA sequencing of the ORF. The mutated plasmid pPSCDR1-GFP,

Table 1
List of plasmids and strains used in this study

Name	Description	Reference
Plasmid		
pPS-CDR1GFP	Plasmid carrying *CDR1-GFP* ORF cloned at *Spe*I site of pSKPDR5PPUS	[21]
pSJCG-C193K	Plasmid pPS-CDR1GFP carrying C193K mutation in *CDR1* ORF	This study
pSJCG-K901C	Plasmid pPS-CDR1GFP carrying K901C mutation in *CDR1* ORF	This study
pSJCG-C193K/K901C	Plasmid pPS-CDR1GFP carrying C193K/K901C mutation in *CDR1* ORF	This study
Strain		
AD1-8u⁻	MATa pdr1-3 his1 ura3Δyor1::hisG Δsnq2::hisG Δpdr5::hisG Δpdr10::hisG Δpdr11::hisG Δycf1::hisGΔpdr3::hisG Δpdr15::hisG	[23]
CDR1GFP	AD1-8u⁻ cells harboring *CDR1GFP* ORF integrated at *PDR5* locus	This study
SJCG-C193K	CDR1GFP cells carrying C193K mutation in *CDR1* ORF and integrated at *PDR5* locus	This study
SJCG-K901C	CDR1GFP cells carrying K901C mutation in *CDR1* ORF and integrated at *PDR5* locus	This study
SJCG-C193K/K901C	CDR1GFP cells carrying C193K/K901C mutation in *CDR1* ORF and integrated at *PDR5* locus	This study

Table 2
List of oligonucleotides used in this study

	Sequence	Purpose
C193K/F	5′-GGGAGACCCGGTGCTGGTAAATCCACATTGTTAAAGACC-3′	Forward primer for mutating C → K at 193 amino acid position
C193K/R	5′-GGTCTTTAACAATGTGGATTTACCAGCACCGGGTCTCCC-3′	Reverse primer for mutating C → K at 193 amino acid position
K901C/F	5′-CATTGTTGAATTGTTTATCTGTGTGAGTCACTACTGGTATTATTA-3′	Forward primer for mutating K → C at 901 amino acid position
K901C/R	5′-TAATAATACCAGTAGTGACTCACACAGATAAACAATTCAACAATG-3′	Reverse primer for mutating K→C at 901 amino acid position
NBD1F	5′-CGGGGATCCTCAGATTCTAAGATGTCG-3′	For sequencing
NBD1R	5′-CGCGGATCCCGACGGATCACCTTTCAT-3′	For sequencing
NBD1F-DWA	5′-CGGGATCCAAGACCATTGCTGTTAAC-3′	For sequencing
NBD1R-DWB	5′-CGGAATTCTCTTTCACCACCGGAAAC-3′	For sequencing
CDF10	5′-CATTACCGTGGTAAAGTTATTTATTCTGC-3′	For sequencing
CDR10	5′-GCAGAATAAATAACTTTACCACGGTAATG-3′	For sequencing

after linearizing with *Xba*I, was used to transform AD1-8u$^-$ cells for uracil prototrophy by a lithium acetate-based method of transformation [20]. Genomic DNA was isolated from the transformants thus obtained using a previously described procedure [21,22]. Genomic DNA was digested with restriction endonuclease (*Eco*RV, *Bam*HI, and *Pst*I; Roche Biochemicals). Digested genomic DNA (10 μg) was separated on a 1%-agarose gel and transferred to a Hybond$^+$ nylon membrane (Amersham). Membranes were hybridized with a α-^{32}P-labeled dATP *CDR1*-specific probe (ORF nucleotides 1–280) under high-stringency conditions [7].

2.3. Preparation of plasma membrane proteins

Purified plasma membrane (PM) fractions of yeast cells were prepared as described previously [23,24]. The PM protein concentration was determined by Bradford assay using bovine serum albumin as the standard.

2.4. Immunodetection of Cdr1p and its mutants

Protein samples (10 μg) were separated by eletrophoresis on sodium dodecyl sulfate-polyacrylamide (SDS) gels (8% acrylamide) and either stained with Coomassie blue or electroblotted onto nitrocellulose membrane (Highbond-C; Amersham). For Western blots, membranes were incubated with a 1:5000 dilution of anti-GFP antibody (BD Biosciences Clontech, Palo Alto, CA, USA), or 1:100 dilution of anti-Pma1p (plasma membrane ATPase) antibody. Immunoreactivity was detected using horseradish peroxidase-labeled antibody with a dilution of 1:5000 using the enhanced chemiluminescence assay system (ECL kit, Amersham).

2.5. Drug susceptibility assay

Drug susceptibilities of yeast strains were measured by three independent methods: filter disk assay, spot assay and minimum inhibitory concentration, as described earlier [25].

2.6. ATPase assay

The amount of inorganic phosphate (P_i) released from the PM fraction of cells expressing either native or mutant variant of Cdr1p was measured as described previously [18,23,26].

2.7. Rhodamine 6G efflux assay

The efflux of rhodamine 6G (R6G) from *S. cerevisiae* cells was determined as previously reported [26]. The R6G fluorescence of the samples was measured with a Cary Eclipse spectrofluorimeter (Varian, Inc., Australia).

3. Results

3.1. Mutations in the Walker A motifs of NBD1 and NBD2 do not affect Cdr1p localization and expression

The significance of the presence of uncommon cysteine and invariant lysine in Walker A of NBD1 and NBD2, respectively, was examined by mutating Cys 193 to Lys (C193K) and Lys 901 to Cys (K901C) (Fig. 1). For this we used a hyper-expression system where Cdr1p was stably overexpressed from the *PDR5* locus in a *S. cerevisiae* mutant, AD1-8u$^-$. The AD1-8u$^-$ was 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 [23,26]. The cells expressing these mutant variants of NBDs were designated as SJCG-C193K, SJCG-K901C and SJCG-C193K/K901C (Table 1). The stable single-copy integration in our heterologous hyper-expression system was confirmed by Southern

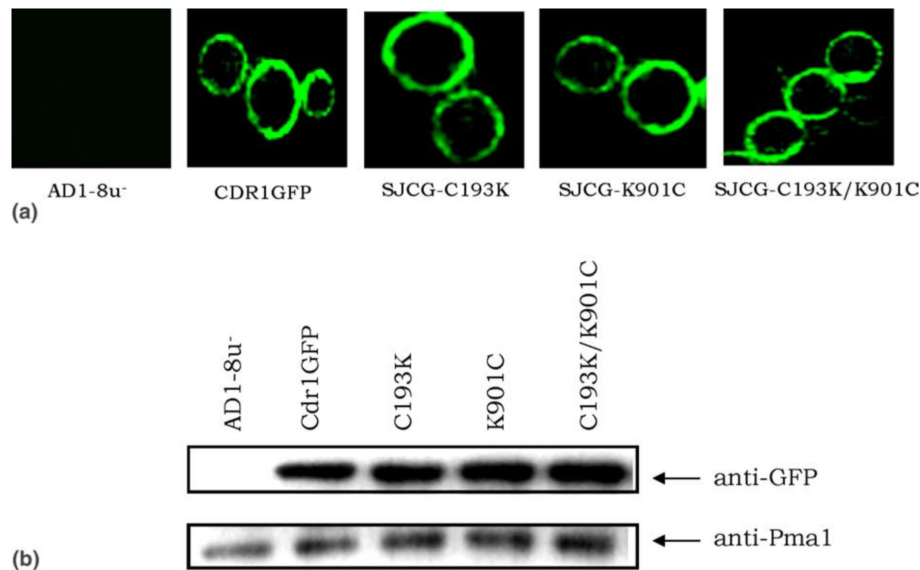

Fig. 2. (a) Confocal images of *Saccharomyces cerevisiae* cells expressing GFP-tagged Cdr1p and its mutant variants. Cells were washed and resuspended in an appropriate volume of 50 mM HEPES, pH 7.0, and placed on the glass slides; a drop of antifade reagent (Fluoroguard™ high performance antifade reagent, Biorad, Hercules, CA) was added to prevent photobleaching. The cells were directly viewed with 100× oil immersion objective on a Biorad confocal microscope (MRC 1024). (b) Expression profile of wild-type and mutant Cdr1 proteins. PM of wild- type and mutant proteins were prepared as described earlier [24]. Immunodetection of the proteins was performed by running the samples (as indicated) on a 8% SDS–PAGE and transferring to nitrocellulose membrane (electrophoretically), followed by probing with anti-GFP antibody and anti-Pma1 antibody and detecting by chemiluminescence using an ECL Kit (Amersham).

hybridization (data not shown). The surface localization of native Cdr1p was confirmed by confocal microcopy. The well-defined green-rimmed appearance in confocal images clearly confirmed that the GFP-tagged Cdr1p and its mutant variants were properly localized to the plasma membrane (PM) (Fig. 2(a)). The localization of Cdr1p in the hyper-expression system was further verified by probing with plasma membrane-specific antibody (Pma1). For this, equal amounts of purified PM proteins isolated from SJCG-C193K, SJCG-K901C and SJCG-C193K/K901C cells were loaded and separated on 8% SDS–PAGE and the proteins were transferred to nitrocellulose membrane (Amersham). The resolved proteins were probed with anti-GFP antibody. The results are depicted in Fig. 2(b), where it is evident that the expression levels of Cdr1p variants in PM of mutants and wild type were similar. Stripping and reprobing of the same blot with anti-Pma1 antibody (kind gift from R. Serrano, Valencia) validated the purified PM preparations (Fig. 2(b), lower panel). The confocal data and Western blotting excluded the possibility of mislocalization and altered expression of Cdr1p due to mutation within Walker A of NBD1 and NBD2.

3.2. Mutations in NBD1 and NBD2 differentially affect the drug susceptibility of C. albicans

We examined the effect of mutations in Walker A of Cdr1p on drug sensitivity of cells expressing mutant proteins by three independent drug susceptibility assays: filter disc, spot assay, and MIC.

As can be judged from the size of inhibition zones in filter disc assays (Fig. 3) the host cells (AD1-8u$^-$) were expectedly sensitive to all the tested drugs, i.e. fluconazole (FLC), miconazole (MIC), cycloheximide (CYH) and ansiomycin (ANS). In contrast, the transformants harboring Cdr1p (CDR1-GFP) wild-type protein showed a high degree of resistance, evident from the zone of inhibition which was either reduced (ANS and MIC) or completely absent (FLC and CYH) at the indicated concentrations (Fig. 3). Compared to the wild-type cells (CDR1-GFP), the C193K mutants showed a modest increase in sensitivity to FLC and ANS, and a $\leqslant$50% rise in sensitivity to MIC and CYH. Interestingly, the cells expressing the Walker A mutant (K901C) of NBD2 were hypersensitive to all the tested drugs, and their level of drug susceptibility was comparable to that of host cells (AD1-8u$^-$). The simultaneous mutations in both NBDs (C193K/K901C) resulted in hypersensitivity to all the tested drugs, to the level comparable to that of host cells (AD1-8u$^-$).

In order to further validate the above-mentioned observation, drug susceptibilities of native and mutant protein-expressing cells was examined by employing two other independent methods, i.e. spot and MIC assays. In spot assays, the host strain (AD1-8u$^-$) was expectedly sensitive to all the drugs when compared to the growth control (without drug). Conversely, substantial growth in presence of drugs was observed for the

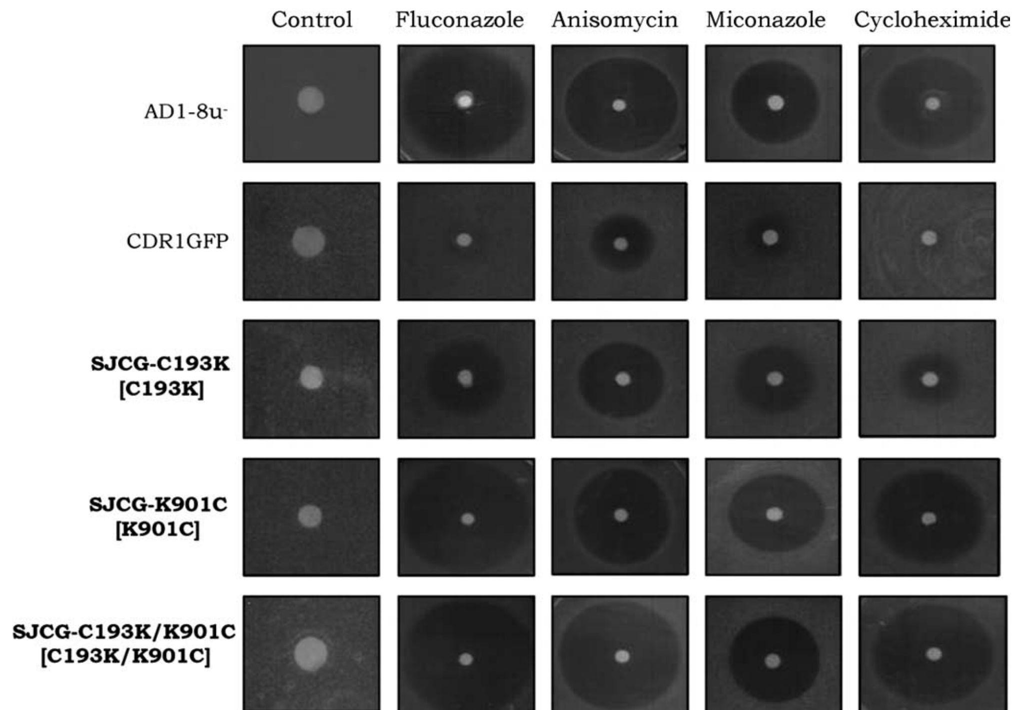

Fig. 3. Drug resistance profiles of wild-type and Walker A mutants determined by filter disc assay as described earlier [25]. For filter disc assay, the drugs were spotted in a volume of 1 to 5 μl at the indicated amounts: fluconazole (64 μg ml^{-1}), anisomycin (32 μg ml^{-1}), miconazole (0.25 μg ml^{-1}) and cycloheximide (0.25 μg ml^{-1}). Cells were incubated at 30 °C for 48 h and the zone of inhibition for each drug was measured.

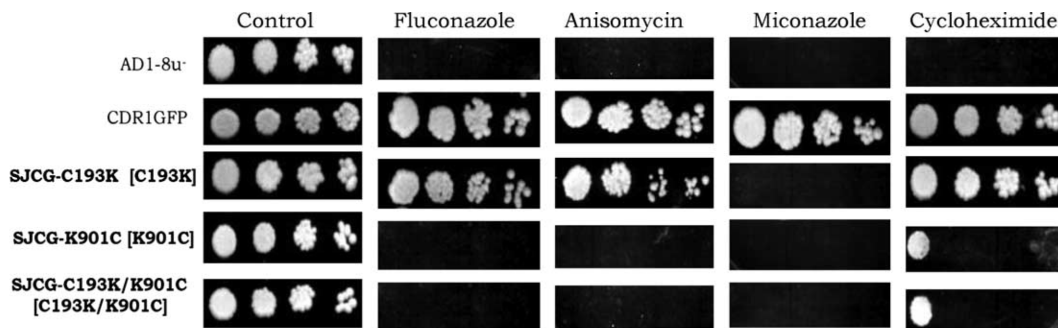

Fig. 4. Drug resistance profiles of wild-type and Walker A mutants determined by spot assays and minimum inhibitory concentration as described earlier [25]. In the spot assay, 5 μl of five-fold serial dilutions of each yeast culture (each with cells suspended in normal saline to an OD of 0.1 (A600)) were spotted on YEPD plates in absence (control) and presence of the following drugs: fluconazole (1 μg ml^{-1}), anisomycin (6 μg ml^{-1}), miconazole (0.1 μg ml^{-1}) and cycloheximide (0.15 μg ml^{-1}).

cells expressing native Cdr1p. It was found that at 1 μg ml^{-1} of FLC, 6 μg ml^{-1} of ANS, 0.1 μg ml^{-1} of MIC and 0.15 μg ml^{-1} of CYH, mutant variant C193K- and K901C-expressing cells showed substantial differences in sensitivity to these drugs. It is evident from Fig. 4 that, while mutant C193K continued to grow at these concentrations of most of the drugs (except MIC where C193K cells did not show any growth), the cells expressing K901C or C193K/K901C showed almost no growth with these drugs, thus displaying hypersensitivity (Fig. 4). The results of the microdilution method also corroborated the drug sensitivity profile of cells expressing native or mutant Cdr1p. Interestingly, there was a difference in minimum inhibitory concentrations between C193K and K901C which indicated the asymmetric affect of these mutations (Table 3). The results of all three independent drug sensitivity tests established that the two mutations in Walker A motifs of NBD1 and NBD2 have differential effect on Cdr1p-mediated drug resistance.

3.3. ATPase activity is affected by NBD mutations

We performed ATPase assays with the PM of wild type and mutants to ask the question whether mutations in NBDs in any way affect the ATPase activity of the full

Table 3
Drug resistance profiles of wild-type Cdr1p and its NBD mutants

	Minimum inhibitory concentration (μg ml^{-1})[a]			
	Fluconazole	Anisomycin	Miconazole	Cycloheximide
AD1-8u⁻	0.25	0.12	0.3	0.007
SJCDR1GFP	64	32	2	1
SJCG-C193K	8	8	0.5	0.125
SJCG-K901C	1	2	0.25	0.016
SJCG-C193K/K901C	0.5	0.25	0.3	0.007

[a] Determined following National Committee for Clinical Laboratory Standards.

protein and hence eventually contribute to the observed drug susceptibility phenotypes. The purified PM fraction from cells expressing native Cdr1p (CDR1-GFP) exhibited a significant increase in the ATPase activity as compared to that of the host cells (AD1-8u^{-}). This substantial and hence measurable difference in oligomycin-sensitive ATPase activity gave us an opportunity to look for the effect of mutations in the divergent Walker

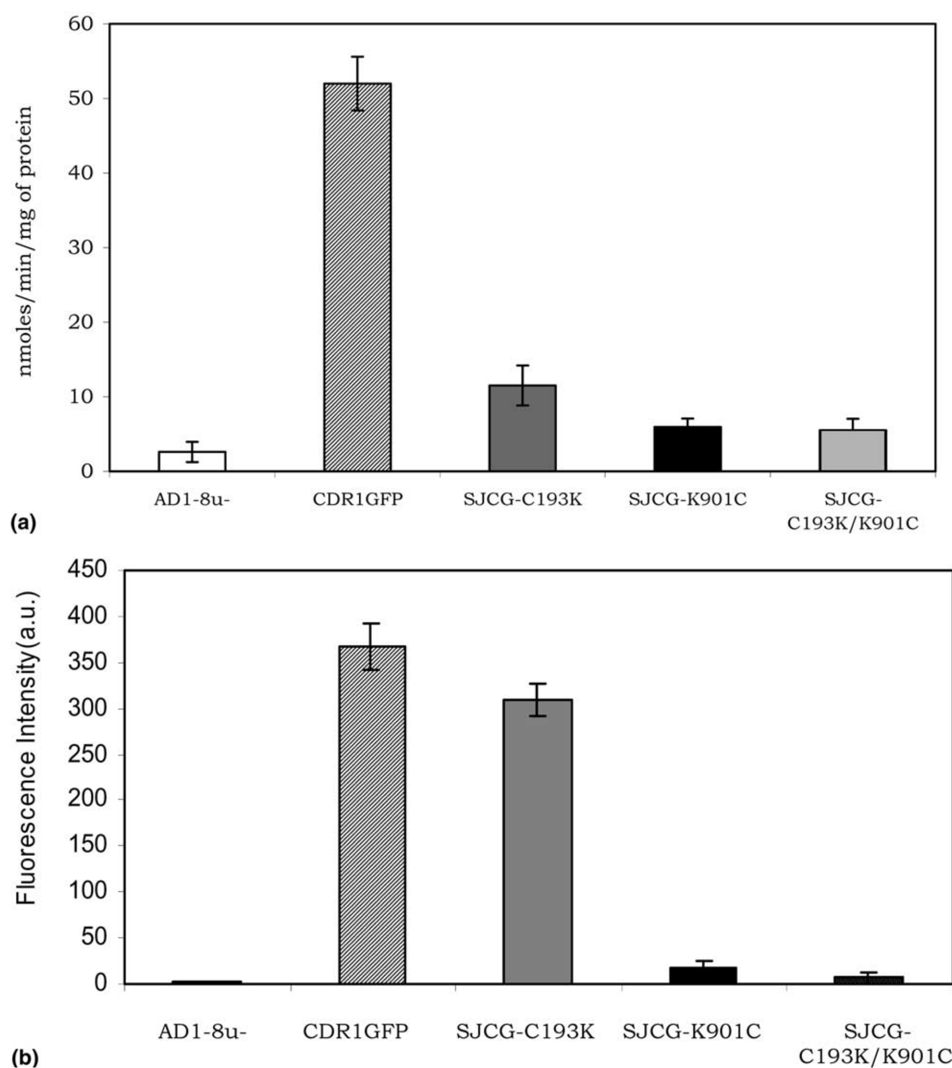

Fig. 5. (a) Comparison of ATPase activity of Cdr1p with its mutants. ATPase activity of the wild type (Cdr1p) and the Walker A mutants (C193K, K901C and C193K/K901C) was assayed and each reaction was performed in triplicate; the values plotted (with ±SD) represent the average of three independent experiments. (b) Comparison of R6G efflux between wild-type and Walker A mutant cells. Cells were de-energized as detailed elsewhere [26] and incubated with R6G at 30 °C for 2 h. Glucose (2%) was added and at different time points cells were rapidly centrifuged and the extracellular concentration of R6G in the supernatant was determined spectrofluorometrically [26]. R6G efflux is represented by bars showing the extracellular concentration of R6G in the supernatant 30 min after the addition of glucose to the cells in wild-type and Walker A mutant cells. The values are the means (indicated by the bars) ± standard deviations of three independent experiments.

A motifs of Cdr1p [23,26]. As depicted in Fig. 5(a), we observed a clear and distinct difference between ATPase activity of the PM prepared from the wild type and from mutants. Substitution of Cys 193 to Lys (C193K) in Walker A motif of NBD1 resulted in a 78% decrease in its ability to hydrolyze ATP, while Lys 901 to Cys (K901C) had a more severe effect as it had only 12% of the native protein activity. The simultaneous substitution of Cys 193 and Lys 901 (C193K/K901C) reduced the ATPase activity to $\leqslant$10% of that of the native protein (Fig. 5(a)). The results of ATPase assays suggested that there is a differential effect, albeit not very large, on the ability to hydrolyze ATP when Cys 193 or Lys 901 are substituted independently (C193K, K901C) or simultaneously (C193K/K901C).

3.4. R6G efflux suggests asymmetry in substrate export ability between NBD1 and NBD2

The difference in the ability to hydrolyze ATP and the sensitivity to drugs further lead us to the question whether there is an asymmetry between NBD1 and NBD2 of Cdr1p with regard to their ability to expel substrates. We monitored rhodamine 6G (R6G) efflux in energy-depleted cells expressing native or mutant variant Cdr1p. The ability of Cdr1p to expel R6G from the cells is ATP-dependent, as has been established earlier by us as well as by others [26]. In this study, when R6G efflux was carried out with cells expressing the variant Walker A mutants of Cdr1p and compared with that of cells expressing the native protein, we observed K901C and K901C/C193K double substitution to significantly affect the R6G efflux ability of Cdr1p, correlated well with the observed hypersensitivity to tested drugs displayed by these mutants. However, drug-sensitive C193K mutant cells showed efflux levels of R6G comparable to those of native-protein-expressing cells (Fig. 5(b)). Thus the substitution of conserved Lys 901 of NBD2 and atypical Cys 193 resulted in an asymmetrical effect on their expulsion ability.

4. Discussion

Multidrug resistance has been linked with ABC transporters in many disease-causing organisms, including several fungal pathogens, and human cancers [1–3]. Although we have a fair understanding of drug-binding and catalytic function of mammalian ABC drug transporters such as human P-gp/MDR1 and MRPs, the same is unfortunately not true for fungal transporters. Considering the importance of ABC transporters like Cdr1p and Cdr2p in azole resistance commonly encountered in clinical isolates of *C. albicans*, we have been functionally characterizing Cdr1p [18,19,23]. Recently, by using a soluble purified N-terminal NBD1 domain of Cdr1p, we have demonstrated that this domain elicits a cation dependent general ribonucleotide triphosphatase activity [18]. We also have found an evolutionary divergence in this domain wherein a conserved variation exists within the catalytically crucial Walker A motif of NBD1 [18]. Reports from other systems suggest that the Walker A motifs of NBDs and especially the well-conserved lysine residue within it are indispensable for ATP hydrolysis, hence its mutation to Arg or Met in either or both NBDs abrogates the drug transport and ATPase activities of mammalian ABC transporters [15]. Our study has highlighted the evolutionary deviation and conservation of cysteine (GxxGxGKS/T) residue instead of a typically found lysine in the Walker A motif of NBD1 of Cdr1p and other fungal transporters. By both biochemical analyses and deduced structural homology modeling, we have demonstrated the indispensability of this evolutionarily divergent Cys 193 in ATP hydrolysis [18,19]. However, since these observations were an outcome of analyses of an in vitro isolated domain, we wanted to understand the significance of variant Walker A motifs of NBD1 and NBD2 in the context of the full protein. The present in vivo study was designed to explore the relative contribution of both the N- and C-terminal NBDs in ATP-binding, hydrolysis and transporter activity of Cdr1p. In order to address these questions, we swapped atypical Cys 193 of Walker A of NBD1 (C193K) and conserved Lys 901 (K901C) of Walker A of NBD2. The effect on ATP hydrolysis and transport activity of such substitution in the full protein of Cdr1p or of its mutant variants was studied using a heterologous hyper-expression system [23]. We observed that mutation within the Walker A of both the NBDs did not affect the expression and the localization of the mutant variant protein, as was evident from Western blots and GFP-tagged fluorescence (Fig. 2). Of note, we earlier had observed that mutant variant C193K in Walker A of the NBD1 domain where Cys 193 was replaced by Lys resulted in an unstable protein ([18] and unpublished data). Interestingly, C193K mutation in the whole protein (this study) did not affect its stability.

The drug resistance profile of Cdr1p C193K or K901C mutants gave an interesting insight into the functioning of the two NBDs. Indeed, cells expressing both mutant variants were sensitive to all the tested drugs but there was a drastic difference in their level of sensitivity, as was revealed by three independent drug susceptibility assays. The cells expressing K901C were hyper-sensitive to drugs when compared to the C193K variant or to native Cdr1p. This clearly established that the two NBDs respond asymmetrically to the substitution of conserved residues of their respective Walker A motifs. The divergence in functioning of the two NBDs was further evident when we compared the export ability of these mutant proteins. It was observed that the export of the fluorescent substrate R6G by a Cdr1p was

severely inhibited in cells expressing K901C as compared to cells expressing the C193K variant. It is intriguing why substitution of C193 does not result in significant impairment of R6G efflux as compared to the K901 mutation. An in-depth analysis of the catalytic cycle, i.e. nucleotide binding, hydrolysis and substrate binding/efflux will be required to answer the questions behind the dichotomy in functioning of the NBDs. This study, however, demonstrates that a diverse N-terminal NBD (GxxGxGC̲S/T) of Cdr1p is functional where uncommon C193 is critical. Considering that all other fungal ABC transporters, including the well-studied Pdr5p of *S. cerevisiae*, have the uncommon cysteine in Walker A of NBD1 (with the exception of Ste6p of *S. cerevisiae*), it is expected that this residue will have an indispensable role in the catalytic cycle [17].

The purified PM isolated from cells expressing mutant variants of Cdr1p showed that replacements of C193K and K901C in Walker A of NBD1 and NBD2 resulted in 78% and 88% impairment of ATPase activity, respectively (Fig. 5). It would mean that the swapping of the cysteine and lysine residues in the Walker A between the two NBDs could not retain the normal ATPase function of the native protein. This further not only signifies the crucial positioning of the C193 and K901 at their respective domains, but also demonstrates that the swapping of the two residues within the two NBDs is intolerable to the Cdr1p function. The difference in loss of ATPase activity between the two mutant proteins was not substantial; therefore, the activity cannot be directly correlated with the differences observed between C193K and K901C mutant proteins with regard to drug resistance and efflux of R6G. Since both mutations occurring independently and simultaneously impaired ATPase function substantially, it is difficult to comment if there are any synergestic contributions of these mutations. Based on overall results, it is clear that the two NBDs of Cdr1p function divergently in vivo. Of note, the C193A mutation and K901A in the full protein also showed impaired ATPase activity (data not shown).

The two NBDs of a number of ABC transporters have been shown to be functionally divergent, playing different roles in the transport process. In prokaryotic ABC type transporters such as the histidine permease of *E. coli*, both NBDs are functionally identical and equally contributive to the protein's activity. Inactivation of either one of these NBDs in the full protein results in a transporter that has its activities reduced to 50%. On the other hand, the NBDs of the eukaryotic transporters such as the human P-gp/MDR1, CFTR (Cystic Fibrosis Transmembrane conductance Regulator) and MRP1, though highly conserved and similar in sequence, do not appear to be functionally complementary, as inactivation of either of them completely abolishes ATPase and transport activities of the protein [15]. The whole issue whether the two NBDs are functionally identical or asymmetric remains to be resolved. In this context our present study clearly demonstrates that the multidrug ABC transporter Cdr1p of pathogenic *C. albicans* possesses functionally divergent NBDs. These results would pave ways to understand the catalytic cycle of drug efflux involving the two NBDs of this medically important ABC drug transporter.

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