Structure and Function Analysis of CaMdr1p, a Major Facilitator Superfamily Antifungal Efflux Transporter Protein of Candida albicans: Identification of Amino Acid Residues Critical for Drug/H\(^+\) Transport
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We have cloned and overexpressed multidrug transporter CaMdr1p as a green fluorescent protein-tagged protein to show its capability to extrude drug substrates. The drug extrusion was sensitive to pH and energy inhibitors and displayed selective substrate specificity. CaMdr1p has a unique and conserved antiporter motif, also called motif C [G(X\(_2\))G(X\(_3\))GP(X\(_2\))GP(X\(_2\))G], in its transmembrane segment 5 (TMS 5). Alanine scanning of all the amino acids of the TMS 5 by site-directed mutagenesis highlighted the importance of the motif, as well as that of other residues of TMS 5, in drug transport. The mutant variants of TMS 5 were placed in four different categories. The first category had four residues, G244, G251, G255, and G259, which are part of the conserved motif C, and their substitution with alanine resulted in increased sensitivity to drugs and displayed impaired efflux of drugs. Interestingly, first category mutants, when replaced with leucine, resulted in more dramatic loss of drug resistance and efflux. Notwithstanding the location in the core motif, the second category included residues which are part of the motif, such as P260, and those which were not part of the motif, such as L245, W248, P256, and F262, whose substitution with alanine resulted in a severe loss of drug resistance and efflux. The third category included G263, which is a part of motif C, but unlike other conserved glycines, its replacement with alanine or leucine showed no change in the phenotype. The replacement of the remaining 11 residues of the fourth category did not result in any change. The putative helical wheel projection showed clustering of functionally critical residues to one side and thus suggests an asymmetric nature of TMS 5.

In recent years, incidences of acquired resistance to azoles in the fungal pathogen Candida albicans, have increased considerably, which poses serious problems in successful chemotherapy of candidiasis. Current evidence suggests that Candida acquires azole resistance by employing multiple mechanisms that include (i) failure of drug accumulation mediated by extrusion pump proteins such as Cdr1p and Cdr2p, belonging to ABC superfamily (MFS), (ii) alterations in the azole resistance mediated by ABC drug exporters. The MFS consists of membrane transport proteins from bacteria to higher eukaryotes involved in symport, antiport, or uniport of various substrates (2, 5, 21, 24, 29). One major cluster of this superfamily consists of proton motive force-dependent drug efflux proteins (14). Bacterial MFS drug transporters are antiporters, which have a unique antiporter motif, also called motif C [G(X\(_2\))G(X\(_3\))GP(X\(_2\))GP(X\(_2\))G], necessary for the drug/H\(^+\) antiport activity (5, 15). Independent of antiporter’s substrate specificities, the antiporter motif in the predicted TMS 5 is conserved in all of the functionally related subgroups in bacteria and plants (14, 25, 29). Although MFS drug exporters in yeast also possess the antiporter motif, its relevance remains to be established. This motif C also has a repetitive GXXXG stretch, which is thought to be important in proper helix packing and dimerization in the ABCG2 transporter of humans (15). Multiple-sequence analysis of the MFS transporters reveals that proteins within this family share greater similarity between their N-terminal halves than in their C-terminal halves, and it is assumed that the later half is responsible for substrate recognition (14). Additionally, the MFS drug antiporter proteins possess many other conserved residues scattered throughout the length of the protein, for exam-

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The yeast strains used in this study are listed in Table S1 in the Materials.

All site-directed mutageneses were carried out using a site-directed mutagenesis kit (Stratagene, La Jolla, CA) as previously described (22). The mutations were introduced into plasmid prCaMDR1-GFP according to the manufacturer’s instructions, and the desired nucleotide sequence alterations were confirmed by DNA sequencing of the ORF. The primers used for the targets are listed in Table S2 in the supplemental material. The mutated plasmid, after being linearized with XbaI, was used to transform AD1-8u− cells for uracil prototrophy by the lithium acetate transformation protocol (22). Integration was confirmed with Southern blot analysis.

Preparation of plasma membrane proteins and immunodetection of the CaMDR1 and its mutant. Purified PM fractions of yeast cells were prepared as described previously (22). The PM protein concentration was determined by bicinchoninic acid assay using bovine serum albumin as the standard. For Western blots, membranes were incubated with a 1:5,000 dilution of anti-GFP antibody, or a 1:1,000 dilution of anti-Pma1p (plasma membrane ATPase) antibody. Immunoreactivity was detected using horseradish peroxidase-labeled antibody with a dilution of 1:5,000 using the enhanced chemiluminescence assay system (ECL kit; Amersham) (22).

Confocal microscopy. The cells were grown to late log phase in SD-ura medium, except for AD1-8u−, where uridine (0.02%) was added to the SD-ura medium. The cells were then washed and resuspended in an appropriate volume of 50 mM HEPES, pH 7.0. The cells were placed on glass slides and then imaged under an oil immersion objective at ×100 magnification on a confocal microscope (Radiance 2100, AGR, 3Q/BLD; Bio-Rad, United Kingdom) (22).

Drug susceptibility assay. Drug susceptibilities of yeast strains were measured by two independent methods: microdilution assay and spot assay. The MIC50 for the strains were determined with a broth microdilution method as described previously (11, 16). For spot assay, 5-μl samples of fivefold 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 onto YEPD plates in the absence (control) or in the presence of the drugs (11, 16).

Drug transport. Accumulation of [3H]MTX (specific activity, 8.60 Ci/mmol) and [3H]FLC (specific activity, 19 Ci/mmol) was determined essentially by the protocol described previously (11, 16). For accumulation assays, 25 μM [3H]MTX and 100 nM [3H]FLC were routinely used. To see the effect of inhibitors on accumulation, different inhibitors were added 10 min before the commencement of the transport. Different inhibitors used included sodium azide (10 mM), sodium orthovanadate (1 mM), carbonyl cyanide m-chlorophenylhydrazone (CCCP) (100 μM), sodium arsenate (1 mM), and N-ethylmaleimide (NEM) (1 mM) (9). The concentrations of inhibitors used were not toxic to cells, and the effects observed were purely due to their metabolic effect. For competition, [3H]MTX was used at a final concentration of 25 μM, and a fivefold concentration for each substrate was used (125 μM) for competition between MTX and various substrates.

For efflux measurements, a 5% yeast cell suspension (2.5 × 105 cells ml−1) was incubated with [3H]MTX at pH 7.4 for 30 min in the presence of CCCP (100 μM) to allow accumulation of drug by passive diffusion. The cells were pelleted and resuspended in MES buffer of indicated pH to initiate the efflux. An aliquot of 100 μl was taken at regular intervals and filtered on a 0.45-μm filter disk (Millipore; India). The cells were washed twice with 3 ml MES-Tris buffer, and the concentration of drug trapped within the cells was measured by counting the filter disks in a liquid scintillation counter (Packard).

RESULTS

Overexpressed CaMDr1p-GFP is properly surface localized. CaMDr1p has recently been overexpressed in C. albicans cells (7). However, to functionally characterize CaMDr1p, in this study, we have overexpressed it in a heterologous system. For this, we have cloned the CaMDr1p-GFP ORF in the plasmid pSK-PDR5PUS (12) and overexpressed it by integrating it at the PDR5 locus downstream from the PDR5 promoter in the S. cerevisiae strain AD1-8u− (12). The host AD1-8u− was derived from a strain containing the pdr1-3 gene with a gain-of-function mutation in the transcription factor Pdr1p, resulting in constitutive hyperinduction of the PDR5 promoter (Fig. 1A) (12). Single-copy integration at the PDR5 promoter was confirmed by Southern hybridization (data not shown). The wild-type CaMDr1p-GFP was transcribed (Fig. 1B), expressed, and properly targeted to the PM, as evident from the Western blot analysis of the PM fraction of cells (Fig. 1C). Expression and localization were also confirmed by confocal microscopy and fluorescence-activated cell sorter (FACS) analysis (Fig. 1A; see Fig. 6B).
Overexpressed CaMdr1p-GFP confers drug resistance. We examined the drug sensitivities of the cells overexpressing wild-type CaMdr1p-GFP (RPCaMDR1-GFP) by two independent drug susceptibility methods, viz., microdilution and spot assays. The microdilution assay revealed that the host strain (AD1-8u⁻/H11002) as expected, was hypersensitive to all of the tested drugs when compared to the growth control (without drug) (see Table S3 in the supplemental material). On the contrary, cells expressing the wild-type CaMdr1p-GFP (RPCaMDR1-GFP) were able to tolerate the same concentrations of the drugs. Compared to the host strain (AD1-8u⁻), MIC₅₀₈ (MIC for 80% inhibition in growth) for the RPCaMDR1-GFP strain
were considerably higher (MIC\textsubscript{80} of 16 \mu g/ml for FLC, 0.5 \mu g/ml for cycloheximide [CYH], 8 \mu g/ml for cerulenin [CER], 1 \mu g/ml for 4-nitroquinoline [4-NQO], and 128 \mu g/ml for MTX) (Fig. 1D). The spot assays confirmed the microdilution results (Fig. 1D; see Table S3 in the supplemental material). The CaMdr1p-GFP chimeric protein was able to confer drug resistance similar to the untagged protein CaMdr1p, indicating that GFP tagging does not affect its ability to function (Fig. 1D; see Table S3 in the supplemental material). In the following experiments, we examined the functions of the RPCaMdr1p-GFP.

CaMdr1p effluxes methotrexate and fluconazole. For checking the ability of drug extrusion by the overexpressed CaMdr1p, we measured the net intracellular accumulated levels of two drug substrates, [\textsuperscript{3}H]MTX and [\textsuperscript{3}H]FLC in RPCaMDR1-GFP cells (Fig. 2A and B). An increased or decreased level of accumulation of the drug, at a given point of time, implies its reduced or enhanced efflux, respectively. It is apparent from Fig. 2A and B that, compared to the host (AD1-8u\textsuperscript{−}) cells, the accumulation of [\textsuperscript{3}H]MTX and [\textsuperscript{3}H]FLC was considerably reduced (more efflux) in cells expressing CaMdr1p-GFP-tagged protein.

**Efflux is sensitive to different inhibitors.** We observed that the accumulation of [\textsuperscript{3}H]MTX and [\textsuperscript{3}H]FLC was sensitive to different energy inhibitors such as sodium azide, sodium orthovanadate, CCCP, and sodium arsenate (Fig. 2A and B). The accumulation of [\textsuperscript{3}H]MTX and [\textsuperscript{3}H]FLC was increased in the presence of these inhibitors, implying reduction in efflux. The sensitivity of [\textsuperscript{3}H]MTX and [\textsuperscript{3}H]FLC accumulation, particularly to proton conductor CCCP, suggests that CaMdr1p function is sensitive to pH and may function as a H\textsuperscript{+} antiporter. Unlike the efflux of drugs mediated by ABC transporter Cdr1p (8), the efflux mediated by MFS CaMdr1p was insensitive to the SH blocker NEM.

**Efflux of MTX and FLC is pH dependent.** To confirm that CaMdr1p is a putative drug/H\textsuperscript{+} transporter, we monitored the efflux of [\textsuperscript{3}H]MTX and [\textsuperscript{3}H]FLC by exposing cells to buffers with different pH values (Fig. 3A and B, respec-
In a typical experiment, cells expressing wild-type CaMdr1p-GFP were treated with proton conductor CCCP (100 μM) and were allowed to accumulate [3H]MTX and [3H]FLC for up to 30 min. These cells were then rapidly pelleted and resuspended in buffers of different pH values to initiate the efflux of accumulated drug against pH gradient. The efflux of the drug was measured in rapid succession, as detailed in Materials and Methods. Our results revealed that MTX efflux is very sensitive to pH (Fig. 3A); for example, RPCaMDR1-GFP cells exposed to acidic pH (3.5 to 5.5) showed maximum efflux (lower accumulation) of the drug. No such pH effect was observed with the host strain AD1-8u/H11002, which did not show any significant efflux of the drugs (Fig. 3A and B). The efflux of [3H]FLC mediated by CaMdr1p was also pH sensitive, which was similar to [3H]MTX and maximum at acidic pH (3.5 to 5.5) (Fig. 3B).

It should be pointed out that ABC drug transporter Cdr1p of C. albicans, when expressed in S. cerevisiae strain AD1-8u/H11002, could also extrude FLC but at a pH optimum of 7.5 (data not shown).

CaMdr1p harbors conserved drug/H+ antiporter domain. The alignment of TMS 5 sequence of CaMdr1p with other MFS transporters revealed extensive conservation of the amino acids (Fig. 4C). Notably, all five glycines of CaMdr1p are conserved in other MFS transporters (Fig. 4C). Considering the fact that, irrespective of the differences in substrate specificities among MFS antiporters, a great level of conservation of amino acid exists in motif C, we analyzed the functional significance of this motif in drug transport by alanine scanning of the entire TMS 5 of CaMdr1p. The TMS 5 sequence was deduced by taking the consensus sequence from the various bioinformatic programs, including HMMTOP, SOSUI, TMHMM, and TopPred programs (18–20, 27, 28).

For alanine scanning of TMS 5, we employed a site-directed mutagenesis approach with mutagenic oligonucleotides (see Table S2 in the supplemental material) where all the amino acids of the TMS 5 were substituted to neutral alanine. To avoid the introduction of new side chains, the three existing alanines in TMS 5 (A246, A247, and A252) were replaced with glycine, while 5 glycines (G244, G251, G255, G259, and G263) were replaced with alanine as well as with long-side-chain amino acid leucine. All 26 mutant variants of CaMdr1p-GFP were stably overexpressed as GFP-tagged variants in a heterologous system, as described in Materials and Methods, and designated as shown in Table S1 in the supplemental material. Single-copy integration was confirmed with Southern blot analysis (data not shown).

Conserved TMS 5 residue substitution results in hypersensitive cells. Confirmed positive mutants were screened for their sensitivity to different substrates by two independent
methods: microdilution assay and spot assay. Based on the
drug sensitivity results of these two methods and the location
of the residues inside or outside the conserved motif, mutant
variants could be placed into four categories. The first
category had four residues, G244, G251, G255, and G259, which are also part of the motif C, and their replace-
ment by alanine (G244A, G251A, G255A, and G259A) led
to variable enhanced sensitivity to tested drugs (Fig. 5A and
Table 1). For example, variants G244A, G251A, and G255A
were only partly sensitive to FLC, CER, and MTX but, in
comparison, were more sensitive toward CYH and 4-NQO.
The variant G259A, however, showed only a slight change in
drug sensitivity profile (Fig. 5). Interestingly, all four glycines of the first category, when substituted with
leucine (G244L, G251L, G255L, and G259L), showed more
dramatic results, as can be seen by complete abrogation
of growth in the presence of the same concentration of the
drugs (Fig. 5B and Table 1). The residues which are con-
served and part of a motif, such as P260, and those which
were not part of a motif, such as L245, W248, P256, and
F262, were included in second category. The replacement
of these residues with alanine only resulted in a more dramatic
increase in drug susceptibility and showed almost no growth
in the presence of tested drugs (Fig. 5C). The third category,
which has one residue, G263, and is a part of the conserved
motif, showed no major change in drug susceptibility levels
when replaced with alanine (G263A) or leucine (G263L)
(Fig. 5D). All 11 residues of TMS 5 belonging to the fourth
category, when replaced with alanine (V243A, A246G,
A247G, S249A, L250A, A252G, V253A, C254A, S257A,
F258A, and F261A), did not affect the drug resistance profile
(Table 1). Considering that the residues of fourth cate-
gory were nonconserved and also not part of antiporter
motif, these residues were not replaced with other amino
acids, such as leucine. Thus, out of the 21 residues of TMS
5, substitution of only nine residues, namely G244, L245,
W248, G251, G255, P256 G259, P260, and F262, affected drug susceptibilities to various degrees (Fig. 5A, B, and C and Table 1). The drug susceptibilities revealed by spot assays matched well with the microdilution results (see Table S3 in the supplemental material).

To exclude the possibility that the observed hypersusceptibility of the mutant variants was not due to poor expression or impaired surface localization, we compared the protein localization of RPCaMdr1p-GFP and variant mutants by Western blot analysis, FACS, and confocal microscopy. Western blot analysis with anti-GFP antibody confirmed similar expression levels of the mutants and the wild type (Fig. 6A). FACS and confocal images also confirmed that there was no difference in cell surface localization of CaMdr1-GFP between the cells expressing wild-type and mutant variants (Fig. 6B).

**Mutant variants display different levels of accumulation (efflux) of radiolabeled drugs.** All the mutants were further analyzed for their functionality by measuring the intracellular accumulation of [³H]MTX and [³H]FLC. The first category of mutant variants, when replaced by alanine, G244A, G251A, G255A, and G259A, showed increased accumulation of [³H]MTX and [³H]FLC (Fig. 7A and B and Table 1). The mutant variants of the second category, including L245A, W248A, P256A, and F262A, also showed increases in their ability to accumulate [³H]MTX and [³H]FLC, thus implying decreased efflux (Fig. 7A and B and Table 1). The third category mutant variant G263, whether replaced with alanine or leucine, had no major impact on drug resistance profile and displayed unaltered accumulation of [³H]MTX and [³H]FLC, comparable to cells expressing wild-type protein (Fig. 7A and B and Table 1).

The mutant variants of the fourth category (V243A, A246G, A247G, S249A, L250A, A252G, V253A, C254A, S257A, F258A, and F261A), which showed no change in the drug resistance profile, also did not display any change in the level of accumulation of the drugs (data not shown). Of note, the reduced ability to efflux drug matched well with the drug susceptibility data for all the categories (Fig. 5 and 7).

**TMS 5 and substrate binding and transport.** The mutant variants which displayed sensitivity in the drug susceptibility assays and were defective in transport were also checked for substrate binding. To examine the effect of TMS 5 mutations on the drug binding sites or ion coupling and transport, [³H]MTX accumulation was competed with fivefold molar excesses of different drugs. The accumulation of [³H]MTX could only be competed out by a molar excess of MTX and 4-NQO, while other tested drugs could not affect the accumulation in wild-type RPCaMDR1-GFP cells (Fig. 8A).
FIG. 6. Comparison of expression of RPCaMDR1p-GFP and different mutant variant localization. (A) Western blot analyses of the PM fractions of mutant variants, with anti-GFP antibody, to confirm their proper targeting. (B) Confocal images and FACS analyses of the mutants of first, second, and third categories to check their expression, proper folding, and localization and comparison with AD1-8u⁻¹ (negative control) and RPCaMDR1-GFP (positive control).
means that MTX and 4-NQO share a common substrate binding site(s). R6G is not a substrate of CaMdr1p-GFP and, expectedly, was unable to affect the accumulation of [3H]MTX (Fig. 8A). Similar to native CaMdr1p-GFP, the accumulation of [3H]MTX by the first category of mutants, G244A, G251A, G255A, and G259A, could be competed out with MTX and 4-NQO, suggesting that these residues are not directly involved in drug transport (Fig. 8B). However, when glycines of the first category were replaced with leucine (G244L, G251L, G255L, and G259L), both MTX and 4-NQO could not compete with the accumulation of [3H]MTX (Fig. 8C). Of note, as a result of substitution of conserved glycines with leucine, the accumulation of [3H]MTX was severely reduced in these variants, and hence, a clear distinction between the inability of MTX and 4-NQO to compete with [3H]MTX and the mutant variants’ inability to participate in drug/H+ transport could not be made. However, based on the fact that the efflux properties do not change after replacement of conserved glycines with alanine, one could cautiously state that these residues may not be directly involved in MTX binding and transport.

As shown in Fig. 8D, the accumulation of [3H]MTX could not be competed out by any drug in the five mutant variants of second category, namely L245A, W248A, P256A, P260A, and F262A. The mutant variant of the third category, G263, behaved as wild-type protein whether replaced with alanine (G263A) or leucine (G263L) (Fig. 8E).

DISCUSSION

CaMdr1p is one of the major efflux pumps of C. albicans belonging to the MFS which is involved in clinically encountered azole resistance. CaMdr1p is functionally identical to ABC drug transporters such as Cdr1p and Cdr2p; however, they differ with regard to the mechanisms of drug extrusion. While the MFS CaMdr1p is a putative antiporter that exchanges H+ with antifungal compounds, the ABC transporters such as Cdr1p and Cdr2p accomplish drug efflux by coupling it directly to the hydrolysis of ATP. Our studies confirmed that an overexpression of CaMDR1-GFP in a heterologous system confers resistance to a variety of tested drugs because of its ability to expel these substrates. The efflux of drug substrates such as MTX and FLC is sensitive to pH, which could be blocked by a proton dissipater like CCCP, confirming that the electrochemical gradient of protons is utilized to extrude the drugs.

The characteristic and well-conserved antiporter motif in TMS 5 was found to be essential for the functionality of CaMdr1p, which was evident from the fact that not even a single substitution of conserved residue within the motif is functionally compensated. Interestingly, all the amino acid residues of CaMdr1p whose substitution resulted in enhanced drug susceptibility and abrogated efflux are clustered in a helical wheel projection of TMS 5 (Fig. 9). The clustering of mutation-sensitive residues on the same face of the helix further confirms that these residues are important for the structural and functional role of the transporter protein (Fig. 9). Importantly, we observed that residues such as W248 and F262 of CaMdr1p, typical of fungal MFS transporters, were critical for enzymatic activity. Previous studies have shown that the aromatic amino acids, such as Phe, Tyr, and Trp, are involved in cationic–π interaction, where aromatic side chains bind to the cations in the aqueous media to pull it out from the water molecule into a hydrophobic
environment, to possibly help in the generation of proton gradient (3). This observation is important because generally negatively charged residues are involved in proton-coupled translocation, but recent reports of MdfA, transporter of \textit{E. coli}, have shown that different MFS transporters might utilize different proton recognition strategies (23). It is thus probable that aromatic amino acids W248 and F262 of \textit{CaMdr1p} and of other fungal MFS transporters may also lie in the glycine-rich pocket and help in utilizing proton gradient. The significance of W248 and F262 in \textit{CaMdr1p} requires further evaluation.

Interestingly, on one hand, both FLC and MTX could be extruded by the transporter but were unable to compete for each other, thus implying that these two drug substrates share different binding sites. This selectivity between the two substrates was retained by all the mutant variants which were capable of extruding MTX. Our competition results further suggest that at least conserved glycines of the core antiporter motif do not appear to affect drug binding to \textit{CaMdr1p}. Thus, observed impairment in efflux by conserved glycine replacement with alanine and the fact that, similar to wild-type protein, MTX and 4-NQO could compete with \textit{[3H]}MTX efflux mediated by these variants suggest that these conserved motif residues do not directly participate in drug binding. The impairment in drug efflux is probably because of the mutant variant’s inability to participate in drug/H$^+$ transport. This issue can, however, be resolved only by direct drug binding studies by employing radio- and photoaffinity-labeled drug substrates.

Taken together, our study supports the prediction that MFS \textit{CaMdr1p} of \textit{C. albicans} functions as a drug/H$^+$ antiporter. The high degree of conservation in TMS 5, along with mutational data, strongly confirms that the motif \textit{G(X$_6$)G(X$_3$)G(X$_3$)GP(X$_2$)G} is essential for drug/H$^+$ trans-
FIG. 9. A helical wheel projection of the primary amino acid sequence was constructed using 3.6 amino acids per turn of the helix by the EMBOSS PEPWHEEL program (17). Mutations that affected the functionality of the CaMdr1p are numbered and highlighted in black.

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