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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 [$G(X_6)G(X_3)GP(X_2)GP(X_2)G$], 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 *A*TP *b*inding *c*assette (ABC), and CaMdr1p, belonging to *m*ajor *f*acilitator *s*uperfamilies (MFS), (ii) alterations in the azole target protein Erg11p, and (iii) up regulation of the *ERG11* gene (11, 13, 16).

The multidrug transporters of *Candida* belonging to the ABC superfamily have been studied extensively. Typically, the predicted topology of Cdr1p and Cdr2p exhibits characteristic features of an ABC transporter; it contains two highly hydrophobic transmembrane domains and two cytoplasm-localized nucleotide binding domains. Each transmembrane domain is comprised of six transmembrane segments (TMS) which are envisaged to confer substrate specificity (22). The nature of Cdr1p and Cdr2p substrates varies enormously, as it includes structurally unrelated compounds such as azoles, lipids, and steroids (10, 26). The structure and function of MFS multidrug transporter CaMdr1p of *C. albicans* is more poorly understood than 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 [$G(X_8)G(X_3)GP(X_2)GG$], 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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ple, motifs A and B are conserved throughout the MFS, while motif C is conserved in only 12- and 14-TMS subfamilies (14).

The CaMdr1p (NCBI database accession no. X53823) of *C. albicans* (formerly *BEN*^{r}, benomyl resistance) is a 564-amino-acid protein with 12 TMSs (1, 4, 6, 9). Based on sequence homology, CaMdr1p is a putative antiporter, with an antiporter motif in TMS 5 [$G(X_6)G(X_3)GP(X_2)GP(X_2)G$] (14). Keeping in view the relevance of the MFS multidrug transporter CaMdr1p in azole resistance in *Candida*, in this study we have examined this protein in terms of its structure and function. For this, we have overexpressed CaMdr1p as a green fluorescent protein (GFP)-tagged protein in a heterologous system. To evaluate the residues of TMS 5 that potentially contribute to drug/H^+ transport, we did alanine scanning of all 21 amino acids of TMS 5 by site-directed mutagenesis. Our results highlighted the importance of the mutant variants of TMS 5, which became hypersensitive to different classes of drugs, with severely impaired efflux activity. Our results for the first time support the prediction that MFS CaMdr1p of *C. albicans* functions as a drug/H^+ antiporter, wherein amino acid residues within conserved motifs as well as outside are crucial for its functioning.

MATERIALS AND METHODS

Materials. Media chemicals were obtained from Difco (BD Biosciences) and HiMedia (Mumbai, India). The drugs cycloheximide, 4-nitroquinoline, methotrexate, cerulenin, and protease inhibitors (phenylmethylsulfonyl fluoride, leupeptin, pepstatin A, and aprotinin) were obtained from Sigma Chemical Co. (St. Louis, MO). Anti-GFP monoclonal antibody was purchased from BD Biosciences Clontech, Palo Alto, CA. [^{3}H]fluconazole ([^{3}H]FLC) was custom prepared, and [^{3}H]methotrexate ([^{3}H]MTX) was procured from Amersham Biosciences, United Kingdom. Ranbaxy Laboratories (New Delhi, India) kindly provided fluconazole.

Bacterial and yeast strains and growth media. Plasmids were maintained in *Escherichia coli* DH5α. *E. coli* was cultured in Luria-Bertani medium (Difco, BD Biosciences) to which ampicillin was added (100 μg/ml). The *Saccharomyces cerevisiae* strain used was AD1-8u$^-$ (*MAT***a** *pdr1-3 his1 ura3* Δ*yor1*:: *hisG* Δ*snq2*::*hisG* Δ*pdr5*::*hisG* Δ*pdr10*::*hisG* Δ*pdr11*::*hisG* Δ*ycf1*::*hisG* Δ*pdr3*::*hisG* Δ*pdr15*::*hisG*), provided by Richard D. Cannon, University of Otago, Dunedin, New Zealand. The yeast strains used in this study are listed in Table S1 in the supplemental material. AD1-8u$^-$, RPCaMDR1-GFP (AD1-8u$^-$ derivative expressing CaMDR1-GFP), RPCaMDR1 (AD1-8u$^-$ derivative expressing CaMDR1), and the mutants were grown in yeast extract-peptone-dextrose (YEPD) broth (Bio101, Vista, CA) or in SD Ura$^-$ dropout media (0.67% yeast nitrogen base, 0.2% dropout mix, and 2% glucose; Difco). For agar plates, 2.5% (wt/vol) Bacto agar (Difco, BD Biosciences) was added to the medium.

Overexpression of CaMDR1-GFP and its integration at the PDR5 locus. For overexpressing and tagging GFP at the C-terminal end of the Ca*MDR1* open reading frame (ORF), the Ca*MDR1* ORF was amplified with primers having the SpeI restriction site, primers CaMdr1-F (5′-ACGCGTCGACGGACTAGTTAGAACTTCACAATGCATTACAG-3′) and GFP-R (5′-ACGCGTCGACGGACTAGTTTATTTGTATAGTTCATCCA-3′) (the SpeI site is underlined), and the PCR product was digested with SpeI and cloned into plasmid pSK-PDR5PPUS (Fig. 1A) (12). Correct orientation of the Ca*MDR1* and GFP ORF was confirmed by restriction enzyme and sequencing. The cloned and sequenced plasmid was named pRP-CaMDR1-GFP, linearized with XbaI, and used to transform *S. cerevisiae* strain AD1-8u$^-$ by the lithium acetate transformation protocol (22) to get strain RPCaMDR1-GFP. The integration of plasmid pRPCaMDR1-GFP was confirmed by Southern analysis (data not shown). Proper expression and targeting of the protein was confirmed with Northern and Western analysis as well (Fig. 1B and C, respectively).

Site directed mutagenesis of pCa*MDR1-GFP* and generation of transformants. 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 pRPCaMDR1-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 purpose 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 Ca*MDR1* 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 MIC_{80}s 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 [^{3}H]MTX (specific activity, 8.60 Ci/mmol) and [^{3}H]FLC (specific activity, 19 Ci/mmol) was determined essentially by the protocol described previously (11, 16). For accumulation assays, 25 μM [^{3}H]MTX and 100 nM [^{3}H]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, [^{3}H]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 (9).

For efflux measurements, a 5% yeast cell suspension (2.5×10^8 cells ml^{-1}) was incubated with [^{3}H]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-PDR5PPUS (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).

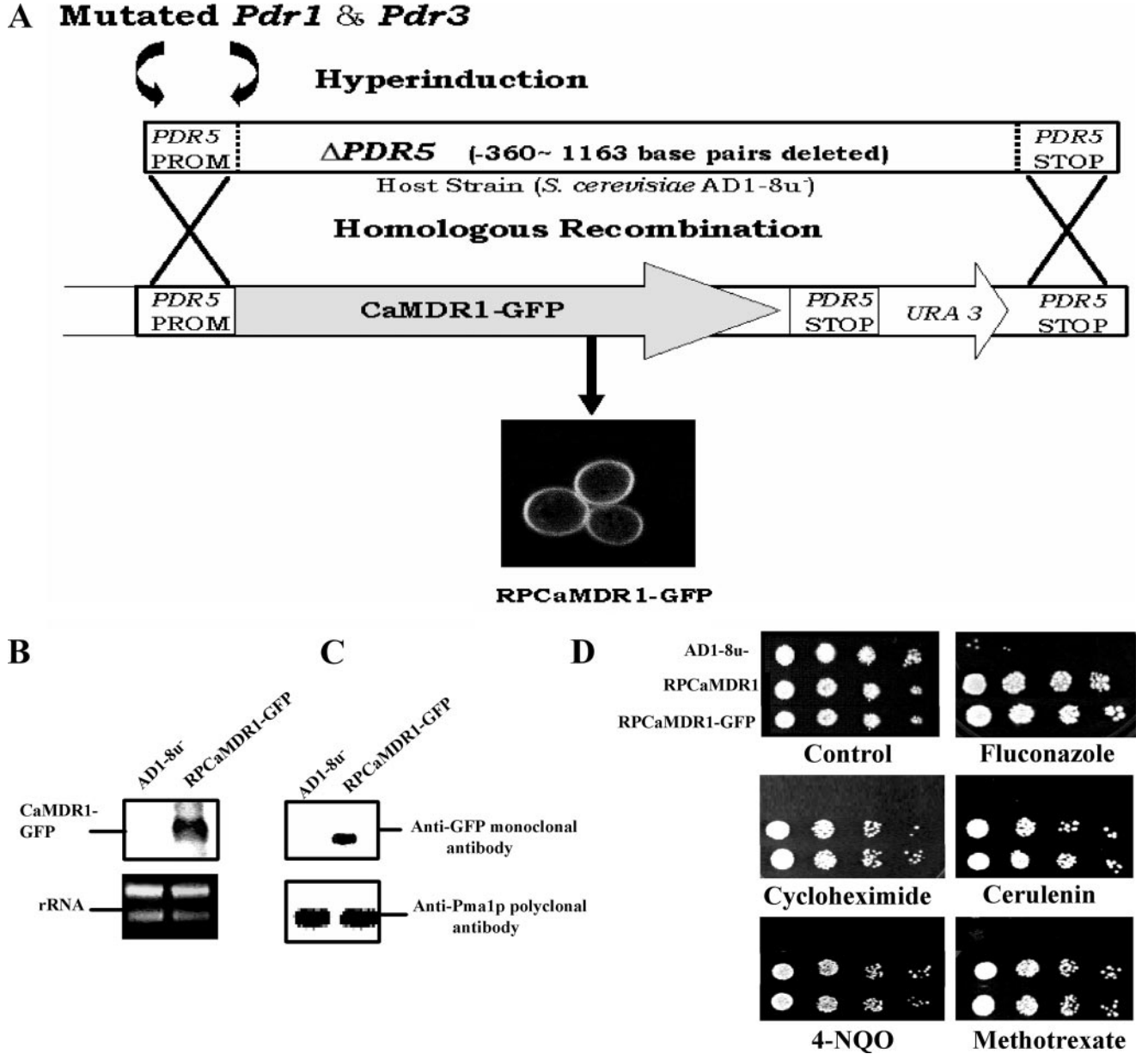

FIG. 1. (A) Strategy showing the cloning and integration of Ca*MDR1* as Ca*MDR1-GFP* at the *PDR5* locus in the overexpression strain of *S. cerevisiae* AD1-8u$^-$, derived from a *pdr1-3* mutant strain with a gain-of-function mutation in the transcription factor Pdr1p, resulting in constitutive hyperinduction of the *PDR5* promoter. (B) Expression of the *C. albicans* Ca*MDR1* mRNA in *S. cerevisiae* strain AD1-8u$^-$ and RPCaMDR1-GFP. Total RNA (30 μg) from the parental strain, AD1-8u$^-$, and RPCaMDR1-GFP was hybridized with a mixture of [α-^{32}P]dATP-labeled *C. albicans* Ca*MDR1* probe. The lower part of the panel shows a loading control to indicate equal loading. (C) Immunodetection of CaMdr1p in the PM of strains AD1-8u$^-$(control) and RPCaMDR1-GFP. The Western blot analyses were done with anti-GFP monoclonal antibody. The purity of the PM fraction was assessed by using anti-Pma1p polyclonal antibody. Confocal pictures of the *S. cerevisiae* cells expressing GFP-tagged wild-type Ca*MDR1*. The cells were viewed directly on a glass slide with a 100× oil immersion objective on a Bio-Rad confocal microscope. (D) Spot test showing that Ca*MDR1* (untagged, without GFP) and Ca*MDR1-GFP* mediated resistance for various drugs. Cells were spotted on YEPD plates in the absence of drug (control) and in presence of the following drugs: FLC (0.17 μg/ml), CYH (0.2 μg/ml), CER (3 μg/ml), 4-NQO (0.2 μg/ml), and MTX (65 μg/ml).

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$^-$) 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$^-$), MIC_{80}s (MIC for 80% inhibition in growth) for the RPCaMDR1-GFP strain

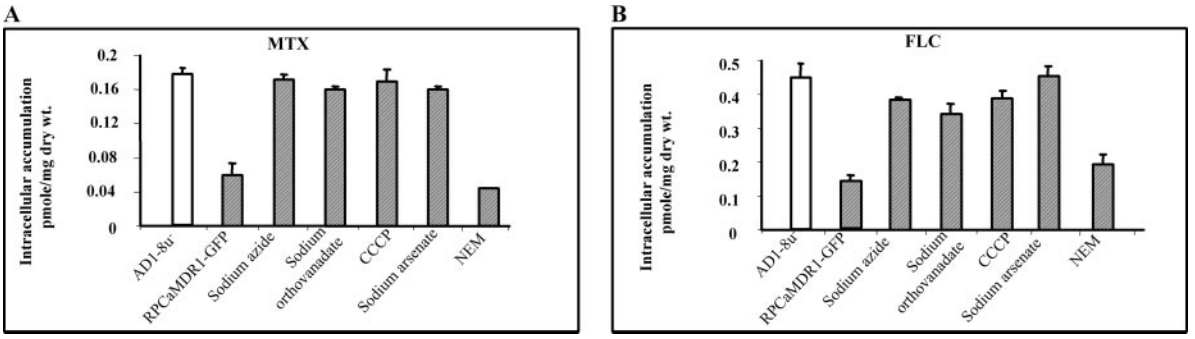

FIG. 2. Functional characterization of the RPCaMdr1p-GFP and effect of different inhibitors on accumulation of radiolabeled drugs. [^{3}H]MTX (A) and [^{3}H]FLC (B) accumulation in RPCaMDR1-GFP cells and host strain AD1-8u$^-$ (control). Effect of different inhibitors on accumulation of [^{3}H]MTX (A) and [^{3}H]FLC (B) in the RPCaMdr1p-GFP strain was monitored as described in Materials and Methods. Different inhibitors were added to various cells 10 min prior to the commencement of transport. Different inhibitors used included sodium azide (10 mM), sodium orthovanadate (1 mM), CCCP (100 μM), sodium arsenate (1 mM), and NEM (1 mM). AD1-8u$^-$ is shown as the control. The values plotted are from 10 min after commencement of transport. The results are the means ± standard deviations from three independent experiments.

were considerably higher (MIC_{80} of 16 μg/ml for FLC, 0.5 μg/ml for cycloheximide [CYH], 8 μg/ml for cerulenin [CER], 1 μg/ml for 4-nitroquinoline [4-NQO], and 128 μg/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, [^{3}H]MTX and [^{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$^-$) cells, the accumulation of [^{3}H]MTX and [^{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 [^{3}H]MTX and [^{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 [^{3}H]MTX and [^{3}H]FLC was increased in the presence of these inhibitors, implying reduction in efflux. The sensitivity of [^{3}H]MTX and [^{3}H]FLC accumulation, particularly to proton conductor CCCP, suggests that CaMdr1p function is sensitive to pH and may function as a H^+ 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^+ transporter, we monitored the efflux of [^{3}H]MTX and [^{3}H]FLC by exposing cells to buffers with different pH values (Fig. 3A and B, respec-

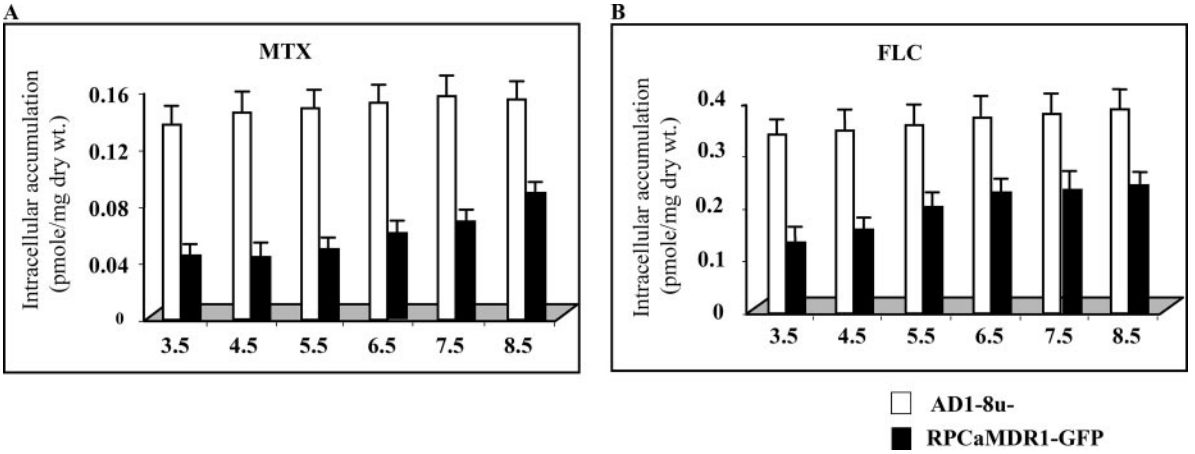

FIG. 3. Effect of pH on [^{3}H]MTX and [^{3}H]FLC efflux. The efflux of [^{3}H]MTX (A) and [^{3}H]FLC (B) was monitored by exposing cells to different pH buffers. In a typical experiment, RPCaMdr1p-GFP cells were treated with CCCP (100 μM) for 10 min and were then allowed to accumulate [^{3}H]MTX for 30 min. These cells were then rapidly pelleted and resuspended in buffers of different pH values. The accumulation of [^{3}H]MTX and [^{3}H]FLC was measured at rapid succession, as detailed in Materials and Methods. The values plotted are from 10 min after commencement of efflux. The results are the means ± standard deviations from three independent experiments.

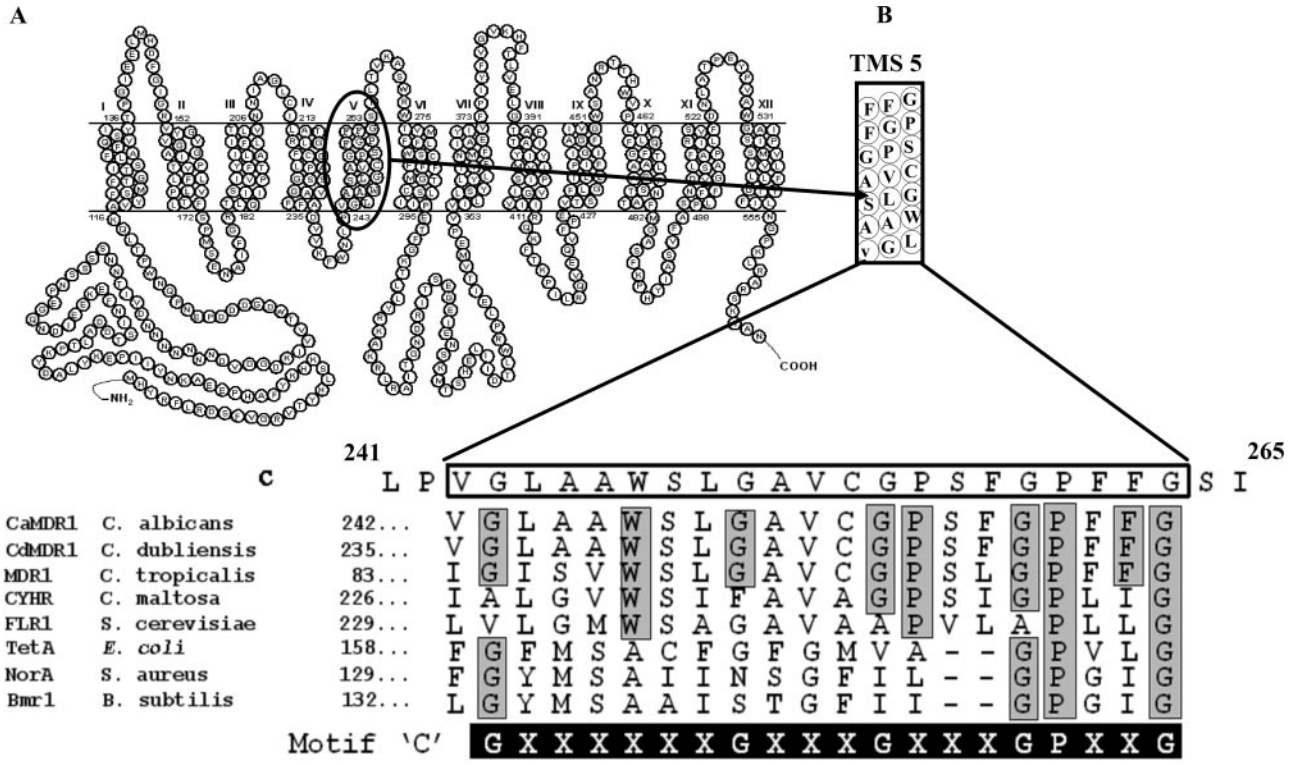

FIG. 4. (A) Predicted topology of the Ca*MDR1* with 12 transmembrane segments. The TMS 5 is circled to show the location of the antiporter motif in the protein. (B) The putative TMS 5 is magnified to show the amino acid residues of TMS 5. (C) Alignment of the protein sequences of the *C. albicans* antiporter Ca*MDR1* TMS 5 with the other fungal and bacterial drug antiporters, showing the presence of the unique and conserved antiporter motif. The amino acid sequences of TMS 5 of CaMdr1p between positions 243 and 263 (boxed) are shown. The sequence of the antiporter motif or motif C is written for comparison, where X can be any amino acid. Residues conserved in all the MFS transporters that are part of the motif are highlighted in gray, whereas residues conserved only in fungal MFS that were found critical for the activity are highlighted in black.

tively). In a typical experiment, cells expressing wild-type CaMdr1p-GFP were treated with proton conductor CCCP (100 μM) and were allowed to accumulate [^{3}H]MTX and [^{3}H]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$^-$, which did not show any significant efflux of the drugs (Fig. 3A and B). The efflux of [^{3}H]FLC mediated by CaMdr1p was also pH sensitive, which was similar to [^{3}H]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$^-$, 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

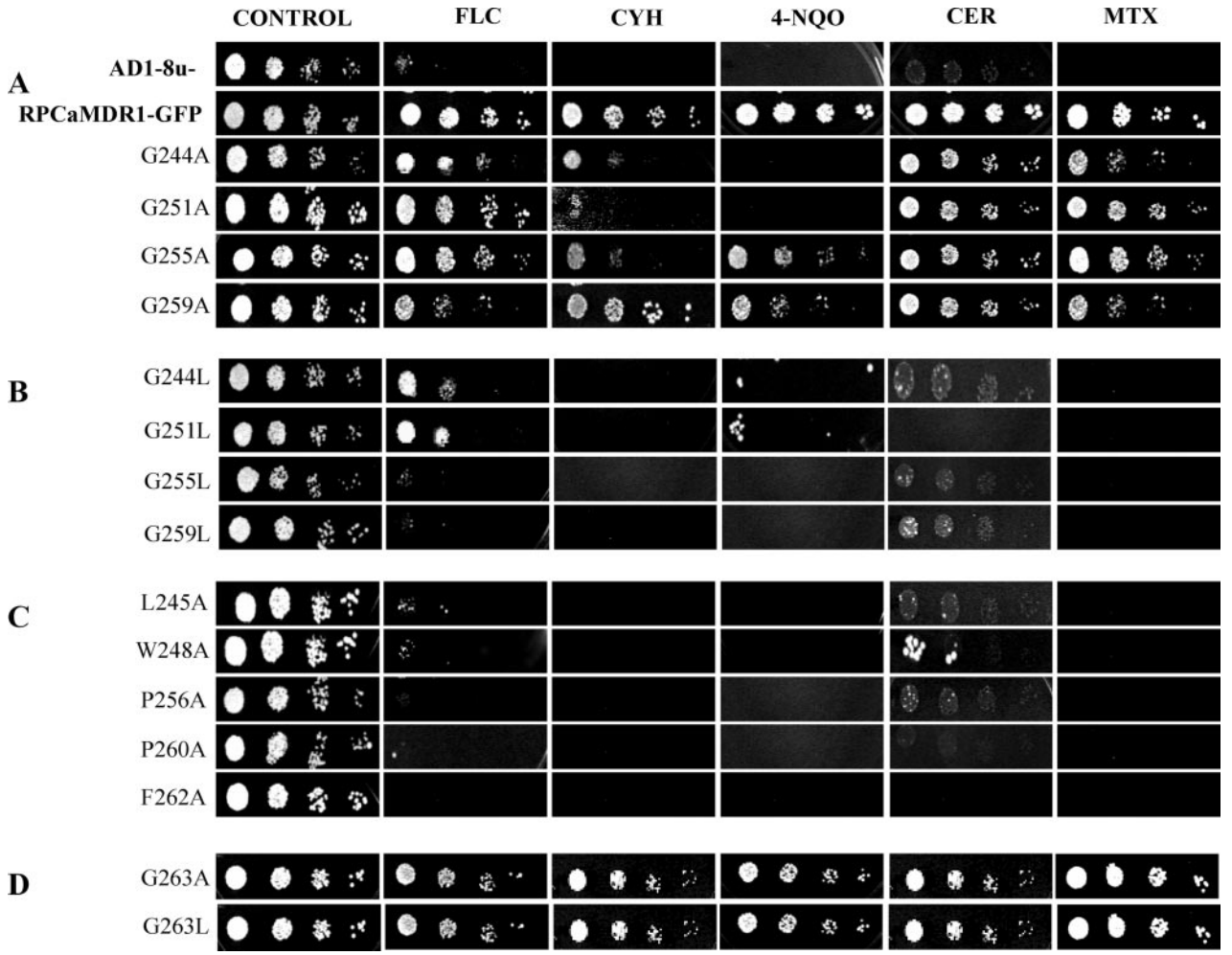

FIG. 5. Drug resistance profile of wild-type and mutant Ca*MDR1* strains determined by the spot assay. Cells were freshly streaked, grown overnight, and then resuspended in normal saline to an A_{600} of 0.1. For spot assay, 5 μl of fivefold serial dilutions, namely 1 (1:5), 2 (1:25), 3 (1:125), and 4 (1:625), of each strain was spotted on to YEPD plates in the absence (control) and presence of the following drugs: FLC (0.17 μg/ml), CYH (0.2 μg/ml), CER (3 μg/ml), 4-NQO (0.2 μg/ml), and MTX (65 μg/ml). Growth differences were recorded following incubation of the plates for 48 h at 30°C. Growth was not affected by the presence of the solvents used for the drugs (data not shown). Panels A and B show the mutant variants of first category of mutant variants substituted with alanine and leucine, respectively. (C) Second category of mutant variants substituted with alanine only. (D) Third category of mutant variants substituted with both alanine and leucine.

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 replacement by alanine (G244A, G251A, G255A, and G259A) led to variable enhanced sensitivity to tested drugs (Fig. 5 A 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 sensitivity toward all the tested drugs (Fig. 5). Interestingly, all four glycines of the first category, when substituted with leucine (G244L, G251L, G255L, and G259L), showed more drastic 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 conserved 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 category 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,

TABLE 1. Summarized representations of the mutant variants[a]

Category	Residue substitution	Drug susceptibility					Drug accumulation (relative %)		Position
		FLC	CYH	4-NQO	CER	MTX	FLC	MTX	
AD1-8u$^-$		−	−	−	−	−	100	100	
RPCaMdr1p-GFP		++++	++++	++++	++++	++++	31	34	
One	G244A	++	+	−	+++	++	55	54	Inside
	G251A	++	+	−	+++	+++	54	54	Inside
	G255A	++	+	+	+++	+++	60	54	Inside
	G259A	+++	++	++	+++	+++	56	54	Inside
	G244L	+	−	−	−	−	72	80	Inside
	G251L	+	−	−	−	−	77	77	Inside
	G255L	−	−	−	−	−	87	86	Inside
	G259L	−	−	−	−	−	92	69	Inside
Two	L245A	−	−	−	−	−	99	94	Outside
	W248A	−	−	−	+	−	85	91	Outside
	P256A	−	−	−	−	−	83	89	Outside
	P260A	−	−	−	−	−	98	94	Inside
	F262A	−	−	−	−	−	85	80	Outside
Three	G263A	++++	++++	++++	++++	++++	35	34	Inside
	G263L	++++	++++	++++	++++	++++	39	37	Inside
Four	V243A	++++	++++	++++	++++	++++	No change	No change	Outside
	A246G	++++	++++	++++	++++	++++			Outside
	A247G	++++	++++	++++	++++	++++			Outside
	S249A	++++	++++	++++	++++	++++			Outside
	L250A	++++	++++	++++	++++	++++			Outside
	A252G	++++	++++	++++	++++	++++			Outside
	V253A	++++	++++	++++	++++	++++			Outside
	C254A	++++	++++	++++	++++	++++			Outside
	S257A	++++	++++	++++	++++	++++			Outside
	F258A	++++	++++	++++	++++	++++			Outside
	F261A	++++	++++	++++	++++	++++			Outside

[a] The drug susceptibility column shows the degree of growth on all of the tested drugs, as follows: ++++, hyperresistant cells; +++, moderately resistant; ++, slightly resistant; +, slight growth; −, hypersensitive variants, with no growth at all. The drug accumulation column shows the relative percent accumulation of the two drugs, [^{3}H]FLC and [^{3}H]MTX. The results are shown as relative percentages with respect to drug accumulation of host strain AD1-8u$^-$ taken as 100%. The position column indicates the position of the variant inside or outside the motif C or antiporter motif.

W248, G251, G255, P256 G259, P260, and F262, affected drug susceptibilities to various degrees (Fig. 5A, B, and C and Table 1). The drug sensitivities 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 mutant variants 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 [^{3}H]MTX and [^{3}H]FLC. The first category of mutant variants, when replaced by alanine, G244A, G251A, G255A, and G259A, showed increased accumulation of [^{3}H]MTX and [^{3}H]FLC (impaired efflux) (Fig. 7A and B and Table 1). Similar to drug sensitivity results, the replacement of glycines of the first category with leucine (G244L, G251L, G255L, and G259L) resulted in a further increase in accumulation of [^{3}H]MTX and [^{3}H]FLC (Fig. 7A and B and Table 1). The mutant variants of the second category, including L245A, W248A, P256A, P260A, and F262A, also showed increases in their ability to accumulate [^{3}H]MTX and [^{3}H]FLC, thus implying decreased efflux of drugs (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 [^{3}H]MTX and [^{3}H]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, [^{3}H]MTX accumulation was competed with fivefold molar excesses of different drugs. The accumulation of [^{3}H]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). It

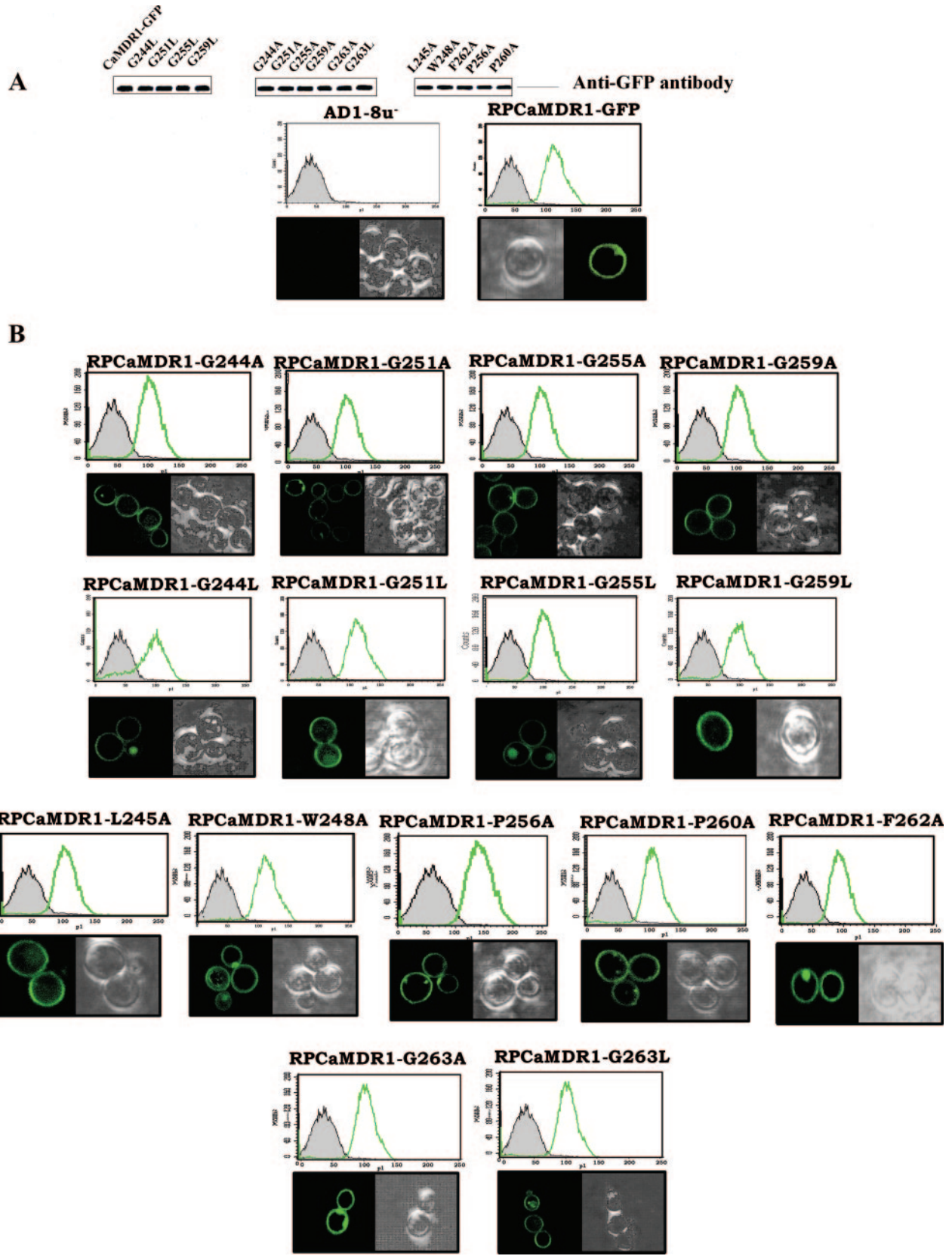

FIG. 6. Comparison of expression of RPCaMDR1p-GFP and different mutant variant localization. (A) Western blot analyses of the PM fractions of mutant variants, with anti-GFP antibody, to confirm their proper targeting. (B) Confocal images and FACS analyses of the mutants of first, second, and third categories to check their expression, proper folding, and localization and comparison with AD1-8u$^-$ (negative control) and RPCaMDR1-GFP (positive control).

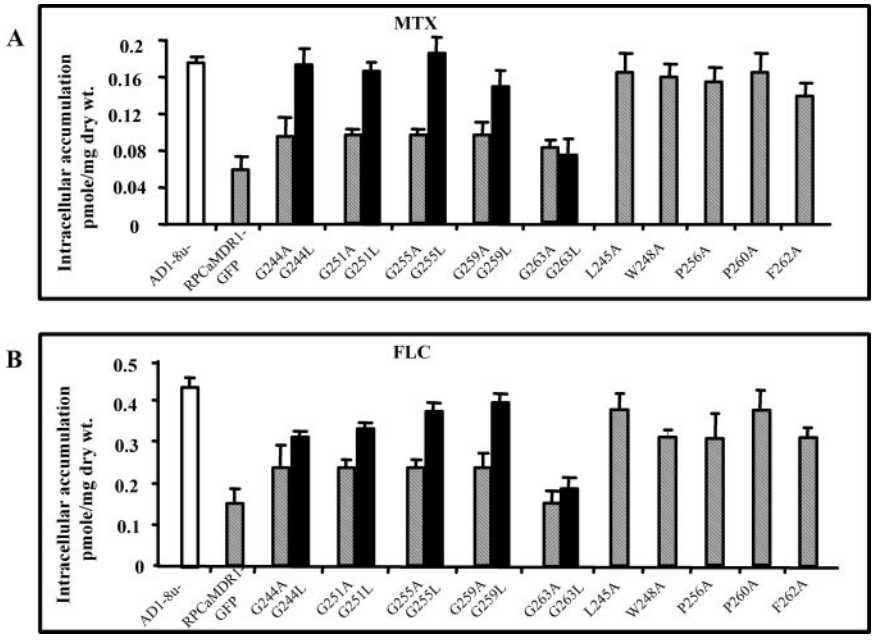

FIG. 7. [^{3}H]MTX and [^{3}H]FLC accumulation in the different mutant variants. [^{3}H]MTX (A) and [^{3}H]FLC (B) accumulation in the first, second, and third categories of mutant variants. The first and third categories of five glycines of antiporter motif substituted with leucine are also shown as black bars for better comparison of glycines substituted with alanine. Controls AD1-8u$^-$ and RPCaMDR1-GFP are also included for comparison. The results are the means ± standard deviations from three independent experiments.

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 [^{3}H]MTX (Fig. 8A). Similar to native CaMdr1p-GFP, the accumulation of [^{3}H]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 [^{3}H]MTX (Fig. 8C). Of note, as a result of substitution of conserved glycines with leucine, the accumulation of [^{3}H]MTX was severely reduced in these variants, and hence, a clear distinction between the inability of MTX and 4-NQO to compete with [^{3}H]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 [^{3}H]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 Ca*MDR1-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

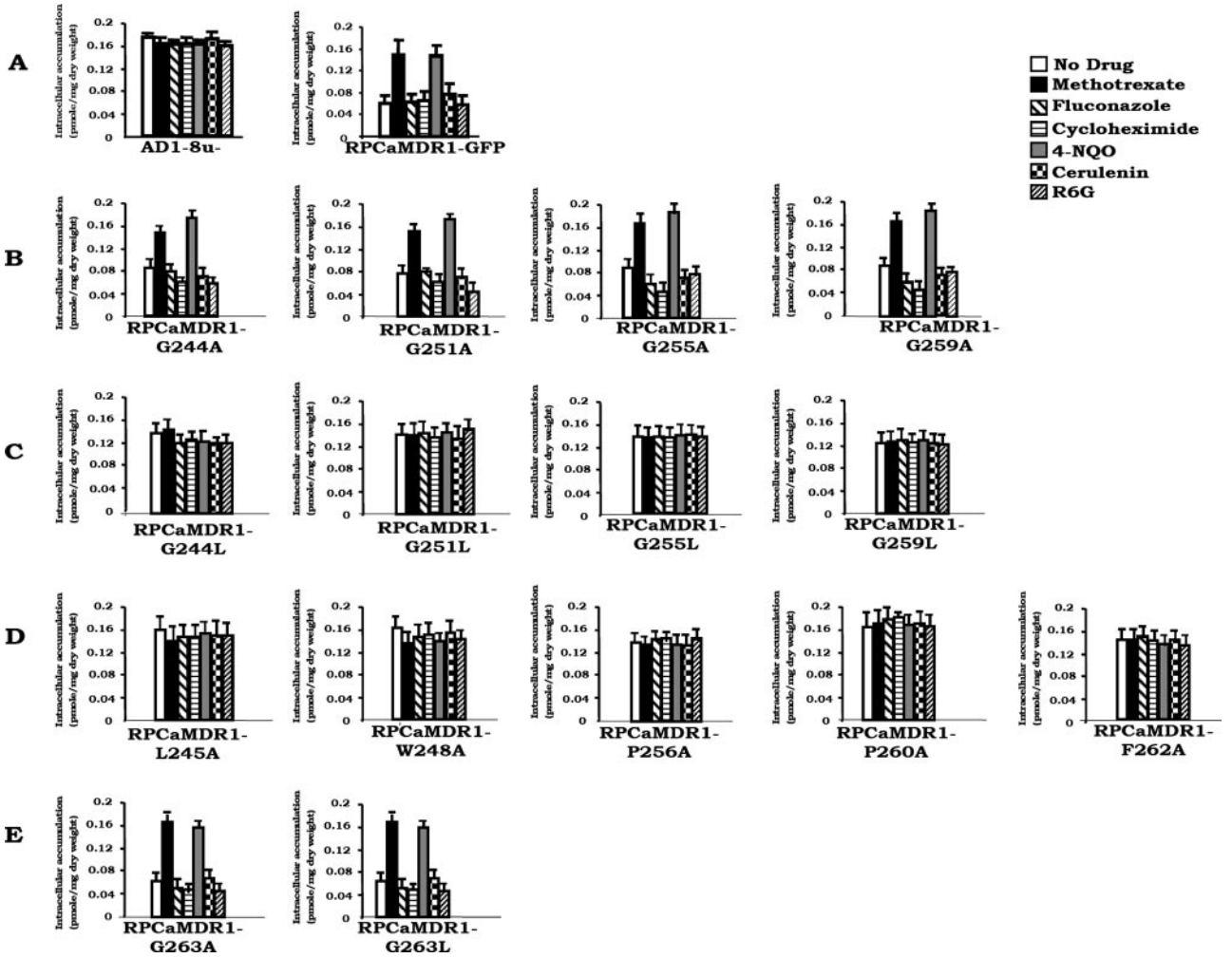

FIG. 8. Competition of various drugs with [^{3}H]MTX for common substrate binding sites. For competition between [^{3}H]MTX and various drugs, [^{3}H]MTX was used at a final concentration of 25 μM, and a fivefold concentration of each drug (125 μM), MTX, FLC, CYH, 4-NQO, and rhodamine 6G (R6G), was used for competition studies. (A) Comparison of the control strains AD1-8u$^-$ (negative) and RPCaMdr1p-GFP (positive). MTX and 4-NQO compete for the [^{3}H]MTX in RPCaMdr1p-GFP. Panels B and C show the first category of the mutant variants, either substituted with alanine or leucine, respectively. (D) Second category of the mutant variants, showing drastic increase in accumulation, implying reduced efflux. (E) Third category of mutant which remained unaffected whether substituted with alanine or leucine. The results are the means from three independent experiments and are represented in pmol/mg (dry weight). The values are derived from the accumulation.

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 *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 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 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 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 [^{3}H]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 CaMdr1p of *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 $G(X_6)G(X_3)G(X_3)GP(X_2)G$ is essential for drug/H^+ trans-

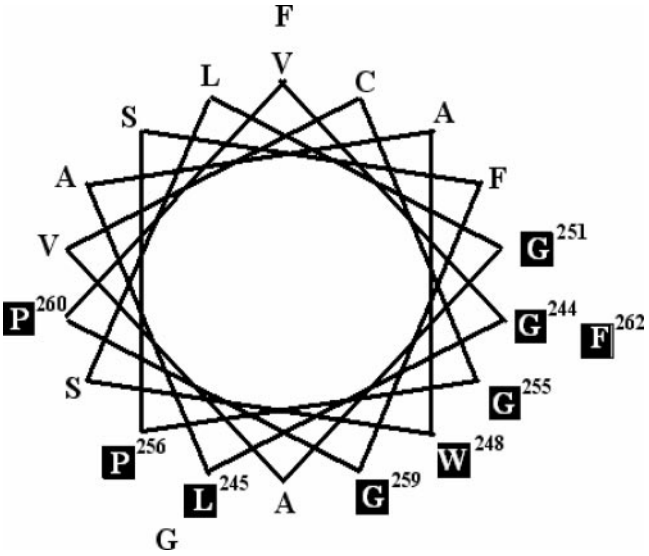

FIG. 9. A helical wheel projection of the primary amino acid sequence was constructed using 3.6 amino acids per turn of the helix by the EMBOSS PEPWHEEL program (17). Mutations that affected the functionality of the CaMdr1p are numbered and highlighted in black.

port. The placements of critical residues which are typical to this fungal transporter do suggest structural and functional differences among MFS drug transporters.

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