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The amino acid residues of transmembrane helix 5 of multidrug resistance protein CaCdr1p of *Candida albicans* are involved in substrate specificity and drug transport☆

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ABSTRACT

In view of the importance of *Candida* Drug Resistance Protein (Cdr1p) of pathogenic *Candida albicans* in azole resistance, we have characterized its ability to efflux variety of substrates by subjecting its entire transmembrane segment (TMS) 5 to site directed mutagenesis. All the mutant variants of putative 21 amino acids of TMS 5 and native CaCdr1p were over expressed as a GFP-tagged protein in a heterologous host *Saccharomyces cerevisiae*. Based on the drug susceptibility pattern, the mutant variants could be grouped into two categories. The variants belonging to first category were susceptible to all the tested drugs, as compared to those belonging to second category which exhibited resistance to selective drugs. The mutant variants of both the categories were analyzed for their ATP catalysis and drug efflux properties. Irrespective of the categories, most of the mutant variants of TMS 5 showed an uncoupling between ATP hydrolysis and drug efflux. The mutant variants such as M667A, F673A, I675A and P678A were an exception since they reflected a sharp reduction in both K_m and V_{max} values of ATPase activity when compared with WT CaCdr1p-GFP. Based on the competition experiments, we could identify TMS 5 residues which are specific to interact with select drugs. TMS 5 residues of CaCdr1p thus not only impart substrate specificity but also selectively act as a communication link between ATP hydrolysis and drug transport.

1. Introduction

An over expression of the drug efflux pump encoding *CDR1* and *CDR2* genes, belonging to the ATP Binding Cassette (ABC) [1–5] and *CaMDR*1, belonging to Major Facilitator (MFS) super families of transporters [6–8], represent one of the most significant mechanisms of azole resistance in pathogenic *Candida albicans*. Among the ABC transporters, Cdr1p is a major drug transporter of *C. albicans* whose elevated expression coincides with an increased efflux of drug substrates in azole resistant clinical isolates [4,9].

CaCdr1p is a 170 kDa multidrug resistance protein that belongs to PDR subfamily of ABC transporter super family [10]. CaCdr1p has 1501 amino acids which are organized as two homologous halves. Each half begins with a cytoplasmic hydrophilic nucleotide binding domain (NBD) that harnesses energy through ATP hydrolysis. Each NBD is followed by a highly hydrophobic transmembrane spanning domain (TMD) comprising of six transmembrane segments (TMS), which confer substrate specificity to CaCdr1p [2]. An enormous range of structurally unrelated substrates compounds such as drugs, lipids and steroids can be exported by CaCdr1p [11–13]. Experiments with purified CaCdr1p have conclusively shown that ATP binding to Cdr1p is not a prerequisite for drug binding and both the mechanisms of drug and ATP binding result in specific conformational changes which take place independent of each other [14]. A direct link between the ability of CaCdr1p to translocate fluorescent glycerophospholipids and efflux drugs has also been demonstrated by Shukla et al. [15].

Several structure and function studies on human ABCB1/P-glycoprotein (P-gp) and its homologues have identified the domains and residues involved in recognition, binding and efflux of drugs. Recent cross linking studies have shown that the cytoplasmic ends of TMS 5 and 8 of human P-gp are in close proximity on one side of the drug binding pocket while those of TMS 2 and TMS 11 are close on the other side of the drug binding pocket, thus forming gates for the entry of drug substrates [16]. The multidrug transporter Pdr5p from *Saccharomyces cerevisiae* is proposed to have at least three different substrate binding sites, each of which appears to use different chemical properties to transport compounds [17]. In addition, several amino acid residues that alter Pdr5p substrate specificity and sensitivity to inhibitors have been shown to be distributed throughout the length of protein [18–20].

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Considering chemically diverse substrates which are expelled by CaCdr1p, the exact number of residues involved in drug binding and transport are far from understood. To understand the mechanism of drug transport mediated by CaCdr1p, we have generated its battery of mutant variants that drastically affect various stages involved in drug extrusion [13]. Shukla et al., 2003 have shown that the deletion of F774 in TMS 6 resulted in mislocalization of the CaCdr1p. In the same study, by employing photo affinity substrate analogs such as [^{125}I] iodoarylazidoprazosin (IAAP) and [^{3}H] azidopine two drug binding sites on CaCdr1p were revealed. Alanine scanning of TMS 11 of CaCdr1p showed that at least seven residues which were critical for determining substrate specificity and drug transport were clustered on the hydrophilic face of the α-helical projection of TMS11 [13]. Together, these studies suggest that the drug binding sites in Cdr1p are scattered throughout the protein and probably more than one residue of different helices are involved in binding and extrusion of drugs. However, there is still insufficient information available to predict where exactly the most common anti fungals, such as azoles bind and how they are extruded.

Based on human P-gp where interactivity of different TMS is well demonstrated [16] and our own study with CaCdr1p-GFP where we have shown that selected residues of TMS 11 involved in drug binding appear to be clustered in a pepwheel projection [13], in this study, we have subjected TMS 5 of Cdr1p to site directed mutagenesis where all the predicted 21 residues were replaced with alanine (existing alanines were replaced by glycine). We show that most of the residues of TMS 5 when replaced displayed decreased resistance to tested drugs. Notwithstanding the variations in ATPase activities, the drug efflux of all the mutant variants was abrogated. Our study for the first time shows that substitution of some of the TMS 5 residues of CaCdr1p results in uncoupling of drug transport from ATP hydrolysis.

2. Experimental procedures

2.1. Materials

Anti-GFP monoclonal antibody was purchased from BD Biosciences Clontech, Palo Alto, Calif. DNA-modifying enzymes were purchased from NEB, USA. Protease inhibitors (Phenylmethylsulfonylfluoride, leupeptin, aprotinin, pepstatin A) and the drugs miconazole (MIC), cycloheximide (CYC), ketoconazole (KTC), anisomycin (ANI), itraconazole (ITR), R6G, β-estradiol (β-EST), progesterone (PRG), oligomycin and other molecular grade chemicals were obtained from Sigma Chemicals Co (St. Louis, Mo.). FLC was generously provided by Ranbaxy Laboratories, India and tritylimidazole (tritylimz) and tricyclohexyltinchloride (hexyltin-Cl) were kind gifts from John Golin. The radio labeled [^{125}I] iodoarylazidoprazosin (IAAP) (2200 Ci/mmol) was purchased from Perkin-Elmer Life Sciences (Boston Mass.) while [^{3}H] Fluconazole ([^{3}H] FLC) was custom prepared by Amersham, UK.

2.2. Media and strains

Plasmids were maintained in *Escherichia coli* DH5α *E. coli* was cultured in Luria-Bertani medium (Difco, BD Biosciences, NJ, USA) to which ampicillin was added (100 μg/ml). The *S. cerevisiae* strain used was AD1-8u^{-} (*MATa pdr1-3 his1 ura3 Δyor1::hisG Δsnq2::hisG Δpdr5::hisG Δpdr10::hisG Δpdr11::hisG Δycf1::hisG Δpdr3::hisG Δpdr15::hisG*), provided by Richard D. Cannon, University of Otago, Dunedin, New Zealand. The yeast strains used in this study are listed in the supplementary data, Table S1. The yeast strains were cultured in Yeast Extract Peptone Dextrose (YEPD) broth (BIO101, Vista, Calif.) or in SD ura^{-} dropout media (0.67% yeast nitrogen base, 0.2% dropout mix, and 2% glucose; Difco). For agar plates, 2.5% (w/v) bacto agar (Difco BD Biosciences, NJ) was added to the medium.

2.3. Methods

2.3.1. Site specific mutagenesis of Cdr1p

Site specific mutagenesis was performed by using the QuickChange Mutagenesis kit from Stratagene (La Jolla, Calif.) as described previously [21]. The mutations were introduced into the plasmid pPSCDR1-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 supplementary data, Table S2. The mutated plasmid, after linearising with *Xba1*, was used to transform AD1-8u^{-} cells for uracil prototrophy by lithium acetate transformation protocol [21]. Integration was confirmed by Southern blot analysis.

2.3.2. Preparation of the plasma membranes and immunodetection of Cdr1p and its mutants

The plasma membranes (PM) were prepared from *S. cerevisiae* cells, as described previously [21]. The PM protein concentration was determined by bicinchonic acid assay using bovine serum albumin as the standard. The Western blot analysis was conducted using anti-GFP monoclonal antibody (1:5000), as described previously [21]. Proteins on immunoblots were visualized using the enhanced chemiluminescence assay system (ECL kit, Amersham Biosciences, Arlington Heights, IL).

2.3.3. 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 washed and resuspended in an appropriate volume of 50 mM HEPES (pH 7.0). The cells were imaged under an oil immersion objective at 100× magnification on a confocal microscope (Radiance 2100, AGR, 3Q/BLD; Bio-RAD, UK) [8].

2.3.4. Flow cytometry

Flow cytometric (FACS) analysis of CaCdr1p-GFP and its mutant variants expressed in *S. cerevisiae* cells was performed with FACsort flow cytometer (Becton-Dickson immunocytometry systems, San Jose, Calif.). Cells were grown to mid log phase and 10^{6} cells were harvested and washed with 50 mM HEPES (pH 7.0). Cells were resuspended in appropriate volume of 50 mM HEPES (pH 7.0)/1× PBS. Ten thousand cells were analyzed in acquisition. Analysis was performed with cell quest software (Becton-Dickson immunocytometry systems) [8,21].

2.3.5. Drug susceptibility

The susceptibilities of *S. cerevisiae* cells to different drugs were determined using spot assays. For spot assay, 3-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 [22].

2.3.6. ATPase assay

The CaCdr1p-GFP associated ATPase activity of the purified PM was measured as an oligomycin-sensitive release of inorganic phosphate as described previously [21]. Briefly, plasma membrane suspension (10 μg of PM protein) was incubated at 30 °C in 0.1 ml of medium containing 60 mM Tris pH 7.5 and 8 mM $MgCl_2$ (ATPase assay buffer) and 20 μM oligomycin where indicated. To eliminate possible contributions from non-specific vacuolar and mitochondrial ATPases, 50 mM KNO_3, and 10 mM NaN_3, respectively were included in the reaction mixture. The reaction was started by addition of 5 mM ATP and was stopped by the addition of 0.1 ml of 5% SDS solution. The amount of inorganic phosphate released was determined immediately as described previously [23].