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Mutations adjacent to the end of transmembrane helices 6 and 7 independently affect drug efflux capacity of yeast ABC transporter Pdr5p

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ABSTRACT

As a mammalian p-glycoprotein homolog, Pdr5p is a major ATP-binding cassette transporter for cellular detoxification in the yeast *Saccharomyces cerevisiae*. In this study, two novel loss-of-function mutations located adjacent to the ends of the predicted transmembrane helices of Pdr5p were identified. C793F and S1230L mutations considerably impaired the transport activity of Pdr5p without affecting the ATPase activity and the expression level of the protein. Our results demonstrate that the size of residue 793 and the hydrophobicity of residue 1230 are important for Pdr5p efflux function. It reveals that amino acid residues located near the end of transmembrane helix play an important role in drug efflux of Pdr5p. Molecular docking results further suggest that these two single mutations might have disturbed interactions between the drugs and Pdr5p, preventing the drugs from approaching the intracellular or extracellular portal and subsequently from being exported by Pdr5p.

1. Introduction

Efflux of xenobiotics by ATP-binding cassette (ABC) transporters is a major mechanism employed by various cells to survive in toxic environment [1]. As the best-studied and most abundant ABC transporter in *Saccharomyces cerevisiae*, Pdr5p mediates cellular detoxification and multidrug resistance (MDR) [2,3]. The predicted topological structure of Pdr5p shows that it contains two nucleotide binding domains (NBDs) and two transmembrane domains (TMDs) which harbor twelve transmembrane helices (TMHs), six extracellular loops (ECLs) and four intracellular loops (ICLs) [4,5].

Pdr5p shares similar secondary topological structure with other homologous ABC transporters and is presumed to extrude substrates by continuous switching between inward-facing and outward-facing conformations, which are believed to have different affinities towards the transported substrates [2]. Although TMDs of ABC transporter are not conserved in length or sequence, it is well accepted that TMDs are essential for substrate recognition, binding and translocation, and the amino acid residues within TMHs might play the important roles [6–9].

To clarify the mechanisms of how Pdr5p recognizes and extrudes a wide variety of compounds out of the cells, numerous mutants had been generated by random or site-directed mutagenesis [10–16]. Despite the effort, the structure–function mechanism of Pdr5p still remains unclear [17–19]. A number of single-point mutations within the TMDs had reportedly altered drug specificity or impaired drug-efflux efficiency of Pdr5p [10–12,14,20]. Some of them were thought to have altered how substrates gain access to or release from the substrate-binding pocket through the portal while the others were shown to be defective in the cross-talk between TMDs and NBDs during the catalytic cycle of Pdr5p.

In this study, we identified two novel loss-of-function mutations C793F and S1230L within the TMDs of Pdr5p. To investigate the potential loss-of-function mechanism, C793 and S1230 were individually substituted with various amino acids and drug susceptibility of each mutant was evaluated. Furthermore, we used a recent published 3D model of Pdr5p to perform an independent docking study, shedding more light into the structure–function relationship of Pdr5p.

2. Materials and methods

2.1. Strains, plasmids and media

The strains and plasmids used in this study are listed in Table 1. *S. cerevisiae* BY4741 cells were grown in synthetic complete medium

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Table 1
Strains and plasmids used in this study.

Strains or plasmids	Relevant characteristics	References
S. cerevisiae BY4741	MATa his3 leu2 metl5 ura3	Yeast Knock-out (YKO) deletion collection
E. coli DH5a	*supE44, AlacU169 (q80lacZAM15), hsdR17, recA1, endA1, gyrA96,thi-1, relA1*	
BY4741Apdr5::YEplac195 (DEL)	*MATa his3 leu2 met 15 ura3 pdr5::loxP*	[18]
BY4741Apdr5::YEplac195-BPDR5 (WT)	*MATa his3 leu2 met 15 ura3 pdr5::loxP PDR5*	[18]
BY4741Apdr5::YEplac195-BPDR5-C793M	*MATa his3 leu2 met 15 ura3 pdr5::loxP PDR5-C793M*	This study
BY4741Apdr5::YEplac195-BPDR5-C793S	*MATa his3 leu2 met 15 ura3 pdr5::loxP PDR5-C793S*	This study
BY4741Apdr5::YEplac195-BPDR5-C793F	*MATa his3 leu2 met 15 ura3 pdr5::loxP PDR5-C793F*	This study
BY4741Apdr5::YEplac195-BPDR5-C793Y	*MATa his3 leu2 met 15 ura3 pdr5::loxP PDR5-C793Y*	This study
BY4741Apdr5::YEplac195-BPDR5-S1230F	*MATa his3 leu2 met 15 ura3 pdr5::loxP PDR5-S1230F*	This study
BY4741Apdr5::YEplac195-BPDR5-S1230Y	*MATa his3 leu2 met 15 ura3 pdr5::loxP PDR5-S1230Y*	This study
BY4741Apdr5::YEplac195-BPDR5-S1230A	*MATa his3 leu2 met 15 ura3 pdr5::loxP PDR5-S1230A*	This study
BY4741Apdr5::YEplac195-BPDR5-S1230N	*MATa his3 leu2 met 15 ura3 pdr5::loxP PDR5-S1230N*	This study
BY4741Apdr5::YEplac195-BPDR5-S1230L	*MATa his3 leu2 met 15 ura3 pdr5::loxP PDR5-S1230L*	This study
BY4741Apdr5::YEplac195-BPDR5-S1230P	*MATa his3 leu2 met 15 ura3 pdr5::loxP PDR5-S1230P*	This study
BY4741Apdr5::YEplac195-BPDR5-E794K	*MATa his3 leu2 met 15 ura3 pdr5::loxP PDR5-E794K*	This study
BY4741Apdr5::YEplac195-BPDR5-E794Q	*MATa his3 leu2 met 15 ura3 pdr5::loxP PDR5-E794K*	This study
Plasmids		
YEplac195		ATCC 87589
YEplac195-BPDR5		[18]
YEplac195-BPDR5-C793M		This study
YEplac195-BPDR5-C793S		This study
YEplac195-BPDR5-C793F		This study
YEp\ac195-BPDR5-C793Y		This study
YEp\ac195-BPDR5-S1230F		This study
YEp\ac195-BPDR5-S1230Y		This study
YEp\ac195-BPDR5-S1230A		This study
YEp\ac195-BPDR5-S1230N		This study
YEp\ac\95-BPDR5-S1230L		This study
YEp\ac\95-BPDR5-C793FS1230L		This study
YEp\ac\95-BPDR5-E794Q		This study
YEp\ac\95-BPDR5-E794K		This study

lacking uracil (SD-ura). *Escherichia coli* strain DH5α was served as the host strain for all plasmid constructions and grown in LB medium with 50 μg/ml ampicillin.

2.2. Chemicals

Cycloheximide (CYH), rhodamine 6G (R6G) and FK-506 monohydrate were dissolved in ethanol, whereas the 2,3,5-Triphenyltetrazolium chloride (TTC) and fluconazole (FLC) stock solutions were prepared with sterile water. All reagents above were purchased from Sigma-Aldrich.

2.3. Random and site-directed mutagenesis

PCR-targeted random mutagenesis was performed as previously described [21] and functional impaired mutants were screened using SD-ura agar plate containing 20 μM FLC. The detailed procedure of random mutagenesis and mutant screening is described in Supplementary materials.

Site-directed mutagenesis was performed using QuickChange kit (Stratagene) and YEplac195-*BPDR5* was used as the template. The resulting mutants were confirmed by DNA sequencing. The primers used in sequencing and mutagenesis are listed in Table S1 (Supplementary materials). All the mutational YEplac195-*PDR5* series were transformed into *pdr5*-null mutant strain following LiAc/SS carrier DNA/PEG method as previously described [22].

2.4. Preparation of purified membrane vesicles and immunoblotting

The plasma membrane (PM) vesicles were purified as previously described [13,16]. The concentration of membrane protein in the vesicles was determined by micro-Bradford assay (Bio-Rad Laboratories). Protein profiles (20 μg/lane) were examined by 7% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie Blue staining. Western blotting was carried out with a Pdr5-specific antibody yN-18 and a Pma1-specific antibody yN-20 (Santa Cruz Biotechnology, Santa Cruz, CA). yN-18 and yN-20 were 600- and 1000-fold diluted in Tris-Buffered Saline Tween-20 (TBST), respectively. HRP labeled donkey anti-goat polyclonal antibody (Beyotime Biotech, China) was diluted 1:3000 in TBST. Proteins were visualized by ECL chemiluminescence detection system (Beyotime Biotech, China) according to the manufacturer's instructions.

2.5. Drug resistance assays

Fresh yeast cells of BY4741Δ*pdr5*::YEplac195-*BPDR5* (WT), BY4741 Δ*pdr5*::YEplac195 (*pdr5*Δ) and BY4741Δ*pdr5*::YEplac195-*PDR5** harboring various *PDR5* mutant genes were inoculated to 5 ml SD-ura media and grown overnight at 30 °C. Then the cell suspensions were diluted to OD_{600} of 0.1 with sterile SD-ura medium. 6 μl of 5-fold serial dilutions was spotted on SD-ura agar plates containing FLC, CYH or TTC. The plates were incubated at 30 °C for 48 h before scanning. Equal amounts of cells (final $OD_{600} = 0.1$) were cultured in 200 μl SD-ura media containing FLC, CYH or TTC and the final OD_{600} were measured by spectrophotometry after incubation at 30 °C with shaking at 250 rpm for 24 h. The mean relative growth rate was determined by OD_{600} ratio of the cell samples cultured in SD-ura media with/without the drugs.

2.6. Determining the inhibitory concentration 50 (IC_{50}) of a drug in liquid culture

Fresh yeast cells were inoculated to 5 ml SD-ura medium and grown overnight at 30 °C. Then the cultures were diluted to OD_{600} of 0.1 with sterile SD-ura medium. 200 μl of above cultures were transferred to a