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Alanine scanning of all cysteines and construction of a functional cysteine-less Cdr1p, a multidrug ABC transporter of *Candida albicans*

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ABSTRACT

Herein, we discuss the role of the native cysteines present in a major multidrug ABC transporter of *Candida albicans*, Cdr1p, and describe the construction of this transporter's functional cysteine-less (cysless) protein version for cross-linking studies. In the experiments in which all 23 cysteines were replaced individually, we observed that most of the cysteine replacements were tolerated by the protein, but the replacement of C1056, C1091, C1106, C1294 or C1336 resulted in an enhanced drug susceptibility together with an abrogated drug efflux. Notably, the ATPase activity was uncoupled, which largely remained unaffected in these variants. The substitution of the critical cysteines with serines restored the normal expression and functionality of Cdr1p because serine can effectively mimic the hydrogen bonding properties of cysteine. Finally, we constructed a functional cysless His-tagged Cdr1p in which all the cysteines of the native protein were replaced with alanines and the critical cysteines were replaced with serines. Notably, cysless GFP-tagged variant of Cdr1p was non-functional. The cysless His-tagged variant of Cdr1p is the first example of a cysless ABC transporter in yeast, and it will lead to a greater understanding of the architecture of this important protein and provide insight into the nature of drug binding and interdomain communication.

1. Introduction

Among the various mechanisms that contribute to the development of multidrug resistance (MDR) in human pathogenic *Candida albicans*, one of the predominant and clinically relevant mechanisms is the overexpression of the genes encoding drug efflux pumps that belong to a superfamily of transporters: ABCs (ATP-Binding Cassettes) like *CDR1* and *CDR2* and MFSs (Major Facilitators) like *MDR1* [1–3]. Various studies have identified Cdr1p as the major drug efflux protein involved in the resistance against azoles that is achieved by rapidly extruding the drug out of the cell at the expense of the energy obtained from ATP hydrolysis [4,5].

The *CDR1* gene encodes an integral plasma membrane (PM) protein with predicted molecular mass of 170 kDa. Cdr1p shares the common architecture of the ABC transporter family, composed of two cytoplasmic nucleotide binding domains (NBD) and two transmembrane domains (TMD) [6]. Compared to its close homologue human MDR1/P-gp, analysis of the primary sequence of Cdr1p predicts that Cdr1p has a reverse topology in which the NBDs precede the TMDs $(NBD–TMD)_2$. Each TMD of Cdr1p is composed of six α-helical transmembrane segments (TMSs) that confer substrate specificity to the protein [7,8]. The NBDs are the sites of the ATP hydrolysis that helps power drug efflux. Conformational changes induced by ATP binding and/or hydrolysis are transmitted from the NBDs to the TMDs, most likely resulting in solute translocation. Cdr1p transports a wide range of structurally unrelated substrates, such as azoles, sterols and lipids as well as various chemically unrelated drugs, such as cycloheximide, anisomycin and organotin compounds [6,9].

To understand the molecular details of drug binding and efflux by Cdr1p, we performed site-directed mutagenesis analyses, which have thus far revealed that several amino acid residues of various TMSs are critical not only for the proper folding and targeting of the Cdr1p to the plasma membrane but also for drug transport [7,8]; however, the precise identity of the drug binding pocket remains largely uncharacterised.

In the absence of a crystal structure of Cdr1p or of any other yeast ABC drug transporter, it is difficult to decipher the shape, size and nature of the drug binding and transport. Recently, the use of cysteine cross-linking in a cysless protein variant of a mammalian ABC transporter has contributed to our understanding of the

Abbreviations: ABC, ATP binding cassette; MFS, major facilitator superfamily; MDR, multidrug resistance; MIC, minimum inhibitory concentration; FLC, fluconazole; CYH, cycloheximide; ITR, itraconazole; KTC, ketoconazole; MIC, miconazole; ANI, anisomycin; DIC, differential interference contrast; YPD, yeast extract peptone and dextrose.

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structural changes that occur upon drug binding and transport. This approach exploits both the ability of cysteines introduced into a cysless variant protein to form a disulphide linkage under oxidative conditions and also the reactivity of the introduced cysteine residues towards various sulfhydryl reagents. Loo and Clarke have successfully carried out cysteine cross-linking studies in the human MDR proteins P-gp and Cftr [10,11]. Their study could predict the residues involved in the formation of the drug binding pocket [12], in the crosstalk between the TMDs and the NBDs [13] and also in ascertaining the dimensions of the drug-binding domain [14]. The recent 3D structure of P-gp validated the data obtained by cysteine cross-linking experiments [15]. The structure of Yor1p, an ABC transporter of *Saccharomyces cerevisiae* that plays a role in oligomycin resistance, has been examined by employing extensive cysteine cross-linking to show that the interactions between specific intracellular loops (ICLs) are influential in shaping the active structure of this transporter [16].

In the present study, we have constructed a cysless Cdr1p for cross-linking experiments. This construction involved two stages. First, we individually replaced each of the 23 cysteines of Cdr1p with an alanine (C-A) to evaluate the structural and functional relevance of each cysteine. We observed that most of the cysteine replacements in Cdr1p were of no functional consequence; however, C1056, C1091, C1106, C1294 and C1336, upon replacement with alanines, yielded proteins with abrogated resistance to drugs. We then constructed a functional cysless variant of His-tagged Cdr1p wherein all 23 cysteines of native protein were replaced simultaneously. Collectively, this study highlights the role of the native cysteines and represents the first report of a cysless yeast ABC transporter, which is expected to become a powerful tool in the study of the dynamic aspects of the structure and function of Cdr1p.

2. Materials and methods

2.1. Materials

All of the molecular-grade chemicals were obtained from Sigma Chemical Co. (MO, USA). The anti-GFP and anti-His monoclonal antibodies were purchased from BD Biosciences Clontech (CA, USA). Fluconazole (FLC) was kindly provided by Ranbaxy Laboratories (New Delhi, India). The oligonucleotides used in this study, as listed in Table 1 of the Supplementary data (ST-1), were commercially procured from Sigma Genosys (Bangalore, India).

2.2. Growth media and strains

The plasmids were maintained in *Escherichia coli DH5α* cells. The *E. coli* was cultured in Luria–Bertani medium from Difco, BD Biosciences (NJ, USA) to which ampicillin was added (100 mg/ml). The yeast strains were cultured in YPD broth from HiMedia (Mumbai, India) or SD-URA$^-$ from Difco, BD Biosciences (NJ, USA) as required. For the agar plates, 2% (w/v) Bacto agar from Difco, BD Biosciences (NJ, USA) was added to the medium. Table 2 in the Supplementary data (ST-2) lists all of the yeast strains used in this study.

3. Methods

3.1. Site-directed mutagenesis

The site-directed mutagenesis was performed using the Quick Change Mutagenesis system from Stratagene (CA, USA). 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 mutated plasmid pPSCDR1-GFP was linearised with Xba I and then used to transform AD1–8u$^-$ as described previously [8].

3.2. Molecular cloning

The pCys-CDR1-GFP plasmid was used to amplify the cysless CDR1-ORF. The CDR1F and HR2 (ST-1) primers that were used for this purpose allowed for the introduction of SpeI restriction sites at the 5′ and 3′ termini, including six consecutives histidines at the 3′ end of the amplicon. The resultant amplicon was digested with SpeI and ligated at the corresponding sites of the linearised pSK-PDR5PPUS vector.

3.3. Drug susceptibility tests

The susceptibilities of *S. cerevisiae* cells were determined using microtitre and spot assays [17]. The following stock solutions were prepared in the solvents indicated in parentheses: 2 mg/ml FLC (water), 0.1 mg/ml cycloheximide (CYH) (water), 1 mg/ml itraconazole (ITR) (DMSO), 1 mg/ml ketoconazole (KTC) (methanol), 1 mg/ml miconazole (MIC) (methanol), 1 mg/ml anisomycin (ANI) (DMSO), 4 mg/ml rhodamine 6G (R6G) (DMSO).

3.4. R6G efflux and ATPase assays

The functionality of Cdr1p was verified by assaying the energy-dependent efflux of its fluorescent substrate rhodamine 6-G (R6G) as described previously [8]. The Cdr1p-associated ATPase activities of the PM from various Cdr1p variants were determined from the oligomycin-sensitive release of inorganic phosphate as described previously [8].

4. Results

4.1. Site-directed mutagenesis of 23 cysteines

To investigate the role of the cysteine residues of Cdr1p, we first evaluated the conservation of these residues. For this evaluation, the sequences of 22 fungal drug and non-drug ABC transporters with reverse topology were aligned, and a sequence logo was generated using "Web Logo" (http://weblogo.berkeley.edu/), which revealed that, of the 23 native cysteines that are distributed throughout the protein molecule, C712 located in TM4 and C1402, C1418, C1441, and C1444 located in extracellular loop 6 (ECL6) showed the highest conservation, whereas the remaining residues showed variable conservation (Supplementary Fig. S1).

To verify whether any of the cysteine residues, including those that are highly conserved, are critical for the proper functioning of Cdr1p, we replaced each cysteine with alanine by employing site-directed mutagenesis. A heterologous hyper-expression system in which GFP-tagged Cdr1p was stably overexpressed from the *PDR5* locus in an *S. cerevisiae* mutant, AD1–8u$^-$, was used [5,8,18]. The cells expressing the variants of Cdr1p were designated as listed in ST-2. The stable, single-copy integration of the variants in the heterologous hyper-expression system was confirmed by Southern hybridisation (data not shown). Two positive clones of each GFP-tagged Cdr1p variant were selected to preclude any clonal variations.

4.2. Certain C-A mutant variants display enhanced susceptibility to drugs

To test the functionality of the 23 cysteine-mutant variants, we performed two independent drug susceptibility tests, namely, the

microtitre and spot assays. The microtitre and spot assays revealed that most of the C-A variants were phenotypically similar to the native Cdr1p (Fig. 1A and B); however, variants such as C1056A, C1091A, C1106A, C1294A and C1336A were highly susceptible to the tested drugs, and their susceptibility profile was comparable to that of the control strain (AD1–8u$^-$) (Fig. 1C). The increased drug susceptibility of cells expressing C1056A, C1091A, C1106A, C1294A and C1336A Cdr1p-GFP was also corroborated by the results of the spot assays (Fig. 1D). Notably, C193, which is present in the Walker A sub domain of NBD1, is a residue that is conserved in all fungal ABC transporters. Our earlier studies have established that this typical C193 residue, which replaces an otherwise well-conserved lysine, is part of the active site where ATP hydrolysis occurs and, hence, is not included in the present study [19].

4.3. Drug susceptible C-A mutant variants are poorly surface localised

Our confocal images of the green fluorescent protein (GFP)-tagged Cdr1p and its mutants confirmed that the expression and localisation of most of the variants of Cdr1p (17 in number) that display unaltered drug susceptibility were similar to those of the cells expressing the native protein (Fig. 1E and F); however, the protein expression was considerably reduced in the variants that became hyper-susceptible to drugs. Thus, variants such as C1056A, C1091A, C1106A and C1294A showed decreased expression and localisation of Cdr1p with the exception of C1336A (Fig. 1G). The FACS analysis of all of the mutant cells corroborated these observations (data not shown). Immunoblotting of the PM of all of the variants using a monoclonal antibody against GFP confirmed that the mutants C1056A, C1091A, C1106A, and C1294A showed decreased expression at the cell surface, whereas the expression of C1336A was similar to that of the wild type (WT) protein (Fig. 2A).

4.4. Drug susceptible C-A mutant variants show severely impaired R6G efflux

One method of monitoring the functionality of Cdr1p is to assess the ability of Cdr1p to mediate the efflux of the fluorescent dye rhodamine 6G, a substrate of this protein [8]. The energy-dependent (glucose-induced) efflux by the C-A mutants was analysed. Because the surface expression of the proteins in the hyper-susceptible variants was considerably reduced relative to the expression of WT Cdr1p (Fig. 2A), the R6G efflux values for these mutants were normalised with respect to their protein expression level in the membrane. It was observed that, after the normalisation of protein expression, all of the hyper-susceptible cysteine mutants elicited impaired R6G efflux (Fig. 2B), whereas the remainder of the resistant C-A variants exhibited R6G efflux activities that were comparable to that of WT Cdr1p-GFP-expressing cells (Fig. 2C).

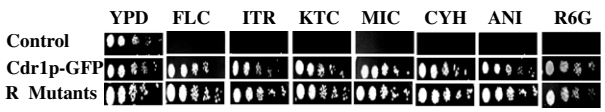
4.5. The ATPase activity of the C-A mutants remains unaltered

Drug transport is powered by coupled ATP hydrolysis at NBDs. We investigated whether the reduced R6G efflux observed in some C-A mutant variants was associated with any loss in ATPase activity by measuring the oligomycin-sensitive release of Pi from

A

STRAIN	FLC	ITR	KTC	MIC	CYH	ANI	R6G
Control	0.5	0.015	0.015	0.015	0.015	0.12	0.25
Cdr1p-GFP	64	4	2	2	1	32	64
R Mutants	64	4	2	2	1	32	64

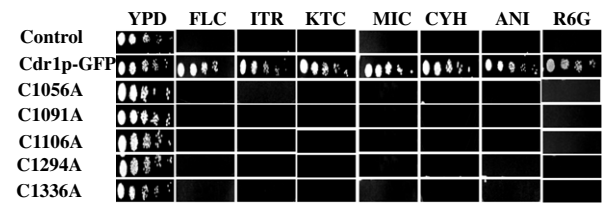
B



C

STRAIN	FLC	ITR	KTC	MIC	CYH	ANI	R6G
Control	0.5	0.015	0.015	0.015	0.015	0.12	0.25
Cdr1p-GFP	64	4	2	2	1	32	64
C1056A	0.5	0.015	0.015	0.015	0.015	0.12	0.25
C1091A	0.5	0.015	0.015	0.015	0.015	0.12	0.25
C1106A	0.5	0.015	0.015	0.015	0.015	0.12	0.25
C1294A	0.5	0.015	0.015	0.015	0.015	0.12	0.25
C1336A	0.5	0.015	0.015	0.015	0.015	0.12	0.25

D



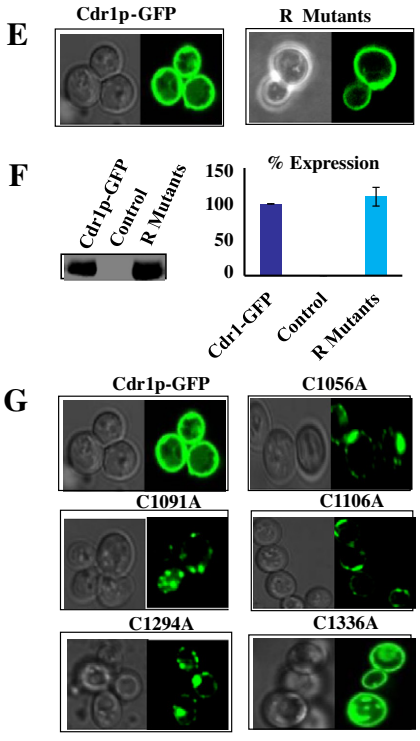


Fig. 1. The phenotypic profile of C-A mutant variants of Cdr1p. Because the profiles of all of the 17 resistant mutant variants were similar, the data of only one typical variant (denoted as the R mutant) are depicted in the figure. (A,B) The results of microtitre (MIC_{80} in μg/ml) and spot assays to show the drug resistance profile of a typical mutant exhibiting the WT phenotype. The cells were spotted either in the absence (YPD) or the presence of different concentrations of the following drugs (FLC, 5 μg/ml; CYH, 0.15 μg/ml; ANI, 4.0 μg/ml; and R6G, 5 μg/ml; ITR, 2 μg/ml; KTC, 0.15 μg/ml and MIC, 0.16 μg/ml). (C,D) Microtitre (MIC_{80} in μg/ml) and spot assay results showing the drug-resistance profiles of the drug-susceptible mutants of Cdr1p-GFP. (E,F) Confocal imaging and immunoblot analysis using an anti-GFP antibody to depict the membrane localisation of the GFP-tagged Cdr1p and its resistant C-A mutant variants as described previously [8]. Bar graphs shown in panel F are the average of percentage values of expression (compared to Cdr1p-GFP which is taken as 100%) from two independent experiments ($n = 2$) and their standard error of mean are shown by error bars. (G) Confocal images of the GFP-tagged Cdr1p and its drug-susceptible mutants. In all of the figures, the right-hand images are the fluorescent images, and the left-hand images depict the DIC images. The control used in all the figures is AD1–8u$^-$ as described in the text.

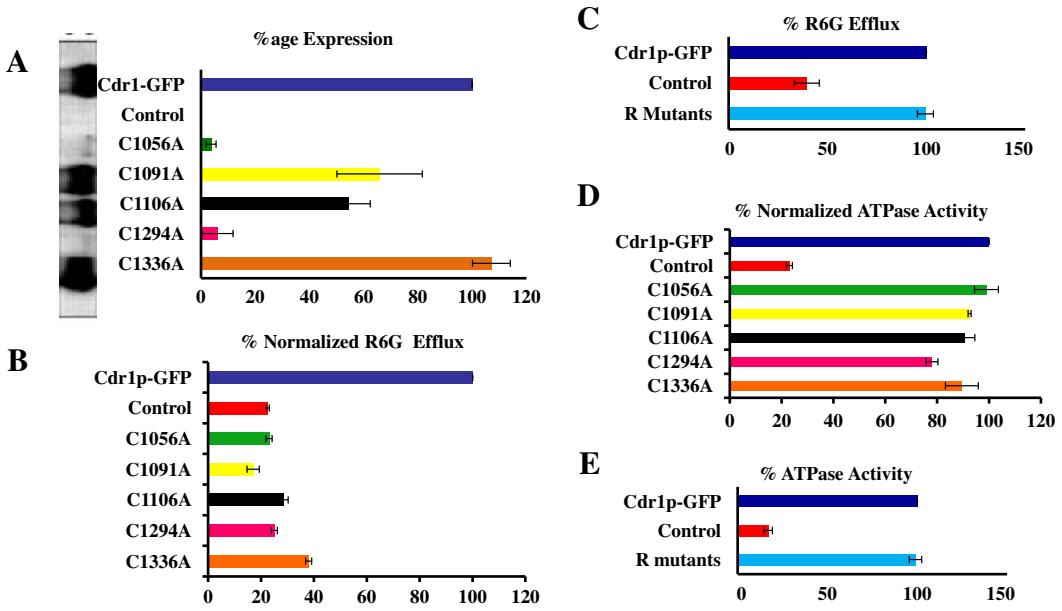


Fig. 2. The functional assays of C-A mutant variants of Cdr1p. (A) A bar graph showing the percentage expression of the drug-susceptible mutant variants of Cdr1p-GFP relative to WT Cdr1p-GFP which is taken as 100%. Values are the average of expression from two independent experiments ($n = 2$) with error bars showing their standard error of mean. (B) The R6G efflux assay of drug-susceptible mutants normalised to the levels of their expression in the membrane. (C) The R6G efflux assay of resistant mutants of Cdr1p-GFP. (D) The ATPase assay of drug-susceptible mutants normalised to the levels of their expression in the membrane. (E) The ATPase assay of resistant mutants of Cdr1p-GFP.

Mg-ATP as described in Section 2. Due to the poor expression of C1056A, C1091A, C1106A, and C1294A, these variants exhibited significantly reduced ATPase activity (data not shown), which, upon normalisation to their protein expression levels, became comparable to that of WT protein (Fig. 2D); however, most of the C-A mutants displayed ATPase activities comparable to that of native protein (Fig. 2E).

4.6. Thiol functional groups of 5 cysteine residues are essential for the functionality of Cdr1p-GFP

Interestingly, when we replaced the C193, C1056, C1091, C1106, C1294 and C1336 residues with serines, the full functionality of Cdr1p could be restored as revealed by their drug susceptibility profile, R6G efflux (Supplementary Fig. S2A–C) and ATPase activity (Supplementary Fig. S2D). These variants of Cdr1p-GFP were also normally expressed, correctly trafficked and recruited to the PM (Supplementary Fig. S2E and F).

4.7. Construction of cysless Cdr1p

Initially, to gain insight into the nature of drug binding and transport and their coupling to ATP catalysis, we attempted to construct a cysless variant of Cdr1p to use in cross-linking experiments. For this construction, the *CDR1* gene was systematically subjected to site-directed mutagenesis to replace all 23 cysteine residues in a single clone. Repeated rounds of site-directed mutagenesis of the pPSCDR1-GFP plasmid were performed, which resulted in the production of a cysless Cdr1p-GFP in which the critical cysteines at positions C193, C1056, C1091, C1106, C1294 and C1336 were replaced with serines (discussed above), whereas the remaining 17 cysteines were replaced with alanines. The resulting cysless Cdr1p-GFP was stably expressed in a heterologous hyper-expression system (described above), and its functionality was evaluated.

4.8. Cysless Cdr1p-GFP is hyper-susceptible to drugs and exhibits poor surface localisation

The cells expressing the cysless Cdr1p-GFP were tested for susceptibility towards drugs such as CYH, ANI, FLC, MIC, ITR, KTC and R6G. We observed that the cysless Cdr1p-GFP was highly susceptible to all of the drugs tested (Fig. 3A). Confocal imaging and Western blotting analysis revealed that cysless Cdr1p-GFP was poorly localised to the cell surface compared to the WT protein (Fig. 3B). Notably, the surface localisation of the cysless Cdr1p-GFP did not improve at any given point of growth. Also, the addition of drug substrates could not rescue the protein to the PM (Supplementary Fig. S3A and B). As expected, the cysless Cdr1p-GFP was unable to efflux R6G or display ATPase activity (Fig. 3C and D). Thus, the cysless-GFP-tagged Cdr1p was not functional.

4.9. The cysless Cdr1p-His is properly localised and displays resistance to drugs

Often, the tagging of a native protein is not tolerated, resulting in a misfolded and non-functional protein. We excluded this possibility by His-tagging Cdr1p at its C-terminus as described in Section 2. The cells harbouring the cysless Cdr1p-His were then tested for their susceptibility towards various drugs. The cysless Cdr1p-His was observed to be resistant to all of the drugs tested, and it behaved similarly to the native protein (Fig. 3E). The results of a filter disc assay confirmed the spot and microtitre results (data not shown). A Western blot analysis of the cysless Cdr1p-His confirmed that, even after the replacement of the 23 native cysteines, the cysless Cdr1p-His was able to be properly recruited to the plasma membrane, albeit at a lower level (~76%) than that of the WT Cdr1p-His (Fig. 3F).

4.10. Drug efflux and ATPase assay of cysless Cdr1p-His

The functionality of the cysless Cdr1p-His was further evaluated by monitoring its ability to efflux R6G. We observed that the efflux

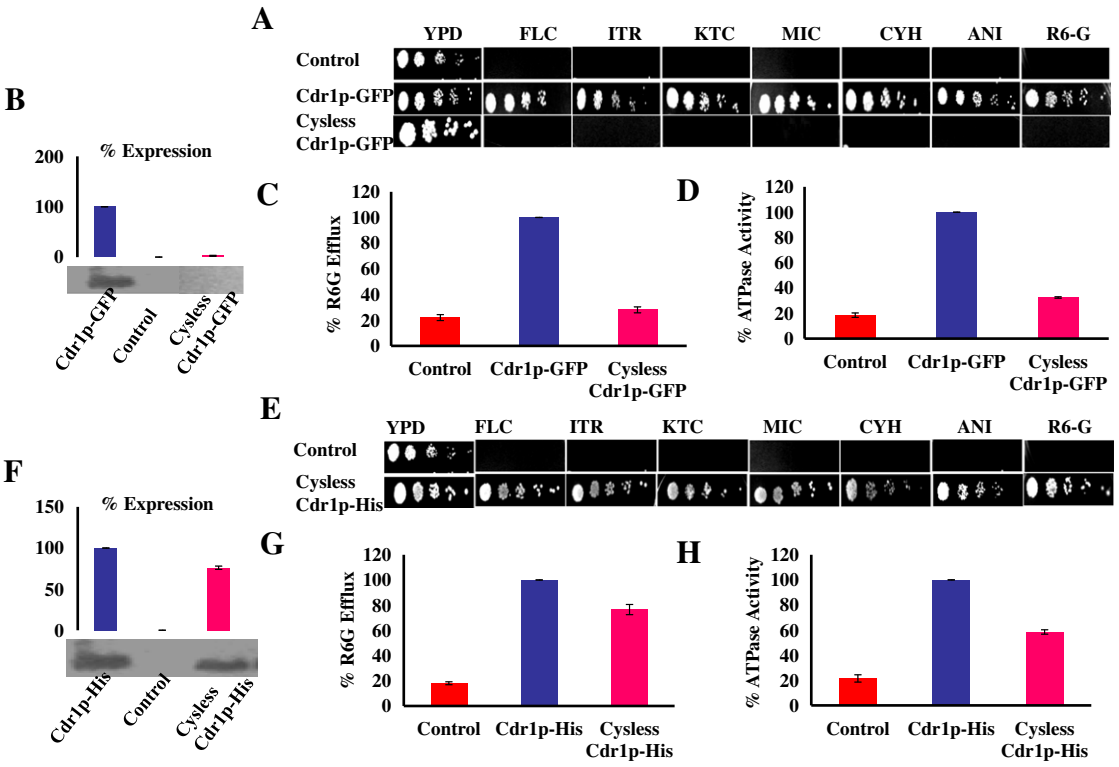


Fig. 3. Comparison of the drug-resistance profiles and functional assays of cysless Cdr1p-GFP and cysless Cdr1p-His. (A) Spot assays of cysless Cdr1p-GFP compared to WT Cdr1p-GFP. (B) Immunoblot analysis showing the membrane localisation of cysless Cdr1p-GFP as compared to WT Cdr1p-GFP performed using an anti-GFP antibody [8]. (C,D) R6G efflux and ATPase assays of cysless Cdr1p-GFP compared to WT Cdr1p-GFP. (E–H) Above set of experiments performed with cysless Cdr1p-His to show its drug-resistance profile, localisation and functionality compared to WT cells. All of these assays were performed as discussed in previous figures. Bar graphs with error bars (showing standard error of mean) in panel B and F are the average of percentage values of expression (compared to Cdr1p-GFP which is taken as 100%) from two independent experiments ($n = 2$).

activity was approximately 76% of that of the WT Cdr1p (Fig. 3G). To exclude the possibility that the cysless Cdr1p-His has, in any way, altered the catalytic cycle of Cdr1p, which might affect the efflux of R6G, we also evaluated the hydrolysis of ATP. For this evaluation, the PM proteins from WT and cysless Cdr1p-His-expressing cells were analysed for their ATPase activity. The ATPase activity of the cysless Cdr1p-His-expressing cells was 60% of that of the cells expressing WT Cdr1p (Fig. 3H).

5. Discussion

In an effort to characterise the nature of the drug binding site of Cdr1p, we have performed an alanine scanning of all the 23 cysteines present in the native protein to generate a cysless protein for cross-linking purposes. Notwithstanding the variable conservation among the cysteines in Cdr1p, most of the cysteines could be replaced with alanines without altering the protein's ability to function. However, the mutant variants C1056A, C1091A, C1106A, C1294A and C1336A exhibited interesting properties, including abrogated efflux activity but normal ATPase activity, implying an uncoupling between ATP catalysis and drug transport. In addition, the four C-A mutations, C1056A, C1091A, C1106A, C1294A appeared to be critical for the surface localisation of Cdr1p. The impaired localisation of the selected cysteine-mutant variants could be due to either trafficking defect, wherein the protein might not be properly folded or the degradation of the protein even after proper localisation. Notably, the variant C1336A which was properly localised to the cell surface displayed hyper susceptibility to drugs and impaired R6G efflux activity. This residue apart from its expected role in H bonding, appears to be critical for drug transport but not for surface localisation. The uncoupling between ATP catalysis and drug efflux in C1336A could be due to defect in its ability to bind or release drugs resulting in susceptible phenotype. However, this remains to be established.

Following the rationale that the cysteine residues C1056, C1091, C1106, C1294 could be important in either the formation of disulphide bridges or in H-bonding, we replaced the C1056, C1091, C1106, C1294 residues with serines. The replacement of cysteine with serine not only restored the surface localisation of Cdr1p, but cells containing the resulting mutants also behaved similarly to the WT cells. Apparently, the –OH group of serine can play similar role to that of the –SH group of cysteine. These findings also lead to the conclusion that none of the cysteine residues is involved in the formation of disulphide linkages, but they are more likely involved in H-bonding with other residues that results in the proper folding of the protein and, thus, help to maintain the native confirmation of the protein.

We have previously shown that at a non-canonical composite catalytic site in Cdr1p, the conserved E327 residue in the Walker A of N-NBD acts as a catalytic base and abstracts a proton from a water molecule present in the active site as part of the Mg–ATP complex. The free -OH group thus formed attacks the γ-phosphate group of an ATP molecule, allowing the bond between the γ- and β-phosphates to weaken and in turn allowing the β-phosphate to abstract a proton from the -SH moiety of the C193 residue. The consequence of the proton abstraction is the cleavage of the phosphodiester bond between the β- and γ-phosphates, allowing the latter to leave. Notably, if C193 is replaced with serine or tyrosine in the full protein, the protein retains its ATPase activity, implying

that the –OH group of serine or tyrosine is sufficient for maintaining proper interactions with ATP [20].

The cysless version of any protein is an important tool that can be used to study various aspects of protein structure using different biochemical methods. Substituted cysteine accessibility mutagenesis (SCAM) is one such powerful tool that has been exploited for structural studies of many eukaryotic and prokaryotic proteins [21]. In this study, we constructed an active His-tagged cysless Cdr1p in which all of the native cysteines are replaced with alanines and those cysteines that affect the function of the protein are replaced with serines. The cysless version of Cdr1p displays drug susceptibility, ATPase activity, and drug transport comparable to those of native Cdr1p, thus paving the way for cross-linking experiments and other related studies.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.11.150.

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