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Chimeras of the ABC drug transporter Cdr1p reveal functional indispensability of transmembrane domains and nucleotide-binding domains, but transmembrane segment 12 is replaceable with the corresponding homologous region of the non-drug transporter Cdr3p

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The molecular basis of the broad substrate recognition and the transport of substrates by Cdr1p, a major drug efflux protein of *Candida albicans*, is not well understood. To investigate the role of transmembrane domains and nucleotide-binding domains (NBDs) of Cdr1p in drug transport, two sets of protein chimeras were constructed: one set between homologous regions of Cdr1p and the non-drug transporter Cdr3p, and another set consisting of Cdr1p variants comprising either two N- or two C-terminal NBDs of Cdr1p. The replacement of either the N- or the C-terminal half of Cdr1p by the homologous segments of Cdr3p resulted in non-functional recombinant strains expressing chimeric proteins. The results suggest that the chimeric protein could not reach the plasma membrane, probably because of misfolding and subsequent cellular trafficking problems, or the rapid degradation of the chimeras. As an exception, the replacement of transmembrane segment 12 (TMS12) of Cdr1p by the corresponding region of Cdr3p resulted in a functional chimera which displayed unaltered affinity for all the tested substrates. The variant protein comprising either two N-terminal or two C-terminal NBDs of Cdr1p also resulted in non-functional recombinant strains. However, the N-terminal NBD variant, which also showed poor cell surface localization, could be rescued to cell surface, if cells were grown in the presence of drug substrates. The rescued chimera remained non-functional, as was evident from impaired ATPase and efflux activities. Taken together, the results suggest that the two NBDs of Cdr1p are asymmetric and non-exchangeable and that the drug efflux by Cdr1p involves complex interactions between the two halves of the protein.

INTRODUCTION

Among ABC transporters, Cdr1p has been shown to play a key role in azole resistance in *Candida albicans* as deduced from its high level of expression in several azole-resistant clinical isolates recovered from patients receiving long-term antifungal therapy (Sanglard *et al.*, 1995). Additionally, a high level of expression of Cdr1p invariably contributes to an increased efflux of fluconazole, thus corroborating its direct involvement in drug efflux (Sanglard *et al.*, 1995; White *et al.*, 1998). Cdr1p thus has not only acquired significant clinical importance but is considered an important player in any design of strategies to combat antifungal resistance.

The *CDR1* gene encodes an integral plasma membrane (PM) protein of 1501 amino acids, with a predicted molecular mass of 169·9 kDa. The topology of Cdr1p exhibits typical characteristic features of an ABC transporter: two highly hydrophobic transmembrane domains (TMDs) and two cytoplasmically localized nucleotide-binding domains (NBDs). Each TMD comprises six transmembrane segments (TMSs), which are envisaged to confer substrate specificity (Shukla *et al.*, 2003, 2004). The NBDs of ABC-type transporter proteins, on the other hand, are the sites of ATP hydrolysis and hence the hub of energy generation to facilitate drug efflux. According to our current understanding, Cdr1p and Cdr2p drug extrusion proteins not only mediate the efflux of azoles and their derivatives but also extrude a

Abbreviations: CM, crude membranes; NBD, nucleotide-binding domain; PM, plasma membrane; TMD, transmembrane domain; TMS, transmembrane segment.

A supplementary figure showing the amino acid sequence alignment of Cdr1p and Cdr3p is available with the online version of this paper.

variety of structurally unrelated drugs and compounds (Dogra *et al.*, 1999; Krishnamurthy *et al.*, 1998a; Prasad *et al.*, 1998; Sanglard *et al.*, 1997; Smriti *et al.*, 2002). However, the molecular mechanism of drug transport mediated by Cdr1p is not yet known.

Although analysis of the *C. albicans* genome reveals that it has 28 putative ABC transporters, only *CDR1* and *CDR2* have been shown to be drug transporters (Braun *et al.*, 2005; Gaur *et al.*, 2005). The well-characterized Cdr3p and Cdr4p, despite having 56 % and 62 % identity, respectively, with Cdr1p, and 55 % and 59 % identity with Cdr2p, and similar predicted domain organization, are not involved in drug efflux and antifungal resistance (Balan *et al.*, 1997; Franz *et al.*, 1998; Sanglard *et al.*, 1999; Smriti *et al.*, 2002). The non-drug transporter Cdr3p is a general phospholipid flippase and translocates membrane phospholipids (Smriti *et al.*, 2002). This situation is reminiscent of the mammalian *MDR* gene family, which encodes the three highly homologous P-gp isoforms in mouse (*mdr1*, *mdr2* and *mdr3*) and two in humans (*MDR1* and *MDR2*) (Ng *et al.*, 1989). While the overexpression of mouse *mdr1* and *mdr3* and human *MDR1* is closely linked to the cell's ability to exhibit multidrug resistance, no such role for mouse *mdr2* or human *MDR2* has been observed. Notably, MDR2, which displays 78 % overall amino acid sequence identity with MDR1, rather functions as a flippase and translocates membrane phospholipids between two monolayers of the lipid bilayer (Ruetz & Gros, 1994; Smit *et al.*, 1993).

To understand the structure and function of individual domains of drug transporter proteins, in the present study we focused on the role and distinctiveness of the NBDs and TMDs of Cdr1p in drug transport. For this we employed two approaches: first we constructed chimeras between Cdr1p and its close homologue Cdr3p and second, to study the functional equivalence of the two cytoplasmic domains, we constructed variants of Cdr1p molecules comprising either the two N-terminal or two C-terminal NBDs of the same protein. Our results demonstrate for the first time that the N- and the C-terminal halves of Cdr1p are largely non-exchangeable. TMS12 was the only part of Cdr1p that could be functionally replaced with the equivalent TMS region of the non-drug transporter Cdr3p. On the other hand, the recombinant strain expressing a Cdr1p variant with two N-terminal NBDs produced a chimeric protein that even upon rescuing to the PM remained non-functional, thus implying that the two NBDs display functional asymmetry and non-equivalence.

METHODS

Materials. Anti-GFP monoclonal antibody was purchased from BD Biosciences Clontech. DNA-modifying enzymes were purchased from Roche Molecular Biochemicals. Protease inhibitors (PMSF, leupetin, pepstatin A, aprotinin) and drugs (miconazole, ketoconazole, itraconazole, cycloheximide, anisomycin), rhodamine 6G and other molecular-grade chemicals were obtained from Sigma. Ranbaxy Laboratories, India, kindly provided fluconazole (FLC).

Media and strains. Plasmids were maintained in *Escherichia coli* DH5α. *E. coli* was cultured in Luria–Bertani medium (Difco) to which ampicillin was added (100 μg ml^{-1}). The bacterial and *Saccharomyces cerevisiae* strains used in this study are listed in Table 1. The yeast strains were cultured in YEPD broth (Bio 101) or SD-ura$^-$ (Bio 101). For agar plates 2 % (w/v) Bacto agar (Difco) was added to the medium.

Vector construction and generation of transformants

Construction of pPS1N/3CGFP. Since plasmid pSK-PDR5PPUS (yeast vector) has a limited choice of unique restriction enzyme sites, initially the chimera consisting of the N-terminal half of *CDR1* and the C-terminal half of *CDR3* (1N/3C) was constructed in pBluescript (pBS) by PCR amplification with a combination of primers (listed in Table 2) using either pPSCDR1GFP (Shukla *et al.*, 2003) or p425-GPD-CDR3* (Table 1) as template and *Pwo* DNA polymerase (Roche Molecular Biochemicals) or *Pfu* DNA polymerase (Stratagene). For construction of pBS1N/3C, primers PS1 (having a *Xba*I restriction site) and 1NR2 were used to amplify the N-terminal half of *CDR1* using pPSCDR1GFP as template, and primers 3CF and PS4 (having a *Sal*I restriction site) were used to amplify the C-terminal half of *CDR3* without the stop codon using p425-GPD-CDR3* as template. The two amplicons were then ligated and the ligated mix was used as template for the final amplification of 1N/3C using primers PS1 and PS4. The amplified fragment was then digested with *Xba*I and *Sal*I (restriction sites introduced in the primers) and cloned into the *Xba*I- and *Sal*I-digested pBS. The GFP ORF was amplified from pPSCDR1GFP using primers GFP-F (having a *Sal*I restriction site) and GFP-R (having *Sal*I and *Xba*I restriction sites). This GFP amplicon was then digested with *Sal*I and cloned into *Sal*I-digested plasmid pBS1N/3C, resulting in plasmid pBS1N/3CGFP. The 1N/3CGFP ORF from plasmid pPBS1N/3CGFP was then taken out with the convenient restriction site *Xba*I and recloned into *Spe*I-digested pSK-PDR5PPUS, resulting in plasmid pPS1N/3CGFP.

Construction of pPS3N/1CGFP. A Similar strategy was employed to construct the vector pPS3N/1CGFP using primers PS5 (having a *Xba*I restriction site) and 3NR to amplify the N-terminal half of *CDR3*, and 1CF and PS8 (having a *Sal*I restriction site) to amplify the C-terminal half of *CDR1* without the stop codon.

Construction of pPS1(1–1173)/3(1154–1501)GFP. For constructing pPS1(1–1173)/3(1154–1501)GFP vector, first the endogenous *Nar*I site at amino acid position 823 in pPS1N/3CGFP was modified by site-directed mutagenesis using primers 1N/3C(*Nar*I823)F and 1N/3C(*Nar*I823)R and the Quickchange mutagenesis system (Stratagene), thus resulting in pPS1N/3CGFP*. Subsequently, *Nar*I sites were created at amino acid positions 789 and 1173 [using primers CDR1P(*Nar*I789)F, CDR1P(*Nar*I789)R and CDR1P(*Nar*I1173)F, CDR1P(*Nar*I1173)R respectively] in pPSCDR1GFP, thus forming plasmid pPSCDR1GFP3, and at amino acid positions 789 and 1165 [using primers 1N/3C(*Nar*I789)F, 1N/3C(*Nar*I789)R and 1N/3C(*Nar*I1165)F, 1N/3C(*Nar*I1165)R respectively] in pPS1N/3CGFP*, resulting in plasmid pPS1N/3CGFP*3 without changing the existing codon by site-directed mutagenesis. The resulting *Nar*I-digested 1·152 kb fragment was taken out from plasmid pPSCDR1GFP3 and ligated to *Nar*I-digested plasmid pPS1N/3CGFP*3.

Construction of pPS1(1–1436)/3(1420–1501)GFP. To construct pPS1(1–1436)/3(1420–1501)GFP vector, *Nar*I and *Mlu*I sites were introduced at amino acid positions 789 and 1436 respectively [using primers CDR1P(*Nar*I789)F, CDR1P(*Nar*I789)R and CDR1P-(*Mlu*I1436)F, CDR1P(*Mlu*I1436)R] in pPSCDR1GFP, thus forming plasmid pPSCDR1GFP2, and similarly, *Nar*I and *Mlu*I sites were introduced at amino acid positions 789 and 1431 respectively

Table 1. Plasmids and strains

Name	Description	Reference
Plasmid		
pSK-PDR5PPUS		Nakamura *et al.* (2002)
pPSCDR1GFP	Carries CDR1GFP ORF	Shukla *et al.* (2003)
p425-GPD-CDR3*	Derived from p425-GPD-CDR3 (kind gift from Martine Raymond) in which two CTG codons were converted to TCG to avoid codon usage problem in *S. cerevisiae*	This study
pBS1N/3CGFP	pBluescript encoding N-terminal half of *CDR1* and C-terminal half of *CDR3* tagged with GFP-encoding gene at the C-terminus	This study
pBS3N/1CGFP	pBluescript encoding N-terminal half of *CDR3* and C-terminal half of *CDR1* tagged with GFP-encoding gene at the C-terminus	This study
pPS1N/3CGFP	Carries 1N/3CGFP ORF cloned at *Spe*I site of pSK-PDR5PPUS	This study
pPS3N/1CGFP	Carries 3N/1CGFP ORF cloned at *Spe*I site of pSK-PDR5PPUS	This study
pPS1N/3CGFP*	pPS1N/3CGFP with modified *Nar*I site in 1N/3CGFP ORF	This study
pPSCDR1GFP2	pPSCDR1GFP with *Nar*I site introduced at 789 amino acid position and *Mlu*I site at 1436 amino acid position in CDR1GFP ORF	This study
pPS1N/3CGFP*2	pPS1N/3CGFP* with*Nar*I site introduced at 789 amino acid position and *Mlu*I site at 1431 amino acid position in 1N/3CGFP* ORF	This study
pPSCDR1GFP3	pPSCDR1GFP with *Nar*I sites introduced at 789 and 1173 amino acid position in CDR1GFP ORF	This study
pPS1N/3CGFP*3	pPS1N/3CGFP* with *Nar*I site introduced at 789 and 1165 amino acid position in 1N/3CGFP* ORF	This study
pPS(1-1173)/3(1154-1501)GFP	pSK-PDR5PPUS encoding Cdr1p up to 1173 amino acid and Cdr3p from amino acid 1154 to 1501, tagged with GFP-encoding gene at the C-terminus	This study
pPS(1-1436)/3(1420-1501)GFP	pSK-PDR5PPUS encoding Cdr1p up to 1436 amino acid and Cdr3p from amino acid 1420 to 1501, tagged with GFP-encoding gene at the C-terminus	This study
pCdr1-1N/1NGFP	pPSCDR1GFP encoding two N-terminal NBDs in CDR1GFP ORF	This study
pPSCDR1GFP*Hin*dIII	pPSCDR1GFP with *Hin*dIII site introduced at 494 amino acid position in CDR1GFP ORF	This study
pCdr1-1C/1CGFP	pPSCDR1GFP encoding two C-terminal NBDs in CDR1GFP ORF	This study
Strains		
AD1-8u$^-$	*MAT**a** pdr1-3 his1 ura3 Δyor1::hisG Δsnq2::hisG Δpdr5::hisG Δpdr10::hisG Δpdr11::hisG Δycf1::hisG Δpdr3::hisG Δpdr15::hisG*	Nakamura *et al.* (2002)
PSCDR1GFP	AD1-8u$^-$ carrying CDR1GFP ORF	Shukla *et al.* (2003)
PS1N/3CGFP	AD1-8u$^-$ carrying 1N/3CGFP ORF	This study
PS3N/CGFP	AD1-8u$^-$ carrying 3N/1CGFP ORF	This study
PS1(1-1173)/3(1154-1501)GFP	AD1-8u$^-$ carrying 1(1–1173)/3(1154–1501)GFP ORF	This study
PS1(1-1436)/3(1420-1501)GFP	AD1-8u$^-$ carrying 1(1–1436)/3(1420–1501)GFP ORF	This study
Cdr1-1N/1NGFP	AD1-8u$^-$ carrying Cdr1-1N/1NGFP ORF	This study
Cdr1-1C/1CGFP	AD1-8u$^-$ carrying Cdr1-1C/1CGFP ORF	This study

[using primers 1N/3C(*Nar*I789)F, 1N/3C(*Nar*I789)R and 1N/3C(*Mlu*I1431)F, 1N/3C(*Mlu*I1431)R] in plasmid pPS1N/3CGFP*, resulting in plasmid pPS1N/3CGFP*2 by site-directed mutagenesis without changing the existing codons. The resulting *Nar*I- and *Mlu*I-digested 1·941 kb fragment was taken out from plasmid pPSCDR1GFP2 and ligated to *Nar*I- and *Mlu*I-digested plasmid pPS1N/3CGFP*2.

Construction of pCdr1-1N/1NGFP. For constructing pCdr1-1N/1NGFP vector, the DNA encoding a hydrophilic region including the N-terminal NBD was amplified using pPSCDR1GFP as template and primers CDR1F(1210) and CDR1R(2657), which allowed the introduction of a *Nar*I restriction site at the 5′ and 3′ ends of the amplicon. The resultant amplicon was then digested with *Nar*I-and ligated to *Nar*I digested pPSCDR1GFP3 vector (*Nar*I digestion of pPSCDR1GFP3 results in deletion of the C-terminal NBD).

Construction of pCdr1-1C/1CGFP. For constructing pCdr1-1C/1CGFP, a *Hin*dIII restriction site was introduced at amino acid position 494 (after the N-terminal NBD) in plasmid pPSCDR1GFP by site-directed mutagenesis using primers *Hin*dIII 494F and *Hin*dIII 494R, resulting in plasmid pPSCDR1GFP*Hin*dIII. The DNA encoding a hydrophilic region including the C-terminal NBD was amplified using pPSCDR1GFP as template and primers 1CF *Hin*dIII

Table 2. Oligonucleotides

Name	Sequence (5′–3′)	Purpose
GFP-F	ACGCGTCGACATGAGTAAGGGAGAAGAA	Forward primer with *Sal*I site for amplifying and cloning GFP ORF
GFP-R	ACGCGTCGACGCTCTAGATTATTTGTATAGTTCATCCA	Reverse primer with *Sal*I and *Xba*I site for amplifying and cloning GFP ORF
PS1	GCTCTAGAGCGAAAAAAATTATGTCAGATTCTA	Forward primer with *Xba*I site for amplifying and cloning N-terminal half of *CDR1*
1NR2	AGCACCTTTATTGAATTCAG	Reverse primer for amplifying and cloning N-terminal half of *CDR1*
3CF	TCAAGTATTTCGAAAGGTGAAACT	Forward primer for amplifying and cloning *CDR3* C-terminal half
PS4	CCATGTCGACAACGTTTTCATGATTGG	Reverse primer with *Sal*I site for amplifying and cloning *CDR3* C-terminal half
PS5	GCTCTAGAGCCAAAAAATGGCCAAGACATCACA	Forward primer with *Xba*I site for amplifying and cloning *CDR3* N-terminal half
3NR	TTTGTTGGTTTGAACGAAAAATA	Reverse primer for amplifying and cloning N-terminal half of *CDR3*
1CF	ATGCAAAAGGGGGAAATTGTTTTGTTCC	Forward primer for amplifying and cloning C-terminal half of *CDR1*
PS8	CCATGTCGACTTTATTTCTTATTTTTTTTCTCTCTGTTACCCT	Reverse primer with *Sal*I site for amplifying and cloning C-terminal half of *CDR1*
1N/3C(*Nar*I823)F	CCTATATGGATGGTATGGCACCATTAGATTTTAGTGG	Forward primer for modifying *Nar*I site at 823 amino acid position in 1N/3CGFP ORF
1N/3C(*Nar*I823)R	CCACTAAAATCTAATGGTGCCATACCATCCATATAGG	Reverse primer for modifying *Nar*I site at 823 amino acid position in 1N/3CGFP ORF
1N/3C(*Nar*I789)F	CTGAATTCAATAAAGGCGCCTCAAGTATTTCGAAAGGTG	Forward primer for introducing *Nar*I site at 789 amino acid position in 1N/3CGFP ORF
1N/3C(*Nar*I789)R	CACCTTTCGAAATACTTGAGGCGCCTTTATTGAATTCAG	Reverse primer for introducing *Nar*I site at 789 amino acid position in 1N/3CGFP ORF
1N/3C(*Nar*I1165)F	GATACCAACCAAGCATATGCGGCGCCATTCTGGAAACAATATATC	Forward primer for introducing *Nar*I site at 1165 amino acid position in 1N/3CGFP ORF
1N/3C(*Nar*I1165)R	GATATATTGTTTCCAGAATGGCGCCGCATATGCTTGGTTGGTATC	Reverse primer for introducing *Nar*I site at 1165 amino acid position in 1N/3CGFP ORF
1N/3C(*Mlu*I1431)F	GGTTATTTATTGAATACGCGTGCCACTGATAACTG	Forward primer for introducing *Mlu*I site at 1431 amino acid position in 1N/3CGFP ORF
1N/3C(*Mlu*I1431)R	CAGTTATCAGTGGCACGCGTATTCAATAAATAACC	Reverse primer for introducing *Mlu*I site at 1431 amino acid position in 1N/3CGFP ORF
CDR1P(*Nar*I789)F	GAATTCAATAAAGGCGCCATGCAAAAGGGG	Forward primer for introducing *Nar*I site at 789 amino acid position in Cdr1GFP ORF

Table 2. Oligonucleotides

Name	Sequence (5′–3′)	Purpose
CDR1P(*Nar*I789)R	CCCCTTTTGCATGGCGCCTTTATTGAATTC	Reverse primer for introducing *Nar*I site at 789 amino acid position in Cdr1GFP ORF
CDR1P(*Nar*I1173)F	GCACTTTTGAAATATGCGGCGCCACTTTGGAAACAATAC	Forward primer for introducing *Nar*I site at 1173 amino acid position in Cdr1GFP ORF
CDR1P(*Nar*I1173)R	GTATTGTTTCCAAAGTGGCGCCGCATATTTCAAAAGTGC	Reverse primer for introducing *Nar*I site at 1173 amino acid position in Cdr1GFP ORF
CDR1P(*Mlu*I1436)F	GGTGGGTATTTTGAAACGCGTAATGATGGATCTTGTG	Forward primer for introducing *Mlu*I site at 1436 amino acid position in Cdr1GFP ORF
CDR1P(*Mlu*I1436)R	CACAAGATCCATCATTACGCGTTTCAAAATACCCACC	Reverse primer for introducing *Mlu*I site at 1436 amino acid position in Cdr1GFP ORF
CDR1F(1210)	GGCGCCATGTCAGATTCTAAGATGTCGTCG	Forward primer for amplifying N-terminal NBD of *CDR1* with *Nar*I site
CDR1R(2657)	GGCGCCGTATATGGAGATGCTGGTCTTGT	Reverse primer for amplifying N-terminal NBD of *CDR1* with *Nar*I site
*Hin*dIII494F	GTATCATTCTTTAAGCTTATGCAAGTGAGG	Forward primer for introducing *Hin*dIII site at 494 amino acid position in Cdr1GFP ORF
*Hin*dIII494R	CCTCACTTGCATAAGCTTAAAGAATGATAC	Reverse primer for introducing *Hin*dIII site at 494 amino acid position in Cdr1GFP ORF
1CF(*Hin*dIII)	GCCCAAGCTTATGCAAAAGGGGGA	Forward primer for amplifying C-terminal NBD of *CDR1* with *Hin*dIII site
1CR(*Hin*dIII)	GCCCAAGCTTTGGTGCAGCATATTT	Reverse primer for amplifying C-terminal NBD of *CDR1* with *Hin*dIII site

and 1CR *Hin*dIII, which allowed the introduction of a *Hin*dIII restriction site at the 5′ and 3′ ends of the amplicon. The resultant amplicon was then digested with *Hin*dIII and ligated to *Hin*dIII-digested pPSCDR1GFP*Hin*dIII (*Hin*dIII digestion of pPSCDR1GFP *Hin*dIII results in deletion of the N-terminal NBD).

After every cloning, the entire chimeric construct was sequenced by using the Big Dye Terminator Cycle sequencing kit (ABI) and an ABI 310 DNA sequencer to confirm that the construct remained in-frame and no mutation had been introduced. Restriction enzyme digestions further confirmed the orientation of each construct. Each plasmid, after linearizing with *Xba*I, was used to transform AD1-8u$^-$ cells as described previously (Shukla *et al.*, 2003). Transformation of yeast cells was performed by the lithium acetate method using routine laboratory protocols (Shukla *et al.*, 2003). Single-copy integration of each transformant at the *PDR5* locus was confirmed by Southern hybridization (data not shown). Two positive clones of each chimera were selected for initial screening to rule out clonal variations.

Immunodetection of Cdr1p. Plasma membranes (PMs) were prepared from *S. cerevisiae* as described previously (Shukla *et al.*, 2003). Briefly, cells were broken with glass beads. The crude membranes (CM) were recovered by centrifugation at 1000 ***g*** to remove unbroken cells and pelleting the CM by ultracentrifugation at 100 000 ***g*** for 1 h. The CM were then resuspended in resuspension buffer and applied to a discontinuous gradient made of an equal volume of 53·5 % (w/v) sucrose and 43·5 % (w/v) sucrose. The purified PM was recovered at the interface of the 43·5 % and 53·5 % sucrose layers, following centrifugation for 5 h at 100 000 ***g***. The Western blot analysis was done using anti-GFP monoclonal antibody (1 : 1000 dilution) and anti-Pma1p polyclonal antibody (1 : 10 000 dilution) as described previously (Shukla *et al.*, 2003). Proteins on immunoblots were visualized using the enhanced chemiluminescence assay system (ECL kit, Amersham Biosciences).

Confocal microscopy. The cells were grown to late exponential phase in SD-ura$^-$ medium, except for AD1-8u$^-$, where uridine (0·02 %) was supplemented 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 directly viewed with a 100× oil-immersion objective on a confocal microscope (Radiance 2100, AGR, 3Q/BLD; Bio-Rad).

Drug susceptibility and other functional parameters. The susceptibilities of *S. cerevisiae* cells to different drugs were tested

by microtitre plate assay and spot assay as described earlier (Mukhopadhyay *et al.*, 2002). The Cdr1p-associated ATPase activity of the purified PM was measured as oligomycin-sensitive release of inorganic phosphate as described previously (Shukla *et al.*, 2003). Efflux of rhodamine 6G and accumulation of [^{3}H]fluconazole were determined essentially as described elsewhere (Kohli *et al.*, 2002; Shukla *et al.*, 2003). Approximately 10^7 cells from an overnight culture were inoculated in 100 ml YPD and grown for 5–6 h at 30 °C with shaking. The cells were pelleted and washed three times with phosphate-buffered saline (PBS) buffer without glucose. The cells were subsequently resuspended as a 2 % cell suspension in de-energization buffer (5 mM dinitrophenol and 5 mM 2-deoxy-D-glucose in PBS without glucose) and incubated for 2 h at 30 °C with shaking. The cells were then washed, resuspended in PBS without glucose and divided into four parts; rhodamine 6G was added to a final concentration of 10 μM to each part, followed by incubation for 2 h at 30 °C. After washing, the cells were suspended in PBS with 2 % glucose. An aliquot of 1 ml was taken after 45 min and centrifuged at 9000 ***g*** for 2 min. The absorbance of the supernatant was measured at 527 nm. To check the accumulation of [^{3}H]fluconazole, the de-energized cells were incubated with 100 nM [^{3}H]fluconazole (0·7 TBq mmol^{-1}) for 2 h. The cells were then washed, and suspended in PBS with 2 % glucose. An aliquot of 1 ml was removed after 45 min, rapidly filtered and washed three times with ice-cold PBS without glucose. The radioactivity that accumulated in filtered cells and that adhered to filter disks was measured in a liquid scintillation counter with a scintillation liquid (tri-Carb 2900TR; Packard). The radioactivity that adhered to the filter disk, which was not significant, was subtracted from the experimental values.

Photoaffinity labelling with [^{3}H]azidopine. PM (25 μg) protein was photoaffinity labelled with 0·5 μM [^{3}H]azidopine (60 Ci mmol^{-1}; 2·2 TBq mmol^{-1}) as described previously (Shukla *et al.*, 2003).

RESULTS

Swapping the N- and C-terminal halves of Cdr1p and Cdr3p yields non-functional recombinant strains

Pair-wise sequence comparisons indicate that Cdr3p displays 56 % sequence identity and 74 % similarity with Cdr1p (see supplementary Fig. S1 available with the online version of this paper). Despite the observed topological (http://ca.expasy.org/tools/, Prasad *et al.*, 1995; Shukla *et al.*, 2003) (Fig. 1A) and sequence similarity, the two proteins are functionally distinct (Balan *et al.*, 1997; Prasad *et al.*, 1995; Smriti *et al.*, 2002). For example, we as well as others (Sanglard *et al.*, 1995; Prasad *et al.*, 1995; Shukla *et al.*, 2003) have shown a direct involvement of Cdr1p in efflux of drugs while Cdr3p has no such activity and is thus a non-drug transporter (Smriti *et al.*, 2002). Cdr3p does possess ATPase activity but this is not coupled to drug efflux; rather it powers translocation of phospholipids between the two lipid monolayers of the PM. The inhibitors which block

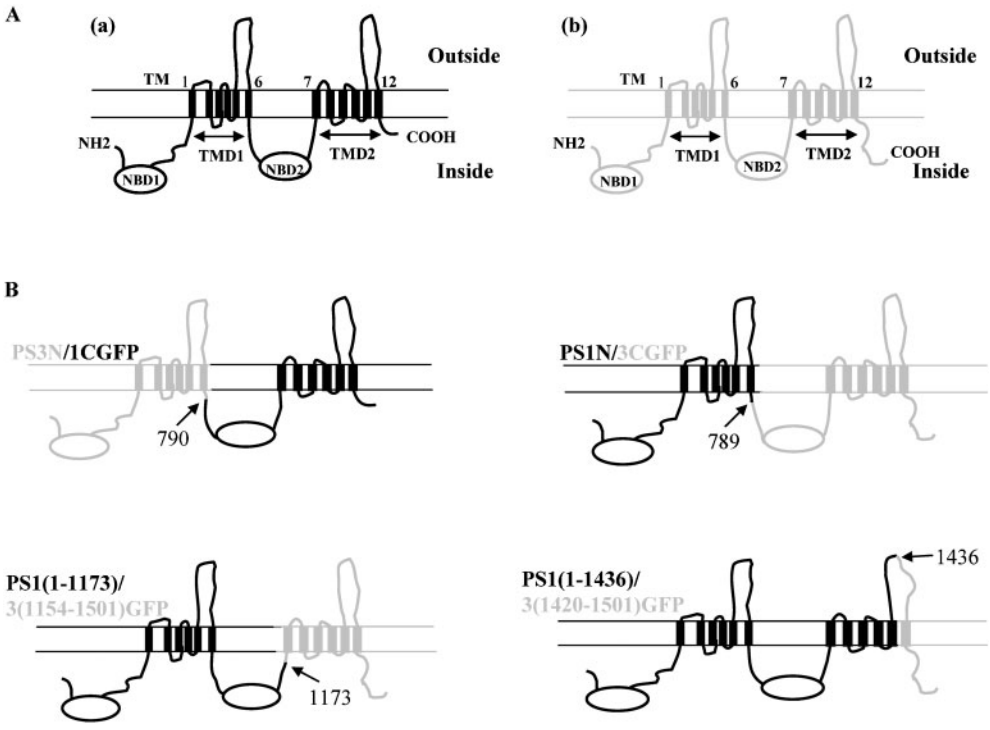


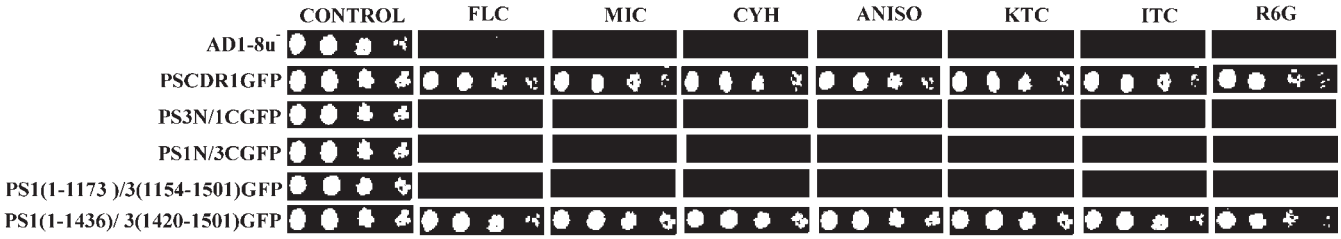
Fig. 1. Predicted topology of Cdr1p and Cdr3p and schematic representation of CDR1/CDR3 chimeric proteins. (A) Proposed secondary structure of Cdr1p (a) and Cdr3p (b) with two TMDs and two NBDs. (B) Illustration of the CDR1/CDR3 chimeras. The position of Cdr1p where the splice with the corresponding sequence of Cdr3p occurs is indicated. The amino acid numbers refer to the segment of Cdr1p present in each construct.

ATPase activity of Cdr3p also block phospholipid translocation mediated by it. Interestingly, Cdr3p appears to be functionally oppositely oriented in the PM as compared to Cdr1p (Smriti *et al.*, 2002).

To determine the domain(s) of Cdr1p involved in drug resistance, we constructed a series of *CDR1*/*CDR3* chimeras (Fig. 1B) by exchanging the homologous regions of *CDR1* with *CDR3* as described in Methods. In order to check the localization of chimeric proteins, each construct, at the C-terminus, was also tagged with the green fluorescent protein (GFP). For functional analysis of the *CDR1/CDR3* chimeras, a heterologous hyper-expression system, where Cdr1p is stably overexpressed from a genomic *PDR5* locus in a *S. cerevisiae* mutant AD1-8u$^-$, was used (Nakamura *et al.*, 2002). The host AD1-8u$^-$, from which seven major ABC transporters have been deleted, was derived from a *Pdr1-3* mutant strain with a gain-of-function mutation in the transcription factor Pdr1p, resulting in a constitutive hyperinduction of the *PDR5* promoter. The new overexpressing strains harbouring *CDR1/CDR3* chimeras were designated PS3N/1CGFP, PS1N/3CGFP, PS1(1–1173)/3(1154–1501)-GFP and PS1(1–1436)/3(1420–1501)GFP (see Table 1 for details).

Interestingly, expression of proteins with homologous substitution of either the N- or the C-terminal half of Cdr1p with Cdr3p (PS3N/1CGFP, PS1N/3CGFP) resulted in nonfunctional recombinant strains. In contrast to wild-type Cdr1p, cells expressing either of the chimeras showed no resistance to drugs on spot and MIC_{80} assays (Fig. 2A, B). This enhanced supersensitivity of recombinant strains expressing either of the chimeras may be linked to their poor expression and localization. To check this, we examined the localization of wild-type Cdr1p and CDR1/CDR3GFP chimeric proteins by confocal microscopy. The confocal images revealed that unlike the rimmed appearance of Cdr1pGFP in cells expressing wild-type Cdr1p, small patches of chimeric PS3N/1CGFP protein on the cell surface were apparent, whereas surface localization of PS1N/3CGFP protein was more severely affected since its fluorescence appeared trapped intracellularly (Fig. 3A). Western blot analysis of PM proteins isolated from PS3N/1CGFP and PS1N/3CGFP chimeras confirmed the confocal microscopy results. None of the PM fractions isolated from these chimeras showed any detectable protein (Fig. 3B, a). However, a faint band was observed with CM preparations of PS3N/1CGFP (Fig. 3B, c). PM-ATPase was used as a marker to check the purity of PM fraction (Fig. 3B, b).

A



B

	MIC_{80} (μg ml^{-1})						
STRAIN	FLC	MIC	CYH	ANISO	KTC	ITC	R6G
AD1-8u⁻	0.5	0.015	0.015	0.12	0.015	0.015	0.03
PSCDR1GFP	64	2	1	32	2	4	32
PS1N/3CGFP	0.5	0.015	0.015	0.12	0.015	0.015	0.03
PS3N/1CGFP	0.5	0.015	0.015	0.12	0.015	0.015	0.03
PS1(1-1173)/3(1154-1501)GFP	0.5	0.015	0.015	0.12	0.015	0.015	0.03
PS1(1-1436)/3(1420-1501)GFP	64	2	1	32	2	4	32

Fig. 2. Drug-resistance profile of *S. cerevisiae* cells expressing wild-type Cdr1GFP and CDR1/CDR3GFP chimeric proteins determined by the spot and MIC assays. (A) Spot assay: 5 μl samples of fivefold serial dilutions of each yeast strain (cells suspended in a normal saline to OD_{600} 0·1) were spotted on YEPD plates in the absence (control) or in the presence of fluconazole (FLC; 1 μg ml^{-1}), miconazole (MIC; 90 ng ml^{-1}), cycloheximide (CYH; 80 ng ml^{-1}), anisomycin (ANISO; 800 ng ml^{-1}), ketoconazole (KTC; 125 ng ml^{-1}), itraconazole (ITC; 100 ng ml^{-1}) and rhodamine 6G (R6G; 6 μg ml^{-1}). Cell growth was monitored after 48 h incubation of plates at 30 °C. (B) MIC assay: determined following National Committee For Clinical Laboratory Standards.

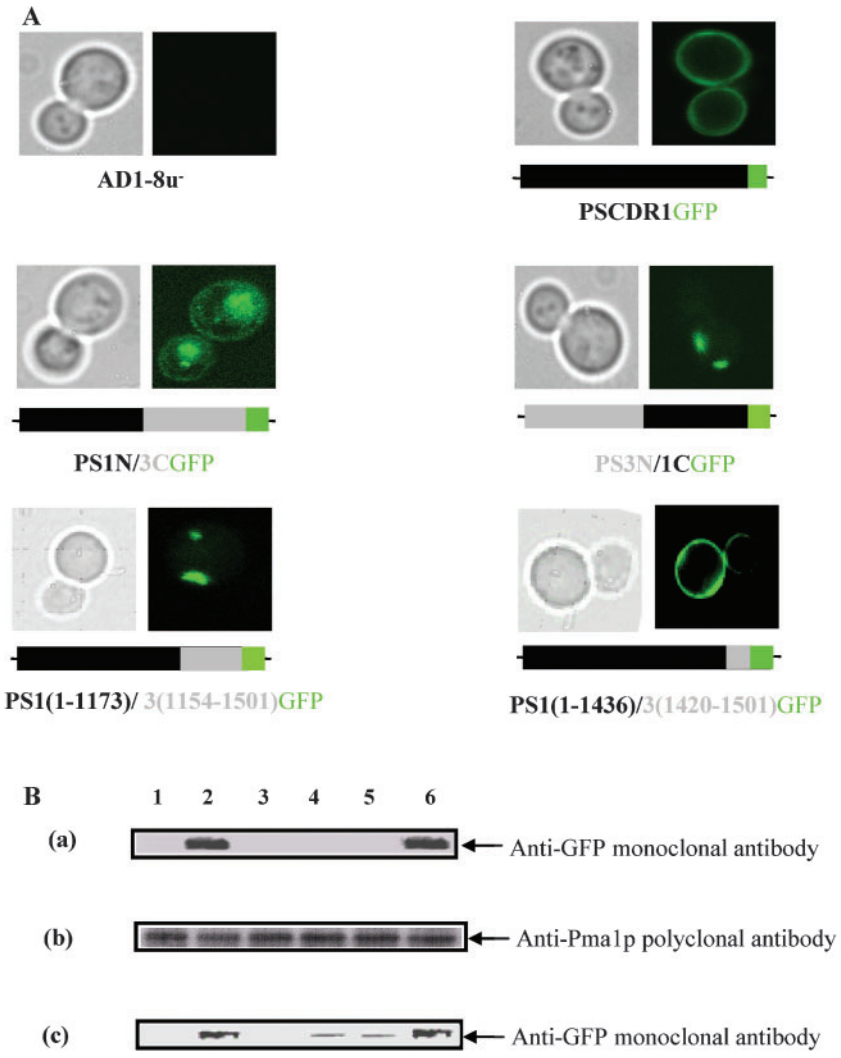


Fig. 3. Localization and expression of wild-type Cdr1GFP and CDR1/CDR3GFP chimeric proteins. (A) Confocal images of AD1-8u$^-$ and cells expressing wild-type Cdr1GFP and CDR1/CDR3GFP chimeric proteins. (B) Expression of wild-type Cdr1GFP and CDR1/CDR3GFP chimeric protein in *S. cerevisiae*: PM (20 μg) proteins from AD1-8u$^-$ (lane 1), PSCDR1GFP (lane 2), PS1N/3CGFP (lane 3), PS3N/1CGFP (lane 4), PS1(1–1173)/3(1154–1501)GFP (lane 5) and PS1(1–1436)/3(1420–1501)GFP (lane 6) were separated on an 8% SDS-PAGE, electroblotted onto a nitrocellulose membrane and probed with monoclonal anti-GFP antibody (a) and rabbit polyclonal anti-Pma1p antibody (b). (c) CM (30 μg) proteins from AD1-8u$^-$ (lane 1), PSCDR1GFP (lane 2), PS1N/3CGFP (lane 3), PS3N/1CGFP (lane 4), PS1(1–1173)/3(1154–1501)GFP (lane 5) and PS1(1-1436)/3(1420-1501)GFP (lane 6) probed with monoclonal anti-GFP antibody. Proteins were immunodetected by chemiluminescence using an ECL kit (Amersham).

Functional replacement of TMS12 of Cdr1p with the homologous TMS of Cdr3p

Since swapping of the N- and C-terminal homologous halves of Cdr1p with Cdr3p yielded non-functional recombinant strains (Figs 2 and 3), we sequentially replaced the Cdr3p region with Cdr1p, selecting the inactive chimera, PS1N/3CGFP, as a framework to restore Cdr1p-like function. Consequently, two chimeras were constructed, one comprising Cdr1p up to the C-terminal NBD [PS1(1–1173)/3(1154–1501)GFP] and the other comprising Cdr1p up to TMS11 [PS1(1–1436)/3(1420–1501)GFP]. As shown in Fig. 2, recombinant strains expressing a chimeric protein comprising the Cdr1p segment up to the C-terminal NBD in PS1(1–1173)/3(1154–1501)GFP remained non-functional. The poor functioning of the PS1(1–1173)/3(1154–1501)-GFP chimera in the recombinant strain was apparently due to its poor cell surface localization as was revealed by confocal and Western analysis (Fig. 3). These results further emphasize the importance of the entire TMD region for the synthesis and assembly of active protein. Interestingly, the replacement of TMS12 of Cdr1p with the homologous TMS of Cdr3p, as in PS1(1–1436)/3(1420–1501)GFP, resulted in complete restoration of Cdr1p activity, which was evident from the drug resistance profile of the recombinant strain (Fig. 2). Thus cells expressing the PS1(1–1436)/3(1420–1501)GFP chimera displayed increased resistance to all the tested drugs, which was comparable to that seen with the wild-type protein (Fig. 2).

The functionality of PS1(1–1436)/3(1420–1501)GFP was further confirmed by analysing ATPase activity, efflux of the fluorescent substrate rhodamine 6G and accumulation of radiolabelled fluconazole. For this, the purified PM protein fractions isolated from PSCDR1GFP and CDR1/CDR3GFP chimeras were analysed for their oligomycin-sensitive ATPase activity. Cells expressing wild-type CDR1GFP and chimeric 1(1–1436)/3(1420–1501)GFP protein showed comparable oligomycin-sensitive ATPase activity, in contrast to the rest of the chimeric proteins (Fig. 4A). In addition, both PSCDR1GFP and PS1(1–1436)/3(1420–1501)GFP cells showed similar levels of rhodamine 6G efflux and fluconazole accumulation (Fig. 4B, C). As expected, confocal images and Western blot analysis of PS1(1–1436)/

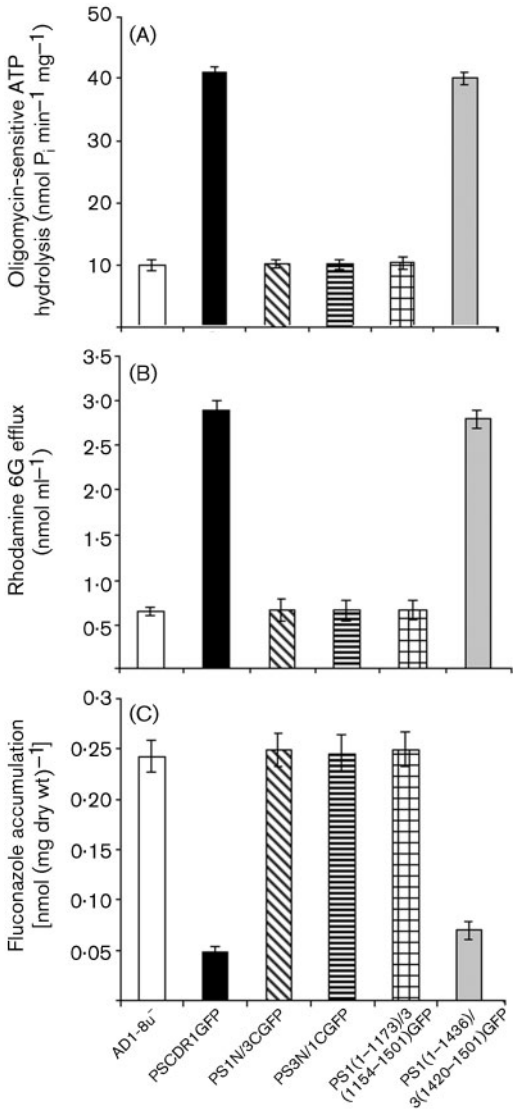


Fig. 4. Functional characterization of *CDR1/CDR3* GFP chimeras. (A) Oligomycin-sensitive ATPase activity in the PM proteins of cells expressing wild-type Cdr1GFP and CDR1/CDR3 GFP chimera. ATPase activity was determined as described in Methods. The results are the means ± SD of three independent experiments. (B, C) Comparison of rhodamine 6G efflux (B) and fluconazole accumulation (C) in cells expressing wild-type Cdr1GFP and CDR1/CDR3GFP. Rhodamine 6G efflux and fluconazole accumulation were determined as described in Methods. The values are the means ± SD of four independent experiments.

3(1420–1501)GFP confirmed the proper surface localization and expression of this chimeric protein (Fig. 3).

The N-terminal and C-terminal NBDs of Cdr1p are non-exchangeable

One of the unresolved issues concerning the function of Cdr1p is whether both NBDs are equivalent, and thus interchangeable, or whether each functions only within the context of a specific membrane environment provided by TMDs. Our previous study has shown (Jha *et al.*, 2003a, b, 2004) that the unique positioning of the catalytically important residues C193 in Walker A of NBD1 and K901 in Walker A of NBD2 could not be exchanged. Hence a construct like CDR1-1C/1NGFP was not used; instead, to address this question, two constructs were instead made, one having Cdr1p with cytoplasmic hydrophilic domains comprising two N-terminal NBDs and another with two C-terminal NBDs (Fig. 5A). These constructs were integrated in *S. cerevisiae* mutant AD1-8u$^-$ as described previously for *CDR1/CDR3* chimeras and checked for single-copy integration (data not shown). The resultant variant proteins Cdr1-1N/1NGFP and Cdr1-1C/1CGFP were analysed for their functionality using two independent drug sensitivity assays. As shown in Fig. 5(B), recombinant strains expressing both types of NBD variants were hypersensitive to all the tested drugs as compared to those expressing wild-type CDR1GFP. The drug sensitivities revealed by spot assay generally matched well with the MIC_{80} test (data not shown). Although both variants of NBDs were hypersensitive to the tested drugs, confocal images revealed differences in their surface localization. While the variant Cdr1-1N/1NGFP, consisting of hydrophilic domains with two N-terminal NBDs, revealed poor cell surface localization, the variant having two C-terminal NBDs showed altogether very poor GFP fluorescence (Fig. 5C).

The Cdr1-1N/1NGFP variant can be brought to the cell surface by growing cells in the presence of Cdr1p substrates

We had earlier observed that a non-functional TMS6 mutant variant (ΔF774) of Cdr1p could be functionally restored to the cell surface if the cells were exposed to drug substrates which act as powerful 'chaperones' for processing misfolded proteins (Shukla *et al.*, 2003). We therefore checked whether the mislocalization of the CDR1/CDR3 chimeric proteins and Cdr1p NBDs variants could be similarly improved. For this, we added different drug substrates of Cdr1p separately, immediately after the lag phase of the *S. cerevisiae* cells (4–5 h). The cells were grown for a further 7 h, then checked by confocal microscopy for the localization of the protein. Of the two NBD variants only Cdr1-1N/1NGFP protein showed improved surface localization with increasing concentrations of drugs (shown for cycloheximide in Fig. 6A, a–f). The surface expression level of the Cdr1-1N/1NGFP variant could be restored to that of wild-type CDR1GFP at 50 ng cycloheximide ml^{-1} (Fig. 6A, a–h). Interestingly, PM protein isolated from Cdr1-1N/1NGFP cells grown in increasing concentrations of cycloheximide also showed a concentration-dependent increase in the amount of variant protein in the PM fraction (Fig. 6B, a), while the presence of drug had no effect in the cells expressing wild-type CDR1GFP (Fig. 6A, g, h; Fig. 6B, a), thus suggesting that there was no inhibition of protein synthesis at the concentration of cycloheximide used for rescue experiments. Additionally, other substrates of Cdr1p, such as fluconazole,

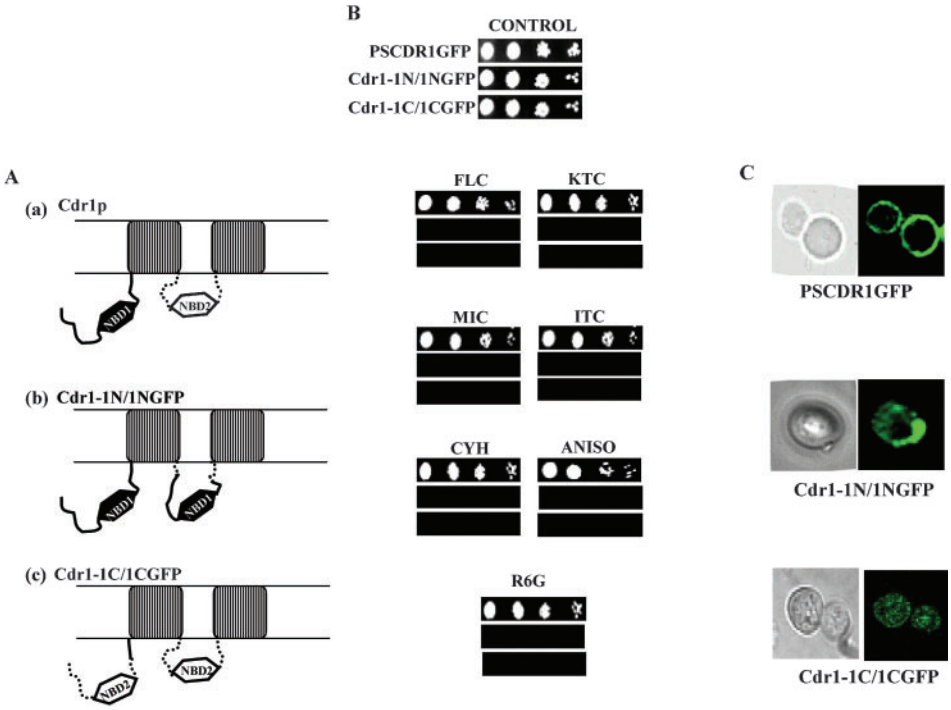


Fig. 5. Schematic representation, drug sensitivity and localization of NBD variants of Cdr1p. (A) Topological model of wild-type Cdr1p comprising N- and C-terminal NBDs (a) and NBD variants of Cdr1p with cytoplasmic hydrophilic domains comprising either two N-terminal NBDs (b) or two C-terminal NBDs (c). The N-terminal NBD is depicted by a filled hexagon and C-terminal NBD by an open hexagon. (B) Drug resistance profile of wild-type and NBD variants of Cdr1GFP determined by spot assay. Samples (5 μl) of fivefold serial dilutions of each yeast strain (cells suspended in a normal saline to OD_{600} 0·1) were spotted on YEPD plates in the absence (control) or in the presence of fluconazole (FLC; 1 μg ml^{-1}), ketoconazole (KTC; 125 ng ml^{-1}), miconazole (MIC; 90 ng ml^{-1}), itraconazole (ITC; 100 ng ml^{-1}), cycloheximide (CYH; 80 ng ml^{-1}), anisomycin (ANISO; 800 ng ml^{-1} and rhodamine 6G (R6G; 6 μg ml^{-1}). Cell growth was monitored after 48 h incubation of plates at 30 °C. (C) Confocal images of *S. cerevisiae* cells expressing GFP-tagged Cdr1p and its NBD variants.

ketoconazole, miconazole, itraconazole and anisomycin, were also tested to improve the localization of chimeric proteins. Of all the substrates tested, only anisomycin could fully rescue the Cdr1-1N/1NGFP variant protein to the PM (Fig. 6A, k), while miconazole and itraconazole were able to rescue the Cdr1-1N/1NGFP variant protein partially to the PM (Fig. 6A, i, j). The presence of drugs in cells expressing variant protein with both C-terminal NBDs (Cdr1-1C/1CGFP) or CDR1/CDR3 chimeric proteins did not improve localization (data not shown). It is noteworthy that no growth difference was observed between strains harbouring variant protein, chimeric protein and wild-type CDR1GFP protein in the absence or presence of the concentrations of the drugs used for the rescue experiments (data not shown).

Rescued Cdr1-1N/1NGFP protein remains non-functional

To test whether improved localization of Cdr1-1N/1NGFP variant protein led to the resumption of its transport function, the efflux of rhodamine 6G was measured. The control AD1-8u$^-$, PSCDR1GFP and Cdr1-1N/1NGFP cells were grown in the presence and absence of cycloheximide (50 ng ml^{-1}) for 7–8 h, and efflux of rhodamine 6G was assayed as described in Methods. The results depicted in Fig. 6(C) show that rescued Cdr1-1N/1NGFP protein was unable to mediate efflux of rhodamine 6G in the presence of cycloheximide. In contrast to this, as observed earlier, SS6G (ΔF774) cells when grown in the presence of drug showed not only improved localization (Shukla *et al.*, 2003) but also restoration of rhodamine 6G efflux. These results suggest that although Cdr1p substrates can rescue the folding defects in one of the NBD variants Cdr1-1N/1NGFP, the protein remains non-functional. Notably, there was some reduction in the efflux of rhodamine 6G by wild-type CDR1GFP cells grown in the presence of cycloheximide. The decrease in efflux by the cells expressing wild-type Cdr1p is probably due to the fact that cycloheximide, which is also a substrate for the Cdr1p transporter, competes with rhodamine 6G.

Rescued Cdr1-1N/1NGFP protein is capable of binding Cdr1p substrates but unable to hydrolyse ATP

To address the question whether the rescued Cdr1p variant having two identical N-terminal NBDs is non-functional

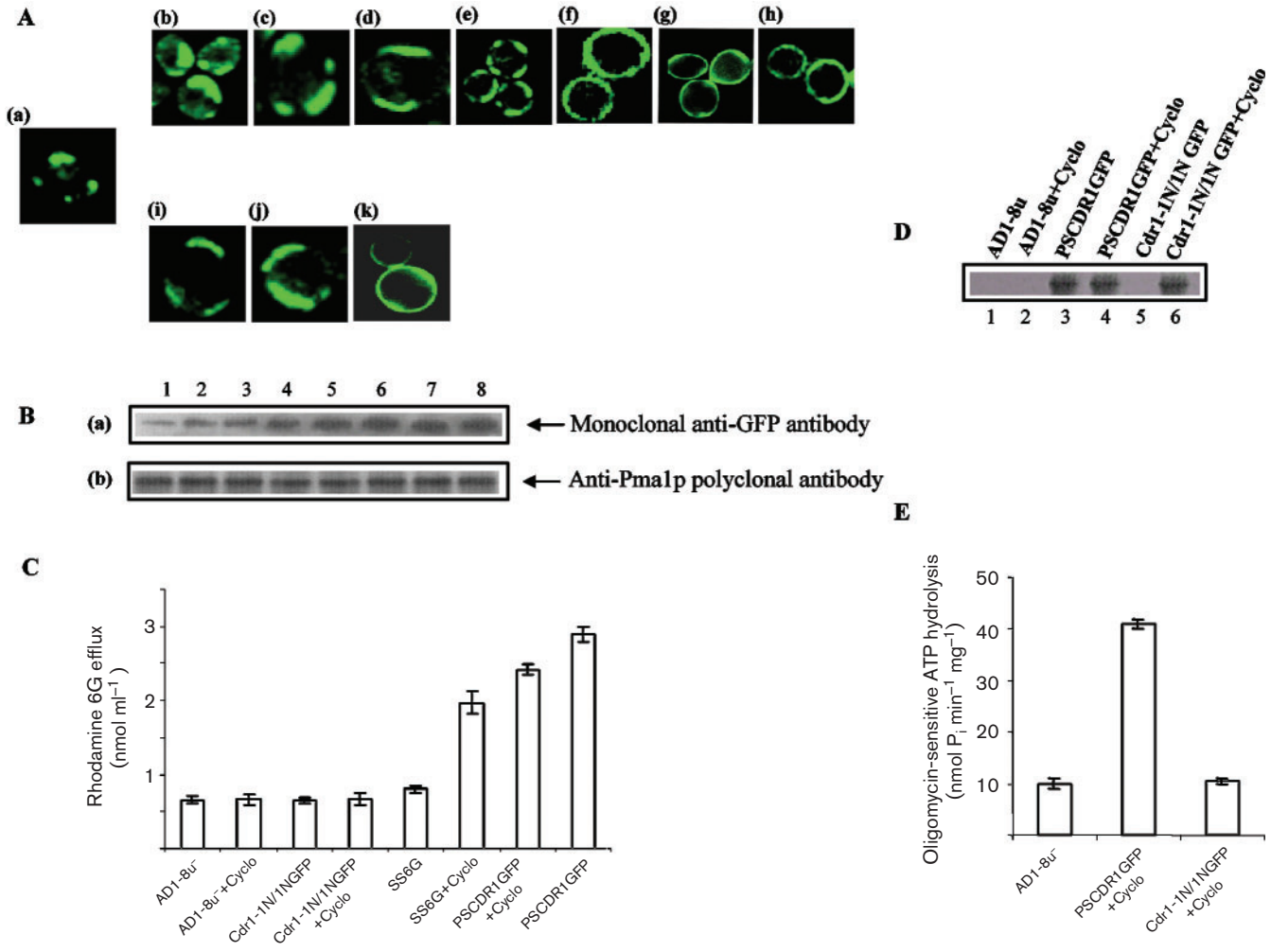


Fig. 6. Properties of the Cdr1-1N/1N variant. (A) (a–f) Confocal pictures of cells expressing Cdr1-1N/1N variant grown in the presence of increasing concentrations of cycloheximide. Cells expressing Cdr1-1N/1N variant grown without drug substrate (a) or in the presence of the following concentrations of cycloheximide (ng ml^{-1}): 10 (b), 20 (c), 30 (d), 40 (e), 50 (f). (g, h) PSCDR1GFP cells expressing wild-type Cdr1GFP grown in the absence and presence of 50 ng cycloheximide ml^{-1} respectively. (i–k) Cells expressing Cdr1-1N/1N variant grown with 50 ng ml^{-1} of miconazole, itraconazole and anisomycin respectively. (B) Expression of Cdr1-1N/1N variant grown in the presence of increasing concentrations of cycloheximide. The PM proteins from Cdr1-1N/1N variant (lane 1), Cdr1-1N/1N variant grown in the presence of increasing concentrations of cycloheximide [10 (lane 2), 20 (lane 3), 30 (lane 4), 40 (lane 5) and 50 (lane 6) ng ml^{-1}], and PSCDR1GFP cells grown in the absence and presence of cycloheximide (50 ng ml^{-1}) (lane 7 and 8 respectively), were separated on a 8% polyacrylamide gel, electroblotted onto a nitrocellulose membrane and incubated with mouse anti-GFP monoclonal antibody (a) and rabbit polyclonal anti-Pma1p antibody (b). Proteins were detected by chemiluminescence using an ECL kit (Amersham). (C) Rhodamine 6G efflux from Cdr1-1N/1NGFP-expressing cells grown in the presence of cycloheximide. The rhodamine 6G efflux by AD1-8u$^-$, Cdr1-1N/1NGFP, SS6G (ΔF774) and PSCDR1GFP cells was determined as described in Methods. The results are the means ± SD of three independent experiments. (D) [^{3}H]Azidopine labelling of AD1-8u$^-$, Cdr1-1N/1NGFP and PSCDR1GFP. PM protein (25 μg) was labelled with azidopine as described in Methods. (E) Oligomycin-sensitive ATPase activity in the PM proteins of cells expressing Cdr1-1N/1NGFP and wild-type Cdr1GFP. The ATPase activity of AD1-8u$^-$, wild-type Cdr1GFP and Cdr1-1N/1N variant protein was determined in the PM fraction as described in Methods. The results are the means ± SD of three independent experiments.

because of impairment in the substrate binding or ATP hydrolysis, we performed photoaffinity labelling with azidopine, a dihydropyridine analogue, and also measured ATPase activity with the PM protein fraction isolated from the wild-type and the Cdr1p N-terminal NBDs variant grown in the presence of cycloheximide (50 ng ml^{-1}). As shown in Fig. 6(D), comparable azidopine labelling was observed with PM isolated from wild-type CDR1GFP and

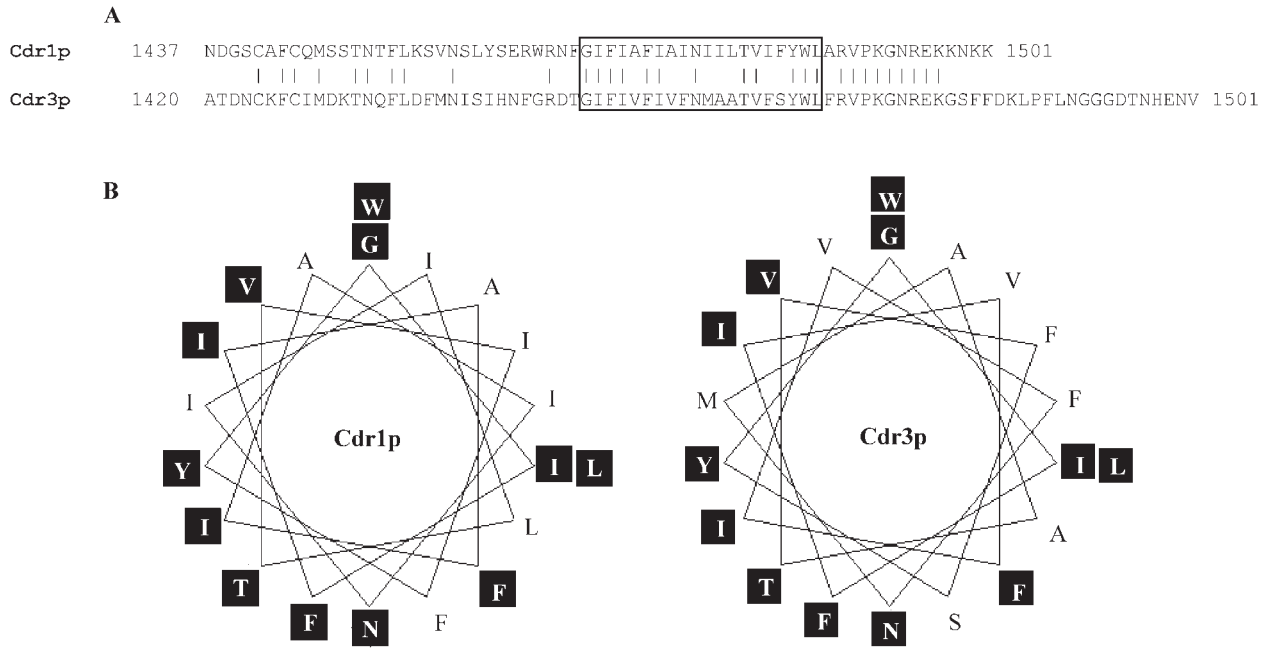


Fig. 7. Sequence alignment and helical wheel projection of the predicted TMS12 of Cdr1p with Cdr3p. (A) Sequence alignment of amino acid residues of Cdr1p replaced with homologous residues of Cdr3p in PS1(1–1436)/3(1420–1501)GFP chimera. The putative TMS12 is boxed. Identical residues are linked by vertical bars. (B) The TMSs of the amino acid sequences of Cdr1p and Cdr3p were determined with the program HMMTOP (Tusnady & Simon, 1998, 2001). The 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 (Rice *et al.*, 2000). White letters on a black background show amino acids that are identical between Cdr1p and Cdr3p.

the Cdr1-1N/1NGFP variant. However, the Cdr1-1N/1NGFP variant showed impaired ATPase activity as compared to wild-type CDR1GFP (Fig. 6E).

DISCUSSION

The molecular mechanisms which govern the function of Cdr1p or Cdr2p as efflux pumps for azoles are not well understood, and information is needed (i) to understand how the protein can bind a structurally diverse range of compounds including different azoles, (ii) to define the drug-substrate binding and (iii) to determine how ATP binding and hydrolysis are linked to the drug transport. In an effort to develop an understanding of the molecular details of the drug binding and transport, we recently overexpressed Cdr1p as a GFP-tagged fusion protein in a heterologous hyper-expression system of *S. cerevisiae* and, by employing site-directed mutagenesis, characterized drug and nucleotide binding (Shukla *et al.*, 2003, 2004). We observed that several point mutations resulted in mutant variants of Cdr1p that were hypersensitive to different drugs (Jha *et al.*, 2004; Shukla *et al.*, 2003, 2004).

Our current study focused on examining the functional relevance of the two NBDs of Cdr1p and identifying candidate protein segments that are important for substrate recognition and binding. To address these questions, we exchanged homologous segments of Cdr1p with corresponding segments of the non-drug transporter Cdr3p. This study shows that any exchange of either the N- or the C-terminus of Cdr1p with the homologous portion of Cdr3p results in non-functional recombinant strains (Fig. 2). It seems that the loss of activity of CDR1/CDR3GFP chimeric proteins could be attributed to the poor cell surface localization of these proteins (Figs 2 and 3), which could also contribute to their enhanced degradation. These results, however, do emphasize that although Cdr1p and Cdr3p have great topological and sequence similarities, this resemblance is not symmetrically distributed between the two halves of the proteins; positioning of both the halves of the individual proteins is probably essential for their distinct functions. Although the sequence identity between Cdr1p and Cdr3p at the N-terminus and C-terminus is as high as 54 % and 58 %, respectively, it is distinct enough to make the former a drug transporter and latter a phospholipid translocator (Prasad *et al.*, 1995; Smriti *et al.*, 2002). By employing a similar chimeric approach, Zhou *et al.* (1999) earlier reported that while the N-terminal halves of human Mdr1p and Mdr2p could be switched, this degree of exchangeability was not found between the C-terminal halves. In the present case, unlike *MDR1/MDR2* chimeras, even the replacement of the N-terminal part of Cdr1p with the corresponding

region of Cdr3p did not yield functional recombinant strains. This implies that in spite of the high similarities between the two homologous proteins, the TMDs of Cdr1p and of Cdr3p cannot be exchanged.

Interestingly, replacement of residues 1437–1501 (which include TMS12) of Cdr1p by the corresponding stretch of Cdr3p resulted in a recombinant strain expressing a functional chimeric Cdr1p variant. It would thus seem that this extreme C-terminus probably does not contribute to the functional difference detected between Cdr1p and Cdr3p. We had earlier observed that the deletion of a 79 amino acid stretch from the C-terminal end of Cdr1p, which encompasses TMS12 of this transporter, resulted in impaired resistance to certain drugs (Krishnamurthy *et al.*, 1998b). Combining the two observations, it seems likely that TMS12 of Cdr1p does harbour drug-binding site(s), which can be exchanged with TMS12 of Cdr3p in the PS1(1–1436)/3(1420–1501)GFP chimeric protein. A closer look at the TMS12 sequence of the two ABC proteins suggests that this may be the case. Comparison of the amino acid sequence of Cdr1p with that of Cdr3p in this residue 1437–1501 segment revealed an overall 38 % identity and the sequence identity within TMS12 of the two proteins becomes as high as 57 % (Fig. 7A). The evident functional exchangeability of TMS12 between Cdr1p and Cdr3p provides clues about residues present in the TM12 that may be important for substrate-binding funtion. A site-directed mutagenesis approach could identify such common residues between the two homologous proteins.

Studies on various ABC transporters have revealed that ATP hydrolysis and substrate transport are strongly dependent on cooperation between NBD1 (N-terminal) and NBD2 (C-terminal) (Walmsley *et al.*, 2003). Interestingly, although NBD1 of Cdr1p contains the conserved Walker A motif (GRPGAGCST), the commonly conserved lysine residue within the Walker A motif is replaced by an uncommon cysteine that appears to be a unique feature of most of the fungal ABC transporters (Jha *et al.*, 2004). Conversely, NBD2 of Cdr1p contains the commonly conserved lysine (GASGAGKT) at the equivalent position in its Walker A motif. There is a complete lack of understanding with regard to the functional equivalence of both the NBDs of Cdr1p and the significance of the variation in their Walker A amino acid sequence (Jha *et al.*, 2003a, b). Our more recent results show that both the NBDs are essential for Cdr1p function, albeit asymmetrically, and the positioning of the cysteine and lysine residues within the respective Walker A motifs is functionally not interchangeable (Jha *et al.*, 2004). In order to further probe the role of NBDs in Cdr1p, in this study we constructed Cdr1p variants possessing either both N-terminal NBDs or both C-terminal NBDs. Our results show that the substitution of either NBD yielded non-functional recombinant strains. Such a situation has previously been encountered with mammalian MDR proteins. For example, CFTR chimeric molecules comprising either two N-terminals or C-terminal NBDs having core NBD sequence or the flanking sequence in addition to core NBD were non-functional because of trafficking defects (Pollet *et al.*, 2000). Similarly, mouse mdr3 and human MDR1 chimeric proteins comprising two C-terminal NBDs resulted in the synthesis of non-functional protein owing to decreased accumulation or altered targeting of the protein to the membrane (Beaudet & Gros, 1995; Hrycyna *et al.*, 1999). Of note, recently a Walker A mutation (K86M) of an ABC half-transporter ABCG2 was shown to affect oligomerization and surface targeting followed by retrieval to the endoplamic reticulum (Henriksen *et al.*, 2005). Interestingly, in the present study, one of the NBD variants of Cdr1p (having both N-terminal NBDs) could be rescued to the PM if cells expressing this protein were exposed to drug substrates. The exact mechanism by which these specific drug substrates facilitate processing of probably misfolded protein is not known. A possible explanation, as suggested in case of P-gp and CFTR protein, is that the drug-binding site(s) in the protein are formed early in the folding intermediates during biosynthesis. It is possible that the occupation of these drug-binding site(s) in the early stages of folding may reduce the concentration of the intermediate that is prone to self-aggregation, thus stabilizing the folding intermediates in a conformation that resembles the wild-type protein, which in turn helps in escaping the cell's quality control mechanism (Loo & Clarke, 1997; Morello *et al.*, 2000).

It is noteworthy that the non-functionality of rescued N-terminal NBD variant protein was not due to any impairment in substrate binding since [^{3}H]azidopine labelling remained unaffected by this substitution of NBDs. Therefore, the functional defect in spite of proper localization was not due to impaired drug binding but rather to a poor ability to hydrolyse ATP, which could be attributed to the loss of communication between the two NBDs or between NBDs and TMDs when exchanged. One must not ignore the flanking sequences as well as the sequence stretches between the Walker A, Walker B and signature C motifs of NBDs, which if exchanged may also contribute to the non-functionality of the variants.

The two NBDs of a number of ABC transporters have been shown to be functionally dissimilar. Interestingly, in the case of human P-gp, which is a close homologue of Cdr1p, the two NBDs were partially interchangeable. For example Pgp-1N/1N, containing two N-terminal NBDs, was functional and was also asymmetric with respect to 8-azido-ATP labelling, suggesting that the context of the ATP site rather than its exact sequence is an important determinant for ATP binding. Pgp-1C/1C and Pgp-1C/1N variants were, however, defective in cell surface expression and function (Hrycyna *et al.*, 1999). In prokaryotic ABC-type transporters such as the histidine permease of *E. coli*, both NBDs are functionally identical and contribute equally to the protein's activity. Inactivation of either one of these NBDs in the full protein resulted in 50 % of the activity (Nikaido & Ames, 1999). On the other hand, there is clear evidence to indicate

asymmetry of function of the NBDs of MRP1 and CFTR (Aleksandrov *et al.*, 2002; Gao *et al.*, 2000).

In conclusion, our analysis of Cdr1p/Cdr3p chimeras shows that neither the N- nor the C-terminal half of Cdr1p can be exchanged with the homologous portion of Cdr3p. The exchange of domains results in altered interfaces leading to non-functional recombinant strains expressing chimeric proteins. We have also confirmed our earlier observation that the N- and C-terminal NBDs of Cdr1p are functionally asymmetric. These results provide important clues to our understanding of the complex interactions between the NBDs and their neighbouring TMDs and should help in resolving mechanisms of drug efflux of structurally unrelated compounds mediated by Cdr1p.

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