Chapter 18
Structure–Function Analyses of Multidrug Transporters

Erwin Lamping, Golnoush Madani, Hee Ji Lee, Masakazu Niimi and Richard D. Cannon

Abstract Proteins catalysing the transport of molecules across biological membranes are vital for organisms in all kingdoms of life. These proteins are needed for the uptake of nutrients and the efflux of signalling molecules and toxic compounds. In the human opportunistic pathogen *Candida albicans,* efflux proteins can translocate antifungal drugs, such as fluconazole, and confer drug resistance. There are two main families of membrane proteins involved in drug transport, the major facilitator superfamily (MFS) and the ATP-binding cassette (ABC) proteins. Both types of protein possess multiple membrane spanning α-helices in transmembrane domains (TMDs), and ABC proteins, in addition, contain cytosolic nucleotide-binding domains (NBDs) involved in ATP hydrolysis. ABC proteins, and to a lesser extent MFS proteins, have broad substrate specificities that are determined by the structure and arrangement of the transmembrane α-helices. The multidrug transporter most often associated with drug resistance of *C. albicans* clinical isolates is ABC protein Cdr1. This is a pleiotropic drug resistance (PDR) ABC protein with domain arrangement NBD_1-TMD_1-NBD_2-TMD_2 and unique large extracellular loops between transmembrane α-helices that may be important for pump function. There is no crystal structure for Cdr1, but X-ray structures of related proteins indicate that unique interactions between conserved elements of the TMDs and the NBDs are important for pump function.

E. Lamping · G. Madani · H.J. Lee · M. Niimi · R.D. Cannon (✉)
Sir John Walsh Research Institute, Faculty of Dentistry, University of Otago, PO Box 56, Dunedin 9054, New Zealand
e-mail: richard.cannon@otago.ac.nz

E. Lamping
e-mail: erwin.lamping@otago.ac.nz

M. Niimi
e-mail: masa.niimi@otago.ac.nz

M. Niimi
Mycology Unit, Department of Microbiology, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand

18.1 Introduction

Fungi possess a variety of integral membrane proteins, the functions of which are to transport substances across biological membranes (Perlin et al. 2014). They are present in many cellular membranes, including vacuolar, mitochondrial, peroxisomal and endoplasmic reticulum membranes, with many important transporters located in the plasma membrane. The plasma membrane is a vital permeability barrier for cellular homeostasis. This barrier stops the free flow of ions, small molecules and macromolecules across the membrane and maintains chemical and electrochemical gradients between the inside and the outside of the fungal cell. In order to transport molecules across this barrier, the fungal cell utilises integral membrane proteins. If the molecule moves down a concentration gradient facilitated by membrane protein channels or pores, then the transport protein simply accelerates passive diffusion, and no energy is required for this process. In many instances, however, molecules are transported against a concentration gradient, and energy is required.

Energy-dependent transport is carried out predominantly by proteins belonging to one of two large protein families, either the ATP-binding cassette (ABC) transporter family, or the major facilitator superfamily (MFS). ABC transporters are primary active transporters that use the energy released by ATP hydrolysis to power transport, whereas MFS proteins are secondary active transporters that utilise electrochemical gradients (e.g. Na^+ or H^+ gradients established by primary active transporters) across the membrane as their energy source. Fungi generate many compounds that are toxic to the cell unless effluxed such as secondary metabolites or endpoints of metabolic pathways including ethanol and organic acids. Fungi also encounter xenobiotics from their environment, which are kept outside the cell by multiple efflux proteins in plasma membranes. Multidrug efflux proteins transport a variety of substrates, and the range of substrates for different pumps can overlap. It is interesting to note that fungi that inhabit a variety of environmental niches, such as *Cryptococcus neoformans,* contain a large repertoire of 33 ABC (Kovalchuk and Driessen 2010) and 150–170 MFS proteins (Janbon et al. 2014). Another example of species that inhabit a variety of niches are *Aspergillus* spp. with 50–70 ABC proteins (Kovalchuk and Driessen 2010) and 275–356 MFS transporters (Coleman and Mylonakis 2009). In contrast, the fungus *Candida albicans*, which is almost entirely associated with mammalian hosts, possesses a more limited range of 26 ABC (Kovalchuk and Driessen 2010) and 85–95 MFS transporters (Coleman and Mylonakis 2009; Gaur et al. 2008), possibly because of its exposure to a smaller range of xenobiotics.

A complication of treating fungal infections with antifungal drugs is that fungal genomes encode transporters that are able to export these drugs out of the cells

thereby rendering the fungi resistant. It has been found that the strong selective pressure exerted by antifungal drugs can select for mutations in transcriptional regulators that result in over-expression of ABC or MFS transporters. For example, the high-level azole resistance of *C. albicans* has been shown to be due to the over-expression of MFS protein Mdr1 or, most frequently, the over-expression of the ABC proteins Cdr1 and Cdr2 (Cannon et al. 2009). Over-expression of Mdr1 can be caused by gain-of-function mutations in transcription factor Mrr1 (Dunkel et al. 2008), whereas the over-expression of Cdr1 and Cdr2 is frequently caused by gain-of-function mutations in transcription factor Tac1 (Coste et al. 2007; Coste et al. 2004). This chapter describes the multidrug transporters of *C. albicans*, focusing on the clinically more significant ABC proteins, and current knowledge of structure–function relationships for these proteins.

18.2 Multidrug Efflux Transporters of *C. albicans*

18.2.1 MFS Transporters

MFS transporters are integral membrane proteins with, most commonly, either 12 or 14 transmembrane spans (TMSs); exceptions do exist with one MFS transporter predicted to have 6 and another small MFS family predicted to have 24 TMSs (Law et al. 2008; Reddy et al. 2012). *C. albicans* possesses an estimated 95 MFS transporters (Gaur et al. 2008) that can be divided into 17 separate phylogenetic lineages. The four major MFS families are the SP (sugar porter), the DHA1 (drug: H^+ antiporter 1), the DHA2 and the ACS (anion:cation symporter) family with 22, 22, 9 and 16 representatives, respectively (Gaur et al. 2008). SP-, DHA1- and ACS-type transporters are predicted to have 12 TMSs and DHA2 members have 14 TMSs. The *C. albicans* genome encodes between 26 (Costa et al. 2014) and 31 (Gaur et al. 2008) DHA1 and DHA2 MFS transporters. Few of these ORFs have been characterised and only one, Mdr1, is thought to be a clinically relevant MFS multidrug efflux pump. *C. albicans* Mdr1, a DHA1 protein (Fig. 18.1 and Table 18.1) also called Ben[r], was originally identified by its ability to confer benomyl and methotrexate resistance on *Saccharomyces cerevisiae* (Fling et al. 1991). It prefers generally less hydrophobic and less branched substrates than *C. albicans* Cdr1 (Puri et al. 2010), and it transports compounds such as fluconazole (FLC) and ketoconazole (KTZ), but not miconazole (MCZ) or itraconazole (ITC) (Lamping et al. 2007). High levels of *MDR1* expression have been detected in certain azole-resistant clinical isolates of *C. albicans* (Sanglard et al. 1995; White et al. 2002; Wirsching et al. 2000). In general, MFS multidrug efflux transporters efflux a narrower range of substrates than ABC multidrug efflux proteins (Puri et al. 2010; Sa-Correia et al. 2009). Mdr1 expression is up-regulated in clinical isolates by gain-of-function mutations in transcription factor Mrr1 (Dunkel et al. 2008; Morschhauser et al. 2007). For further details on the transcriptional control of
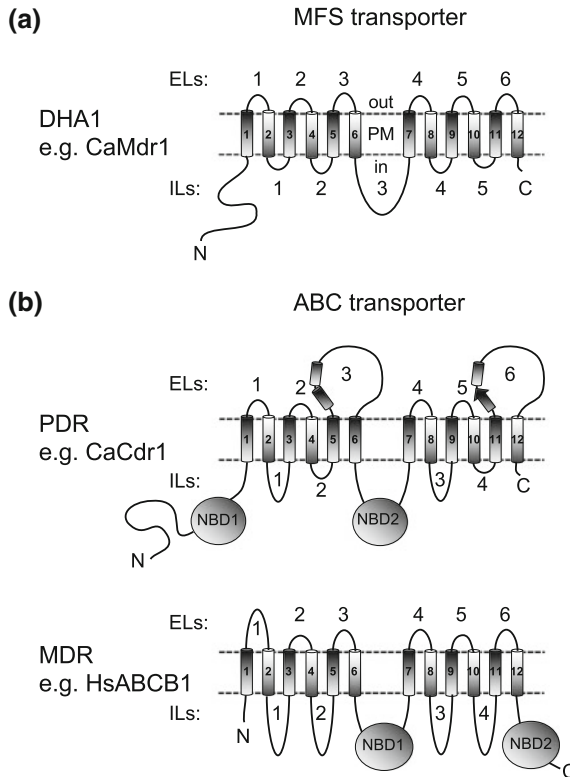
Fig. 18.1 Basic topologies of MFS, and PDR and MDR ABC transporters. *Cylinders* indicate α-helices, the *arrow* indicates a *β*-sheet, *ovals* indicate cytosolic NBDs, and the plasma membrane (PM) separating the inside (*in*) from the outside (*out*) of the cell is represented by two *dashed lines*. **a** General topology of DHA1 MFS transporters such as *C. albicans* Mdr1 (Ca Mdr1). DHA1 MFS transporters typically comprise ∼500 amino acids including 12 TMSs that are connected by short intracellular loops (ILs) or extracellular loops (ELs). The core MFS transporter fold consists of four TMS repeats (i.e. TMS1-3, TMS4-6, TMS7-9, TMS10-12) that originated from two separate tandem-duplications of a three TMS ancestor; i.e. TMS1/4/7/10, TMS2/5/8/11 and TMS3/6/9/12 share the same TMS ancestor (also see Fig. 18.2a). **b** The prototypic fungal PDR family multidrug efflux transporters *S. cerevisiae* Pdr5 and *C. albicans* Cdr1 (Ca Cdr1; top panel), are characterised by a topology with the NBDs NBD1 and NBD2 preceding TMD1 (TMS1-6) and TMD2 (TMS7-12), respectively. All other ABC transporter families, including the MDR family such as the human multidrug efflux pump HsABCB1 (Hs ABCB1; bottom panel), exhibit the typical ABC transporter topology with the TMDs preceding the respective NBDs

C. albicans MFS transporters see the next chapter. Another DHA1 MFS transporter with a potential role in drug resistance is Flu1 (Table 18.1). Although there is no evidence of Flu1 over-expression in azole-resistant clinical isolates, disruption of *FLU1* in *C. albicans* caused susceptibility to mycophenolic acid but it had no effect on FLC sensitivity (Calabrese et al. 2000). Flu1 is thought to efflux the human

fungicidal peptide histatin 5 (Hst 5), although Hst 5 did not induce over-expression of *FLU1* mRNA (Li et al. 2013).

Two other genes with homology to DHA1 MFS transporters, *NAG3* (also known as *TMP1*) and *NAG4* (also known as *TMP2*), may also be involved in drug transport (Table 18.1). When these genes in *C. albicans* are disrupted the cells become more susceptible to cycloheximide (CHX) (Yamada-Okabe and Yamada-Okabe 2002) but their over-expression has not been associated with clinical drug resistance.

18.2.2 ABC Transporters

As their name suggests, ABC proteins contain nucleotide-binding domains (NBDs). These domains contain highly conserved amino acid motifs and are characterised by the Walker A and the Walker B motifs (Walker et al. 1982) and the hallmark ABC signature motif or C-loop. The 26 *C. albicans* ABC proteins (Braun et al. 2005) can be classified into the following subgroups: PDR (pleiotropic drug resistance), MDR (multidrug resistance), MRP (multidrug resistance-associated protein), ALDp (adrenoleukodystrophy protein), EF3 (elongation factor EF-3) and RL1 (RNAse L inhibitor) (Gaur et al. 2005). The EF3 and RL1 ABC proteins lack TMSs and so, despite the ability to hydrolyse ATP, are not involved in transport.

The two ABC proteins most often associated with drug resistance in clinical *C. albicans* isolates are Cdr1 and Cdr2 (Maebashi et al. 2001; Sanglard et al. 1995; White 1997) (Table 18.1). These are 'full-size' ABC transporters in that they contain two homologous halves each with a NBD and a TMD (Cannon et al. 2009). The two TMDs each contain six TMSs and together they are thought to form the substrate transport channel. Cdr1 and Cdr2 are PDR-type ABC proteins with an inverted order of the domains [NBD-TMD]$_2$—only found in fungi and plants (Crouzet et al. 2006)—as opposed to the typical [TMD-NBD]$_2$ arrangement found in all other ABC proteins (Fig. 18.1) (Lamping et al. 2010).

Cdr1 and Cdr2 have a broad substrate specificity and, when heterologously expressed in *S. cerevisiae*, they confer resistance to FLC, KTZ, MCZ, ITC, rhodamine 6G (R6G), CHX and cerulenin (CER) among many other compounds (Puri et al. 2010; Tanabe et al. 2011). Both Cdr1 and Cdr2 are often co-expressed at elevated levels in azole-resistant clinical *C. albicans* isolates because their promoters contain drug-responsive elements CGGN$_4$CGG (de Micheli et al. 2002; Liu et al. 2007); the sequence recognised by transcription factor Tac1 (Coste et al. 2004). These azole-resistant isolates possess gain-of-function mutations in Tac1 (Coste et al. 2007; Coste et al. 2004). The use of Cdr1- and Cdr2-specific antibodies, Cdr1-specific inhibitors, and gene deletions, has shown that Cdr1 contributes more to FLC efflux than Cdr2 in FLC-resistant clinical isolates (Holmes et al. 2008; Tsao et al. 2009).

Table 18.1 *C. albicans* MFS and ABC proteins that may be involved in multidrug resistance

Transporter type	ORF[a]	Gene	Number of amino acids	MW (kDa)	Potential substrates[b]	Evidence for role in drug transport	
						in vitro	in vivo
MFS[c]	orf19.5604	MDR1	564	62.9	FLC, KTZ, BEN, CHX	(Fling et al. 1991) (Lamping et al. 2007)	(Wirsching et al. 2000) (Sanglard et al. 1995) (White et al. 2002) (Morschhauser et al. 2007)
	orf19.6577	FLU1	610	67.6	FLC, CHX, MPH, Hst5	(Calabrese et al. 2000)	
	orf19.2158	NAG3	561	62.6	CHX	(Yamada-Okabe and Yamada-Okabe 2002)	
	orf19.2160	NAG4	581	64.6	CHX	(Yamada-Okabe and Yamada-Okabe 2002)	
ABC	orf19.6000	CDR1	1501	170	FLC, KTZ, MCZ, ITC, R6G, CHX, CER	(Lamping et al. 2007)	(Maebashi et al. 2001) (Sanglard et al. 1995) (Tsao et al. 2009) (White 1997)
	orf19.5958	CDR2	1499	169	FLC, KTZ, MCZ, ITC, R6G, CHX, CER	(Lamping et al. 2007)	(Maebashi et al. 2001) (Tsao et al. 2009) (White et al. 2002)
	orf19.1783	YOR1	1488	168		(Znaidi et al. 2008)[d]	

[a] ORF identifier from *Candida* Genome Database: http://www.candidagenome.org/

[b] FLC, fluconazole; KTZ, ketoconazole; BEN, benomyl; CHX, cycloheximide; MPH, mycophenolic acid; Hst5, histatin 5; MCZ, miconazole; ITC, itraconazole; R6G, rhodamine 6G; CER, cerulenin

[c] all MFS transporters are DHA1 type consisting of 12 predicted TMSs

[d] expression of *YOR1* (and *CDR1* and *MDR1*) induced by Upc2, substrates are unknown

18.3   Topology of *C. albicans* Multidrug Efflux Transporters

18.3.1   *MFS Transporter Mdr1*

C. albicans Mdr1 has 12 predicted TMSs connected by hydrophilic loops (Fig. 18.1a) (Pasrija et al. 2007). Unlike other MFS transporters such as human glucose transporters GLUT1, -3 or -5 or *Escherichia coli* lactose transporter LacY, multidrug efflux transporter EmrD, and tetracycline transporter TetA, most fungal MFS transporters, including *S. cerevisiae* Flr1 and *C. albicans* Mdr1, Flu1, Nag3 and Nag4, have a ∼100–160 amino acid N-terminal extension (Fig. 18.1a; data not shown), the function of which remains to be determined. Early topology studies of DHA1 transporters using reporter fusions revealed that the N- and C-termini of these proteins are cytoplasmic (Law et al. 2008). There is a degree of homology between the N- and C-terminal halves of MFS proteins indicating that they may have arisen by duplication of an ancestor protein with 6 TMSs (Paulsen and Skurray 1993). These two halves are separated by the extended cytoplasmic intracellular loop 3 (IL3; Fig. 18.1a), which permits a relatively large, rigid, movement between the two domains during the proposed 'rocker-switch'-type substrate translocation through the centre of the transporter (Law et al. 2008; Yan 2013; Yan 2015). Further structural and functional analyses revealed that an even earlier duplication of a 3-TMS ancestor possibly gave rise to this 6 TMS precursor protein. Thus, the 12 TMS core of most MFS transporters consists of four inverted 3-TMS repeats (Fig. 18.1a) (Quistgaard et al. 2016; Yan 2015).

18.3.2   *ABC Transporters Cdr1 and Cdr2*

All ABC transporters consist of four core sub-domains: two TMDs that provide a channel for the passage of compounds across the lipid bilayer, and two cytoplasmic NBDs that provide the necessary energy for active transport (Fig. 18.1b). Prokaryotes commonly express these individual sub-domains as separate polypeptide chains, whereas eukaryotes usually express half-size transporters (TMD-NBD) that form functional homo- or hetero-dimers, or full-size transporters with all four sub-domains [TMD-NBD]$_2$ expressed as a single polypeptide (Fig. 18.1b) (Hollenstein et al. 2007a, b; Jones et al. 2009). As mentioned above, PDR transporters like Cdr1 and Cdr2 are the only ABC sub-group with an inverted domain arrangement where the two NBDs precede their respective TMDs [NBD-TMD]$_2$ (Fig. 18.1b). They also have a ∼150 amino acid N-terminal extension rich in hydrophilic (S, T, N, Q) and charged (D, E, R, K) residues (Lamping et al. 2010), similar to the fungal MFS transporters mentioned above. This N-terminal domain may be important for protein–protein interactions that regulate PDR transporter function and/or expression. Our analysis of 244 PDR

sequences also predicted four small (EL1, -2, -4, -5) and two large ELs (EL3, -6) with PDR family-defining helical and beta-sheet motifs near the exits of TMS5 and TMS11, and two small (IL2, -4) and two large ILs (IL1, -3) connecting 12 predicted TMSs (Fig. 18.1b) (Lamping et al. 2010). Plant and human PDR transporters exhibit the same topology with the same conserved EL3 and EL6 helical and beta-sheet motifs near the exits of TMS5 and TMS11 (Fig. 18.1b; unpublished data). MDR ABC proteins, like human ABCB1 (also known as P-glycoprotein or Pgp), also have 12 TMSs but, unlike PDR transporters such as Cdr1 and Cdr2, they have equally large IL1-IL4 that extend deep into the cytosol, and they do not have large ELs (Fig. 18.1b). As discussed below, this has significant structural and functional consequences for these different types of ABC exporter.

18.4   Structural Analyses of Multidrug Transporters

The structure of proteins, and protein complexes, provides pivotal clues about how proteins fulfil their function. There are several techniques that can generate structural information at atomic or near-atomic resolution—two of the most frequently used techniques being X-ray crystallography and cryo-electron microscopy (CryoEM) (Delmar et al. 2015; Ford et al. 2009; Vinothkumar 2015). There are hundreds of thousands of structures of soluble proteins deposited in the protein data bank (pdb; http://www.rcsb.org/pdb/home/home.do), but high-resolution structures for integral membrane proteins are much harder to obtain and account for less than 1% of all deposited structures. The amphiphilic nature of membrane proteins makes it very difficult to obtain enough mono-disperse protein and to produce the well-ordered three-dimensional (3-D) crystals that are required for X-ray crystallography. However, recent advances in CryoEM technology (i.e. improved detector technology and software analysis tools (Cheng et al. 2015; Delmar et al. 2015; Henderson 2015; Nannenga et al. 2013; Rigaud et al. 2000; Vinothkumar 2015)), which do not require well-ordered 3-D crystals for the structural analysis of proteins, has helped to increase the pace for solving membrane protein structures, including structures for MFS and ABC transporters.
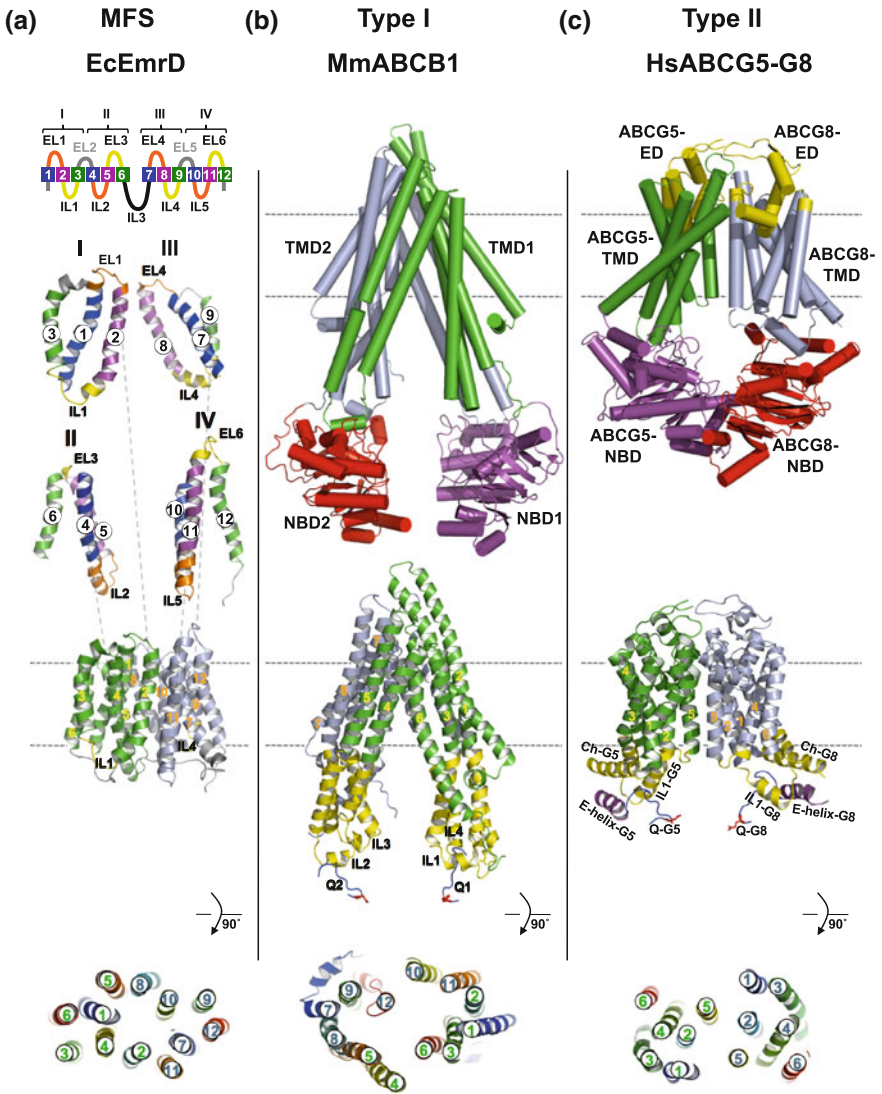
18.4.1   MFS Transporters

There are currently 64 solved structures for 23 MFS transporters representing 10 different MFS transporter families and that display all four major pump conformations (inward open—inward occluded—outward occluded—outward open) of the recently proposed 'clamp and switch model' for MFS transporter function (Quistgaard et al. 2016).

It would appear that all MFS transporters, irrespective of their substrate specificities and transport coupling mechanisms, share a common structural fold known

as the MFS fold (Yan 2013). The canonical MFS fold comprises 12 TMSs which are organised into two discreetly folded N- and C-terminal domains displaying a two-fold pseudosymmetry (Yan 2013). Each domain further divides into a pair of inverted 3 + 3 TMS repeats, the basic functional unit of MFS proteins (Yan 2013). Interestingly, the corresponding TMSs (i.e. TMS1, -4, -7, -10; TMS2, -5, -8, -11; and TMS3, -6, -9, -12; blue, magenta and green, respectively; Fig. 18.2a) of two 3-TMS repeats are always positioned next to each other in opposite transmembrane orientations (Fig. 18.2a). The substrate-translocation cavity at the centre of the transporter is enclosed by the blue helices (TMS1, -4, -7 and -10; Fig. 18.2a). Major interactions between TMS1 and TMS7 near the extracellular space and between TMS4 and TMS10 near the cytosol are thought to control access of solutes to the substrate-binding cavity (Nomura et al. 2015; Quistgaard et al. 2016; Yan 2015). Some residues of the magenta helices (TMS2, -5, -8 and -11) that typically mediate the interface between the N- and C-terminal domains (Fig. 18.2a), also face the substrate-binding cavity and may participate in substrate binding/transport and coupling of substrate transport to the symport or antiport of H^+ or other chemiosmotic solutes. The green helices (TMS3, -6, -9 and -12; Fig. 18.2a) are positioned on the outside of the protein, mostly stabilising MFS transporter structure. Most MFS transporters also have a conserved hallmark A-motif $Gx_3(D/E)(R/K)xG$ [x](R/K)(R/K) (x indicates any residue) between TMS2 and -3 and/or TMS8 and -9 (Quistgaard et al. 2016). These motifs are important for gating interactions in the four different MFS pump conformations (Quistgaard et al. 2016; Yan 2013; Yan 2015). Some drug: H^+ MFS antiporter families, including *C. albicans* Mdr1, also have a conserved C-motif Gx_3GPx_2G near the end of TMS5 (Paulsen et al. 1996; Paulsen and Skurray 1993), which has been proposed to be involved in linking H^+ translocation to export as it is only conserved among export proteins (Paulsen and Skurray 1993).

### 18.4.2   ABC Transporters

ABC transporters are divided into three types of importers (type I, type II and energy-coupling factor (ECF) importers; Fig. 18.3), only found in prokaryotes, and two types of exporters (type I and type II exporters; Fig. 18.2b, c), found in all kingdoms of life. While the NBDs have generally well-conserved structures, their TMD folds vary significantly, as demonstrated in the centre and bottom panels in Figs. 18.2b, c and 18.3—possibly a reflection of their large substrate diversity (Jones et al. 2009; Kerr et al. 2010; Locher 2004; Rees et al. 2009). Many ABC transporters possess additional domains responsible for regulating transporter activity, necessary for protein–protein interactions or important for protein targeting to the correct membrane. Type I and type II ABC importers, for example, require an additional high-affinity substrate-binding protein component (yellow domains in Fig. 18.3a, b) that delivers a specific ligand to the transporter, while others have

(a) MFS EcEmrD
(b) Type I MmABCB1
(c) Type II HsABCG5-G8

◀**Fig. 18.2** Structures of a typical MFS transporter and type I and type II ABC exporters. All structures were prepared with PyMOL Molecular Graphics System. The lipid bilayer is indicated with dashed grey lines. **a** *E. coli* multidrug efflux transporter EcEmrD (pdb: 2GFP; (Yin et al. 2006)), like *C. albicans* Mdr1 an H⁺/drug antiporter. Because all MFS transporters share the same core fold, it is likely that *C. albicans* Mdr1 folds like EcEmrD. The *top* panel in **a** shows how a typical MFS transporter contains four core subunits. Subunit I comprises TMS1/2/3, subunit II TMS4/5/6, subunit III TMS7/8/9 and subunit IV comprises TMS10/11/12. MFS transporters possibly experienced two separate gene duplications of a common 3 TMS ancestor. Thus, TMSs with the same colour (*blue*, *magenta* or *yellow*) are equivalent and ELs and ILs with the same colour (*orange*, *yellow* or *grey*) are also equivalent. The centre panel in **a** shows the entire EcEmrD molecule with the N-terminal half (TMS1-6) in *green*, the C-terminal half (TMS7-12) in *pale blue*, the IL3 linker between TMD1 and TMD2 in *black* and the conserved IL1 and IL4 highlighted in *yellow*. **b** and **c** Nucleotide-free conformations of type I and type II ABC exporters. **b** Type I *Mus musculus* multidrug efflux pump MmABCB1 (pdb: 3G5U; (Aller et al. 2009)); and **c** Type II *Homo sapiens* cholesterol ABC exporter HsABCG5-G8 (pdb: 5DO7; (Lee et al. 2016)). MmABCB1 is a single polypeptide, whereas HsABCG5-G8 is a heterodimer of two separate polypeptide chains. Their N- and C-terminal TMDs are *green* and *pale blue* and their N- and C-terminal NBDs *magenta* and *red*, respectively. The *centre* panels in **b** and **c** highlight the major contact points between the TMDs (ILs and coupling helices (Ch) in *yellow*) and the Q-loops (*dark blue*; conserved Qs are shown as *red* sticks) of the NBDs for type I (**b**) and type II (**c**) ABC exporters. The four ILs of type I exporters provide 'asymmetric' contact with the two NBD Q-loops (centre panel), whereas IL1 and a conserved positively charged cytosolic Ch preceding TMS1 provide 'symmetric' contact with their respective NBDs; i.e. the Ch and IL1 of ABCG5 and ABCG8 are in contact with the Q-loop and a conserved E-helix (*magenta*; *centre panel*) of their own NBDs (*centre right*). Visible TMS helices are numbered in *yellow* (N-terminal TMD) and *orange* (C-terminal TMD). The *bottom panels* show cross-sections through the centres of the TMDs for MFS (**a**), type I (**b**) and type II (**c**) ABC transporters viewed from the extracellular side after tilting the TMDs depicted in the *centre panels* forward by 90˚. The N- and C-terminal TMDs are rainbow colour-coded and numbered in *green* (N-terminal TMD) or *blue* (C-terminal TMD), respectively. Each transporter has a unique TMS topology with unique arrangements of TMSs surrounding a centrally located substrate-binding pocket

N-terminal TMD extensions that cause transporter dimerization and/or help target the transporter to its correct destination.

Due to the lack of any type II exporter structure, *S. cerevisiae* Pdr5 was initially modelled on the structures of type I exporters (Rutledge et al. 2011). However, the first type II ABC exporter structure for the open conformation of the human cholesterol transporter HsABCG5-G8 (Fig. 18.2c) has recently been solved (Lee et al. 2016). Perhaps not surprisingly, given the unique features characterising PDR transporters (Lamping et al. 2010), HsABCG5-G8 exhibited a unique TMD fold (bottom of Fig. 18.2c), quite different from that of type I exporter MmABCB1 (bottom of Fig. 18.2b). Lee and colleagues noticed a unique TMD-NBD cis-interface with novel connecting-helices (Ch; yellow) and E-helices (magenta) just before TMS1 and after the Q-loop of ABCG5 and ABCG8, respectively (centre panel; Fig. 18.2c). Conserved salt-bridges stabilised cis contacts between the TMDs (Ch and IL1) and NBDs (Q-loop and E-helix) of HsABCG5-G8 (centre panel in Fig. 18.2c) (Lee et al. 2016). We previously reported PDR transporter-specific positively charged helices just before TMS1 and TMS7 (Lamping et al. 2010) that align with the two Chs of ABCG5 and ABCG8 (data not shown), and we can

(a) Type I
ModAB$_2$C$_2$

(b) Type II
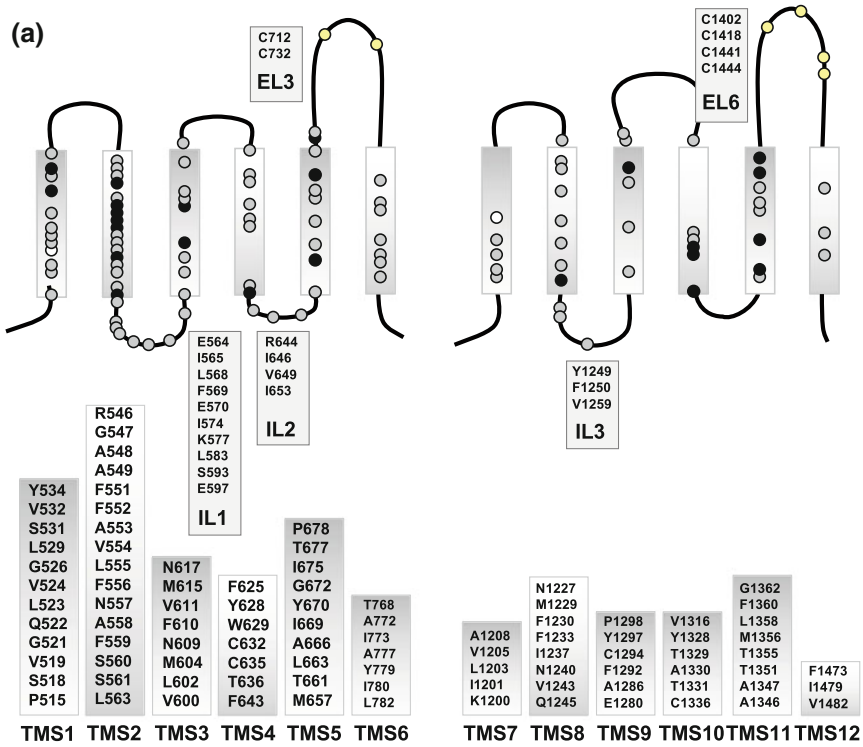BtuC$_2$D$_2$F

(c) ECF-type
Ecf-folate
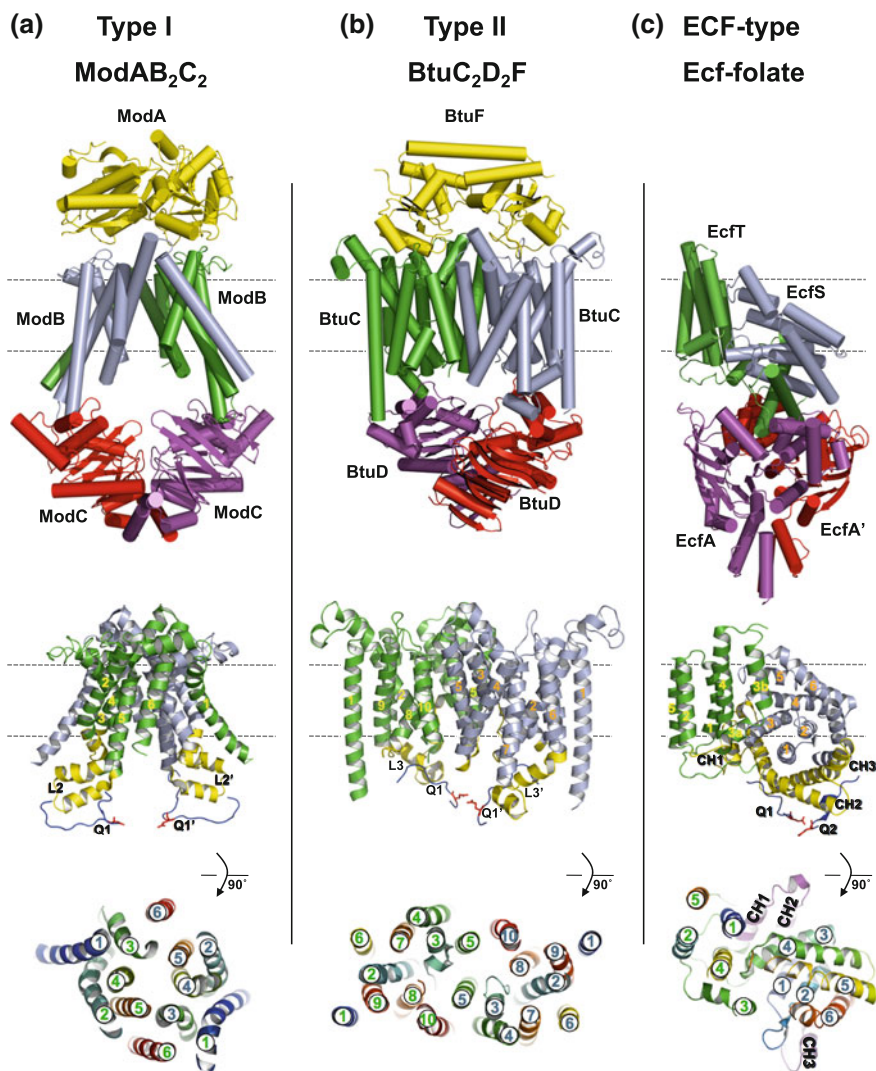
confirm that all fungal PDR transporters also have the conserved E-helices adjacent to their respective Q-loops (Fig. 18.4b) suggesting that Pdr5 and Cdr1 have the same structural/functional constraints as HsABCG5-G8. Also of significant interest was how the unique extracellular domains of HsABCG5-G8 fold. The previously identified PDR transporter EL motifs (Lamping et al. 2010) are visualised in ABCG5 (PDRA = ^{557}I-F^{565}; PDRB = ^{569}K-F^{580}) and ABCG8 (EL6 motif = ^{584}W-S^{591}; EL6 helix = ^{595}F-S^{609}) as four separate helices (yellow; top

◄**Fig. 18.3** Structures of type I, type II and ECF-type ABC importers. Nucleotide-free conformations of three ABC importers with the same annotations used in Fig. 18.2b, c. **a** Type I *Archaeoglobus fulgidus* molybdate ABC importer ModAB$_2$C$_2$ (pdb: 2ONK; (Hollenstein et al. 2007a, b)). **b** Type II *E. coli* vitamin B12 ABC importer BtuC$_2$D$_2$F (pdb: 2QI9; (Hvorup et al. 2007)). **c** *Lactobacillus brevis* energy-coupling factor (EFC) ABC importer Ecf-folate transporter (pdb: 4HUQ; (Xu et al. 2013)). ModAB$_2$C$_2$ and BtuC$_2$D$_2$F each consist of five separate domains that are encoded by three separate genes, the periplasmic molybdate and vitamin B12 binding proteins ModA and BtuF (*yellow*), the N- (*green*) and C-terminal (*pale blue*) TMDs ModB and BtuC and the N- (*magenta*) and C-terminal (*red*) NBDs ModC and BtuD, respectively. In contrast, the *L. brevis* Ecf-folate transporter consists of four domains, each encoded by a separate gene: EcfA (*magenta*) and EcfA' (*red*) are the N- and C-terminal NBDs, and EcfT (*green*) and the folate binding protein EcfS (*pale blue*) are the N- and C-terminal TMDs. The centre panels highlight the major contact points of the TMDs with the Q-loops of the NBDs. ModB-IL2 (L2 and L2'; *left*) and BtuC-IL3 (L3 and L3'; *centre*) provide the major contact points with the Q-loops of ModC and BtuD, respectively, ECF importers like the *L. brevis* Ecf-folate transporter, however, interact with the Q-loops of EcfA and EcfA' via three intracellular coupling helices (CH1, CH2 and CH3) that are part of the large EcfT-IL2 (*right*). The *bottom* panels highlight the unique TMD folds of these three different types of ABC importer. Note how EcfS is toppled over towards EcfT—this conformation reflects the structure of the Ecf-folate transporter after substrate release

panel of Fig. 18.2c). Both helices of each monomer bend towards each other and form a critical part of the TMD by dipping into the central binding cavity of the transporter (top panel; Fig. 18.2c).

Most of the current knowledge about the transport and energy coupling of ABC exporters relates to studies with type I ABC exporters. *S. aureus* Sav1866, homolog of one of the best-studied multidrug efflux pumps, human Pgp, was one of the first type I ABC exporters for which the structure was solved (Dawson and Locher 2006; Dawson and Locher 2007). The structures obtained were for the closed conformation with the substrate-binding cavity accessible to the extracellular space. Most other type I ABC exporter structures solved since then have been for open conformations with the substrate-binding cavity open to the cytosol (Fig. 18.2b). These structures revealed important insights into how type I ABC exporters couple ATP-binding and hydrolysis with substrate transport. Typically, the first IL of each TMD (IL1 and IL3) is in close contact with its proximal NBD Q-loop (Q-loop1 and Q-loop2, respectively; Fig. 18.2b centre), whereas the second loop (IL2 and IL4) reaches across the opposite NBD by contacting Q-loop2 and Q-loop1, respectively (centre panel of Fig. 18.2b). Computer simulations of the transport cycle of type I exporters suggested large, rigid, conformational changes of the NBDs upon ATP-binding that move the two NBDs close together and the cylindrical, barrel-like, structure formed by the two TMDs, especially TMS4, -6, -10 and -11 that surround a central substrate-binding cavity (bottom panel of Fig. 18.2b), becomes exposed to the outer leaflet and the extracellular space (Aller et al. 2009; Dawson and Locher 2006; Jin et al. 2012; Li et al. 2014; Prajapati and Sangamwar 2014; Ravna et al. 2009; Rosenberg et al. 2003; Velamakanni et al. 2008; Ward et al. 2007; Ward et al. 2013). This twisting motion of the TMDs induced by movement of the hinge-like contacts between IL1-4 with the Q-loops of NBD1 and NBD2 (Fig. 18.2b) causes helix rotation (*Helix Rotation Model*) (Gutmann et al.

(a)

EL3: C712, C732

EL6: C1402, C1418, C1441, C1444

IL1: E564, I565, L568, F569, E570, I574, K577, L583, S593, E597

IL2: R644, I646, V649, I653

IL3: Y1249, F1250, V1259

TMS1	TMS2	TMS3	TMS4	TMS5	TMS6	TMS7	TMS8	TMS9	TMS10	TMS11	TMS12
Y534	R546	N617	F625	P678	T768	A1208	N1227	P1298	V1316	G1362	F1473
V532	G547	M615	Y628	T677	A772	V1205	M1229	Y1297	Y1328	F1360	I1479
S531	A548	V611	W629	I675	I773	L1203	F1230	C1294	T1329	L1358	V1482
L529	A549	F610	C632	G672	A777	I1201	F1233	F1292	A1330	M1356	
G526	F551	N609	C635	Y670	Y779	K1200	I1237	A1286	T1331	T1355	
V524	F552	M604	T636	I669	I780		N1240	E1280	C1336	T1351	
L523	A553	L602	F643	A666	L782		V1243			A1347	
Q522	V554	V600		L663			Q1245			A1346	
G521	L555			T661							
V519	F556			M657							
S518	N557										
P515	A558										
	F559										
	S560										
	S561										
	L563										

(b)

Walker A   Q-loop / E helix   ABC signature   Walker B / D-loop   H-loop

N ———————————————————————————— C

NBD1

	Walker A1*	Q-loop1*	E-helix1	ABC1	Walker B1*	D-loop1	H-loop1*
CaCdr1	187GRPGAGC	236SAETDVH	248VGDTLE	303VSGGE	323IQCWDN	329ATRGLD	361YQCSQ
ScPdr5	193GRPGSGC	242NAEADVH	254VFETLV	309VSGGE	329FQCWDN	335ATRGLD	367YQCSQ

NBD2

	Walker A2	Q-loop2	E-helix2	ABC2*	Walker B2	D-loop2	H-loop2
CaCdr1	895GASGAGK	940VQQQDVH	952VREALQ	1001LNVEQ	1022LLFLDE	1028PTSGLD	1059HQPSA
ScPdr5	905GASGAGK	949CQQQDLH	961VRESLR	1010LNVEQ	1031LVFLDE	1037PTSGLD	1068HQPSA

◄**Fig. 18.4** Summary of Cdr1 mutagenesis studies. **a** Cdr1 TMD residues that have been shown to be important for Cdr1 efflux pump function (Kolaczkowski et al. 2013; Rawal et al. 2013; Shah et al. 2015) are identified. TMSs are shown as light grey boxes and residues that selectively affected transport of some, but not all, Cdr1 test substrates are shown as *grey circles*, and residues with severe functional defects (i.e. no drug efflux and reduced ATPase activity) are shown as *black circles*. A list of these residues is shown underneath the corresponding TMS or next to the corresponding IL. Residues G521 (*centre* of TMS1) and A1208 (*centres* of TMS7) that, when mutated, improved efflux of small compounds such as resazurin, an Snq2 substrate (Kolaczkowski et al. 2013), are shown as *white circles* and the six conserved EL3 and EL6 cysteines (Lamping et al. 2010) are indicated as *yellow circles*. **b** A graphical representation of the conserved Cdr1/Pdr5 NBD motifs highlighted in different colours with a list of the Walker A, Walker B, Q-loop, E-helix, D-loop, H-loop and ABC signature motif residues for Cdr1 and Pdr5 shown underneath. Red residues are critical for ATP-binding and/or hydrolysis as discussed in the text. * = non-canonical NBD motifs typical for PDR transporters

2010) of TMSs surrounding the substrate-binding cavity and thus changes the affinity of substrates so that they can be released to the cell exterior after transporter closure. Like efflux pump substrates, inhibitors also bind to the substrate-binding cavity (Aller et al. 2009) but, unlike Pgp substrates that can induce the ATPase activity of the efflux pump up to 100-fold by helping bring the two NBDs closer together and thus increase the affinity for ATP and induce their own transport—also referred to as the *Induced Fit Model*—inhibitors seem to keep the NBDs apart and freeze the transporter in an open conformation (Aller et al. 2009; Gutmann et al. 2010; Ma and Biggin 2013). ATP hydrolysis is believed to then reset the transporter in the inward facing conformation so that another transport cycle can begin. The *Switch Model* proposed that after ATP hydrolysis the two NBDs separate by >20 Å (Higgins and Linton 2004), while others suggested that the large gap separating the NBDs in the open conformation of most type I ABC exporter structures is not a biologically viable state. Some studies have shown that the NBDs indeed remain in contact at all times during an alternating ATP-binding and hydrolysis cycle (George and Jones 2012; Jones and George 2009; Sauna et al. 2007). In the *Constant Contact Model,* the ATP-bound active site is closed while the second ATP-binding site is empty and slightly further apart (George and Jones 2012). After transport, ATP hydrolysis and the release of Pi and ADP, the empty low-affinity site switches to a high-affinity site and another ATP hydrolysis cycle begins (Fribourg et al. 2014; George and Jones 2012; Ravna et al. 2009).

However, despite all these valuable contributions, details of the ATP kinetics of type I ABC exporters remain unresolved. Electron microscopy single particle analysis of *E. coli* MsbA and Pgp revealed remarkable differences between the two pumps, not only in their conformational changes in the presence of nucleotides and substrates, but also in the separation between their two NBDs. Therefore, perhaps more than one model is required to describe type I ABC exporter function (Moeller et al. 2015).

18.5   Structure–Function Activity Relationships Deduced from *C. albicans* Multidrug Transporter Mutagenesis

18.5.1   *MFS Transporter Mdr1*

Structural and functional analyses of *C. albicans* Mdr1 have indicated that TMS5 and IL3 residues are critical for drug transport. Alanine substitutions of 21 TMS5 residues showed clustering of functionally critical residues, including all conserved C-motif residues (G251, G255, P256, G259), to one face of TMS5 (Pasrija et al. 2007). The functional importance of IL3 residues was also established by site-directed mutagenesis (SDM) (Mandal et al. 2012). This region contains a number of charged residues, half of which were critical for Mdr1 function (Mandal et al. 2012). Yan proposed a model whereby IL3 helices provide a latch that strengthens the intracellular gate in the outward open conformation of GLUT1 and related SPs (Yan 2015). *C. albicans* Mdr1 also has remnants of the 'hallmark' A-motif (174-SPMSENAIFGRT-185; Paulsen et al. 1996) with three of the six conserved residues (i.e. E178, G183 and R184) found between TMS2 and -3, and, like most other MFS transporters, it has a conserved D235 at the end of TMS4 and a conserved 296-P-E-(T/S) motif at the cytosolic end of TMS6 (Nomura et al. 2015; Paulsen et al. 1996). Based on existing structures (Nomura et al. 2015; Quistgaard et al. 2016; Yan 2013; Yan 2015), Mdr1 R184 is likely to form important salt-bridges with D235 and E297 at different stages of the transport cycle. The critical contribution of E297 to Mdr1 drug transport was confirmed by Mandal and colleagues (Mandal et al. 2012).

An important question is why does Mdr1 have a wider substrate specificity than most other MFS pump homologues? MFS proteins often have conserved gating residues lining the central binding cavity, which are responsible for their substrate specificity. For example, a single-point mutation Q166E in TMS5 of the substrate-binding cavity of GLUT5 was enough to switch the substrate-binding preference from fructose to glucose (Nomura et al. 2015). Similarly, the *E. coli* multidrug efflux pump EmrD has two long helical regions composed of TMS4, -5, -10, -11 and slightly extended IL2 and IL4 that, together with a run of three positively charged residues at the end of TMS4, are proposed to act as a substrate-specificity filter (Yin et al. 2006). Another example of the importance of TMS1, -4, -7, -10 residues (blue helices on Fig. 18.2a) and, in particular, residues of the proton-dependent oligopeptide transporter (POT) family-defining motif Ex$_2$ERFxYY of TMS1, in substrate selectivity, proton binding and oligopeptide symport was revealed by the structural analysis of the *Streptococcus thermophilus* POT oligopeptide symporter PepTSt (Solcan et al. 2012). These structural and mechanistic principles appear to hold true for most, if not all, MFS transporters, and, in the absence of an Mdr1 structure, they provide a model for how *C. albicans* multidrug efflux pump Mdr1 works.

18.5.2 ABC Transporter Cdr1

18.5.2.1 TMDs and ELs

Early random mutagenesis studies with *S. cerevisiae* Pdr5 identified several residues that were critical for drug transport. It was not clear, however, how these residues, which were distributed throughout the molecule, caused reduced efflux of some or all drugs and/or affected the ATPase activity of Pdr5 (Egner et al. 2000; Egner et al. 1998; Tutulan-Cunita et al. 2005). Both MDR and PDR ABC exporters appear to have multiple overlapping substrate-binding sites encompassing a large substrate-binding cavity within their TMDs, with Pdr5 and Cdr1 possessing at least three separate substrate-binding sites (Golin et al. 2003; Golin et al. 2007; Puri et al. 2009; Shukla et al. 2003). There are, however, differences in the preferred substrates of ABC proteins. Pgp for instance prefers hydrophobic substrates with hydrogen-bond acceptor groups (Seelig and Landwojtowicz 2000), whereas size (optimum $\sim$ 200–225 Å^3), but not hydrophobicity, seemed the major determinant for a good Pdr5 substrate (Golin et al. 2003; Golin et al. 2007). Cdr1, on the other hand, seemed to prefer hydrophobic substrates and molecular-branching (Puri et al. 2010).

In the absence of a high-resolution structure, bioinformatics and biochemistry are the best ways to identify residues involved in drug binding and/or transport. Heterologous expression of efflux pumps in *S. cerevisiae* provides many advantages for studying structure–function relationships. *S. cerevisiae* can be easily genetically manipulated and a vast mutant collection is available. Cdr1/Pdr5 variants can be rapidly generated and expressed at high levels at the correct cellular location (Lamping and Cannon 2010; Niimi et al. 2012; Tanabe et al. 2011). In addition, one can quickly select for naturally occurring suppressor mutants of transport defective variants by isolating resistant colonies that appear after prolonged incubation at 30 °C on plates containing high (Niimi et al. 2012) concentrations of toxic efflux pump substrates (Golin and Ambudkar 2015). SDM of all 252 Cdr1 TMS residues indicated that almost half of them ($\sim$42%) affected substrate transport and/or ATPase activity (Fig. 18.4a) (Rawal et al. 2013), making interpretation of the effects of mutations of individual residues very challenging. Interestingly, most residues affecting substrate transport (grey circles; Fig. 18.4a) and/or ATPase activity (black circles; Fig. 18.4a) were located in TMS1, -2, -5, -8 and -11, whereas TMS4, -6, -10 and -12 contributed least to substrate transport and/or ATPase activity (Fig. 18.4a) (Rawal et al. 2013). Given this result, it was perhaps a little surprising that only $\sim$ 18% of all IL mutants affected Cdr1 drug transport and none of them affected the Cdr1 ATPase activity (Fig. 18.4a) (Shah et al. 2015). The observed phenotypes of TMS5 mutant residues affecting Cdr1 pump function did not relate to which face of the helix they belonged to, the degree of conservation of the residues or the nature of their side chains (Puri et al. 2009; Rawal et al. 2013). In contrast, TMS11 mutations of both Cdr1 and Pdr5 that affected drug efflux were mainly clustered on the hydrophilic face of the helix, suggesting a direct

contribution of TMS11 to a centrally located drug-binding cavity (Egner et al. 2000; Kueppers et al. 2013; Rawal et al. 2013; Saini et al. 2005; Shukla et al. 2004).

The role of EL residues in protein assembly, cell surface localisation and substrate specificity has been highlighted in a random mutagenesis study of Pdr5 (Egner et al. 1998). A single substitution of one of the six conserved EL3/EL6 cysteines (yellow residues in Fig. 18.4a) of fungal PDR transporters (Lamping et al. 2010), C1427Y, caused mislocalization of the mutant Pdr5 to the endoplasmic reticulum. The equivalent residue in Cdr1, C1418, was found to be essential for pump function (Shukla et al. 2003). These studies highlighted the importance of the conserved extracellular cysteines that possibly form three extracellular disulfide bonds that stabilise the structure of the highly conserved extracellular domains of PDR transporters (Lamping et al. 2010). Molecular mapping and biochemical characterization of more than 50 Pdr5/Cdr1 suppressor mutants that had lost their ability to respond to efflux pump inhibitors RC21v3, a Cdr1 specific D-octapeptide inhibitor (Niimi et al. 2012), macrolides such as FK506 (Egner et al. 1998; Kralli and Yamamoto 1996; Lamping et al. 2007; Tanabe et al. 2011) and milbemycins (Lamping et al. 2007; Lee et al. 2001; Niimi et al. 2012; Silva et al. 2013), depsipeptides enniatin (Hiraga et al. 2005; Holmes et al. 2008; Lamping et al. 2007; Lee et al. 2001; Tanabe et al. 2011) and beauvericin (Lee et al. 2001; Tanabe et al. 2011) further highlighted the importance of the conserved EL3 and EL6 in Cdr1/Pdr5 efflux pump function ((Lamping et al. 2014) and unpublished results). How these extracellular domains contribute to type II exporter function is an important question that needs to be answered in order to understand how PDR multidrug efflux pumps work.

18.5.2.2 NBDs and ILs

The highly conserved NBDs are defined by the Walker A and the Walker B motifs (Walker et al. 1982) and the hallmark ABC signature motif which are critical for ATP-binding and hydrolysis (Fig. 18.4b; (Prasad and Goffeau 2012)). Typically, ABC transporters have two canonical ATP-binding sites, also referred to as composite nucleotide-binding domains (CNBD), comprising Walker A and Walker B motifs of one NBD and the ABC signature motif of the other. CNBD1 and CNBD2 of most ABC proteins are believed to alternatingly bind and hydrolyse ATP (see Sect. 18.4.2). However, PDR transporters have a high, non-inducible, basal ATPase activity (Ernst et al. 2008) and they are characterised by one canonical CNBD2 (i.e. typical Walker A2, Walker B2 and ABC1 signature motifs; Fig. 18.4b) and one non-canonical CNBD1 with its Walker A1 K residue replaced by a C (C193 in Cdr1), its Walker B1 catalytic E replaced by an N (N328 in Cdr1), the conserved H-loop H of NBD1 replaced by a Y (Y361 in Cdr1) and the C-loop G of ABC2 replaced by a E (E1004 in Cdr1; red residues in Fig. 18.4b).

SDM of C199 of Walker A1 and K911 of Walker A2 (Fig. 18.4b) indicated that the Walker A2 K911, but not the Walker A1 C199, was critical for Pdr5 ATPase activity and drug transport (Ernst et al. 2008). SDM of Cdr1 Walker B1 (D327,

N328), Walker B2 (D1026, E1027) as well as all five ABC1 (VSGGE) and ABC2 (LNVEQ; Fig. 18.4b) residues found that all four Walker B residues (Rai et al. 2006) and ABC residues S304, G306 and E307 of ABC1 and the equivalent ABC2 residues N1002 and E1004, but not Q1005 (Kumar et al. 2010), were critical for both the ATPase activity as well as drug efflux, indicating the importance of both CNBDs in ATP-binding and/or hydrolysis (Kumar et al. 2010). In contrast, alanine substitution of the Pdr5 D-loop residues D340A and D1042A (red residues; Fig. 18.4b) 'uncoupled' the ATPase activity from drug transport by affecting drug efflux but not the ATPase activity of Pdr5, indicating that D-loop residues are important for the crosstalk between the NBDs and the TMDs of PDR transporters (Furman et al. 2013). Surprisingly, mutation of the conserved H-loop H1068A had no effect on Pdr5 ATPase activity, instead it selectively abolished R6G transport without affecting the transport of any other substrates (Ernst et al. 2008). This led to the *Kinetic Substrate Selection* model for Pdr5 transport, whereby the authors proposed that the H1068A mutation changed the transport kinetics in such a way that only the transport of 'slow' substrates such as R6G was impaired because the average time it took 'slow' substrates to enter or diffuse into the transporter far exceeded the time the mutant transporter was open for substrates to enter (Ernst et al. 2010).

A study of the Pdr5 S558Y (Cdr1 A548; Fig. 18.4a) mutation at the top of TMS2 provided important clues about how TMS2 may be critical for the crosstalk between the NBDs and the TMDs (Sauna et al. 2008). The phenotype of yeast with this mutation was drug hypersensitivity despite wild-type ATPase activity or, in other words, this mutant had a pump defect because its ATPase activity could not be translated into the conformational changes needed for drug efflux. This defect could be recovered by second-site suppressor mutations in Q-loop1 (N242 K and E244G; Fig. 18.4b) and the centre of IL1 (S597I, S597T (Cdr1 S587)) (Ananthaswamy et al. 2010). Similarly, second-site Pdr5 N242 K suppressor mutants with improved CHX transport were found in the IL2-TMS5 (M649I, V656L, A666G) region and just C-terminal of ABC2 (K1016I) (Ananthaswamy et al. 2010). The same V656L second-site suppressor mutation was also found in a search for improved CHX transport (i.e. increased CHX resistance) of yeast over-expressing wild-type Pdr5 (Downes et al. 2013). Alanine substitution of Pdr5 V656, however, caused the Pdr5 ATPase activity to become uncoupled from drug efflux and led to drug hypersensitivity of the strain over-expressing the mutant protein. Taken together, these data support a model whereby Pdr5 IL2 may possibly be part of a cis-transmission interface between TMD1 and NBD1, unlike MmABCB1 where IL2 is a critical part of a trans-transmission interface by being in direct contact with Q-loop2 of NBD2 (Fig. 18.2b). The critical importance of the TMS2-IL1 region for the crosstalk between the NBDs and TMDs of PDR transporters was further supported by the Cdr1 SDM studies described in Sect. 18.5.2.1 with 7 of 24 functionally critical Cdr1 TMD residues (black residues; Fig. 18.4a) located in TMS2 and almost all remaining TMS2 residues affecting efflux, and more than 50% of all IL residues affecting Cdr1 drug efflux were located in IL1 (Fig. 18.4a) (Rawal et al. 2013; Shah et al. 2015).

Taken together, these studies suggest a critical role for the deviant CNBD1 in intra-domain signalling of ATP-binding at the canonical CNBD2 (Ananthaswamy et al. 2010; Furman et al. 2013) which causes the NBDs to move closer together and, in turn, leads to large conformational changes at the TMDs followed by drug release in the closed conformation of the transporter. In addition, TMS2-IL1, and possibly IL2, seem to play a critical role in the cis-transmission crosstalk between the NBDs and the TMDs. Despite all these findings, however, it is still not clear whether CNBP1 can also bind and/or hydrolyze ATP, or whether it has evolved an alternative 'regulatory' function and is only able to bind—possibly at all times—but not hydrolyze ATP (Furman et al. 2013; Gupta et al. 2011; Prasad and Goffeau 2012).

18.6 Model of Fungal PDR Transporters

It is evident that there has been a plethora of studies dissecting Cdr1/Pdr5 structure and function. The vast amount of biochemical data accumulated over the past two decades combined with PDR transporter phylogeny and the first type II ABC exporter structure, HsABCG5-G8, provide evidence for the following tentative model of how PDR transporters function. Cdr1/Pdr5 TMS1, -2, -5, -7, 8, -11 (yellow to red; Fig. 18.5c) form a large central substrate-binding cavity with TMS3, -4, -6, -9, -10, -12 (green to blue; Fig. 18.5c) stabilising the protein during the transport cycle. Salt-bridges help stabilise critical contacts between the positively charged Ch near TMS1 and IL1, Q-loop1 and E-helix1 of NBD1. The homologous parts of the C-terminal half provide similar cis interactions between NBD2 and TMD2. CNBD1 is possibly bound to ATP at all times while CNBD2 provides the energy required for the large conformational changes associated with drug efflux by binding and hydrolysing ATP. Openings between TMS1 and TMS11 (gate 1; Fig. 18.5c) and TMS5 and TMS7 (gate 2; Fig. 18.5c) may provide gates for substrates to enter, in contrast to type I exporters where substrates are thought to enter between TMS4 and TMS6 and TMS10 and TMS12 (Fig. 18.5a). The extracellular domain also plays a critical part in this process, as evidenced by phylogeny and the HsABCG5-G8 structure. How these extracellular domains contribute to drug efflux, however, remains to be explored.

18.7 Concluding Remarks

C. albicans possesses many membrane transport proteins which perform vital cellular functions. ABC and, to a lesser extent, MFS transporters have broad substrate specificities that can include antifungal drugs and xenobiotics. Increased expression of pumps, particularly Cdr1, is selected for by growth in the presence of drugs and confers drug resistance. Because of their promiscuity, the normal
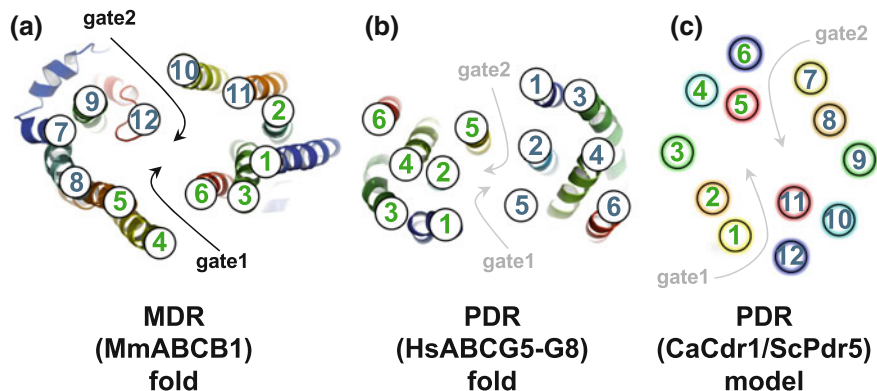
Fig. 18.5 Fungal PDR transporters are predicted to fold like HsABCG5-G8. The TMD folds for MmABCB1 (**a**) and HsABCG5-G8 (**b**) are the same as shown in Fig. 18.2b, c. This figure shows how MDR transporters (**a**) have TMS4, -5, -6 and TMS10, -11, -12 lining the substrate-binding pocket, whereas the PDR transporter homolog HsABCG5-G8 (**b**) has TMS1, -2, and -5 of ABCG5 (*left half*) and ABCG8 (*right half*) lining the substrate-binding pocket. Annotations are the same as in Fig. 18.2**c**. The predicted TMS topology for Cdr1 and Pdr5 (Lamping et al. 2014) (**c**). This topology model was created by careful consideration of all published and unpublished data relating to the structure and function of Cdr1 and Pdr5. The colours of TMSs: *red*, *orange*, *yellow*, *green*, *turquois* and *blue* indicate homologous N- (*lighter* colours) and C-terminal (*darker* colours) TMS pairs from the most to the least 'important' (i.e. the most important TMSs had the most number of residues affecting Cdr1/Pdr5 ATPase activity and/or drug efflux; also see Fig. 18.4a). Proposed (**a**) and possible gates (**b**; **c**) where pump substrates may enter the transporter from the inner leaflet of the lipid bilayer are indicated with *black* (**a**) and *grey* (**b**; **c**) *arrows*. Note how the predicted PDR TMS topology closely resembles that of ABCG5-G8 (**b**) but has little semblance to type I exporters (**a**)

biological function of multidrug efflux pumps is an unanswered question. In vitro and in vivo studies have demonstrated that inhibition of Cdr1 can sensitise azole-resistant *C. albicans* clinical isolates to FLC and ITC. Further advances in the determination of the 3D structures of multidrug transporters will help design more potent specific and broad spectrum pump inhibitors and may also elucidate the reaction cycle for an important class of membrane transporters. Molecular modelling and site-directed mutagenesis is beginning to reveal that the arrangement of external loops, transmembrane helices and interactions between these helices and the NBDs are important for pump function and are potential drug targets.

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