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Molecular modeling of the human multidrug resistance protein 1 (MRP1/ABCC1)

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Abstract

Multidrug resistance protein 1 (MRP1/ABCC1) is a 190 kDa member of the ATP-binding cassette (ABC) superfamily of transmembrane transporters that is clinically relevant for its ability to confer multidrug resistance by actively effluxing anticancer drugs. Knowledge of the atomic structure of MRP1 is needed to elucidate its transport mechanism, but only low resolution structural data are currently available. Consequently, comparative modeling has been used to generate models of human MRP1 based on the crystal structure of the ABC transporter Sav1866 from *Staphylococcus aureus*. In these Sav1866-based models, the arrangement of transmembrane helices differs strikingly from earlier models of MRP1 based on the structure of the bacterial lipid transporter MsbA, both with respect to packing of the twelve helices and their interactions with the nucleotide binding domains. The functional importance of Tyr^{324} in transmembrane helix 6 predicted to project into the substrate translocation pathway was investigated.

Keywords: Multidrug resistance protein; ABC transporter; Organic anion transporter; Homology modeling; MRP1; Sav1866; Site-directed mutagenesis

Multidrug resistance protein 1 (MRP1/ABCC1) is a 190 kDa transport protein that belongs to the ATP-binding cassette (ABC) superfamily of transmembrane proteins. It confers resistance on cancer cells by using the energy of ATP binding and hydrolysis to efflux anticancer drugs. Originally cloned from a doxorubicin-selected lung cancer cell line, MRP1 has since been found to be expressed in a range of solid and hematological tumours, and has been demonstrated to transport a wide array of structurally diverse substrates [1–4]. In addition to the anticancer drugs doxorubicin, vincristine and methotrexate (MTX), MRP1 transports many glutathione, glucuronide and sulfate conjugated organic anions, such as leukotriene C_4 (LTC_4), 17β-estradiol 17-(β-D-glucuronide) ($E_217\beta G$), and estrone 3-sulfate (E_13SO_4), respectively [2,3]. MRP1 is also found in normal tissues throughout the human body, where together with other ABC transporters, it plays a significant role in tissue defense from toxic agents [4,5]. Thus the expression levels and activity of drug transporting ABC proteins such as MRP1 are important considerations in drug development and chemical toxicity [5].

Transport proteins belonging to the ABC superfamily share several common structural components, including multiple transmembrane (TM) helices arranged in polytopic membrane spanning domains (MSDs), as well as nucleotide binding domains (NBDs) that are the site of ATP binding and hydrolysis [6,7]. Evidence from both biochemical and computational analyses indicates that MRP1 contains 17 TM helices distributed among three MSDs: MSD0 (TMs 1–5), MSD1 (TMs 6–11) and MSD2 (TMs 12–17). MSD1 and MSD2 are each followed by a NBD. This 4-domain arrangement is commonly observed among eukaryotic ABC transporters

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encoded as a single polypeptide. However, the presence of the NH_2-proximal MSD0 occurs in only 7 human ABC proteins, all of which, like MRP1, are in the ABC 'C' subfamily [4].

Transport by ABC proteins, including MRP1, remains a partially understood multistep process involving substrate recognition, binding, transport and release, which are coupled to ATP binding and hydrolysis, and ADP release [6–8]. Elucidation of the precise details of the transport mechanism(s) of ABC proteins would be greatly facilitated by high resolution structural information on the conformation of the transporters in the presence and absence of substrates and/or nucleotides. Unfortunately, high resolution structures of full-length ABC transporters remain extremely limited. In the case of the 190 kDa MRP1, currently available structural data is of low resolution (22 Å) [9], largely due to the challenges associated with expression and crystallization of such a large and hydrophobic mammalian membrane protein.

In the absence of high resolution structural data, we previously used comparative modeling techniques to generate two models of the core MSDs of MRP1 based on the crystal structure of the bacterial lipid A transporter MsbA homodimer [10]. The two monomers of MsbA were aligned to MSD1–NBD1 and MSD2–NBD2 of MRP1 to model the 4-domain core region of the transporter that has been demonstrated to be capable of LTC_4 transport [11]. However, it is now recognized that our published models of MRP1 (and those of several other mammalian ABC transporters) are likely incorrect. This realization stems at least in part from the recent 3.0 Å crystal structure of the Sav1866 transporter from *Staphylococcus aureus* reported by Dawson and Locher [8,12]. Thus the Sav1866 structure has been shown to be incompatible with the previously published MsbA crystal structures. This has resulted in a re-examination of the data and subsequent retraction of these MsbA structures that had formed the basis for atomic homology models of several mammalian ABC transporters [13].

Sav1866 is a homodimeric multidrug exporter that was crystallized in an outward conformation, reflecting the ATP-bound state [12,14]. In the present study, we have used the Sav1866 structure as a template for comparative modeling of the core 4-domain region of MRP1, and show that although the Sav1866-based models display some similarities to earlier models, there are significant differences particularly with respect to the packing of the TM helices and their interactions with the NBDs. The revised Sav1866-based models of MRP1, unlike the previous models, also position an aromatic residue, Tyr^{324} (TM6), so that it projects into the putative substrate translocation pathway formed by the MSDs. Consequently, we have tested the functional importance of this residue by using site-directed mutagenesis to replace Tyr^{324} with Ala, Phe and Trp, and characterizing the transport activity of the resulting mutants.

Materials and methods

Generation of homology models of MRP1. To generate the models, two alignments of MRP1 with Sav1866 were produced by ClustalW (v1.82; http://www.ebi.ac.uk/clustalw/) using the default settings. In the first alignment, the sequences of MSD1–NBD1 (amino acids 300–871) and MSD2–NBD2 (amino acids 971–1531) of human MRP1 were aligned separately to the Sav1866 sequence by a multiple sequence alignment that included corresponding regions of closely related ABCC family members (Supplementary Material). In the second alignment, only the sequences of MSD1–NBD1 and MSD2–NBD2 of MRP1 were aligned with Sav1866, and the resulting alignment optimized manually. For each alignment, Modeller 7v7 [15] was used to generate 100 models of MRP1 using the Protein Data Bank coordinates of Sav1866 as the template structure (http://www.rcsb.org/pdb; PDB ID: 2HYD). The five models from each alignment with the lowest objective function values were selected for energy minimization in GROMACS 3.3 [16]. Energy minimization was by steepest gradient descent, with an initial step size of 0.01 nm and a maximum of 1000 steps; each of the models minimized converged within the allowed number of steps. Energy minimized models were evaluated for stereochemical quality by PROCHECK [17] and ranked according to their score. The models produced by the multiple sequence alignment received higher PROCHECK scores than those produced by the single sequence alignment. For the purposes of this study, the highest ranking model produced by the multiple sequence alignment was used (Supplementary Material).

Vector construction and site-directed mutagenesis. To create the single mutants Y324A, Y324F and Y324W, the QuikChange™ site-directed mutagenesis kit (Stratagene Inc., La Jolla, CA), was used. The template used for mutagenesis was a 1.9 kb BamHI/SphI-MRP1 fragment in pBluescriptSK(+) (Stratagene) [18]. The mutations were introduced using the following sense primers (mutated residues are underlined, silent mutations to introduce restriction sites are italicized, and restriction enzymes used are indicated in parentheses): Y324A, 5′-GAAGAAGCT CATGAGGAATGCGGGCCCAAAGGTC-3′ (BsmI); Y324F, 5′-GCT CATGAGGAAG*A*ACGGCCCAAAGGTCTTG-3′ (ApaI); Y324W, 5′-GAAGAAGCTCAT*C*AGGAACCAGGGCCCAAAGGTC-3′ (BspHI). A 352 bp BamHI/PshAI-fragment containing the mutation was subcloned back into pcDNA3.1(–)-$MRP1_K$ [19]. The 352 bp fragments were sequenced.

MRP1-mediated transport of organic anions in membrane vesicles prepared from transfected cells. Wild-type and mutant pcDNA3.1(–)-$MRP1_K$ expression vectors were transfected into SV40-transformed human embryonic kidney cells (HEK293T), and membrane vesicles prepared as previously described [20]. MRP1 protein levels were determined by immunoblot analysis using mAb QCRL-1 [20]. ATP-dependent uptake of the ^{3}H-labeled MRP1 substrates into inside-out membrane vesicles was measured using a rapid filtration technique adapted to a 96-well plate format [20].

Results and discussion

We previously used comparative modeling techniques to generate two models of MRP1; one based on the crystal structure of the lipid A transporter MsbA from *Vibrio cholera*, and the other based on a model of P-glycoprotein (ABCB1) derived from the crystal structure of MsbA from *Escherichia coli* [10,13,21,22]. It has since been shown, based on the structure of Sav1866, that the derived structures of MsbA which were used as templates for our earlier MRP1 models are incorrect in both the hand of the structure, and in the arrangement of the TM helices. Thus these structures and the publications describing them have been retracted [13]. In the present study, we describe revised

models of the core region of MRP1 based on the crystal structure of the homodimeric exporter Sav1866.

It is generally held that greater than 30% identity between the target and template sequence is desirable for comparative modeling, at least for soluble proteins [23]. Each monomer of Sav1866 is composed of a MSD containing six TM helices followed by a NBD and has approximately 22% sequence identity to MSD1–NBD1 and MSD2–NBD2 of MRP1. As expected, however, a good portion of the identity between Sav1866 and MRP1 is found in the highly conserved NBDs [6,24]. Nevertheless, in representing the relationships among membrane proteins, sequence homology may not be as important as similarity in overall transmembrane domain topology, as studies have shown that functionally unrelated proteins with sequence homology as low as 9% may show an unexpectedly high degree of structural similarity [25,26]. This is consistent with recent reports that the majority of helix–helix packing arrangements may be divided into a relatively small number of clusters [27].

The new models of the functional core of MRP1 presented here resemble the Sav1866 structure, with the TM helices in an outward facing conformation that exposes the putative substrate translocation pathway to the extracellular space (Fig. 1). The TM helices exhibit considerable twist, and interact with TM helices of the opposite MSD (Fig. 1A and B). In particular, TMs 6 and 7 (MSD1) and TMs 12 and 13 (MSD2) twist to interact in the opposite MSD with TMs 14–17 (MSD2) and TMs 8–11 (MSD1), respectively, creating two distinct groups of TM helices. The models of MRP1 do not include the first five NH_2-proximal TM helices (MSD0) because of the lack of an appropriate homologous template structure. It is not known precisely how MSD0 physically interacts with the MRP1 core described in the models here; however, the protein expressed without this domain is transport competent [11].

The TM helices in the Sav1866-based MRP1 models are arranged quite differently from the TM helices in the MsbA-based MRP1 models. In these latter models, the TM helices are relatively perpendicular to the membrane and consequently, the same subset of TM helices lines the substrate translocation pathway through both the inner and outer leaflets of the membrane [10]. In contrast, in the Sav1866-based MRP1 models, all of the TM helices contribute residues to the surface of the pathway. However, because of the bending and twisting of the TM helices in the Sav1866-based models, different subsets of TM helices line the translocation pathway depending on the depth of their location within the lipid bilayer. Thus, TMs 6, 8,

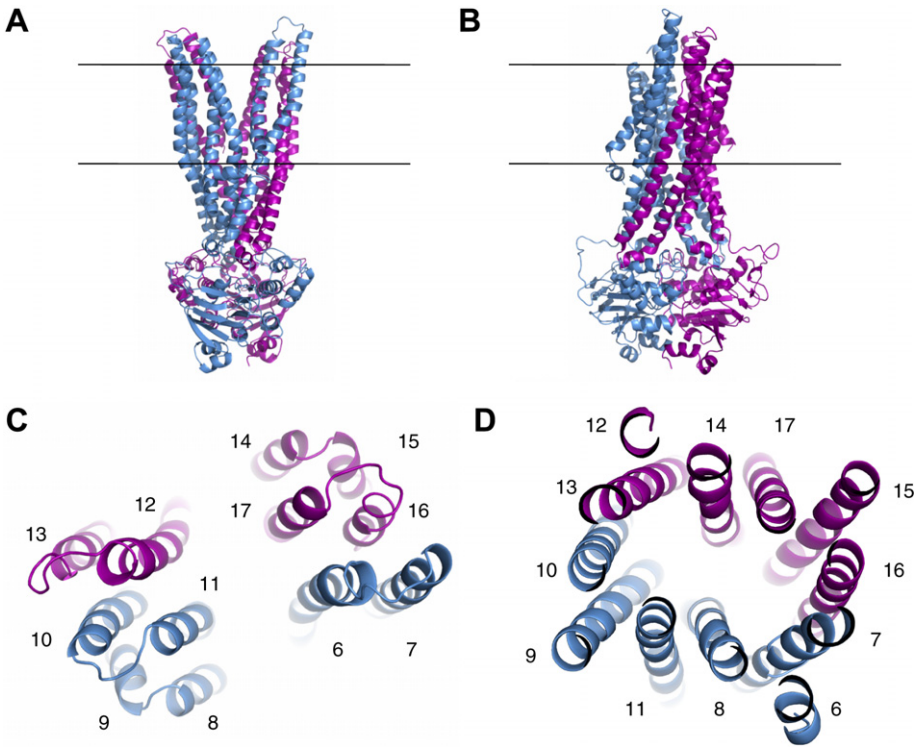

Fig. 1. Model of the core region of MRP1 based on the crystal structure of *S. aureus* Sav1866. The model is comprised of the second (MSD1; TM helices 6–11) and third (MSD2; TM helices 11–17) MSDs and the two NBDs of MRP1. (A) Shown is the α-carbon backbone in ribbon representation, as viewed from the plane perpendicular to the lipid bilayer. MSD1–NBD1 (blue), MSD2–NBD2 (purple). (B) Shown is a view of (A) rotated 90 degrees around a vertical axis. (C) Shown is the arrangement of TM helices as viewed from the extracellular face of the membrane. TM helices 6, 8, 11, 12, 14, and 17 line the pore. (D) Shown is the arrangement of TM helices as viewed from the extracellular face of the membrane. As a result of bending, TM helices 7–10 and 13–16 line the pore. (For interpretation of color mentioned in this figure the reader is referred to the web version of the article.)

and 11 of MSD1 and TMs 12, 14, and 17 of MSD2 line the pore close to the extracellular side of the lipid bilayer (Fig. 1C), while TMs 7–10 (MSD1) and TMs 13–16 (MSD2) line the pore at the membrane-cytosol interface (Fig. 1D).

The TM helices of the homodimeric exporter Sav1866 exhibit considerable twist to interact with the TMs and NBD of the opposite subunit, challenging the widely accepted model of an ABC transporter structure wherein the MSDs interact solely with the NBD of the same subunit [6,12]. This interaction is mediated by two short "coupling helices" in the cytoplasmic loops of Sav1866 (designated ICL1 and ICL2) that interact physically with the NBDs of the opposite subunit [12]. Cysteine mutagenesis and chemical cross-linking experiments support a similar architecture in the ABC multidrug exporter LmrA from *Lactococcus lactis* and the human multidrug exporter P-glycoprotein (ABCB1) [28,29]. It has been proposed that these coupling helices are functionally important as a conserved means of coordinating conformational changes in the MSDs and NBDs of ABC transporters [7,8]. Sequence alignments of MRP1 and Sav1866 indicate that the sequence corresponding to the second coupling helix is relatively well conserved in MRP1, while the sequence for the first coupling helix is not. The conservation of residues in cytoplasmic loop 7 (CL7) of MRP1 corresponding to the second coupling helix in Sav1866 is consistent with strong biochemical evidence that firmly establishes the critical role of CL7 in mediating signaling between the MSDs and NBDs, as well as influencing substrate specificity [30–32]. The new models of MRP1 should be useful in designing additional biochemical studies of this conserved region of MRP1 to elucidate more precisely the mechanisms by which conformational changes in the MSDs are coupled to ATP binding and hydrolysis, and release of ADP/P_i by the NBDs.

It is important to remember that even with a clearer understanding of the conformational changes governing coordination of the MSDs and NBDs in ABC proteins, differences in the physiological activities and cellular environments of Sav1866 and MRP1 are likely to limit the extent to which the structure and mechanism of these two proteins may be compared. Sav1866 is not yet well characterized biochemically; however, when purified and reconstituted in liposomes, it has been shown that the anticancer drugs doxorubicin and vinblastine stimulate its ATPase activity [12]. These compounds are known substrates of MRP1 [3,4] although by themselves, they do not detectably stimulate the ATPase activity of this transporter [33]. Furthermore, it is not known whether Sav1866 can transport any of the conjugated or unconjugated organic anions that are well established substrates of MRP1. In addition, lipid composition has been shown to directly affect the activity of several ABC transporters and it is well known that membrane lipids can vary by composition in different species, tissue types, and even location within the same cell membrane [34,35]. Thus, differences in the membrane environments of bacterial and human proteins, combined with a lack of overlapping substrate specificity and substrate-stimulated ATPase activity, are almost certain to affect the extent to which the structure and transport mechanism of Sav1866 may be compared to that of MRP1.

Despite these limitations, our Sav1866-based models of MRP1 should provide a useful base for guiding biochemical analyses of MRP1. Towards this end, these models show that Tyr^{324} (TM6), located approximately halfway through the predicted membrane bilayer, faces into the putative substrate translocation pathway where it might

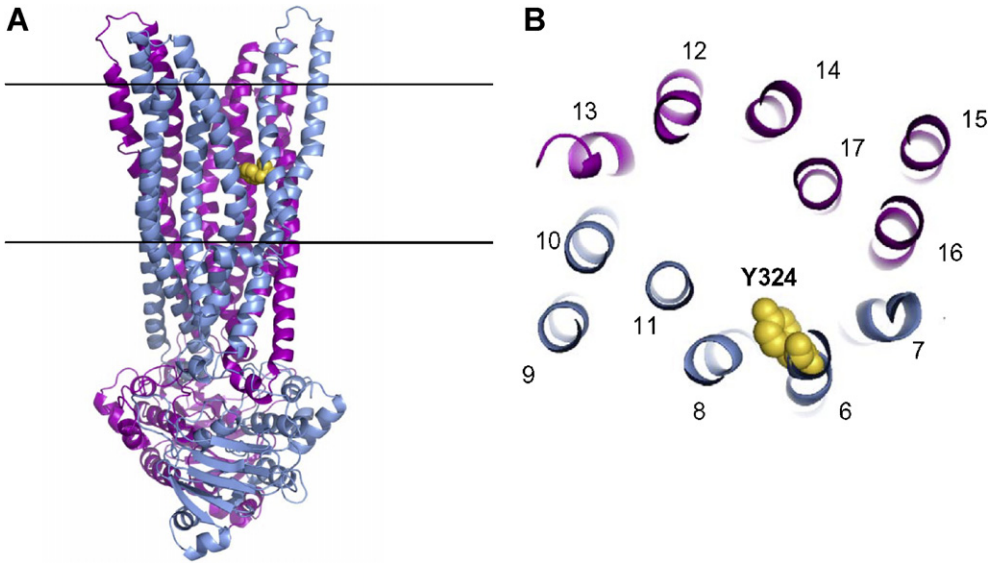

Fig. 2. Location of Tyr^{324} in a Sav1866-based model of MRP1. (A) Tyr^{324} (Y324) (yellow), is located in TM6, approximately halfway through the predicted membrane bilayer; (B) Tyr^{324} is predicted to project into the substrate translocation pathway formed by MSD1 (blue) and MSD2 (purple) of MRP1. (For interpretation of color mentioned in this figure the reader is referred to the web version of the article.)

Table 1
Effect of substituting Tyr^{324} on organic anion transport by MRP1

Substrate	% Wild-type MRP1 transport activity[a]		
	Y324A	Y324F	Y324W
$E_2$17βG	114 ± 25	162 ± 5*	98 ± 12
$E_1$3SO_4	116 ± 42	181 ± 16*	94 ± 4
LTC_4	112 ± 29	171 ± 1*	102 ± 16
MTX	94 ± 31	152 ± 17*	95 ± 21

[a] Data are means (±SD) of values obtained in three independent experiments.
* $P < 0.05$.

interact with one or more substrates of MRP1 (Fig. 2). Consequently, to test the functional importance of Tyr^{324}, this residue was replaced with Ala, Phe, and Trp and the transport activity was determined. Both conservative and non-conservative substitutions of Tyr^{324} had little or no effect on MRP1 expression levels (not shown) or on its organic anion transport activity with the exception of the phenylalanine mutation (Table 1). In the case of Y324F, the transport of all four organic anion substrates tested was increased 1.5- to 1.8-fold relative to wild-type MRP1. Thus, while the polar aromatic properties of the residue at position 324 in MRP1 are not critically important for substrate interactions, they do appear to have some influence on the overall activity of the transporter.

Several other aromatic residues have been shown previously to be important determinants of MRP1 substrate specificity [10,18,19]. In the MsbA-based models of MRP1, at least four of these residues (Trp^{553}, Phe^{594}, Trp^{1198}, and Trp^{1246}) were predicted to line the substrate translocation pathway [10]. In the Sav1866-based models, the functionally important Phe^{594} (TM11) and Trp^{1246} (TM17), like Tyr^{324}, still remain in a position where they project into the pore (Fig. 3). In contrast, Trp^{553} (TM10) is now shielded from the translocation pathway by TM11, and TM16 is rotated such that Trp^{1198} (TM16) is now facing away from the pore (Fig. 3). Though the positions of Trp^{553} and Trp^{1198} in the Sav1866-based models of MRP1 suggest that their interaction with substrate is unlikely, it is important to consider that this model is based on a crystal structure that represents only one structural conformation in the transport cycle of Sav1866, that is, the outward conformation consistent with the presence of bound ATP [12,14]. *In vivo*, it is expected that ATP hydrolysis and/or ADP release shifts the MSDs to an inward facing conformation to reveal a high-affinity substrate binding site(s) that is accessible from the interior of the cell [12,14]. Thus, it is reasonable to expect that homology models based on inward facing structures are likely to have different sets of residues lining the substrate translocation pathway than outward facing structures. Finally, the homology models presented here, as elsewhere, are typically based on one sequence alignment. Alternate alignments are certainly possible, particularly in the MSD regions, and the moving of a single gap may shift residues such that they turn to face into, or away from, the pore.

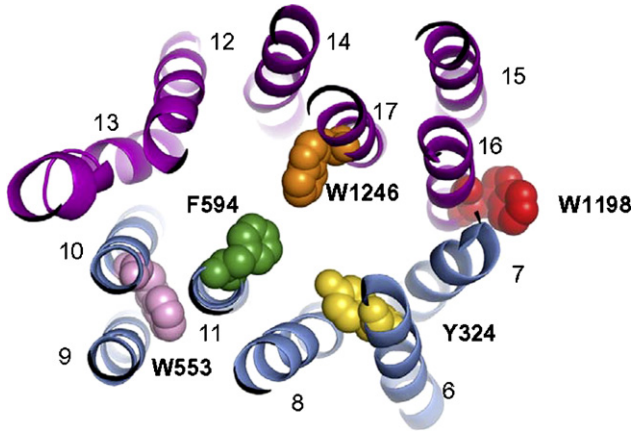

Fig. 3. Location of functionally important aromatic residues in MSD1 and MSD2 of MRP1. Tyr^{324} (Y324) (yellow), Phe^{594} (F594) (green) and Trp^{1246} (W1246) (orange) line the substrate translocation pathway in the Sav1866-based MRP1 model. In contrast, Trp^{1198} (W1198) (red) is facing away from the pore, and Trp^{553} (W553) (pink) is shielded from the translocation pathway by TM11. (For interpretation of color mentioned in this figure the reader is referred to the web version of the article.)

In conclusion, the objective of this study was to generate homology models of MRP1 using the recently published crystal structure of the bacterial Sav1866 exporter as a template. Not unexpectedly, these Sav1866-based models of MRP1 were found to be significantly different from the previous MsbA-based models of MRP1, particularly with respect to the arrangement of the TM helices. The MRP1 models presented are not inconsistent with the biochemical data against which they have thus far been evaluated. Although higher resolution, experimentally determined structures of MRP1 and other human ABC transporters are still sorely needed, the Sav1866-based models described here should be useful in guiding and interpreting ongoing biochemical studies of MRP1 structure-function relationships.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.10.141.

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