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Membrane insertion and topology of the amino-terminal domain TMD0 of multidrug-resistance associated protein 6 (MRP6)

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ABSTRACT

The function of the ATP-binding cassette transporter MRP6 is unknown but mutations in its gene cause pseudoxanthoma elasticum. We have investigated the membrane topology of the N-terminal transmembrane domain TMD0 of MRP6 and the membrane integration and orientation propensities of its transmembrane segments (TMs) by glycosylation mapping. Results demonstrate that TMD0 has five TMs, an N_{out}–C_{in} topology and that the less hydrophobic TMs have strong preference for their orientation in the membrane that affects the neighboring TMs. Two disease-causing mutations changing the number of positive charges in the loops of TMD0 did not affect the membrane insertion efficiencies of the adjacent TMs.

1. Introduction

ATP-binding cassette (ABC) transporters constitute a superfamily of proteins that transport a wide variety of substances across biological membranes by using ATP hydrolysis as an energy source [1]. These transporters consist of a core structure typically containing two transmembrane domains (TMD1 and TMD2) and two nucleotide-binding domains (NBD1 and NBD2). One of the 48 human members of this superfamily is the multidrug-resistance associated protein 6 (MRP6, ABCC6), which physiological function and substrates are unknown. The protein is predominantly localized to the basolateral membrane in liver and kidney [2,3] and has been shown to transport glutathione conjugates *in vitro* [4]. Mutations in MRP6 cause the autosomal recessive disease pseudoxanthoma elasticum (PXE), which is characterized by a progressive ectopic mineralization of elastic fibers in dermal, ocular and vascular tissues [5,6]. Defective MRP6 has been suggested to decrease plasma pyrophosphate levels, which otherwise counteracts mineralization [7], and to affect the regulation of genes directly engaged in the mineralization process [8]. The majority of mutations causing PXE are found in the vicinity of interdomain interactions [9], especially in the NBDs, which have been shown to cooperate for ATP hydrolysis [10,11]. Many of the disease-causing mutations in other ABC transporters alter protein biosynthesis, trafficking and localization apart from affecting transport function, binding and hydrolysis of ATP [12–14].

Separate from the core structure, MRP6 also contains an additional N-terminal transmembrane domain (TMD0) and a connecting loop (L0) of which the former is only found in a handful of other members of the ABCC subfamily of ABC transporters. The function of TMD0 of MRP6 is not known but studies of this domain in other proteins have been aimed to understand its role. In MRP1, TMD0 has been suggested to stabilize retention of the transporter in the plasma membrane or to play a regulatory role in the interaction with other proteins and substrates [15]. In MRP2, TMD0-L0 is involved in apical localization and stabilization [16]. In SUR1,

Abbreviations: ABC transporter, ATP-binding cassette transporter; ER, endoplasmic reticulum; MRP, multidrug-resistance associated protein; NBD, nucleotide binding domain; PXE, pseudoxanthoma elasticum; TM, transmembrane segment; TMD, transmembrane domain; SP, signal peptidase; SPI, signal peptidase inhibitor

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TMD0-L0 is important for regulating gating of the potassium channel Kir6 [17]. Although there is no high-resolution three-dimensional structure of MRP6, it is possible to create three-dimensional homology models of the core domains of MRP6 based on structures of NBDs and TMDs of ABC transporter homologues [9,11]. However, there is no structural information on TMD0, which is predicted to contain five transmembrane segments (TMs) by TMHMM [18].

In mammals, membrane proteins destined for the secretory pathway, such as MRP6, utilize a co-translational pathway for insertion into the endoplasmic reticulum (ER) membrane that is mediated by the translocon Sec61 [19–21]. The hydrophobicity of the TMs and the flanking positively charged residues play crucial roles during membrane insertion and folding. Previously, the membrane insertion of the ABC transporters CFTR (ABCC7) and P-glycoprotein (ABCB1), that both lack TMD0, have been studied [22]. In this study, we have determined experimentally that the membrane topology of TMD0 of MRP6 consists of five TMs with the N- and C-termini on the external and cytoplasmic side, respectively. Furthermore, we have investigated the ER membrane insertion and orientation propensities of the TMs of TMD0 and if they were affected by two PXE-causing mutations. The results suggest that all of the TMs of TMD0 have the ability to be inserted into the membrane independently of each other and it seems that the less hydrophobic TMs have a stronger preference for their orientation in the membrane, which are unaffected by the two PXE-causing mutations investigated.

2. Materials and methods

Detailed protocols for the methods can be found in Supplemental materials.

2.1. DNA manipulations

TMD0-L0 of MRP6 (301 residues) and its deletion mutants were cloned into the pGEM1 vector as previously described [23,24]. For the truncation mutants of TMD0-L0, the following deletions were made: $A_{71} - I_{94}$ (ΔTM2), $A_{101} - E_{125}$ (ΔTM3), $V_{130} - A_{152}$ (ΔTM4) and $V_{165} - P_{191}$ (ΔTM5). N- and C-terminal constructs corresponding to signal peptidase (SP) cleaved fragments were made by introduction of stop codons at the predicted cleavage site ($AA_{151-152}$) and by cloning the C-terminal region (from position 151) with the addition of an initial start codon into pGEM1, respectively. The individual TMs of TMD0 were introduced into two *Escherichia coli* leader peptidase (Lep) model proteins (LepH2 and LepH3) expressed from pGEM1 [25–27]. R_{60}GY deletion was introduced in constructs where TM1 is in LepH2 and in LepH3 and E125K was introduced in constructs where TM3 and TM4 are in both Lep model proteins. Site-specific mutagenesis was performed using the QuikChange™ Site-Directed Mutagenesis protocol from Stratagene. All mutants were confirmed by sequencing of plasmid DNA at Eurofins MWG Operon (Ebersberg, DE).

2.2. In vitro expression

Constructs cloned in pGEM1 were transcribed and translated in the TNT® SP6 Quick Coupled System as previously described [26,27] in the presence and absence of dog pancreas rough microsomes (CRMs) [28].

2.3. Endo H and SPI treatment

To demonstrate N-linked glycosylation by oligosaccharyl transferase, Endo H treatment was performed as previously described [26]. To demonstrate cleavage by signal peptidase, the signal peptidase inhibitor SPI was used as previously described [26,29].

2.4. Analysis and quantifications

Translation products were analyzed by SDS–PAGE, visualized in a Fuji FLA-3000 phosphorimager and quantified with the MultiGauge (Fujifilm) software.

3. Results

3.1. Localization of the N- and C-termini of TMD0-L0 expressed in the ER membrane

The N-terminal 301 residues of wild-type human MRP6, corresponding to TMD0 and the following loop L0, contain five predicted TMs and three natural N-linked glycosylation sites with acceptor asparagines N_{15}, N_{243} and N_{259} (Fig. 1A). This construct (TMD0-L0) was expressed *in vitro* and labeled with [^{35}S] Met in the absence and presence of ER microsomes. SDS–PAGE analysis of the resulting radioactive protein shows two products: one non-glycosylated and one migrating slower and shifted up by approximately 2.5kDa on the gel, which corresponds to a single N-linked glycosylation modification (Fig. 1B). Protein bands were quantified and the percentage of singly glycosylated product was calculated to 57% with respect to the non-glycosylated product indicating that at least 57% is targeted and inserted into the ER membrane. From here onwards we will only consider proteins inserted in the membrane, i.e. with at least one glycosylation, similar to what has been used previously [30] (see Supplemental materials for details of quantifications and calculations). Therefore, the TMD0-L0 construct was considered 100% singly glycosylated. To deduce if the only glycosylation modification detected corresponded to that in the N-terminal tail, an additional acceptor site was introduced between N_{15} and the N-terminus at position 11 in the protein (N11 TMD0-L0), of which the expression resulted in 52 ± 6% singly and 48 ± 6% doubly glycosylated. These results therefore indicate that the N-terminal tail of TMD0 is located in the ER lumen (Fig. 1B).

While the N-terminal glycosylation site that was modified by the oligosaccharyl transferase enzyme complex had the sequence N_{15}QT, the unmodified C-terminal sites had N_{243}SS and N_{258}RS. To exclude the possibility that the lack of glycosylation in the C-terminal tail was due to the presence of a serine instead of a threonine in the glycosylation site, one of them was mutated into threonine (S245T TMD0-L0). This mutant displayed the same amount of singly glycosylated product (100%) as TMD0-L0 and no double or triple modifications (Fig. 1B), which suggests a cytosolic localization of the C-terminal tail of TMD0.

In addition, the loop between TM4 and TM5 was elongated with an additional N-linked glycosylation site (for exact sequence see Supplemental materials) introduced in the TMD0-L0 construct (N166 TMD0-L0, Fig. 1C). This construct displayed 17% singly and 24% doubly glycosylated products as well as two cleaved products, which might be explained by a potential signal peptidase (SP) cleavage site predicted by SignalP [31] in the sequence following TM4 between A151 and A152 (Fig. 1A). This cleavage site is normally not accessible to SP, as seen from the uncleaved wild type TMD0-L0 construct, but only when the sequence of the TM4–TM5 loop is modified. It has been reported previously that SP, which normally only cleaves signal peptides, sometimes cleaves in the ER luminal C-terminal flanking region of TMs in recombinant sequences as demonstrated using an SP inhibitor (SPI) [26,29,32,33]. The active site of SP is located close to the luminal surface of the ER membrane and therefore only translocated