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Phenylalanine 368 of multidrug resistance-associated protein 4 (MRP4/ABCC4) plays a crucial role in substrate-specific transport activity

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ABSTRACT

Multidrug resistance-associated protein 4 (MRP4) is a membrane transporter that mediates the cellular efflux of a wide range of anionic drugs and endogenous molecules. MRP4 transport can influence the pharmacokinetics of drugs and their metabolites, therefore more knowledge about the molecular determinants important for its transport function would be of relevance. Here, we substituted amino acids Phe^{368}, Trp^{995}, and Arg^{998} with conservative or non-conservative residues, and determined the effect on transport of the model substrates estradiol 17-β-D-glucuronide ($E_2 17\beta G$), cyclic guanosine monophosphate (cGMP), methotrexate (MTX), and folic acid into membrane vesicles isolated from baculovirus transduced HEK293 cells overexpressing the mutant MRP4 proteins. This revealed that all Arg^{998} mutations appeared to be deleterious, whereas the effect of a Phe^{368} or Trp^{995} replacement was dependent on the amino acid introduced and the substrate studied. Substitution of Phe^{368} with Trp (F368W) induced a gain-of-function of $E_2 17\beta G$ transport and a loss-of-function of MTX transport, which could not be attributed to an altered substrate binding. Moreover, we did not observe any modification in ATP or ADP handling for F368W. These results, in combination with docking of substrates in a homology model of MRP4 in the inward- and outward-facing conformation, suggest that Phe^{368} and Trp^{995} do not play an important role in the initial binding of substrates. They, however, might interact with the substrates during rearrangement of helixes for substrate translocation, funneling the substrates to the exit site in the outward-facing conformation.

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1. Introduction

Multidrug resistance-associated protein 4 (MRP4/ABCC4) is a member of the C subfamily of ATP-binding cassette (ABC) transporters. This drug efflux transporter translocates substrates across the plasma membrane of the cell in an ATP-dependent manner [1]. MRP4 is known to transport a wide variety of drugs, including antiviral, antibiotic, cardiovascular, and cytotoxic agents, and endogenous molecules that are involved in cellular signaling and communication, such as cyclic nucleotides, ADP, eicosanoids, uric acid, conjugated steroid hormones, folic acid, bile acids, and glutathione [1]. MRP4 is widely expressed in various blood cells, neurons, cardiovascular tissue, and epithelia of organs that are involved in determining the disposition of drugs, including kidney, liver, blood–brain-barrier and intestine, where it can be located either apically or basolaterally depending on the cell type [1–4]. Because of its localization and its broad substrate specificity, MRP4 plays a role in the disposition of various drugs and their metabolites, and could have a key function in cellular protection and extracellular signaling pathways [1]. MRP4 is encoded by the *ABCC4* gene, comprising a protein of 1325 amino acids with a typical ABC transporter core structure. It is composed of two membrane spanning domains (TMDs), each consisting of six transmembrane helices (TM) important for drug binding, and two nucleotide binding domains (NBDs), which bind and hydrolyze ATP to drive transport. *ABCC4* is a highly polymorphic gene, and several non-synonymous polymorphisms have been found in different

Abbreviations: MRP, multidrug resistance-associated protein; eYFP, enhanced yellow fluorescent protein; HEK293, human embryonic kidney 293 cells; DMEM, Dulbecco Eagle's modified medium; F368/Phe^{368}, phenylalanine at position 368 of MRP4; W995/Trp^{995}, tryptophan at position 995 of MRP4; R998/Arg^{998}, arginine at position 998 of MRP4; Ala/A, alanine; Asp, asparagine; Leu/L, leucine; Lys/K, lysine; Ser/S, serine; Tyr/Y, tyrosine; TM, transmembrane helix; TMD, transmembrane domain; NBD, nucleotide binding domain; $E_2 17\beta G$, estradiol 17-β-D-glucuronide; MTX, methotrexate; cGMP, guanosine 3′,5′-cyclic monophosphate; P-gp, P-glycoprotein; LTC_4, leukotriene C_4.

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MRP1  586 LALFNILRFPLN-ILPMVISSIVQ . . . . SYSLQVTTYLNWLVRMSSEMETNIVAV 1261
MRP2  583 ITLFNILRFPLS-MLPMMISSMLQ . . . . SNALNITQTLNWLVRMTSEIETNIVAV 1269
MRP3  572 VSLFNILRLPLN-MLPQLISNLTQ . . . . SYSLQVTFALNWMIRMMSDLESNIVAV 1257
MRP4  355 VTLYGAVRLTVTLFFPSAIERVSE . . . . SYALTLMGMFQWCVRQSAEVENMMISV 1010
MRP5  437 VTVFNSMTFALK-VTPFSVKSLSE . . . . SYAVQLTGLFQFTVRLASETEARFTSV 1160
MRP6  571 LTVLNILNKAQA-FLPFSIHSLVQ . . . . SAALQVTQTLQWVVRNWTDLENSIVSV 1233
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Fig. 1. Alignment (ClustalW2) of amino acid sequences of human MRP1-MRP6. The marked amino acids, F368 in TM6, and W995 and R998 in TM12 of MRP4 have been substituted in this study.

human populations. Previous studies showed that some of the polymorphisms in *ABCC4* affect transport of substrates (e.g. antiviral agents and chemotherapeutic drugs), mainly by affecting the MRP4 protein expression and localization, and might thereby affect the pharmacokinetics of these drugs [5–7].

Because MRP4 plays an important role in the distribution and excretion of drugs and their metabolites, it would be interesting to gain more knowledge about the molecular determinants important for its transport function. To understand the molecular basis of transport, mutational studies of functionally important amino acids are indispensable. In contrast to MRP1, 2, and 3, for MRP4 only one study has been performed that focused on identification of amino acids important for substrate recognition and binding. In that study, we revealed that Phe^{368}, Phe^{369}, Glu^{374}, Arg^{375}, and Glu^{378} in TM6, and Arg^{998} in TM12 of MRP4 are important for its transport function [8]. In addition, we demonstrated that Arg^{375} is involved in MTX transport, but not in cGMP transport.

To further explore which amino acids are important for the transport functionality of MRP4, we investigated the effect of Phe^{368} (TM6), Trp^{995} and Arg^{998} (TM12) amino acid substitutions on the substrate-dependent transport activity of MRP4. We selected Trp^{995} and Arg^{998} because these amino acids are highly conserved within the MRP family (Fig. 1). Moreover, our previously described homology model showed that Phe^{368} was located opposite to Trp^{995} in the substrate binding cavity of MRP4 [8]. Hence, Phe^{368}, Trp^{995}, and Arg^{998} mutants were expressed in human embryonic kidney 293 (HEK293) cells. Several functional characteristics of the transporters were investigated using membrane vesicles isolated from these cells. The results from this study suggest that sequential to initial substrate binding, the aromatic amino acids in TM6 and TM12 can induce substrate-dependent conformational changes, which funnel the substrates to their exit sites.

2. Materials and methods

2.1. Materials

[6,7-^{3}H(N)]-estradiol 17-β-D-glucuronide ($E_2 17\beta G$) (41.8 Ci/mmol) was purchased from PerkinElmer (Groningen, The Netherlands). [3,5,7-^{3}H(N)]-methotrexate disodium salt (MTX) (23 Ci/mmol), [8-^{3}H]-guanosine 3′,5′-cyclic monophosphate ammonium salt (cGMP) (13.5 Ci/mmol), and [3,5,7,9-^{3}H(N)]-folic acid diammonium salt (48.6 Ci/mmol) were purchased from Moravek, Inc. (Brea, USA). Bac-to-Bac and Gateway system, DMEM + Glutamax-I culture medium, Tris, and fetal calf serum were purchased from Life Technologies (Bleiswijk, The Netherlands). Triple flasks (500 cm^2) were purchased from Sanbio BV Biological Products (Uden, The Netherlands). Unlabeled $E_2 17\beta G$, cGMP, folic acid and MTX, sodium butyrate, HEPES, DL-dithiothreitol (DTT), adenosine 5′-triphosphate magnesium salt (ATP) (from bacterial source), and adenosine 5′-monophosphate monohydrate (AMP) (from yeast) were purchased from Sigma–Aldrich (Zwijndrecht, The Netherlands). Sodium monovanadate (anhydrous) was from E. Merck (Darmstadt, Germany) and $MgCl_2$ was from BOOM (Meppel, The Netherlands). D(+)-saccharose was purchased from VWR (Leuven, Belgium). Protein concentrations were determined with a Bio-Rad protein assay kit from Bio-Rad Laboratories (Veenendaal, The Netherlands).

2.2. Site-directed mutagenesis of MRP4 and generation of expression vectors and baculovirus

The previously described Gateway entry vector containing the human MRP4 coding sequence [8] was used as a template for site-directed mutagenesis of the following amino acids: F368W, F368Y, F368A, W995F, W995Y, W995A, R998S, R998K, R998Y, and R998L. The mutagenesis was performed as described previously [8]. Briefly, forward and reverse primers (Biolegio, Nijmegen, The Netherlands) were designed for the specific amino acid substitutions and were used to perform a site-directed mutagenesis PCR on the MRP4 entry vector. After confirmation of the mutations by sequence analysis, the MRP4 mutant entry vectors were cloned into a VSV-G improved pFastBacDual vector for mammalian cell transduction using the Gateway system [8]. Baculoviruses were produced as described in the Bac-to-Bac manual (Life Technologies, Bleiswijk, The Netherlands).

2.3. Cell culture and transduction of HEK293 cells with baculovirus of MRP4 and mutants

HEK293 cells were grown in DMEM-Glutamax-I supplemented with 10% fetal calf serum at 37 °C under 5% CO_2-humidified air. For transduction, HEK293 cells were seeded in 500 cm^2 triple flasks at a confluence of 20%. After 24 h, the culture medium was removed and a mixture of 25 ml medium and 10 ml of the negative control (enhanced yellow fluorescent protein/eYFP), wild type MRP4, or MRP4 mutant baculovirus was added. After an incubation of 15 min at 37 °C, another 40 ml medium was added. Sodium butyrate (5 mM) was added 6 h after transduction and the cells were further incubated at 37 °C under 5% CO_2-humidified air. Three days after transduction, the cells were harvested by spinning them down at 3500 × *g* for 10 min. Pellets we stored at −80 °C until further use.

2.4. Isolation of membrane vesicles

Isolation was performed according to a previously described method, with slight modifications [9]. Briefly, cells were lysed in ice-cold hypotonic buffer supplemented with protease inhibitors and centrifuged at 100,000 × *g*. The pellet was homogenized in ice-cold TS buffer (10 mM Tris-HEPES, and 250 mM sucrose, pH 7.4) with protease inhibitors using a Dounce homogenizer and the suspension was centrifuged at 2000 × *g*. Membranes (in supernatant) were spinned down by a 100,000 × *g* centrifugation step and vesicles were produced using passage through a 27-gauge needle. Protein concentrations were determined by Bio-Rad protein assay kit, and crude membrane vesicles were dispensed in aliquots, frozen in liquid nitrogen, and stored at −80 °C until further use.

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