Characterization of MOAT-C and MOAT-D, New Members of the MRP/cMOAT Subfamily of Transporter Proteins

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Background: Multidrug resistance-associated protein (MRP) and canicular multispecific organic anion transporter (cMOAT) are transporter proteins that pump organic anions across cellular membranes and have been linked to resistance to cytotoxic drugs. We previously identified MOAT-B, an MRP/cMOAT-related transporter, by use of a polymerase chain reaction approach. However, analysis of expressed sequence tag (EST) databases indicated that there may be additional MRP/cMOAT-related transporters. To further define the MRP/cMOAT subfamily of transporters, we used EST probes to isolate complementary DNAs for two related transporter proteins, MOAT-C and MOAT-D. Methods: MOAT-C and MOAT-D expression patterns in human tissues were determined by RNA blot analysis, and chromosomal localization of the genes was determined by fluorescence in situ hybridization. Results: MOAT-C is predicted to encode a 1437-amino-acid protein that, among eukaryotic transporters, is most closely related to MRP, cMOAT, and MOAT-B (about 36% identity). However, MOAT-C is less related to MRP and cMOAT than MRP and cMOAT are to each other (about 48% identity). Like MOAT-B, MOAT-C lacks an N-terminal membrane-spanning domain, indicating that the topology of this protein is similarly distinct from that of MRP and cMOAT. MOAT-D is predicted to encode a 1527-amino-acid protein that is the closest known relative of MRP (about 58% identity). MOAT-D is also highly related to cMOAT (about 47% identity). The presence of an N-terminal membrane-spanning domain indicates that the topology of MOAT-D is quite similar to that of MRP and cMOAT. MOAT-C transcripts are widely expressed in human tissues; however, MOAT-D transcript expression is more restricted. The MOAT-C and MOAT-D genes are located at chromosomes 3q27 and 17q21.3, respectively. Conclusions: On the basis of amino acid identity and protein topology, the MRP/cMOAT transporter subfamily falls into two groups; the first group consists of MRP, cMOAT, and MOAT-D, and the second group consists of MOAT-B and MOAT-C. [J Natl Cancer Inst 1998;90:1735–41]

Cellular resistance is a major obstacle to the successful use of anticancer chemotherapeutic agents. Studies of cell lines selected in vitro for resistance to natural product drugs suggest that at least two transporters of the adenosine triphosphate (ATP)-binding cassette (ABC) family represent important components of the cellular drug resistance machinery. P-glycoprotein (Pgp), the first transporter shown to confer natural product drug resistance, functions as an ATP-dependent pump that transports diverse lipophilic drugs across the plasma membrane (1). The second transporter, multidrug resistance-associated protein (MRP), also confers resistance to natural product drugs (2–4). Although Pgp and MRP confer similar resistance phenotypes (5–7), their structures and substrate specificities are distinct. MRP shares only 23% amino acid identity with Pgp and, in contrast to Pgp, is capable of transporting organic anions such as glutathione S-conjugates (8). More recently, isolation of canicular multispecific organic anion transporter (cMOAT), a liver-specific MRP-related transporter, was reported to confer enhanced sensitivity to cytotoxic drugs (13). In addition, a cMOAT-deficient rat was reported to exhibit reduced biliary clearance of methotrexate (14). These observations suggest that cMOAT may confer resistance to some cytotoxic drugs and that it may also be involved in the hepatobiliary excretion of anticancer agents.

The important functions of MRP and cMOAT led us to attempt the isolation of other related transporters. Using a degenerate polymerase chain reaction (PCR) approach, we previously isolated MOAT-B, an MRP/cMOAT-related transporter (15). Analyses of expressed sequence tag (EST) database sequences in our laboratory (Belinsky MG, Kruh GD: unpublished data) and in other laboratories (12,16) suggest that additional human MRP-related transporters may exist, but their full-length coding sequences have not been reported.

The purpose of this study was to isolate the complementary DNAs (cDNAs) of two additional MRP/cMOAT family members, MOAT-C and MOAT-D, and to compare their predicted amino acid sequences, topologies, and expression patterns with those of the known subfamily members, MRP, cMOAT, and MOAT-B. This comparison helps to define the MRP/cMOAT subfamily with regard to protein structure and tissue-specific expression.

Materials and Methods

Isolation of cDNAs of MOAT-C and MOAT-D

MOAT-C cDNA clones were isolated from bacteriophage libraries prepared from human ovarian cancer cell line A2780 and human leukemia cell line HL60 by plaque hybridization, with the use of Integrated Molecular Analysis of Genomes (I.M.A.G.E.) consortium cDNA clone 113196 as the initial probe (17). The 5’ end of the MOAT-C-coding sequence was obtained by rapid amplification of cDNA ends (RACE) by use of a Marathon cDNA amplification kit (Clontech Laboratories, Inc., Palo Alto, CA) and cDNA prepared from a human ovarian cancer cell line. MOAT-D cDNA clones were isolated from bacteriophage libraries prepared from human liver and monocytes by use of the I.M.A.G.E. consortium cDNA clone 208097 as the initial probe. Nucleotide sequence analysis was performed with an ABI 377 DNA sequencer, and sequences were assembled with the use of the Sequencer program (Gene Codes Corp., Ann Arbor, MI).

Protein sequence was predicted by use of the Wisconsin Genetics Computer Group Package version

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9.1 (Madison, WI). To generate hydrophobicity plots, we used the Kyte-Doolittle algorithm with a window of seven residues. To generate amino acid sequence alignments, we used the PILEUP command (gap weight 3.0; length weight 0.1). Transmembrane-spanning segments, which are approximately 20-amino-acid stretches of nonpolar residues, were assigned by inspection and with the assistance of the TMAP program (18). Nucleotide-binding folds, which are approximately 150- to 170-amino-acid hydrophilic domains characteristic by the presence of Walker A (GXXG/AXGKS/T) and Walker B (dβ,D, where β is a hydrophobic residue) motifs, and the signature C sequence (LSGGQ) of ABC transporters (19), were identified in amino acid alignments with known ABC transporters. The accession numbers of proteins described in the text are as follows: MOAT-B, AF071202; MRP, P33527; cMOAT, U63970; YCF1 (i.e., yeast cadmium resistance factor 1), P39109; SUR (i.e., sulfonil urea receptor), Q09428; CFTR (i.e., cystic fibrosis transmembrane conductance regulator), P13569; and MDR1, P08183.

RNA Blot Analysis

Blots containing poly A+ RNA isolated from human tissues were purchased from Clontech Laboratories, Inc., and hybridized with MOAT-C, MOAT-D, or actin cDNA probes, according to the manufacturer’s directions.

Chromosomal Localization

Preparation of metaphase spreads from phytohemagglutinin-stimulated lymphocytes of a healthy female donor and fluorescence in situ hybridization to chromosomes were carried out as previously described (20). Segments of the MOAT-C or MOAT-D cDNAs inserted in plasmid pBluescript were biotinylated by nick translation in a reaction mixture containing 1 μg DNA, 20 μM each of deoxyadenosine triphosphate, deoxycytidine triphosphate, and deoxyguanosine triphosphate, 1 μM deoxythymidine triphosphate, 25 mM Tris-HCl buffer (pH 7.5), 5 mM MgCl2, 10 mM β-mercaptoethanol, 10 μM biotin-16-deoxyuridine triphosphate (Boehringer Mannheim, Indianapolis, IN), 2 U DNA polymerase I/deoxyribonuclease I (Life Technologies, Inc. [GIBCO BRL], Gaithersburg, MD), and water to a total volume of 50 μL. The probe was denatured and hybridized overnight at 37 °C to chromosomes in the metaphase spreads. Hybridization sites were detected by use of fluorescein-labeled avidin (Oncor, Inc., Gaithersburg, MD) and amplified by addition of anti-avidin antibody (Oncor, Inc.) and a second layer of fluorescein-labeled avidin. The chromosome preparations were counterstained with diamidino-2-phenylindole (DAPI) and observed with a Zeiss Axiopt fluorescence microscope equipped with a cooled charge coupled device camera (Photometrics, Tucson, AZ) operated by a Macintosh computer workstation. Digitized images of DAPI staining and fluorescein signals were captured, pseudo-colored, and merged by use of Oncor Image version 1.6 software.

RESULTS

Isolation of cDNAs of MOAT-C and MOAT-D

A BLAST search (21) of the EST database revealed two clones with significant similarity to MRP and cMOAT. The first clone (I.M.A.G.E. consortium clone 113196) was 1.2 kilobases (kb) in length, 800 base pairs (bp) of which encoded an MRP-related peptide. A segment of clone 113196 was used as a probe to screen bacterial libraries. The resulting cDNA clones contained 2 kb of additional coding sequence and 1 kb of additional 3′ untranslated sequence. An additional 1.7 kb of 5′ sequence was obtained by RACE, yielding a total of about 5.9 kb of cDNA. Nucleotide sequence analysis revealed an open reading frame of 4311 bp that was preceded by an in-frame stop codon located at position −93 and encoding a protein of 1437 residues, which we designated MOAT-C. A cDNA encoding a partial peptide of MOAT-C (residues 493–1438) was described recently (22). The second sequence identified in our database search (I.M.A.G.E. consortium clone 208097) was 1.2 kb in length, of which 588 bp encoded an MRP-related peptide. A segment of this clone was used as the initial probe to screen bacterial libraries, and a total of about 5.2 kb of cDNA sequence was isolated. Nucleotide sequence analysis revealed an open reading frame of 4581 bp, which we designated MOAT-D. An upstream in-frame stop codon was not present in the cDNA clones, and attempts to obtain additional upstream sequences by RACE were unsuccessful. The most upstream ATG, located at nucleotide position 6, was therefore designated as the putative translational initiation site.

Analysis of MOAT-C- and MOAT-D-Predicted Proteins

Typical features of ABC transporters were present in the predicted MOAT-C and MOAT-D proteins (Fig. 1, A; Fig. 1, B). The proteins were composed of hydrophobic domains containing potential transmembrane-spanning helices (α helical stretches of about 20 nonpolar residues) and two nucleotide-binding folds (conserved hydrophobic domains of about 150–170 amino acids that are present in all ABC family transporters). Conserved Walker A and B motifs, as well as conserved C motifs, the signature sequence of ABC transporters, were present in the nucleotide-binding folds. Computer-assisted analysis (18) of potential transmembrane-spanning helices of MOAT-C predicted 12 transmembrane helices with six helices in each of two membrane-spanning domains. This 6 + 6 configuration is in agreement with topological models proposed for several other ABC transporters (23,24) and is shown in Fig. 1, A. Alternative predictions of transmembrane segments were obtained with the use of different program parameters or input alignments. Comparison of the hydrophathy profiles of MOAT-C with other MRP/cMOAT-related transporters (Fig. 2, A) indicated that its structure is similar to that of MOAT-B. Like MOAT-B, MOAT-C has two membrane-spanning domains, each of which is appended N-terminal to an ATP-binding fold. Neither MOAT-C nor MOAT-B has a hydrophobic extension of about 200 amino acids that is present in the organic anion transporters MRP, cMOAT, MOAT-D, and YCF1, as well as in the SUR, a related ABC transporter that is not an organic anion pump. MOAT-C is distinguished from MOAT-B, as well as from CFTR and MDR1, by the presence of an N-terminal hydrophilic extension of 88 amino acids.

In contrast to MOAT-B and MOAT-C, hydrophobicity analysis of MOAT-D indicated that it has three membrane-spanning domains (Fig. 1, B; Fig. 2, A). Similar to MRP, cMOAT, YCF1, and SUR, MOAT-D has an additional hydrophobic domain located at its N-terminus. A 5 + 6 + 6 configuration of transmembrane-spanning helices has been proposed for MRP, in which the N-terminal extension harbors five helices, and six helices are present in both the second and third membrane-spanning domains (23,25–27). Inspection of an alignment of the MOAT-D and MRP amino acid sequences with the use of the GAP program indicated that proposed MRP transmembrane segments were conserved in MOAT-D. This 5 + 6 + 6 model for MOAT-D is shown in Fig. 1, B. An alternative configuration (5 + 6 + 4) was predicted by use of a computer-assisted analysis. MRP has been reported to have two N-linked glycosylation sites in its N-terminus (Asn-19 and Asn-23) and another site located between the first and second transmembrane-spanning helices of its third membrane-spanning domain (Asn-1006) (26). Potential N-terminal (Asn-18) and distal N-glycosylation (Asn-1006/1007) sites were conserved in analogous positions in MOAT-D. Only
The distal N-glycosylation site of MRP is potentially conserved in MOAT-C (Asn-890) and MOAT-B (Asn-746/754) (15).

The degree of relatedness of the nucleotide-binding folds of ABC transporters is considered to be an indication of potential functional conservation. Comparison of the predicted amino acid sequences of the nucleotide-binding folds of MOAT-C and MOAT-D with other ABC transporters indicated that they were most closely related to those of the MRP/cMOAT subfamily transporters MRP, cMOAT, MOAT-B, and YCF1. As shown in Table 1, the nucleotide-binding fold 1 of MOAT-C was about equally related to the other five transporters, with 33.1%–36.5% amino acid identity. Aside from these transporters, MOAT-C was most closely related to SUR, with which its first and second nucleotide-binding folds shared about 49%/51% identity, and the CFTR, with which its nucleotide-binding folds shared about 44%/42% identity (data not shown in Table 1).

The nucleotide-binding folds of MOAT-D were clearly most closely related to those of MRP and cMOAT (67.3%–73.8%) but were slightly more related to those of MRP. In contrast, the nucleotide-binding folds of MOAT-D shared only 54.1%–57.3% identity with those of MOAT-C and MOAT-B. Overall, MOAT-D was again most closely related to MRP (57.6%) and cMOAT (46.8%), but it was substantially more related to MRP. Consistent with the analysis of nucleotide-binding folds, MOAT-D was much less related overall to MOAT-C and MOAT-B, with which it shared only 33.1% and 35.3% identity, respectively. An insertion (Fig. 2, B) of the amino acid sequences of the MOAT-C and MOAT-D nucleotide-binding folds with those of related transporters revealed that nucleotide-binding fold 1 of the MRP/cMOAT-related transporters is distinguished from that of SUR and MDR1 by the presence in the latter of 26 and 13 amino acid insertions, respectively. An insertion is also absent in CFTR.

Expression Pattern of MOAT-C and MOAT-D in Human Tissues

The tissue expression patterns of MOAT-C and MOAT-D were examined by RNA blot analysis. As shown in Fig. 3, A (upper panels), a MOAT-C transcript of the distal N-glycosylation sites that are conserved with reported N-glycosylation sites in multidrug resistance-associated protein (MRP). The indicated MOAT-C transmembrane-spanning helices were predicted by use of the TMAP program and an input alignment of MOAT-B and MOAT-C. The indicated MOAT-D transmembrane helices are based on inspection of an alignment with MRP and hydrophathy analysis with the use of the Kyte–Doolittle algorithm (31).
approximately 6.6 kb (at arrow) was readily detected in several tissues, with highest levels in skeletal muscle, intermediate levels in kidney, testis, heart, and brain, and low levels in most other tissues, including spleen, thymus, prostate, ovary, and placenta. Prolonged exposures were required for detection in lung and liver. MOAT-D (middle panels) was expressed as an approximately 6-kb transcript (at arrow). Compared with the expression pattern of MOAT-C,
the MOAT-D expression pattern was more restricted, with high transcript levels in the colon, pancreas, liver, and kidney and lower levels in the small intestine, placenta, and prostate. Prolonged exposures were required to detect MOAT-D in the testis, thymus, spleen, and lung.

**Chromosomal Localization of MOAT-C and MOAT-D Genes**

The MOAT-C and MOAT-D gene localizations on chromosomes were determined by fluorescence in situ hybridization (Fig. 3, B). Hybridization of the MOAT-C probe to spreads of human lymphocytes in metaphase revealed specific labeling at human chromosome band 3q27 (upper panel). Fluorescent signals were detected on chromosome 3q in each of 22 metaphase spreads scored. Of 75 signals observed, 43 (57%) were on 3q. Paired signals were seen only at band 3q27. Hybridization of the MOAT-D probe revealed specific labeling at human chromosome band 17q21.3 (Fig. 3, B; lower panel). Fluorescent signals were detected on chromosome 17q in each of 21 metaphase spreads scored. Of 83 signals observed, 34 (41%) were at 17q21.3. Paired signals were seen only at band 17q21.3.

**DISCUSSION**

The isolation of MOAT-C and MOAT-D cDNAs extends to five, the number of human MRP/cMOAT subfamily members for which full-length coding sequences are known. On the basis of the degree of amino acid similarity and overall topology, these proteins fall into two groups. The first group is composed of MOAT-D, MRP, and cMOAT. These three transporters are highly related, sharing about 47%–58% amino acid identity. MOAT-D is more closely related to MRP (about 58% identity) than is cMOAT to MRP (about 48% identity) and is thus the closest known relative of MRP. This group of transporters also has in common an N-terminal membrane-spanning domain that is predicted to harbor approximately five transmembrane helices. This N-terminal extension is also present in

![Figure 3](image-url)
YCF1, a closely related transporter, and SUR, a more distantly related protein involved in the regulation of potassium channels (28). The second group of MRP/cMOAT-related transporters is composed of MOAT-B and MOAT-C. Like MOAT-D, MOAT-B and MOAT-C are more closely related to MRP (39.4% and 35.8%, respectively) and cMOAT (36.8% and 36.2%, respectively) than to other human transporters. However, they share considerably less similarity with MRP, cMOAT, and MOAT-D than the latter three transporters share with each other. In addition, MOAT-B and MOAT-C do not have N-terminal membrane-spanning domains, and their topologies are therefore more similar to those of many other eukaryotic ABC transporters that also have only two membrane-spanning domains (e.g., MDR1 and CFTR).

The functions of MOAT-B, MOAT-C, and MOAT-D remain to be elucidated. Both MRP and cMOAT transport glutathione, glucuronide, and sulfate conjugates. Conservation of the MRP/cMOAT substrate specificity is more likely for MOAT-D than for MOAT-B or MOAT-C, given the higher degree of amino acid identity of MOAT-D. One possibility is that MOAT-D may transport a class of organic anions that is distinct from the conjugates transported by MRP and cMOAT. For MOAT-B and MOAT-C, it is difficult to speculate at this time on the implications of the absence of the N-terminal membrane-spanning domain, since the contribution of this domain to substrate specificity and function has yet to be established in MRP and cMOAT. However, the observation that this structural feature is found in all of the known organic anion transporters suggests that it may be a structural hallmark of organic anion transporters. The only known exception to this is SUR. Thus, the absence of an N-terminal membrane-spanning domain in MOAT-B and MOAT-C, as well as their weaker amino acid similarities to MRP and cMOAT, suggest that their functions and substrate specificities may be different from the latter two transporters.

The expression patterns of the three MRP/cMOAT homologues also suggest that their physiologic functions are distinct from those of MRP and cMOAT. We previously found that the MOAT-B transcript is highly expressed in prostate but is also expressed in many other tissues (15). In this study, we found that MOAT-D is predominantly expressed in the colon, pancreas, liver, and kidney. This pattern is distinct from that of MRP, which is widely expressed (29), and cMOAT, which is highly expressed in the liver but is also expressed in the kidney and small intestine (10, 11, 30). In contrast, MOAT-C is expressed at highest levels in skeletal muscle, kidney, testis, heart, and brain and is also expressed in most other tissues but is barely detectable in the lung and liver. Further studies designed to define the substrate specificities of these MRP/cMOAT-related transporters should help to elucidate their physiologic functions as well as their possible contributions to cellular resistance to cytotoxic drugs and the pharmacokinetics of these agents.

References


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NOTES

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