Expression of Human MRP6, a Homologue of the Multidrug Resistance Protein Gene MRP1, in Tissues and Cancer Cells

Marcel Kool, Marcel van der Linden, Marcel de Haas, Frank Baas, and Piet Borst

Division of Molecular Biology, The Netherlands Cancer Institute, 1066 CX Amsterdam [M. K., M. v. d. L., M. d. H., P. B.], and Department of Neurology, Academic Medical Center, 1105 AZ Amsterdam [F. B.], the Netherlands

ABSTRACT

The human multidrug resistance protein (MRP) family contains at least six members: MRP1, the gene encoding the multidrug resistance protein; cMOAT or MRP2, encoding the canicular multispecific organic anion transporter; and four homologues, called MRP3, MRP4, MRP5, and MRP6. The most recently discovered member of the family, MRP6, is analyzed in this report. The MRP6 gene is located on chromosome 16, immediately next to MRP1, and encodes a protein of 1503 amino acids with a predicted molecular weight of M, 165,000. The 3′ end of the MRP6 protein was found to be almost identical with the anthracycline resistance associated (ARA) protein identified previously in epirubicin-selected leukemia cells. Using both 3′- and 5′-derived MRP6 probes, we found that MRP6 is highly expressed in liver and kidney and to a low or very low extent in a few other tissues. No evidence was obtained for an independent expression of the ARA part of the MRP6 gene in normal tissues. To assess a possible role for MRP6 in multidrug resistance, we examined a large panel of resistant cell lines for the (over)expression of MRP6. We found overexpression of the complete MRP6 gene or part of it only in those cell lines with high overexpression and amplification of the MRP1 gene. DNA blot (Southern) analysis showed that MRP6 or a part of it is also amplified in these cell lines. Our results suggest that MRP6 does not play a role in the resistance of the resistant cells analyzed, and that MRP6/ARA is only coamplified with MRP1 because of its location immediately next to it on the same chromosome.

INTRODUCTION

Inherent or acquired resistance to multiple chemotherapeutic agents often prevents the successful treatment of common malignancies. In human cancer cells, MDR2 can be caused by an enhanced drug efflux mediated by members of the large family of ABC transporter proteins, such as the MDR1 P-glycoprotein (reviewed in Ref. 1) and MRP1 (reviewed in Ref. 2). Both membrane proteins are able to transport a range of drugs with different cellular targets. P-glycoprotein transports these drugs in an unmodified form, whereas MRP1 can transport drugs either conjugated to acidic ligands such as GSH, glucuronide, or sulfate, or transport them in an unmodified form together with GSH (3–7). Both proteins confer resistance by decreasing the intracellular concentration of cytotoxic drugs (8, 9).

Other members of the ABC transporter family that might contribute to drug resistance in human cancer cells include cMOAT or MRP2 (10–13), ABC-C (14), TAP (15), ARA (16, 17), and ABC-2 (18). The genes encoding these proteins are overexpressed in some MDR or cisplatin-resistant tumor cell lines. Studies with mutant MRP2 (10–13), ABC-C (14), TAP (15), ARA (16, 17), and ABC-2 contribute to drug resistance in human cancer cells include cMOAT or sulfate, or transport them in an unmodified form together with GSH, whereas MRP1 can transport these drugs in an unmodified form, whereas MRP1 can transport drugs either conjugated to acidic ligands such as GSH, glucuronide, or sulfate, or transport them in an unmodified form together with GSH (3–7). Both proteins confer resistance by decreasing the intracellular concentration of cytotoxic drugs (8, 9).

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Besides MRP1 and MRP2, there are at least four more MRP homologues expressed in humans, called MRP3, MRP4, MRP5, and MRP6 (11). We found MRP3 and MRP5, but not MRP4, to be overexpressed in some resistant cell lines, but no correlation was found thus far between their expression and the resistance of these cell lines (11). An additional MRP gene, here called MRP6, was uncovered recently by the ongoing human genome sequencing project.4 The MRP6 gene is located immediately next to MRP1 on chromosome 16p13. The 3′ end of the predicted protein sequence of MRP6 is almost identical to the recently identified ARA protein (17). To investigate a possible role of MRP6 in drug resistance, we examined the same panel of (multi)drug-resistant cell lines analyzed previously for MRP1–5 (11). We found that the overexpression of MRP6 or the ARA part of this gene is invariably associated with amplification of the adjacent MRP1 gene. MRP6 does not appear to contribute to the resistance of the cell lines analyzed.

MATERIALS AND METHODS

Cell Lines. All cell lines used in this study have been described before in Kool et al. (11) and references therein. All cell lines were grown in DMEM or RPMI 1640 (Life Technologies, Inc.), supplemented with 10% FCS, 2 mM glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin. All cells were free of Mycoplasma as tested by the use of the Gene-Probe rapid Mycoplasma detection system (Gene-Probe, San Diego, CA).

Cloning and Sequencing of MRP6 cDNA. One MRP6 cDNA clone (no. 200946) was obtained from the I.M.A.G.E. consortium (23). Additional MRP6 cDNA clones were isolated with RT-PCR using total RNA isolated from human liver and MRP6-specific primers based on the genomic sequence of MRP6 in the database (accession number U91318). The following primers were used to check expression of specific parts of the MRP6 gene: mk78, 5′-GAGGCTGAACCTGGCGGCAC-3′; mk100, 5′-CTCTTCTCTCGGTGGACTC-3′; mk113, 5′-GAATCGAAGACACTGGAAG-3′; mk114, 5′-CCCTGAGAGAGACTGG-3′; mk115, 5′-GCTATTGCCTTGTTGTGC-3′; and mk116, 5′-ATGCCATGCGCAGGGAACAG-3′. cDNA clones were sequenced using the ABI 377 automatic sequence. Sequence analysis was done using the GCG package of Wisconsin University, version 9.1 (24). The MRP6 cDNA sequence has been deposited with GenBank under accession number AF076622.

RNA. Cytoplasmic RNA from cell lines was isolated by a NP40 lysis procedure (25). Total cellular RNA from tissue samples obtained during surgery or autopsy was isolated by acid guanidinium thiocyanate-phenol-chloroform extraction (26). Poly(A)+ RNA was purified using Dynabeads (Dynal, Oslo, Norway).

RNase Protection Assays. By PCR amplification of MRP6 cDNA, two fragments were generated that were used as RNase protection probes. The 5′-cDNA probe of 259 bp was generated using the MRP6 cDNA clone no. 200946 from the I.M.A.G.E. consortium and primers mk78 5′-GAGGCTGAACCTG-
GCGGCCCCAC-3’ (forward primer) and mk79 5’-GGAGTCTACTTTAGACAGTTTATACCC-3’ (reverse primer). This MRP6 cDNA clone contains, besides correct exon sequences, part of the intron sequence between exons 2 and 3 and represents part of an incomplete spliced MRP6 mRNA. The 5’ probe was made such that when this incomplete spliced mRNA is present in cells or tissues, a fragment of 259 bp will be detected, but when the mRNA is spliced correctly, a fragment of 168 bp, corresponding to nucleotides 73–242 (GenBank accession number AF076622) will be detected. The 3’ cDNA probe of 237 bp, corresponding to nucleotides 3019–3255, was generated with RT-PCR using total RNA isolated from human liver and the primers mk80 5’-GGGCTGTTTGCCTCC-3’ (forward primer) and mk81 5’-GGCTGACCTCCAGGAGTCTACTTTA-3’ (reverse primer). This 3’ probe also maps within the ARA cDNA sequence (GenBank accession number X95715). Both fragments were cloned into pGEM-T (Promega Corp., Madison, WI), and the sequences were confirmed. For RNase protection assays, α-32P-labeled RNA transcripts were transcribed from either Ncol (3’)- or SphI (5’)-linearized plasmids, using Sp6 RNA polymerase. For MRP1 RNA detection, a 244-bp MRP1 cDNA fragment was used [nucleotide positions 239–483 (27)]. RNase protection assays were carried out according to Zinn et al. (28), modified by Baas et al. (29). Protected probes were visualized by electrophoresis through a denaturing 6% acrylamide gel, followed by autoradiography. In all experiments, a probe for γ-actin was included as control for RNA input. The amount of MRP1 and MRP6 RNA relative to the amount of γ-actin RNA was calculated using a phosphorimager (Fuji BAS 2000, TINA 2.08h).

Southern Blot Analysis. Genomic DNA was isolated from cell lines as described by Miller et al. (31). DNA (10 μg) was digested with the restriction enzyme BamHI, and 10 μg of each digest were separated by electrophoresis in a 0.8% agarose gel. Separated DNA was transferred to Hybond-N nylon membrane, and hybridization was carried out as described previously (25) using either MRP1 or MRP6 cDNA probes.

RESULTS

MRP6 cDNA Sequence. The human MRP6 gene is located on chromosome 16p13, immediately next to MRP1. The genes are in opposite orientation, and the 3’ ends are only 9 kb apart (Fig. 1).4 The complete genomic sequence of MRP6 contains 99806 bp, and Adams et al.4 concluded that the 29 predicted exons would encode a protein of 1401 amino acids (GenBank accession no. U91318). However, multiple sequence alignments of the predicted protein sequence of MRP6 with the protein sequences of the other human MRP homologues MRP1 (27, 32), MRP2 (10, 33), MRP3,5 and MRP5 showed that several stretches of amino acids were missing in the predicted MRP6 sequence in comparison with the other MRP homologues [Fig. 2; missing amino acids in the MRP6 sequence of Adams et al.4 are indicated in bold]. This suggested that the actual protein sequence of MRP6 is longer than predicted. We confirmed this with RT-PCR using total human liver RNA and specific MRP6 primers. An extra exon was found in the genomic sequence of MRP6 between the predicted exons 2 and 3 as verified by RT-PCR using the primers mk78 and mk113. Another extra exon is present between exons 5 and 6 as shown with primers mk114 and mk115. The sequence of this extra exon was also found in an EST clone (no. 108190) derived from the MRP6 gene and found in dbEST. Furthermore, sequence alignments and RT-PCR (primers mk100 and mk116) showed that exon 25 encodes 16 amino acids more than predicted. On the basis of our analyses, we conclude that the total number of exons in the MRP6 gene is 31 and is equal to the number in the MRP1 gene (34). In addition, all of the intron-exon boundaries appear to be conserved between both genes. However, the sizes of most of the introns are not conserved and are much larger in the MRP1 gene (34).6 This explains why the MRP1 gene spans at least 200 kbp, about twice the size of the MRP6 gene.

The 31 exons of MRP6 encode a protein of 1503 amino acids, with a predicted molecular weight of M, 165,000. After our analysis was completed, Hirohashi et al. (35) reported the sequence of a rat cDNA, corresponding to a protein of 1502 amino acids, called MLP-1. MLP-1 and MRP6 are 79% identical in sequence and nearly identical in size, and we infer that MLP-1 is the rat homologue of MRP6. The homology of the human MRP6 protein with other known ABC transporters is shown in Table 1. The highest overall identity was found with human and mouse MRP1 (45.0 and 45.5% identity, respectively). Our hydrophobicity analysis (not shown) of the MRP6 amino acid sequence also indicates a conserved organization of putative transmembrane domains, resulting in a similar topology as suggested for MRP1. The MRP1 protein probably spans the membrane 17 times, with the NH2 terminus being extracellular (36–38). This unusual topology is supported by the demonstration that the Asn19 and Asn23 sites on the NH2 terminus of MRP1 are indeed glycosylated in vivo (36). There is an additional glycosylated site in MRP1, at Asn1006. All three sites are conserved in human MRP2. In human and rat MRP6, only one of the sites at the NH2 terminus is conserved; the others are absent (Ref. 35 and Fig. 2).

ARA protein. By comparing the protein sequence of MRP6 with other known ABC transporters, we found that the 3’ end of MRP6 is almost identical to the sequence of ARA (Ref. 17; Fig. 2). ARA was found to be overexpressed in a human leukemia T-cell line CCRF-CEM/E1000 selected for resistance against epirubicin (39). The 1936-bp ARA cDNA contains an open reading frame encoding a protein of 453 amino acids with a predicted molecular weight of M, 49,600. When first published, ARA showed the highest identity with human MRP1 (51% identity). However, it is clear now from our data that ARA represents the 3’ end of MRP6. The last 438 amino acids of ARA (of 453) are encoded by exons 23 and exons 25–31 of MRP6. Exon 24 of MRP6 (66 amino acids) is completely missing in the ARA sequence (Fig. 2). Exon 23 is present in the ARA cDNA sequence, but the translated amino acid sequence is partly in another frame. This could be due to a sequence error in the ARA cDNA sequence. The first 15 amino acids of ARA are not encoded by any sequence in the MRP6 gene. However, the first eight amino acids of ARA were found to be identical to the first eight amino acids of MRP1. The other seven amino acids (amino acids 9–15) at the 5’ end of ARA were also encoded by the 5’ end of MRP1 but came from another reading frame. Moreover, comparison of the 5’ nucleotide sequence of the ARA cDNA with the nucleotide sequence of the MRP1 cDNA showed that the first 160 nucleotides of the ARA cDNA (115 nucleotides 5’ untranslated and 45 nucleotides 5’ coding region) are nearly identical to the 5’ nucleotide sequence of MRP1 cDNA.

EST Database. Our search of the EST database (dbEST; Ref. 40) with the MRP6 cDNA sequence yielded no EST sequences derived from the 3’ end of the coding region of MRP6 cDNA. This is remarkable because most cDNA libraries in the EST database were made with oligo-dT primers. As a consequence, most EST
Fig. 2. Multiple sequence alignment of the human MRP1, MRP2, MRP6, and ARA proteins. Protein sequences were aligned using the PILEUP program of GCG (25). The GenBank accession numbers for the proteins used in this comparison are the following: MRP1, L05628; MRP2, U49248; MRP6, AF076622; and ARA, X95715. The Walker A and B motifs and the nucleotide binding domain-specific signature sequences are doubly underlined. The 17 putative transmembrane domains are singly underlined. Asterisks above the alignment indicate N-glycosylation sites. Additional amino acids of the MRP6 protein that were not predicted in the database sequence of Adams et al. (23) are shown in bold.
sequences in dbEST correspond to the 3' ends of genes. This was also found when we searched the database for the other MRP homologues, MRP2–5. Only a few EST sequences were found derived from the 3’ untranslated region of MRP6, which overlap with the 3’ untranslated region sequence of ARA (17), but most EST sequences came from the 5’ end of MRP6. Almost all overlapping EST sequences containing exons 1 and 2 of MRP6 (9 of 10 clones, all from a fetal liver spleen cDNA library) also contained part of the sequence of intron 2. This could be due to incomplete splicing of the MRP6 RNA.

**Tissue Distribution of MRP6 RNA.** To examine the expression of MRP6 RNA in various human tissues and to see whether ARA and MRP6 are differentially expressed, we made two different RNase protection probes to detect ARA and/or MRP6 RNA expression. The 3’ probe (C in Fig. 1) detected both ARA and MRP6 RNA, whereas the 5’ probe (D in Fig. 1) detected only MRP6 RNA. The 5’ probe allowed us to see both the incompletely spliced MRP6 RNA, containing part of intron 2, found in the fetal liver cDNA library, and the completely spliced MRP6 RNA.

With both probes, a similar expression pattern was obtained in all tissues (Fig. 3), indicating that ARA is not expressed separately from the MRP6 gene. High expression of MRP6 RNA was found in kidney and liver and low to very low expression levels in most other tissues. Only a few tissues (spleen, testis, bladder, heart, brain, and tonsil) contained no detectable MRP6. In all tissues with MRP6 RNA expression, both the incompletely and completely spliced forms were detected with the 5’ probe, mostly in more or less equal amounts (Fig. 3B). Some tissues contained high levels of incompletely spliced MRP6 (stomach, salivary gland, thyroid gland, and ovary), others (liver and kidney) had lower levels. Besides the two protected fragments mentioned, we always detected in all tissues with the 5’ probe a third protected fragment with a size between the other two fragments (Fig. 3B). This probably comes from another incompletely spliced form of the MRP6 RNA. Expression of MRP6 is relatively low, even in liver, and we have been unable to detect full-length mRNA of the expected size on blots, using either total RNA or poly(A)+ RNA.

**Expression of MRP6 RNA in Human Cancer Cells.** For the expression of MRP6 RNA in human cancer cell lines, we studied the same panel of cell lines analyzed previously for MRP1–5 expression (11). In most cell lines no (2008 series) or only very low levels of MRP6 RNA were detected (most other cell lines; data not shown). Significant levels of MRP6 RNA were only found in human lung cancer cell lines, which also overexpress MRP1 and which were selected for high levels of doxorubicin resistance (Fig. 4 and Table 2).

Using the 3’ probe, increased levels of MRP6 RNA were detected in the GLC4/ADR cells, and some increase was detected in the HL60/ADR cells. A decrease was found in the COR-L23/ADR cells (Fig. 4A). With the 5’ probe, overexpression was detected again in the GLC4/ADR cells and a little in the MOR/R and HL60/ADR cells. We also detected overexpression in the COR-L23/ADR cells, not of the correctly spliced fragment of 168 bp, but only of the two incompletely spliced fragments (Fig. 4B and Table 2).

**Amplification of MRP1 and MRP6.** Southern blots made with BamHI-digested genomic DNA of the cell lines overexpressing MRP1 and MRP6 were hybridized with 5’ and 3’ cDNA probes of MRP1 and MRP6. The MRP1 gene is amplified in all doxorubicin-resistant cell lines (GLC4/ADR, COR-L23/ADR, HL60/ADR, and MOR/R), as reported before (27, 41–43). Fig. 5A shows the results with the 3’ probe. The results with the 5’ probe were similar (data not shown and Table 2).

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**Table 1 Sequence comparison of human MRP6 protein (1503 amino acids) with related ABC transporters**

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with the results obtained with the 3' cDNA probe, which detected no amplification in the COR-L23/ADR cells (Fig. 5B and Table 2). The amplified BamHI fragment in the COR-L23/ADR cells is a little smaller than in the parental COR-L23 cells, indicating a possible rearrangement in the MRP6 gene in these cells as well. This amplification pattern agrees with the detected overexpression of MRP6 using the 5' probe for RNase protection (Fig. 4B and Table 2).

**DISCUSSION**

**The MRP Gene Family.** Six members of the MRP family have thus far been identified in humans. Because the human genome has not been sequenced completely yet, it is possible that more MRP-related genes will be found. If more MRP genes exist, it is unlikely that these are widely expressed, because no additional MRP-like sequences are detectable in the large number of EST sequences present in the current dbEST database. Multiple MRP-related genes have been identified, not only in humans but also in the yeast *Saccharomyces cerevisiae* (five), in the nematode *Caenorhabditis elegans* (at least four), and in the plant *Arabidopsis thaliana* (at least five; Refs. 44–46). The encoded proteins have only been characterized in a few cases. Human MRP1 and MRP2, yeast YCF1, and *A. thaliana* MRP1 and MRP2 function as GS-X pumps (3, 4, 13, 47–51). A potential role in drug resistance has only been shown for human MRP1 and MRP2, yeast YCF1 and YOR1, and *C. elegans* MRP1 (12, 27, 34, 45, 52–54). We have found previously that MRP1, MRP2, MRP3, and MRP5, but not MRP4, were up-regulated in a few drug-resistant human tumor cell lines. Thus far, correlations with drug resistance were only found for MRP1 and MRP2 expression (11).

**Expression of MRP6 in Normal Tissues.** The analysis of MRP6 expression is complicated by the fact that this gene yields multiple transcripts. As we have shown here, the recently identified ARA gene actually represents the 3' end of the MRP6 gene. In theory, the ARA cDNA could therefore have been derived from a MRP6 splice variant. However, we find with 3' and 5' probes similar levels of MRP6 RNA in normal tissues (Fig. 3), making it unlikely that the ARA part is expressed separately from the complete MRP6 gene. In addition, our analysis of the EST database showed that several 5' end-derived MRP6 transcripts contain unspliced intron sequences. Our 5' RNase protection probe was constructed to allow detection of both incompletely and completely spliced transcripts. In all normal tissues and cell lines with MRP6 expression, both forms of MRP6 RNA were detected with this probe (Fig. 3B). Only the ratio between the unspliced and spliced forms differed between tissues. It is unlikely that the unspliced form gives rise to a functional protein, because there is no open reading frame in the sequence of intron 2. The physiological role of MRP6 remains unknown. The high level of MRP6 expression in the excretory organs liver and kidney is compatible with a role in cellular detoxification by secretion of GSH S-conjugates or other organic anions, as has been shown for MRP1 and MRP2.

**Expression of MRP6 in Resistant Cell Lines.** Our analysis of a panel of MDR and cisplatin-resistant cell lines has provided no indications for involvement of MRP6 in drug resistance. Most cell lines showed no or only very low MRP6 RNA levels. Overexpression of MRP6 was only detected in doxorubicin-resistant lung cancer cell lines known to have high overexpression of MRP1 (27, 42).
In each case, however, the overexpression is associated with coamplification of MRP1 and MRP6. In the GLC4/ADR and MOR/R cell lines, the entire MRP6 gene appears to be amplified, and the amplified gene yielded equal levels of transcript detected with 3' and 5' RNase protection probes (Fig. 4 and Table 2), although the levels are very low in the MOR/R cell line. The amplicon in the GLC4/ADR gene is large enough to contain both genes (41, 42). In the other cell lines, the MRP6 gene is also amplified but rearranged in some (HL60/ADR) or all (COR-L23/ADR) amplicons (Fig. 5 and Table 2). This follows from the abnormal restriction fragments derived from the amplicon (Fig. 5), the unequal levels of transcript detected by 3' and 5' probes (Fig. 4 and Table 2), and amplification of only the 3' part (HL60/ADR) or the 5' part (COR-L23/ADR) of the MRP6 gene (Fig. 5 and Table 2). In other doxorubicin-resistant cell lines that overexpress MRP1 without amplification of the gene, like the 30.3M cell line (42), we did not detect any overexpression of MRP6 (data not shown). On the basis of these results, we conclude that overexpression of (part of) MRP6 is only the consequence of its coamplification with the MRP1 gene and that MRP6 does not contribute to resistance in the cell lines analyzed here.

The ARA Gene. Our results also clarify the nature and significance of the ARA gene (16, 17). We show here that ARA mainly contains the 3' end of the MRP6 gene. Its amplification in resistant cells can be explained by coamplification with MRP1. Moreover, the published ARA cDNA sequence (17) starts with 160 nucleotides derived from MRP1. Therefore, it represents a jumbled MRP1-MRP6 fusion mini-cDNA. It is possible that amplification of the MRP1 and MRP6 genes in the CCRF-CEM/E1000 cell line, used to identify ARA (17), was accompanied by complex rearrangements resulting in some amplicons encoding the fusion mini-gene. Cloning/sequencing artifacts could also provide an explanation. Whatever the origin of ARA, it is unlikely that it could encode any protein contributing to drug resistance, because the mRNA, if translated, would only encode a fragment of...

![DNA and RNA levels of the cell lines analyzed](image)

**Table 2** DNA and RNA levels of the cell lines analyzed

| Cell lines | Probe A | | | Probe B | | | Probe C | | | Probe D |
|---|---|---|---|---|---|---|---|---|---|
| | DNA | RNA | DNA | RNA | DNA | RNA | DNA | RNA | DNA | RNA |
| GLC4 | +** | + | + | nd | | | | | |
| GLC4/ADR | +++ | +++ | +++ | nd | | | | | |
| COR-L23 | + | + | + | nd | | | | | |
| COR-L23/ADR | +++ | +++ | +++ | nd | | | | | |
| MOR/P | + | | + | nd | | | | | |
| MOR/R | ++ | + | ++ | nd | | | | | |
| HL60 | + | + | + | nd | | | | | |
| HL60/ADR | +++ | +++ | +++ | nd | | | | | |

**---** +++++, low to high levels of DNA and RNA; nd, not determined; −, no expression.

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MRP6 containing the last five transmembrane domains and one ATP-binding site.

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Expression of Human MRP6, a Homologue of the Multidrug Resistance Protein Gene MRP1, in Tissues and Cancer Cells

Marcel Kool, Marcel van der Linden, Marcel de Haas, et al.


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