Vol. 162, No. 1, 1989 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 14, 1989 Pages 224-231

P-GLYCOPROTEIN GENE (MDR1) cDNA FROM HUMAN ADRENAL: NORMAL P-GLYCOPROTEIN CARRIES Gly^{185} WITH AN ALTERED PATTERN OF MULTIDRUG RESISTANCE

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Received June 2, 1989

SUMMARY: We isolated a full-length MDR1 cDNA from human adrenal where P-glycoprotein is expressed at high level. The deduced amino acid sequence shows two amino acid differences from the sequence of P-glycoprotein obtained from colchicine-selected multidrug resistant cultured cells. The amino acid substitution Gly→Val at codon 185 in P-glycoprotein from colchicine resistant cells occurred during selection of cells in colchicine. As previously reported, cells transfected with the MDR1 cDNA carrying Val^{185} acquire increased resistance to colchicine compared to other drugs. The other amino acid substitution Ser→Ala at codon 893 probably reflects genetic polymorphism. The MDR1 gene, the major member of the P-glycoprotein gene family expressed in human adrenal, is sufficient to confer multidrug-resistance on culture cells.

The human MDR1 gene encodes a 170-kDa transmembrane glycoprotein (P-glycoprotein) (1) that was initially isolated from human multidrug-resistant cells (2, 3). P-glycoprotein works as an efflux pump to remove a variety of structurally dissimilar antitumor drugs from multidrug-resistant cells (4). When introduced in drug sensitive cells, the human MDR1 gene confers multidrug resistance on these cells (5).

The MDR1 RNA is found at substantial level in the liver, kidney, colon, and small intestine and at high level in the adrenal (6). Immunohistochemical analysis (7) has demonstrated the specific localization of P-glycoprotein at the apical surface of biliary hepatocytes, columnar epithelial cells of the colon and small intestine and on the brush border of proximal tubule cells in the kidney. P-glycoprotein is also found on specialized capillary endothelial cells of the brain and testis (8, 9). These results suggest that P-glycoprotein helps excrete metabolites and natural cytotoxic substances in the diet (4) or act as part of the blood-brain or blood-testis barrier. In the adrenal, however, P-glycoprotein was found to be diffusely distributed (7), suggesting that it might have a role different from in other organs.

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0006-291X/89 $1.50

Recent studies revealed high levels of MDR1 expression in tumors derived from adrenal, colon and kidney which are known to be intrinsically drug resistant (6, 10, 11). These results indicate that high level of expression of P-glycoprotein can be associated with multidrug-resistance in human tumors.

One strategy to circumvent multidrug resistance in tumors is to inhibit the function of P-glycoprotein. It is very important to elucidate the physiological function of P-glycoprotein in human organs in order to foresee the possible side effects caused by preventing its activity. As a first step in studying the function of P-glycoprotein in human adrenal which is of special interest because of its high expression and diffuse distribution, we isolated a full-length MDR1 cDNA from normal human adrenal.

MATERIALS AND METHODS

cDNA cloning and sequencing

Poly(A)$^+$ RNA from human adrenal was isolated by standard procedure. cDNA was synthesized essentially as described by Gubler and Hoffman (12) except for using an oligo (dT) primer with a NotI site at its 5'-end. After blunt-end synthesis, cDNA was digested with NotI and inserted into SmaI and NotI digested and dephospholylated Bluescript vector (Stratagene). The library was screened with the MDR1 cDNA clones pMDR5A and pMDR10 (5). Sequencing of cDNA clones was done by the dideoxy sequencing procedure (13) after subcloning into M13 mp18 and M13 mp19.

Dried-gel Southern hybridization

To detect single copy genes with oligonucleotide probes, we used dried-gel hybridization (14). EcoRI digested genomic DNA was denatured in 0.5% agarose after electrophoresis. The dried-gel was hybridized in a solution containing 2 x 10^6 cpm/ml of ^{32}P-labeled oligonucleotide at 37^{o}C. Oligonucleotide probes were labeled by synthesizing complementary strands from a primer with Klenow polymerase and ^{32}P-dCTP (3000Ci/mmol). The gel was then washed in a solution containing 3M tetramethylammonium chloride at 2^{o}C-5^{o}C below the postulated Td for the oligonucleotides (15).

Construction of chimera plasmids and drug resistance assay of transfectants

The plasmid pHaMDR1/A contains an MDR1 cDNA derived from the colchicine-selected KB cell line KB-C2.5, carrying valine at codon 185 and alanine at codon 893 (16). The plasmid pHaMDRA1 carrying glycine at codon 185 and serine at codon 893 was constructed by replacing the 3.5 kb SacII-PstI fragment of pHaMDR1/A with the equivalent fragment of pMDRA1. The plasmid pHaMDRGA, encoding glycine at codon 185 and alanine at codon 893, was obtained by replacing the 2.2 kb SacII-HindIII fragment of pHaMDR1/A with the equivalent fragment of pMDRA1.

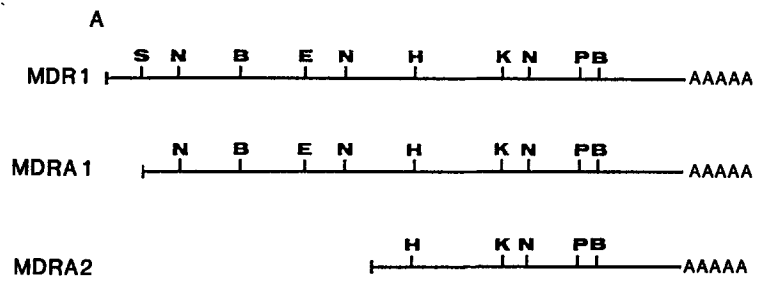
Plasmids were cotransfected into drug-sensitive NIH3T3 cells with pSV2neo by the calcium phosphate coprecipitation method as described (5). Transfected cells were first enriched by selection in medium containing G418 at 0.8 mg/ml; then pooled G418-resistant cells (about 800 colonies each) were selected in medium containing colchicine at 60 ng/ml. After two weeks of selection, about 500 colonies per 10 μg of pHaMDR1/A and 200 colonies per 10 μg of pHaMDRA1 or pHaMDRGA were obtained. Five independent clones from each transfection were picked and expanded for further analysis.

The colony-forming ability of transfectants was measured by plating aliquots of 300 cells in duplicate or triplicate into 60 mm dishes. After 10 days of incubation in 4 ml medium containing different concentration of the drugs, colonies were stained with methylene blue and counted.

RESULTS

Cloning and sequencing of full-length cDNA from human adrenal

A cDNA library from human normal adrenal was constructed and screened as described in MATERIALS AND METHODS. Two positive clones pMDRA1 and pMDRA2 were isolated (Fig. 1A). Sequence analysis revealed that the larger clone, pMDRA1, contained the entire



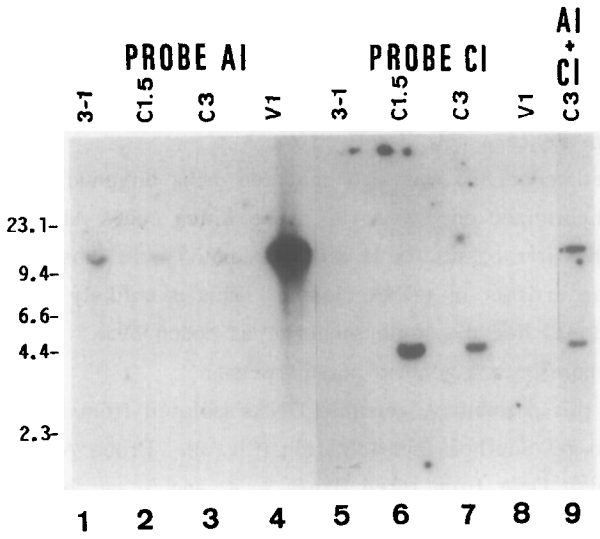
B

MDR1	MDRA1
554	554
AAT GAA GTT ATT GGT	AAT GAA GGA ATT GGT
Val(185)	Gly(185)
2677	2677
GAA GGT GCT GGG AAG	GAA GGT TCT GGG AAG
Ala(893)	Ser(893)
-43	-43
CTTCCAAGATTTCAC	CTTCCAAAATTTCAC
540	540
ATGTCTCTAAGATTA	ATGTCTCCAAGATTA
1236	1236
TGAAGGGCCTGAACC	TGAAGGGTCTGAACC
1641	1641
TGGTTCGCAACCCCA	TGGTTCGTAACCCCA
3435	3435
AAGAGATCGTGAGGG	AAGAGATTGTGAGGG
4036	4036
AGAAATCATAGTTTA	AGAAATCGTAGTTTA

Fig. 1 (A) Restriction maps of KB-C2.5 derived MDR1 cDNA and the MDR cDNA clones isolated from human adrenal. B:BalI, E:EcoRI, H:HindIII, K:KpnI, N:NcoI, P:PstI, S:SacI. (B) The different nucleotides and deduced amino acid sequences between MDR1 and pMDRA1. The different nucleotides and amino acids are underlined. Nucleotides are numbered from the putative translation initiation site.

coding region. The 5'-noncoding region of pMDRA1 was 137 bases long and was shorter than the previously isolated MDR1 cDNA. The MDR1 cDNA was initially isolated from the colchicine-selected multidrug resistant KB carcinoma cell line, KB-C2.5, and appeared to represent a transcript from the upstream promoter of the MDR1 gene (2). The downstream promoter is mainly used in human organs including adrenal (17). The 5'-end of pMDRA1 corresponded to the transcription start site of the downstream promoter of MDR1 gene. These results indicate that pMDRA1 is a full-length MDR1 cDNA from the human adrenal.

There were, however, nine nucleotide differences between the sequence of KB-C2.5 derived cDNA and pMDRA1 (Fig. 1B). Recently a full-length MDR1 cDNA was isolated from the vinblastine-selected KB line, KB-V1, and was found to have three nucleotide differences compared to the sequence of KB-C2.5 derived cDNA (18). These three nucleotide differences were found also in pMDRA1 at positions 540, 554 and 555 and these nucleotides in pMDRA1 were identical to those in KB-V1 derived cDNA. The differences at positions 554 and 555 resulted in a single amino acid substitution (Val→Gly) at codon 185. The nucleotide difference at position 2677 also resulted in an amino acid substitution



A1; 5' TT AAT GAA GGA ATT GGT GAC 3'
C1; 5' TT AAT GAA GTT ATT GGT GAC 3'
P1; 3' AA CCA CTG 5'

Fig. 2 Dried-gel Southern hybridization of DNA from KB cell lines. The oligonucleotides used for preparing the ^{32}P-labeled probes are shown at the bottom. A1 and C1 correspond to positions 545 to 564 of the adrenal derived MDR1 cDNA and the KB-C2.5 derived MDR1 cDNA, respectively. P1 was used as a primer for preparing the ^{32}P-labeled antisense probes. 10 µg of EcoRI digested DNA from KB3-1 (lanes 1 and 5), 2.5 µg of EcoRI digested DNA from KB-C1.5 (lanes 2 and 6), KB-C3 (lanes 3, 7 and 9), and KB-V1 (lanes 4 and 8) were analyzed by dried-gel Southern hybridization with probes A1 (lanes 1-4), C1 (lanes 5-8), and mixed probes of A1 and C1 (lane 9). The upper signal in lane 9 looks strong because the A1 probe contains three cytidine sites which can incorporate ^{32}P-dCTP, but the probe C1 has only two.

(Ala→Ser) at codon 893. One of nine differences was found in the 5'-noncoding region at position -43. This sequence was confirmed by analyzing the first exon of the MDR1 gene in genomic DNA (Ueda, K. et. al. unpublished results). Other nucleotide differences were in the third position of each codon or in the 3'-noncoding region and did not result in amino acid substitutions.

Analysis of two amino acid substitutions in adrenal derived cDNA

As noted above, there were two amino acid substitutions between adrenal derived and KB-C2.5 derived MDR1 cDNAs. Dried-gel Southern hybridization analyses were performed using oligonucleotide probes (Fig. 2) specific to each sequence to determine which amino acids are found in the human genome and in DNA from multidrug-resistant KB cells. The probe A1 (Gly at codon 185) hybridized to a fragment of approximately 10 kb DNA from multidrug-resistant cells (Fig. 2) and human kidney (data not shown). This fragment was amplified in vinblastine-selected multidrug-resistant KB cells, KB-V1 (lane 4). The probe C1 (Val at codon 185) hybridized to DNAs only from colchicine-selected multidrug-resistant KB cells (KB-C1.5 and -C3) (lanes 6 and 7). The hybridized fragment was about 4.4 kb and was amplified in these cells. These results indicate that the MDR1 gene in the human genome has glycine at codon 185 and that one allele of the MDR1 gene was mutated during

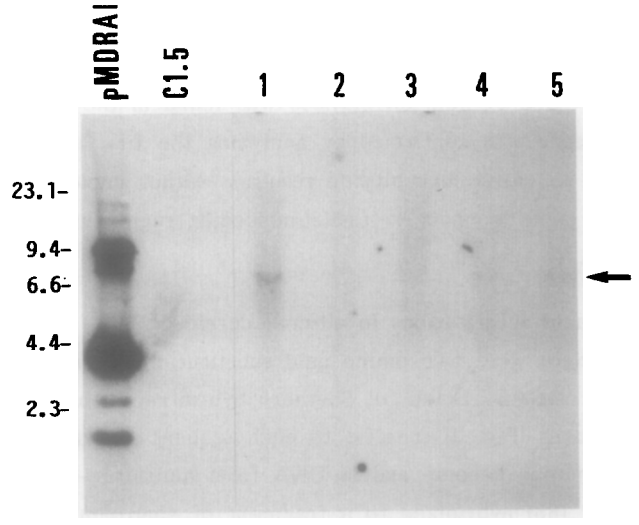
selection and amplified in colchicine-selected multidrug-resistant KB cells. Furthermore, to account for the different size of the EcoRI fragment, one allele of the MDR1 gene in colchicine-selected multidrug-resistant KB cells must have another mutation or rearrangement in an intron to create a new EcoRI site.

The sequence at codon 893 was also analyzed using oligonucleotide probes A3. DNAs from KB cell lines hybridized only with the probe which codes Ala at codon 893, suggesting two possibilities. The first possibility is that the amino acid substitution Ala→Ser at codon 893 in pMDRA1 is an artifact of cDNA cloning. This is unlikely because two independent clones (pMDRA1 and A2) had the same sequence at codon 893. The second possibility is that this mutation represents a genetic polymorphism.

To investigate this possibility, genomic DNAs isolated from human leukocytes were investigated by dried-gel Southern hybridization (Fig. 3). Probe A3 (Ser at codon 893) hybridized to leukocyte DNA from three (lanes 1, 4, and 5) out of five persons. Under these conditions, probe A3 did not hybridized to the amplified MDR1 gene from KB-C1.5, but did hybridized to the plasmid pMDRA1 (Fig. 3). These results indicate that the differences at position 2677 could result from genetic polymorphism.

Functional analysis of two amino acid substitutions

To determine whether the amino acids at codons 185 and 893 have functional roles, we constructed three expression vectors. These expression plasmids were cotransfected with pSV2neo into NIH3T3 cells. Transfectants were first selected with G418 and then with colchicine.



A3; 5' CTA GAA GGT TCT GGG AAG A 3'
P3; 3'A CCC TTC T 5'

Fig. 3 Detection of the sequence at position 2677 of human leukocyte DNA. A3 corresponds to the position 2668 to 2686 of the adrenal derived MDR1 cDNA. P3 was used as a primer for preparing the ^{32}P-labeled antisense probe. 10 ng of pMDRA1, 2.5 µg of EcoRI digested DNA from KB-C1.5, and 10 µg of EcoRI digested DNA from human leukocytes (lanes 1-5) were hybridized with probe A3. The position of the hybridized band is shown by an arrow.

TABLE I RELATIVE RESISTANCE OF TRANSFECTANTS

Transfected cell line[a]	Relative resistance[b]			MDR mRNA[c]
	Colchicine	Adriamycin	Vinblastine	
NVA1 (pHaMDR1/A)	9.3(1.0)	2.2(1.0)	4.7(1.0)	1.0
NVA2	12.1(1.3)	2.5(1.1)	5.3(1.1)	0.96
NGS1 (pHaMDRA1)	6.8(0.73)	5.1(2.3)	14.0(3.0)	2.6
NGS2	7.7(0.83)	5.0(2.3)	15.0(3.2)	3.7
NGA1 (pHaMDRGA)	5.7(0.61)	3.2(1.5)	11.0(2.3)	3.6
NGA2	6.5(0.70)	3.2(1.5)	12.0(2.6)	2.7
NIH 3T3	1.0(0.11)	1.0(0.45)	1.0(0.21)	ND[d]

[a]Two independent clones transfected by the plasmid in parentheses are shown. The plasmids pHaMDR1/A, pHaMDRA1 and pHaMDRGA carry Val^{185} and Ala^{893}, Gly^{185} and Ser^{893}, and Gly^{185} and Ala^{893}, respectively.
[b]Relative resistance is the ratio of IC_{50} of the transfected cell lines relative to that of NIH3T3. Values in parentheses are the relative resistance to the cell line NVA1.
[c]Relative amount of MDR mRNA expressed from transfected chimeric MDR genes was determined using an RNase protection assay (5). The amounts are presented relative to that in the cell line NVA1.
[d]Not detected.

The relative resistance of transfectants to colchicine, vinblastine and adriamycin were analyzed by a quantitative colony formation assay and compared to the expression levels of the transfected MDR gene (Table I). The expression levels of MDR mRNA in transfectants corresponded well to the relative resistance to vinblastine. The resistance to colchicine relative to the MDR mRNA expression level, however, drastically decreased in cells transfected with pHaMDRA1 (NGS1 and NGS2) and pHaMDRGA (NGA1 and NGA2) which contain wild type MDR1 cDNAs compared to cells transfected with pHaMDR1/A (NVA1 and NVA2). These results confirm previous finding of Choi et. al. (18) using a cDNA from vinblastine-selected multidrug resistant cells and indicate that the amino acid substitution at codon 185 from glycine to valine, which occurred during the selection by colchicine, increases the resistance to colchicine but has a little effect on the resistance to vinblastine. The amino acid substitution at codon 893 from alanine to serine does not affect the resistance to colchicine but may slightly increase resistance to adriamycin.

DISCUSSION

To begin studying the physiological function of P-glycoprotein in the adrenal, we isolated a full-length cDNA of the MDR1 gene from the human adrenal in which P-glycoprotein is extensively expressed. Human MDR1 cDNA has been isolated from multidrug-resistant mutant sublines of KB epidermoid carcinoma cells (3, 17, 18). Therefore pMDRA1 is the first full-length cDNA clone isolated from human normal tissue and analyzed.

The RNase protection analysis (unpublished results) revealed that the MDR1 gene is the major member of the P-glycoprotein gene family expressed in human adrenal and its

expression level is at least 10 times higher than that of MDR2 (MDR3), another member of this gene family. The MDR2 gene is expressed in human liver but not in colon or multidrug-resistant KB cells. These results suggest that the products of MDR1 gene and MDR2 gene may have different physiological functions and that the product of the MDR1 gene plays the important role in the human adrenal and multidrug-resistant cells.

Sequence analysis revealed that the cDNA clone isolated from the human adrenal has nine nucleotide differences compared to the sequence of a previously isolated KB-C2.5 derived cDNA. These nucleotide differences result in two amino acid substitutions. One of them is identical to the amino acid substitution found in KB-V1 derived cDNA (18) and has been reported to alter the cross-resistance patterns. This was confirmed by our results.

The nucleotide difference which causes the amino acid substitution at codon 893 resulted from genetic polymorphism. Recently, another polymorphism has been reported in the MDR1 gene affecting a HindIII site (19). We also found a mutation or rearrangement in an intron of the MDR1 gene from colchicine-selected multidrug-resistant KB cells (Fig. 2). Mutations in KB-C2.5 derived cDNA has been presumed to occur spontaneously during exposure to the selecting drug (18). It might be important to investigate whether exposure to antitumor agents during chemotherapy causes spontaneous mutations in this gene. These could result in the alteration of sensitivity of cancer cells to antitumor agents.

We reported here the deduced amino acid sequence of the wild type MDR1 cDNA from the normal adrenal. We believe that this will facilitate the studies on the physiological function of P-glycoprotein in the adrenal.

ACKNOWLEDGMENT

This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan.

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