cDNA Cloning of the Hepatocyte Canalicular Isoform of the Multidrug Resistance Protein, cMrp, Reveals a Novel Conjugate Export Pump Deficient in Hyperbilirubinemic Mutant Rats*

(Received for publication, March 7, 1996)

Markus Bühler†, Jörg König‡, Manuela Bromt, Jürgen Kartenbeck*, Herbert Springer†, Toru Horiës, and Dietrich Keppler†

From the 1Deutsches Krebsforschungszentrum, D-69120 Heidelberg, Federal Republic of Germany and the 2Eisai Research Laboratories, Ibaraki 300-21, Japan

ATP-dependent transport of glutathione and glucuronate conjugates from hepatocytes into bile is mediated by a distinct member of the ATP-binding cassette superfamily. We have cloned and sequenced the canalicular isoform of the multidrug resistance protein from rat liver, and termed it cMrp. This membrane glycoprotein is composed of 1541 amino acids with an identity of 47.8% with the human multidrug resistance protein (MRP) and of 41.9% with the yeast cadmium factor (YCF1). The carboxyl-terminal 130 amino acids of the human hepatocyte canalicular isoform of MRP (cMRP) were 80.2% identical with rat cMrp.

cMrp was not expressed in the liver of two mutant rat strains, the Eisai hyperbilirubinemic rat and the GY/TR− mutant, which are deficient in the ATP-dependent transport of conjugates across the canalicular membrane. Immunoblotting using an antibody raised against the carboxyl terminus of cMrp detected the glycoprotein of about 190 kDa only in the canalicular membrane from normal liver. Double immunofluorescence and confocal laser scanning microscopy localized cMrp exclusively to the canalicular membrane domain of hepatocytes and demonstrated its loss in the hyperbilirubinemic mutant rat. The results identify cMrp as a canalicular transport protein with a novel sequence and with a function similar to the one of the MRP.

The multidrug resistance protein (MRP)1 was originally cloned and sequenced from multidrug-resistant human lung cancer cells and identified as an integral membrane glycoprotein of about 190 kDa belonging to the superfamily of ATP-binding cassette transporters (1). Primary active ATP-dependent transport of amphiphilic anions, particularly of conjugates of lipophilic substances with glutathione, glucuronate, or sulfate, has been recognized as the function of MRP as studied in plasma membrane vesicles (2–6). An isoform of MRP has been identified in the hepatocyte canalicular membrane by immunoblotting, immunofluorescence microscopy, and sequencing of cDNA fragments (7). The hepatocyte canalicular membrane is a site of active transport of substances from the liver into bile. The canalicular isoform of MRP was not detected in the mutant rat strain GY/TR− (7) which is deficient in the secretion of anionic conjugates from the liver into bile (8). Reverse transcription PCR indicated the apparent loss of one MRP-related mRNA species in the liver of the GY/TR− mutant rats (7). These studies provided the basis for the cloning and sequencing of the full-length cDNA encoding the rat hepatocyte canalicular isoform of Mrp (cMrp) reported in the present study. We describe, in addition, the absence of cMrp in another strain of mutant rats, the Eisai hyperbilirubinemic rat (EHBR). Similar to the GY/TR− mutant rats (8), the EHBR mutants are characterized by an impaired secretion of anionic conjugates, including bilirubin glucuronide, from the hepatocytes into bile (9, 10) and by the lack of ATP-dependent conjugate transport by canalicular plasma membrane vesicles (11).

The sequence of rat cMrp reported below represents a novel ATP-binding cassette transporter, which we compare to the related MRP sequence determined by Cole et al. (1) as well as to the yeast cadmium resistance transport protein YCF1 (12). Moreover, we deduced the carboxyl-terminal sequence of the human hepatocyte canalicular MRP isoform, cMRP, from a cDNA clone isolated from human liver. This has been of particular interest since the hereditary deficiency of the hepatocyte canalicular conjugate export pump in humans is considered to be the basis of the Dubin-Johnson syndrome (13) which is characterized by a selective abnormality in the excretion of conjugated anions into the bile and by a chronic conjugated hyperbilirubinemia (14).

EXPERIMENTAL PROCEDURES

Materials—[14,15,19,20-3H4]LTC4 (4.7 Tbid/mmol), [α-32P]dCTP (111 Tbid/mmol), and α-35S-dATP (37 Tbid/mmol) were obtained from DuPont NEN. The Rediprime DNA labeling system was from Amersham-Buchler (Braunschweig, Germany). The LTD4 receptor antagonist and transport inhibitor MK 571 was kindly provided by Dr. A. W. Ford-

---

*This work was supported in part by the Deutsche Forschungsgemeinschaft through SFB 352/B3, by the Forschungsschwerpunkt Transplantation, Heidelberg, and by the Fonds der Chemischen Industrie, Frankfurt, Germany. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

†To whom correspondence should be addressed: Division of Tumor Biochemistry, Deutsches Krebsforschungszentrum, Im Neuenheimer Feld 280, D-69120 Heidelberg, Germany. Tel.: 49/6221-422400; Fax: 49/6221-422402; E-mail: d.keppler@dkgz-heidelberg.de.

‡The abbreviations used are: MRP, human multidrug resistance protein; Mrp, gene encoding the rat multidrug resistance protein; MRP, rat multidrug resistance protein; ABC, ATP-binding cassette; cMrp, human canalicular isoform of the multidrug resistance protein; cMrp, rat canalicular isoform of the multidrug resistance protein; cMrp, gene encoding the rat canalicular multidrug resistance protein; DPPIV, dipeptidyl peptidase IV (CD26; EC 3.4.14.5); EHBFR, Eisai hyperbilirubinemic rat (9, 10); GY, Groningen Yellow; GY/TR−, transport-deficient Wistar rat (8); LT, leukotriene; MK 571, 3-[(S)-2-[7-chloro-2-quinoxalinyl]thienyl]phenyl]-3-[(S)-3-dimethyl-amino-3-oxapropylyl]-thio)-methyl(thio)propanic acid; RACE, rapid amplification of cDNA ends; RT, reverse transcription; PCR, polymerase chain reaction; TM, transmembrane; bp, base pair(s); kb, kilobase pair(s).
Hutchinson (Merck-Frost Centre for Therapeutic Research, Pointe Claire-Dorval, Quebec, Canada). The protein standard mixture (M, 43,000–200,000) for SDS-polyacrylamide gel electrophoresis was from Merck (Darmstadt, Germany). Nitrocellulose filters (pore size 0.2 μm) were from Schleicher & Schuell (Dassel, Germany).

Antibodies—The EAG5 polyspecific antibody was raised against the 12-amino acid sequence (reverse transcription of rat mRNA) at the carboxyl terminus of the rat mrf sequence. The peptide was synthesized automatically (ABI 430A peptide synthesizer, Applied Biosystems) under previously published conditions (15) with an additional cysteine residue at the amino-terminal end of the peptide. After coupling to maleimide-activated keyhole limpet hemocyanin (Perbio) according to the protocol of the manufacturer, rabbit polyclonal anti-rat mrf antibodies were produced. Monoclonal rat anti-dipeptidyl peptidase IV (DPP IV) antibody, De 13.4 (16) was kindly provided by Dr. W. Reutter (Freie Universität, Berlin, Germany). Goat anti-rabbit secondary antibodies coupled to Texas Red were purchased from Dianova (Hamburg, Germany), and goat anti-mouse secondary antibodies coupled to Cy3 (cyanin-2-OSU) were from Biotrend (Köln, Germany).

Immunofluorescence Microscopy—Small pieces of rat liver were removed after anesthetized animals and snap-frozen in isopentane, pre-cooled in liquid nitrogen. For single- and double-labeled immunofluorescence microscopy 4–5 μm thick tissue sections were prepared with a cryotome (Leica, Wetzlar, Germany). Confluent scanning fluorescence microscopy was performed with an LSM 410 apparatus (Carl Zeiss, Jena, Germany). The microscope, equipped with appropriate filter combinations, operated with an argon ion (488 nm) and a helium/neon laser (543 nm). Prints were taken from optical sections of 0.8-μm thickness.

Rat and Human Tissues—Male Sprague-Dawley rats (200–300 g) and female (250–350 g) Wistar rats were purchased from Charles-Benton Wiga (Sulzfeld, Germany). EHB, defective in the secretion of glutathione S-conjugates and structurally related amphiphilic anions (9–11), were provided by Dr. T. Horie from the Eias Pharmaceutical Company, Ibaraki, Japan. This mutant rat strain was derived from a Sprague-Dawley rat colony. Another mutant rat strain, the GY/TR mutant (8, 17, 18), was derived from a Wistar rat colony. Based on cross-breeding experiments, the conclusion has been drawn that the mutations in the EHSR and GY/TR mutants are at least allelic (19). Male GY/TR mutant rats were provided by Dr. F. Kuipers (University of Groningen, Groningen, The Netherlands). Animals were maintained on a standard diet with free access to food and water.

The human liver sample was obtained perioperatively from excised liver of a patient suffering from primary hepatocellular carcinoma. Only healthy liver tissue was used for isolation of total RNA.

Membrane Vesicle Preparation and Measurement of ATP-dependent (3H)LTC4 Transport—Plasma membrane vesicles enriched in hepatocyte canalicular or basolateral membrane domains from Sprague-Dawley and Wistar rat liver were prepared as described elsewhere (7, 20). Transport of (3H)LTC4 (50 nM) into membrane vesicles was determined for 2 min and discontinued by the rapid filtration method as described previously (3, 20).

Isolation of RNA from Tissues—Total RNA was isolated from freeze-dried tissues by a guanidinium thiocyanate lysis procedure with subsequent centrifugation in cesium chloride solution (21). In brief, brain and liver tissues (up to 4 g) were homogenized in guanidinium thiocyanate and subsequently homogenized (Ultroturrax T25, Ika & Kunkell IKA-Labortechnik, Staufen i. Br., Germany) for 1 min in 10 ml of lysis buffer containing 4 mM guanidinium thiocyanate, 0.5% (w/v) sodium laurylsarcosine, 25 mM trisodium citrate, 0.1 mM β-mercaptoethanol. After centrifugation at 10,000 × g for 15 min, 8–9 ml of the supernatants were layered on top of a cesium chloride cushion, prepared in 0.5 M calcium chloride, 10 mM EDTA, pH 8.0 and subsequently centrifuged at 180,000 × g in a SW 40 Ti Beckman rotor for 20 h at 18°C. The clear pellet was dissolved in 200–500 μl of diethyl pyrocarbonate-treated water. The RNA was once precipitated with ethanol, washed with 70% ethanol, and finally dissolved in 100–200 μl of diethyl pyrocarbonate-treated water.

Poly(A)+-enriched RNA was isolated from frozen rat liver using the Stratagene mRNA isolation kit according to the manufacturer’s instructions (Stratagene, La Jolla, CA).

3′-RACE Studies and RT-PCR Experiments—RT-PCR was performed as described in detail recently (7). In order to analyze the 3′-ends of rat liver mrf and of human liver dmrP (cDNAs), 5 μg of total liver RNA from each liver were reverse transcribed in a 50 μl of transcription buffer (50 mM Tris, pH 8.3, 75 mM KCl, 10 mM dithiothreitol, 1 mM dUTPs, 40 units of RNasin) in the presence of 5 μg of oligo(dT)-adapter primer (OWM 30) with 50 units of StrataScript RT-PCR enzyme (Stratagene). The reaction mixture was subsequently diluted by addition of 2 ml of water and concentrated to a final volume of about 100 μl by the use of Microcon100 (Amicon, Inc., Beverly, MA). First round PCR was performed with 1 μl of total volume of DNA polymerase (PK-3-5′) as reaction buffer as provided by the manufacturer) containing 1.5 mM MgCl2, 1.25 units of Taq DNA polymerase, 0.25 μM sense and antisense primer, and 5 μl of reverse transcription mixture.

Primers used for 3′-RACE experiments were as follows: oligo(dT)-adapter primer (OWM30), 5′-GAGATCGTCGATCGAATATC-3′, adapter primer 1 (OWM31), 5′-GAGATCGTCGACTGCTG-3′, adapter primer 2 (OWM32), 5′-CGACTGAGATTCAAGACT-3′, the two rat mrf-specific sense primers (ForI-4440), 5′-TGCTGCAAGAATGACCCAT-3′, and (ForI/I-4467), 5′-CAGAAAGGGAGAAATGTAGT-3′, the two human mrf-specific sense primers (cmrP-N1), 5′-GAGGTGCTGACCTCA-3′, and (cmrP-N2), 5′-CTCAAGCTTTTGTGGGACCAACA-3′.

Downloaded from http://www.jbc.org/ by guest on July 23, 2018

Northern Blot Analysis—To not stated otherwise, formamide-denatured poly(A)+-RNA was fractionated by electrophoresis on a 1.2% formaldehyde-containing agarose gel, transferred to Duralon-UV membranes (Stratagene) with 10 × SSC buffer (1.5 M NaCl, 0.15 sodium citrate, pH 7.0), prehybridized for 1 h at 68°C, and hybridized for 18 h at 68°C with the nick-translated 70-mer d probe from rat liver (7), (cmrP corresponding to seq1) prepared by RT-PCR as described recently (7). A rat β-actin probe served as a control. The size of RNA was estimated by the Molecular Weight Marker II (Boehringer Mannheim, Mannheim, Germany). Nick-translation was performed with the Rediprime DNA labeling system (Amersham-Buchler, Braunschweig, Germany). According to the instructions which were provided by the manufacturer, rabbits were immunized with this conjugate.

Construction and Screening of a Rat Liver cDNA Library—A unidirectional cDNA library in the Uni-ZAP XR vector was made using 6 μg of rat liver poly(A)+-enriched RNA (mRNA) and the ZAP-cDNA® system (Stratagene, Heidelberg, Germany) according to the manufacturer’s instructions. In brief, the mRNA was reverse transcribed by means of an XhoI-linked poly(dT) primer in the presence of a nucleotide mixture with 10 mM dNTPs and then ligated under low stringency conditions. The resulting double-stranded cDNA were filled in with cloned T4 DNA polymerase, and then nick-translated with [γ-32P]ATP. After isolation of the double-stranded cDNA by the use of DNA polymerase I and RNase H, unevolved termini of double-stranded cDNAs were filled in with cloned Pfu DNA polymerase, and then EcoRI adapters were ligated to the blunt ends. Xho digestion released the EcoRI adapter and residual linker-primer from the 3′-end of the cDNA. cDNA was size-fractionated by using a 0.5% agarose gel, stained with 5.7 μl of ethidium bromide, and EcoRI-restricted Uni-ZAP XR vector. Following digestion, the library was packaged into λ phages by the use of Gigapack III Gold packaging extract (Stratagene, Heidelberg, Germany). The primary library, containing approximately 1.7 × 108 independent clones with an average insert size of about 2 kb, was amplified without delay. This amplified library was screened in a first round by plaque hybridization using the recently described 347-bp RT-PCR fragment from rat liver cDNA seq (7, see also “Results”). The fragment was labeled as described under “Northern Blotting.” The plaque hybridizations were performed at
68°C in hybridization buffer (6 × SSC, 0.1% SDS, 0.1% SDS) followed by three high stringency washing steps (0.1% SDS; 0.1% SDS; 0.1% SDS; each at 68°C for 20 min). Positive clones were then plaque-purified by secondary and tertiary screening and the Bluescript phagemid, containing the cmrp insert was obtained by the in vivo excision procedure. The first round of hybridization yielded several cmrp clones with sizes up to 3.4 kb. A second rat liver cDNA library screening was performed using the EcoRI-BamHI 5'9'-restriction fragment (693 bp) of the longest first-round cmrp clone as probe. This screening yielded a full-length cmrp clone of 4.9 kb.

DNA Sequencing—The cDNA clones were sequenced by the dideoxynucleotide chain termination method of Sanger (22) using [α-35S]dATP and the sequencing kit from Pharmacia Biotech Inc. (Freiburg, Germany). Dried gels were exposed to Kodak BioMax MR-1 film (Sigma).

The HUSAR program (23) based on the Wisconsin Genetics Computer Group (GCG) program package (24) was used for computer analysis during this study. Further details are given under "Results."

Immunoblot Analysis of Proteins—Membrane fractions (25 mg protein) were loaded onto a 7.5% (w/v) SDS-polyacrylamide gel electrophoresis (25), without boiling, and subjected to electrophoresis. After electrotransfer onto nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany) the blots were blocked with Tris-buffered saline containing 0.05% Tween 20 and 5% bovine serum albumin for 1 h at room temperature and probed overnight with the polyclonal anti-cMrp antibody EAG15 (dilution 1:40,000), raised against the carboxyl-terminal peptide of cMrp. Antibody binding was visualized with horseradish peroxidase-conjugated goat anti-rabbit antibody (Bio-Rad, München, Germany) diluted 1:1000, followed by enhanced chemiluminescence detection (Amersham-Buchler, Braunschweig, Germany) with exposure on Hyperfilm-MP.

RESULTS

Cloning of the cDNA Encoding the Hepatocyte Canalicular Conjugate Export Pump—The rat liver λ-ZAP cDNA library was screened for isolation of the cDNA encoding the hepatocyte canalicular export pump and several clones were isolated by

![Fig. 1. Deduced amino acid sequence of rat cmrp.](http://www.jbc.org/)

**Table 1**

| Protein      | Amino acids | Identity | Similarity  |
|--------------|-------------|----------|-------------|
| cmrp rat     | 1541        | 100      | 100         |
| MRP human    | 1531        | 47.8     | 67.9        |
| YCF1        | 1515        | 41.9     | 63.2        |
| YHD5        | 1592        | 32.9     | 57.8        |
| Sur          | 1582        | 32.4     | 54.8        |
| CFTR human   | 1481        | 30.1     | 55.7        |
| Mr2 rat      | 1278        | 23.7     | 50.0        |
| MDR1 human   | 1280        | 22.8     | 50.0        |

* a Sequence alignments were generated using the BEST program from the HUSAR program package (23).
* b See Fig. 1.
* c Cole et al. (1).
* d Szczypka et al. (12).
* e Johnston et al. (30).
* f Aguilar-Bryan et al. (31).
* g Riordan et al. (32).
* h Brown et al. (33).
* i Chen et al. (34).
protein YCF1 from yeast (12), the putative yeast permease YHDS5 (30), the sulfonamide receptor (31), the cystic fibrosis transmembrane conductance regulator CFTR (32), and with lower similarity and identity scores, with the Mdr2 P-glycoprotein (33), and with the MDR1 P-glycoprotein (34) (Table I). The amino acid sequence deduced from the cMrp cDNA contained the structural features consistent with a protein composed of two homologous halves, typical for the ABC transporter proteins. Each half contained the transmembrane domains (TM I and TM II) in Table I and the structural motifs (28, 29) for putative ATP-binding sites including the Walker A and B motifs and the putative consensus pattern for the ABC transporter family signature (C) (Fig. 1). The high sequence similarity, especially in the two nucleotide-binding domains and in the carboxyl-terminal part, between cMrp, MRP (1), and YCF1 (12) is outlined in Table II. The pattern in the second nucleotide-binding domain (amino acids 1433–1447) also fulfilled the consensus pattern for the ABC transporter family signature (C) except for the isoleucine in position 1435. Analysis by the use of the TMAP program for prediction of transmembrane segments revealed at least 13 transmembrane segments for the cMrp sequence (Fig. 1). This analysis was based on a CLUSTAL sequence alignment file (35) and included cMrp (Fig. 1), human MRP (1), and the rat sulfonamide receptor (Sur) protein (31). Interestingly, the TMAP program (26) led to a prediction of a 9 plus 4 distribution of the transmembrane segments (Fig. 1), rather than a 8 plus 4 distribution described earlier for MRP by the use of a different algorithm (1, 37). The 9 plus 4 distribution corresponds to the transmembrane segment pattern for the sulfonamide receptor (Sur) protein (31) suggesting an extracellular location of the N-terminal domain of the protein (37). TMAP analysis using alignments which included the yeast cadmium resistance factor YCF1 (12) and the supposed ATP-dependent permease YHDS5 from yeast (30) led to a prediction of 14 transmembrane segments. However, only the positions and the number of the first two transmembrane segments are affected by these different TMAP predictions. The cMrp sequence contained 11 predicted N-glycosylation sites of which three were located on the predicted extracellular loop between transmembrane segments 10 and 11, and, as in the human MRP and the rat sulfonamide receptor (Sur) protein, a single N-glycosylation site was predicted on the extracellular domain near the amino terminus of cMrp (Fig. 1). In addition, the sequence between the first nucleotide-binding domain and the TM II domain, defined in Table II and commonly referred to as the linker region, is characterized by a high content of about 38% charged amino acids and several consensus sequences for phosphorylation by

**Table I**

| Domain (peptide) | Amino terminus (1–96) | TM I (97–648) | NBD1 (649–798) | Linker region (799–960) | TM II (961–1301) | NBD2 (1302–1474) | Carboxyl terminus (1475–1541) |
|-----------------|----------------------|--------------|---------------|-------------------------|-----------------|-----------------|-----------------------------|
| cMrp rat        | Identity or similarity | 100          | 100           | 100                     | 100             | 100             | 100                        |
| MRP human       | Identity             | 29.8         | 40.2          | 66.7                    | 36.2            | 47.8            | 73.3                        |
|                 | Similarity           | 57.4         | 61.3          | 82.7                    | 57.1            | 70.3            | 84.9                        |
| YCF1 yeast      | Identity             | 21.8         | 34.0          | 54.0                    | 27.1            | 46.3            | 63.4                        |
|                 | Similarity           | 57.5         | 56.9          | 70.0                    | 51.4            | 66.5            | 77.9                        |
| Sur rat         | Identity             | 27.3         | 25.3          | 43.6                    | 25.2            | 32.5            | 51.2                        |
|                 | Similarity           | 56.8         | 49.4          | 66.4                    | 45.7            | 53.3            | 71.5                        |

a Domains for this multisequence alignment are defined with respect to the amino acid position in cMrp (Fig. 1). TM I designates the peptide comprising transmembrane segments 1–9 and TM II comprises transmembrane segments 10–13. NBD1 and NBD2 designate the first and second nucleotide-binding domains, respectively.

b Cole et al. (1).
c Szczypka et al. (12).
d Aguilar-Bryan et al. (31).

**Fig. 2. Northern blot analysis of cMrp expression in normal and mutant rat livers.** Each lane contains 5 μg of poly(A)+ RNA from liver of EHBR and GY/TR− mutant rats and the corresponding wild-type strains Sprague-Dawley (SD) and Wistar (Wi). The RNA was first hybridized with the 347-bp cDNA fragment of cMrp (seq1) (7) under high-stringency conditions and reprobed with a rat β-actin cDNA fragment as outlined under “Experimental Procedures.” Autoradiographs are 4-day and 1-day exposure for cMrp and β-actin, respectively. Only RNA from the wild-type rat strains (Wi and SD) gave two distinct bands.

using the 347-bp cDNA fragment seq1 (7). Restriction analyses indicated that clone CM23 showed the largest insert with about 3.4 kb in length. A second plaque hybridization was performed using the 5′-located EcoRI-BamHI restriction fragment of clone CM23. This screening led to clone CM2,1 with an insertion length of 4.9 kb, and to clone CM2,17 with an insertion length of 4.6 kb. The entire insert of clone CM2,1 was sequenced from both strands and revealed a first in-frame ATG codon located at nucleotides 50–52, followed by a stop codon located at positions 4673–4675. The sequence context around the ATG translational start codon was −3ATTATGG+4 and fulfilled the Kozak consensus (27) with the invariant A at position –3 and the usual G at position +4. Two putative polyadenylation sites with the consensus sequence AATAAA were located at positions 4866–4781.
and diamonds, respectively. Numbers indicate the amino acid position the polyclonal antibodies 6KQ (36) and EAG15 are indicated by dots.

Fig. 3. Deduced carboxyl-terminal sequences of rat cMrp and Mrp, and human cMRP and MRP. The corresponding nucleotide sequences of the rat mrp and the human dMrp were obtained by the 3′-RACE technique. The rat cMrp sequence (see Fig. 1) was donated under “Experimental Procedures”; the human MRP sequence (1) is from the EMBL Data Bank (L05628). The sequences were aligned by the CLUSTAL program from the HUSAR program package (23, 24, 35). Boxes (black background) indicate that at least two amino acids are identical at this position. Similarity scores for these carboxyl-terminal sequences relative to cMrp are indicated (Sim.) in the figure; identity scores relative to cMrp are as follows: human cMRP, 80.2%; rat Mrp, 65.8%; and human Mrp, 66.7%. The peptide sequences recognized by the polyclonal antibodies 6KQ (36) and EAG15 are indicated by dots and diamonds, respectively. Numbers indicate the amino acid position corresponding to cMrp.

cAMP-dependent protein kinase A and protein kinase C (Fig. 1).

Northern Blot Analysis of cmrP Expression in Normal and Mutant Rat Liver—The question whether the lack of amplification of cMrp cDNA in the transport-deficient GY/TR− mutant rat (7) was due to point mutations within the primer target sites or possibly due to a lack of the corresponding mRNA was studied by analysis of the expression of the cMrp gene by Northern blot hybridization (Fig. 2). PolyA+ -enriched RNA from liver tissues from GY/TR− and EBHBR mutant rats, as well as from normal Wistar and normal Sprague-Dawley rats, was probed using the 347-bp cMrp cDNA fragment seq1 (7). The rat cMrp cDNA fragment hybridized to two mRNAs of about 5.5 and 7.5 kb (Fig. 2). Interestingly, the seq1 cDNA fragment hybridized only with mRNA from normal Sprague-Dawley and normal Wistar rat liver, whereas liver of both mutants, EBHR and GY/TR−, lacked the corresponding mRNA (Fig. 2). These findings indicate that the defects in EBHR and GY/TR− mutants rats are caused by a lack of expression or by rapid degradation of the cMrp mRNA detected by the seq1 cDNA probe.

Comparison of the Deduced Carboxyl-terminal Amino Acid Sequences of Rat cMrp, Human cMrp, Rat Mrp, and Human MRP—The purified polyvalent 6KQ antibody, raised against the peptide at the carboxyl terminus of human MRP (36), indicated the absence of the carboxyl isoform of MRP in the GY/TR− mutant rat (7) as well as the absence of the human cMrp in the liver of a patient with Dubin-Johnson syndrome (13). 3′-RACE studies with rat and human liver mRNA, using primer systems targeted against the highly conserved second nucleotide-binding domain of these MRP sequences (see “Experimental Procedures”), served to identify the carboxyl-terminal sequence of rat liver Mrp and human liver cMrp (Fig. 3). Alignment of the carboxyl termini of these 4 ABC transporters of the MRP family indicated a high degree of sequence similarity of Mrp and cmrP from rat liver with the sequences of human MRP (Table II) and human liver cMRP, respectively (Fig. 3). Furthermore, the sequence analysis supported the immunological observations (7) mentioned above on the reactivity of the 6KQ antibody (36), because of the high sequence similarity in the antigenic peptide part against which the 6KQ antibody had been raised (Fig. 3, marked by dots).

Immunoblot Analysis of cMrp in Rat Liver Membranes—We have raised the polyvalent EAG15 antibody against the peptide of 12 amino acids at the carboxyl terminus of cMrp (Fig. 3, closed diamonds) in order to differentiate between rat Mrp and its hepatoocyte canalicular isoform cMrp. Immunoblot analyses were performed with canalicular and basolateral membrane preparations from the GY/TR− and EBHBR mutants and from their corresponding normal Wistar and Sprague-Dawley counterparts. The EAG15 antibody detected a protein of about 190 kDa exclusively in the canalicular membrane of the normal liver (Fig. 4). The protein was not detectable in membranes from GY/TR− and EBHBR mutants, even after enhancement of the sensitivity by an extended exposure time of the blot (Fig. 4). Exposure of the blot for longer time periods in the enhanced chemiluminescence detection system indicated small amounts of the 190-kDa protein in the basolateral membrane preparation which results from its known contamination with canalicular membranes (7). Furthermore, faint bands were visible at 60–70 kDa after long term exposure in canalicular membranes from normal liver but not in the membranes from EBHBR and GY/TR− mutants, suggesting carboxyl-terminal peptide degradation products of cMrp. Deglycosylation of rat liver canalicular membrane preparations by glycopeptide N-glycosidase under conditions described recently (7) reduced the apparent molecular weight of cMrp, detected on the immunoblot by the
ATP-dependent \[^{3}H\]LTC\(_4\) Transport into Membrane Vesicles of Normal and EHBR Mutant Rats—The deficient transport in the EHBR mutants was confirmed by measurement of ATP-dependent transport of \[^{3}H\]LTC\(_4\) into inside-out oriented liver plasma membrane vesicles (Table III). In order to differentiate between binding of the labeled substrate and ATP-dependent uptake into the vesicles, transport was performed by incubation of the membrane vesicles with 50 nM \[^{3}H\]LTC\(_4\) in the presence of 4 mM ATP or 4 mM 5'-AMP (2, 3, 7, 20). The net ATP-dependent uptake of \[^{3}H\]LTC\(_4\) by canalicular membrane vesicles from EHBR liver was below 3% of normal, and the residual transport was not significantly affected by a potent and selective inhibitor of the MRP-mediated transport (3), the LTD\(_4\)-receptor antagonist MK571 (38). The anionic quinoline derivative MK571 effectively inhibited \[^{3}H\]LTC\(_4\) transport into membrane vesicles prepared from normal liver (Table III). The residual transport activity in the basolateral membrane preparation from normal Sprague-Dawley rat liver most probably resulted from contamination of the preparation with canalicular membrane vesicles (7). This residual transport was also effectively inhibited by MK571.

**DISCUSSION**

Recently we studied the expression of the human MRP gene and of the rat mrp gene in liver by PCR amplification of cDNA reverse transcribed from poly(A)\(^{+}\) RNA using degenerate primer pairs derived from highly conserved regions in the second nucleotide-binding domain of the human MRP gene and the Leishmania \(Ltpgpa\) gene (7). In this recent study we sequenced two different 347-bp cDNA fragments from rat liver, termed seq1 and seq2 (7), which exhibited a high degree of sequence similarity with the human MRP cDNA (1). Our present work demonstrates that the seq1 sequence (7) has been part of a novel cDNA encoding a membrane glycoprotein (Fig. 1) of the ABC superfamily of transporters. Based on the amino acid sequence similarity with other ABC proteins, the canalicular isoform cMrp (Fig. 1) is related next to MRP (1) (67.9% overall similarity).
ATP-dependent leukotriene C4 transport activity in canicular and basolateral membrane vesicle preparations from normal Sprague-Dawley (SD) and EHBR mutant rat liver

| Membrane          | ATP-dependent LTC4 transport |
|--------------------|-------------------------------|
|                    | -MK571 | +MK571 |
| SD (canicular)     | 16.1 ± 0.7 (100) | 3.1 ± 0.5 (19) |
| SD (basolateral)   | 4.8 ± 0.4 (30) | 0.8 ± 0.4 (50) |
| EHBR (canicular)   | 0.4 ± 0.2 (2.5) | 0.3 ± 0.2 (1.9) |
| EHBR (basolateral) | 0.9 ± 0.5 (5.6) | 0.7 ± 0.4 (4.3) |

Mean values ± S.E. from four determinations.

**REFERENCES**

1. Cole, S. P. C., Bhardwaj, G., Gerlach, J. H., Mackie, J. E., Grant, C. E., Almquist, K. C., Stewart, A. J., Kurz, E. U., Duncan, A. M., and Deetley, R. G. (1992) Science 259, 1650–1654

2. Jedlitschky, G., Leier, I., Buchholz, U., Center, M., and Keppler, D. (1994) Cancer Res. 54, 4833–4836

3. Leier, I., Jedlitschky, G., Buchholz, U., Cole, S. P. C., Deetley, R. G., and Keppler, D. (1994) J. Biol. Chem. 269, 27807–27810

4. Müller, M., Meijer, C., Zaman, G. J. R., Borst, P., Schepers, R. J. M., Mulder, N. H., Deetley, R. G., and Jensen, P. L. M. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 13033–13037

5. Leier, I., Jedlitschky, G., Buchholz, U., Center, M., Cole, S. P. C., Deetley, R. G., and Keppler, D. (1996) Biochem. J. 314, 433–437

6. Jedlitschky, G., Leier, I., Buchholz, U., Barnouin, K., Kurz, E. U., and Keppler, D. (1996) Cancer Res. 56, 988–994

7. Mayer, R., Kartenbeck, J., Büchler, M., Jedlitschky, G., Leier, I., and Keppler, D. (1995) J. Cell Biol. 131, 1501–1510

8. Oude Elferink, R. P. J., Meijer, C., Kuipers, F., Jansen, P. L. M., Groen, A. K., and Groothuis, G. M. M. (1995) Biochim. Biophys. Acta 1241, 215–268

9. Takeda, K., Sano, N., Nanta, T., Uchida, Y., Yamakawa, A., Horie, T., Mikami, T., and Tagaya, O. (1991) Hepatology 14, 352–360

10. Hosokawa, S., Taiwagi, O., Mikami, T., Nozaki, Y., Kawaguchi, A., Yanatsu, K., and Shamoto, M. (1992) Lab. Anim. Sci. 42, 27–34

11. Fernández-Checka, C. J., Takikawa, H., Horie, T., Oktents, M., and Kaplitz, N. (1992) J. Biol. Chem. 267, 1667–1673

12. Szczypka, M. S., Wemmerle, J., A. Roy-Mowley, R. S., and Thiele, D. J. (1994) J. Biol. Chem. 269, 22853–22857

13. Kartenbeck, J., Leuschner, U., Mayer, R., and Keppler, D. (1996) Hepatology 24, 1061–1066

14. Roy Chowdhury, J., Roy Chowdhury, N., Wolkoff, A. W., and Arias I. M. (1994) In The Liver: Biology and Pathobiology (Arias, I. M., Boyer, J., Fausto, N., Jakoby, W. B., Schachter, D., and Shafritz, D., eds) pp. 741–504, Raven Press, New York

15. Schröder, M., Aleywood, P., Jones, A., Alewood, D., and Kent, S. B. H. (1992) Int. J. Pept. Protein Res. 40, 180–193

16. Becker, A., Neumeier, R., Hébrard, C., Lo, N., Hartel, S., and Reutter, W. (1992) Biochem. J. 283, 681–688

17. Jansen, P. L. M., Peters, W. H. M., and Lammers, W. H. (1985) Hepatology 5, 573–579

18. Kuipers, F., Ensingink, M., Hovingh, R. Van der Steen, A. M., Haraldon, M., F. Jevon, Y., and Vonk, R. J. (1988) J. Clin. Invest. 81, 1593–1599

19. Nishida, T., Hardenbrook, C., Gatmaitan, Z., and Arias, I. M. (1992) Am. J. Physiol. 262, G629–G635

20. Böhm, M., Müller, M., Leier, I., Jedlitschky, G., and Keppler, D. (1994) Gastroenterology 107, 255–265

21. Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J., and Rutter, W. J. (1979) Biochemistry 18, 5294–5299

22. Sanger, F., Coulson, A. R., Barrett, B. G., Smith, A. J. H., and Roe, B. A. (1980) J. Mol. Biol. 134, 161–178

23. Sanger, M., Giatt, E. H., Ritter, O., and Suhai, S. (1995) Comput. Methods Program Biomed. 46, 131–141

24. Devereaux, J., Haebeler, P., and Smithies, O. (1984) Nucleic Acids Res. 12, 387–395

25. Laemmli, U. K. (1970)

26. Devereux, J., Haeberli, P., and Smithies, O. (1984)

27. Higgins, C. F., Callaghan, M. P., Mimmack, M. L., and Pearce, S. R. (1988) Bioessays 8, 111–116

28. Hyde, S. C., Ensley, P., Hartstorn, M. J., Mimmack, M. L., Geilandi, U., Pearce, S. R., Geilandi, M. P., Gill, D. R., Hubbard, R. E., and Higgins, C. F. (1990) Nature 346, 362–366

29. Johnson, M., Andrews, S., Brinkman, R., Cooper, J., Dingle, H., Dover, J., Du, Z., Favello, J., Fulton, L., Gattung, S., Gesel, C., Kirsten, J., Kucaba, T., Harby, H., & M. Johnson, C., Langston, J., Lehtirissi, P., Louis, J., Macr, C., Mardis, E., Menezes, S., Mouser, L., Nhan, M., Rikfin, L., Riles, L., St. Peptar, H., Trevaslisk, E., Vaughan, K., Vignati, D., Wilcox, L., Wohldman, P., Waterston, R., Wilson, R., and Vaudin, M. (1994) Science 265, 2077–2082

30. Aguilar-Bryan, L., Nichols, C. G., Weidler, S. W., Clement, J. P., Boyd, A. E., III, Gonzalez, G., Herrera-Sosa, H., Ngy, K., Bryan, J., and Nelson, D. A. (1995) Science 268, 423–426

31. Riordan, J. R., Rommens, J. M., Kerem, B., Alon, N., Ramahel, R., Grzelczak, Z., Zielenski, J., Lok, S., Plaisaic, N., Choi, J. L., Drum, M. L., Iannuzzi, M. C., Collini, F. S., and Rana, J. G. (1989) Science 245, 1057–1067

32. Brown, P. C., Thorgeirsson, S. S., and Silverman, J. A. (1993) Nucleic Acids Res. 21, 3885–3891

33. Chen, C., Chin, J. E., Ueda, K., Clark, D. P., Pastan, I., Gottesman, M. M., and Roninson, I. B. (1986) Cell 47, 381–389
35. Higgins, D. G., and Sharp, T. M. (1989) Comput. Appl. Biosci. 5, 151–153
36. Krishnamachary, N., Ma, L., Zheng, L., Safa, A. R., and Center, M. S. (1994) Oncol. Res. 6, 119–127
37. Philipson, L. H., and Steiner, D. F. (1995) Science 268, 372–373
38. Jones, T. R., Zamboni, R., Belley, M., Champion, E., Charette, L., Ford-Hutchinson, A. W., Frenette, R., Gauthier, J. Y., Leger, S., Masson, P., McFarlane, S., Piedchuta, H., Rokach, J., Williams, H., Young, R. M., De-Haven, R. N., and Pong, S. S. (1989) Can. J. Physiol. Pharmacol. 67, 17–28
39. Ishikawa, T., Müller, M., Klünemann, C., Schaub, T., and Keiper, D. (1990) J. Biol. Chem. 265, 19279–19286
40. Huber, M., Guhlmann, A., Jansen, P. L. M., and Kepler, D. (1987) Hepatology 7, 224–228
41. Arias, I. M., Che, M., Gatmaitan, Z., Leveille, C., Nishida, T., and St. Pierre, M. (1993) Hepatology 17, 318–329
42. Böhme, M., Buchler, M., Muller, M., and Kepler, D. (1993) FEBS Lett. 333, 193–196
43. Cole, S. P. C., Sparks, K. E., Fraser, K., Loe, D. W., Grant, C. E., Wilson, G. M., and Deeley, R. G. (1994) Cancer Res. 54, 5902–5910
cDNA Cloning of the Hepatocyte Canalicular Isoform of the Multidrug Resistance Protein, cMrp, Reveals a Novel Conjugate Export Pump Deficient in Hyperbilirubinemic Mutant Rats
Markus Büchler, Jörg König, Manuela Brom, Jürgen Kartenbeck, Herbert Spring, Toru Horie and Dietrich Keppler

J. Biol. Chem. 1996, 271:15091-15098.
doi: 10.1074/jbc.271.25.15091

Access the most updated version of this article at http://www.jbc.org/content/271/25/15091

Alerts:
  • When this article is cited
  • When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 42 references, 15 of which can be accessed free at http://www.jbc.org/content/271/25/15091.full.html#ref-list-1