Functional expression of the rat liver canalicular isoform of the multidrug resistance-associated protein

Jerzy Madon, Uta Eckhardt, Thomas Gerloff, Bruno Stieger, Peter J. Meier*

Division of Clinical Pharmacology and Toxicology, Department of Medicine, University Hospital, CH-8091 Zurich, Switzerland

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Abstract The rat hepatocanalicular isoform (called mrp2) of the human multidrug resistance-associated protein (MRP) has been cloned and transiently expressed in COS-7 cells and in *Xenopus laevis* oocytes. In both systems mrp2 expression induced a markedly increased efflux of intracellularly formed [^{14}C]2,4-dinitrophenyl-*S*-glutathione. Injection of mrp2 cRNA into oocytes also stimulated efflux of [^{3}H(N)]leukotriene C_4. Furthermore, mrp2 mRNA was markedly decreased in the liver of the transport mutant TR$^-$ rat, which has a congenital defect in the biliary excretion of glutathione-*S* conjugates and of other divalent organic anions. The study provides a direct demonstration of mrp2-mediated transport function and supports the concept that mrp2 represents the canalicular multispecific organic anion transporter (cMOAT) of mammalian liver.

Key words: Multidrug resistance-associated protein; Rat liver; Canalicular transport; Organic anion; Glutathione conjugate

1. Introduction

The canalicular plasma membrane domain of mammalian liver localizes a variety of ATP-dependent transport systems for excretion of endogenous and exogenous lipophilic compounds and drug conjugates into bile [1,2]. One example of these canalicular ATP-binding cassette proteins is the so-called canalicular multispecific organic anion transporter (cMOAT) that mediates ATP-dependent biliary excretion of different organic anions including bilirubin diglucuronide, glutathione conjugates of chlorodinitrobenzene (DNP-GS) and of bromosulfophthalein, leukotriene C_4 (LTC_4) and sulfated and glucuronidated bile salts [3]. Defective cMOAT causes conjugated hyperbilirubinemia in three mutant rat strains, called the TR$^-$, GY (Groningen Yellow), and EHBR (Esai HyperBilirubinemic Rat) rats [1–4]. Furthermore, failure to express cMOAT forms the basis of the so-called Dubin-Johnson syndrome, which represents a benign hereditary form of conjugated hyperbilirubinemia in man [5]. Recently, the rat and human liver cMOAT has been cloned [6–9] and shown to represent a liver-specific isoform of the human multidrug resistance-associated protein (MRP1) [10]. This so-called mrp2 [2,6] or cMrp [7] is absent from the canalicular membrane of the hyperbilirubinemic mutant rat strains [6–8]. In TR$^-$ rat liver a single-nucleotide deletion in the *mrp2* gene results in a frame shift leading to a stop codon [6]. Although these studies suggest a correlation between the presence of the *cMOAT* gene, the absence of the gene product from the hepatocanalicular membrane, and the defined congenital transport defect in TR$^-$ and EHBR liver tissue, no direct transport function has so far been shown for mrp2 or cMrp. Here we demonstrate that expression of mrp2 in COS-7 cells and in *Xenopus laevis* oocytes resulted in a marked induction of [^{14}C]DNP-GS and [^{3}H(N)]leukotriene C_4 ([^{3}H]LTC_4) efflux, thus providing direct proof for the suggested transport function of the hepatocanalicular *mrp2* gene product.

2. Material and methods

2.1. Materials

[^{14}C(U)]1-Chloro-2,4-dinitrobenzene ([^{14}C]CDNB) (10 mCi/mmol) was obtained from Amersham International, Little Chalfont, UK. [^{14}C]2,4-Dinitrophenyl-*S*-glutathione ([^{14}C]DNP-GS) was synthesized by incubating [^{14}C]CDNB) and GSH in the presence of glutathione-*S*-transferase at 37°C as described [11]. [^{3}H]Taurocholic acid (2.1 Ci/mmol) and [^{3}H]LTC_4 (165 Ci/mmol) were obtained from DuPont-New England Nuclear, Boston, MA, USA.

2.2. Animals

Male Sprague-Dawley rats (SUT:SDT) were obtained from the Institut für Labortierkunde, University of Zürich (Zürich, Switzerland). Male GY/TR$^-$ rats were provided by Drs. Peter L.M. Jansen and F. Kuipers from the University of Groningen (Groningen, The Netherlands). Mature *Xenopus laevis* females were purchased from H. Kähler (Hamburg, Germany) and kept under standard conditions [12].

2.3. Cell lines

The following cell lines were used: CHO (Chinese hamster ovary), COS-1 and COS-7 (African green monkey kidney; SV40 transformed), HeLa (human cervix epithelioid carcinoma), HuH7 (human hepatoma), 3LL (mouse lung), MDCK (dog kidney), MH1C1 (rat Morris hepatoma) and V79 (Chinese hamster lung). Unless stated otherwise, the cells were grown in Iscove's modified Dulbecco's medium (IMDM), supplemented with 10% v/v fetal calf serum (FCS), at 37°C and 5% v/v CO_2 atmosphere in a humidified incubator.

2.4. Cloning of mrp2

Total RNA was isolated from human HuH7 cells. Two cDNA fragments were amplified by RT-PCR covering 2.5 kb (positions 156–2654) and 2.7 kb (positions 2080–4765) of the original human MRP1 sequence [10]. The identity of these PCR fragments with MRP1 was verified by sequence analysis. The fragments were then used to screen a random and poly-dT primed λgt-10 rat liver cDNA library from Clontech (Palo Alto, CA). The cDNA clones were hybridized at 65°C using standard conditions [13]. Filters were washed with 2×SSC at 55°C. A total of 12 positive clones were identified and analyzed by sequencing. One clone of 2.0 kb showed 57% homology with MRP1. This clone was amplified and used to rescreen the rat liver cDNA library under high stringency conditions. Among 25 positive clones, a 4.2 kb clone was identified that contained an identical sequence as the probe. Further screenings eventually identified an additional 2.5 kb overlapping clone. Together, these two overlapping clones covered the entire open reading frame (4623 nucleotides, 1541 amino acids) of mrp2 [6] or cMrp [7] as well as 94 nucleotides at the 5′- and 224 nucleotides at the 3′-untranslated regions. The entire mrp2 cDNA was cloned into the *Eco*RI restriction site of the expression vector pcDNAI/Amp (Invitrogen, San Diego, CA). Successful subcloning of this new plasmid construct, called

*Corresponding author. Fax: (41) (1) 255 4411.

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pKEH1, was confirmed by restriction enzyme analysis and sequencing of the ligation sites.

2.5. Northern blotting

Total RNA was isolated from rat liver and various cell lines using the Trizol reagent from Gibco BRL (Life Technologies Ltd., Paisley, UK). Poly A^+ RNA was isolated from total rat liver RNA with the Oligotex mRNA kit from Qiagen (Hilden, Germany). RNA samples were electrophoresed and transferred to a Hybond N membrane (Amersham) as described [14]. Hybridizations were carried out with a 0.68 kb fragment of the cloned mrp2 (positions 1–680) and a 0.7 kb RT-PCR fragment of rat liver β-actin mRNA generated with commercial primers (Clontech). The blots were hybridized under high stringency conditions at 65°C in 6×SSC/5×Denhardt's solution/0.6% w/v SDS containing denatured calf thymus DNA at 100 μg/ml. After hybridization the filters were washed three times at 65°C in 2×SSC/0.2% w/v SDS for 20 min and once more in 0.1×SSC/0.1% w/v SDS for 10 min.

2.6. Functional expression of mrp2 in COS-7 cells

COS-7 cells were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco), supplemented with 10% v/v FCS, 100 U/ml penicillin and 100 μg/ml streptomycin, at 37°C and 5% v/v CO_2 atmosphere in a humidified incubator. In preliminary experiments it was ascertained that COS-7 cells rapidly converted [^{14}C]CDNB to its glutathione conjugate [^{14}C]DNP-GS as has been described in other cell lines [1,15]. COS-7 cells were transfected in 35 mm dishes with the pKEH1 vector (see above) or with the *mrp2*-free control vector pCI-Neo from Promega (Madison, WI) using a DEAE-dextran transfection method [16]. After an expression time of 68 h, the cells were washed three times with transport buffer (3 ml, 37°C) consisting of (in mmol/l) 116.4 NaCl, 5.3 KCl, 0.8 $MgSO_4$, 26.2 $NaHCO_3$, 1.0 NaH_2PO_4, 5.5 D-glucose, pH 7.4. Subsequently, they were incubated with 1 ml of transport buffer containing 10 μM [^{14}C]CDNB, which enters cells by passive diffusion. Efflux of [^{14}C]DNP-GS was determined at 37°C by removing 100 μl aliquots of the transport buffer at the indicated time points. Samples were immediately extracted with 100 μl ethylacetate and centrifuged for 2 min (12 000×*g*) to separate [^{14}C]CDNB from the intracellularly formed [^{14}C]DNP-GS [15]. Radioactivity in 80 μl of both phases was determined by liquid scintillation counting. The amount of radioactivity was corrected for the decrease in volume of the transport medium. At the end of the experiment cell monolayers were washed three times with ice-cold transport buffer. The cells were then solubilized with 1 ml 1% w/v Triton X-100 and the remaining cell-associated radioactivity also determined.

2.7. Functional expression of mrp2 in Xenopus laevis oocytes

Frogs were anesthetized in a bath containing 0.1% of ethyl *m*-amino benzoate (MS-222) for 15 min. Oocytes were removed from the ovary by laparotomy and transferred into a Ca^{2+}-free OR-2 solution [17] supplemented with 1.5 mg/ml collagenase type D (Boehringer, Mannheim, Germany). After an incubation period of 40 min at room temperature, oocytes were washed in modified Barth's solution consisting of (mmol/l): 88 NaCl, 15 HEPES-NaOH, pH 7.6, 2.4 $NaNCO_3$, 1.0 KCl, 0.82 $MgSO_4$, 0.41 $CaCl_2 \cdot 2H_2O$, 0.3 $Ca(NO_3)_2$ and 50 μg/ml gentamicin. Stage V and VI oocytes were selected. After an overnight incubation at 18°C, vital oocytes were injected either with 5 ng of mrp2 cRNA (0.1 μg/μl) or with 50 nl water (controls). After 3 days in culture at 18°C, the oocytes were injected with 60 nl of 10.3 mM [^{14}C]DNP-GS, 50 nl of 2.1 μM [^{3}H]LTC_4 or 60 nl of 476 μM [^{3}H]taurocholate [18]. Subsequently they were washed once in ice-cold Na^+-free efflux medium consisting of (in mmol/l): 100 choline chloride, 2 KCl, 1 $CaCl_2$, 1 $MgCl_2$, and 10 HEPES, adjusted to pH 7.5. For efflux studies single oocytes were incubated at 25°C in 0.5 ml efflux medium. Aliquots of 50 μl were removed after the indicated time intervals and the effluxed radioactivity determined by liquid scintillation counting. The amount of radioactivity was corrected for the decrease in volume of the efflux medium. At the end of the experiment each oocyte was washed once in 8 ml of ice-cold efflux medium and dissolved in 0.5 ml of 10% w/v SDS. The remaining oocyte associated radioactivity was also determined and used for the estimation of relative and absolute efflux rates.

2.8. Other methods

Protein was determined with the BCA protein assay kit (Pierce, Rockford, IL) [19].

3. Results and discussion

Using the strategy described in Section 2, we cloned and sequenced an isoform of the human MRP1 [10] from a λgt-10 rat liver cDNA library. The open reading frame of the cloned cDNA contained 4623 nucleotides encoding a protein of 1541 amino acids with a calculated molecular mass of 173 kDa. The deduced amino acid sequence is 100% identical with the recently cloned rat hepatocyte canalicular MRP1 isoform mrp2 [2,6] or cMrp [7]. In addition to the open reading frame, the cloned cDNA contains 94 nucleotides at the 5′- and 224 nucleotides at the 3′-untranslated regions.

Northern blot analysis with a 0.68 kb mrp2 fragment from the 5′-end confirmed the presence of two strongly reacting mRNA species of approximately 5.4 and 7.5 kb in normal rat liver (Fig. 1, lanes 1, 3) [7], whereas no reactivity with an additional 9.5 kb transcript was found as previously suggested [6]. Only a very weak hybridization reaction with a 7.5 kb transcript was obtained with poly(A)$^+$ RNA isolated from TR^- rat liver (Fig. 1, lane 2) supporting the concept of a close correlation between defective mrp2 expression and defective canalicular organic anion excretion in the transport mutant TR^-, GY and EHBR rat strains [6–8]. Since no cMOAT protein could be detected with a specific monoclonal antibody at the canalicular membrane of TR^- rat liver in an earlier study [6], the weak hybridization signal may have resulted from residual unstable (defective) mrp2 mRNA not yet degraded or from mRNA encoding an additional, as yet unidentified mrp homologue. Interestingly, a similar weak hybridization reaction was also observed with total RNA isolated from the rat Morris hepatoma cell line MH1C1 (Fig. 1, lane 4). In contrast, all nonhepatic cell lines tested did not show

Table 1
Efflux of DNP-GS, LTC_4 and taurocholate (TCA) in mrp2 cRNA- and water-injected *Xenopus laevis* oocytes

Substrate	Time (min)	Efflux		
		mrp2-cRNA-injected	water-injected	*P*
[^{14}C]DNP	5	8.5 ± 0.7 pmol	10.4 ± 0.6 pmol	NS
	30	76.3 ± 10.2 pmol	20.1 ± 1.8 pmol	< 0.001
[^{3}H]LTC_4	5	8.3 ± 3.7 fmol	4.2 ± 0.4 fmol	NS
	30	24.6 ± 6.1 fmol	9.6 ± 0.9 fmol	< 0.01
[^{3}H]TCA	5	0.7 ± 0.2 pmol	0.5 ± 0.2 pmol	NS
	30	2.8 ± 0.2 pmol	3.1 ± 0.3 pmol	NS

Oocytes were injected either with 5 ng/50 nl of mrp2 cRNA or with 50 nl of water. After 3 days in culture at 18°C substrate efflux was measured as described in Section 2. Data are given as the means ± S.E.M. of separate measurements in 10–13 different oocytes. NS, not significant.

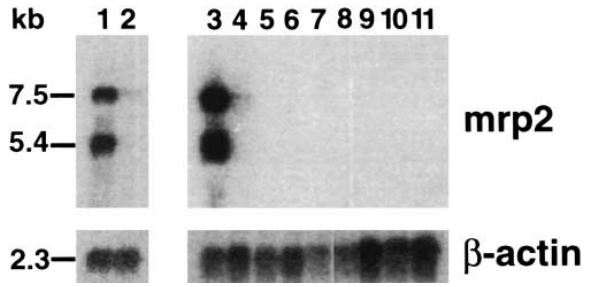

Fig. 1. Northern blot analysis of rat liver poly(A)$^+$ RNA (1 μg; lanes 1, 2) and of total RNA (5 μg) isolated from rat liver (lane 3) and from various cell lines (lanes 4–11). Hybridizations were performed with a 0.68 kb fragment of the cloned mrp2 (positions 1–680) and a 0.7 kb RT-PCR fragment of rat liver β-actin mRNA generated with commercial primers (Clontech). Lane 1, normal Sprague-Dawley rat liver. Lane 2, TR$^-$ rat liver. Lane 3, normal rat liver. Lane 4, MH1C1 cells. Lane 5, COS-1 cells. Lane 6, COS-7 cells. Lane 7, MDCK cells. Lane 8, HeLa cells. Lane 9, V79 cells. Lane 10, 3LL cells. Lane 11, CHO cells.

any hybridization reactivity with the mrp2 probe suggesting further that mrp2-mediated organic anion transport is predominantly occurring in hepatocytes and liver-derived cell lines.

Cloning of the entire cDNA region into the *Eco*RI site of the expression vector pcDNAI/Amp yielded the new plasmid construct pKEH1. To directly demonstrate the transport function of mrp2, we transiently transfected COS-7 cells with the mrp2-containing plasmid construct pKEH1. As illustrated in Fig. 2, the transfected COS-7 cells showed a markedly increased efflux of endogenously formed [^{14}C]DNP-GS as compared to control cells (transfected with the 'empty' vector pCI-Neo). This mrp2-mediated efflux increased linearly during the first 4 min. Thereafter, [^{14}C]DNP-GS efflux remained constant in the transfected cells, while it slowly increased further in mock-transfected cells. Since analysis of the [^{14}C] CDNB contents in mrp2-transfected cells at the end of the efflux experiments still showed the presence of 34% of [^{14}C]CDNB initially added, intracellular conjugation of CDNB with reduced glutathione might have become rate limiting in the transfected cells after 4 min of incubation (data not shown). Since DNP-GS is a typical substrate of cMOAT [1], these results provide direct evidence that mrp2 can mediate canalicular organic anion transport in normal rat liver. This conclusion was further corroborated by additional studies in *X. laevis* oocytes. As depicted in Table 1, mrp2 cRNA injected *X. laevis* oocytes also exhibited an increased [^{14}C]DNP-GS efflux rate during the first 30 min of incubation. Similar results were also obtained with [^{3}H]LTC$_4$ (Table 1) which represents a typical high-affinity substrate of cMOAT [20]. After 45 min of incubation, the content of initially injected [^{3}H]LTC$_4$ decreased to 82% and 43% in water- and mrp2 cRNA-injected oocytes respectively (Fig. 3). These results provide further proof for mrp2-mediated active outward transport of divalent organic anions. In contrast, efflux of taurocholate, which is not a substrate of cMOAT [1], was similar in mrp2-cRNA and water injected oocytes (Table 1), thus supporting that mrp2 is not involved in canalicular secretion of monoanionic bile acids.

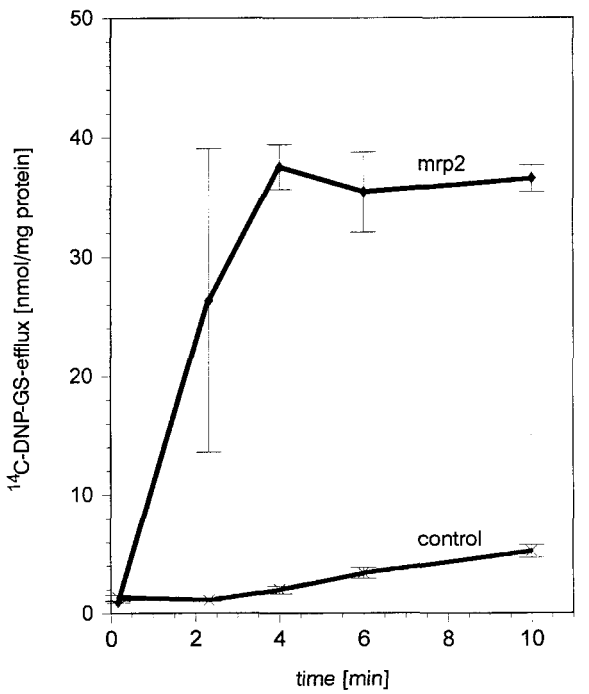

Fig. 2. Efflux of [^{14}C]DNP-GS from COS-7 cells transfected with the mrp2 vector (pKEH1; 0.5 μg) or with the control vector pCI-Neo (0.5 μg). The cells were incubated with 10 μM [^{14}C]CDNB and efflux of [^{14}C]DNP-GS determined as described in Section 2. The results represent the means ± S.E.M. of a triplicate determination.

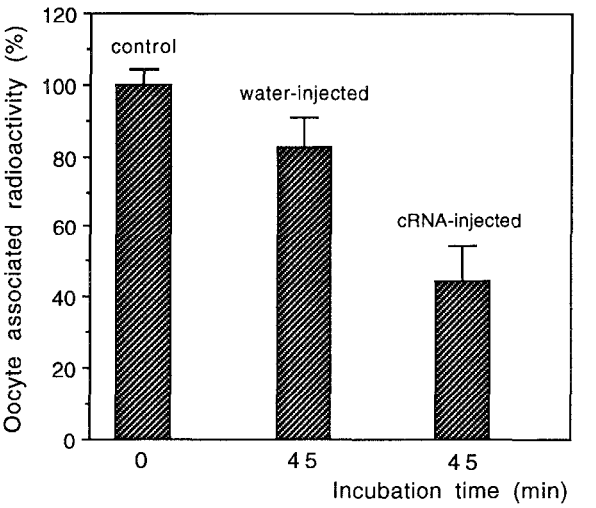

Fig. 3. Efflux of [^{3}H]LTC$_4$ from water- and mrp2 cRNA-injected *Xenopus laevis* oocytes. Oocytes were injected with 50 nl of 2.1 μM [^{3}H]LTC$_4$ and incubated for 40 min in standard efflux medium at 25°C (see Section 2). At the end of the experiment each oocyte was washed twice in ice-cold efflux medium and dissolved in 0.5 ml of 10% w/v SDS for determination of the oocyte associated radioactivity. Results are given as percent of controls (= initially injected amounts of radioactivity) and represent the means ± S.D.

In conclusion, we have cloned the protein involved in canalicular secretion of glutathione-conjugates and LTC$_4$ (mrp2) in liver parenchymal cells and verified by its functional expression in two independent systems that mrp2 is indeed functionally active as a glutathione conjugate and LTC$_4$ export pump. Hence, the study complements recent findings in transport mutant rat strains [7,8] and provides additional functional evidence for the qualification of mrp2 as the canalicular multispecific organic anion transporter (cMOAT) in rat liver.

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References

[1] R.P.J. Oude Elferink, D.K.F. Meijer, F. Kuipers, P.L.M. Jansen, A.K. Groen, G.M.M. Groothuis, Biochim. Biophys. Acta 1241 (1995) 215–268.
[2] M. Müller, H. Roelofsen, P.L.M. Jansen, Semin. Liver Dis. 16 (1996) 211–220.
[3] R.P.J. Oude Elferink, P.L.M. Jansen, Pharmacol. Ther. 64 (1994) 77–97.
[4] M. Yamazaki, H. Suzuki, Y. Sugiyama, Pharmaceut. Res. 13 (1996) 497–513.
[5] J. Kartenbeck, U. Leuschner, R. Mayer, D. Keppler, Hepatology 23 (1996) 1061–1066.
[6] C.C. Paulusma, P.J. Bosma, G.J.R. Zaman, C.T.M. Bakker, M. Otter, G.L. Scheffer, R.J. Scheper, P. Borst, R.P.J. Oude Elferink, Science 271 (1996) 1126–1128.
[7] M. Büchler, J. König, M. Brom, J. Kartenbeck, H. Spring, T. Hories, D. Keppler, J. Biol. Chem. 271 (1996) 15091–15098.
[8] K. Ito, H. Suzuki, T. Hirohashi, K. Kume, T. Shimizu, Y. Sugiyama, Int. Hepatol. Commun. 4 (1996) 292–299.
[9] K. Taniguchi, M. Wada, K. Kohno, T. Nakamura, T. Kawabe, M. Kawakami, K. Kagotani, K. Okumura, A. Shin-ichi, M. Kuwano, Cancer Res. 56 (1996) 4124–4129.
[10] S.P. Cole, G. Bhardwaj, J.H. Gerlach, J.E. Mackie, C.E. Grant, K.C. Almquist, A.J. Stewart, E.U. Kurz, A.M. Duncan, R.G. Deeley, Science 258 (1992) 1650–1654.
[11] Y.C. Awasthi, H.S. Garg, D.D. Dao, C.A. Partridge, S.K. Srivastava, Blood 58 (1981) 733–738.
[12] Colman, A. (1986) in: Transcription and Translation: A Pratical Approach (Hames, B.D. and Higgins, S.J., Eds.), pp. 271–302, IRL Press, Oxford.
[13] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Plainview, NY.
[14] E. Jacquemin, B. Hagenbuch, B. Stieger, A.W. Wolkoff, P.J. Meier, Proc. Natl. Acad. Sci. USA 91 (1994) 133–137.
[15] R. Evers, G.J.R. Zaman, L. van Deemter, H. Jansen, J. Calafat, L.C.J. Oomen, R.P.J. Oude Elferink, P. Borst, A.H. Schinkel, J. Clin. Invest. 97 (1996) 1211–1218.
[16] Kriegler, M. (1992) Gene Transfer and Expression: A Laboratory Manual, MacMillan, London.
[17] R.A. Wallace, D.W. Jared, J.N. Dumont, M.W. Sega, J. Exp. Zool. 184 (1973) 321–333.
[18] B.J. Shneider, M.S. Moyer, J. Biol. Chem. 268 (1993) 6985–6988.
[19] K. Wiechelmann, R. Bran, J. Fitzpatrick, Anal. Biochem. 175 (1988) 231–237.
[20] M. Huber, A. Guhlmann, P.L.M. Jansen, D. Keppler, Hepatology 7 (1987) 224–228.