The Role of Prostaglandin E Receptor-Dependent Signaling via cAMP in Mdr1b Gene Activation in Primary Rat Hepatocyte Cultures


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Received August 12, 2005; accepted January 12, 2006

ABSTRACT

Multidrug resistance (mdr) proteins of the mdr1 type function as multispecific xenobiotic transporters in hepatocytes. In the liver, mdr1 overexpression occurs during regeneration, cirrhosis, and hepatocarcinogenesis and may contribute to primary chemotherapy resistance. Cultured rat hepatocytes exhibit a time-dependent "intrinsic" increase in functional mdr1b expression, which depends on cyclooxygenase-catalyzed prostaglandin E2 release. In the present study, the prostaglandin E (EP) receptor agonist misoprostol (1–10 μM) further enhanced intrinsic mdr1b mRNA expression in primary rat hepatocytes. On the other hand, [1α,2β,5α](-(+) -7-[5-[1,1'-biphenyl]-4-yl]methoxy]-2-(4-morpholino)-3-oxocyclopentyl]-4-heptenoic acid (AH23848B) (30 μM), an antagonist of the cAMP-coupled EP4 receptor, and the protein kinase A (PKA) inhibitor, N-[2-[bromocinnamylamino]-ethyl]-5-isooquinolinesulfonamide (H89) (10 nM), repressed intrinsic mdr1b mRNA up-regulation, whereas the stable cAMP analog 8-bromo-cAMP (10 μM) and the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) (100 μM) further enhanced intrinsic mdr1b expression. Primary rat hepatocytes, transiently transfected with reporter gene constructs controlled by mdr1b 5'-gene-flanking regions [−1074 to +154 base pairs (bp)], demonstrated pronounced mdr1b promoter activity, already without the addition of exogenous modulators. Nevertheless, activity was further stimulated by misoprostol, 8-bromo-cAMP, or IBMX. Cotransfection with expression vectors for PKI, an inhibitor protein of cAMP-dependent PKA, or KCREB, a dominant-negative mutant of the cAMP-responsive element-binding protein (CREB), decreased high-intrinsic mdr1b promoter activity. KCREB also counteracted misoprostol-induced mdr1b promoter activation. In conclusion, these data provide evidence for a pivotal role of EP receptor-stimulated, cAMP-dependent gene activation in primary rat hepatocytes. Thus, these data might offer potential new target structures for the reversal of primary drug resistance, for example, of liver tumors.

Supplemental material to this article can be found at: http://jpet.aspetjournals.org/content/suppl/2006/01/13/jpet.105.094193.DC1

P-glycoproteins (P-gps) of the multidrug resistance transporter 1 (mdr1) type (MDR1 in humans; mdr1a and mdr1b in rodents) are plasma membrane-located proteins involved in ATP-dependent extrusion of a broad spectrum of hydrophobic or amphiphilic compounds, including numerous drugs. Overexpression of mdr1-type transporters in tumor cells has been found to contribute to chemotherapy resistance. Physiologically, mdr1 expression in tissues with excretory or barrier functions suggests that mdr1 transporters participate in excretion of and/or protection against potentially toxic endo- and xenobiotics (Thiebaut et al., 1987; Chan et al., 2004). The
extent of mdr1 expression, e.g., in intestine or liver, would be expected to affect pharmacokinetics of therapeutically relevant mdr1 substrates (Chan et al., 2004). In the liver, mdr1 proteins are located in the canalicular membrane of hepatocytes. Mdr1 expression is elevated under particular (patho)physiological conditions, e.g., during rodent liver regeneration or rodent (Teeter et al., 1993) and human hepatocarcinogenesis (Huang et al., 1992; Takanishi et al., 1997). Human hepatocytes in diseased livers exhibiting primary biliary cirrhosis, chronic hepatitis C virus infection, or regeneration after submassive cell necrosis are also characterized by MDR1 up-regulation (Ros et al., 2003). Notably, induction of cyclooxygenase (COX)-2 has been demonstrated in regenerating rat liver after partial heptectomy (Casado et al., 2001). Furthermore, an increase in COX-2 protein expression has been observed in well-differentiated human hepatocellular carcinoma and during progression of human liver fibrosis (Koga et al., 1999; Cheng et al., 2002; Qiu et al., 2002), suggesting a regulatory link between mdr1 overexpression and the COX system. Indeed, association of mdr1 expression with COX-2 expression/activity has been shown for a human hepatocellular carcinoma cell line (Fantappie et al., 2002), for rat mesangial cells after adenovirus-mediated transfection with COX-2 cDNA (Paweletz et al., 2002), and, finally, in doxorubicin (synonymous with adriamycin hydrochloride and hydroxydaunorubicin)-treated human myeloid leukemia cells (Puhlmann et al., 2005).

Primary cultures of rat hepatocytes exhibit a time-dependent “intrinsic” increase in mRNA expression of the mdr1b isoform (Lee et al., 1993; Hirsch-Erns et al., 1995). Because this overexpression resembles mdr1b gene activation during liver regeneration or early phases of hepatocarcinogenesis, primary rat hepatocytes have been used to investigate mechanisms of hepatic mdr1 gene regulation.

We have recently shown that intrinsic mdr1b mRNA up-regulation during rat hepatocyte culture is, at least in part, attributable to the action of products of COX-dependent arachidonic acid metabolism, e.g., prostaglandin E2 (PGE2), which accumulates in the culture supernatant in a time-dependent manner. Furthermore, COX inhibition repressed which accumulates in the culture supernatant in a time-dependent manner. Furthermore, COX inhibition repressed which accumulates in the culture supernatant in a time-dependent manner. Furthermore, COX inhibition repressed which accumulates in the culture supernatant in a time-dependent manner. Furthermore, COX inhibition repressed which accumulates in the culture supernatant in a time-dependent manner. Furthermore, COX inhibition repressed which accumulates in the culture supernatant in a time-dependent manner. Furthermore, COX inhibition repressed which accumulates in the culture supernatant in a time-dependent manner. Furthermore, COX inhibition repressed which accumulates in the culture supernatant in a time-dependent manner. Furthermore, COX inhibition repressed which accumulates in the culture supernatant in a time-dependent manner. Furthermore, COX inhibition repressed which accumulates in the culture supernatant in a time-dependent manner. Furthermore, COX inhibition repressed which accumulates in the culture supernatant in a time-dependent manner. Furthermore, COX inhibition repressed which accumulates in the culture supernatant in a time-dependent manner. Furthermore, COX inhibition repressed which accumulates in the culture supernatant in a time-dependent manner. Furthermore, COX inhibition repressed which accumulates in the culture supernatant in a time-dependent manner. Furthermore, COX inhibition repressed which accumulates in the culture supernatant in a time-dependent manner. Furthermore, COX inhibition repressed which accumulates in the culture supernatant in a time-dependent manner. Furthermore, COX inhibition repressed which accumulates in the culture supernatant in a time-dependent manner. Furthermore, COX inhibition repressed which accumulates in the culture supernatant in a time-dependent manner. Furthermore, COX inhibition repressed which accumulates in the culture supernatant in a time-dependent manner. Furthermore, COX inhibition repressed which accumulates in the culture supernatant in a time-dependent manner. Furthermore, COX inhibition repressed which accumulates in the culture supernatant in a time-dependent manner. Furthermore, COX inhibition repressed which accumulates in the culture supernatant in a time-dependent manner. Furthermore, COX inhibition repressed which accumulates in the culture supernatant in a time-dependent manner. Furthermore, COX inhibition repressed which accumulates in the culture supernatant in a time-dependent manner. Furthermore, COX inhibition repressed which accumulates in the culture supernatant in a time-dependent manner. Furthermore, COX inhibition repressed which accumulates in the culture supernatant in a time-dependent manner. Furthermore, COX inhibition repressed which accumulates in the culture supernatant in a time-dependent manner. Furthermore, COX inhibition repressed which accumulates in the culture supernatant in a time-dependent manner. Furthermore, COX inhibition repressed which accumulates in the culture supernatant in a time-dependent manner. Furthermore, COX inhibition repressed which accumulates in the culture supernatant in a time-dependent manner. Furthermore, COX inhibition repressed which accumulates in the culture supernatant in a time-dependent manner. Furthermore, COX inhibition repressed which accumulates in the culture supernatant in a time-dependent manner. Furthermore, COX inhibition repressed which accumulates in the culture supernatant in a time-dependent manner. Furthermore, COX inhibition repressed which accumulates in the culture supernatant in a time-dependent manner. Furthermore, COX inhibition repressed which accumulates in the culture supernatant in a time-dependent manner. Furthermore, COX inhibition repressed which accumulates in the culture supernatant in a time-dependent manner. Furthermore, COX inhibition repressed which accumulates in the culture supernatant in a time-dependent manner. Furthermore, COX inhibition repressed which accumulates in the culture supernatant in a time-dependent manner. Furthermore, COX inhibition repressed which accumulates in the culture supernatant in a time-dependent manner. Furthermee...
One-Step RT-PCR kit (Invitrogen, Groningen, The Netherlands). Primers were used at 500 nM each (Table 1). In brief, 1 μg of total RNA and the primer pair were denatured for 10 min at 70°C. Reaction buffer and the reverse transcriptase/Taq polymerase mix were added, and reverse transcription was performed for 45 min at 45°C. Reverse transcriptase was subsequently inactivated during a 2-min incubation step at 94°C. Samples were then subjected to 35 amplification cycles, each consisting of 20 s at 96°C, 1 min at 61°C, and 1 min at 72°C followed by 7 min at 72°C after the last cycle. PCR products were analyzed on a 1% agarose gel and visualized by ethidium bromide staining. The EP4 primer set yielded a 659-bp PCR-product.

**Immunoblot of P-gps.** Hepatocyte fractions were enriched for plasma membranes by sucrose gradient centrifugation (Hirsch-Ernst et al., 1998). Twelve micrograms of protein per lane were separated electrophoretically through 7.5% sodium dodecyl sulfate-polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Millipore, Eschborn, Germany) by semidyry blotting (Ziemann et al., 1999). P-gps were detected using the primary polyclonal antibody PC03 (Oncogene Research Products, Cambridge, MA) directed against a protein region conserved in all rat P-gps according to Ziemann et al. (1999).

**Construction of pGL3-mdr1b Promoter Reporter Gene Plasmids.** A fragment representing ~1.2 kilobases of the native sequence flanking the Wistar rat mdr1b gene (~1074 to +154 bp) was amplified using a primer pair (rat-mdr1b-forward and -reverse) (Table 1) corresponding to sites of the Fisher 344 rat mdr1b promoter (Silverman and Hill, 1995) (GenBank accession no. 1.16546.1). Primers contained an additional 5'-NheI recognition site. The PCR fragment was ligated into the pCR-XL-TOPO vector (Invitrogen). Sequence analysis of three individual clone inserts revealed slight but consistent sequence differences in our sequence of the Wistar rat mdr1b promoter compared with the promoter of the Fisher 344 rat strain. The Wistar rat mdr1b promoter sequence (EMBL accession no. AJ312410.1) was used for further experiments. The mdr1b promoter fragment was cloned from the pCR-XL-vector into the NheI site of the pGL3-Basic firefly luciferase expression vector (Promega, Mannheim, Germany) and designated as plG3-mdr1b (−1074 to +154). A 5'-deletion construct bearing the region from −250 to +154 base pairs (bp) of the mdr1b promoter was generated by PCR using pGL3-mdr1b (−1074 to +154) as a template and the rat mdr1b reverse primer and an internal forward primer (mdr1b/400 bp-forward) (Table 1) as primers. The PCR fragment was further processed as described above and the resulting firefly luciferase construct was designated pGL3-mdr1b (−250 to +154). Identity and insert orientation in constructs were validated by cycle sequencing.

**Hepatocyte Transfection and Luciferase Reporter Gene Assay.** Rat hepatocytes, plated onto six-well plates, were transiently transfected 24 h after seeding with the firefly luciferase constructs pGL3-Basic (Promega), pGL3-mdr1b (−1074 to +154), pGL3-mdr1b (−250 to +154), or pGL3C2B1 (Bauer et al., 2004) in combination with one of the Renilla reniformis luciferase constructs pRL-SV40, pRL-CMV, or pRL-TK (Promega) using Effectene (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. All R. reniformis luciferase constructs exhibited high promoter activities in primary rat hepatocytes. Cells were further cotransfected with the expression plasmids pPKI, pPKImut, or pKCREB as indicated. Equivalent DNA content of transfection controls without pKCREB was ensured by addition of pUC 19 DNA. Six hours later, the medium was replaced with fresh MX-83 medium with or without modulators. After 48 h, hepatocytes were lysed, and firefly and R. reniformis luciferase activities were measured according to Bauer et al. (2004). Firefly luciferase activities were normalized to R. reniformis luciferase activities (serving as internal standard for transfection efficiency) in the same sample ("relative luciferase activity").

### Results

**Enhancement of Intrinsic Mdr1b Up-Regulation and Intracellular cAMP by Misoprostol.** We previously demonstrated that prostaglandins participate in intrinsic mdr1b up-regulation in primary rat hepatocyte cultures (Ziemann et al., 2002). Accordingly, in the present study, the PGE2 receptor agonist misoprostol further enhanced intrinsic mdr1b mRNA expression in a time- and concentration-dependent manner (Fig. 1). Induction amounted to a significant 2.35 ± 0.51-fold increase (n = 4, P ≤ 0.01, Student’s t test for paired values) at 10 μg/ml after 72 h of incubation compared with untreated controls (Fig. 1). However, an effect was already evident after 24 h (1.22 ± 0.007-fold increase; n = 2; data not shown) and 48 h (1.68 ± 0.45-fold increase; n = 5; P ≤ 0.05, Student’s t test for paired values; data not shown). Stimulation of primary rat hepatocytes for 5 min with 10 μg/ml misoprostol led to an ~2-fold increase in intracellular cAMP, as measured by a competitive enzyme immunoassay (Supplemental Fig. 1).

**Inhibition of Intrinsic Mdr1b Up-Regulation by AH23848B.** Treatment of primary rat hepatocytes with PGE2 has previously been shown to enhance production of cAMP as a second messenger (Qu et al., 1999). In the present study, incubation of rat hepatocytes with the EP receptor agonist misoprostol also increased intracellular cAMP levels (Supplemental Fig. 1), indicating that the Gs-coupled EP receptors EP2 and EP4 might be involved in PGE2-dependent signal transduction in rat hepatocytes. Indeed, incubation of primary rat hepatocytes with the EP4 receptor agonist AH23848B concentration-dependently inhibited intrinsic mdr1b mRNA expression. With respect to the concentration range tested, inhibition was maximal at 30 μM after 24 h of incubation, amounting to a reduction by 73 ± 2.6% (Fig. 2A) (n = 3, P ≤ 0.001, Student’s t test for paired values). AH23848 at a concentration of 30 μM also inhibited misoprostol-induced increase in mdr1b mRNA expression (C. Ziemann, A. Riecke, and G. Rüdell, unpublished data). The fluorescent mdr1 substrate Rho123 was used to examine

### Table 1

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer Sequence: 5' → 3'</th>
<th>Primer Site(^a)</th>
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</thead>
<tbody>
<tr>
<td>Rat-mdr1b-forward</td>
<td>AttgctagcAGGAGCAGTACGAGGTAAGAGTCAG(^b)</td>
<td>−1074 to −1055(^e)</td>
</tr>
<tr>
<td>Rat-mdr1b-reverse</td>
<td>AttgctagcCTCCGTGACTACGACCTCC(^b)</td>
<td>+154 to +135(^e)</td>
</tr>
<tr>
<td>Mdr1b/400 bp-forward</td>
<td>AttgctagcATGCCACCGTTGACATCTCGAG(^a)</td>
<td>−250 to −228(^c)</td>
</tr>
<tr>
<td>Rat-EP4-forward</td>
<td>TTCCTTACATGATCGGGCTCTCA</td>
<td>+731 to +754(^d)</td>
</tr>
<tr>
<td>Rat-EP4-antisense</td>
<td>GTCTCCGAGTAGATGAAAGCTCTG</td>
<td>+1369 to +1369(^d)</td>
</tr>
</tbody>
</table>

\(^a\) Relative to transcription start site.

\(^b\) Additional 5'-NheI recognition site and the three-base overhang are written in lowercase letters.

\(^c\) GenBank accession no. L1.6546.1.

\(^d\) European Molecular Biology Laboratory accession no. AJ312410.1.

\(^e\) GenBank accession number D28869.1.
enhanced mdr1b mRNA expression) resulted in significantly reduced Rho123 accumulation compared with vehicle controls (Fig. 2B), pointing to an increase in mdr1-dependent transport activity and thus in functionally active mdr1 protein. In contrast, preincubation of cells for 48 h with AH23848B enhanced Rho123 accumulation (n = 3, 1.5 ± 0.03-fold, P ≤ 0.01, Student’s t test for paired values) compared with untreated controls, reflecting reduction in Rho123 efflux/mdr1-dependent transport activity (Fig. 2B). In addition, AH23848B also counteracted the misoprostol-induced increase in mdr1-dependent transport activity (Fig. 2B). Concomitant incubation of hepatocytes with AH23848B and Rho123 during the Rho123 accumulation assay did not interfere with the attainment of steady-state Rho123 levels (Supplemental Fig. 2), indicating that AH23848B did not directly inhibit mdr1b-dependent transport activity. Thus, the decrease in transport activity in AH23848B-precultured cells was most likely due to repression of mdr1 expression. One-step reverse transcription-PCR analysis indicated that primary rat hepatocyte cultures expressed EP4 mRNA (Supplemental Fig. 3). Thus, the EP4-receptor antagonist AH23848B repressed functional intrinsic and misoprostol-induced mdr1b up-regulation.

Further Enhancement of Intrinsic Mdr1b Up-Regulation by 8-Bromo-cAMP and IBMX. Because EP4 receptor activation is coupled to an increase in cAMP, the effects of cAMP elevation and of a stable cAMP analog on mdr1b mRNA expression were examined to confirm participation of a cAMP-coupled EP-receptor in mdr1b gene regulation and to further investigate the involved signal transduction pathway. Primary rat hepatocytes were precultured for 24 to 72 h with or without the cAMP analog 8-bromo-cAMP or the phosphodiesterase inhibitor IBMX, which prevents cAMP degradation. Concentration-dependent up-regulation of intrinsic mdr1b mRNA overexpression was observed for both modulators; maximal effects were demonstrated after 24 h of incubation, with 10 μM 8-bromo-cAMP amounting to a 2.7 ± 0.29-fold increase (n = 4, P ≤ 0.01, Student’s t test for paired values) (Fig. 3A) and 100 μM IBMX amounting to a 2.6 ± 0.19-fold increase (n = 3, P ≤ 0.01, Student’s t test for paired values) (Fig. 3A). Higher concentrations were less effective. This is in accordance with a concentration dependence of cAMP-elicited effects in hepatocytes, e.g., with stimulation of DNA synthesis at low cAMP concentrations and inhibition of DNA synthesis at high concentrations, as reviewed by Servillo et al. (2002). In addition, IBMX markedly enhanced P-gp expression after 72 h of incubation (Fig. 3B). This IBMX-dependent overexpression was accompanied by reduced Rho123 accumulation (reduction to 51 ± 0.2%, mean value of three independent experiments, P ≤ 0.05, Student’s t test for paired values), indicating up-regulation of functionally active mdr1-type P-gp expression (Fig. 3C). Accordingly, preculture with 8-bromo-cAMP for 48 h also resulted in decreased intracellular Rho123 levels (Fig. 3C). Thus, these data support the conclusion that induction of mdr1b mRNA expression by modulators of cAMP-dependent pathways was of functional relevance.

Strong Transcriptional Activity of the Mdr1b Promoter in Primary Rat Hepatocyte Cultures and Further Enhancement of Promoter Activity by Misoprostol, 8-Bromo-cAMP, and IBMX. To investigate whether mdr1b gene transcription contributes to intrinsic mdr1b functional relevance of reduced mdr1b mRNA expression. Attainment of high steady-state levels of the dye was interpreted in terms of low mdr1-dependent transport activity and vice versa. In agreement with Fig. 1, pretreatment of cells for 48 h with 10 μg/ml misoprostol (concentration leading to

**Fig. 2.** Inhibition of intrinsic mdr1b mRNA overexpression and enhancement of Rho123 accumulation by AH23848B. A, quantitative Northern blot analysis and a representative Northern blot. Total RNA was extracted from rat hepatocytes precultured for 24 h with or without AH23848B. Blots were hybridized to an mdr1b-specific oligonucleotide, and mdr1b mRNA expression was quantified. Data represent mean values ± S.E. of three independent experiments. Control values were set to 100%. *, P ≤ 0.05; ***, P ≤ 0.001 versus medium; Student’s t test for paired values. B, representative Rho123 accumulation assay. Cells were precultured for 48 h with or without AH23848B in the presence (●) or absence (■) of 10 μg/ml misoprostol. Rho123 accumulation was normalized for protein content per dish. Data represent mean values ± S.D. of an experiment performed in triplicate. ***, P ≤ 0.01; ****, P ≤ 0.001 versus medium with DMSO as vehicle control; ++++, P ≤ 0.001 versus misoprostol-treated cells; Student’s t test for unpaired values.

**Fig. 1.** Enhancement of intrinsic mdr1b mRNA overexpression in misoprostol-treated rat hepatocytes. Representative Northern blot of rat hepatocyte mRNA is shown. Cells were cultured for 72 h with or without misoprostol. The blot was hybridized to an mdr1b-specific probe and rehybridized to a GAPDH mRNA-specific oligonucleotide.
up-regulation, primary rat hepatocytes were transiently transfected with a firefly luciferase expression vector under the control of a fragment of the Wistar rat mdr1b promoter (from −1074 to +154 bp), designated pGL3-mdr1b(−1074 to +154). Transfected cells exhibited very strong intrinsic mdr1b promoter activation (Table 2). This was reflected by high relative luciferase activities compared with cells transiently transfected with the pGL3-Basic vector (lacking an active promoter sequence) or the pGL3C2B1 construct, consisting of the pGL3-Basic vector with inserted part of the native 5′-sequence flanking the phenobarbital-inducible cytochrome P-450 CYP2B1 gene (Bauer et al., 2004).

High intrinsic luciferase expression was further up-regulated by incubation of pGL3-mdr1b(−1074 to +154)-transfected hepatocytes with 10 µg/ml misoprostol (Fig. 4A), 10 to 100 µM 8-bromo-cAMP, or 10 to 100 µM IBMX for 48 h (Fig. 4B). The increase in mdr1b promoter activity was 1.2 ± 0.27-fold for misoprostol, 1.5 ± 0.35- and 1.5 ± 0.32-fold for 8-bromo-cAMP, and 1.7 ± 0.24- and 1.3 ± 0.44-fold for IBMX, respectively, compared with untreated controls. These results support the conclusion that enhanced mdr1b gene

![Fig. 3. Induction of mdr1b mRNA expression and mdr1-dependent transport activity by 8-bromo-cAMP and IBMX. A, quantitative Northern blot analysis. Total RNA was extracted from rat hepatocytes precultured for 24 h with or without 8-bromo-cAMP or IBMX. Data represent mean values ± S.E. of three (1, µM 8-bromo-cAMP and 10 µM 1 mM IBMX) or four (10 and 100 µM 8-bromo-cAMP) independent experiments. Control values were set to 100%. **, P ≤ 0.01 versus culture medium (8-bromo-cAMP) or culture medium with DMSO (IBMX) as vehicle controls; Student’s t test for unpaired values. B, representative Western blot. P-gp expression in cells cultured for 72 h with or without 10 or 100 µM IBMX. C, representative Rho123 accumulation assays. Cells were precultured for 48 h with or without 10 µM 8-bromo-cAMP or for 72 h with or without 100 µM IBMX. Rho123 accumulation was normalized for protein content per dish. Data represent mean values ± S.D. of experiments each performed in triplicate. **, P ≤ 0.01; ***, P ≤ 0.001 versus culture medium (8-bromo-cAMP) or culture medium with DMSO (IBMX) as vehicle controls; Student’s t test for unpaired values.](#)

![Fig. 4. Mdr1b promoter activation by misoprostol, 8-bromo-cAMP, or IBMX. Hepatocytes precultured for 24 h were transiently cotransfected with 0.25 µg of the mdr1b promoter firefly luciferase gene construct pGL3-mdr1b(−1074 to +154) and 0.005 or 0.5 µg of the R. reniformis luciferase constructs pRL-TK (misoprostol and IBMX) or pGL-SV40 (8-bromo-cAMP). After treatment of cells for 48 h with misoprostol, 8-bromo-cAMP, or IBMX, luciferase activities were determined. Control values were set to 100%. A, misoprostol: data represent mean values ± S.E. Relative to pGL3-Basic. pGL3-Basic 0.0024 ± 0.0003 1
pGL3C2B1 0.0276 ± 0.0029 11
pGL3-mdr1b(−1074 to +154) 3.2613 ± 0.2221 1356

B, IBMX and 8-bromo-cAMP: data represent mean values ± S.E. of triplicate (8-bromo-cAMP) or quintuplicate (IBMX) determinations of representative experiments. **, P ≤ 0.01 versus culture medium (8-bromo-cAMP) or culture medium with DMSO (IBMX); Student’s t test for unpaired values.](#)
transcription contributes to intrinsic and also to misoprostol-, 8-bromo-cAMP-, and IBMX-induced mdr1b mRNA overexpression.

**Involvement of PKA in Regulation of Intrinsic Mdr1b Up-Regulation.** To examine whether cAMP-dependent PKA might represent the next player downward in the regulation of intrinsic mdr1b up-regulation, hepatocytes were cotransfected with pGL3-mdr1b(−1074 to +154) and an expression construct for the PKA inhibitor protein PKI (pPKI) or a control construct, harboring the gene for an ineffective PKI mutant (pPKImut) (Day et al., 1989). Cotransfection of hepatocytes with pPKI significantly reduced mdr1b promoter activity compared with cotransfection with pPKImut (Fig. 5A). In addition, incubation of cells for 48 h with a 10 nM concentration of the PKA inhibitor H89 reduced intrinsic mdr1b mRNA up-regulation to 69.1 ± 5.78% (%n = 4, P ≤ 0.01) of vehicle controls (Fig. 5B). H89 also almost completely inhibited IBMX-induced mdr1b mRNA up-regulation (Supplemental Fig. 4).

**Inhibition of High Intrinsic Mdr1b Promoter Activity by Overexpression of KCREB.** Because EP4 receptor-dependent activation of PKA seemed to participate in the regulation of PGE_2-dependent intrinsic mdr1b up-regulation in rat hepatocyte cultures and the transcription factor CREB is a well known substrate of PKA (Mayr and Montmyn, 2001), with relevance in the liver, we investigated whether CREB might be involved in signal transduction pathway(s) resulting in PGE_2-dependent intrinsic mdr1b gene activation. Therefore, hepatocytes were cotransfected with pGL3-mdr1b(−1074 to +154) and an expression construct for KCREB (pKCREB), a dominant-negative CREB variant that dimerizes with CREB-like proteins and prevents DNA binding of the heterodimers (Walton et al., 1992). Overexpression of KCREB inhibited to similar reduction levels both intrinsic and misoprostol-induced mdr1b promoter activation (Fig. 6A), suggesting that CREB or proteins dimerizing with CREB might contribute to regulation of intrinsic and miso-

![Fig. 5. Influence of PKA inhibition on mdr1b promoter activity and intrinsic mdr1b mRNA overexpression. A, representative experiment of five. Primary rat hepatocytes precultured for 24 h were transiently cotransfected with 0.1 μg/well mdr1b promoter construct pGL3-mdr1b(−1074 to +154), 0.005 μg/well R. reniformis luciferase construct pRL-SV40, and 0.4 μg/well expression constructs for the PKA inhibitor protein PKI (pPKI) or its inactive mutant PKImut (pPKImut), serving as control. Cells were cultured for an additional 48 h, and luciferase activities were determined. Data represent mean values ± S.E. of a 5-fold determination. * + P ≤ 0.05 versus pPKImut-transfected cells; Student’s t test for unpaired values. B, quantitative Northern blot analysis. Total RNA was extracted from cells cultured for 48 h with or without the PKA inhibitor H89 (10 nM). Data represent mean values ± S.E. of four independent experiments. Control values were set to 100%. ** + P ≤ 0.01 versus control medium with ethanol as solvent control; Student’s t test for paired values.](image)

![Fig. 6. Inhibition of intrinsic and misoprostol-induced mdr1b promoter activity by KCREB overexpression. A, representative experiment of three. Primary rat hepatocytes precultured for 24 h were transiently cotransfected with 0.1 μg/well mdr1b promoter luciferase-reporter gene construct pGL3-mdr1b(−1074 to +154) and 0.003 μg/well of the R. reniformis luciferase construct pRL-CMV with or without (control) 0.1 μg/well expression construct for the dominant-negative CREB mutant KCREB (pKCREB) or pUC 19 DNA (without pKCREB, control). Firefly and R. reniformis luciferase activities were determined, with subsequent calculation of relative luciferase activities, following culture of cells for an additional 48 h with or without misoprostol. Data represent mean values ± S.E. of a triplicate determination. * + P ≤ 0.05 versus pUC 19 DNA-cotransfected cells with culture medium + ethanol as solvent control; ** + P ≤ 0.001; *** + P ≤ 0.001 versus pUC 19 DNA-cotransfected cells, misoprostol-treated; Student’s t test for unpaired values. B, cells were transiently cotransfected with 0.1 μg/well pGL3-mdr1b(−1074 to +154) or the deleted construct pGL3-mdr1b(−250 to +154) and 0.003 μg/well pRL-CMV in the absence (control) or presence of 0.1 μg/well pKCREB. Relative luciferase activities were determined 48 h later. Data represent mean values of relative luciferase activities ± S.E. of eight (pGL3-mdr1b(−1074 to +154)) and seven (pGL3-mdr1b(−250 to +154)) independent experiments or mean values of only R. reniformis luciferase activities ± S.E. of eight (pRL-CMV) independent experiments, each performed in triplicate. *** + P ≤ 0.001 versus pUC 19 DNA-cotransfected cells; Student’s t test for paired values.](image)
prostol-induced mdr1b overexpression. Intrinsic promoter activity was reduced to 52 ± 4.4% (n = 8, P ≤ 0.001, Student’s t test for paired values) of controls. The inhibitory capacity of KCREB on mdr1b promoter activity was displayed to a similar extent in cells transfected with the pGL3-mdr1b(-250 to +154) construct bearing the shorter mdr1b promoter fragment (Fig. 6B). Thus, regulatory elements sensitive to KCREB-dependent repression of mdr1b gene activation appeared to be localized within these 400 bp of the mdr1b promoter. In contrast, R. reniformis luciferase activities, resulting from R. reniformis expression from the pRL-CMV construct, were not significantly reduced by KCREB overexpression, pointing to specific inhibition of mdr1b promoter activity by KCREB (Fig. 6B).

**Discussion**

Although mdr1-type P-gps are expressed in hepatocytes of normal liver, overexpression has been shown to occur during liver regeneration or in liver disease, e.g., during hepatocarcinogenesis. Primary rat hepatocyte cultures exhibit a time-dependent intrinsic increase in mdr1b mRNA expression and thus have been used as a model to study mechanisms of mdr1b gene regulation during (patho)physiological processes in the liver. A previous study demonstrated that pronounced intrinsic mdr1b expression during hepatocyte culture is, at least in part, attributable to background levels of prostaglandins, e.g., to a release of PGE2 into the culture medium (Ziemann et al., 2002).

PGE2 exerts its effects via binding to EP receptors. Participation of EP2/EP4 has been demonstrated in PGE2-stimulated oxygen uptake in hepatic parenchymal cells (Qu et al., 1999). Substantial inhibition of intrinsic and misoprostol-induced mdr1b overexpression in primary rat hepatocytes by the EP4 receptor antagonist AH23848B, as observed in the present study, is in accordance with a pivotal role of the widely distributed EP4 receptor in mediating misoprostol- and PGE2-dependent mdr1b overexpression. Basal levels of EP4 mRNA have been demonstrated previously in cultured rat hepatocytes (Perez et al., 2004). As reported by Fennekohl et al. (2000), EP2 and EP4 mRNA expression was undetectable in purified rat hepatocytes but was induced during hepatocyte culture by the proinflammatory cytokine interleukin 6. In addition to our results concerning inhibition of mdr1b expression by the EP4 receptor antagonist, we also found the EP1/EP2 receptor antagonist AH8609 to suppress intrinsic mdr1b mRNA expression. Maximal inhibition amounted to 32 ± 3.0% after 24 h of incubation (n = 3; P ≤ 0.01, Student’s t test for paired values) (C. Ziemann, A. Riecke, and G. Rudell, unpublished data) and thus was less pronounced than the inhibition observed with the EP4 receptor antagonist. These observations indicate that EP4 and EP2 might cooperate in mdr1b gene regulation, although a possible participation of other EP receptors (EP1 and EP3) cannot be ruled out. PGE2-dependent cooperation of EP2 and EP4 has previously been demonstrated for induction of adherent activity in guinea pig gastric mucosal cells (Hoshino et al., 2003). Both EP4 and EP2 receptors are linked to the generation of cAMP as second messenger. Indeed, in the present study, we found the EP receptor agonist misoprostol to lead to elevation of cAMP levels in cultured hepatocytes. Furthermore, intrinsic mdr1b promoter activity and mdr1b mRNA expression were further enhanced in rat hepatocytes by 8-bromo-cAMP and the inhibitor of cAMP degradation, IBMX. Repression of intrinsic mdr1b mRNA expression by H89, an inhibitor of cAMP-dependent PKA, and a decrease in mdr1b promoter activity in hepatocytes overexpressing the PKA inhibitor protein PKI support the conclusion that PKA acts downstream in EP receptor- and cAMP-dependent mdr1b gene regulation. In human tumor cells, e.g., in breast cancer cells (Scala et al., 1995; Rohlf and Glazer, 1998) and prostate tumor spheroids (Wartenberg et al., 2000), an involvement of cAMP and PKA in regulation of MDR1 expression has been demonstrated previously without establishment of a connection to a special receptor or receptor agonist. Interestingly, Puhlmann et al. (2005) recently demonstrated a PGE2-mediated increase in MDR1-dependent transport activity in HL-60 human myeloid leukemia cells. In addition, sensitivity of HL-60 cells toward the MDR1 substrate doxorubicin was enhanced by the EP1/EP2 receptor antagonist AH8609, with the cAMP-coupled EP2 receptor being the predominant EP receptor in HL-60 cells. These data suggest that an EP receptor/cAMP/PKA signaling pathway might also be important in the regulation of MDR1 expression in human tumor cells. However, the present study (using primary rat liver cells) for the first time directly links cAMP- and PKA-dependent mdr1b gene expression to EP receptor activation.

 Pronounced mdr1b promoter activity observed in transiently transfected primary rat hepatocytes suggests involvement of transcriptional regulation in intrinsic mdr1b up-regulation. High intrinsic mdr1b promoter activity was still observed in hepatocytes transfected with the deletion construct encompassing the mdr1b promoter region from -250 to +154 bp (data not shown). Other studies using stably transfected H-4-II-E cells provided evidence that the region between -250 and -163 bp is essential for regulation of basal mdr1b promoter activity, depending on the presence of nuclear factor κB and p53 binding sites (Zhou and Kuo, 1998). Nevertheless, the activity of these transcription factors is not known to be subject to activation by PKA. In contrast, the ubiquitously expressed cAMP-responsive element-binding protein CREB and closely related factors (i.e., activating transcription factor-1 and cAMP-response element modulator-r) represent transcription factors activated by PKA (Mayr and Montminy, 2001). Overexpression of the dominant-negative CREB mutant KCREB in primary rat hepatocytes repressed intrinsic and misoprostol-induced mdr1b promoter activity. These results indicate that CREB or CREB-like proteins might contribute to EP receptor-dependent mdr1b transcription in primary rat hepatocytes. CREB or CREB-like proteins play important roles in liver (patho)physiology, for example, in liver metabolism and regeneration or during progression of hepatocellular carcinoma (Servillo et al., 1997, 2002; Rudnick et al., 2001; Abramovitch et al., 2004). Interestingly, CREB activation during liver regeneration seems to require COX-2-dependent prostaglandin release (Rudnick et al., 2001). The interference of KCREB (Walton et al., 1992), with intrinsic and misoprostol-induced mdr1b promoter activation, may be interpreted either in terms of a direct or an indirect effect. CREB might, on the one hand, bind directly to the mdr1b promoter. If this were true, CREB binding sites would be expected to reside within the mdr1b promoter fragment (-250 to +154 bp), because the...
inhibitory effect of KCREB was also observed with the deleted mdr1b promoter construct. This would be in line with the observation that, in many genes, cAMP-responsive element (CRE) sequences are located in the first 200 bp upstream from the transcription start. Inspection of the mdr1b promoter fragment (~250 to +154 bp) suggested two potential CRE candidate sequences within the proximal mdr1b promoter region. However, it remains to be further investigated whether these putative CRE binding sites are of functional relevance. In addition, CREB might also interact with non-CRE sites in the mdr1b promoter. On the other hand, the effect of CREB on mdr1b gene transcription might be indirect; an example for indirect CREB action has been demonstrated for T cells of mice transgenic for a dominant-negative CREB element (Barton et al., 1996). This was explained with reduced expression of CREB-dependent transcription factors. Concerning the human MDR1 gene, Rohlf and Glazer (1998) demonstrated PKA-dependent regulation of the MDR1 gene promoter, which was ascribed to a PKA-mediated activation of Sp1 and subsequent interaction with an Sp1-response element. Whether the MDR1 gene promoter also contains functional CRE sequences has not been elucidated to date. In any case, striking parallels exist in the regulation of mdr1b and MDR1 expression by cAMP and PKA (Scalà et al., 1995; Wartenberg et al., 2000).

In conclusion, the present study for the first time provides evidence for a prostaglandin-induced, EP receptor-mediated, cAMP- and PKA-related pathway leading to activation of the mdr1b gene. Taking into account the fact that prostaglandin production is enhanced during liver regeneration and hepatocarcinogenesis and that CREB is involved in these processes, our data are consistent with a model in which CREB or related factors interacting with CREB function as pivotal regulators in mdr1b gene activation during (patho)physiological conditions in the liver. Thus, the present study offers a further step for a better understanding of the signal transduction pathways mediating transcriptional activation of the multispecific mdr1 drug transporter in liver cells and thus provides potential new target structures for modulation of multidrug resistance.

Acknowledgments

The excellent technical assistance of S. Blume, C. Schmitz-Saluce, and A. Gregus is gratefully acknowledged. We thank R. A. Maurer for providing pPKI and pPKImut expression plasmids, R. H. Goodman for providing KCREB expression plasmid, and GlaxoSmithKline for providing EP receptor antagonists AII28468B and AII6809.

References


Aknowledgments


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