Identification of a Bile Acid-responsive Element in the Human Ileal Bile Acid-binding Protein Gene

INVolVEMENT OF THE FARNESOId X RECEPTOR/9-cis-RETINOIC ACID RECEPTOR HETERODIMER

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Intestinal bile acid-binding protein (I-BABP) is a cytosolic protein that binds bile acids (BAs) with a high affinity. In the small intestine, its expression is restricted to the ileum where it is involved in the enterohepatic circulation of BAs. Using the enterocyte-like Caco-2 cell line, we have recently shown that BAs increased I-BABP gene expression. To determine whether this regulation occurs in vivo, the effect of BA depletion or supplementation was studied in mice. A dramatic drop in I-BABP mRNA levels was observed in mice treated with the BA-binding resin cholestyramine, whereas an increase was found in animals fed with taurocholic acid. BAs are physiological ligands for the nuclear farnesoid X receptor (FXR). Both FXR and I-BABP are co-expressed along the small intestine and in Caco-2 cells. To determine the role of FXR in the regulation of I-BABP expression, the promoter of the human I-BABP gene was cloned. In Caco-2 cells, cotransfection of FXR and RXRa is required to obtain the full transactivation of the I-BABP promoter by BAs. Deletion and mutation analyses demonstrate that the FXR/RXRa heterodimer activates transcription through an inverted repeat bile acid responsive element located in position −160/−148 of the human I-BABP promoter. In conclusion, we show that FXR is a physiological BA sensor that is likely to play an essential role in BA homeostasis through the regulation of genes involved in their enterohepatic circulation.

Primary BAs¹ are synthetized from cholesterol in the liver where they are conjugated with glycine or taurine prior to secretion into bile (1). In most mammals, bile is stored in the gall bladder. During a meal, BAs are released into the duodenum where they are required for the efficient absorption of dietary fat and lipid-soluble vitamins. In the distal gut, conjugated BAs may undergo bacterial modifications leading to the formation of secondary BAs. In humans, more than 90% of BAs are reabsorbed throughout the intestine and return, via the portal blood, to the liver where they are secreted again into bile. This enterohepatic circulation is essential for the maintenance of BA and cholesterol homeostasis (1).

Intestinal absorption of BAs takes place as a function of their chemical form (hydrophobicity index) through three complementary mechanisms: passive nonionic diffusion and facilitated and active protein-mediated transports. After bacterial deconjugation, passive diffusion of protonated BAs occurs in the ileum and colon. Passive absorption of glycine-conjugated BAs has also been recently reported in the jejunum of guinea pig (2). Conjugated dihydroxy-BAs are primarily absorbed at the jejunal level via facilitated transport, whereas taurine and glycine trihydroxy-BA are actively transported in the ileum (3). The relative contribution of jejunal carrier-mediated transport under physiological conditions remains to be determined. By contrast, the active ileal absorption of conjugated BAs has been the subject of extensive research. BA uptake in the ileum is mediated by an ileal sodium-dependent bile acid transporter (IBAT) located in the brush border membrane of ileocytes. This 38-kDa integral plasma membrane protein has been cloned in various species including humans (4). The physiological importance of IBAT for the maintenance of the enterohepatic circulation of BAs, and hence, cholesterol homeostasis, is supported by the observation that patients with mutations in the IBAT gene fail to absorb BAs efficiently and have reduced low density lipoprotein cholesterol levels (5, 6). Once in the cell, BAs are bound to the intestinal bile acid-binding protein (I-BABP), a 14–15-kDa cytoplasmic protein whose expression is restricted to the ileum (7). The I-BABP gene has been cloned and characterized from mouse (8) and rabbit (9). I-BABP binds BAs with a high affinity and may be involved in cellular BA uptake and trafficking because it has been found to be transiently associated with the IBAT in the brush border membrane (10). Therefore, the synthesis of I-BABP may constitute a rate-limiting step in the enterohepatic circulation of BAs. In agreement with this hypothesis, we have recently shown that BAs are potent inducers of I-BABP gene expression in enterocyte-like Caco-2 cells (11).

Recent findings have shown that BAs bind (12) and activate (12–14) the farnesoid X receptor (FXR), a member of the nuclear receptor superfamily. FXR can bind DNA sequences comprised of two inverted repeats separated by one nucleotide (IR-1) as a heterodimer with the 9-cis-retinoic acid receptor.
and the last 15 cm (segments 4–5) were considered to be the ileum. The following 25 cm (segments 2–4) were considered to be the jejunum, and the small intestine is known to start usually only after the Treitz’s ligament. The efficiency of treatments were assessed by the evaluation of hepatic (w/w) cholestyramine or 0.5% (w/w) TCA and CDCA, respectively. The additional 10 min. DNA-protein complexes were resolved on a 4% gel. The longest 5′-flanking region was characterized by variable gene expression along the gastric axis at the origin of functional specialization. If the small intestine is 50% of the total length, it is known that changes in BA pool size result in marked alterations in FXR-mediated Regulation of the Human I-BABP Gene.

**Materials and Methods**

**Chemicals**—Cholic acid, glycocholic acid, deoxycholic acid, taurocholate, taurochenodeoxycholic acid, and lithocholic acid were purchased from Steraloids, whereas chenodeoxycholic acid (CDCA) and taurocholic acid (TCA) were purchased from Sigma.

**Animals and Experimental Treatments**—French guidelines for the use and care of laboratory animals were followed. Male Swiss mice (30 ± 2 g) purchased from Centre d’Elevage R. Janvier (France) were used. Animals were housed individually in a controlled environment (constant temperature and humidity, darkness from 8 p.m. to 8 a.m.) and fed ad libitum a standard chow containing 3% (w/w) lipid (UAR A-04, Usine d’Alimentation Rationnelle, France). To study the effect of BA depletion or supplementation on intestinal I-BABP expression in vivo, mice were fed laboratory chow for 10 days containing either 4% (w/w) cholestyramine or 0.5% (w/w) TCA and CDCA, respectively. The efficiency of the treatments was assessed by the evaluation of hepatic 7α-hydroxylase mRNA levels (17). All sacrifice, intestinal and hepatic tissues were snap frozen in liquid nitrogen and stored at −80 °C until RNAs were extracted. In adult Swiss mice, the length of the small intestine is 50 ± 5 cm. To determine whether I-BABP and FXR are co-expressed in the gut, the small intestine was divided in five segments of 10 ± 0.5 cm from the pylorus to the ileocecal valvula. The jejunum is known to start usually only after the Treitz’s ligament. The first 10 cm (segment 1) were arbitrary considered to be the duodenum, the following 25 cm (segments 2–4) were considered to be the jejunum, and the last 15 cm (segments 4–5) were considered to be the ileum.

**Northern Blotting**—Total RNAs were extracted from liver and intestinal mucosa by the phenol-chloroform-LiCl method (18). Poly(A)+ RNAs were prepared using Oligotex suspension (Qiagen) according to the manufacturer’s instructions. Total RNAs (20–50 μg) or poly(A)+ (4.5 μg) were electrophoresed on a 1% agarose gel and transferred to Gene Screen membranes (NEN Life Science Products) using previously published procedures (19). DNA from rat I-BABP (20), rat L-FABP (21), human FXR, human IBAT (5), and rat cholesterol 7α-hydroxylase were used as probes. Probes were labeled with [α-32P]dCTP (3000 Ci/mmol; Amersham Pharmacia Biotech) by the megaprime kit (Amersham Pharmacia Biotech). A 24-residue oligonucleotide specific for rat 18 S RNA was used as probe to ensure that equivalent amounts of RNAs were loaded and transferred. This oligonucleotide was 5′ end-labeled using T4 polynucleotide kinase and [γ-32P]ATP (3000 Ci/mmol, Amersham Pharmacia Biotech).

**Molecular Cloning of the Human I-BABP Promoter**—A human genomic library (CLONTECH, HL1111j) was screened using an EcoRI fragment of human I-BABP cDNA as hybridization probe (22). Approximately 1 million plaque-forming units were screened, and several positive clones were isolated then purified. A clone containing the longest 5′-flanking region was characterized by subcloning in pUC19 and then sequencing on both strands by the dideoxy chain termination method. The 5′-flanking DNA fragment of 3.7-kilobase DNA genomic fragment of human I-BABP was used as a template for polymerase chain reaction-based generation of different deletion and mutation constructs of the promoter region. Polymerase chain reactions were performed with a proofreading DNA polymerase (Pfu DNA polymerase, Stratagene) and a common antisense primer that ended at +4 bp downstream the transcription start site. The −2769/+4 and −1204/+4 bp fragments were cloned upstream the cholangemophilic acetyltransferase (CAT) gene in the pCAT3-1 basic vector (Promega). The −148/+4 bp fragment was obtained by AvaI digestion of the −2769/+4 bp fragment. Mutation and deletion of the IR-1 sequence was generated by polymerase chain recombination using the following oligonucleotides: 5′-TCCCAAGCTTGATAAATGAGTAAGG-3′ (mutations underlined) and 5′-GGCAATGGGGTGACAGCACTTGGGGCTTGTCCCTCCAGGT-3′, respectively. All constructs were confirmed prior to use by both restriction digestions and sequencing with the dideoxy chain termination method.

**Cotransfection Assays**—The enterocyte-like Caco-2 cells were used for the transfection studies. They were plated in 6-well plates in Dulbecco’s modified Eagle’s medium in the absence of phenol red supplemented with 10% charcoal-stripped fetal calf serum at 50–60% confluency. In general, transfection mixtures contained 250 ng of human FXR and/or human RXRs expression vectors, 4 μg of I-BABP-CAT reporter plasmid, 500 ng of human IBAT expression vector (generous gift of Dr P. Dawson, Wake Forest University, Winston-Salem, NC), 500 ng of β-galactosidase expression vector. Cells were transfected overnight by calcium phosphate precipitation. The medium was changed by Dulbecco’s modified Eagle’s medium (without phenol red) supplemented with 10% delipidated calf serum and BAs, and the cells were incubated for an additional 24 h. Cell extracts were prepared and assayed for CAT and β-galactosidase activities.

**Results**

**Effects of a BA Depletion or Supplementation on I-BABP mRNA Levels in Mice**—An in vivo study was designed to assess the effect of alterations in the luminal BA levels on I-BABP gene expression. The experiments were conducted in mice rather than in rats, because their intestines are subject to intermittent fluxes of BAs released from the gall bladder. BA depletion was achieved pharmacologically by the addition of the BA sequestrant resin cholesteryamine in the diet. The BA loading was performed by supplementation of the diet with either TCA or CDCA. No significant changes in body mass or in food intake were found between controls and treated mice through the course of the experiment (data not shown). The effectiveness of the treatments was demonstrated by an increase or a decrease in hepatic cholesterol 7α-hydroxylase gene expression in response to cholesteryamine and BAs, respectively (17) (data not shown). In ileum, a dramatic drop in I-BABP mRNA levels was seen in BA-depleted mice, whereas a significant increase occurred after chronic TCA feeding. By contrast, the unconjugated BA CDCA exerted no effect on I-BABP expression under these conditions (Fig. 1). These data demonstrate that changes in BA pool size result in marked alterations in I-BABP gene expression in vivo.

**I-BABP and FXR Are Co-Expressed in Both Small Intestine and Caco-2 Cells**—The small intestine is a heterogeneous organ characterized by variable gene expression along the gastroscopic axis at the origin of functional specialization. If the nuclear receptor FXR is involved in the regulation of I-BABP gene expression, these proteins should be coexpressed within this tissue. As shown in Fig. 2A, ileum is the exclusive intestinal segment where FXR, I-BABP, and IBAT are expressed. By contrast, L-FABP, an I-BABP-related protein known to also bind BA (23), exhibits a overlapping pattern of expression with FXR (Fig. 2A). It is noteworthy that L-FABP expression is not regulated by BA (11). Coexpression of I-BABP was detected in undifferentiated Caco-2 cells used in the transfection studies. By contrast, IBAT mRNA were undetectable in these cells. As previously described (11), an induction of I-BABP mRNA levels is triggered when Caco-2 cells were sub-
FXR-mediated Regulation of the Human I-BABP Gene

FIG. 1. I-BABP expression is regulated by BAs in vivo. Male Swiss mice were fed for 10 days with either a BA sequestrant (cholestyramine) or with TCA or CDCA as described under “Materials and Methods.” A, Northern blot hybridization of I-BABP mRNA and 18 S rRNA levels. 20 μg of total RNA from mouse ilea were resolved on a 1% agarose gel containing 2.2 M formaldehyde, transferred to a nylon membrane, and fixed by UV irradiation. B, quantification by densitometric scanning. *, p = 0.05; **, p = 0.01, n = 5.

FIG. 2. I-BABP and FXR are coexpressed in the small intestine. Northern blot hybridization of I-BABP, FXR, IBAT, and L-FABP mRNA. 4.5 μg of poly(A+) RNA were resolved on a 1% agarose gel containing 2.2 M formaldehyde, transferred to a nylon membrane, and fixed by UV irradiation. A, the small intestine was divided into five segments of 10 ± 0.5 cm from the pylorus to the ileoceleal valvula as described under “Materials and Methods.” Segment 1 is duodenum, from segment 2 to the middle of segment 4 is jejunum, and from middle of segment 4 to segment 5 is ileum. B, mRNA from liver and kidney. C, mRNA from Caco-2 cells treated with either vehicle alone (V) or 250 μM CDCA.

Promoter was cloned upstream of a CAT reporter gene. This reporter was transiently transfected into Caco-2 cells together with human FXR and/or human RXRα expression vectors in the presence or absence of 100 μM CDCA and/or 1 μM 9-cis-retinoic acid. As shown in Fig. 3A, reporter gene activity was induced efficiently in presence of FXR and CDCA. Weaker activation was seen with RXRα and its ligand, 9-cis-retinoic acid. Maximum transactivation was obtained when both FXR and RXRα expression vectors were co-transfected, and the cells were treated with CDCA and 9-cis-retinoic acid. These data demonstrate that the I-BABP gene is regulated by the FXR/RXR heterodimer. We next evaluated the ability of different physiologically relevant BAs to activate transcription of the human I-BABP promoter. Both CDCA and the secondary bile acid deoxycholic acid were efficacious activators of the I-BABP promoter-reporter construct (Fig. 3B). CDCA-mediated induction of reporter gene expression was dose-dependent with a half-maximal effective concentration (EC50) of about 3 μM. In contrast to CDCA and deoxycholic acid, lithocholic acid, and the hydrophilic BA cholic acid exhibited relatively weak activation (Fig. 3B), whereas the conjugated BAs failed to activate transcription through the I-BABP promoter. These last data are paradoxical because (i) the glyco- and tauro-conjugated BAs are the major forms found in vivo and (ii) TCA feeding significantly increases the I-BABP mRNA levels in the mouse (Fig. 1). We previously showed (12) that conjugated BAs require the presence of the membrane transporter (IBAT) to activate FXR in CV-1 cells. As shown in Fig. 2A, IBAT is absent in the Caco-2 cells. To overcome this limitation, Caco-2 cells were cotransfected with a human IBAT expression vector together with FXR and RXRα. Under these conditions, the I-BABP promoter-reporter gene was strongly activated by 5 μM cholic acid, TCA, or glycocholic acid (Fig. 3D). Thus, the major physiological BAs regulate expression of the human I-BABP gene promoter.

Identification of a Positive BARE in the Human I-BABP Gene Promoter—The FXR/RXRα heterodimer recognizes the insect ecdysone responsive element that consists of an inverted repeat of the nuclear receptor half-site sequence AG(G/T)TCA separated by 1 nucleotide (IR-1) (15). Sequence alignment of the proximal promoters of the human, rabbit, and mouse I-BABP genes revealed a highly conserved but imperfect IR-1 sequence (Fig. 4). To determine whether the FXR/RXRα heterodimer can bind to this IR-1 motif, electrophoretic mobility shift assays were performed using the 32P-labeled IR-1 from the human I-BABP promoter in the presence of human FXR and/or human RXRα. Neither FXR nor RXRα alone bound to the probe (Fig. 5A, lanes 1 and 2). However, when mixed, the two proteins bound efficiently to the I-BABP IR-1 (Fig. 5A, lane 3). This binding was specific, as demonstrated by competition with an excess of either wild type IR-1 motif or an idealized IR-1 sequence containing two consensus half-sites (Fig. 5A, lanes 4–7). A mutated IR-1 motif failed to compete (Fig. 5A, lanes 8 and 9).

To explore the functional role of this FXR/RXRα-binding site in the regulation of human I-BABP gene by BAs, Caco-2 cells were cotransfected with human FXR and human RXRα expression vectors and I-BABP promoter-CAT reporter plasmids in which the IR-1 sequence was mutated. The −120/4 +44 construct containing the native IR-1 sequence was transactivated about 30-fold in the presence of CDCA (Fig. 5B, lane 1). BA transactivation of the I-BABP gene was abolished in the −148/−44 construct in which the IR-1 sequence was partially deleted (Fig. 5B, lane 2). Notably, mutation or deletion of the IR-1 motif resulted in the complete loss of reporter gene induction in response to CDCA (Fig. 5B, lanes 3 and 4). These data establish
this IR-1 motif in the proximal human I-BABP promoter to be a functional BARE.

**DISCUSSION**

I-BABP is a small cytoplasmic protein that belongs to the fatty acid-binding protein family (24). In the small intestine, it is found exclusively in the ileum where it binds BAs with high affinity. Although the cellular function of I-BABP is not yet fully understood, it may facilitate BA uptake and trafficking and/or serve as an intracellular buffer for protecting cells from the detergent effects of excess BAs. We have recently shown that I-BABP gene expression in enterocyte-like Caco-2 cells is tightly regulated by BAs, especially CDCA (11). To establish the physiological relevance of these data, an *in vivo* study was conducted in mice chronically subjected to a BA depletion or excess. Adapative up- and down-regulation of the I-BABP expression appears to depend on the size of the BA pool because the BA sequestrant cholestyramine triggered a dramatic drop in I-BABP mRNA levels while supplementation of the diet with TCA increased I-BABP expression levels. Surprisingly, CDCA exerted no regulatory action when it was added in the diet. It is possible that CDCA does not reach the ileum in a sufficient concentration to regulate I-BABP expression under these conditions. Indeed, it is known that protonated, unconjugated BAs passively diffuse along the small intestine. The acidic microclimate found in the unstirred water layer overlying the microvilli of enterocytes (25) must favor the protonation of dietary CDCA and thus its passive uptake. The positive feedback reported here was not found by Arrese and co-workers (26) in the rat because neither common bile duct ligation nor pharmacological sequestration of BAs led to a change in the expression of IBAT and I-BABP. The origin of this discrepancy is likely due to differences in the regulation of BA metabolism and transport between species that have gall bladders (e.g. humans and mice), and those that do not (e.g. rats). Thus in humans and other mammals with gall bladders, it may be necessary to modulate the expression of I-BABP and other genes involved in BA homeostasis to cope with the fluxes that occur in BA levels within the enterohepatic circulation.

The nuclear receptor FXR was recently proposed to be a physiological BA sensor (12–14). FXR is found in the tissues known to have significant BA metabolism, i.e. liver, kidney, adrenals, and gut (15). Our detailed examination of its expression along the mouse small intestine shows that FXR displays an overlapping expression pattern with the BA-transporters (I-BABP and IBAT) in the ileum. This finding suggests that FXR could play a physiological role in the regulation of BA flux in the ileal segment of the small intestine. Indeed, analysis of the human I-BABP promoter reveals the existence of a specific

**FIG. 3.** The I-BABP promoter is activated by FXR and bile acids. A, the −2769/+44 I-BABP-CAT construct was cotransfected in Caco-2 cells with an empty vector (NT) or with expression vectors for human FXR and/or human RXRa. Cells were treated either with the RXRa ligand 9-cis-retinoic acid (1 μM) or the FXR ligand CDCA (100 μM) or both ligands for 24 h. B, the −2769/+44 I-BABP-CAT construct was cotransfected with FXR and RXRa expression vectors in Caco-2 cells. Cells were treated for 24 h with 100 μM concentrations of the indicated BAs. C, dose response of CDCA, −2769/+44 I-BABP construct was cotransfected with RXRa and FXR expression vectors into Caco-2 cells that were treated with increasing concentrations of CDCA for 24 h. D, Caco-2 cells were cotransfected with the human IBAT expression vector as well a FXR and RXRa expression vectors and were treated for 24 h with 5 μM of the indicated BAs.

**FIG. 4.** A conserved IR-I sequence in the human, rabbit, and mouse I-BABP gene promoters. The first 200 bp of the human, rabbit and mouse gene promoters were aligned using the ClustalW algorithm. Numbering starts from the transcription start site of each promoter (A in bold). Asterisks show nucleotides that are conserved between the three species.

**FIG. 3.** The I-BABP promoter is activated by FXR and bile acids. A, the −2769/+44 I-BABP-CAT construct was cotransfected in Caco-2 cells with an empty vector (NT) or with expression vectors for human FXR and/or human RXRa. Cells were treated either with the RXRa ligand 9-cis-retinoic acid (1 μM) or the FXR ligand CDCA (100 μM) or both ligands for 24 h. B, the −2769/+44 I-BABP-CAT construct was cotransfected with FXR and RXRa expression vectors in Caco-2 cells. Cells were treated for 24 h with 100 μM concentrations of the indicated BAs. C, dose response of CDCA, −2769/+44 I-BABP construct was cotransfected with RXRa and FXR expression vectors into Caco-2 cells that were treated with increasing concentrations of CDCA for 24 h. D, Caco-2 cells were cotransfected with the human IBAT expression vector as well a FXR and RXRa expression vectors and were treated for 24 h with 5 μM of the indicated BAs.
BA responsive element, which interacts with the FXR/RXRα heterodimer. Because BAs exist predominantly in their conjugated form in vivo, it is noteworthy that physiological concentrations of both glyco- and tauro-conjugated BAs are able to transactivate the human I-BABP promoter when Caco-2 cells are cotransfected with FXR and RXRα expression vectors and the different I-BABP promoter construct as indicated. Lane 1, the −1204/+44 I-BABP construct contains the IR-1 sequence located between −160 and −148; lane 2, the −148/+44 I-BABP construct is a deletion of the 5′ end of the promoter that lacks the IR-1; lanes 3 and 4, the −1204/+44 I-BABPmut and I-BABPdel constructs mutate or delete the IR-1 (mutation underlined). Cells were treated with 100 μM CDCA for 24 h. Data are expressed as fold activation relative to cells treated with the vehicle alone and represents mean ± S.E.

In conclusion, we have presented the first data demonstrating that I-BABP gene expression is physiologically regulated by BA flux. The conservation of this regulatory pathway from mice to humans is consistent with the role of FXR as a physiological BA sensor in the gut. Because the loss of BAs is a major way for the elimination of cholesterol, identification of FXR antagonists may provide a new therapeutic approach for the treatment of hypercholesterolemia and hence, of cardiovascular diseases.

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