FXRE can function as an LXRE in the promoter of human ileal bile acid-binding protein (I-BABP) gene

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Abstract **Ileal bile acid-binding protein (I-BABP) is a 14 kDa cytosolic protein which binds bile acids with a high affinity. It is thought to be implicated in the enterohepatic circulation of bile acids and, hence, in cholesterol homeostasis. Using a combination of in vivo and in vitro experiments, we have recently shown that I-BABP gene expression can be indirectly up-regulated by cholesterol through the activation of sterol-responsive element-binding protein 1c (SREBP1c) by liver X-receptor (LXR). We report here that I-BABP can be also a direct target for LXR. I-BABP regulation by LXR is maintained when the SREBP binding site is deleted in the I-BABP promoter and occurs, in the absence of conventional LXRE sequences, through an IR1 sequence previously identified as a farnesoid X-receptor-responsive element (FXRE). Electrophoretic mobility shift assays demonstrated that the LXR/RXR heterodimer specifically recognizes the FXRE. Collectively, these data strongly suggest that LXR can regulate the I-BABP gene by both direct and indirect mechanisms.**

Key words: Ileal bile acid-binding protein; Liver X-receptor; Farnesoid X-receptor; Cholesterol; Bile acid; Fatty acid-binding protein; Small intestine; Nuclear receptor

1. Introduction

Excessive cholesterol content in the Western diet greatly contributes to the development of vascular strokes of which the human and social costs are dramatic. Therefore, considerable energy has been devoted for several years to determining the players responsible for the control of cholesterol (CS) homeostasis. CS balance is dependent on the equilibrium between dietary supplies, de novo cellular synthesis and fecal elimination, essentially as bile acids (BA). Primary BA are synthesized from CS in the liver in which they are conjugated with taurine or glycine prior to being secreted into bile. In the intestinal lumen, they facilitate fat digestion and absorption. In healthy humans, more than 95% of BA are reabsorbed along the small intestine and return by the portal route to the liver to be secreted again into bile. This enterohepatic circulation is crucial for the maintenance of CS homeostasis. Indeed, BA not reclaimed by the small intestine constitute the main physiological way of CS elimination. BA reabsorption is essentially supported by a specific transport system constituted of a set of membrane and soluble BA carriers located in the distal part of the gut (i.e. ileum) [1]. BA uptake mainly takes place through an active transport supported by the ileal sodium-dependent bile acid transporter (I-BAT) located in the brush border membrane of ileocytes [2]. Once in the cell, BA are reversibly bound to a soluble 14 kDa protein, the ileal bile acid-binding protein (I-BABP) [3], prior to being secreted into portal blood by a basolateral transport system constituted of the multidrug resistance protein 3 [4] and a truncated form of I-BAT [5].

Photoaffinity labeling of ileal tissue demonstrated that I-BABP is the only physiologically relevant BA carrier found in the cytosol of ileocytes [6]. Therefore, it is likely that I-BABP expression levels can be critical for an efficient enterohepatic circulation of BA. Indeed, I-BABP is thought to be involved in BA desorption from the apical plasma membrane, intracellular trafficking and targeting on the basolateral BA transport system. In good agreement with this putative critical role, we have recently shown that the I-BABP gene is tightly controlled by different molecular players considered as cellular sterol sensors. Indeed, I-BABP gene expression is up-regulated by BA [7,8] through the activation of the nuclear receptor farnesoid X-receptor (FXR, NR1H4). This regulation requires the binding of an FXR/9-*cis*-retinoic acid receptor (RXR) heterodimer to a specific bile acid-responsive element (BARE or FXRE) constituted of an inverted hexanucleotide repeat spaced by one nucleotide (IR1) identified in the proximal part of the human I-BABP promoter [9]. The dramatic decrease in the expression levels of the I-BABP gene found in FXR null mice indicates that this nuclear receptor is a major regulator for this gene under normal physiological conditions [10].

I-BABP gene expression has also been found to be induced by a CS-enriched diet in mice [11]. It is now well established that CS is a gene modulator. Indeed, the intracellular level of CS controls the proteolytic activation of transcription factors termed sterol-responsive element-binding proteins (SREBP). The activated SREBP recognizes a specific nucleotide sequence, the sterol-responsive element (SRE), in the target genes leading to their transcriptional rate [12]. Moreover, CS provides metabolic derivatives, BA and oxysterols, which

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Abbreviations: BA, bile acids; CS, cholesterol; I-BABP, ileal bile acid-binding protein; FXR, farnesoid X-receptor; LXR, liver X-receptor; RXR, retinoid X-receptor; FXRE, FXR-responsive element; LXRE, LXR-responsive element; SRE, sterol-responsive element; I-BAT, ileal bile acid transporter; FAS, fatty acid synthase; PLTP, phospholipid transfer protein; SREBP, sterol regulatory element-binding proteins; CAT, chloramphenicol acetyltransferase

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are ligand-mediated activators for the nuclear hormone receptors FXR and liver X-receptors (LXRα, NR1H3 or LXRβ, NR1H2), respectively [13]. After identification of an SRE sequence in the proximal promoter of the human I-BABP gene, we have recently shown that I-BABP regulation by CS occurs via an indirect pathway involving the LXR-mediated induction and activation of the SREBP1c isoform [11]. Such indirect regulation by LXR has also been reported for genes of the lipogenic pathway, stearoyl-CoA desaturase-I [14] and fatty acid synthase (FAS) [15]. LXRs associated with RXR preferentially recognize a DNA hormone response element, termed LXRE, that consist of two direct hexameric repeats separated by four nucleotides (DR4) found in several target genes including the SREBP1c gene. The recent demonstration that LXR and SREBP-binding sites independently but additively confer LXR responsiveness to the FAS gene [15] led us to explore the possibility of a direct control of the I-BABP gene by LXR.

In the current report, we show that I-BABP is also a direct LXR target gene. This regulation is achieved, in the absence of a conventional LXRE, by the binding of an LXR/RXR heterodimer to the FXR-responsive element found in the proximal promoter of human I-BABP.

2. Materials and methods

2.1. Cell culture and organ culture of ileal explants

Caco-2 cells were cultured in a controlled environment (37°C, 5% CO_2) in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) containing 4 mM glutamine, 1% non-essential amino acids, 100 U/ml penicillin, 100 μg/ml streptomycin and supplemented with 20% fetal calf serum (FCS). The medium was changed every 2 days.

Male Swiss mice were fasted overnight and ileal explants were prepared then cultured as previously described [16]. In brief, ileal samples were rapidly removed, washed, then sliced into strips of which the serosa was stripped off. Ileal explants were pre-cultured for 4 h at 37°C under an oxygenated atmosphere in HEPES-buffered DMEM containing 10% NCTC-135, 10% FCS, 1% fungizone and 0.1 mg/ml gentamicin (all from Gibco-Life Technology). The explants were cultured for 16 h in the same medium supplemented with 5% lipoprotein-free medium in the presence of LXR agonist (50 μM GW3965, generous gift of Dr. T.M. Willson, GlaxoSmithKline, Research Triangle Park, NC, USA). Control cultures received the vehicle alone (2 μl/ml dimethyl sulfoxide).

2.2. Northern blot analysis

Total RNAs were isolated with the RNeasy mini kit (Qiagen). 10 μg of RNAs were electrophoresed on a 1% agarose gel and transferred to GeneScreen membrane (NEN Life Science Products) using previously published procedures [9]. cDNA from human I-BABP was used as probe [17]. The cDNA from murine 18S rRNA was used to ensure that equivalent amounts of RNAs were loaded and transferred. Probes were labeled with [α-^{32}P]dCTP (3000 Ci/mmol; ICN) by a megaprime kit (Amersham Pharmacia Biotech).

2.3. Plasmid construction and transfection assays

Wild type −2769/+44 (I-BABP 2.8^{wt}) and −1204/+44 (I-BABP 1.2^{wt}) bp fragments of the human I-BABP promoter were cloned upstream of the chloramphenicol acetyltransferase (CAT) gene in the pCAT3-basic vector (Promega). Deletions of SRE (I-BABP 1.2$^{del\ SRE}$) and FXR-responsive element (I-BABP 1.2$^{del\ FXRE+SRE}$) were generated by site-directed mutagenesis (Quickchange™ site-directed mutagenesis kit, Stratagene) using the following oligonucleotides: 5′-caggacaggaggagaagaagcctcagcaactgggagag-3′ and 5′-ggcaatggggtgacagcacttggggcttgtccctccaggt-3′ respectively. All constructs were confirmed prior to use by restriction digestion.

Caco-2 cells were used for transfection studies (passage 35–40). They were plated in 6-well plates in DMEM supplemented with 10% FCS at 40–50% confluence. Transfection mixes contained 4 μg of I-BABP-CAT reporter plasmid and 500 ng of β-galactosidase expression vector. Cotransfection mixes contained 4 μg of I-BABP-CAT reporter plasmid, 250 ng of human LXRα or human LXRβ expression vectors, 250 ng of human RXRα expression vectors and/or 100 ng of human SREBP1c expression vectors (generous gift from Dr. T.F. Osborne, University of California, Irvine, CA, USA) and 500 ng of β-galactosidase expression vector. Cells were transfected overnight by the calcium phosphate precipitation method. In transfection studies, the medium was changed to DMEM supplemented with 10% delipidated serum. The cells were incubated for an additional 24 h. Cell extracts were prepared and assayed for CAT and β-galactosidase activities.

2.4. Electrophoretic mobility shift assays

Human LXRα and human RXRα were synthesized in vitro using the TNT rabbit reticulocyte lysate coupled in vitro transcription/translation system (Promega) according to the manufacturer's instructions. Gel mobility shift assays (20 μl) contained 20 mM HEPES (pH 7.8), 120 mM KCl, 0.4% Nonidet P-40, 12% glycerol, 2 mM dithiothreitol, 0.2 μg poly(dI-dC), and freshly synthesized LXRα and RXRα proteins. Competitor oligonucleotides including the wild type or mutated I-BABP FXRE sequences gatcggccaGGGTGAATAACCTcggg and gatcggccaGG**AA**GAATA**TT**CTcgggg (mutations in bold) were added at a 50-fold excess. After 10 min incubation on ice, 10 ng of 5′ end-labeled [γ^{32}P]ATP oligonucleotide (I-BABP FXRE^{wt} or I-BABP FXRE^{mut}) was added, and the incubation continued for an additional 10 min. DNA–protein complexes were resolved on a 4% polyacrylamide gel in 0.5 M TBE (90 mM Tris, 90 mM boric acid, 2 mM EDTA). Gels were dried then subjected to autoradiography at −70°C.

3. Results and discussion

3.1. An LXR agonist induces I-BABP gene expression ex vivo

Mice subjected to an LXR agonist (GW3965) exhibit an increase in I-BABP mRNA levels [11]. Nevertheless, this effect is likely, at least in part, indirect since the LXR-mediated induction of cholesterol 7α-hydroxylase leads to a rise in BA synthesis [18]. Therefore, to explore whether the I-BABP gene may be up-regulated by LXR independently of the BA/FXR pathway, an ex vivo experiment was performed using mouse ileal explants cultured in the presence of an LXR agonist. According to previously published data [11], this treatment leads to a slight but significant rise in I-BABP mRNA levels demonstrating that I-BABP is an LXR target gene (Fig. 1).

3.2. LXR can transactivate the human I-BABP promoter independently of the SREBP-binding site

In a previous study, we have shown that LXR-mediated regulation of the I-BABP gene takes place through a cascade of events involving the induction of SREBP1c by LXR producing, in turn, the transactivation of the I-BABP gene. This last step requires an SREBP-binding site (SRE) identified in the proximal promoter of human I-BABP [11]. In the present report, we have hypothesized that the I-BABP promoter might also be a target for a direct regulation by LXR as recently depicted for the FAS gene [15].

In a first experiment, to explore the respective influence of LXRα and LXRβ on the transcriptional activity of the I-BABP gene, Caco-2 cells were transiently co-transfected with a large human I-BABP promoter fragment (I-BABP2.8^{wt}) cloned upstream of the CAT reporter gene and with expression vectors for human LXRα or LXRβ and/or human RXRα. As shown in Fig. 2A, no activity of the reporter gene was observed with LXRα, LXRβ or RXRα alone. By contrast, a strong transactivation of the reporter gene was found when LXRα or LXRβ was co-transfected together with RXRα. Since no quantitative difference was

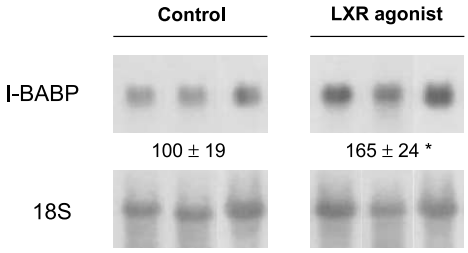

Fig. 1. LXR agonist induces I-BABP expression in mouse ileal explants. Ileal explants from male Swiss mice were cultured for 16 h in medium supplemented with 5% lipoprotein-free serum in the presence of 50 μM LXR agonist (GW3965). Control received the vehicle alone. I-BABP mRNA levels were evaluated by Northern blotting using 10 μg of total RNA and normalized to 18S rRNA. Mean ± S.E.M.

observed whatever the LXR isoform used, the further experiments were performed with LXRα only. To determine the promoter regions containing the sequences responsible for LXR induction, a set of 5′ deletion constructs were next realized (Fig. 2B). Interestingly, the LXR-mediated CAT activity was still fully conserved when the shorter construct was used demonstrating that the sequence between −183 and +44 mediates the response of the I-BABP promoter to LXR. As this region contains the SRE site responsible for the indirect regulation of I-BABP by LXR [11], a deletion experiment was undertaken to establish if LXR is able to transactivate the I-BABP promoter independently of the $SRE^{-72/-62}$ sequence (Fig. 2C). The 12-fold induction of CAT activity mediated by the LXRα/RXRα heterodimer on the wild-type promoter construct was reduced by 50% when the SRE was deleted. This gives the first demonstration that LXR can up-regulate the I-BABP promoter in the absence of the $SRE^{-72/-62}$ sequence.

3.3. LXR/RXR heterodimer activates the I-BABP promoter through the SRE and FXRE sequences

This finding raises the question of the existence of an LXRE in the proximal promoter of the human I-BABP gene. The direct regulation of LXR target genes occurs via a direct hexamer repeat separated by four nucleotides (DR4 sequence) [19]. Computer analysis of the human $I\text{-}BABP^{-183/+44}$ promoter sequence has not made it possible to identify such a typical DR4 site suggesting the existence of a non-conventional LXRE. It is noteworthy that an FXRE known to be a major regulatory site for the I-BABP gene is found in this promoter sequence [9]. Moreover, it has recently been shown in the context of phospholipid transfer protein

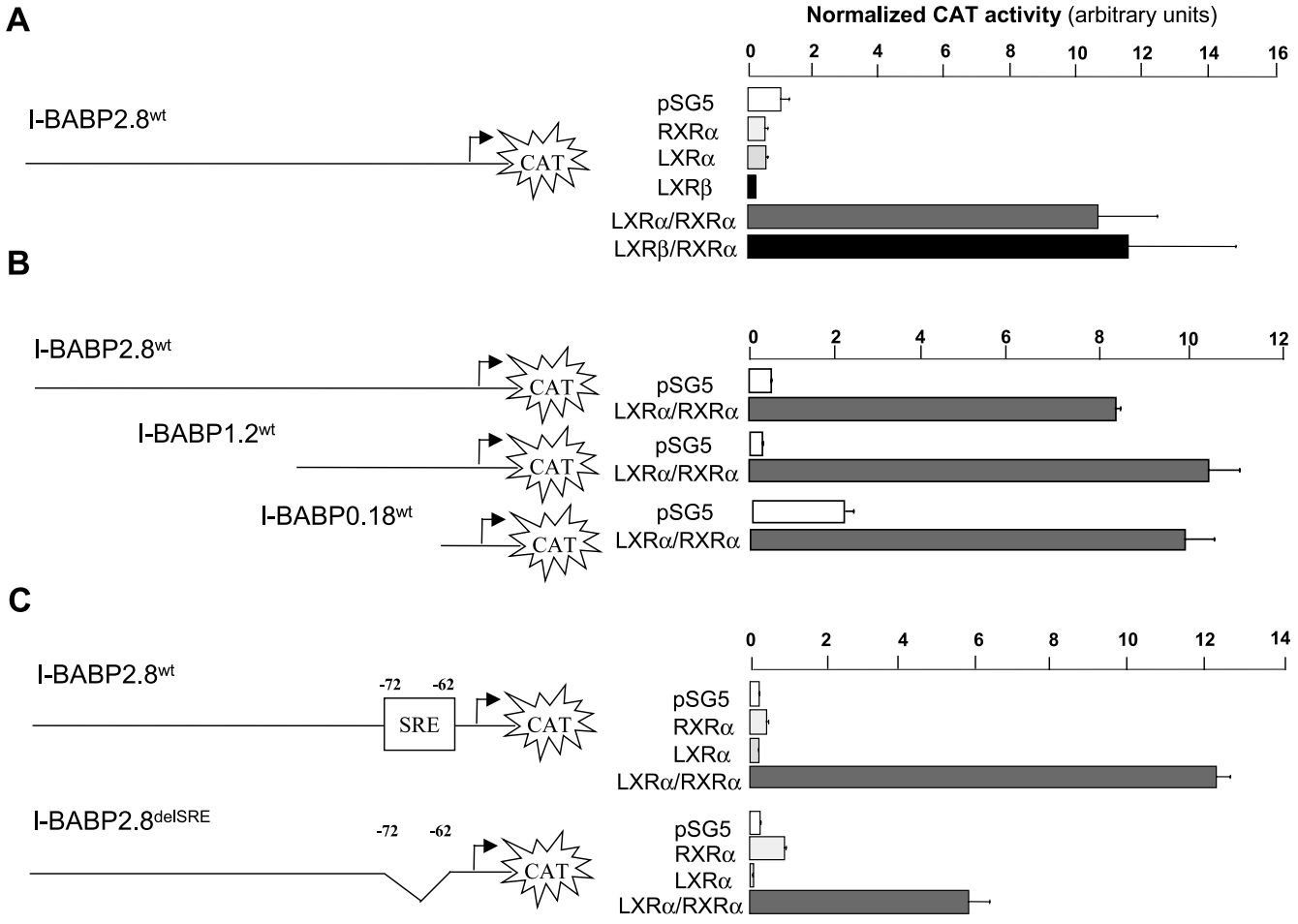

Fig. 2. Human I-BABP promoter is an LXR target gene. Human enterocyte-like Caco-2 cells (passage 35–40) were transiently transfected in a 10% FCS medium. Twelve hours after the transfection, the cells were cultured for an additional 24 h in medium containing 10% delipidated serum. A: LXRα and LXRβ activate the $I\text{-}BABP2.8^{wt}$ promoter (−2769 to +44 bp). B: LXR responsiveness is located in the proximal human I-BABP promoter. 5′ deletion analysis of the I-BABP promoter. C: Activation of the I-BABP promoter is maintained when the SREBP-binding site is deleted. CAT activity normalized to β-galactosidase activity. Mean ± S.E.M., $n = 3$.

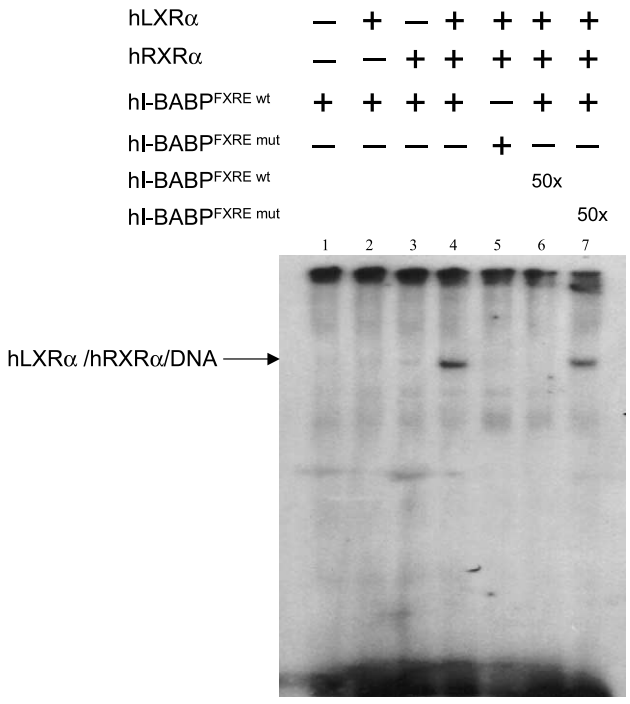

Fig. 3. LXR/RXR heterodimer specifically binds to the FXR-binding site found in the proximal promoter of the I-BABP gene. Electrophoretic mobility shift assay was performed with in vitro translated LXRα and RXRα and radiolabeled wild type hI-BABP$^{FXR\ wt}$ or mutated hI-BABP$^{FXR\ mut}$ as probe. Competition analysis was performed with a 50-fold excess of unlabeled hI-BABP$^{FXR\ wt}$ or hI-BABP$^{FXR\ mut}$.

(PLTP) promoter that the FXRE is a possible binding site for the LXR/RXR heterodimer [20]. To investigate whether LXR can also bind to the I-BABP FXRE$^{-160/-148}$ sequence, electrophoretic mobility shift assays were next carried out (Fig. 3). In vitro translated LXRα and RXRα proteins and radiolabeled oligonucleotides corresponding to the wild type FXRE (hI-BABP$^{FXR\ wt}$) or mutated FXRE (hI-BABP$^{FXR\ mut}$) were used. Only the LXRα/RXRα heterodimer binds to hI-BABP$^{FXR\ wt}$ (lane 4). Introduction of mutations in the IR1 sequence (hI-BABP$^{FXR\ mut}$) fully abolished the binding (lane 5). Competition assay using unlabeled oligonucleotides in excess demonstrated the specificity of the LXR/RXR binding to hI-BABP$^{FXR\ wt}$ since the formation of the complex was suppressed in the presence of a 50-fold excess of wild type hI-BABP$^{FXR\ wt}$ (lane 6), whereas it is conserved in the presence of hI-BABP$^{FXR\ mut}$ (lane 7). All together these data demonstrate that the FXRE can function as an LXRE in the promoter of the human I-BABP gene.

The existence of different binding sites for both LXR/RXR and SREBP in the human I-BABP promoter suggests that these two families of transcription factors can cooperate in the regulation of I-BABP gene expression. Co-transfections of expression vectors encoding LXRα, RXRα and/or active form SREBP1c in the presence of the wild type I-BABP/CAT promoter/reporter system were undertaken to explore this hypothesis. As shown in Fig. 4A, the wild type sequence of the I-BABP promoter (I-BABP 1.2^{wt}) was responsive to LXRα/RXRα and SREBP1c alone. The combination of these two transcription factors led to a 25-fold increase in I-BABP 1.2^{wt} promoter activity. Deletion of the SRE sequence (I-BABP 1.2$^{del\ SRE}$) fully abolished the SREBP1c-mediated transactivation of the reporter gene and reduced the magnitude of

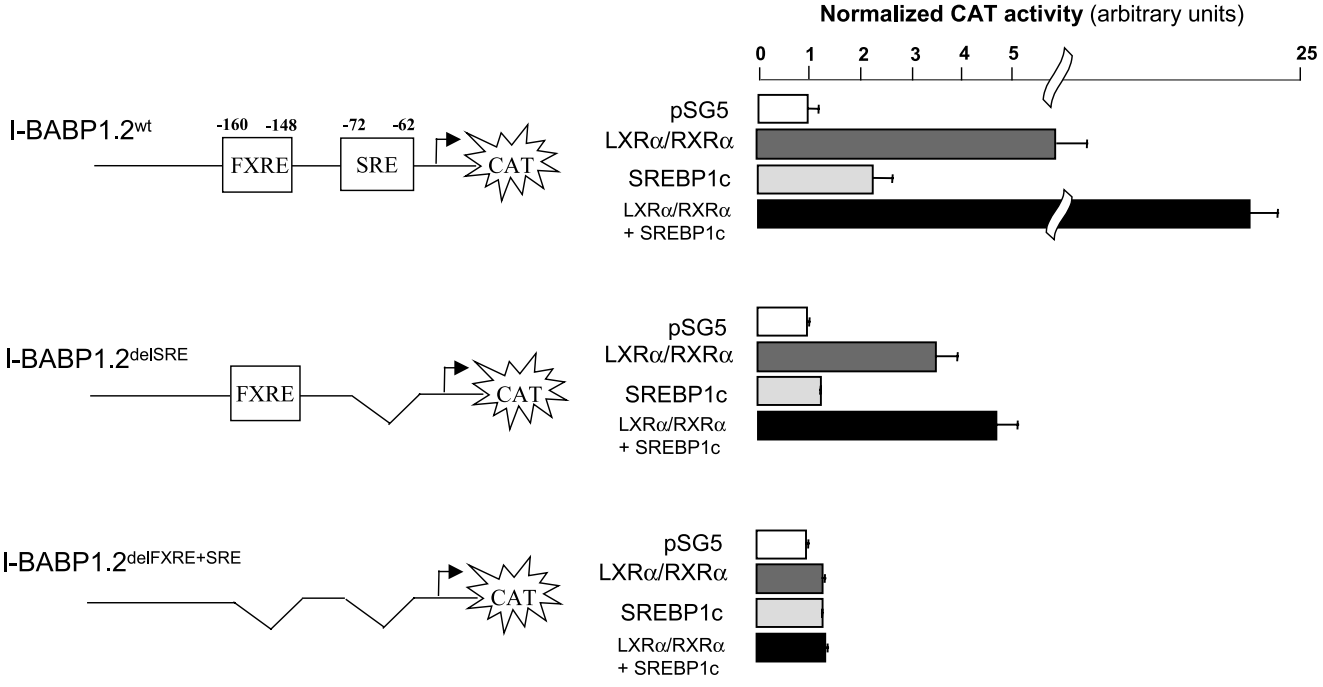

Fig. 4. LXR/RXR activates I-BABP promoter through SRE and FXRE. Caco-2 cells were transiently transfected with either the I-BABP1.2^{wt}, I-BABP1.2$^{del\ FXRE}$ or I-BABP1.2$^{del\ FXRE+SRE}$ promoter–reporter gene constructs, together with expression vectors for LXRα, RXRα and/or expression vector for SREBP1c. The cells were transfected in 10% FCS medium. Twelve hours after the transfection, the cells were cultured for an additional 24 h in 10% FCS medium. CAT activity normalized to β-galactosidase activity. Mean ± S.E.M., $n = 3$.

LXRα/RXRα activation. The double deletion of FXRE and SRE sequences (I-BABP $1.2^{\text{del FXRE+SRE}}$) suppressed all promoter response (Fig. 4B). Collectively, these data are consistent with a synergistic regulation of the I-BABP promoter by LXR/RXR and SREBP1c.

In conclusion, the I-BABP gene can be directly as well as indirectly up-regulated by LXR. The coexistence of these two LXR regulatory pathways has already been found for the FAS gene which encodes a rate-limiting enzyme in de novo lipogenesis [15]. Nevertheless, in contrast to FAS, the direct effect of LXR on I-BABP occurs by the binding of the LXR/RXR heterodimer to an IR1 sequence previously identified as an FXR-binding site in the promoter of the human I-BABP gene [9]. It is noteworthy that similar data have recently been reported for the PLTP [20] suggesting that the FXRE sequence can function as an LXR-binding site in different genes. The physiological significance of this finding remains to be determined. It might constitute a redundant system contributing to the maintenance of I-BABP gene sensitivity to CS changes via the activation of LXR by oxysterols when FXR is lacking (e.g. in FXR null mice) or a cross-talk between LXR and FXR in the regulation of I-BABP gene expression. Further investigations are required to answer to these questions. The present result confirms that I-BABP gene expression is tightly regulated by various transcription factors and nuclear hormone receptors (SREBP, FXR, LXR) considered to be CS sensors. Although the precise physiological function(s) of this soluble BA carrier in the ileocyte is not yet fully established, this new finding strongly suggests that I-BABP protein is important for the maintenance of CS homeostasis.

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