α-Crystallin Is a Target Gene of the Farnesoid X-activated Receptor in Human Livers*

Received for publication, March 23, 2005, and in revised form, July 11, 2005
Published, JBC Papers in Press, July 11, 2005, DOI 10.1074/jbc.M503182200

Florence Y. Lee‡, Heidi R. Kast-Woelbern‡‡, Jenny Chang‡, Guizhen Luo‡, Stacey A. Jones¶, Michael C. Fishbein¶, and Peter A. Edwards‡‡‡‡

From the ‡Department of Biological Chemistry and ‡Department of Pathology and Laboratory Medicine, David Geffen School of Medicine at UCLA and the ‡‡Molecular Biology Institute, UCLA, Los Angeles, California 90095 and ‡‡‡GlaxoSmithKline Research and Development, Research Triangle Park, North Carolina 27709

α-Crystallins comprise 35% of soluble proteins in the ocular lens and possess chaperone-like functions. Furthermore, the αA subunit (αA-crystallin) of a crystallin is thought to be “lens-specific” as only very low levels of expression were detected in a few non-lenticular tissues. Here we report that human αA-crystallin is expressed in human livers and is regulated by farnesoid X-activated receptor (FXR) in response to FXR agonists. αA-Crystallin was identified in a microarray screen as one of the most highly induced genes after treatment of HepG2 cells with the synthetic FXR ligand GW4064. Northern blot and quantitative real-time PCR analyses confirmed that αA-crystallin expression was induced in HepG2-derived cell lines and human primary hepatocytes and hepatic stellate cells in response to either natural or synthetic FXR ligands. Transient transfection studies and electrophoretic mobility shift assays revealed a functional FXR response element located in intron 1 of the human αA-crystallin gene. Importantly, immunohistochemical staining of human liver sections showed increased αA-crystallin expression in cholangiocytes and hepatocytes. As a member of the small heat shock protein family possessing chaperone-like activity, αA-crystallin may be involved in protection of hepatocytes from the toxic effects of high concentrations of bile acids, as would occur in disease states such as cholestasis.

Although the in vivo function and mechanism of action of α-crystallins are not well understood, in vitro studies suggest that they possess chaperone-like properties (9, 10). The prevalent hypothesis is that α-crystallins function as molecular chaperones to sequester unfolded proteins in the lens to maintain the refractive power essential for normal lens function (2). Besides its chaperone activity, α-crystallins have also been shown to possess autokinase activity (11, 12), but the significance of this activity is unknown. In vitro studies have also demonstrated that α-crystallins associate with cytoskeletal elements, consistent with a possible structural role for this protein in the lens (13–15). Mutations in both αA- and αB-crystallin have been linked to cataractogenesis in humans (16–19). Consistent with this finding, αA-crystallin knock-out mice developed mild cataracts at as early as 7-weeks of age, which further progressed into severe lens opacification as a result of formation of inclusion bodies composed mainly of αB-crystallin (20). However, αB-crystallin knock-out mice surprisingly did not develop cataracts (21).

The farnesoid X-activated receptor (FXR, also NR1H4)1 is a member of the nuclear hormone receptor superfamily (22, 23). Nuclear hormone receptors are ligand-activated transcription factors that regulate transcription of target genes to control key physiological processes including reproduction, development, and metabolism. FXR expression is specific to liver, kidney, adrenal gland, and intestine, with low levels of expression in fat and the heart (24, 25). Recent studies identified four FXR isoforms that are produced from the single human or murine gene as a result of use of alternative promoters and alternative RNA splicing (25, 26). The four isoforms (FXRα-1–α4) are expressed in different tissues in the above-mentioned tissues. More importantly, they have been shown to regulate transcription of some target genes in an isoform-specific manner (25, 27).

The discovery of bile acids as the natural physiological ligands for FXR (28–30) and the subsequent generation of the FXR-null mice (31) led to the identification of a number of FXR target genes. Many of these genes are involved in bile acid or cholesterol homeostasis, lipoprotein, and triglyceride metabo-

* This work is supported by National Institutes of Health Grants HL30568 and HL68445 (to P. A. E.), a grant from the Laubisch Fund (to P. A. E.), and United States Public Health Service National Research Service Award GM07185 (to F. Y. L). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Present address: Ligand Pharmaceuticals, 10275 Science Center Drive, San Diego, CA 92121.

‡‡ To whom correspondence should be addressed: Dept. of Biological Chemistry, David Geffen School of Medicine at UCLA, 10833 Le Conte Ave, 33-257 CHS, Los Angeles, CA 90095. Tel.: 310-206-3717; Fax: 310-794-7345; E-mail: pedwards@mednet.ucla.edu.

1 The abbreviations used are: FXR, farnesoid X-activated receptor; FXRE, FXR response element; CRYAA, α-A-crystallin; ABC, ATP binding cassette; BSEP, ABCB11, bile salt export pump; MRP2, ABCC2, multidrug resistance-related protein 2; MDR3, ABCB4, multidrug resistance protein 3; CYP7A1, cholesterol 7α-hydroxylase; SHP, small heterodimer partner; RXR, retinoid X receptor; RXRα, retinoid X receptor α; h, human; r, rat; mpC-II, apolipoprotein C-II; CDCA, chenodeoxycholic acid; IR-1, inverted repeat with 1-bp spacer; EMA, electrophoretic mobility shift assay; GW4064, 3-(2,6-dichlorophenyl)-4-3-carboxy-2-chloro-stilben-4-yl-oxy-methyl-5-isopropyl-isoxazole; PLTP, phospholipid transfer protein; HSP, heat shock protein; Neo, neomycin; HSC, hepatic stellate cells.

31792
Bile acid homeostasis through coordinated regulation of hepatic bile acid transporters and catabolism of cholesterol into bile acids (33–35). In response to elevated levels of bile acids in the liver, FXR is activated and induces a number of ATP binding cassette (ABC) transporters, including the bile salt export pump (BSEP, ABCB11), multidrug resistant-associated protein 2 (MRP2, ABCB2), and the human multidrug resistant protein 3 (MDR3, murine mdr2, ABCB4) (36-38). BSEP and MRP2 efflux bile salts from hepatocytes into the bile duct, whereas MDR3/mdr2 flips phospholipids across the bile canicular membrane before their dissociation into the bile, thus aiding in the desorption and solubilization of bile salts and cholesterol in the bile (39–41). In addition, activation of FXR leads to the repression of cholesterol 7α-hydroxylase (CYP7A1), the rate-limiting enzyme in the bile acid synthesis pathway, via complex feedback inhibition mechanisms involving the small heterodimer partner (SHP) protein (42) and/or the human fibroblast growth factor 19 (43). Moreover, bile acids also repress their own biosynthesis through FXR-independent pathways (44).

Under normal conditions the regulatory events mentioned above are sufficient to protect the liver from accumulation of bile acids. Perturbation of this intricate regulatory cascade either as a result of certain disease states or genetic mutations leads to accumulation of bile acids in the liver and subsequent liver damage, a condition known as cholestasis (45, 46). Recent studies have demonstrated that FXR activation may protect the liver from cholestasis-induced damage (47). Here we provide evidence that human αA-crystallin is expressed in the human livers and is a direct target gene of FXR. We propose that the induction of αA-crystallin expression in response to bile acid-activated FXR contributes to cellular defense against bile acid-induced hepatotoxicity.

**EXPERIMENTAL PROCEDURES**

**Materials**—The synthetic FXR-specific ligand GW4064 was a gift from Dr. Patrick Maloney (GlaxoSmithKline) (48). Mammalian expression vectors for rat FXR (pCMX-FXR) and human RXRα (pCMX-hRXRa) were gifts from Dr. Ron Evans (Salk Institute, La Jolla, CA), and that for human FXRα was a gift from Dr. Bryan Goodwin (GlaxoSmithKline). Primary cultures of human hepatocytes and hepatic stellate cells were obtained from ADMET Technologies (Durham, NC). Dexamethasone was from the Sigma. Insulin transfection reagent (Roche-Boehringer) and all other tissue culture reagents were from Invitrogen. Mammalian expression vectors for individual human FXR isoforms (pcDNA3.1-hFXRα, -α2, -α3, and -α4) were generated using standard procedures. Polyornucleotide antibodies to recombinant bovine αA-crystallin, the amino-terminal peptide of αA-crystallin, and the carboxyl-terminal peptide of αA-crystallin were generous gifts from Dr. Joseph Horwitz (UCLA Jules Stein Eye Institute) (20, 49). The sources of other reagents and all other tissue culture reagents were from Invitrogen. Mammalian expression vectors for individual human FXR isoforms (pcDNA3.1-hFXRα, -α2, -α3, and -α4) were generated using standard procedures. Polyornucleotide antibodies to recombinant bovine αA-crystallin, the amino-terminal peptide of αA-crystallin, and the carboxyl-terminal peptide of αA-crystallin were generous gifts from Dr. Joseph Horwitz (UCLA Jules Stein Eye Institute) (20, 49). The sources of other reagents and all other tissue culture reagents were from Invitrogen.

**Cell Culture and Stable Cell Lines**—The generation and maintenance of stably infected HepG2-Neo and HepG2-FXR cells have been described (50). CV-1 cells were cultured in modified Eagle's medium supplemented with 10% fetal bovine serum at 37 °C and 5% CO2. Primary cultures were maintained with 155 mM fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin G, and 100 μg/ml streptomycin. After overnight culture, cells were either collected for RNA isolation or treated with 300 nM GW4064 or vehicle (0.1% Me2SO) for the indicated times. After treatment, total RNA was isolated using Trizol reagent, and real-time PCR was performed as described above.

**RNA Isolation and Northern Blot Assay**—Unless otherwise indicated, HepG2 cells were cultured in medium containing super-striped fetal bovine serum for 24 h before the addition of vehicle (Me2SO) or ligand. After an additional 24–48 h, total RNA was isolated. Northern blot assays were carried out as described previously (37). All membranes were hybridized to a control probe (18 S or 36B4) to correct for small differences in the amount of RNA loaded in each lane. The RNA levels were quantitated using a PhosphoImager (ImageQuant software; Amersham Biosciences).

**Western Blot Analysis**—HepG2-FXR cells were either untransfected or transfected with expression plasmids encoding FXR and RXR and treated with Me2SO or GW4064 for 24 h before protein isolation. Cells were lysed in buffer (50 mM Tris-Cl, pH 7.4, 25 mM KCl, 5 mM MgCl2, 1 mM EDTA) containing 1 mM phenylmethylsulfonil fluoride and Protease Inhibitor Cocktail (Roche-Boehringer) by 3 freeze-thaw cycles. Protein concentrations of the lysates were determined with the Bradford-based assay (Bio-Rad) using bovine serum albumin as the standard. Fifty micrograms of total protein were loaded onto a 15% SDS-PAGE mini-gel followed by electrophoresis and transfer to a polyvinylidine difluoride membrane (Millipore, MA). Anti-αA-crystallin polyclonal antibodies were used at 1:50 dilution followed with horseradish peroxidase-conjugated anti-rabbit IgG (Amer sham Biosciences) at 1:10,000 dilution. Signal was detected using ECL Plus reagent (Amer sham Biosciences).

**Reporter Genes**—A 557-bp fragment from the human αA-crystallin proximal promoter (−523 to +33 relative to the transcription start site) was amplified from human genomic DNA using the primers 5′-ggtaggctcgcgaattctgggtgc-3′ (−523 forward) and 5′-gctagcaggtcgggaatttcg-3′ (−523 reverse) and cloned into the pGL3 basic vector (Promega, Madison, WI) to generate the pGL3-500 reporter plasmid. The first intron of the αA-crystallin gene (−275 to +1461) was also amplified from human genomic DNA using the primers 5′-ggtccctgggtcgtctgggttc-3′ (−275 forward) and 5′-gctagctcgcgaattctgggtgc-3′ (−1461 reverse) and cloned into pEYFP in the pZK7-Luc vector, generating the pZK7-αA-crystallin reporter construct.

**Transient Transfections and Reporter Gene Assays**—Transient transfections of CV-1 cells were performed in triplicate in 48-well plates using the MBS mammalian transfection kit (Stratagene) with minor modifications. The cells were transfected with 100 ng of the reporter construct of interest and 50 ng of the pCMV-β-galactosidase plasmid together with plasmids encoding either rat (r) or human (h) FXR (50 ng of either pCMX-FXRα or pDNA-hFXRα, pcDNA-hFXRα, or pCMX-hFXRα, or pSG5-hFXRα) and/or 5 ng of pCMX-hRXRa, as indicated in the specific figures. After transfection, cells were incubated in media supplemented with 10% “superstriped” fetal bovine serum (HyClone, Logan, UT) containing either vehicle or the synthetic FXR-specific ligand GW4064. After 48 h, cells were lysed, and the luciferase activities were measured with the Promega luciferase assay system and normalized to β-galactosidase activity (50).

**Protein Mobility Shift Assays (EMSAs)**—EMSAs were performed essentially as described (51, 37). Human RXRs and human FXRα were synthesized from CMX-hRXRa and pSG5-hFXRα expression vectors, respectively, using the TNT T7-Coupled Reticulocyte System (Promega). Unprogrammed lysate was prepared using the empty pCMX vector. Binding reactions contained 10 mM HepES, pH 7.6, 40 mM NaCl, 2.5 mM MgCl2, 0.5 mM Nonidet P-40, 10% glycerol, 0.5 mM Dithiothreitol, 2 μg of poly(dI-dC), 25 μg of bovine serum albumin, and
1–4 μl of each receptor protein. Control incubation received unprogrammed lysate alone. Oligonucleotide probes were end-labeled with \(^{32}\)P using polyadenylate kinase (New England Biolabs). Specific activities of end-labeled oligonucleotide probes were determined by scintillation counting. Volumes of \(^{32}\)P-labeled oligonucleotide probes equivalent to 25,000 cpm were used in each reaction. Reactions were incubated on ice for 15 min before the addition of radioactively labeled oligonucleotide probes and where indicated in the presence of antibodies against FXRs (Santa Cruz, CA). In competition assays, competitor oligonucleotides were added at 50-, 100-, and 250-fold molar excess. Samples were held on ice for another 5 min, and the protein-DNA complexes were resolved on a pre-electrophoresed 5% polyacrylamide gel in 1× TBE (45 mM Tris borate, 1 mM EDTA) at 4 °C. Gels were dried and autoradiographed at −70 °C for 8 h. The double-stranded oligonucleotides were annealed from the following single-stranded oligonucleotide and an oligonucleotide corresponding to the complementary sequence; FXRE1 WT, 5′-gacagaaggaggctgcatctgagacaggca-3′; FXRE WT, 5′-catgacaccaagggcagtgacctcatc-3′; SHP2 WT, 5′-cattccacag-3′; IR-1 elements are indicated in boldface, and mutations are capitalized.

Immunohistochemical Studies—Fresh-frozen, cryoircthic, and cholestatic human liver sections were obtained from the Tissue Procurement Core Laboratory of the Department of Pathology and Laboratory Medicine at UCLA. The protocol was approved by the Institutional Review Board of UCLA. Hematoxylin and Esin staining were performed by the core laboratory using standard protocols. Immunohistochemical studies using anti-αA-crystallin polyclonal antibodies against recombinant bovine protein, amino- or carboxyl-terminal peptide, were performed as follows; frozen sections were thawed at room temperature for 30 min, fixed in 100% methanol for 10 min at −20 °C, and then air-dried at room temperature for 20 min. Sections were then blocked with hydrogen peroxide (0.3%) to quench endogenous peroxidase activities. After rinsing with PBS, the sections were in turn blocked with 10% normal goat serum (20 min), avidin (15 min), and biotin (15 min) (Avidin/Biotin Blocking Kit, Vector laboratories, CA) at room temperature. Sections were then incubated with one of the anti-αA-crystallin antibodies for 1.5 h at room temperature in a humidity chamber, washed thoroughly with phosphate-buffered saline for 20 min. Secondary antibodies were added for 30 min, and the sections were washed with phosphate-buffered saline. The sections were developed using the VECTORSTAIN ABC peroxidase system and either the 3,3-diaminobenzidine or the 3-amino-9-ethylcarbazole substrates from Vector Laboratories, counterstained with hematoxylin, and dehydrated, and the slides were mounted in xylene-based mounting medium.

RESULTS

αA-Crystallin Expression Is Induced in HepG2 Cells and Primary Human Hepatocytes in Response to FXR Ligands—In an attempt to identify target genes that are regulated by FXR, a microarray screen was carried out with RNAs isolated from HepG2 cells that stably express the neomycin resistance gene (HepG2-Neo) or the neomycin resistance gene and rat FXRα2 (HepG2-FXR) that had been treated for 24 h with vehicle (Me2SO) or CDCA (100 μM). Total RNA was isolated, separated on a 1% agarose/formaldehyde gel, transferred to a nylon membrane, and sequentially hybridized to radioactively labeled cDNA probes for CRYAA, SHP, and 36B4, as described under “Experimental Procedures.” The relative CRYAA and SHP mRNA levels are indicated. B, HepG2-FXR cells were treated for 24 h with vehicle (Me2SO) or 1 μM GW4064. Total RNA was isolated, and Northern blot analysis was performed as described using cDNA probes for CRYAA, HSP27, HSP70, and 36B4. The relative CRYAA mRNA levels are indicated. C, primary human hepatocytes were treated for 48 h with Me2SO or 10 μM GW4064. Northern blot analysis was performed as described using cDNA probes for CRYAA, SHP, and 18 S ribosomal RNA. D, Western blot analysis of total protein lysates (50 μg) from HepG2-Neo and HepG2-FXR cells either untransfected or co-transfected with expression plasmids expressing both FXR and RXR and subsequently treated for 24 h with vehicle (Me2SO) or 1 μM GW4064. The membrane was probed with polyclonal anti-bovine αA-crystallin antibodies. Recombinant purified αA-crystallin (50 μg) was loaded as a positive control (lane 5).

Fig. 1. Induction of αA-crystallin mRNA and protein by both natural and synthetic ligands of FXR in HepG2 cells and primary human hepatocytes. A, HepG2-Neo and HepG2-FXR cells were treated with vehicle (Me2SO) or CDCA (100 or 250 μM). Total RNA was isolated, separated on a 1% agarose/formaldehyde gel, transferred to a nylon membrane, and sequentially hybridized to radioactively labeled cDNA probes for CRYAA, SHP, and 36B4, as described under “Experimental Procedures.” The relative CRYAA and SHP mRNA levels are indicated. B, HepG2-FXR cells were treated for 24 h with vehicle (Me2SO) or 1 μM GW4064. Total RNA was isolated, and Northern blot analysis was performed as described using cDNA probes for CRYAA, HSP27, HSP70, and 36B4. The relative CRYAA mRNA levels are indicated. C, primary human hepatocytes were treated for 48 h with Me2SO or 10 μM GW4064. Northern blot analysis was performed as described using cDNA probes for CRYAA, SHP, and 18 S ribosomal RNA. D, Western blot analysis of total protein lysates (50 μg) from HepG2-Neo and HepG2-FXR cells either untransfected or co-transfected with expression plasmids expressing both FXR and RXR and subsequently treated for 24 h with vehicle (Me2SO) or 1 μM GW4064. The membrane was probed with polyclonal anti-bovine αA-crystallin antibodies. Recombinant purified αA-crystallin (50 μg) was loaded as a positive control (lane 5).

Downloaded from http://www.jbc.org/ by guest on July 18, 2018

To rule out the possibility that the induction of CRYAA mRNA was due to a general effect of FXR ligands on the expression of heat shock proteins, HepG2-FXR cells were treated with either vehicle (Me2SO) or the synthetic FXR-specific ligand GW4064 (1 μM) for 24 h before RNA isolation. The data of Fig. 1B illustrate the specificity of CRYAA mRNA induction in response to FXR ligand treatment. In contrast to CRYAA, neither heat shock protein 27 (HSP27) nor heat shock protein 70 (HSP70) mRNA levels were induced after activation of FXR (Fig. 1B). These data suggested that FXR ligand treatment specifically increased CRYAA mRNA levels but not that of other heat shock proteins.

The increase in CRYAA mRNA expression in response to FXR ligand treatment in HepG2 cells resulted in a concomitant increase in CRYAA protein level. Western blot analysis using polyclonal antibodies toward recombinant bovine αA-crystallin demonstrated that αA-crystallin protein levels also increased...
in response to an FXR-specific ligand (Fig. 1D, compare lane 2 versus lane 1). Furthermore, this induction is FXR-dependent since transient transfection of plasmids encoding FXR and RXR further augmented the level of αA-crystallin protein and the subsequent response to FXR ligand treatment (Fig. 1D, compare lanes 3 and 4 versus lanes 1 and 2). We also noted that the apparent molecular weight of αA-crystallin in HepG2 cells is higher than that of bacterially expressed purified recombinant bovine αA-crystallin (Fig. 1D, lane 1–4 versus lane 5). To rule out the possibility that the bands in lanes 1–4 of Fig. 1D correspond to αB-crystallin, a duplicate blot was hybridized with anti-αB-crystallin antibodies. No signal was detected, although the antibody was shown to react strongly with recombinant αB-crystallin (data not shown). Therefore, we hypothesize that the difference in molecular weight may be due to previously uncharacterized post-translational modifications of αA-crystallin in HepG2 cells.

To investigate whether CRYYA mRNA was expressed and regulated in rodent livers, we isolated RNAs from livers of wild-type mice gavaged with either vehicle or GW4064 (30 mg/kg twice a day for 4 days). Northern blot analysis indicated that CRYYA mRNA was present at very low levels that were unchanged after the GW4064 treatment (data not shown). In contrast, the hepatic levels of SHP mRNA were highly induced (data not shown). Taken together, these findings suggested that regulation of CRYYA mRNA by FXR is human-specific since it was only observed in human-derived hepatocytes but not in murine livers.

The First Intron of the αA-Crystallin Gene Confers FXR-dependent Transcription Activation in Transient Transfection Assay—With one reported exception (53), FXR activation of known target genes requires that FXR binds to an FXR response element (FXRE) as an FXR/RXR heterodimer (24, 54). Most known FXREs consist of two half-sites with the consensus sequence AGGTCA arranged as an inverted repeat separated by one nucleotide (IR-1) (22). Nevertheless, recent studies have identified a few FXR target genes that possess FXREs other than the classic IR-1 motif (37, 27, 55). Analysis of the published nucleotide sequence of the proximal promoter and first intron of the CRYYA gene revealed a number of putative IR-1 motifs that are similar to previously identified FXR-response elements. To identify which if any of these putative IR-1 sequences are functional, we initially generated two luciferase reporter gene constructs. A 557-bp region of the proximal promoter that includes the transcription start site was cloned into the pGL3 luciferase reporter vector (pGL3–500). A second reporter construct was generated by cloning the first intron of the CRYYA gene upstream of the minimal pTK promoter-luciferase reporter vector (pTK-CryAAint1) (Fig. 2A).

CV-1 cells were transiently transfected with one of these reporter gene constructs in the presence or absence of expression plasmids encoding human RXRα and rat FXR (corresponding to the FXR2 isoform) (24, 25). Transfected cells were subsequently treated with either vehicle (Me2SO) or the synthetic FXR ligand GW4064 for 48 h. Both the pGL3–500 construct and the empty pGL3 vector exhibited low and unregulated transcriptional activities (Fig. 2A). In contrast, the pTK-CryAAint1 reporter gene exhibited specific and potent transcriptional activation after treatment with GW4064 and co-expression of FXR and RXR (Fig. 2B). Notably, the luciferase activity and fold induction were far greater than that seen with the positive control reporter gene driven by two copies of a well characterized IR-1 element (pTK-2X IR-1) from the hepatic control region of the human apolipoprotein E/C-I/C-IV/C-II gene cluster (Fig. 2B) (52). These results suggested that a potent and functional FXRE is present in the first intron of the CRYYA gene.

Identification of Two Putative FXREs in the First Intron of the CRYYA Gene—The transient transfection assays described in Fig. 2B suggest that the first intron of the CRYYA gene contains one or more functional FXRE. Analysis of the genomic sequence of this region identified two putative FXREs (Fig. 3A, FXRE1 and FXRE2).

To determine whether FXR/RXR heterodimers bound to these putative FXREs, we performed EMSAs using in vitro transcribed and translated rat FXR and human RXRα proteins and radioactively labeled oligonucleotide probes corresponding to either FXRE1 or FXRE2. As is evident in Fig. 3B, FXR/RXR heterodimers bound to both FXREs in vitro (lanes 4 and 10). However, FXRE2 led to the formation of a more robust shifted complex than FXRE1, suggesting that FXR/RXR heterodimers may bind to FXRE2 with higher affinity. An antibody specific to human RXRα supershifted the complexes (lanes 5 and 11), whereas an antibody to YY1 (Yin Yang 1) has no effect (lanes 6 and 12). The well characterized IR-1 from the phospholipid transfer protein (PLTP) served as a positive control (Fig. 3B, lanes 13 and 14) (50, 56).
FXR and αA-Crystallin

Fig. 3. The RXRα/FXR heterodimer binds to two putative FXREs in the first intron of the αA-crystallin gene. A, location and sequence of the two IR-1 elements (putative FXREs) in the first intron of the αA-crystallin gene. The asterisk marks the translation start site. The IR-1 elements in FXRE1 and FXRE2 are in boldface, whereas the arrows indicate the orientation of each half-site. B, electrophoretic mobility shift assays were performed as described under "Experimental Procedures." 32P-labeled oligonucleotide probes containing sequences of FXRE1, FXRE2, or the previously characterized IR-1 element from the promoter of the human phospholipid transfer protein (hPLTP) gene (98, 56) were incubated with in vitro transcribed and translated hFXRα1 and/or hRXRα, as indicated. Antibodies against RXRα were also included in the reaction in lanes 5, 11, and 14. The FXR/RXRα/32P-labeled oligonucleotide probe complex and the supershifted anti-RXRα/FXRα/FXRα/IR-1 complex are indicated.

Competition EMSAs were also carried out using non-radioactively labeled oligonucleotides corresponding to FXRE2 from the CRYAA gene, a mutated FXRE2 containing four transversions, or the IR-1 from the PLTP promoter (Fig. 4). FXRE2 was chosen as the competitor because the data in Fig. 3 suggest that it binds FXRα/RXRα heterodimer with higher affinity than FXRE1. As seen in Fig. 4, increasing concentrations of oligonucleotides corresponding to either unlabeled FXRE2 or IR-1 from the PLTP promoter reduced the formation of the FXR/RXR heterodimer with the radioactive probe (lanes 5–7 and lanes 11–13). In contrast, no competition was observed when the mutant FXRE2 was used as a competitor (Fig. 4, lanes 8–10). These data demonstrated that the interaction between FXRα/RXRα and FXRE2 is specific.

FXRE2 Is a Functional Enhancer Element in the Natural Context of the CRYAA Gene—To determine whether the FXREs present in the first intron of the human CRYAA gene are functional in their natural context, a luciferase reporter gene construct was generated that under the control of a genomic fragment containing the proximal promoter region plus exon 1, intron 1, and part of exon 2 (1320 to +1581) of the CRYAA gene (Fig. 5A). To ensure the production of a functional luciferase protein, the start codon located in exon 1 of the CRYAA gene was cloned in-frame with the ATG of the luciferase gene in the vector (pGL3-pE1E2 in Fig. 5A). Translation of the resulting transcript should produce a fusion protein containing 94 amino acids of the amino-terminal CRYAA sequence (derived from exons 1 and 2) in-frame with luciferase.

CV-1 cells were transiently transfected with the pGL3-pE1E2 promoter-reporter gene construct in the presence or absence of plasmids expressing human RXRα and human FXRα1 (Fig. 5A). Transfected cells were subsequently treated with either vehicle (Me2SO) or the synthetic FXR ligand (GW4064) for 48 h. The data of Fig. 5A demonstrated that pGL3-pE1E2 is highly induced by GW4064 after co-transfection of FXR-encoding expression plasmids. We conclude that the putative FXRE(s) in intron 1 retained its enhancer activity in the natural context of the gene. Induction of luciferase activity was unaffected by a mutation in FXRE1 (compare Fig. 5B to 5A). However, mutation of FXRE2 completely abolished induction of reporter gene activity (Fig. 5C), and luciferase levels declined to those of the empty vector (Fig. 5D). Taken together, these data clearly demonstrate that FXRE2 in intron 1 of the human CRYAA gene functions as a bona fide FXR response element.

Because we have previously demonstrated that certain FXR target genes are equally activated by each of the four FXR isoforms, whereas other genes are responsive to specific FXR isoforms (25, 27, 57), we performed transient transfection using the wild-type pGL3-pE1E2 reporter gene in the presence or absence of co-transfected plasmids encoding hRXRα, hFXRα2, or individual human FXR isoforms. Each human FXR isoform was expressed from the same vector (pcDNA3.1) to ensure similar expression and allow for a direct comparison. The data of Fig. 6A show that under these conditions all four human FXR isoforms are able to induce αA-crystallin reporter gene transcription activity. However, hFXRα2 and hFXRα4, the isoforms lacking the MYTG motif adjacent to the DNA binding domain (25), induce a far more robust transcriptional activation than the two isoforms that contain this motif (Fig. 6A).

Competitive protein binding assays were performed under "Experimental Procedures." Unlabeled oligonucleotide probes containing sequences of FXRE2, mutated FXRE2, or IR-1 from the hPLTP promoter (a previously characterized high affinity binding site for FXR/RXR) were added at 50-, 100-, and 250-fold molar excess to binding reaction mixtures containing 32P-labeled FXRE2 oligonucleotide probes and in vitro translated and transcribed hFXRα1 and/or hRXRα as indicated.

Immunohistochemical Staining of Human Liver Sections Reveals αA-Crystallin Expression in Hepatocytes and Cholangiocytes—Because we have shown that CRYAA is an FXR target gene and its expression in human hepatocytes is induced in response to FXR ligands, we performed immunohistochemical staining of human liver sections using antibodies raised against a degenerate peptide corresponding to the amino ter-
FXR and αA-Crystallin

minus of bovine, rodent, and human αA-crystallin (49). Hema-toxylin and eosin staining was performed on human liver samples that were either normal (Fig. 7A), cirrhotic (Fig. 7B), or cholestatic (Fig. 7C). As evident in Fig. 7D, specific staining of αA-crystallin is seen in cholangiocytes, the epithelial cells that line the bile ducts, of the normal human liver. In sections of the human cirrhotic liver, specific staining of αA-crystallin is seen in cells located at the periphery of regenerative modules encircled by fibrotic tissues (Fig. 7, E and F, arrows). There is also strong staining of cells located within the fibrotic tissues (Fig. 7, E and F, arrowheads). These latter cells likely correspond to differentiating liver progenitor cells that form reactive bile ductules in response to hepatic damage (58) or to myofibroblasts derived from activated hepatic stellate cells (HSCs) (59, 60). In addition, in cholestatic liver sections taken from a patient suffering from primary biliary atresia, hyperprolifera-tion of bile ducts is evident, and αA-crystallin expression is seen in cholangiocytes that line these bile ducts (Fig. 7G, arrow). Strikingly, the staining is specifically found on the lumen-facing side of these cholangiocytes. Bile droplets can be seen within some of these latter bile ducts (Fig. 7H, arrow). Although the antibody used in these stainings has been shown previously to recognize both αA- and αB-crystallin (49), as expected, immunohistochemical stainings of these same liver sections using antibodies specific to αB-crystallin failed to de-tect any specific signal (data not shown). Taken together, these data suggest that αA-crystallin is specifically expressed in hepatocytes, cholangiocytes, and possibly activated HSCs in human livers by FXR under pathological conditions that are associated with increased hepatic levels of bile acids.

Real-time Quantitative PCR Confirms Expression and Regulation of αA-Crystallin by an FXR Agonist in Primary Human Hepatic Cell Cultures—Because immunohistochemical staining of human liver sections revealed the presence of αA-crystallin in multiple hepatic cell types, we carried out real-time quantitative PCR analysis using RNAs isolated from primary cultures of human hepatocytes, cholangiocytes, and HSCs. The data confirm that αA-crystallin is both expressed in primary human hepatocytes (Fig. 8B) and HSCs (Fig. 8A) and is induced after treatment with GW4064. Moreover, the results in Fig. 8B demonstrated that αB-crystallin is not responsive to FXR ligand treatment. Repression of CYP7A1 mRNA in the primary human hepatocytes was used as a positive control for FXR-ligand responsiveness. To date, we have been unable to demonstrate αA-crystallin expression in primary human cholangiocyte cultures, although FXR is present both in these cells (data not shown) (61) and in

Fig. 5. FXRE2 is the bona fide FXR response element required for FXR-dependent transactivation of the αA-crystallin gene in its natural context. CV-1 cells were transiently transfected in triplicate with the indicated pGL3 reporter gene constructs under the control of genomic DNA sequence containing the proximal promoter, exon 1, intron 1, and part of exon 2 of the αA-crystallin gene with either no mutation (A), a mutated FXRE1 (B), or a mutated FXRE2 (C). The empty pGL3 plasmid serves as the negative control (D). The presence and absence of expression plasmids for human FXRα and human FXRα1 (expressed from a pSG5 vector) are indicated. Cells were treated with either vehicle (MeSO (DMSO)) or 1 μM GW4064 (GW) for 48 h post-transfection. Relative light units (RLU) were shown in each panel after normalization to β-galactosidase activities to control for differences in transfection efficiency. E1 and E2 indicate exon 1 and exon 2 of the αA-crystallin gene, respectively. The initiating methionine in exon 1 is indicated by an asterisk (*). The open circle (○) designates FXRE1, and the closed circle (●) designates FXRE2. The cross (×) over either the open or the closed circle signifies mutation in either FXRE1 or FXRE2, respectively. Data are given as the mean ± S.D.; p values were determined by unpaired two-tailed Student’s t test. *, p < 0.001; **, p < 0.002. The data are representative of three separate experiments.

Fig. 6. αA-crystallin promoter-reporter gene construct is preferentially activated by FXR isoforms lacking the MYTG motif. CV-1 cells were transiently transfected in triplicate with either the pGL3 reporter gene under the control of genomic DNA sequence containing the proximal promoter, exon 1, intron 1, and part of exon 2 of the αA-crystallin gene (A) or the empty pGL3 plasmid (B) and expression plasmids encoding RXRα and the indicated human FXR isoforms in pcDNA. The initiating methionine in exon 1 is indicated by an asterisk (*). Cells were treated with either vehicle (MeSO (DMSO)) or 1 μM GW4064 (GW) for 48 h post-transfection. Relative light units (RLU) were shown in each panel after normalization to β-galactosidase activities to control for differences in transfection efficiency. Data are given as mean ± S.D.; p values were determined by unpaired two-tailed Student t test; *, p < 0.05.
murine cholangiocytes (62). This discrepancy may result from de-differentiation and de-polarization of cholangiocytes in culture and/or difference in growth environment.

**DISCUSSION**

The present study identifies the human CRYAA gene as a direct target of FXR and shows that H9251 A-crystallin protein is expressed in various cell types of cirrhotic and cholestatic human livers. This finding is intriguing because H9251 A-crystallin was generally considered to be a "lens-specific" protein. The murine H9251 A-crystallin gene, in contrast to its human counterpart, is not regulated by FXR. Consistent with this observation, analysis of the murine H9251 A-crystallin genomic sequence reveals that the functional FXRE identified in the first intron of the human gene was not conserved in the murine gene. Although nuclear receptor response elements are usually located in the proximal promoters of target genes, there have been several examples in which the response elements are found in the distal enhancers (52) or in the intron of the target genes (63, 43). In addition, other differences have been noted between the human and rodent H9251 A-crystallin genes; for example, the murine gene encodes alternative transcripts as a result of alternative mRNA splicing of an exon located in the first intron of the rodent H9251 A-crystallin gene (64, 65). Other human-specific FXR target genes have also been identified; these include syndecan-1 (27) and fibrinogen (57). In addition, activation of the closely related nuclear receptor liver X receptor (LXR) is known to activate the murine but not the human genes encoding CYP7A1 (66, 67) and LXR itself (68). Detailed studies demonstrated that the liver X receptor response element in the promoter of the murine Cyp7a1 gene is not conserved in the human gene. This provides a mechanism by which rodents can eliminate excess dietary cholesterol through the activation of Cyp7a1 expression and the resulting enhanced conversion of cholesterol to bile acids. This renders rodents less susceptible to diet-induced hypercholesterolemia than humans, who lack this compensatory response (69–72).

H9251 -Crystallins are stress-related and cell-protective proteins. Their ability to bind partially denatured proteins to prevent...
FXR and α-A-Crystallin

Further denaturation or aggregation has been well established (1–3). α-Crystallins have also been shown to aid in the refolding of partially denatured proteins in vitro (73, 74). Notably, Hatters et al. (75) reported on the ability of α-crystallins to inhibit amyloid formation by lipid-poor apoC-II, one of the known target genes of FXR (52). In the absence of lipids, apoC-II was shown to assume a less stable conformation in vitro, contributing to a higher probability of self aggregation and fiber formation (75).

In vitro studies with α-A- and α-B-crystallin led to the suggestion that each protein has independent chaperone-like activity and functions (76–78). For example, α-B-crystallin is normally expressed at relatively high levels in the heart, and the lack of α-B-crystallin expression has been implicated in desmin-related myopathy (79–81). More recently, α-B-crystallin was shown to associate with the perinuclear Golgi in a human glioblastoma cell line, suggesting a possible role for α-B-crystallin in the Golgi reorganization during cell division (82).

The induction of α-A-crystallin expression in the liver by ligand-activated FXR, reported herein, likely implies a novel role for α-A-crystallin in bile acid homeostasis and/or protection of hepatocytes and cholangiocytes from excess bile acids. Immunohistochemical staining demonstrated that α-A-crystallin is expressed in cells that border the regenerative nodule of hepatocytes and the fibrotic portal tract in human cirrhotic liver (Fig. 7). These cells are thought to be derived from liver progenitor cells (also known as oval cells), which are able to differentiate into hepatocytes or cholangiocytes upon liver injury as part of the liver regeneration mechanism (58, 83). The fact that α-A-crystallin is induced in isolated primary hepatic stellate cell cultures also suggests a role in injury response, as stellate cells are involved in repair of damaged liver (59, 60). Taken together, these data suggest a new role for α-A-crystallin as a stress-induced hepatoprotective protein.

Excess hepatic bile acid levels are highly detrimental to normal liver function (84). A number of FXR-dependent pathways have been identified that ensure hepatocytes are protected from excess bile acids. For example, activated FXR down-regulates the rate-limiting enzyme in bile acid synthesis, CYP7A1, by mechanisms that include activation of SHP and/or fibroblast growth factor 19 (42, 43) and induces numerous transporter proteins in the liver, including BSEP, MRP2, and MDR2/3, to facilitate the transport of bile acids and phospholipids into the bile. Mutations in these latter transporters have been linked to various forms of cholestatic liver disease associated with increased hepatic levels of bile acids (85–87).

Although the precise mechanism mediating the cytotoxic effect of bile acids remains obscure, it has been generally attributed to their hydrophobicity which likely disrupts membrane integrity and denatures proteins (88). As a result, hepatocytes have developed a number of mechanisms to protect themselves from the toxic effects of bile acids. For example, toxic secondary bile acids such as lithocholic acid also activate the pregnane X-receptor, a nuclear receptor known to be important in regulating hepatic xenobiotic metabolism and detoxification (89–91).

The results presented in this study demonstrate that a specific heat shock protein, α-A-crystallin, is a direct target of FXR in human liver. These data suggest that α-A-crystallin induction represents yet a third mode of defense for cells exposed to excess bile acids. To our knowledge, α-A-crystallin expression has not been shown previously to respond to stress-induced stimuli outside of the lens. Immunohistochemical staining of human liver sections demonstrate that α-A-crystallin is expressed in hepatocytes and/or differentiating liver progenitor cells of cholestatic and cirrhotic human livers in addition to cholangiocytes of both normal and pathological human livers (Fig. 7). Consequently, we hypothesize that α-A-crystallin functions to prevent protein denaturation and cell damage in the face of excess intracellular bile acids. The data presented here suggest that activation of FXR might also protect human livers from the deleterious effects that result from excessive intracellular bile acid levels.

Acknowledgments—We thank members of the Edwards laboratory for critical comments throughout these studies. We also thank Dr. Charles Laasman and Dr. Hal Yee for helpful discussions. We especially thank Dr. Joseph Horwitz and members of his laboratory at the Jules Stein Eye Institute, UCLA for many helpful discussions and the generous gifts of antibodies.

REFERENCES
1. Derham, B. K., and Harding, J. J. (1999) Prog. Retin. Eye Res. 18, 463–509
2. Horwitz, J. (2003) Exp. Eye Res. 76, 145–153
3. Narberhaus, P. (2002) Microbiol. Mol. Biol. Rev. 66, 64–93
α-Crystallin Is a Target Gene of the Farnesoid X-activated Receptor in Human Livers
Florence Y. Lee, Heidi R. Kast-Woelbern, Jenny Chang, Guizhen Luo, Stacey A. Jones, Michael C. Fishbein and Peter A. Edwards

J. Biol. Chem. 2005, 280:31792-31800.
doi: 10.1074/jbc.M503182200 originally published online July 11, 2005

Access the most updated version of this article at doi: 10.1074/jbc.M503182200

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

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

This article cites 91 references, 40 of which can be accessed free at http://www.jbc.org/content/280/36/31792.full.html#ref-list-1