Ligand-dependent Activation of the Farnesoid X-receptor Directs Arginine Methylation of Histone H3 by CARM1*

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Meenakshisundaram Ananthanarayanan‡§, SiDe Li‡, Natarajan Balasubramaniyan, Frederick J. Suchy, and Martin J. Walsh¶

From the Department of Pediatrics, Mount Sinai School of Medicine, New York, New York 10029

In this study we demonstrate that the class II nuclear hormone receptor, farnesoid X-receptor (FXR), incorporates histone methyltransferase activity within the gene locus for bile salt export pump (BSEP), a well-established FXR target gene that functions as an ATP-dependent canalicular bile acid transporter. This methyltransferase activity is directed specifically to arginine 17 of histone H3. We demonstrate that FXR is directly associated with co-activator-associated arginine methyltransferase 1 (CARM1) activity. Furthermore, we show by chromatin immunoprecipitation that the ligand-dependent activation of the human BSEP locus is associated with a simultaneous increase of FXR and CARM1 occupation. The increased occupation of the BSEP locus by CARM1 also corresponds with the increased deposition of Arg-17 methylation and Lys-9 acetylation of histone H3 within the FXR DNA-binding element of BSEP. Consistent with these findings, CARM1 led to increased BSEP promoter activity with an intact FXR regulatory element, whereas CARM1 failed to transactivate the BSEP promoter with a mutated FXRE. Induction of endogenous BSEP mRNA and Arg-17 methylation by FXR regulatory element ligand, CDCA, requires CARM1 activity. Therefore, histone methylation at Arg-17 by CARM1 is a downstream target of signaling through ligand-mediated activation of FXR. Our studies provide evidence that FXR directly recruits specific chromatin modifying activity of CARM1 necessary for full potentiation of the BSEP locus in vivo.

An important component in the architecture of chromatin is the dynamic alteration in the post-translational modification of nucleosomal histones (1). As a result of these changes in histone modification, recent studies have confirmed the existence of a code embodied within the enzymatic post-translational conjugation of histones that provide the appropriate chromatin template for many nuclear processes that include the regulation of transcription, DNA replication, and repair (2). Studies of nuclear hormone receptors have provided evidence for the direct interactions between the activation of nuclear hormone receptors and the remodeling of chromatin through histone modification (3). In this study we demonstrate the association of histone H3 methyltransferase activity with the farnesoid X-receptor (FXR).1 FXR is a member protein of the class II nuclear hormone receptor gene family (4). Recent studies have shown FXR to have several ligands that activate FXR through the intracellular heterodimerization with the retinoid X-receptor (RXR) (5). Despite the capacity of FXR function to regulate many aspects of bile acid metabolism and transport through transcriptional activation of a number of target genes (6), little is known about how FXR achieves a transcriptionally active state. Furthermore, less is known about the nuclear protein components associated with the FXR/RXR heterodimer as a higher order ternary complex. To begin to understand the biochemical basis of how FXR may function to direct transcription, we tested whether FXR can associate with histone methyltransferase activity in vivo. Many studies have now confirmed that histone methyltransferases can directly imprint a code onto the NH2-terminal region of the core histone with methyl groups that may elicit either an active or repressive state within the chromatin architecture (7–9). Along with the acetylation of key residues along the NH2-terminal end of histone H3, further studies have demonstrated that an important process necessary for ligand-dependent nuclear hormone receptor activation of target genes is the mono- and dimethylation of a discrete arginine within the NH2-terminal end of histone H3 positioned at Arg-17 (10). Consistent with this model is the fact that the post-translational modification of histone H3 is important spatially and temporally to appropriately transform the conformation of chromatin structure of genes poised for transcriptional activation (11, 12).

Conjugated bile acids are excreted into bile via the bile salt export pump (BSEP, gene name Abcb11), an ATP-binding cassette transporter localized to the canalicular membrane of hepatocytes. Recent studies (6, 13) have revealed that BSEP promoters from human, mouse, and rat all contain a conserved FXR element AGGTCA(n)/TGACCT (FXRE). Cotransfection of BSEP with FXR/RXR cDNAs followed by addition of bile acids and 9-cis-retinoic acid (ligands for FXR and RXR, respectively) leads to a significant activation of the promoter activity. These studies are consistent with studies using FXR−/− mice showing decreased murine Bsep expression as well as altered bile acid, cholesterol, and triglyceride levels in these animals that were aggravated by bile acid feeding (14–16).

An important component in the architecture of chromatin is the dynamic alteration in the post-translational modification of nucleosomal histones (1). As a result of these changes in histone modification, recent studies have confirmed the existence of a code embodied within the enzymatic post-translational conjugation of histones that provide the appropriate chromatin template for many nuclear processes that include the regulation of transcription, DNA replication, and repair (2). Studies of nuclear hormone receptors have provided evidence for the direct interactions between the activation of nuclear hormone receptors and the remodeling of chromatin through histone modification (3). In this study we demonstrate the association of histone H3 methyltransferase activity with the farnesoid X-receptor (FXR).1 FXR is a member protein of the class II nuclear hormone receptor gene family (4). Recent studies have shown FXR to have several ligands that activate FXR through the intracellular heterodimerization with the retinoid X-receptor (RXR) (5). Despite the capacity of FXR function to regulate many aspects of bile acid metabolism and transport through transcriptional activation of a number of target genes (6), little is known about how FXR achieves a transcriptionally active state. Furthermore, less is known about the nuclear protein components associated with the FXR/RXR heterodimer as a higher order ternary complex. To begin to understand the biochemical basis of how FXR may function to direct transcription, we tested whether FXR can associate with histone methyltransferase activity in vivo. Many studies have now confirmed that histone methyltransferases can directly imprint a code onto the NH2-terminal region of the core histone with methyl groups that may elicit either an active or repressive state within the chromatin architecture (7–9). Along with the acetylation of key residues along the NH2-terminal end of histone H3, further studies have demonstrated that an important process necessary for ligand-dependent nuclear hormone receptor activation of target genes is the mono- and dimethylation of a discrete arginine within the NH2-terminal end of histone H3 positioned at Arg-17 (10). Consistent with this model is the fact that the post-translational modification of histone H3 is important spatially and temporally to appropriately transform the conformation of chromatin structure of genes poised for transcriptional activation (11, 12).

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‡ Both authors contributed equally to this work.
§ To whom correspondence may be addressed: Dept. of Pediatrics, Box 1664, The Mount Sinai Medical Center, One Gustave L. Levy Place, New York, NY 10029. Tel.: 212-241-9714; Fax: 212-426-1972; E-mail: meena.ananth@mssm.edu.
¶ To whom correspondence may be addressed. Tel.: 212-241-9714; Fax: 212-426-1972; E-mail: martin.walsh@mssm.edu.

1 The abbreviations used are: FXR, farnesoid X-receptor; CDCA, chenodeoxycholic acid; OST, glutathione S-transferase; FXRE, FXR regulatory element; HA, hemagglutinin; wt, wild type; RXR, retinoid X-receptor; ChIP, chromatin immunoprecipitation; DMEM, Dulbecco’s modified Eagle’s medium; RA, retinoic acid; BSEP, bile salt export pump; AdoMet, S-adenosylmethionine.
Recently, several enzymes that can catalyze either arginine or lysine methylation have been identified and partially characterized (17, 18). Despite the ability of protein-arginine methyltransferases to direct the methylation of the NH2-terminal regions of the various core histones, it has been a recent finding that these proteins exist as components of larger complexes within the active transcriptional apparatus (19, 20). Of those known to target the methylation of arginine along the NH2-terminal end of histones, co-activator-associated arginine methyltransferase 1 (CARM1) is associated with the glucocorticoid receptor-interacting protein 1 (GRIP1) in activating transcription through ligand-dependent regulation by estrogen receptors (10, 17). CARM1 preferentially methylates histone H3 at the arginine residue at position 17 from the NH2 terminus, a modification associated with active transcription (10). It was shown that CARM1 requires arginine methyltransferase activity to coactivate estrogen receptor-mediated transcription (17, 21). Furthermore, it was noted that the interplay between the specific substrate of CARM1, Arg-17, was further induced by 21). Furthermore, it was noted that the interplay between the specific substrate of CARM1, Arg-17, was further induced by acetylation of specific lysines residues along the NH2 terminus of histone H3 particularly, Lys-18 (22). These results argue for the coordinated interaction between acetylation of histone H3 and methylation of arginine 17 as suggested previously (23).

In this report, we demonstrate the association of FXR with histone H3 methyltransferase activity in vivo. This activity is preferentially localized to the methylation of arginine 17 and potentially affects methylation of lysine 4 of histone H3 in vitro. We determined that FXR is associated with CARM1, and this association is mediated through the central arginine methyltransferase domain of CARM1. To determine the consequence of the association between CARM1 and FXR, we examined cross-linked immunoprecipitates of chromatin to determine that the simultaneous occupation of the target gene BSEP by the nuclear hormone receptor FXR and the arginine methyltransferase CARM1 are enhanced upon ligand-dependent activation of FXR. Our results show that ligand-dependent activation of BSEP is associated with methylation of histone H3 at arginine 17 and acetylation of lysine 9.

**MATERIALS AND METHODS**

**Plasmid DNA and Antibodies**—All expression vectors for wild-type and mutant NH2-terminal hemagglutinin (HA)-tagged CARM1 were described previously (24). Expression vectors for HA-tagged GRIP1 (SRC-2) and SRC-1a were also described previously (25). Expression vector for human FXR1 (wt and AF2) was kindly provided by D. Mangelsdorf (UT Southwestern Medical Center, Dallas) and was described previously (6). Antibodies against the HA tag were purchased from Sigma. Antibodies against human FXR (H-130) were purchased as rabbit polyclonal antisera (catalog number sc-13063) (Santa Cruz Bio-technology). Antiserum against GRIP-1 (SRC-2) and SRC-1 were described previously (26). Antibody for the GST tag was purchased as mouse monoclonal antibody (Sigma). Antiserum against histone H3 was purchased (Upstate Biotechnology, Inc.) as purified rabbit polyclonal antisera.

**Cell Cultures**—Hepatocellular carcinoma (HepG2) cells were provided by G. Acs (Mount Sinai School of Medicine, New York). HepG2 cells were cultured in 90% Dulbecco's modified Eagle's medium (DMEM) and 10% heat-inactivated fetal bovine serum with 1× gentamycin (Invitrogen). Cell cultures were maintained at 37 °C in 5% CO2 in a humidified cell culture incubator.

**BSEP Promoter Analysis**—Transfection of HepG2 cells with the human BSEP 0.2-kb wild-type and FXRE mutant promoter and cotransfection with wild-type (wt) FXR, AF2-deleted FXR, and FXR and RXR were carried out as described by us previously (6). 3XFXRE-TK-Luc was constructed by cloning three copies of the FXRE from the rat Bsep promoter upstream of the TK promoter. CV-1 cells were transfected with this construct and cotransfected with wtFXR, AF2-deleted FXR, RXX, and CARM1 using LipofectAMINE 2000 (Invitrogen) according to the manufacturer's directions. Addition of 100 μM DDA and further details are included in the figure legends. All transfections were normalized by assay of β-galactosidase activity elicited by cotransfection with pCMV-βgal. All data were analyzed by Student's t test, and a p value of ≤0.05 is considered significant.

**Real Time PCR**—BSEP levels were quantitated by real time PCR on an Applied Biosystems 7900HT Sequence Detection Systems Analyzer using Brilliant SYBR Green QPCR kit from Qiagen as described previously (13) by using the following BSEP primers: forward, 5′-caacctgtgccagacactta-3′; reverse, 5′-ggagttgctggcagcagta-3′. 18 S levels were quantitated using the ribosomal control reagent kit from Applied Biosystems according to manufacturer's directions. Ct values for each sample were normalized by subtracting the Ct value for 18 S from the obtained Cts values, i.e. ΔCt sample = Ct experimental − Ct 18S. Fold change in BSEP mRNA over untreated control (which was set to 1) was obtained by the comparative method as 2−ΔΔCtSample (Applied Biosystems, User Bulletin 2).

**Immunoprecipitation and Immunoblotting**—Transfection of HepG2 cells was performed on 1 × 105 cells/cm2 in 10-cm diameter dishes as described previously (6) with a total of 5 μg of pSGS.HA plasmid encoding wild-type or mutants of human CARM1 as described previously (27). Transfections or cotransfections with pSGS.HA-GRIP1 were also performed similarly. All transfections were performed by cationic liposome-mediated transfection using FuGENE6 (Roche Applied Science) according to the manufacturer's recommended protocol. All reagents were purchased using immunoblotting Western blots were described previously (28). Immunoblots of immunoprecipitated cellular lysates were performed using 20% of the total immunoprecipitated material from transfected cells and were analyzed on 12.5% SDS-polyacrylamide gels. Immunoblotting was carried out with mouse monoclonal antibody against the HA epitope tag (CA18-A) or with an antibody against the respective antigens for FXR or GST.

**CARM1 Reactions**—Immunoprecipitates were collected by centrifugation in microtubes washed four times in MLB buffer and finally resuspended in MTS buffer (containing 20 mM Tris-HCl (pH 8.0), 200 mM NaCl, 0.4 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride). Methyltransferase assays were conducted on 3 μg of purified histone H3 (Roche Applied Science) or on GST fusions with wild-type and with mutated human histone H3 in which specific residues were mutated as indicated in the figure legends. Some of these reactions were briefly described as gifts (Y. Shinkai, Kyoto University, Kyoto, Japan, and T. Kouzarides, Cambridge University/Wellcome Trust UK Cancer Research Institute, Cambridge, UK) and are described previously (12, 29, 30). Purified and glutathione agarose-purified histone H3 proteins were incubated with individual immunoprecipitates as shown (Fig. 2) with 2 μM S-adenosyl-l-methyl-31C(1)Chimethionine (Amersham Biosciences, catalog number CFA860). 31C(M) was in 3°C. Reactions were terminated by addition of a protein gel loading buffer containing 6 μM urea and SDS and analyzed by 15% SDS-PAGE and fluorographed. Corresponding immunoblots indicative of the input used for each of the mutations tested were confirmed against the GST tag.

**Chromatin Immunoprecipitation (ChIP)**—Chromatin immunoprecipitations were described previously (13) by using antibodies against human FXR (H-130) (Santa Cruz Biotechnology). Antiserum against histone H3 was purchased (Upstate Biotechnology, Inc.) as purified rabbit polyclonal antisera.

**DNA methylation analysis**—Hepatocellular carcinoma (HepG2) cells were provided by G. Acs (Mount Sinai School of Medicine, New York). HepG2 cells were cultured in 90% Dulbecco's modified Eagle's medium (DMEM) and 10% fetal bovine serum with 1× gentamycin (Invitrogen). Cell cultures were maintained at 37 °C in 5% CO2 in a humidified cell culture incubator.
FIG. 1. Histone H3 methyltransferase activity is associated with FXR. Panel A, HepG2 cells (kindly provided by G. Acs, Mount Sinai School of Medicine) were cotransfected with expression vectors for the human FXR cDNA, GRIP1 cDNA, and full-length HA-tagged CARM1 cDNA and immunoprecipitated from nuclear lysates with either anti-HA (12CA5), anti-CARM1 (Abcam), anti-GRIP1 (M. R. Stalleep, University of Southern California School of Medicine), or anti-FXR (Santa Cruz Biotechnology) antisera and protein-A agarose (Sigma). Immunoprecipitated lysates were then used to conduct an in vitro methyltransferase assay using total purified histones (Roche Applied Sciences) and [methyl-\(^{14}\)C]AdoMet. Methylated histone H3 was detected by SDS-PAGE and autoradiography. Coomassie stain of total input of histones (bottom panel) used for the autoradiograph (top panel). Panel B, comparative measurement of histone methyltransferase activity from immunoprecipitates as described in panel A. Values represent the mean of the total counts/min of methyl-\(^{14}\)C-labeled histone were evaluated minus the total counts from immunoprecipitates from normal rabbit serum ± S.D. Panel C, various point mutants directed within the NH\(_2\) terminal region of the human histone H3 were used to map the methylation of histone H3 by FXR immunoprecipitates by in vitro methylation assays. Mutations directed at specific residues within the NH\(_2\) terminal region of the human histone H3 cDNA are indicated above the gel. Immunoprecipitation (IP) of nuclear lysates from HepG2 cells, transfected with both HA-tagged CARM1 and human FXR, were used in a methyltransferase assay in the presence of the various affinity-purified histone H3 mutants fused to GST and [methyl-\(^{14}\)C]AdoMet. Reactions were incubated for 1 h at 30 °C with immunoprecipitates by either anti-HA or anti-FXR as indicated. Reactions were terminated and analyzed by 12.5% SDS-PAGE and fluorographed. Input amount of the GST histone H3 variants were monitored by immunoblot with antisera directed against GST (bottom panel).

extraction, ethanol precipitation, and resuspension into 50 μl of nuclease-free water. DNA was recovered from a percentage of chromatin after proteinase K digestion and phenol chloroform extraction as a control. Amplification of specific regions of BSEP promoter was performed by PCR with DNA recovered from immunoprecipitations and performed using three dilutions of DNA containing 2, 1, and 0.5 μl with 34 cycles of amplification. The primers selected for PCR are as follows: Bsep site 1, (forward) 5’-tttcccaacagcatgtgttgtt-3’, and (reverse) 5’-gaggaaggccagaaatgttg-3’; Bsep site 2, (forward) 5’-ccacatgctttattt-3’; and (reverse) 5’-gattgctgtggaatacctc-3’. PCRs were analyzed on 1.5% agarose gels and were visualized by ethidium bromide staining and ultraviolet light transillumination. For quantitative real time PCR, DNA was dissolved in 50 μl of water and 2 μl used from each sample with Brilliant SYBR Green PCR kit from Qiagen as described previously. All chromatin immunoprecipitates analyzed by real time PCR were performed at least three times, and the difference for the mean was less than ~15%. Three microliters of PCR were extracted at 24, 34, and 40 complete cycles for visualization on agarose gels and stained with ethidium bromide. The values indicated represent specific binding efficiency (total nanograms detected with antibody and PCR minus the nanogram quantity detected with a control rabbit serum and PCR) as a fraction of total DNA input. Values represent S.D. from the mean.

RESULTS AND DISCUSSION

FXR Is Associated with H3 Histone Methyltransferase Activity in Vivo—To determine whether histone H3 methyltransferase activity is associated with FXR, we tested a number of immunoprecipitates from HepG2 cells cotransfected with expression vectors for the FXR and CARM1 cDNAs. We used a recombinant HA-tagged CARM1 as one of a number of candidate arginine methyltransferases to monitor methyltransferase activity associated with FXR. Results shown in Fig. 1 (panel A) demonstrate that the expression of FXR is accompanied with histone methyltransferase activity (shown in the last lane). Methyltransferase activity on histone H3 was confirmed by the immunoprecipitation of transfected HA-tagged CARM1 with an anti-HA and anti-CARM1 antisera. Although robust...
methyltransferase activity was identified and expected with anti-HA and anti-CARM1 antisera, significant levels of histone H3 methyltransferase activity were retained by anti-FXR antisera. Additionally, we tested the immunoprecipitates for GRIP1, because GRIP1 was shown previously to interact directly with CARM1 (20, 22). Results shown demonstrate significant histone H3 methyltransferase associated with the anti-GRIP1 immunoprecipitates (Fig. 1). These results suggest that histone H3 methyltransferase expression is associated with FXR.

To determine the amino acid substrate position of methyltransferase activity associated with FXR, we used affinity-purified GST-tagged human histone H3 as substrate. Wild-type histone H3 and mutated H3 with mutations at specific arginine and lysine residues along the NH₂-terminal axis of histone H3 were used. In these mutants, the lysines at residues 9 and 4 were replaced with arginines. Arginine at position 17 was replaced with lysine. Recombinant GST-tagged histone H3 was affinity-purified and used in an in vitro histone methyltransferase activity assay. Results indicate that a majority of histone methyltransferase activity is conferred onto the wild-type histone H3 protein with immunoprecipitated HA-tagged CARM1, Fig. 1 (panel B, 1st lane). However, significant histone H3 methyltransferase activity was retained from immunoprecipitates of nuclear lysates with an anti-FXR antiserum (Fig.1, panel B, 2nd lane). The effect of each of the mutations was compared by the intensity of the labeling of the GST-tagged histone H3 mutant with [methyl-14C]AdoMet and immunoprecipitates of FXR. The replacement of arginine 17 with a lysine failed to label as intensely with [methyl-14C]AdoMet when compared with either the wild-type or with a replacement at lysine 9. Most interestingly, the replacement of lysine 4 also caused a significant loss in the signal of labeled GST-tagged histone H3. This result suggests that FXR may also be associated with methylation directed at lysine 4. We demonstrate that the majority of the methylation of histone H3 from immunoprecipitates of nuclear lysates by anti FXR likely exists within arginine 17 and lysine 4.

The Association of FXR and CARM1 within the BSEP Locus—Because of the methyltransferase activity associated with FXR, we wanted to determine whether CARM1 is directly associated with FXR in vivo. Plasmids expressing various deletions of the HA-tagged CARM1 encoding specific deletions of CARM1 indicated above. Nuclear extracts were prepared and immunoprecipitated (IP) with an anti-HA antibody. Immunoprecipitates were loaded onto a 12.5% SDS-PAGE, and Western blots were prepared using a rabbit polyclonal antiserum directed against FXR (top immunoblot). Input levels of the HA-tagged CARM1 deletions were monitored by an immunoblot with anti-HA antibody from whole nuclear lysates from identically transfected HepG2 cells. Immunoblots were visualized with a chemiluminescent substrate (SuperSignal®, Pierce) and exposed to x-ray film. Panel B, ChIP was performed with HepG2 cells transfected with the HA-tagged full-length CARM1. HepG2 cells were fixed in formaldehyde and chromatin sonicated into fragments (~500 bp). Chromatin fragments were then immunoprecipitated with rabbit antiserum directed against FXR, histone H3, dimethylated Arg-17 histone H3, CARM1, or acetylated Lys-9 of histone H3. Immunoprecipitates were then used to amplify two regions of the human BSEP promoter, hBSEP site 1 (nucleotides −147 to +58) as a 205-bp fragment and hBSEP site 2 (nucleotides −587 to −353) as a 234-bp fragment (as indicated) of the human BSEP locus by PCR. Molecular weight markers and normal rabbit serum controls were used but not shown. ± refers to presence/absence of FXR-activating ligand CDCA at 100 μM.

**Fig. 2.** FXR is associated with CARM1 and can occupy the BSEP locus in vivo. Panel A, HepG2 cells were transfected with expression vectors for HA-tagged CARM1 encoding specific deletions of CARM1 indicated above. Nuclear extracts were prepared and immunoprecipitated (IP) with an anti-HA antibody. Immunoprecipitates were loaded onto a 12.5% SDS-PAGE, and Western blots were prepared using a rabbit polyclonal antiserum directed against FXR (top immunoblot). Input levels of the HA-tagged CARM1 deletions were monitored by an immunoblot with anti-HA antibody from whole nuclear lysates from identically transfected HepG2 cells. Immunoblots were visualized with a chemiluminescent substrate (SuperSignal®, Pierce) and exposed to x-ray film. Panel B, ChIP was performed with HepG2 cells transfected with the HA-tagged full-length CARM1. HepG2 cells were fixed in formaldehyde and chromatin sonicated into fragments (~500 bp). Chromatin fragments were then immunoprecipitated with rabbit antiserum directed against FXR, histone H3, dimethylated Arg-17 histone H3, CARM1, or acetylated Lys-9 of histone H3. Immunoprecipitates were then used to amplify two regions of the human BSEP promoter, hBSEP site 1 (nucleotides −147 to +58) as a 205-bp fragment and hBSEP site 2 (nucleotides −587 to −353) as a 234-bp fragment (as indicated) of the human BSEP locus by PCR. Molecular weight markers and normal rabbit serum controls were used but not shown. ± refers to presence/absence of FXR-activating ligand CDCA at 100 μM.
tested a second site of the human in Fig. 2, the transfection with the expression vector for CARM1 (shown generates an increased signal in the methylation of histone H3 that Arg-17 of histone H3, as a substrate of CARM1, and CARM1 co-occupy the same region of the human ChIP is clearly detectable. These results suggest that both FXR and CARM1 exists in vivo, we explored the prospect that a well characterized target gene of FXR, BSEP, may also be a target of CARM1 action. To answer this question, we used ChIP studies to verify the occupation of the human BSEP locus by FXR and CARM1. ChIP experiments demonstrate the presence (or absence) of protein(s) within a specified region of a target locus. Essentially, DNA-protein and protein-protein interactions within the chromatin architecture are cross-linked by formaldehyde. This is followed by release of the protein cross-links, and nuclei are then isolated. The remaining chromatin is sheared by sonication into a discrete size (>500 bp), and the sheared chromatin is immunoprecipitated with a suitable antiserum and protein-A-agarose to pull down IgG. The remaining immunoprecipitates are extensively washed, and DNA was extracted from the immunoprecipitates. Residual DNA is then used in PCR for identifying the genomic region of interest (human BSEP). Results shown in Fig. 2 (panel B) indicate that the human BSEP locus containing the FXR binding consensus (6) (shown as hBSEP site 1) is occupied by endogenous FXR under native conditions both in the absence and presence of the FXR-activating ligand CDCA. However, it was apparent that a 24-h treatment with the FXR ligand 100 μM CDCA results in an increased occupation by endogenous FXR. It should be stressed that these ChIP experiments are demonstrating endogenous FXR but utilize the recombinant HA-tagged CARM1. To establish that equivalent amounts of input chromatin were used, we examined the input levels of immunoprecipitated histone H3 from a ChIP experiment using antisera against histone H3 as a positive control. Further examination of both CARM1 and the substrate for CARM1 (arginine at position 17 of histone H3) indicates that CARM1 and arginine (Arg-17) methylation of histone H3 occupy the identical site as shown. To demonstrate the ability of the ChIP to distinguish between specific regions of the BSEP locus, we tested a second site of the human BSEP promoter region between −587 and −353 lacking any putative consensus cis element for FXR binding as a negative control (site 2). In this region, ChIP fails to identify any occupation by FXR or CARM1. However, the signal generated from the histone H3 ChIP is clearly detectable. These results suggest that both FXR and CARM1 co-occupy the same region of the human BSEP locus.

Our results further substantiate the function of CARM1 by the fact that Arg-17 of histone H3, as a substrate of CARM1, generates an increased signal in the methylation of histone H3 at Arg-17 within the hBSEP site 1. This is a result from the treatment of HepG2 with the FXR-activating ligand following the transfection with the expression vector for CARM1 (shown in Fig. 2, panel B). Because acetylation of histone H3 is an important cue for the methylation of Arg-17 (23), we also tested whether acetylation of histone H3 at Lys-9 is affected. Results indicate by ChIP experiments that Lys-9 acetylation of histone H3 also corresponds with activation of BSEP by CDCA. These findings suggest that FXR utilizes CARM1 to generate an important imprint in the histone code necessary for the remodeling of chromatin architecture poised for the activation of the human BSEP locus.

Increased Methylation of Arg-17 Is Ligand-dependent and Requires CARM1 Activity—As a way to determine whether there is arginine methylation of histone H3 by CARM1, we tested the extent of Arg-17 methylation within the endogenous BSEP locus associated with FXR activity in hepatocellular carcinoma HepG2 cells in the presence of wild-type and methyltransferase-deficient mutants of human CARM1. Cells transfected with wild-type and a mutant CARM1 were used to test for the relative quantity of Arg-17 methylation of histone H3 within the BSEP locus directly associated with FXR occupation within the hBSEP site 1 (as described in Fig. 2, panel B). Essentially, ChIP experiments were performed and quantitated by real time PCR to determine the relative binding efficiency of antibodies directed against histone H3 that carry dimethyl groups on Arg-17. To control for the level of background associated with the antibodies, we tested a corresponding antibody that recognizes all histone H3 species. Results shown in Fig. 3, panel A, demonstrate the dramatic increased deposition of Arg-17 dimethyl groups from the (BSEP site 1) PCR product in the presence of transfected wild-type CARM1 when compared with native HepG2 cells and cells transfected with methyltransferase-deficient CARM1 when treated with 100 μM CDCA. Corresponding levels of histone H3 from rabbit antisera were monitored for 30 cycles (Fig. 3, panel A, lower panel) to determine the relative input of chromatin DNA used to verify the relative amount of Arg-17 dimethyl groups of histone H3 (upper panel).

To measure the extent of the deposition of Arg-17 dimethyl groups directly associated with the FXR regulatory element of the BSEP locus, we performed quantitative real time PCR from ChIPs of HepG2 cells transfected with wild-type and a mutant CARM1 lacking methyltransferase activity. Results shown (Fig. 3, panel B) indicate an increased deposition of dimethyl groups of Arg-17 H3 is both ligand-dependent and associated with FXR activity in hepatocellular carcinoma, whereas mutant CARM1 failed to achieve the same levels in the deposition of Arg-17 dimethyl groups within histone H3. This result confirms that arginine methyltransferase activity of CARM1 is directly associated with ligand-dependent induction of Arg-17 methylation of the BSEP promoter in HepG2 cells.

CARM1 Augments FXR Transactivation of the BSEP Promoter Both the in the Native as Well as in a Heterologous Promoter Context—To verify that the above data with ChIP assays have in vivo relevance, we transfected HepG2 cells with a 0.2-kb BSEP promoter upstream of firefly luciferase as a reporter (Fig. 4, panel A) as well as a TK-3XFRE-Luc into CV-1 cells (Fig. 4, panel C) in addition to cotransfection with FXR/RXR and SRC1a. Cotransfection with FXR/RXR and SRC1a resulted in a 32-fold stimulation of luciferase activity over control in the absence of CARM1 cotransfection. Most importantly, when increasing amounts of CARM1 (50–200 ng) were transfected in addition to FXR/RXR and SRC1a, there was a further stimulation increasing to a maximum of 39-fold over control (23% increase compared with absence of CARM1; p < 0.05) at 200 ng of CARM1. This effect was even more dramatic when a construct with 3XFRE from rat Bsep promoter in front of TK promoter was used. In this case, as shown in Fig. 4, panel C, cotransfection with CARM1 led to 117.2-, 129.5-, and 211.1-fold increase of reporter gene activity com-
pared with empty vector transfection at 50, 100, and 200 ng, respectively, in contrast to a 98-fold increase (significant with \( p < 0.05 \) compared with absence of CARM1) in the absence of CARM1. These data lend support to the ChIP analysis and demonstrate that CARM1 participates in arginine methylation of histone H3 at the \( BSEP \) locus resulting in enhanced transcription.

CARM1 Activity Is Specifically Associated with the FXRE—To determine the specificity of CARM1 activity for FXR, HepG2 cells were transfected with the \( BSEP \) promoter construct carrying the mutated FXR element (FXRE). We show that FXR/RXR or CARM1 fail to activate through a mutated FXRE (Fig. 5). However, cotransfection with the wild-type FXRE-containing \( BSEP \) promoter led to an increase in luciferase activity from CARM1 under the same conditions. These results indicate that FXR/RXR heterodimers cannot bind to a mutated FXRE and activate the mutated \( BSEP \) promoter. Consistent with this finding is the fact that CARM1 activity on the \( BSEP \) promoter required an intact FXR target site. To determine whether transactivation of \( BSEP \) by CARM1 was specific to FXR activity, the AF2 domain of FXR was deleted and used as a dominant-negative protein for FXR activity. We have shown previously that AF2-deleted FXR fails to transactivate the \( BSEP \) promoter with an intact FXRE (6). Similar to the experiments as described above, we cotransfected the \( BSEP \) promoter with an intact FXRE or a 3XFXRE-Tk-Luc construct.
FIG. 4. CARM1 augments wild-type but not AF2-deleted FXR activation of the \textit{BSEP} promoter. A schematic of the reporter construct is shown at the top (panels A–D). Panel A, $2 \times 10^5$ HepG2 cells were transfected in 12-well plates with 0.2-kb \textit{BSEP} promoter containing the FXRE and cotransfected with wtFXR (25 ng), FXR\textsubscript{a} (25 ng), SRC1\textsubscript{a} (50 ng), and varying amounts of CARM1 using FuGENE 6 as described by us previously (6). pCMV-\textit{b}Gal was transfected as a control. Medium was changed to charcoal-adsorbed phenol red-free DMEM the next day, and ligands for FXR (100 \(\mu\)M CDCA) and RXR (1 \(\mu\)M 9-cis-RA) were added to wells transfected with FXR/RXR and solvent (Me\textsubscript{2}SO) to control wells. The next day, firefly luciferase and \(\beta\)-galactosidase activities were assayed using kits from Promega and Stratagene, respectively. Data are represented as relative luciferase activity with the activity of the promoter in the absence of cotransfections being 1. Panel B, transfections were done similar to those described in A except that AF2-deleted FXR was used. Panel C, $2 \times 10^5$ CV-1 cells in 12-well plates were transfected with TK-3xFXRE-Luc along with wild-type FXR, RXR\textsubscript{a}, SRC1\textsubscript{a}, and various concentrations of CARM1. Replacement of medium, addition of ligands for nuclear receptors and reporter gene, and \(\beta\)-galactosidase assays were performed as in A. Panel D, transfections were similar to those in panel C except for cotransfection with AF2-deleted FXR. All experiments were performed twice with each condition in triplicate, and data as mean \(\pm\) S.E. from a representative experiment is shown. In all panels *, \(p < 0.05\) when compared with promoter alone without CARM1 but in the presence of FXR/RXR and SRC1\textsubscript{a}.

into HepG2 and CV-1 cells, respectively, together with FXR/ RXR, SRC-1a, and increasing concentrations of CARM1, and the results are shown in Fig. 4 (panels \textit{B} and \textit{D}). As seen in Fig. 4 (panel \textit{D}), using CV-1 cells, wild-type FXR transactivated \textit{BSEP} promoter 93.2-fold over control which was further stimulated to 118.7-fold over control with 200 ng of CARM1 (\(p \leq 0.05\) compared with absence of CARM1). In contrast, cotransfection with an AF2-deleted FXR construct failed to stimulate
**Fig. 5.** CARM1 does not augment BSEP 0.2-kb promoter in which the FXRE has been mutated. Panel A, schematic of the reporter construct is shown at the top. Panel B, 2 × 10⁶ HepG2 cells were transfected in 12-well plates with 0.2-kb BSEP promoter containing the FXRE and also cotransfected with wtFXR (25 ng), RXRα (25 ng), SRC1α (50 ng), and varying amounts of CARM1 using FuGENE 6 as described under “Materials and Methods.”

**Fig. 6.** Arginine methyltransferase-deficient CARM1 (CARM1 Mut) fails to augment BSEP promoter activity in the presence of FXR/RXR and SRC1 in the presence of ligand. Panel A, a schematic of the reporter construct containing 3xFXRE fused into the BamHI and BglII site of TK promoter is shown. Panel B, CV-1 cells were plated in 12-well plates and transfected with wild-type and mutant CARM1 plasmids along with FXR/RXR and SRC1α-encoding plasmids using FuGENE 6 as described under “Materials and Methods.” The cells were also transfected with pCMV-βGAL for normalization of transfection efficiency. Twenty four hours later, the medium was changed and ligands added and assayed at 48 h for luciferase activity as described in Fig. 4. *, p ≤ 0.05 compared with transfection in the absence or with mutant CARM1.

**BSEP promoter** in the presence of CARM1 (67.0-, 67.3-, 70.9-, and 77.6-fold over control with 0, 50, 100, and 20 ng of CARM1, respectively). These data further confirm that the effect of CARM1 on the BSEP promoter is mediated through FXR and required functional FXR protein.

**Protein-arginine Methyltransferase Activity of CARM1 Is Necessary for Enhancement of FXR/RXR Activation of the BSEP Promoter**—We next wanted to determine whether a mutant that is deficient in methyltransferase activity would be effective in the augmentation of the BSEP promoter as seen in earlier experiments. For this purpose, CV-1 cells were transfected with a plasmid containing 3×FXRE upstream of the TK promoter driving luciferase and cotransfected with wild-type or a CARM1 deletion mutant lacking amino acids 1–461, which has been shown to be necessary for enzyme activity as well as coactivator binding. As seen in Fig. 6, whereas cotransfection with wild-type CARM1 led to a 135-fold increase in luciferase activity over control (compared with 104.6-fold increase in the absence of CARM1; p < 0.05), mutant CARM1 failed to augment BSEP promoter-driven luciferase activity in the presence of FXR, RXR, and SRC1α (107.2-fold over control; not significant compared with absence of CARM1). These data led us to conclude that methyltransferase activity as well as the coactivator association domain of CARM1 (amino acids 1–461) are necessary requirements for the effect of CARM1 on the BSEP promoter, which is consistent with the coimmunoprecipitation data shown in Fig. 2, panel A.

**Synergistic Activation of Endogenous BSEP by FXR and CARM1**—To further verify that CARM1 activates the BSEP promoter, we measured endogenous BSEP mRNA levels in HepG2 cells after cotransfection with FXR/RXR and 400 ng of CARM1 by real time PCR with additional controls as indicated in the figure. The results of the real time PCR are shown in Fig. 7. Cotransfection of FXR/RXR increased the mRNA levels 5.3-fold, whereas additional cotransfection with coactivator SRC-1 increased BSEP mRNA 5.4-fold over untreated cells. In HepG2 cells that were further cotransfected with 400 ng of CARM1, the mRNA levels for BSEP as measured by real time PCR increased 47.8-fold, showing a significant increase in BSEP mRNA. Cotransfection with SRC1 alone, CARM1 alone in the absence of FXR/RXR or with FXR/RXR, and CARM1 without SRC1 resulted in minimal increase of BSEP message to 2.1, 2.5, and 2.0, respectively. Furthermore, cotransfection with all the components (FXR/RXR, SRC1, and CARM1) in the absence of the ligands 1 μM 9-cis-retinoic acid and 100 μM CDCA resulted in a slight increase in message to 7.5-fold over untreated controls. Therefore, our studies indicate that the effect of CARM1 can be demonstrated on the FXR target gene BSEP as seen from the increased promoter activity as well as increased mRNA synthesis as measured by real time PCR.

We have demonstrated that FXR is an important mediator of CARM1 activity within the BSEP promoter. Additionally, we have demonstrated the occupation of FXR on the endogenous BSEP. These are the first studies of this kind that has estab-
lished a direct relationship between FXR with an endogenous target gene in vivo, in this case BSEP. Although the occupation of BSEP by FXR and the direct association of CARM1 with FXR activation were anticipated, it is of interest how FXR may either establish a specific context within chromatin or may be recruited to an already established conformation. These studies provide insight into a growing number of genes influenced by the specific post-translational modification of histone H3 and help to establish this as a biochemical mechanism utilized by FXR. The influence of CARM1 on the transcription of the BSEP locus followed by transition to a transcriptionally competent locus indicated by the arrow.

We demonstrate that in vitro methylation of histone H3 is dramatically affected when a mutation is directed at lysine 4 to create a mutant arginine within histone H3 (Fig. 1, panel B). Although this was unexpected, it is consistent with other activities associated with CARM1 (23, 30). It is plausible to consider that based on the notion that a histone code prevails in directing either transcriptional repression or activation (34), methylation of Lys-4 could be a requisite for either recognition by CARM1 or coactivator activity associated with CARM1 such as that of SRC-1. Although several lysine methyltransferases that target K4 methylation have been identified among a number of highly conserved gene families (35), we have yet to address the role of this activity on FXR activation. There are a number of examples whereby the Set domain proteins encoding lysine methyltransferase such as Set7 may participate in gene activation by nuclear hormone receptors (31, 36). However, it is not yet clear if Set7 is an intrinsic component of this activation mechanism (31).

In summary, we have confirmed previous speculation that FXR constitutively occupies the BSEP locus using chromatin immunoprecipitation analysis (Fig. 2, panel B). This result suggests that FXR mediates contrasting activities through recruitment of coactivator and corepressor activities or that FXR may remain poised depending on the presence of activating ligand in a basal conformation. This finding is consistent with the function of nuclear hormone receptors, in general, and has been described from many earlier seminal studies based on this model (37). However, our studies now provide evidence that FXR directly recruits specific chromatin modifying activity in vivo.
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Ligand-dependent Activation of the Farnesoid X-receptor Directs Arginine Methylation of Histone H3 by CARM1

Meenakshisundaram Ananthanarayanan, SiDe Li, Natarajan Balasubramaniyan, Frederick J. Suchy and Martin J. Walsh

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