Histone H3K4 trimethylation by MLL3 as part of ASCOM complex is critical for NR activation of bile acid transporter genes and is downregulated in cholestasis

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hepatoma cells; microarray; epigenetics; gene regulation; activating signal cointegrator-2-containing complex; H3 lysine 4; nuclear receptors; mixed lineage leukemia 3

LIGAND-ACTIVATED NUCLEAR RECEPTORS (NRs), such as Farnesoid x receptor (FXR) and glucocorticoid receptor (GR), initiate transcription by binding to specific DNA regulatory elements in promoters of target genes and recruiting coactivator proteins to modify chromatin structure and induce assembly of the transcription complex containing RNA polymerase II (PolII) (2, 3, 6, 18). The primary coactivators including the p160 coactivators such as TIF2/GRIP-1/SRC-2 and related members of the p300 family bind directly to ligand-activated NRs and recruit an array of secondary coactivators such as p300/cAMP response element binding protein (CREB)-binding protein (CBP) and coactivator-associated arginine methyltransferase (CARM1), which acetylate and methylate (respectively) histones and other proteins in the transcription complex (16, 33). Secondary coactivators act synergistically in a requisite sequence to enhance transcriptional activation by binding to the p160 coactivators or other primary coactivators (25, 34).

We have been interested in how the expression of NRs and various coactivators are altered in cholestasis leading to downregulation of major bile acid transporters, bile salt export pump (BSEP), multidrug resistance associated protein 2 (MRP2), and sodium taurocholate cotransporting polypeptide (NTCP) (7, 8, 24, 26). We have found that several critical components of a steady-state complex called activating signal cointegrator-2 (ASC-2)-containing complex (ASCOM) are altered in mouse after bile duct ligation. This complex contains the transcriptional coactivator nuclear receptor coactivator 6 (NCOA6) and the histone H3 lysine 4 (H3K4) methyltransferase mixed lineage leukemia 3 (MLL3) and its paralog MLL4. Our studies in the normal mice and human liver cell lines show that these components interact directly with the bile acid receptor FXR and with GR and are involved in expression of the their target genes, BSEP, MRP2, and NTCP. Moreover, in the common bile duct ligated mouse impaired expression of critical coactivators and disruption of histone modifications in cholestasis can adversely affect FXR/GR transactivation, even if NR expression is not altered.

MATERIALS AND METHODS

Cells and cell culture. HepG2 cells were cultured in MEM with 10% FBS and antibiotics. Huh-7 cells were cultured in RPMI with FBS and antibiotics. HEK293 cells were grown in DMEM with the same supplements. All cells were grown in 10% CO2 in a humidified incubator maintained at 37°C. When cells were treated with FXR/RXR ligands, chenodeoxycholic acid (CDCA), and 9-cis retinoic acid,
respectively, the medium was changed to DMEM without phenol red and contained charcoal-adsorbed FBS.

**Chemicals.** All chemicals were obtained from Sigma unless stated otherwise. Small interfering (siRNAs) for NCOA6, MLL3, and MLL4 were obtained from Dharmacon or Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies to NCOA6 (400A-1) were from Bethyl Labs, while MLL3 and MLL4 (ALR) antibodies were from Santa Cruz Biotechnology. Affinity-purified antibodies to MLL4 were obtained from Dr. Kai Ge, Nuclear Receptor Laboratory (National Institute of Diabetes and Digestive and Kidney Diseases).

**Common bile duct ligation.** Common bile duct ligation (CBDL) in C57Bl/6 mice were performed as described earlier using a protocol (8) approved by the Institutional Animal Care and Use Committee of Mount Sinai School of Medicine. Briefly, laparotomy was performed on the mice following which bile duct was ligated proximally and distally and severed in the middle. Serum bile acids were estimated by a kit from Trinity Biotech to ensure that successful cholestasis was achieved (see Supplemental Data; Supplemental Material for this article is available online at the Am J Physiol Gastrointest Liver Physiol website). Sham surgery was performed on control mice in which laparotomy and manipulation of the liver was performed, but bile duct was not ligated. Livers from sham-operated and bile duct-ligated mice were collected at 1, 3, and 7 days postligation. All animals received humane care according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH publication 86–23 revised 1985).

**Plasmid constructs.** Human BSEP promoter-luciferase plasmid was generated as described by us previously (1). Plasmids encoding FXR and RXR were generously supplied by Dr. David Mangelsdorf (Dallas, TX) as described earlier (1). Preparation of full-length and deletion constructs representing various domains of NCOA6 has been recently described (27).

**siRNA-mediated knockdown of NCOA6, MLL3, and MLL4.** siRNAs against NCOA6, MLL3, and MLL4 were obtained from Dharmacon (siGenome pool) or Santa Cruz Biotechnology (for sequences of siRNAs used, see Supplemental Table S3). For knockdown experiments, HepG2 or Huh-7 cells were plated in sixwell plates (1 × 10⁶ cells/well) and incubated 2 days later with 50 nM siRNA using TransIT-LKO (MirusBio) at a ratio of 1:1 (µl/µl) according to manufacturer’s instructions. Six hours later, medium was added to the wells, and 24 h later, spent medium was replaced with fresh DMEM. Forty-eight hours later total RNA was prepared using Trizol kit (Invitrogen, Carlsbad, CA), and real-time PCR analysis was conducted following conversion of mRNA into cDNA. Cells were harvested from the wells and lysates were prepared by resuspending in mammalian protein extraction buffer (Sigma, St. Louis, MO) after 72 h following siRNA transfection as described in Western blotting analysis.

**Transient transfections and luciferase assays.** HepG2 or Huh-7 cells were plated at a concentration of 1 × 10⁶ cells/well in 24-well plates 2 days earlier. They were transfected at day 0 with mouse or human BSEP promoter at 0.5 µg/well (in triplicate/per group) and also cotransfected with 50 ng FXR/RXR and various amounts of NCOA6 expression plasmids in OPTI-MEM (Invitrogen) where indicated. Transfections were carried out using TransIT-LT (MirusBio) at DNA:TransIT ratio of 1:3. On day 1, the medium was changed to DMEM without Phenol Red and with charcoal-adsorbed FBS. Ligated 9-cis retinoic (1 µM) acid and CDCA (100 µM) for RXR and FXR, respectively, were added at this time and luciferase activities were measured 24 h later using Promega kit (Promega, Madison, WI). When siRNA adenoviruses against NCOA6 were used to study their effect on promoter activity, they were infected 6 h after transfection with promoter and FXR/RXR/NCOA6 plasmids as follows: 5 × 10⁹ viral particles/well were (multiplicity of infection of 1:1000) added to the wells in 0.15 ml of OPTI-MEM after removal of the plasmid mixture. After incubation for 1.5 h in the incubator, 0.4 µl of serum containing DMEM were added and returned to the incubator overnight. The next day, phenol red-free DMEM and ligands were added, and luciferase activity was assayed as described above. Normalization of transfection efficiencies in the different wells was achieved by cotransfection with pCMVβ galactosidase and assay of galactosidase activity. All transfection experiments were repeated twice with similar results. Representative experiments are shown in Results due to inter-experiment variations in transfection.

**Glutathione S-transferase pull-down assay.** Partial recombinant proteins of NCOA6 were overexpressed as glutathione S-transferase (GST) fusion proteins in E.coli BL21 as previously described (27). Equal amounts of GST and GST fusion proteins were used for GST pull-down assays by incubating 5 µl of 35S-methionine-labeled full-length FXR obtained by in vitro translation using TNT T7 coupled transcription and translation system in a 500 µl of NETN buffer containing 1 mg/ml fatty acid free BSA in the presence and absence of CDCA (100 µM). The bound proteins were washed with three times with NETN buffer and eluted into SDS-PAGE sample buffer by heat denaturation. The proteins were separated on 4–20% gradient gels, and after the signal was fixed and amplified, the gels were dried and autoradiographed.

**Mammalian two-hybrid analysis.** The mammalian two-hybrid assay was performed by modified Checkmate mammalian two-hybrid system (Promega) following manufacturer’s instructions. HepG2 cells were cotransfected with the pBIND/FXR (containing ligand binding domain), pACT/NCOA6 (coding for 6 different domains), and pSG9/ luc plasmids expressing Lipofectamine 2000 (Invitrogen) according to the manufacturer’s recommended protocol. Briefly, cells were seeded in 24-well plates at a density of 1 × 10⁵ cells/well and cultured to about ~90% confluency. Plasmid constructs were combined at a ratio of 2:1:4 (promoter:NR:coactivator) to a total of 0.8 µg/well, and cells were transfected and cultured for 24 h followed by the addition of ligand CDCA (100 µM) for another 24 h. Finally, luciferase activity was determined with Lumistar Optima luminometer (LMG Biotech) with a commercially available kit (Promega). The transfection efficiency was normalized using Renilla luciferase activity, which was simultaneously expressed from the pBIND plasmids. Further details are available from the authors on request.

**Chromatin immunoprecipitation analysis of cultured cell lines and mouse liver.** Chromatin immunoprecipitation analysis (ChIP) assays were conducted essentially by a combination of previously described protocols (12) and manufacturer’s instructions using EZ-Chip/Magna-ChIP G kit from Upstate Biotechnology. Biotinylated DNA was sonicated in Diagenode Bioruptor Sonicator for 8 × 30 s (twice, with 30-s on/30-s off cycles) resulting in DNA fragments of 200–1,000 bp. In the case of mouse liver, a suspension of nuclei prepared in 0.8 ml in an Eppendorf tube was sonicated for 8 × 10 s placing the tube in ice-cold water using a Misonix XI Series sonicator (Qsonica, Newtown, CT). The fragmented DNA was diluted in ChIP dilution buffer and preadsorbed with ProteinG- sepharose/salmon sperm DNA for 1 h at 4°C. Ten percent of the chromatin was removed and saved as “Input.” It was then incubated overnight at 4°C with 3–5 µg of the appropriate antibodies or normal mouse IgG (control). Antibody–chromatin complexes were captured by incubation with protein G-Sepharose and centrifuged. Protein G-Sepharose beads were washed with low salt, high salt, lithium chloride and finally with Tris-EDTA buffer. DNA from the beads was then eluted. Reversal of protein cross-linking and protease K digestion followed by purification of the DNA was then achieved. An aliquot of the DNA (5 µl) was used in a PCR reaction using specific primers flanking the FXR element (FXRE) site of human and mouse BSEP/Bsep, mouse Mrp2, and GCRE in the human NTCP. Primers flanking a site distant from the FXRE site were used as negative controls. Positive control for the ChIP consisted of immunoprecipitation with RNA PolII antibody. Quantitation of the DNA precipitated was done using quantitative
(q)PCR and expressed as percent relative to the IgG control. ChIP primers used in the studies are provided in Supplemental Table S2.

Methodological details relating to 1) preparation and analysis of customized epigenetic array, 2) real-time PCR analysis, and 3) Western blot analysis can be found at the end of the Supplement Materials.

Statistical analysis. All data are shown as means ± SD (3 animals/ experiments per group). In all the experiments, comparison of data between individual groups was performed using unpaired Student’s t-test. For the sake of clarity, all significant P values in Figs. 1–6 are indicated as P < 0.05, although in some cases they were even lower.

RESULTS

Expression of multiple genes encoding epigenetic modifiers, coactivators, and corepressors is altered in mouse liver during cholestasis. As a first step towards understanding the role of epigenetic modifications in the regulation of bile acid transporter genes, a custom-made array platform using RT² Profiler PCR Array System (SuperArray Biosciences Corp) was used to examine the expression of 84 genes encoding coactivators and histone modifying enzymes (see template with gene names in Supplemental Fig. S2). cDNA from mouse livers after 1 and 3 days post-CBDL was analyzed and compared with sham-operated controls. The results of the analysis plotted as fold change compared with sham (≥2-fold) are shown in Fig. 1, A and B, for 1 and 3 days post-CBDL, respectively. There were a number of genes whose expression was downregulated significantly (P < 0.05) at 1 day ranging from >2-fold up to 11-fold. On day 3, there were many genes whose expression was upregulated that included several nuclear receptors and histone modifying enzymes, suggesting that this response might represent an adaptive mechanism by the liver. We specifically noted that MLL3, a histone H3K4 lysine methyl transferase, was downregulated at both the time points (−6.1 ± 1.4 at 1 day and −2.6 ± 1.9 at 3 days). Expression levels of these genes partially returned to control levels at 7 days after CBDL consistent with partial normalization of transporter message levels. Recent studies (14) have shown that during LXR activation of target genes, a transcription initiation complex termed ASCOM, is recruited to the promoter that contains the coactivator NCOA6 (ASC-2, PRIP) and the histone H3K4 methyl transferases MLL3 or MLL4. Catalytic activity of MLL3/MLL4 results in trimethylation of H3K4, which is an “activation mark.” Since our array did not contain NCOA6 and...
MLL4, their expression by qPCR analysis was assessed separately and is shown in Fig. 1, C and D, respectively. While there was no significant change in expression of NCOA6 mRNA level, there was a marked decrease in MLL4 expression at both time points. qPCR analysis of TNFα and IL-6 mRNA levels was carried out as positive controls for inflammation; both were elevated by CBBD (Fig. 1D) as reported previously (4). Since bile acid transporters (including Ntcp, mrp2, and BSEP/bsep) are downregulated early in rodent and human cholestasis (7, 8, 24, 26), these data together pointed to the potential role of MLL3/MLL4 as part of the ASCOM complex in FXR (BSEP/bsep and mrp2) and GR (NTCP) activation in liver.

**siRNA-mediated silencing of MLL3, MLL4, and NCOA6 results in downregulation of BSEP and NTCP expression in hepatoma cells.** To further assess how MLL3/MLL4 and NCOA6 might regulate bile acid transporter expression, we systematically analyzed the effect of siRNA-mediated silencing of MLL3/MLL4 (individually and together) and NCOA6 (Fig. 2, A, B, and C, respectively) on BSEP and NTCP expression in HepG2 cells. siGenome pools (pools of 4 different siRNAs) representing NCOA6 and MLL3/4 were transfected into HepG2 cells. As shown in Fig. 2A, compared with control siRNA treatment, specific MLL3 siRNA reduced its own message to 45.2% of control while expression of BSEP and NTCP were reduced significantly (P < 0.05) to 24.4 and 40% of control, respectively. Although control siRNA also modestly decreased MLL3 protein, specific MLL3 siRNA reduced MLL3 protein to 16% of control. The reason for the downregulation of MLL3 protein expression by NCOA6 (Fig. 2A, right) is currently unknown. Figure 2B shows the results after treatment with MLL4 siRNA. In this experiment, an additional set of experiments were included in which both MLL3 and MLL4 (also known as ALR) siRNAs were silenced simultaneously to see if MLL3/4 silencing resulted in additive effects on expression of transporter mRNAs. There is considerable confusion in the gene nomenclature for human MLL4, and hence we refer to it as MLL4 (ALR). MLL4 and MLL4 + MLL3 siRNA treatment resulted in a decrease of MLL4 message levels to 26.3 and 30.6% of control, respectively. Under the same conditions, BSEP mRNA levels were reduced to 56.5 and 10.5% of control while NTCP levels were reduced to 39.5 and 29.5% of control, respectively. Studies by Lee et al. (14) have suggested that ASCOM complexes may contain ASC-2 (ASC-2) is a critical determinant of BSEP transcriptional activation by FXR.

Since siRNAs directed against NCOA6 inhibited promoter activity, we wanted to test, conversely, whether overexpression of NCOA6 using plasmid cotransfection will activate the promoter activity dose dependently. We transfected HepG2 cells with BSEP promoter and cotransfected with FXR/RXR in the presence of increasing amounts of NCOA6 full-length cDNA-encoding plasmid (Fig. 3B). While there was no noticeable increase using 250 ng of NCOA6 plasmid DNA compared with its absence, the promoter activity was enhanced to 120 ± 8.4, 140 ± 12, and 198 ± 6.5% of control by 500 ng, 1 µg, and 2 µg of plasmid cotransfection (P < 0.05 compared with controls for 1 and 2 µg of plasmid DNAs). Taken together, these results substantiate our hypothesis that ASCOM complex is a major participant in FXR-mediated activation of BSEP promoter.

**Fxr interacts with NCOA6 NR boxes (LXXLL motif) 1 and 2 in GST pull-down assays and by mammalian two-hybrid analysis.** We next reasoned that if FXR/GR recruited ASCOM complex to BSEP/bsep, mrp2, and NTCP promoters, it is likely that there was direct protein-protein interaction between these proteins. To assess such an interaction, we carried out GST pull-down assays and mammalian two-hybrid analysis employing fragments of NCOA6 and FXR and its LBD and the results are shown in Fig. 4, B and C. Figure 4B shows the results of a
GST pull-down assay where $^{35}$S-FXR was incubated in vitro with fragments 1–6 (NCOA6 I to NCOA6 VI, schematically shown in Fig. 4A) of NCOA6 linked to GST. Shorter exposure of the autoradiograms shows that FXR interacts with NCOA6 III, V, and VI. The fragment V interaction was further enhanced by the addition of CDCA. Of note is the fact that both fragments III and V contain “LXXLL” motifs and hence are named NR boxes 1 and 2. Previous work (28, 32) using a variety of coactivators have shown that NRs interact with coactivators via this motif and mutation of this motif abolishes the binding. Longer exposure of the autoradiogram is shown in Supplemental Fig. S3. Figure 4B, bottom, shows a Coomassie blue-stained gel of the proteins for the various fragments of NCOA6 to indicate that all the corresponding proteins were expressed.

Fig. 2. RNAi-mediated silencing of MLL3 (A), MLL4 (B), and NCOA6 (C) in HepG2 cells results in significant downregulation of bile salt export pump (BSEP) and sodium taurocholate cotransporting polypeptide (NTCP) expression. Genome siRNA pools (Dharmacon and Santa Cruz Biotechnology) against MLL3, MLL4 (ALR), and NCOA6 were obtained and transfected into HepG2 cells as described. mRNA levels for BSEP and NTCP were quantified using qPCR in addition to those for MLL3, MLL4, and NCOA6. Protein levels of cognate genes were also analyzed using specific antibodies. A representative Western blot and relative protein amounts are shown at right. *P value <0.05, compared with control small interfering (si)RNA-treatment.
expressed as GST-fusion proteins. Lanes 1 and 3 represent molecular weight markers and the empty GST, respectively.

To obtain additional evidence for FXR binding to NCOA6, we performed two-hybrid analysis in a mammalian cell line and the results are depicted in Fig. 4C. The slight stimulation of reporter gene activity by pSG9 vector and by FXR-LBD in the presence of CDCA might represent background activity of the system. The subsequent bars show relative luciferase activity (relative to vector alone) of FXR-LBD alone and NCOA6 I-VI (in pACT vector) when cotransfected with pBINDFXR-LBD. Similar to the pull-down data, fragments NCOA6 III and V showed significantly (P < 0.05) enhanced interaction in the absence and presence of CDCA (for NCOA6 V only) that was significant compared with FXR alone and NCOA6 fragments I, II, IV, and VI. Constructs NCOA6 I, II, IV, and VI showed no difference in their stimulation of reporter activity compared with FXR-LBD alone (with and without ligand) and ranged from 8.5-to 10.3-fold compared with vector. Taken in conjunction with the GST pull-down analysis, these results confirm that FXR does indeed interact with NCOA6 by binding to both NR boxes 1 and 2 presumably leading to recruitment of “ASCOM” complex to the BSEP promoter locus.

ChIP analysis of HepG2 cells and mouse liver reveal recruitment of MLL3, NCOA6, and H3K4 trimethylation at the BSEP/mrp2/NTCP promoter loci with no recruitment in 3-day CBDL mouse livers. Having demonstrated that MLL3/MLL4 and NCOA6 are involved in BSEP transcriptional activation, we performed ChIP assays to show recruitment of MLL3 and NCOA6 and histone H3K4 trimethylation to BSEP/Bsep (human and mouse) and Mrp2 (in mouse) FXRE locus in hepatoma cells and in mouse liver. In addition, we also examined the recruitment of ASCOM complex to the GCRE locus in human NTCP promoter since previous studies (6, 15) have demonstrated such a recruitment to GR-target genes. Results of the ChIP data are shown in Fig. 5, A and B, for cells and Fig. 5, C, D, and E for liver tissue. Figure 5A shows a representative gel of a PCR reaction from a ChIP experiment highlighting recruitment of MLL3, NCOA6, and H3K4 trimethylation to BSEP FXRE in the presence and absence of CDCA in HepG2 cells. As would be expected, significantly diminished recruitment of the proteins were observed in the absence of ligand. Since there is endogenous synthesis of bile acids in HepG2 cells, it would be predicted that there would be some basal recruitment of these mediators to the BSEP locus even in the absence of added ligand. We also observed similar recruitment of ASCOM components to OSTS promoter FXRE, suggesting this could be a general mechanism of activation for other FXR target genes (see Supplemental Fig. S4). We were also able to demonstrate recruitment of ASCOM components to the GCRE locus in human NTCP promoter as seen in Fig. 5A, bottom, consistent with previous reports in the literature that GR target genes also recruit ASCOM complex (15). Figure 5B shows qPCR results of the ChIP assay of the BSEP FXRE locus showing a 19.5-, 9.5-, 12.3-, and 54.5-fold increase (P < 0.05, compared with the absence of ligand) in the signal relative to IgG control when antibodies to NCOA6, MLL3, H3K4, and RNA PolIII (as a positive control) were used for ChIP. Fold increases (relative to IgG) of 5.6, 1.05, 4.5, and 4.4 for NCOA6, MLL3, H3K4, and PolIII were obtained in the absence
of ligand (P < 0.05, compared with IgG except for MLL3). ChIP assay for MLL4 could not be carried out, as the commercially available antibodies were found unsuitable for the assay.

Although these studies used hepatoma cells, we wanted to verify that the recruitment of these coactivators also occurred in vivo in mouse liver and whether changes in their recruitment could explain downregulation of Bsep and Mrp2 during cholestasis. The data in Fig 5, C–E show a representative gel and qPCR quantitation of the mouse liver ChIP assay. The data in Fig. 5C reveal that NCOA6, MLL3, and PolII are recruited to Bsep and Mrp2 FXRE locus in the mouse promoter followed by H3K4 methylation. qPCR evaluation of the precipitates obtained with the specific antibodies in sham and 3-day CBDL
mouse liver are shown in Fig. 5, D and E, for Bsep and Mrp2, respectively. We chose the 3-day CBDL because previous studies (including our own: Ananthanarayanan M, Li Y, Suchy, FJ, unpublished observations) in rats and mice have shown downregulation of Bsep [at 5 days in mice by Park et al. (24)] and Mrp2 at 1–3 days post-CBDL (7, 8, 24). BSEP message has also shown to be downregulated in humans during cholestasis (26). Significantly enhanced recruitment (P < 0.05
relative to IgG) to the BSEP FXRE locus of 2.1-, 2.9-, 3.7-, and 9.4-fold over IgG for NCOA6, MLL3, PolII, and H3K4 methylation, respectively, was seen in sham-operated livers while no or “negative” enrichment was seen in the CBDL livers. For the Mrp2 FXRE locus, the enrichments were 2.1-, 3.2-, 3.2-, and 2.7-fold over IgG (P < 0.05 vs IgG) for NCOA6, MLL3, PolII, and H3K4 methylation, respectively, in the sham livers while no enrichment was observed in the livers of 3-day CBDL mice. As the FXRE site is not conserved in the human MRP2 while no enrichment was observed in the livers of 3-day CBDL mice. As the FXRE site is not conserved in the human MRP2 animals with various forms of cholestasis (11, 31), there is little information on how assembly of coregulators and histone modifications are involved in perpetuating and adapting to cholestasis (9, 29, 30). Several mechanisms are likely to be operative: 1) the production of coactivators may be impaired or induced in cholestasis, 2) their recruitment to the nucleosome may be disrupted by alterations in chromatin structure, and 3) there may be active recruitment of corepressors and histone deacetylases to the promoters of these genes.

In an initial effort to understand how the expression of coregulators that regulate transporters involved in bile formation may be altered in liver disease, a custom RT²PCR Profiler array was designed to obtain an unbiased view of changes during the cholestatic process induced by common bile duct ligation. The expression profile was composed of a panel of 84 genes that include many of the known liver coactivators, corepressors, and histone modifying enzymes (see gene list in Supplement Material). We realized that although many genes will be altered during cholestasis, most may have no role in regulation of liver transporters. However, these studies could also lead to the discovery of novel coactivators whose role in regulating FXR-dependent genes had not been established. An important limitation of this approach is that it only examines coregulator gene expression that changes in cholestasis. Alternatively, recruitment of a coactivator and its translocation into the nucleus may be impaired and/or there could be formation of a repressor complex without a change in expression of the individual components.

The RT²PCR Profiler array showed that H3K4 methyltransferase MLL3 was significantly downregulated in the CBDL livers. MLL3 has recently been implicated in regulation of LXR- and FXR-mediated gene expression(13, 14). MLL3 or its paralog MLL4 belongs to a steady-state complex called ASCOM (for ASC-2 complex) containing NCOA6 (ASC-2) that serves as a coactivator of multiple nuclear receptors and transcription factors. UTX, a H3K27 demethylase, is also part of this complex. NCOA6 binds to many nuclear receptors in a ligand-dependent manner through its two LXXLL motifs or NR boxes. The first LXXLL motif of NCOA6, located near the centrally located activation domain AD2, interacts with many nuclear receptors and in a recent study (13) showed relatively weak but specific interaction with the nuclear receptor FXR. LXXL-2 located towards the C-terminal region had been thought to be restricted in its specificity to LXR.

DISCUSSION

Ligand-induced activation of nuclear receptors for bile acids and other biliary constituents plays an essential role in regulation of genes involved in bile formation (22). This complicated process involves the cyclical assembly and disassembly of transcriptional complexes on DNA promoter elements (5). These multisubunit complexes contain a great variety and number of coregulator proteins and histone modifying enzymes that are recruited by NRs and modulate target gene activity (19). It is also clear that coactivators are differentially used by transcription factors, including NRs, in a cell-, tissue-, and promoter-specific manner (20, 21). Multiple complexes of coactivator proteins participate in local remodeling of chromatin and in recruitment and activation of RNA PolII. As the basic mechanisms and diversity of coregulators underlying ligand-dependent transcriptional activation by nuclear receptors has become better defined, it was natural to propose that their expression and function might be altered in disease. This has proven to be the case in cancer and in some genetic disorders (17, 23). However, although the expression of some nuclear receptors has been studied in humans and experimental animals with various forms of cholestasis (11, 31), there is little information on how assembly of coregulators and histone modifications are involved in perpetuating and adapting to cholestasis (9, 29, 30).

Fig. 6. Schema of the current working model for the role of ASCOM complex in FXR/GR-transactivation of BSEP/Bsep, Mrp2, and NTCP promoters. Ligand binding to FXRE/GCRE at the cognate promoter locus leads to recruitment of ASCOM complex to the response element because of the interaction between FXR/GR and NCOA6. MLL3/MLL4, which form part of the ASCOM complex then methylates histone H3K4 to the trimethylated state, which is an activation mark. This modification of histones results in transcriptional activation of BSEP/Bsep, Mrp2, and NTCP promoters. Conversely, downregulation of MLL3/MLL4 during cholestatic disease could lead to decreased recruitment of ASCOM complex resulting in downregulation of corresponding gene transcription of ASCOM components (such as MLL3/MLL4) resulting in decreased transporter mRNAs.

The down-regulation of MLL3 after 1 and 3 days of CBDL was confirmed by RT-PCR. Its paralog MLL4, which potentially could provide redundant or compensatory activity, was also decreased. NCOA6 was not represented on the RT²PCR Profiler array, but its expression at the mRNA level was not changed on analysis by qPCR. Thus a critically important function of the ASCOM complex in mediating histone H3K4 trimethylation, a mark for transcriptionally active chromatin, was disrupted by CBDL.
The importance of both NCOA6 and MLL3/4 for expression of bile acid transporters was examined in HepG2 cells. Knockdown of MLL3, MLL4, or NCOA6 expression using either siRNA or siRNA adenoviruses led to a significant decrease in mRNA and protein levels for these components of the ASCOM complex and to a marked decrease in BSEP and NTCP mRNA levels. Knockdown of either MLL3 or MLL4 led to a similar decrease in BSEP and NTCP mRNA levels, and siRNA knockdown of both methyltransferases (MLL3/4) was additive. Conversely, cotransfection of varying amounts NCOA6 into HepG2 cells along with the BSEP promoter linked to luciferase, FXR, RXR plasmids, and ligand stimulation led to a dose-dependent increase in the luciferase activity. The effects of NCOA6 and MLL3 knockdown on NTCP expression are of interest, as NCOA6 has been shown recently to interact with HNF4α (27), which we have shown previously to be critical for activation of the mouse Ntcp gene (10).

While our article was in preparation, another study (13) showed that the first LXXLL motif of NCOA6 interacted weakly, but specifically, with FXR in a GST pull-down and yeast two-hybrid assays. In contrast, we found both the LXXLL-1 and LXXLL-2 motifs bind to FXR in the GST pull-down assay, but binding to LXXLL-2 occurred more avidly and was enhanced in the presence of CDCA. These results were confirmed using the mammalian two-hybrid analysis. The reasons for this discrepancy with the work of Kim et al. (13) are not entirely clear, although it might be related to the fact that we used a mammalian two-hybrid system while Kim et al. used a yeast system. Our data thus suggest an important role for both LXXLL-1 and 2 of NCOA6 in tethering the ASCOM complex to FXR.

Next, we examined whether NCOA6 and associated methyltransferases MLL3 and MLL4 were recruited to the promoters of the FXR-target genes BSEP/Bsep, Mrp2, and OSTA and NTCP, a target gene for GR (6), using ChIP assays. In HepG2 cells NCOA6, MLL3 were recruited to FXR response elements (FXRE) of BSEP and OSTA (see Supplemental Material) in the presence of CDCA and to the GCARE in NTCP promoter confirming previous reports for other GR targets (15). H3K4 trimethylation of the histoneH3 surrounding BSEP-FXRE and NTCP-GCARE was also detected (Fig. 5). We then examined the recruitment of these ASCOM complex components to the mouse liver FXRE in Bsep and Mrp2 promoters. While we observed significant enrichment of the proteins in 3-day sham livers, no such enrichment was seen in the CBDL livers, suggesting that the downregulation of these genes observed during cholestasis in some studies in rodents and humans (7, 8, 24, 26) can be partly due to the decreased recruitment resulting in altered H3K4 methylation at these loci. Our data are consistent with preliminary studies in Mll3-null mice that exhibit (due to redundancy between Mll3 and Mll4) symptoms of mild cholestasis (Dr. Jae Woon Lee, Baylor College of Medicine, Houston, personal communication). Taking these data together, we have schematically illustrated in Fig. 6 our current working model of how ASCOM complex associates with its partners MLL3/MLL4 at the FXRE/GCARE site and activates transcription on ligand binding and the changes that occur during cholestasis.

A limitation of our work is that current approaches to the study of coactivators and epigenetic modification of histones fail to recognize the dynamic nature of changes that occur rapidly as protein complexes are recruited and others are released on ligand binding. These processes could be disrupted in disease even if the expression of these proteins is not altered and their presence on the promoter of a gene of interest is not altered in ChIP assays.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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