Transcriptional corepressor SHP recruits SIRT1 histone deacetylase to inhibit LRH-1 transactivation

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ABSTRACT

Orphan nuclear receptor Small Heterodimer Partner (SHP; NR0B2) is a transcriptional corepressor of a wide variety of nuclear receptors (NRs). Here, we report that SHP recruits SIRT1, a class III histone deacetylase, in an NR-specific manner to inhibit transcriptional activity. SHP interacts and co-localizes specifically with SIRT1 in vivo and inhibition of SIRT1 activity leads to a recovery from the intrinsic repressive activity of SHP but not of DAX1. Furthermore, we observed that SIRT1 does not deacetylate SHP or LRH1. However, inhibition of either SIRT1 or SHP significantly diminished the repressive effect of SHP on LRH1 transactivity. LRH1-mediated activation of CYP7A1 and SHP gene transcription was significantly repressed by both SHP and SIRT1 whereas inhibition of SIRT1 activity by inhibitors or dominant negative SIRT1 or knockdown of SHP led to a significant release of this inhibitory effect. ChIP assays revealed that SHP recruits SIRT1 on LRH1 target gene promoters and SIRT1 deacetylated template-dependent histone H3 and H4 to inhibit transcription of LRH1 target genes. Finally, we demonstrated that inhibition of SIRT1 activity significantly reversed SHP-mediated inhibition of bile-acid synthesis by LRH1 overexpression, thereby suggesting a novel mechanism of SHP-mediated inhibition of LRH1-dependent bile-acid homeostasis via recruitment of SIRT1 histone deacetylase protein.

INTRODUCTION

The orphan nuclear receptor small heterodimer partner (SHP) protein is a unique member of the mammalian nuclear receptor (NR) superfamily that lacks a conventional DNA-binding domain but contains a putative ligand-binding domain (1). SHP is highly expressed in liver and predominantly functions as a transcriptional corepressor of a wide array of NRs and transcription factors (2,3). Recent studies indicate that SHP may repress its targets via direct binding and/or interference with the coactivator interaction interface of its target NRs, or by antagonizing coactivator functions on NRs via recruiting corepressor complexes that include histone deacetylases (HDAC) 1, 3 and 6, Sin3A, and mammalian histone methyltransferase (G9a) (2–6). SHP interacts and regulates transcriptional activities of a large number of NRs, including both ligand regulated receptors, such as estrogen receptor (ER), GR, TR, AR, RAR and RXR (retinoid X receptor), and orphan receptors, such as LRH-1 (liver receptor homolog 1), HNF-4 (hepatic nuclear factor 4), Nur77, ERR, CAR, LXR, PPAR and thus, has been implicated in regulating diverse biological activities, including cholesterol/bile acid (BA), lipid and glucose/energy metabolic pathways (2,3).

The sirtuins are a highly conserved family of NAD-dependent enzymes that regulate lifespan in lower organisms (6–8). Recently, the mammalian sirtuins have been connected to an ever widening circle of activities that encompass cellular stress resistance, genomic stability, tumorigenesis and energy metabolism. The founding member of the sirtuin family, yeast Sir2 (silent information regulator 2), was originally isolated in a screen for silencing factors (8). To date, seven mammalian homologs have been identified, with mammalian SIRT1 evolutionarily closest to yeast Sir2. Cell biological studies have further demonstrated different subcellular compartments for each family member, with SIRT1, SIRT6 and SIRT7 being nuclear proteins, SIRT3, SIRT4 and SIRT5 mitochondrial proteins, and SIRT2 being found both in the nucleus and the cytoplasm, in a cell and tissue-dependent context (6,7). SIRT1 is a nuclear class III deacetylase and
regulates homeostatic gene-expression programs by deacetylating key transcription factors and coregulators including LXR\(\text{a}\), PPAR\(\gamma\), FXR, PGC-1\(\text{a}\), p300/CBP, Foxo1, NF-xB and p53 (6–8). The NAD-dependent deacetylase SIRT1 has been shown to regulate lipid and carbohydrate metabolism and has been shown to extend life span in several species (6–8).

SHP has been reported to play a key role in the negative feedback regulation of cholesterol 7\(\alpha\) hydroxylase gene (CYP7A1) expression in the liver (10,11). This hepatic enzyme catalyzes the first and rate-limiting step of the neutral pathway for the conversion of cholesterol into BAs and thus plays a crucial role in enterohepatic cholesterol-BA homeostasis (12). BAs also feedback-regulate BA biosynthesis, where activated FXR induces SHP gene expression, and SHP in turn inhibits LRH-1 and/or HNF4\(\alpha\) activities on the BA response elements (BAREs) of CYP7A1 promoter (10–12). Previous studies have suggested that SHP mediates recruitment of mSin3A-Swi/Snf and Gp2 (G protein pathway suppressor 2), a subunit of the NR corepressor (N–CoR) complex, to the CYP7A1 promoter, resulting in chromatin remodeling and gene repression (5.6). A recent study has demonstrated that SIRT1 knockdown in Type II diabetes mellitus (T2DM) rat model is associated with significant induction of CYP7A1 gene expression, thereby indicating a crucial role of SIRT1 in regulating hepatic cholesterol metabolism via modulation of CYP7A1 transcriptional activity (13). Although the key regulatory transcription factors like FXR, LRH-1 and SHP, and chromatin remodeling factor like SIRT1 have been identified, the precise molecular events that occur at the level of CYP7A1 promoter chromatin and gene repression by a possible involvement of SHP and SIRT1 are yet to be studied.

In the current study, we examined the mechanism of regulation of BA homeostasis by SHP and SIRT1. Using CYP7A1 gene, the key and rate-limiting enzyme for BA homeostasis, as a major target, we studied the repressive effect of SHP and SIRT1 on LRH1-mediated CYP7A1 transactivation at the native chromatin level in HepG2 cells. We analyzed the effect of SHP-SIRT1 on chromatin structure at the endogenous human CYP7A1 promoter and the possible involvement of chromatin remodeling and histone de-acetylation. Here, we report that SHP interacts with and recruits SIRT1 histone deacetylase to the endogenous human CYP7A1 promoter and represses LRH1 activity, thereby highlighting a novel mechanism of SHP repressive action which involves recruitment of novel factors on its targets and also elucidating the role of SIRT1 in regulating LRH1 transactivation via SHP.

**MATERIALS AND METHODS**

**Chemicals and antibodies**

Nicotinamide (NAM) was obtained from Calbiochem and all other chemicals were obtained from Sigma. Antibodies used in this work were as follows: anti-Flag M2 (Stratagene), anti-HA (12CA5, Roche), anti-GST (Santa Cruz), anti-SHP (H160, Santa Cruz), and anti-SIRT1, anti-acetyl-histone H3 (Ac-H3), anti-acetyl-histone H4 (Ac-H4), anti-acetylated lysine (Ac-Lysine), anti-Myc, anti-tubulin, anti-HDAC1 and anti-HDAC3 antibodies were obtained from Cell Signaling Technology (CST). The primary antibodies were used at a dilution ranging from 1:200 to 1:1000 in western blot analysis, and at a dilution of 1:200 in immunoprecipitation.

**Plasmid and DNA construction**

The plasmids of pCMV–\(\beta\)-gal, pSG5–HA–ERR\(\gamma\), pcDNA3–HA–LRH1, pSG5–RAR\(\alpha\), pcDNA3–HA–Nur77, pcDNA3–HA–CAR, pcDNA3–ER\(\alpha\), pcDNA3–HA–SHP, pcDNA3–Flag–SHP, pcDNA3–Flag–SIRT1, pcDNA3–Myc–SIRT1, pcDNA3–Myc–SIRT1H363Y, pcDNA3–HA–HNF4\(\alpha\), pEGFP–SHP (GFP–SHP), Gal4–DBD, Gal4DBD–SHP, Gal4DBD–DAK1, Gal4DBD–LRH1, the luciferase reporter constructs of Sfl4–Luc, RARE–Luc, NurRE–Luc, (NR)\(_3\)–TK–Luc, ERE–Luc, Gal4–TK–Luc (Gal4–Luc) and the human SHP (hSHP–Luc) and the human CYP7A1 (hCYP7A1–Luc) gene promoter luciferase constructs have been described elsewhere (14–19). pcDNA3–Flag–SIRT6,–SIRT7 has been described elsewhere (17). pEBG, pEBG–SHP (1–260, full-length) constructs has been described elsewhere (14,15). Deletion constructs of pEBG–SHP (1–148), (1–92), (160–260) and (92–148) were constructed from pEBG–SHP (1–260) construct and pcDNA3–Flag–SIRT1 (1–499), (1–243), (243–747) and (499–747) were constructed from pcDNA3–Flag–SIRT1 (1–747, full length) construct and all constructs were confirmed by DNA sequencing. pSUPER and pSUPER–siSHP constructs were kindly provided by Dr J.K. Kemper. pSUPER–siSIRT1 and pSUPER–siHDAC1 constructs had been described elsewhere (17).

**Cell culture, transient transfection and luciferase assay**

HepG2, HEK293T (293T) and HeLa cells were obtained from the American Type Culture Collection. Maintenance of cell lines and transient transfection assays were performed using Lipofectamine2000 transfection reagent (Invitrogen) according the manufacturer’s instructions as described elsewhere (15). Briefly, cells were transfected with indicated reporter plasmids together with expression vectors encoding various transcription factors or treated with various chemicals. Total cDNA used for each transfection was adjusted to 1 \(\mu\)g/well by adding appropriate amount of empty vector and pCMV–\(\beta\)-gal plasmid was used as an internal control. Cells were harvested 40–48 h post-transfection for luciferase and \(\beta\)-galactosidase assays. The luciferase activity was normalized to \(\beta\)-galactosidase activity and expressed as relative luciferase units (RLU).

**In vitro and In vivo GST pulldown assay, coimmunoprecipitation (Co-IP) assay and western blot analysis**

**In vitro** and **In vivo** GST pull-down experiments were performed as described elsewhere (15). In brief, 293T cells were transfected in 60-mm dishes with the indicated plasmids. After 48 h of transfection, the whole-cell extracts were harvested and subjected to GST pulldown assay and coimmunoprecipitation (Co-IP) analysis. The complexes were resolved on SDS-PAGE and the antigens were detected by immunoblotting.
were prepared and equal amounts of total protein were used for in vivo GST pull-down assays followed by western blot analysis with indicated antibodies. Co-IP and western blot analysis were performed as described previously (15). For Co-IP from tissue extracts, C57BL/6J mice \( (n = 3) \) were maintained ad libitum for 3 days and sacrificed. Liver tissue samples were used for Co-IP assay. In western blot analysis of immunoprecipitated proteins, conventional HRP-conjugated anti-rabbit IgG was replaced with rabbit IgG TrueBlot (eBioscience, #18-8816) to minimize/eliminate signal interference by the immunoglobulin heavy and light chains.

Confocal microscopy

Confocal microscopy assays were carried out as described elsewhere (15). In brief, the HeLa cells grown on gelatin-coated coverslips were transfected with indicated plasmids using Lipofectamine2000 transfection reagent (Invitrogen) according to the manufacturer’s instructions. After 24 h of transfection, the cells were fixed with 2% formaldehyde followed by immunostaining and finally the cells were subjected to observation by confocal microscopy.

Preparation of recombinant adenovirus

For ectopic expression of the genes, adenoviral delivery system was used. Briefly, the cDNA encoding HA–LRH1 was cloned into pAdTrack shuttle vector. Recombination of AdTrack–CMV–HA–LRH1 with adenoviral gene carrier vector was performed by transformation into pretransformed AdEasy-BJ21 competent cells. Adenoviruses (Ad) encoding GFP only (Ad–GFP), Ad–SHP, Ad–sSHP, Ad–SIRT1 and Ad–SIRT1H355A were described elsewhere (17,18).

RNA interference

Knockdown of SHP, SIRT1 and HDAC1 was performed using the pSuper vector system (17,18). HepG2 cells were transfected with siRNA constructs using Lipofectamine2000 (Invitrogen) according to the manufacturer's instructions. siRNA treated cells were subjected to reverse transcription PCR (RT–PCR) for confirmation of knockdown, and siRNA constructs were further used for the transient transfection assay as indicated in the figure legends.

Reverse transcriptase PCR and quantitative real-time PCR analysis

Total RNA was isolated using the TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. The mRNAs of CYP7A1 and SHP were analyzed by reverse transcriptase PCR (RT–PCR) or quantitative real-time PCR (qPCR) as indicated. DNA samples from total RNA reverse transcription or from chromatin immunoprecipitation (ChIP) assays served as the templates for qPCR experiments, which were performed with QuantiTect SYBR GreenER PCR Kit (Qiagen) and the Roter-Gene 6000 real-time PCR system (Australia) in triplicate. Median cycle threshold values were determined and used for analysis. ChIP data are presented as fold enrichments over the values obtained with immunoprecipitations using control antibody (IgG). mRNA expression levels were normalized to those of β-actin (ACTB). The RT–PCR and qPCR primer sequences are available upon request.

Chromatin immunoprecipitation assay

Formaldehyde cross-linking of cells, chromatin immunoprecipitations (ChIPs), and real-time PCR analyses were performed as described elsewhere (17). The immunoprecipitates were analyzed by qPCR. ChIP data are presented as fold enrichments over the values obtained with immunoprecipitations using control (IgG). Primer sequences are available upon request.

BA analysis

After treating cells (as described in figure legend of Figure 6B) in serum-free media, the media was collected for analysis of BAs. Sep–Pak C18 reversed phase cartridges (Waters Associates, Inc., Milford, MA) were used for BA extraction from media as described elsewhere (20). Total BA concentration was analyzed by enzymatic 3α-hydroxysteroid dehydrogenase method using total BA assay kit (Bio-quant Inc., San Diego, CA) according to the manufacturer’s instruction.

Statistical analysis

Student's t-test was performed using GraphPad Prism 4.0 and results were considered to be statistically significant when \( P < 0.05 \).

RESULTS

SHP differentially recruits SIRT1 or HDAC in an NR-specific manner

SHP has been demonstrated to repress transcriptional activity of several liganded and orphan NRs that include LRH1, ERRγ, RARz, Nur77, CAR and ERz (2,3). It has also been shown that SHP recruits various chromatin remodeling factors including HDACs, Swi/Snf cofactor complex, and GPS2 to exhibit transcriptional corepressor activity on its targets (4–6,21). To elucidate the exact mechanism involved behind involvement of SHP in chromatin remodeling and repression of transcriptional activity of its targets we first performed transient transfection assay with SHP and LRH1, ERRγ, RARz, Nur77, CAR and ERz in the presence or absence of inhibitors for class I and II HDACs, TSA (trichostatin A) and class III HDAC (SIRT1), NAM (Figure 1A–C). Interestingly, we found that the repressive pattern of SHP and the recruitment of HDACs (class I or II and class III) are dependent on the target transcription factor involved. Our result indicates that SHP-mediated repression of LRH1, ERRγ and RARz (Figure 1A) was not reversed by TSA, whereas NAM treatment significantly released the inhibitory effect of SHP on these transcription factors. On the other hand, SHP-mediated repression of Nur77, CAR and ERz was reversed upon TSA treatment, whereas NAM
showed no effect on this repressive pattern (Figure 1B). Next, RNA interference (siRNA) demonstrated that knockdown of endogenous SIRT1 (by siSIRT1), but not knockdown of HDAC1 (by siHDAC1), significantly released SHP-mediated repression of LRH1 transactivity (Figure 1C, left). Similarly, like our previous observation (Figure 1B), siHDAC1 cotransfection, but not siSIRT1 cotransfection, significantly reversed SHP-mediated repression of CAR transactivity (Figure 1C, right). RT–PCR analysis of SIRT1 and HDAC1 gene expression confirmed a strong inhibitory effect of siSIRT1 and siHDAC1 on SIRT1 and HDAC1 gene expression respectively (Figure 1D). Overall, our initial results suggest that SHP-mediated repression of target transcription factor

Figure 1. Utilization of differential repressive mechanism by SHP. Reporter assays (A–C) were performed as described in ‘Materials and Methods’ section. HepG2 cells were cotransfected with indicated reporter luciferase vectors (100 ng each) and expression vectors (200 ng each). β-gal expression vector (100 ng) was used as an internal control for each transfected well. Cells were treated with RA (1 μM; panel A, right) or E2 (10 nM; panel B, right) for 12 h followed by TSA (100 nM) or NAM (20 μM) treatments for further 12 h in the absence or presence of RA or E2. Cells were treated only with TSA or NAM (panel A, B, left, middle) for 12 h prior to the measurement of luciferase activity. (D) Effect of siRNAs of siSIRT1 and siHDAC1 on the expression of SIRT1 and HDAC1 respectively. HepG2 cells were transfected with pSuper- siSIRT1 or siHDAC1 or pSuper [control (−)] and cells were collected for RNA isolation, by RT–PCR analysis, 72 h post-transfection. β-actin (ACTB) gene expression was shown as control. Data is representative of at least three independently performed experiments.
activity is dependent on the differential recruitment of HDAC (class I and II histone deacetylase) or SIRT1 (class III histone deacetylase).

SHP interacts and colocalizes with SIRT1 in vivo

Since cell biological studies have demonstrated that among sirtuins family members (SIRT1-7), SIRT1, SIRT6 and SIRT7 are localized predominantly/exclusively in the nucleus (7–9), we next analyzed the interaction pattern of SIRT1, SIRT6 and SIRT7 with NR SHP (Figure 2A). In vivo GST pull-down assay demonstrated that exogenous SHP interacted specifically with exogenous SIRT1 (left panel) but not with either SIRT6 or SIRT7 (right panel). To reconfirm the interaction between SHP and SIRT1, we performed coimmunoprecipitation (Co-IP) assay to detect endogenous interaction between SHP and SIRT1 in human HepG2 cells and C57BL/6J mouse liver extracts (Figure 2B). Co-IP with specific antibody for SHP and SIRT1 detected endogenous interaction and complex formation between SIRT1 and SHP in both HepG2 cells (left panel) as well as in mouse liver tissue extracts (right panel). Next, to investigate whether SHP and SIRT1 are co-localized in the same subcellular compartment, we performed confocal microscopy in HeLa cells (Figure 2C). Our results demonstrated that both SHP and SIRT1 are co-localized in the nucleus as can be evidenced from the merged image. Collectively, these results demonstrate that SHP interacts and co-localizes with SIRT1 in vivo.

Interaction domain mapping of SHP and SIRT1

Next we attempted to ascertain the interaction domain between SHP and SIRT1 using in vivo GST pull-down assays (Figure 3). The schematic diagrams (Figure 3A and B, top) depict the domains into which both SHP and SIRT1 protein can be broadly divided. First, using
several deletion mutant constructs for SIRT1 protein, we found that SHP interacted with the C-terminus domain of SIRT1 protein (Figure 3A, bottom and C), suggesting that SIRT1 C-terminus domain is necessary for interaction with SHP. Next, we used different SHP mutant constructs and our results demonstrated that the N-terminus domain of SHP was responsible for interaction with SIRT1 protein (Figure 3B, bottom and D). Overall, these results demonstrated that the amino-acid residues 499–747 of SIRT1 protein, corresponding to the C-terminus domain, is required for interaction with SHP whereas the amino-acid residues 1–92 of SHP protein is sufficient to interact with SIRT1.

SHP recruits SIRT1 to inhibit LRH1 transcriptional activity

NR DAX-1 is the closest relative to SHP in the NR family and both share considerable structural and functional similarities and have been previously demonstrated to possess intrinsic repressive effect (2,3). Therefore, we tried to investigate whether the involvement of SIRT1 is specific for SHP-mediated transcriptional repressive activity. Using Gal4-fusion proteins of SHP and DAX-1, we performed transient transfection assays in the absence or presence of siSIRT1 and SIRT1 inhibitor NAM (Figure 4A). Both SHP and DAX-1 showed significant repression of basal Gal4 luciferase reporter (Gal4–Luc) transactivity. However, upon cotransfection with siSIRT1 (left) or treatment with NAM (right) we found that the intrinsic repressive effect of SHP was significantly reversed whereas no reversal of DAX-1 intrinsic repressive effect was observed in any of those treatments. These results suggest that SIRT1 plays an important role in mediating the repressive effect of SHP specifically.

Previous studies have shown that SIRT1 interacts and deacetylates several transcription factors (7–9). Thus, we were interested to know whether the interaction of SIRT1 with SHP (Figure 2) has any effect on the acetylation status of SHP protein. We performed Co-IP assays to determine the acetylation/deacetylation level of SHP by SIRT1 (Figure 4B) using specific antibody to detect acetylated lysine residues (Ac-Lysine). Interestingly, no significant change in the acetylation/deacetylation level...
of SHP was observed upon co-transfection with SIRT1, indicating that SIRT1 may not directly affect the acetylation/deacetylation status of SHP protein.

Next, we tried to ascertain the involvement of SHP and SIRT1 on LRH1-mediated transcriptional activity by transient transfection assay using Gal4–luc reporter and Sft4–luc (containing multiple LRH1/SF-1-binding elements) reporter constructs (Figure 4C and G). Gal4-fused LRH1 protein significantly activated the reporter luciferase activity and cotransfection with SHP

Figure 4. SHP recruits SIRT1 to inhibit LRH1 transactivation. Reporter assays (A, C and G) were performed as described in ‘Materials and Methods’ section. HepG2 cells were cotransfected with indicated expression vectors (200 ng each) and Gal4–Luc (A and C) or Sft4–Luc (G) luciferase reporter vector (100 ng). β-gal expression vector (100 ng) was used as an internal control for each transfected well. Cells were treated with NAM (20 μM), resveratrol (100 nM) or piceatannol (20 μM) for 12 h prior to the measurement of luciferase activity. Data is representative of at least three independently performed experiments and shown as mean ± SD; *P < 0.05 using Student’s t-test. (B, D and F) HepG2 cells were cotransfected with Flag–SHP and Myc–SIRT1 (B) or HA–LRH1 (D), HA–HNF4α (F) and Flag–SIRT1. Forty-eight hours post transfection, Flag–SHP, HA–LRH1 or HA–HNF4α were immunoprecipitated and analyzed by western blot analysis using indicated antibodies. Data is representative of at least three independently performed experiments. (E) In vitro GST pull-down assays. 35S-radiolabeled SIRT1 protein (upper panel) or 35S-radiolabeled SHP protein (lower panel) were incubated with GST, or GST–LRH1 fusion proteins. The input lane represents 10% of the total volume of in vitro-translated proteins used for binding assay. Protein interactions were detected via autoradiography.
dramatically repressed this transactivation (Figure 4C, left). The repressive effect of SHP was significantly reversed by either cotransfection with siSIRT1 expression vector or upon treatment with SIRT1 inhibitor NAM. NAM treatment or siSIRT1 cotransfection, in the absence of overexpression of SHP, also significantly activated Gal4–LRH1 mediated transactivity. Conversely, treatment with SIRT1 activators, Resveratrol or Piceatannol, showed similar repressive effect on Gal4–LRH1 transactivation (right), similar to SHP. Cotransfection with siSHP expression vector dramatically reversed the strong repressive effect of SIRT1 activators on Gal4–LRH1 transactivation. We further confirmed these effects in Sft4–luc reporter constructs (Figure 4G) using wild-type SIRT1 and a deacetylase-defective SIRT1 (H363Y) mutant (17) on LRH1 transactivation (left). Overexpression of SIRT1 alone inhibited LRH1 activity (~70–80%) which was significantly released by siSHP overexpression. However, the dominant negative SIRT1 mutant SIRT1H363Y showed no observable effect of LRH1 transactivity, thereby suggesting that SIRT1 deacetylase activity is needed for the repression of LRH1 by SHP. Finally, endogenous SIRT1 inhibition by siSIRT1 or NAM significantly recovered LRH1 activity from SHP repressive effect in Sft4–luc (right), similar and consistent with our previous observations (Figure 4C, left).

SHP plays a well-documented role in inhibiting LRH1-mediated transcriptional activation (10–12). Knockdown of endogenous SIRT1 has also been demonstrated to increase LRH1 gene transcription (13). Consistent with these reports, our earlier observations (Figure 1A and C, left) suggest that SIRT1 is involved, at least in part, in the SHP-mediated repression of LRH1 transcriptional activity. Therefore, initially we investigated the acetylation/deacetylation level of LRH1 protein by SIRT1 (Figure 4D and F). Co-IP assay results using cotransfection of LRH1 or HNF4α (positive control) in the absence or presence of SIRT1 clearly demonstrated that SIRT1 has no observable effect on the acetylation/deacetylation status of LRH1 protein (Figure 4D) but physically interacts with and deacetylated HNF4α protein (Figure 4F). Interestingly, we could not detect SIRT1 protein after immunoprecipitation with anti-HA antibody (for HA–LRH1), suggesting that LRH1 does not physically interact with SIRT1. Additionally, we performed in vitro GST pull-down assays using 35S-labeled in vitro translated SHP and SIRT1 proteins and GST-fused LRH1 protein and our results demonstrated that LRH1 directly interacts with SHP but not with SIRT1 (Figure 4E). Taken together, these results indicate that SHP recruits SIRT1 and utilizes SIRT1 deacetylase activity to inhibit LRH1 mediated transcriptional activation.

SIRT1 potentiates SHP-mediated repression of LRH1 target gene transcription

SHP is a key player in hepatic BA biosynthesis via negative regulation of CYP7A1 mainly by repression of LRH1 (10–12). LRH1 is also a well known activator of SHP gene transcription (10,11), thereby, leading to an auto-regulatory loop of gene regulation by SHP. Thus, we attempted to elucidate the role of SHP–SIRT1 interaction in the inhibition of LRH1 target gene transcription (Figure 5). Using human CYP7A1 and SHP gene promoter luciferase constructs, we performed transient transfection assay in the absence (Figure 5A) or presence of LRH1 overexpression (Figure 5B). The basal promoter activity was significantly increased upon endogenous knockdown of SHP (by siSHP) or by SIRT1 knockdown using siSIRT1 or NAM treatment (Figure 5A), thereby suggesting the involvement of both SHP and SIRT1 in transcriptional repression of CYP7A1 and SHP gene promoter activity, possibly via inhibition of several transcription factors, including LRH1, which are common targets of both SHP and SIRT1, like HNF4α. Next, we tried to evaluate specifically the LRH1-mediated effect on these gene promoters and the role of SHP and SIRT1. LRH1 overexpression led to a significant activation of these gene promoters (~5–7-fold). Both SHP and SIRT1 independently inhibited the promoter transactivation by LRH1 and endogenous knockdown of SIRT1 or SHP significantly reversed the repressive effect of SHP or SIRT1, respectively (Figure 5B). Expectedly, repression of CYP7A1 and SHP promoter transactivation by SHP overexpression was released upon treatment with NAM, and resveratrol-mediated inhibition of the promoter activities was reversed by siSHP cotransfection (Figure 5B).

Next, we examined the changes in the mRNA levels of CYP7A1 and SHP genes under basal condition (Figure 5D) or using adenovirus (Ad) LRH1 infection with combinatorial treatments of either Ad–SHP infection in absence or presence of Ad–SIRT1H355A (dominant negative SIRT1) (17) or NAM as well as Ad–SIRT1 infection with or without Ad–siSHP infection (Figure 5E). First, we confirmed the protein expression of LRH1, SIRT1 and SHP after the respective adenovirus infection (Figure 5C). Under basal condition, Ad–SHP and Ad–SIRT1 significantly repressed CYP7A1 and SHP gene expression and these repressive effects were recovered considerably upon co-infection with Ad–SIRT1H355A or NAM treatment (with Ad–SHP) as well as by Ad–siSHP (with Ad–SIRT1) (Figure 5D). These results demonstrate a similar trend to the promoter activity observed in Figure 5A and suggests the possibility of repressive effect of SHP and SIRT1 on various transcription factors which are known to induce CYP7A1 and SHP gene expression. Therefore, we attempted to investigate the effect of SIRT1 and SHP in LRH1-specific induction of CYP7A1 and SHP gene expression (Figure 5E). RT–PCR analysis (Figure 5E, top) and qPCR quantification (Figure 5E, bottom) results clearly demonstrated the induction of CYP7A1 and SHP mRNA levels after Ad–LRH1 infection. Subsequently, Ad–SHP infection led to a dramatic decrease in CYP7A1 and SHP mRNA levels. Ad–SIRT1H355A infection or NAM treatment released the Ad–SHP mediated repression of CYP7A1 as well as SHP mRNA level. Similarly, Ad–SIRT1 co-infection with Ad–LRH1 significantly repressed mRNA level of both CYP7A1 and SHP genes and knockdown of endogenous SHP levels by Ad–siSHP led to a recovery in the CYP7A1
mRNA expression but, had no observable effect on SHP mRNA level. Taken together, these results suggest that SHP and SIRT1 works co-operatively to inhibit LRH1-mediated target gene induction, though, under basal conditions the role of various other transcription factors which are reported to be common target of both SHP and SIRT1, like HNF4α, cannot be ruled out completely.

Figure 5. Involvement of SHP and SIRT1 in the repression of LRH1 target genes. Reporter assay (A and B) was performed as described in ‘Materials and Methods’ section. HepG2 cells were cotransfected with luciferase constructs of human CYP7A1 (hCYP7A1–Luc) and human SHP (hSHP–Luc) gene promoters (100 ng each) and indicated expression vectors (200 ng each). β-gal expression vector (100 ng) was used as an internal control for each transfected well. Cells were treated with NAM (20 μM) or resveratrol (Resv, 100 nM) for 12 h prior to the measurement of luciferase activity. (C) HepG2 cells were transfected with pcDNA3 only (−) or pcDNA3–HA–LRH1 (400 ng each) or infected with indicated adenovirus vectors (50 MOI) for 36–72 h and cells extracts were for western blot analysis using indicated antibodies. (D and E) HepG2 cells were treated with indicated adenovirus vectors (50 MOI) for 36–72 h. Twelve hours prior to RNA extraction cells were treated with NAM (20 μM, lane 3 and 6) and total RNA isolated were used for RT–PCR (top) and qPCR (bottom) analysis of CYP7A1 and SHP gene expression. β-actin (ACTB) gene expression was shown as control. Data is representative of at least three independently performed experiments and shown as mean ± SD; *P < 0.05 using Student’s t-test.
SHP recruits SIRT1 and utilizes SIRT1 deacetylase activity to inhibit LRH1-mediated gene transcription

Since gene repression is often associated with decreased histone acetylation (22), we determined whether the recruitment of SIRT1 by SHP results in template-associated histone (H3 and H4) deacetylation. HepG2 cells were treated with indicated adenovirus vectors (50 MOI) for 36–72 h. Twelve hours prior to preparation of cell lysates for ChIP assay cells were treated with NAM (20 μM). Chromatin fragments were prepared and immunoprecipitated with the indicated specific antibodies. DNA fragments covering BARE-I and BARE-II element on CYP7A1 (left) and LRH1-binding regions on SHP promoter (right) were PCR-amplified as described in the ‘Materials and Methods’ section. HepG2 cells were infected with adenovirus vectors as indicated for 36–72 h and for the last 12 h cells were treated with NAM (20 μM) as indicated. Media was collected for total bile synthesis using Sep-Pak cartridges as described in the ‘Materials and Methods’ section. Data is representative of at least three independently performed experiments and shown as mean ± SD; *P < 0.05 using Student’s t-test. (C) HepG2 cells were infected with adenovirus vectors as indicated for 36–72 h and for the last 12 h cells were treated with BA (50 μM) as indicated and total RNA isolated were used for RT–PCR (top) and qPCR (bottom) analysis of CYP7A1 mRNA level. β-actin (ACTB) gene expression was shown as control. Data is representative of at least three independently performed experiments and shown as mean ± SD; *P < 0.05 versus untreated, **P < 0.05 versus BA treatment and #P < 0.05 versus adenovirus infected lanes alone, using Student’s t-test. (D) Schematic representation of an auto-regulatory loop controlling the expression of SHP by LRH1 and inhibition of LRH1 activity by SHP itself via recruitment of SIRT1 histone deacetylase activity on target gene promoters of LRH1.

Figure 6. SHP recruits SIRT1 deacetylase to inhibit LRH1-mediated target gene activation. (A) The recruitment of SIRT1 by SHP on CYP7A1 (left) and SHP (right) gene promoters is associated with template-associated histone (H3 and H4) deacetylation. HepG2 cells were treated with indicated adenovirus vectors (50 MOI) for 36–72 h. Twelve hours prior to preparation of cell lysates for ChIP assay cells were treated with NAM (20 μM). Chromatin fragments were prepared and immunoprecipitated with the indicated specific antibodies. DNA fragments covering BARE-I and BARE-II element on CYP7A1 (left) and LRH1-binding regions on SHP promoter (right) were PCR-amplified as described in the ‘Materials and Methods’ section. (B) HepG2 cells were infected with adenovirus vectors as indicated for 36–72 h and for the last 12 h cells were treated with NAM (20 μM) as indicated. Media was collected for total bile synthesis using Sep-Pak cartridges as described in the ‘Materials and Methods’ section. Data is representative of at least three independently performed experiments and shown as mean ± SD; *P < 0.05 using Student’s t-test. (C) HepG2 cells were infected with adenovirus vectors as indicated for 36–72 h and for the last 12 h cells were treated with BA (50 μM) as indicated and total RNA isolated were used for RT–PCR (top) and qPCR (bottom) analysis of CYP7A1 mRNA level. β-actin (ACTB) gene expression was shown as control. Data is representative of at least three independently performed experiments and shown as mean ± SD; *P < 0.05 versus untreated, **P < 0.05 versus BA treatment and #P < 0.05 versus adenovirus infected lanes alone, using Student’s t-test. (D) Schematic representation of an auto-regulatory loop controlling the expression of SHP by LRH1 and inhibition of LRH1 activity by SHP itself via recruitment of SIRT1 histone deacetylase activity on target gene promoters of LRH1.

SHP recruits SIRT1 and utilizes SIRT1 deacetylase activity to inhibit LRH1-mediated gene transcription

Since gene repression is often associated with decreased histone acetylation (22), we determined whether the recruitment of SIRT1 by SHP results in template-associated histone (H3 and/or H4) deacetylation at the LRH1 target gene promoters (CYP7A1 and SHP) by using ChIP assays (Figure 6A). Adenoviral overexpression of LRH1 increased acetylation of H3 (Ac–H3) and H4 (Ac–H4) on the LRH1-responsive region of CYP7A1 and SHP promoters. This H3–H4 acetylation was severely compromised upon Ad–SHP or Ad–SIRT1 infection and the decrease of histone acetylation was significantly recovered upon treatment with Ad–SIRT1H355A or NAM on Ad–SHP infected cells or by Ad–siSHP infection on Ad–SIRT1 infected cells. No signal was detected after PCR amplification of control regions of either CYP7A1 or SHP promoters which are non-responsive to LRH1 occupancy and transactivation (data not shown). Additionally, we performed similar set of experiments in absence of Ad–LRH1 overexpression to assess the basal promoter acetylation status (Supplementary Figure S1A). Decreased acetylation of H3 and H4 were observed upon Ad–SHP and Ad–SIRT1 infection and this decrease was recovered
upon inhibition of SIRT1 activity or by endogenous knockdown of SHP, suggesting the involvement of other transcription factors regulating CYP7A1 and SHP gene promoters, along with LRH1, that are targets of SHP and/or SIRT1. A previous report demonstrated the co-occupancy of HDAC1, HDAC3 and SHP on CYP7A1 and SHP gene promoter under BA-treated condition (5). Therefore, we investigated the occupancy of HDAC1 and 3 on CYP7A1 gene promoter under our experimental conditions (Supplementary Figure S1B). Interestingly, both HDAC1 and 3 were detected in these conditions, though various combinatorial treatments showed no significant change in the occupancy status (fold occupancy) of either HDAC1 or 3, thereby suggesting them to be enzymatically inactive under these conditions and a promoter-specific activity of HDACs in general. Collectively, these results demonstrate that the recruitment of SIRT1 by SHP on the LRH1 target gene promoters stimulates deacetylation of template-associated histone H3 and H4 and this chromatin remodeling phenomenon ultimately leads to repression of LRH1 target gene expression by SHP in a SIRT1-dependent manner.

To confirm the effect of SIRT1 recruitment by SHP on total BA synthesis in the absence or presence of LRH1 overexpression, we analyzed the cell culture media for BA synthesis (Supplementary Figure S1C and Figure 6B). In the absence of LRH1 overexpression, Ad–SHP mediated repression of basal BA synthesis was significantly recovered by SIRT1 inhibition (using NAM or Ad–SIRT1H355A) (Supplementary Figure S1C). Overexpression of LRH1 resulted in a significant increase in total BA synthesized and SHP overexpression led to ~50% decrease in the total bile synthesis. Overexpression of dominant negative SIRT1 or SIRT1 inhibitor NAM resulted in a significant recovery in the total BA synthesis in the culture media, suggesting that SHP and SIRT1 co-ordinates the negative regulation of BA synthesis. BAs are known to be a negative regulator of CYP7A1 gene expression (10–12). Therefore, to evaluate the importance of SIRT1 in BA metabolism and CYP7A1 gene repression, we treated cells with BA (Figure 6C). BA mediated repression of CYP7A1 gene expression was recovered to a considerable extent by overexpression of Ad–SIRT1H355A or Ad–siSHP. Combinatorial overexpression of dominant negative SIRT1 and siRNA SHP recovered CYP7A1 mRNA level to a significant extent (~80%) after BA treatment, thereby confirming a key role of SIRT1, along with SHP in regulating CYP7A1 gene expression and subsequently BA metabolism.

DISCUSSION

In the current study, we demonstrated that SHP differentially recruits HDACs of class I (HDAC1) or class III (SIRT1) in a NR specific manner and inhibits transcriptional activity of these NRs. We also evidenced in vivo exogenous and endogenous interaction and colocalization of SIRT1 with SHP in the nucleus. The specificity of interaction between SHP and SIRT1 was further confirmed from the observations that neither SIRT6 nor SIRT7, two other nuclear-localized members of the sirtuins family, interacted with SHP. On the other hand, unlike SHP, the intrinsic repressive effect of NR DAX1, the closest family member to SHP, was not dependent on SIRT1-recruitment to its target transcription factors. Interestingly, neither SHP nor its target transcription factor and NR LRH1 were directly deacetylated by SIRT1. However, we demonstrated that SHP-mediated repression of LRH1 transcriptional activity as well as inhibition LRH1 target gene promoter activity and mRNA levels were significantly dependent on SIRT1 deacetylase activity. Detailed elucidation of the molecular mechanism of SHP–SIRT1 effect suggested template-dependent chromatin remodeling of LRH1 target gene promoters by SHP via utilization of SIRT1 deacetylase activity. Overall, our current finding reveals a novel molecular mechanism employed by nuclear corepressor SHP to inhibit transcriptional activity of its target transcription factor and provides evidences of a new role of SIRT1 in working concertedly with NRs and affecting chromatin remodeling in target gene promoters.

Histone acetylation and deacetylation are essential factors in modifying chromatin structure and regulating gene expression in euukaryotes (2,3). Many studies have demonstrated that methylation of H3 lysine9 (H3–K9) is associated with transcriptionally inactive chromatin (23,24). Recently, it was also reported that SHP could interact not only with the G9a H3–K9 methyltransferase but also with lysine 9-methylated histone 3, pointing out the role of SHP in negative regulation of gene expression via chromatin remodeling (21). In our study, we initially observed that the inhibition of transcriptional activity by SHP is via utilization of both class I HDAC recruitment (as in case of Nur77, CAR and ERx) as well as class III HDAC, i.e., SIRT1 recruitment (as in case of LRH1, ERRγ and RARx). This interesting NR-specific recruitment of different class of HDACs is a novel phenomenon observed for SHP and is reminiscent of a previous report from our group demonstrating similar NR-specific repression mechanism by SMILE (CREBZF), a SHP-interacting protein (14, 15 and 17). Interaction of SHP specifically with SIRT1 and not with any other nuclear-localized SIRT family members (SIRT6 and SIRT7) further enhances the significance of SIRT1 recruitment by SHP to target transcription factors. We further observed that SIRT1 contributes significantly to the intrinsic repressive effect of SHP, but not for DAX1. This further underlines the importance of SIRT1 in SHP-mediated repression of transcription factors. Though inhibition of SIRT1 activity by NAM was not completely able to recover the intrinsic repressive effect of SHP exerted on Gal4DBD fused protein alone, however, involvement of various other coactivator complexes with SHP which are TSA-sensitive proteins (class I and II HDACs, mSin3A and GPS2) should be taken into consideration. Along with this hypothesis, a recent report demonstrated the interaction between SHP and a non-HDAC protein, E1D1 and the mechanism of action of E1D1 was via antagonizing the CBP-dependent coactivator functions (25). Previous report from our group also suggested the role of helices H6 and H7 in transcriptional repression.
Among mammalian Sirtuins, SIRT1 is the closest homolog to yeast Sir2p and the best studied by far (7–9). The first noteworthy feature about the mechanism of action of SIRT1 is that while the majority of its associated functions are exerted through chromatin, however, as opposed to other HDACs, SIRT1 is a coordinator of simultaneous events, evidenced by its capacity to bind myriad factors and to target for deacetylation of the histone marks H4K16Ac and H3K9Ac, as well as transcription factors, and even other enzymes such as the histone acetyltransferase p300 or the histone methyltransferase Suv39h1. Sirtuins in general and histone acetyltransferase p300 or the histone deacetylases HDAC1 and 3 have been shown to interact with and recruit HDACs or other non-HDAC cofactors (like mSin3A–EID1) to repress transcriptional activation of its targets (4–6,21). Therefore, an obvious question is how SIRT1 differentially recruits these chromatin remodeling complexes to ultimately repress transcription. A plausible explanation behind this interesting phenomenon might be the conformational changes that may occur during the interaction of SIRT1 with various NRs, which might decide the complex formation of SIRT1 with a wide variety of chromatin remodeling complex. In fact, our results demonstrate that HDAC1 and 3 could be detected in the chromatin complex, but are irrespective to the CYP7A1-specific LRH1–SHP–SIRT1 complex, thereby suggesting that the HDAC activity might be a promoter-specific phenomenon. Co-crystallization of SHP–NR complexes in future studies will be able to provide with a detailed insight into this phenomenon. On the other hand, SIRT1-mediated inhibition of LRH1 transactivity has been previously attributed to mSin3A and Swi/Snf complex formation with SHP as well as GPS2–SHP interaction (5,6). Our findings suggest that SIRT1 leads to deactivation of HNF4α along with possibly ERRα, consistent with these features of SIRT1, our study demonstrates that NR corepressor SHP recruits SIRT1 to inhibit LRH1, along with possibly ERRα and RARα, transactivation (as we observed in Figure 1A). Previous reports investigating the role of SIRT1 have demonstrated that it can interact with and deacetylate specific transcription factors, thereby modulating their ability to activate or repress gene transcription (7–9,27–30). Among NRs, SIRT1 has been shown to directly deacetylate and activate LXRα (27) whereas deacetylation of HNF4α by SIRT1 leads to deactivation of HNF4α (28). A recent study demonstrated that FXRα is dynamically deacetylated/acetylated by SIRT1 and p300 to modulate the activity of FXRα under various metabolic states (29). SIRT1 has also been demonstrated to interfere with the activity of PPARγ by binding to the corepressors NCoR and SMRT (30). In that context, our findings, for the first time, suggest recruitment of SIRT1 by a NR corepressor (SHP) in a NR-specific manner, leading to template-dependent histone H3 and H4 deacetylation without direct deacetylation of either SHP or the transcription factor (LRH1) itself. However, SHP is also known to recruit HDACs or other non-HDAC cofactors (like EID1) to repress transcriptional activation of its targets (4–6,21). Therefore, an obvious question is how SIRT1 differentially recruits these chromatin remodeling complexes to ultimately repress transcription. A plausible explanation behind this interesting phenomenon might be the conformational changes that may occur during the interaction of SHP with various NRs, which might decide the complex formation of SHP with a wide variety of chromatin remodeling complex. In fact, our results demonstrate that HDAC1 and 3 could be detected in the chromatin complex, but are irrespective to the CYP7A1-specific LRH1–SHP–SIRT1 complex, thereby suggesting that the HDAC activity might be a promoter-specific phenomenon. Co-crystallization of SHP–NR complexes in future studies will be able to provide with a detailed insight into this phenomenon. On the other hand, SHP-mediated inhibition of LRH1 transactivity has been previously attributed to mSin3A and Swi/Snf complex formation with SHP as well as GPS2–SHP interaction (5,6). Our findings suggest that SIRT1 recruits SIRT1 to inhibit LRH1 transactivity. The possibility of recruitment of various chromatin remodeling complexes to the same NR by SHP can be explained by taking into account the upstream chromatin remodeling complexes involved in regulating SHP gene expression and protein stability. In that context, BAs have been shown to regulate SHP gene expression and protein stability via ERK-signaling pathway (29), whereas the kinase LKB1, a tumor suppressor that is required for AMPK activation under energy-deficient conditions, is regulated by SIRT1 (8) and AMPK in turn has been reported by our group to induce SHP gene expression (18,31–33) and may also affect post-translational modifications of SHP protein.

Previous studies showed that along with the BA-activated NR FXR, LRH1 activates SHP gene promoter and transcription (10–12). The BA-induced SHP then interacts with and inhibits LRH1 and/or HNF4α bound to the BA response element (BARE) in the CYP7A1 promoter, resulting in a negative feedback auto-regulatory loop (10–12). A recent study demonstrated that Brm or Brg-1 ATPases (contained in the Swi/Snf complexes) play an important role in regulation of BA metabolism under physiological conditions by Fxr and SHP. Brg-1 interacted with Fxr and enhanced Fxr-mediated transactivation of SHP, whereas Brm interacted with SHP and enhanced SHP-mediated repression of CYP7A1 and auto-repression of SHP (34). SIRT1 is critically involved in liver metabolic regulation (7–9,13) and a recent report suggests that SIRT1 knockdown increases levels of CYP7A1 mRNA (13). Consistent with these reports, our current observations suggest that recruitment of SIRT1 by SHP leads to the decreased promoter activity and mRNA levels of LRH1-induced CYP7A1 and SHP gene. Interestingly, our results also demonstrate that BA-mediated repression of CYP7A1 gene expression was significantly recovered by inhibition of SIRT1 activity. Thereby, it suggests the involvement of a novel cofactor, SIRT1, in the negative feedback autoregulatory loop of SHP gene (Figure 6C). Recruitment of SIRT1 deacetylase activity by SHP to regulate BA homeostasis as well its own gene expression therefore provides with a new mechanism of tight control exerted by SHP in liver metabolic pathways. Overall, our current findings provide insight into a novel chromatin remodeling strategy utilized by SHP and from a broader perspective it may be relevant, not only for the regulation of cholesterol and BA metabolism, but also for glucose homeostasis and other important biological processes in which both SHP and SIRT1 plays well-documented key regulatory roles.

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online.

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