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AMPK-dependent Repression of Hepatic Gluconeogenesis via Disruption of CREB·CRTC2 Complex by Orphan Nuclear Receptor Small Heterodimer Partner*[S]

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Orphan nuclear receptor small heterodimer partner (SHP) plays a key role in transcriptional repression of gluconeogenic enzyme gene expression. Here, we show that SHP inhibited protein kinase A-mediated transcriptional activity of cAMP-response element-binding protein (CREB), a major regulator of glucose metabolism, to modulate hepatic gluconeogenic gene expression. Deletion analysis of phosphoenolpyruvate carboxykinase (PEPCK) promoter demonstrated that SHP inhibited forskolin-mediated induction of PEPCK gene transcription via inhibition of CREB transcriptional activity. *In vivo* imaging demonstrated that SHP inhibited CREB-regulated transcription coactivator 2 (CRTC2)-mediated cAMP-response element-driven promoter activity. Furthermore, overexpression of SHP using adenovirus SHP decreased CRTC2-dependent elevations in blood glucose levels and PEPCK or glucose-6-phosphatase (G6Pase) expression in mice. SHP and CREB physically interacted and were co-localized *in vivo*. Importantly, SHP inhibited both wild type CRTC2 and S171A (constitutively active form of CRTC2) coactivator activity and disrupted CRTC2 recruitment on the PEPCK gene promoter. In addition, metformin or overexpression of a constitutively active form of AMPK (Ad-CA-AMPK) inhibited S171A-mediated PEPCK and G6Pase gene expression, and hepatic glucose production and knockdown of SHP partially relieved the metformin- and Ad-CA-AMPK-mediated repression of hepatic gluconeogenic enzyme gene expression in primary rat hepatocytes. In conclusion, our results suggest that a delayed effect of metformin-mediated induction of SHP gene expression inhibits CREB-dependent hepatic gluconeogenesis.

Glucose homeostasis is regulated by the opposing actions of insulin and glucagon (1–3), and glucose production in the liver is controlled primarily by gluconeogenesis (4). The regulation of hepatic gluconeogenesis involves the transcriptional regulation of key metabolic enzymes, including PEPCK[6] and G6Pase. The gluconeogenic program is largely regulated at the level of transcription and the process is coordinated by CREB via its direct binding to the cAMP-response element (CRE) site on the promoter of PEPCK, G6Pase, or PGC-1α (PPARγ coactivator-1α) (5).

Metformin has been shown to activate AMP-activated protein kinase (AMPK) via an LKB1-dependent mechanism (6). AMPK is a serine/threonine kinase that functions as an intracellular energy sensor and has been implicated in the modulation of glucose and fatty acid metabolism (7). AMPK is activated by physiological stimuli, including exercise, muscle contraction, and hormones, such as adiponectin and leptin, as well as by physiological stresses, glucose deprivation, hypoxia, oxidative stress, and osmotic shock conditions (8, 9). In the liver, activation of AMPK suppresses hepatic gluconeogenesis acutely by direct phosphorylation of its substrates, including CREB-binding protein (CBP) (10), CRTC2 (11), and GSK3β (glycogen synthase kinase 3β) (12). Recent studies also suggest that AMPK induces SHP gene expression and inhibits hepatic gluconeogenic gene expression in animal models (13, 14).

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[6] The abbreviations used are: PEPCK, phosphoenolpyruvate caboxykinase; G6Pase, glucose-6-phosphatase; AMPK, AMP-activated protein kinase; SHP, small heterodimer partner; CREB, cAMP-responsive element-binding protein; Ad, adenovirus; CRE, cAMP-response element; CREB, CRE-binding protein; CBP, CREB-binding protein; ER, endoplasmic reticulum; PKA, protein kinase A; CA-AMPK, constitutively active AMPK; AICAR, 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside; siSHP, siRNA specific to mouse SHP; siCRTC2, siRNA specific to mouse CRTC2.

Orphan nuclear receptor SHP (NR0B2) lacks a typical nuclear receptor DNA-binding domain and is expressed predominantly in the liver, whereas nominal expression is also detected in the heart, lung, pancreas, spleen, kidney, smooth muscle, testis, and ovary (15, 16). SHP is a transcriptional repressor of a number of nuclear receptors and transcription factors, including estrogen receptor (ER) (17, 18), ER-related receptor (19), glucocorticoid receptor (20), androgen receptor (21), forkhead transcription factor FoxA2 (HNF3) (22), HNF4 (hepatocyte nuclear factor 4) (23), HNF6 (hepatocyte nuclear factor 6) (24), CCAAT/enhancer-binding protein α (25), and BETA2/NeuroD (26). SHP expression is regulated by several other members of the nuclear receptor superfamily, including the orphan nuclear receptors SF-1, LRH-1, ER-related receptor γ, and the bile acid receptor FXR (16). SHP plays a crucial role in regulating glucose metabolism (13, 27). Mutations in the SHP gene are associated with mild obesity in Japanese subjects (28). Metformin, hepatocyte growth factor, and sodium arsenite increase SHP gene expression and inhibit the PEPCK and G6Pase gene expression; fenofibrate inhibits PAI-1 expression through induction of SHP (13, 14, 29, 30).

The basic leucine zipper (bZIP) protein CREB binds to CREs that contain the 5′-TGACGTCA-3′ consensus motif and activate the transcription of CRE-bearing genes, such as G6Pase and PEPCK (31). CREB coactivator CRTC2 significantly contributes to the CRE-dependent transcriptional activation of hepatic gluconeogenesis (11). Under feeding or in the presence of insulin, CRTC2 is located in the cytoplasm via its phosphorylation at Ser^{171} by members of the AMPK family of Ser/Thr kinase, including AMPK and SIK1 (salt-inducible kinase 1) (11, 32). Fasting triggers activation of cAMP-dependent protein kinase (PKA) to promote dephosphorylation and nuclear entry of CRTC2, which results in the increased occupancy of CRTC2 over promoters of PEPCK, G6Pase, or PGC-1α and activation of the entire gluconeogenic program in mouse liver or in rat primary hepatocytes (33, 34). Indeed, knock-out of CRTC2 decreases circulating glucose concentrations during fasting, due to the attenuation of the gluconeogenic gene expression (35).

In this study, we have demonstrated that AMPK inhibits phosphorylation-defective mutant CRTC2 (S171A)-dependent hepatic gluconeogenesis. SHP inhibits CRE promoter activity by direct interaction with CREB, thus inhibiting the recruitment of CRTC2 on the chromatin. Metformin or constitutively active AMPK inhibits not only WT CRTC2 but also S171A-dependent activation of hepatic gluconeogenic genes, whereas knockdown of SHP negated these effects, suggesting that AMPK could regulate CREB·CRTC2-dependent gluconeogenesis via an alternative mechanism by activating transcription of SHP. Taken together, our result suggests that SHP is an important contributor of AMPK-dependent suppression of CREB·CRTC2-mediated hepatic gluconeogenic gene expression.

EXPERIMENTAL PROCEDURES

Materials and Plasmids—Metformin (1,1-dimethylbiguanide hydrochloride, Sigma), 8-bromoadenosine 3,5-cyclic monophosphate (Sigma), forskolin (Calbiochem), 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) (Biomol), and insulin (Norvolin R, Green Cross) were purchased from the indicated companies and dissolved in the recommended solvents. The pGL3basic-G6Pase (−1227/+57) and PEPCK (−2.0 kb) promoter constructs were previously described (29). 8× CRE-luc constructs were constructed by inserting into the pGL2-promoter vector between BglII sites. pRC-RSV-PKA (catalytic subunit) was kindly provided by Dr. Richard A. Maurer (Oregon Health and Science University, Portland, OR). The plasmids pcDNA3-HA-SHP and pGEX4T1-SHP (25), pEBG-SHP (16), Gal4-TK-luc, and constitutively active form of AMPK (CA-AMPK) (13) constructs were previously described. The Gal4 DNA-binding domain was linked to CREB (Gal4-CREB) and CRTC2 (Gal4-CRTC2) via the EcoRI-XhoI cDNA fragments. The pcDNA3-FLAG-CREB and pcDNA3-FLAG-CRTC2 plasmids were constructed via subcloning of the EcoRI-XhoI cDNA fragments of rat CREB and mouse CRTC2 into pcDNA3-FLAG vector. All plasmids were confirmed via sequencing analysis.

Cell Culture and Transient Transfection Assay—Human hepatoma HepG2 cells were maintained in DMEM (Invitrogen), supplemented with 10% fetal bovine serum (FBS; Cambrex Bioscience Walkersville, Inc., Walkersville, MD) and antibiotics (Invitrogen). Cells were split into 24-well plates at densities of $2–8 \times 10^4$ cells/well the day before transfection. Transient transfections were performed using the SuperFect transfection reagent (Qiagen, Valencia, CA) according to the manufacturer's instructions. Cells were co-transfected with the indicated reporter plasmids together with expression vectors encoding various transcription factors. Total DNA used in each transfection was adjusted to 1 μg/well by adding an appropriate amount of empty vector, and cytomegalovirus (CMV)-β-galactosidase plasmids were co-transfected as an internal control. Cells were harvested 40–48 h after the transfection for luciferase and β-galactosidase assays. The luciferase activity was normalized with β-galactosidase activity.

Preparation of Recombinant Adenovirus—Adenoviruses for control GFP, CRTC2, siCRTC2, CRE-luc, SHP, siSHP, and CA-AMPK were described previously (13, 33). The cDNA sequence (^{239}GACAGTAGCCTTCCTCAGA^{259}) of an siRNA specific to mouse SHP was incorporated into the AdTrack-CMV shuttle vector, and a recombinant vector was generated using the Ad-Easy adenoviral vector system. The recombinant viruses were amplified in 293AD cells and isolated via cesium chloride density centrifugation (Sigma). The viruses were collected and desalted, and the titers were measured using Adeno-X^{TM} rapid titer (BD Bioscience) according to the manufacturer's instructions.

Isolation and Culture of Primary Rat Hepatocytes—Rat primary hepatocytes were isolated from Sprague-Dawley rat livers as described previously (36). After attachment, primary hepatocytes were infected with adenovirus for 48 h in DMEM. Cells were treated with 10 μM forskolin (FSK) for 2 h or 100 nM insulin plus 2 mM glucose for 18 h and 1 mM metformin for 12 h in DMEM.

Isolation and Culture of Primary Human Hepatocytes—Primary Human Hepatocytes were obtained from the Liver Tissue and Cell Distribution System of the National Institutes of

Health (S. Strom, University of Pittsburgh). Hepatocytes were cultured as described previously (37).

Quantitative RT-PCR—Total RNA from rat primary hepatocytes and human primary hepatocytes was extracted using an RNeasy minikit (Qiagen, Valencia, CA) or Easy-spin™ minikit (Intron). 1 μg of total RNA was used for generating cDNA with Superscript II enzyme (Invitrogen). cDNAs were analyzed by quantitative PCR using a SYBR Green PCR kit and TP800, Thermal Cycler DICE real time system (Takara).

Western Blot Analysis—Liver tissues were homogenized in RIPA lysis buffer and rat primary hepatocytes lysis with RIPA buffer. The proteins (50–100 μg/lane) were separated via SDS-PAGE on 6–12% gel and then transferred to pure nitrocellulose blotting membrane. The membranes were probed with an HA-HRP antibody for TORC2 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and SHP (H-160) antibody, monoclonal phospho-AMPK-α antibody (phosphorylated at Thr^{172}; Cell Signaling Technology), and a polyclonal AMPK-α antibody (Cell Signaling Technology) and then developed using an ECL Western blot detection kit (Pierce). Each of the membranes was also probed with β-tubulin antibody (Santa Cruz Biotechnology, Inc.) to verify equal protein loading.

Confocal Microscopy—HeLa cells were grown on coated glass coverslips and transfected with pEGFP-SHP and pCDNA3 FLAG-CREB using Lipofectamine 2000™ reagent. 24 h after transfection, the cells were fixed for 10 min in 3.7% formaldehyde, mounted on glass slides, and observed using a laser-scanning confocal microscope (Olympus Corp., Lake Success, NY) (24).

In Vivo Imaging Assay—For *in vivo* imaging, mice infected with Ad-CRE luciferase virus (0.5×10^9 pfu) together with Ad-GFP, Ad-CRTC2, or Ad-SHP virus 5 days postinfection, were injected intraperitoneally with 100 mg/kg glucagon and 100 mg/kg sterile firefly D-luciferin. After 10 min, mice were anesthetized and imaged on the IVIS SPECTRUM Imaging System (Xenogen).

GST Pull-down Assay—Briefly, CREB and CRTC2 were labeled with [^{35}S]methionine using the TNT-coupled reticulocyte lysate system (Promega Corp., Madison, WI) according to the manufacturer's instructions. GST alone and GST-fused SHP (GST-SHP) proteins were prepared as previously described (24).

In Vivo Interaction Assay—293T cells in DMEM supplemented with 10% FBS were seeded into 6-well flat-bottomed microplates at a concentration of 2×10^5 cells/well 1 day before transfection, as described previously (24). In brief, 293T cells were transfected with 1 μg of each of the indicated plasmids by calcium phosphate precipitation. Twenty-four hours after transfection, the cells were collected and solubilized. GST pull-down was performed as described previously (24).

Chromatin Immunoprecipitation Assay—ChiP assays were performed as described previously (24). HepG2 cells were transfected with 3 μg of pcDNA3-FLAG-CRTC2 (S171A) and pcDNA3-HA-SHP, and whole cell lysates were immunoprecipitated with monoclonal antibody against FLAG and DNA sequences analyzed by PCR using primer sets that encompassed the proximal (−200 to +10 bp) or distal (−1700 to −1500 bp) region of the human PEPCK promoter. The primer sequences were as follows: proximal, forward (5′-TCCCTTC-TCATGACCTTTGG-3′) and reverse (5′-GCTTGGTAGCT-AGCCCTCCT-3′); distal, forward (5′-GAGGGTTTCCAGG-ACAACAA-3′) and reverse (5′-GCCCTGATCTTTGGACT-CAA-3′); distal, forward (5′-GCTGTGGTGGTGAAGCT-GTA-3′) and reverse (5′-AGCCATGTACGTAGCCATCC-3′) (PEPCK, G6Pase, and actin).

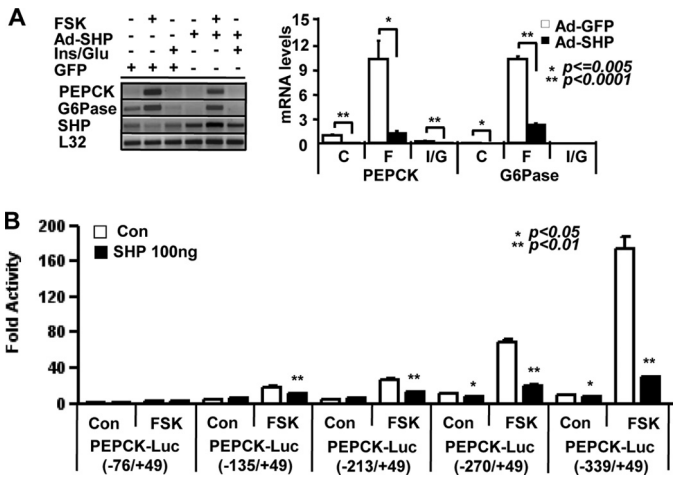

FIGURE 1. **SHP inhibits PEPCK gene promoter activity through CRE.** *A*, RT-PCR analysis showing the effects of SHP expression on mRNA levels for PEPCK and G6Pase in rat primary hepatocytes. After 48 h of adenoviral infection, cells were treated with 10 μM FSK for 2 h or 100 nM insulin for 18 h (*, $p \leq 0.005$; **, $p < 0.0001$) ($n = 3$). All mRNA levels were normalized to ribosomal L32 expression. *B*, transient assays of HepG2 cells transfected with serial deletion PEPCK luciferase constructs (−339, −270, −213, −135, −76/+49) to show the effects of SHP expression on PEPCK transcription. Cells were co-transfected with 200 ng of pXP2-rPEPCK-luc together with 200 ng of pcDNA3-FLAG-SHP or 100 ng of pcDNA3 empty vector for 40 h and then treated with 10 μM FSK for 4 h (*, $p < 0.05$; **, $p < 0.01$) ($n = 3$). *Con*, control; *error bars*, S.E.

Glucose Output Assay—Glucose production from primary rat hepatocytes was measured according to the manufacturer's protocol, using a colorimetric glucose oxidase assay (Sigma). Briefly, after the experimental time period as indicated, the cells were washed three times with phosphate-buffered saline. Then the cells were incubated for 3 h at 37 °C, 5% CO_2, in glucose production buffer (glucose-free DMEM, pH 7.4, containing 20 mmol/liter sodium lactate, 1 mmol/liter sodium pyruvate, and 15 mmol/liter HEPES, without phenol red). The glucose assays were performed in triplicate, and the intra-assay coefficient of variation was 5% as described previously (33).

Statistical Analysis—Data are expressed as means ± S.E. Analysis of variance was used to determine significant differences as detected by Duncan's multiple comparison test. Values of $p < 0.05$, $p < 0.001$ were considered to be statistically significant. All experiments were performed three times.

RESULTS

SHP Inhibits PEPCK Gene Promoter Activity via Regulation of CREB—Previously, it has been reported that SHP inhibits hepatic gluconeogenesis, although the exact mechanism is still controversial to date. Indeed, adenovirus-mediated overexpression of SHP (Ad-SHP) greatly reduced forskolin-induced PEPCK and G6Pase gene expression in rat primary hepatocytes (Fig. 1*A*). To elucidate the mechanism of SHP-dependent repression on the PEPCK promoter, we attempted to identify

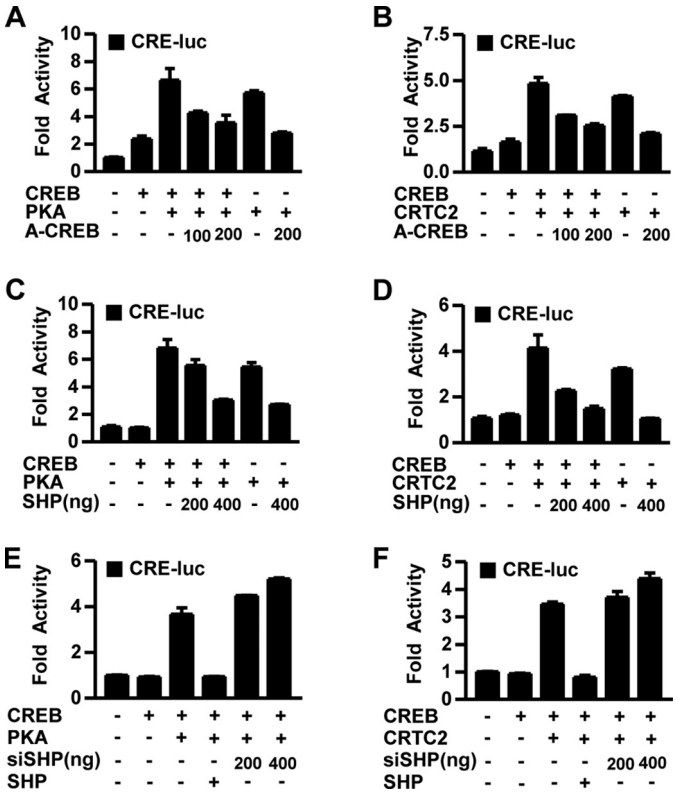

FIGURE 2. **SHP inhibits PKA- and CRTC2-mediated CREB transcriptional activity.** *A* and *B*, HepG2 cells were transfected with 200 ng of CRE-luc reporter and pcDNA3-FLAG-CREB (10 ng), pRC-RSV-PKA (100 ng) (*A*), pcDNA3-FLAG-CRTC2 (100 ng) (*B*), indicated amounts of pRC-RSV-A-CREB (dominant negative form) or pcDNA3 empty vector, respectively. Effects of SHP on PKA and CRTC2 mediated CRE-luc promoter activity were shown. *C* and *D*, HepG2 cells were transfected with 200 ng of CRE-luc reporter and pRC-RSV-PKA (100 ng) (*C*), pcDNA3-FLAG-CRTC2 (100 ng) (*D*), and the indicated amounts of pcDNA3-HA-SHP or pcDNA3 empty vector, respectively. *E* and *F*, HepG2 cells were transfected with 200 ng of CRE-reporter plasmid (pGL2-CRE-luc) and pcDNA3-FLAG-CREB (10 ng), pRC-RSV-PKA (100 ng) (*E*), pcDNA3-FLAG-CRTC2 (100 ng) (*F*), and the indicated amounts of pSUPER-siSHP or pSUPER empty vector, respectively. *Error bars*, S.E.

the SHP-responsive region on forskolin-induced PEPCK promoter activity. First, we performed co-transfection assays using several PEPCK reporter deletion constructs with SHP in the presence or absence of FSK in HepG2 cells. Deletion analysis revealed that the inhibitory effect of SHP on the PEPCK gene promoter is observed in the PEPCK-luc (−339/+49) construct, and this repressive effect is maintained upon further deletion up to −135, a region that contains the CRE (Fig. 1*B*). These results suggest that SHP may inhibit FSK-mediated activation of PEPCK gene promoter via repression of CREB transactivity.

SHP Inhibits CRTC2-mediated CREB Transcriptional Activity—To confirm the mechanism by which PKA and CRTC2 regulate CREB transcriptional activity, we performed transient transfection assays in HepG2 cells using CRE-luciferase reporter plasmid. Overexpression of PKA significantly increased CRE-luciferase reporter activity. However, co-transfection with A-CREB (dominant negative form) inhibited CREB-mediated CRE-luciferase reporter activity (Fig. 2*A*). CRTC2-mediated activation of CRE-luciferase reporter activity was also dose-dependently inhibited by A-CREB (Fig. 2*B*). Next, to confirm whether SHP has a role on CREB transcriptional activity, we co-transfected CREB and PKA in the presence or absence of SHP. Overexpression of SHP strongly inhibited PKA-induced CRE-luciferase reporter activity (Fig. 2*C*). CRTC2-mediated CRE-luciferase reporter activity was also inhibited by SHP in a dose-dependent manner (Fig. 2*D*). To rule out the possibility that SHP might also target other CREB coactivators in FSK-induced transactivation of CREB, we performed a knockdown experiment of CRTC2 using CRTC2 siRNA. Our transient transfection study indicated that FSK-induced CRE-luc promoter activity was significantly repressed by either SHP overexpression or CRTC2 knockdown. However, SHP overexpression had no further effect on FSK-induced CRE-luc promoter activity upon CRTC2 knockdown (supplemental Fig. 1). To investigate the involvement of endogenous SHP in CREB transactivity, we knocked down endogenous SHP using siSHP expression plasmid. Knockdown of endogenous SHP significantly increased PKA-mediated CREB transactivity (Fig. 2*E*). Similarly, CRTC2-mediated CREB transactivity was also strongly increased upon knockdown of endogenous SHP (Fig. 2*F*). Overall, these results indicate that SHP inhibits PKA- and CRTC2-induced CREB transcriptional activity.

SHP Inhibits CRTC2-mediated and CRE- dependent Hepatic Gluconeogenesis in Vitro and in Vivo—To assess the functional consequence of the down-regulation of CREB-mediated hepatic gluconeogenesis by SHP, we investigated whether overexpression of SHP has any effect on CREB-dependent transactivity in rat primary hepatocytes. Overexpression of SHP with Ad-SHP decreased FSK-mediated CRE-luciferase reporter activity (Fig. 3*A*). Ad-CRTC2- mediated CRE-luc activity was also decreased by Ad-SHP (Fig. 3*B*), which suggests that SHP may decrease CRTC2-induced hepatic glucose production. As shown previously, Ad-CRTC2 enhanced glucose production in rat primary hepatocytes. However, elevated hepatic glucose production was suppressed by Ad-SHP in rat primary hepatocytes (Fig. 3*C*). Next, we wanted to confirm the role of SHP in regulating CREB-dependent hepatic gluconeogenesis *in vivo*. Ad-CRTC2 highly increased CRE-luc activity in mouse liver by *in vivo* imaging analysis. However, CRTC2-mediated CRE-luc activity was significantly inhibited by Ad-SHP (Fig. 3, *D* and *E*). Moreover, CRTC2-mediated induction of PEPCK and G6Pase gene expression was also decreased by Ad-SHP (Fig. 3*F*). Consistent with the decrease in CRTC2-induced gluconeogenic enzyme gene expression by Ad-SHP, Ad-CRTC2-mediated induction of blood glucose level was significantly decreased by Ad-SHP (Fig. 3*G*). These results indicate that SHP inhibits CREB-dependent induction of hepatic gluconeogenic gene expression and glucose production *in vivo*.

SHP Physically Interacts with CREB to Inhibit CREB Transcriptional Activity—Previous reports have demonstrated that SHP represses several transcriptional factors, including HNF3 (22), HNF4 (23), or CCAAT/enhancer-binding protein (25) via direct interaction to decrease hepatic gluconeogenic gene transcription. To investigate whether CREB- and CRTC2-mediated CRE transcriptional activity is suppressed by SHP via direct interaction between SHP and CREB or CRTC2, we performed a GAL4-based transcription assay and *in vivo* GST pull-down assays. Expression vectors encoding the DNA-binding domain of the yeast transcription factor GAL4 fused to CREB and CRTC2 were co-transfected with a GAL4-binding site upstream

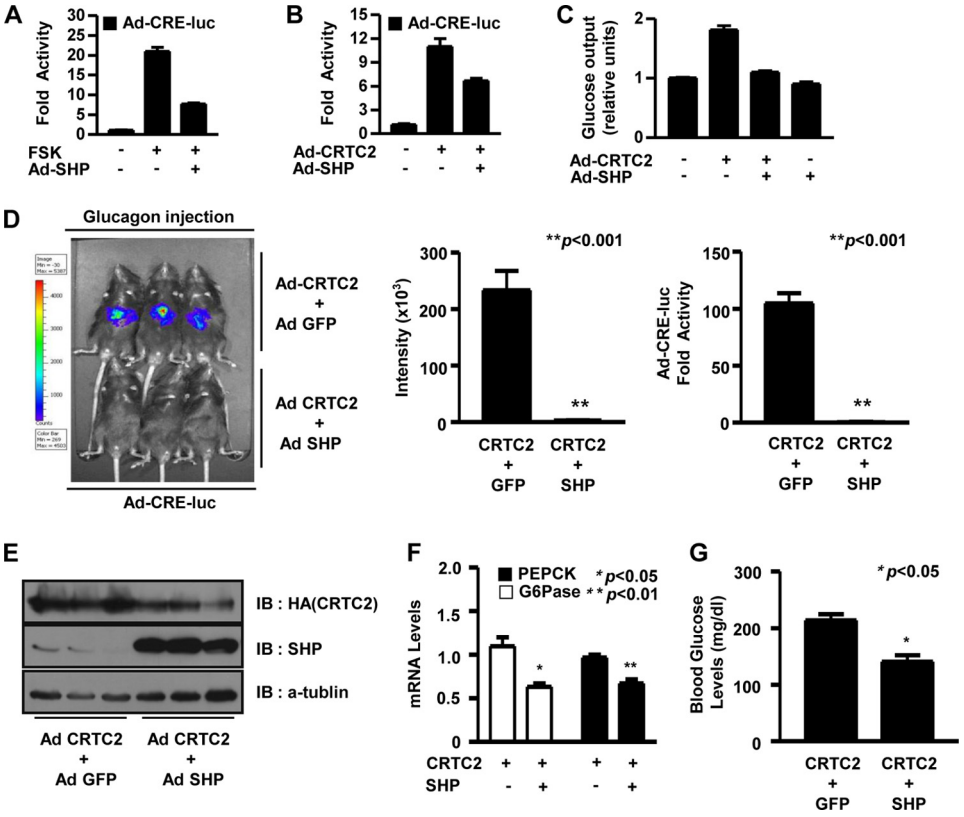

FIGURE 3. **SHP inhibits CRTC2-mediated and CRE-dependent hepatic gluconeogenesis *in vivo* and *in vitro*.** *A* and *B*, effects of Ad-SHP on Ad-CRE-luc activity in rat primary hepatocytes exposed to FSK (*A*) or infected with Ad-CRTC2 (*B*). *C*, SHP effect on hepatic glucose output. A glucose output assay was performed using primary hepatocytes infected with CRTC2 and SHP adenoviral expression for 24 h, using glucose-free media supplemented with gluconeogenic substrate sodium lactate (20 mM) and sodium pyruvate (1 mM). Shown is the effect of Ad-SHP *in vivo*. *D*, live imaging of hepatic CRE-luciferase (Ad-CRE-luc) activity in Ad-CRTC2- and Ad-SHP-infected or Ad-CRTC2- and Ad-GFP-infected mice after insulin and glucagon injection (**, $p < 0.01$, $n = 3$). *E*, protein levels in Ad-CRTC2- and Ad-SHP-infected or Ad-CRTC2- and Ad-GFP-infected mice. *F*, the mRNA amounts of PEPCK and G6Pase in Ad-CRTC2- and Ad-SHP-infected or Ad-CRTC2- and Ad-GFP infected mice (*, $p < 0.05$; **, $p < 0.001$) ($n = 3$). *G*, hepatic blood glucose concentration (*, $p < 0.05$, $n = 3$). *IB*, immunoblot; *Error bars*, S.E.

activator sequence (USA)-driven luciferase reporter construct. The transcriptional activity of CREB was inhibited by SHP in a dose-dependent manner (Fig. 4*A*). Interestingly, SHP also decreased PKA-induced transcriptional activity of CRTC2 (Fig. 4*B*). To determine whether the repression of CREB transcriptional activity by SHP is mediated through a direct physical interaction, we performed *in vivo* GST pull-down assays. We co-transfected 293T cells with expression vectors for FLAG-CREB and GST-SHP (pEBG-SHP) or GST (pEBG) alone, and protein complexes were isolated using glutathione-Sepharose. CREB specifically interacted with GST-SHP but not GST alone (Fig. 4*C*, *top*). The expression level of each protein was verified by Western blot using antibodies specific for GST and FLAG (Fig. 4*C*, *middle* and *bottom*, respectively). Next, to investigate whether SHP and CREB are co-localized in the same subcellular compartment, we performed confocal microscopy analysis in HeLa cells. Consistent with previous reports, the majority of SHP protein was localized in the nuclei of cells (24), and the CREB localization pattern was observed to be identical to that of SHP (Fig. 4*D*). These results demonstrate that SHP inhibits CREB transcriptional activity via physical interaction with CREB.

SHP Inhibits Binding of CRTC2 to CREB—It has been reported that CRTC2 mainly contributes to the activation of CREB transcription activity (11). To investigate the mechanism for SHP-dependent CREB repression in detail, we performed transient transfection assays to determine whether SHP competes with CRTC2 upon CREB transactivation. As shown previously, CREB transcriptional activity was significantly increased by PKA, which was decreased by SHP. In transient transfection assays, reduction of CREB transcriptional activity by SHP was significantly relieved by CRTC2 in a dose-dependent manner in HepG2 cells (Fig. 5*A*). Next, we examined whether SHP inhibited transcriptional activity of both wild type CRTC2 and the constitutively active form of CRTC2 (S171A), which is refractory to AMPK-mediated Ser^{171} phosphorylation (11). Interestingly, SHP could inhibit both wild type and S171A-induced CRE-luc promoter activity in a dose-dependent manner (Fig. 5*B*). We then performed an *in vitro* GST pull-down competition assay to confirm competition between SHP and CRTC2 for binding to CREB *in vitro*. Increasing amounts of unlabeled full-length CRTC2 competed with GST-fused SHP for the binding to CREB (Fig. 5*C*). These results indicate that SHP competes with CRTC2 for the binding to CREB to repress its transcriptional activity in a CRTC2 phosphorylation-independent manner.

SHP Inhibits CRTC2-mediated Hepatic Gluconeogenic Enzyme Gene Expression—We next examined whether SHP could inhibit the activities of PKA- and CRTC2-driven CREB activity on gluconeogenic gene promoters. Overexpression of PKA increased G6Pase and PEPCK promoter activity, and these promoter activities were decreased with overexpression of SHP (Fig. 6*A*). Similarly, SHP also inhibited CRTC2-induced G6Pase and PEPCK promoter activity (Fig. 6*B*). Furthermore, to determine whether SHP inhibits DNA-binding of CREB and occupancy of CRTC2 on the PEPCK gene promoter, we performed ChIP assay in the hepatocytes. We observed that SHP inhibited occupancy of the active form of CRTC2 (S171A) on CRE elements of the PEPCK gene promoter (Fig. 6*C*). However, SHP could not abolish the CREB binding on CRE elements of the PEPCK gene promoter (Fig. 6*D*). These results suggest that SHP might compete with CRTC2 for binding to CREB on the PEPCK promoter. Next, we examined whether adenoviral Ad-SHP inhibits Ad-S171A-induced PEPCK and G6Pase gene expression in rat primary hepatocytes. Indeed, Ad-SHP strongly inhibited Ad-S171A-induced PEPCK and G6Pase gene

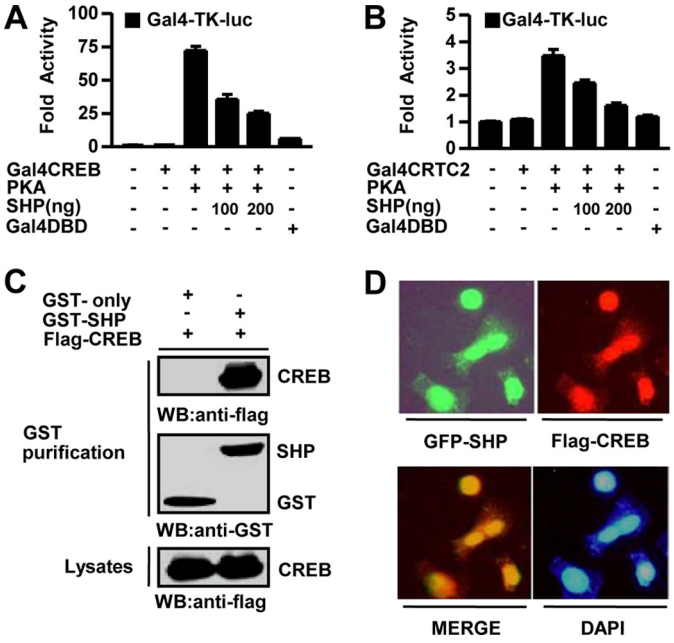

FIGURE 4. **SHP physically interacts with CREB to inhibit CREB transcriptional activity.** *A* and *B*, HepG2 cells were co-transfected with 200 ng of Gal4-TK-luc and pRC-RSV-PKA (100 ng), Gal4 DNA-binding domain (100 ng), Gal4-CREB (100 ng) (*A*), and Gal4-CRTC2 (*B*) with the indicated amounts of pcDNA3/HA-SHP. *C*, 293T cells were co-transfected with expression vectors for pcDNA3-FLAG-CREB with GST-SHP (pEBG-SHP), or GST alone (pEBG) as a control. To check subcellular localization of SHP and CREB, HeLa cells were transiently transfected with pEGFP-SHP and pcDNA3-FLAG-CREB. *D*, to check subcellular localization of SHP and CREB, HeLa cells were transiently transfected with pEGFP-SHP and pcDNA3-FLAG-CREB. Immunofluorescence confocal microscopy showed subcellular localization of SHP and CREB. DAPI staining was used to visualize nuclei, and the *yellow color* in the *merged image* represents the co-localization of SHP and CREB. *WB*, Western blot; *error bars*, S.E.

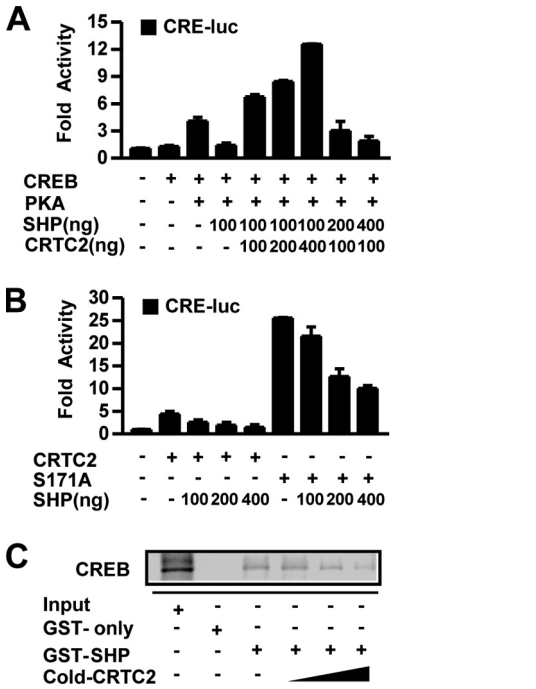

FIGURE 5. **SHP inhibits binding of CRTC2 to CREB.** SHP competes with CRTC2 on CRE-dependent transcription. *A*, HepG2 cells were transfected with CRE-luc reporter plasmid (200 ng), pcDNA3-FLAG-CREB (10 ng), pRC-RSV-PKA (100 ng), and the indicated amount of expression vectors for pcDNA3-FLAG-CRTC2 and pcDNA3-HA-SHP. *B*, HepG2 cells were transfected with CRE-luc reporter (200 ng) and pcDNA3-FLAG-CRTC2 (100 ng), pcDNA3-FLAG-CRTC2 (S171A) (100 ng), and the indicated amount of pcDNA3-HA-SHP expression vectors. pcDNA3 empty vector was used to adjust the total amount of DNA used in each transfection. *C*, *in vitro* translated ^{35}S-labeled CREB and unlabeled CRTC2 was incubated with bacterially expressed GST-SHP, and then a GST pull-down assay was performed. *Error bars*, S.E.

expression (Fig. 6*E*). Consistent with the inhibition of Ad-S171A-induced PEPCK and G6Pase gene expression by SHP, Ad-S171A-induced hepatic glucose production was also strongly decreased by Ad-SHP in rat primary hepatocytes (Fig. 6*F*). These data demonstrated that SHP could down-regulate the CREB-mediated PEPCK and G6Pase gene expression via inhibition of CREB·CRTC2 complex formation on PEPCK and G6Pase gene promoters.

Metformin Inhibits CRTC2-mediated Hepatic Gluconeogenesis through Induction of SHP Gene Expression—Previous reports have ascertained that acute effect of AMPK on hepatic gluconeogenesis through a direct phosphorylation of CRTC2 at Ser^{171} (11, 34, 38). Because our previous study demonstrated that metformin increased SHP gene transcription and expression via AMPK (13), we hypothesized that metformin might inhibit both wild type CRTC2 and S171A-mediated CREB-dependent gluconeogenesis through induction of SHP expression. First, we investigated whether metformin can inhibit both wild type CRTC2 and constitutively active CRTC2 (S171A)-induced CRE-luc activity. As expected, metformin dose-dependently inhibited both CRTC2- and S171A-induced CRE-luc promoter activity (Fig. 7*A*). We also confirmed that metformin could inhibit both CRTC2- and S171A-mediated PEPCK and G6Pase gene promoter activity in hepatic cells (Fig. 7*B*). However, the inhibitory effect of metformin on S171A-induced PEPCK and G6Pase gene promoter activity was significantly reduced by knockdown of SHP gene expression using siSHP in HepG2 cells, suggesting that metformin-dependent regulation of CRTC2 may be elicited by increased SHP production (Fig. 7*C*). To confirm the effect of metformin or AICAR-induced SHP on association of CRTC2 with the promoters of PEPCK and G6Pase, we performed chromatin inmmunoprecipitation assays in primary hepatocytes. Treatment of FSK significantly increased occupancy of CRTC2 over CRE of the PEPCK and G6Pase promoters, whereas metformin or AICAR strongly abolished it. In accordance with the inhibitory effect on CRTC2, both metformin and AICAR increased the occupancy of SHP via its enhanced binding with CREB on CRE elements of the PEPCK (Fig. 7*D*) and G6Pase (Fig. 7*E*) gene promoters. Therefore, these results show that the metformin- and AICAR-mediated induction of SHP gene expression inhibits CREB transcriptional activity via inhibition of CRTC2 DNA occupancy.

Consistent with previous reports, metformin significantly induced SHP mRNA levels in a dose-dependent manner in rat primary hepatocytes (Fig. 7*F*). Furthermore, metformin significantly increased SHP mRNA levels in a time- and dose-dependent manners in human primary hepatocytes, demonstrating that this regulatory mechanism is conserved among species (supplemental Fig. 2, *A* and *B*). We then attempted to confirm whether Ad-S171A-induced gluconeogenic gene expression could be repressed by metformin after knockdown of the SHP

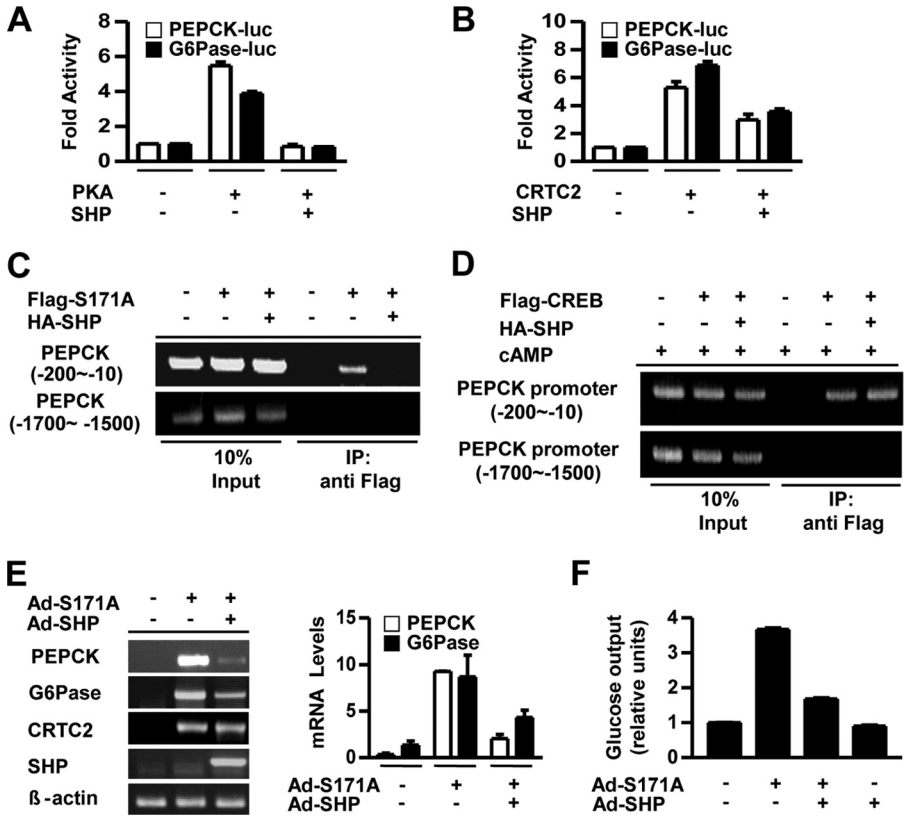

FIGURE 6. **SHP inhibits CRTC2-mediated gluconeogenic gene expression.** *A* and *B*, HepG2 cells were co-transfected with 200 ng of PEPCK and G6Pase promoter together with pRC-RSV-PKA (100 ng) (*A*), pcDNA3-FLAG-CRTC2 (100 ng) (*B*), pcDNA3-HA-SHP (100 ng), or 100 ng of pcDNA3 empty vector. The ChIP assay shows that SHP inhibits CRTC2 DNA binding to the PEPCK promoter. *C* and *D*, HepG2 cells were transfected with 3 μg of pcDNA3-HA-SHP and pcDNA3-FLAG-CRTC2 (*C*) and with pcDNA3-FLAG-CREB and cAMP analog (*D*). Whole cell lysates were immunoprecipitated with monoclonal antibody against FLAG and DNA sequences analyzed by PCR. *E*, RT-PCR analysis showing the effects of adenoviral SHP expression on CRTC2 (S171A)-mediated mRNA levels for PEPCK and G6Pase in rat primary hepatocytes. Primary hepatocytes were infected with adenovirus CRTC2 (S171A) and SHP. After 24 h of adenoviral infection, cells were harvested. *F*, glucose output assay was performed using primary hepatocytes infected for 24 h with constitutive active CRTC2 (S171A) and SHP adenovirus, using glucose-free media supplemented with gluconeogenic substrate sodium lactate (20 mM) and sodium pyruvate (1 mM). *IP*, immunoprecipitation; *error bars*, S.E.

gene. Knockdown of SHP using Ad-siSHP significantly relieved the metformin-mediated inhibition of PEPCK and G6pase gene expression (Fig. 7*G*). Moreover, CRTC2-dependent induction of hepatic gluconeogenic gene expression was significantly repressed by metformin treatment, and the reduction of gluconeogenic gene expression by metformin was recovered by the knockdown of the SHP gene using SHP siRNA (supplemental Fig. 3). In addition, metformin inhibited Ad-S171A-induced hepatic glucose production significantly, and this effect was negated by knockdown of SHP (Fig. 7*H*). These results demonstrate that metformin inhibits CRTC2-induced hepatic gluconeogenesis via induction of SHP gene expression.

AMPK Inhibits CRTC2-mediated Hepatic Gluconeogenic Gene Expression via Induction of SHP-mediated Hepatic Gluconeogenic Gene Expression—Metformin-mediated AMPK activation increases SHP gene transcription (13), which prompted us to evaluate the effect of AMPK on CRTC2-mediated hepatic gluconeogenesis. First, we analyzed the phosphorylation of the AMPK and subsequent changes in SHP gene expression pattern in primary rat hepatocytes following AICAR treatments. Treatments with 1 mM AICAR significantly induced phosphorylation of AMPK. Consistent with this, AICAR-treated primary rat hepatocytes showed an increase in SHP protein level at 3 h, which was maintained at 24 h (Fig. 8*A*). As expected, AICAR-induced raised SHP protein levels was restored by Ad-siSHP (Fig. 8*B*). Consistent with these changes, S171A-mediated raised PEPCK proteins were also restored by provision of AICAR, whereas knockdown of SHP reversed inhibitory effects of AICAR. To avoid potential nonspecific effects that are associated with chemical treatment, we utilized CA-AMPK for the further study. Using transient transfection in HepG2 cells with the PEPCK and G6Pase gene promoters, we demonstrated that S171A-mediated promoter activity of PEPCK and G6Pase was significantly decreased by CA-AMPK. In contrast, siRNA-mediated knockdown of SHP blocked the ability of CA-AMPK to inhibit S171A-mediated PEPCK and G6Pase promoter activity (Fig. 8*C*). Finally, to further confirm the role of SHP in AICAR-mediated inhibition of S171A-mediated PEPCK and G6Pase gene expression, we used adenovirus-mediated overexpression of the constitutively active form of AMPK (Ad-CA-AMPK) and Ad-siSHP. Notably, Ad-CA-AMPK inhibited S171A-mediated PEPCK and G6Pase gene expression in primary rat hepatocytes, which was reversed by Ad-siSHP (Fig. 8*D*). Taken together, these results indicate the importance of SHP in mediating inhibitory effects of AMPK on CRTC2-mediated gluconeogenesis.

DISCUSSION

In this study, we found that the inhibitory effect of chronic metformin treatment on CREB-mediated hepatic gluconeogenic enzyme gene expression and hepatic glucose production was mediated by induction of SHP. SHP decreases CREB-dependent induction of gluconeogenic gene expression and hepatic glucose production via direct interaction with CREB. We suggest that the acute response of metformin-mediated activation of AMPK inhibits CREB-dependent hepatic gluconeogenesis through CRCT2 phosphorylation, whereas chronic treatment of metformin inhibited CREB-dependent hepatic gluconeogenesis through induction of SHP gene expression.

AMPK suppresses CREB-mediated hepatic gluconeogenesis by regulation of CREB-binding protein (10) and CRTC2 (11). Moreover, it has been previously reported that PXR

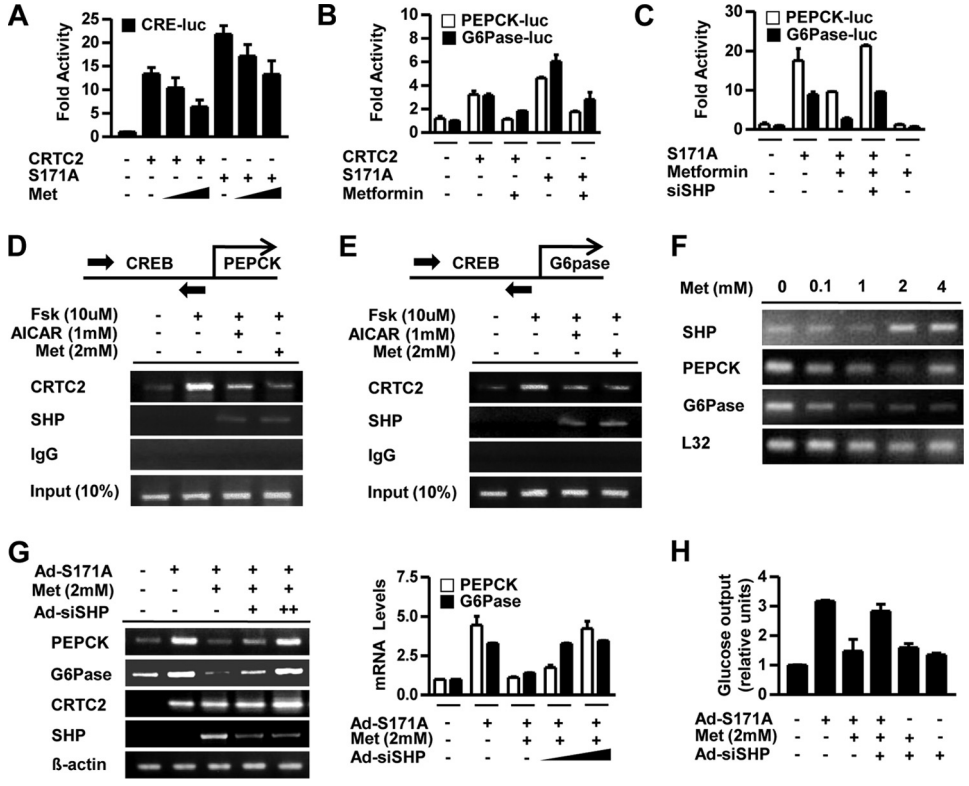

FIGURE 7. **Metformin inhibits CRTC2-dependent hepatic gluconeogenesis via induction of SHP.** *A*, HepG2 cells were transfected with 200 ng of CRE-luc and pcDNA3-FLAG-CRTC2 (WT) (100 ng), pcDNA3-FLAG-CRTC2 (S171A) (100 ng) or 100 ng of pcDNA3 empty vector and then treated with 0.1 or 1 mM metformin for 12 h. *B*, HepG2 cells transfected with 200 ng of PEPCK-luc, G6Pase-luc and pcDNA3-FLAG-CRTC2 (100 ng), pcDNA3-FLAG-CRTC2 (S171A) (100 ng), pcDNA3-HA-SHP (100 ng), or 100 ng of pcDNA3 empty vector, respectively, for 24 h and then treated with 0.1 or 1 mM metformin for 12 h. *C*, HepG2 cells were transfected with pSUPER human SHP siRNA expression vector. Twenty-four hours after the transfection, the cells were co-transfected with 200 ng of PEPCK-luc, G6Pase-luc, and pcDNA3-FLAG-CRTC2 (S171A) (100 ng) for 24 h and then treated with 1 mM metformin for 12 h. *D* and *E*, rat primary hepatocytes were pretreated with forskolin for 6 h preceding metformin and AICAR treatment for 12 h. Soluble chromatin was prepared and immunoprecipitated with polyclonal antibody against SHP and CRTC2 or IgG only as indicated. 10% of the soluble chromatin was used as input. Quantitative PCR was performed to determine and quantify the occupancy of SHP and CRTC2 to endogenous PEPCK (*D*) and G6Pase promoter (*E*). *F*, RT-PCR analysis showing the effects of metformin on mRNA levels of SHP, PEPCK, and G6Pase in rat primary hepatocytes treated with the indicated amount of metformin for 12 h. *G*, RT-PCR analysis showing the effects of metformin through SHP expression on mRNA levels of PEPCK and G6Pase in rat primary hepatocytes. Twenty-four hours after the infection with Ad-CRTC2 (S171A) and Ad-siSHP, cells were treated with 2 mM metformin for 12 h. Total RNA was isolated for quantitative PCR analysis SHP mRNA expression and was normalized to β-actin expression. *H*, glucose output assay was performed using primary rat hepatocytes infected with Ad-CRTC2 (S171A) and Ad-siSHP for 24 h and then treated with 1 mM metformin for 12 h, using glucose-free media supplemented with gluconeogenic substrate sodium lactate (20 mM) and sodium pyruvate (1 mM). *Error bars*, S.E.

represses glucagon-activated transcription of the G6Pase gene by directly binding to CREB (39), and direct inactivation of GSK3β also inhibits transcriptional activity of CREB through AMPK-induced phosphorylation of GSK3β (12). In this study, we demonstrated a novel mechanism by which the AMPK signaling pathway inhibits CREB-mediated hepatic gluconeogenic gene expression. Our results demonstrated that SHP directly interacted with CREB (Fig. 4) and inhibited CRTC2-mediated CREB transcriptional activity (Fig. 2). These results suggest that SHP might inhibit the activities of both CREB and CRTC2.

In obese and diabetic *db/db* mouse liver, a higher expression level of CRTC2 protein was observed due to the defects in refeeding-dependent mechanisms for CRTC2 phosphorylation and degradation (33). CRTC2 null mice displayed low circulating blood glucose concentrations and improved insulin sensitivity in the context of diet-induced obesity, suggesting that regulation of CREB·CRTC2 activity is critical in the maintenance of glucose homeostasis (35). Therefore, we suggest that SHP may inhibit CRTC2-induced hepatic gluconeogenic gene expression and blood glucose concentrations in insulin-resistant conditions and may improve CRTC2-mediated insulin resistance via increasing insulin sensitivity.

CBP/p300 is required for the recruitment of CRTC2 following exposure to cAMP, and CRTC2 promotes CBP recruitment to the CREB target gene, indicating that the CRTC2·CBP complex has reciprocal effects on the recruitment of both proteins to CREB target gene promoter (40). Previous studies reported that AMPK regulated hepatic gluconeogenesis through phosphorylation of CBP (10), and SHP repressed the expression of gluconeogenic enzyme genes through the dissociation of FOXO1 or HNF4 from CBP (41). In this study, we demonstrated that SHP competed with CREB transcriptional coactivator CRTC2 to inhibit the CREB·CRTC2 complex (Figs. 5 and 6). These results suggest that SHP plays an additional role in suppression of CREB-dependent hepatic gluconeogenesis via co-factor competition. However, we do not rule out the possibility that SHP competed with CBP. We have found that recruitment of CRTC2 onto the PEPCK promoter was significantly inhibited by SHP without affecting CREB recruitment (Fig. 7). This phenomenon is similar to the previous observation that CRTC2 recruitment does not appear to modulate CREB DNA binding activity but rather enhances the interaction of CREB with the TAF-(II)-130 component of TFIID following its recruitment to the promoter (42). Therefore, our study suggests the possibility that SHP may inhibit the CREB·CBP·CRTC2 complex via inhibition of stable CBP occupancy over the promoter and prevent recruitment of other transcription components via a cofactor competition mechanism.

CRTC2 was shown to be phosphorylated at Ser^{171} by AMPK agonist AICAR and relocated from the nucleus to the cytoplasm even in the presence of FSK (11). Our previous studies have demonstrated that AMPK elevated SHP gene expression via USF-1 (upstream stimulatory factor) (29). AMPK-mediated

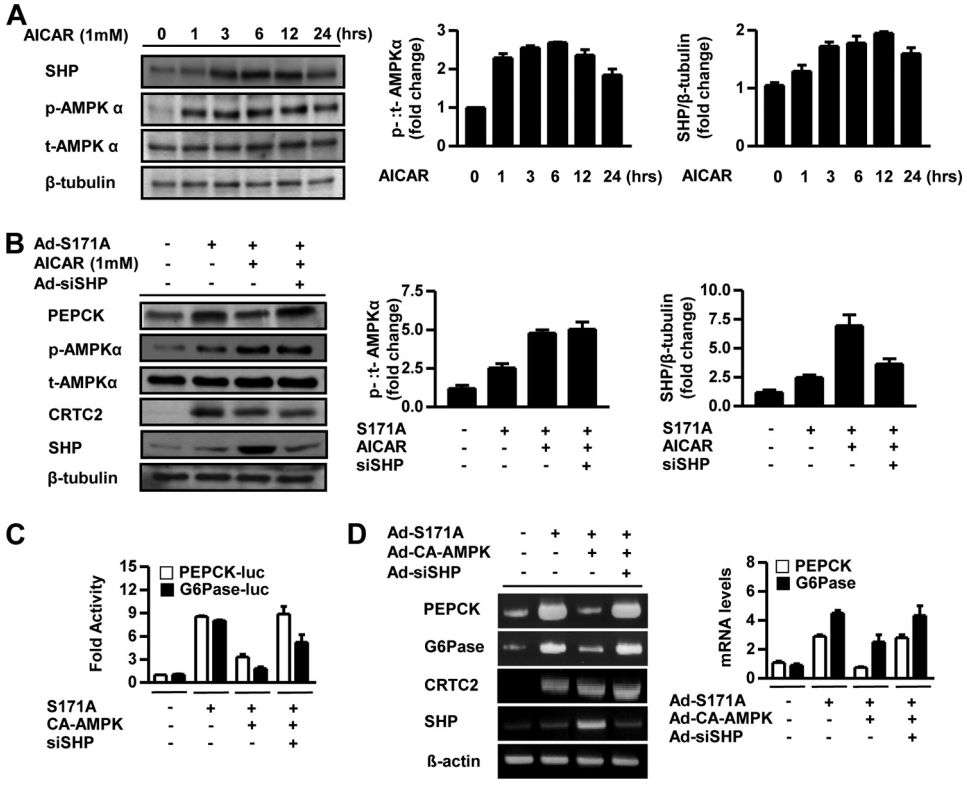

FIGURE 8. **AMPK inhibits CRTC2-mediated hepatic gluconeogenic gene expression via induction of SHP.** *A*, Western blot analysis showing the effects of AICAR on phosphorylation of AMPK and induction of SHP in rat primary hepatocytes treated with 1 mM of AICAR for the indicated times. *B*, primary rat hepatocytes were infected with Ad-siSHP and constitutively active CRTC2 (Ad-S171A) for 48 h followed by AICAR treatment and harvested for Western blot analysis using the indicated antibodies. *C*, HepG2 cells were transfected with pSUPER human SHP siRNA expression vector and, after 24 h, subsequently co-transfected with 200 ng of PEPCK-luc, G6Pase-luc, and pcDNA3-FLAG-CRTC2 (S171A) (100 ng) for 24 h and then treated with 1 mM AICAR for 12 h. *D*, RT-PCR analysis showing the effects of AMPK through SHP expression on mRNA levels of PEPCK and G6Pase in rat primary hepatocytes. Twenty-four hours after the knockdown of SHP using Ad-siSHP in the cells, infected with Ad-CRTC2 (S171A) and Ad-AMPK, total RNA was isolated for quantitative PCR analysis of SHP mRNA expression and was normalized to *β*-actin expression.

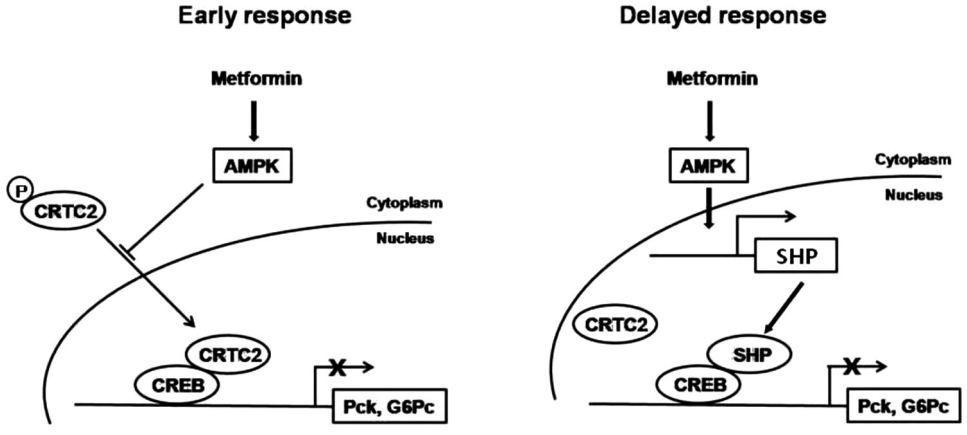

FIGURE 9. **Schematic diagram of SHP-mediated inhibition of CREB·CRTC2 complex-dependent gluconeogenesis.** Early response (*left*) of metformin phosphorylates CRTC2 at Ser^{171} and leads to its nuclear exclusion through AMPK activation. On the other hand, delayed response (*right*) of metformin antagonizes the stimulatory effects of CREB·CRTC2 complex-mediated gluconeogenesis through induction of SHP expression. SHP directly interacts with CREB, which subsequently inhibits CREB-dependent gluconeogenic enzyme gene expression via competition with CRTC2.

suppression of hepatic gluconeogenic genes is likely to be mediated quickly by CRTC2 phosphorylation. Here, we explored the role of the delayed effect of metformin-induced AMPK to inhibit hepatic gluconeogenesis via SHP. In this study, chronic treatment of AMPK activator, metformin, and overexpression of AMPK suppress phosphorylation-defective CRTC2 (S171A)-mediated hepatic gluconeogenic enzyme gene expression and hepatic glucose production by induction of SHP gene expression, and this effect was totally abolished by depletion of SHP using Ad-siRNA. Consistent with these results, prolonged provision of AICAR also decreased PEPCK protein levels through AMPK-mediated SHP gene expression. These results suggest that metformin- and AICAR-mediated chronic activation of AMPK disrupts CREB-dependent hepatic gluconeogenesis via induction of SHP gene expression. Mouse models of hepatic insulin resistance display increased dephosphorylation and nuclear accumulation of CRTC2, but metformin is still effective in treating the hyperglycemia in these conditions. Therefore, we suggest that SHP can be a potential regulator of metformin-mediated anti-diabetic effects. However, a more detailed study and elucidation of SHP is required in animal models, like SHP knock-out mice as well as insulin-resistant rodent models.

In conclusion, we suggest that metformin regulates the CREB-mediated hepatic gluconeogenic process by both CRTC2 phosphorylation and direct inhibition of CREB via modulation of SHP expression. Metformin-dependent activation of AMPK could acutely phosphorylate CRTC2 at Ser^{171} and leads to its nuclear exclusion. Chronic treatment of metformin antagonizes the stimulatory effects of CREB·CRTC2 complex-mediated hepatic gluconeogenesis through induction of SHP expression, suggesting that SHP has an important role in delayed metformin action (Fig. 9). Our study provides a novel insight into the dual molecular mechanisms by which AMPK affects CREB-mediated glucose metabolism. Further studies are required to elucidate the process by which other metabolic regulators and inducers of SHP gene may improve CREB-mediated hyperglycemia and provide therapeutic benefit for treatment of insulin resistance conditions prevalent in diabetes.

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