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Protein Kinase A Suppresses Sterol Regulatory Element-binding Protein-1C Expression via Phosphorylation of Liver X Receptor in the Liver*

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Sterol regulatory element-binding protein (SREBP)-1c is a transcription factor that controls synthesis of fatty acids and triglycerides in the liver and is highly regulated by nutrition and hormones. In the current studies we show that protein kinase A (PKA), a mediator of glucagon/cAMP, a fasting signaling, suppresses SREBP-1c by modulating the activity of liver X receptor α (LXRα), a dominant activator of SREBP-1c expression. Activation of PKA repressed LXR-induced SREBP-1c expression both in rat primary hepatocytes and mouse livers. Promoter analyses revealed that the LXRα-binding site in the SREBP-1c promoter is responsible for PKA inhibitory effect on SREBP-1c transcription. *In vitro* and *in vivo* PKA directly phosphorylated LXRα, and the two consensus PKA target sites (195, 196 serines and 290, 291 serines) in its ligand binding/heterodimerization domain were crucial for the inhibition of LXR signaling. PKA phosphorylation of LXRα caused impaired DNA binding activity by preventing LXRα/RXR dimerization and decreased its transcription activity by inhibiting recruitment of coactivator SCR-1 and enhancing recruitment of corepressor NcoR1. These results indicate that LXRα is regulated not only by oxysterol derivatives but also by PKA-mediated phosphorylation, which suggests that nutritional regulation of SREBP-1c and lipogenesis could be regulated at least partially through modulation of LXR.

In mammals, carbohydrates are an essential energy resource. When consumed in excess, carbohydrates are converted to lipids by lipogenic enzymes in preparation for times of energy deficiency. These processes are known to be regulated at the transcription level, and several different transcription factors are known to contribute to this regulation. Sterol regulatory element-binding protein (SREBP),[2] a transcription factor belonging to the basic helix-loop-helix-leucine zipper family, regulates triglyceride synthesis and cholesterol metabolism (1). Three different SREBP isoforms have been identified. SREBP-1c plays a role primarily in triglyceride synthesis (2), whereas SREBP-2 regulates cholesterol metabolism (3). SREBP-1a is expressed in growing cells and activates an entire array of genes involved in both triglyceride and cholesterol synthesis (4). In liver SREBP-1c controls transcription of genes involved in fatty acid and triglyceride synthesis (2, 5). SREBP-1c expression is highly regulated in response to the nutritional condition. Expression of SREBP-1c is undetectable during fasting, whereas its expression is strongly induced in a fed state followed by similar adapted nutritional changes in lipogenic genes (6).

To understand the molecular mechanism of nutritional regulation of SREBP-1c expression, the SREBP-1c gene promoter has been extensively studied. In the proximal region of the mouse SREBP-1c promoter, Sp1, NF-Y, USF, SREBP, and LXR-binding sites have been identified (7–9). It has been observed that insulin (10) and glucose (11, 12) are factors that induce hepatic SREBP-1c *in vivo* and *in vitro*, although precise mechanisms are yet to be elucidated. Adenoviral overexpression in rat primary hepatocytes suggested that the signal transducer, Akt, could be a potential regulator of SREBP-1c transcription (13).

Liver X receptors (LXR) belong to a nuclear receptor superfamily. The LXR subfamily consists of two members, LXRα and LXRβ, which are activated by oxysterols (14). The expression pattern of LXRα is restricted mainly to liver, adipocytes, small intestine, and macrophages, whereas LXRβ is expressed ubiquitously. Although early reports revealed involvement of LXRs in cholesterol homeostasis (15, 16), recent studies suggest that LXR negatively regulates gluconeogenesis (17) and inflammatory responses (18, 19). While investigating the pharmacological effect of LXR in rodent models, it has been observed that LXR ligands are protective against the development of atherosclerotic lesions (20) and ameliorates conditions of high blood glucose and impaired glucose tolerance (21). LXRs directly bind the cis element on the SREBP-1c promoter as heterodimers with RXR, leading to transcriptional activation (8). Several studies have established LXRs as dominant activators of SREBP-1c expression. LXRα/β double knock-out mice

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[2] The abbreviations used are: SREBP, sterol regulatory element-binding protein; dbcAMP, dibutyryl-cAMP; NID, nuclear receptor interaction domain; DBD, DNA binding domain; LBD, ligand binding domain; LXR, liver X receptor α; PKA, protein kinase A; CREB, cAMP-response element-binding protein; CBP, CREB-binding protein; HA, hemagglutinin; CMV, cytomegalovirus; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay; ChIP, chromatin immunoprecipitation; LXRE, LXR responsive element; RE, responsive element.

revealed dramatically reduced expression of SREBP-1c (9). We have used an expression cloning strategy to show that LXRs are the primary activators of the SREBP-1c promoter (8). Polyunsaturated fatty acids are the only known dietary lipid capable of negatively regulating hepatic SREBP-1c expression and lipogenesis (22). A portion of these effects is mediated at the transcriptional level through repression of LXR activity (23).

Considering that hepatic SREBP-1c expression is dominated by LXRs and eliminated by fasting, it is probable that there is a mechanism by which LXR mediates the repressed SREBP-1c expression during fasting. Consistently, it has been reported that glucagons and its signal mediator, cAMP, suppresses SREBP-1c in rat primary hepatocytes (24). Protein kinase A (PKA) is a cAMP-dependent protein kinase that consists of both a catalytic and regulatory subunit and regulates numerous cellular functions in eukaryotic cells by phosphorylating target proteins. In regard to energy metabolism, PKA is subordinated to glucagon and adrenalin and, therefore, is classically recognized as a fasting signal to activate gluconeogenesis and β-oxidation and to oppose triglycerides synthesis and glucose utilization. It is known that several nuclear receptors (*e.g.* estrogen receptor, retinoic acid receptor, peroxisome proliferator-activated receptor, and hepatocyte nuclear factor-4α) are phosphorylated by PKA leading to modification of their trans activities via diverse mechanism (25–28). In the current study we investigated effects of cAMP/PKA on the SREBP-1c expression and LXR signaling system.

EXPERIMENTAL PROCEDURES

Materials—Anti-LXRα, anti-RXR, and anti-SREBP-1 antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Anti-CREB and anti-phospho-CREB (Ser-133) antibodies were purchased from Cell Signaling Technology Inc. (Beverly, MA). Anti-His 5 antibody was purchased from Qiagen Inc. Anti-HA and anti-Myc antibodies were purchased from Roche Applied Science. Anti-FLAG antibody was purchased from Promega (Madison, WI). Dibutyryl-cAMP was purchased from Promega. T0901317 as synthetic LXR ligand was purchased from WAKO.

Animals—Male mice (C57BL/6J) were obtained from CLEA Japan (Tokyo, Japan). For fasting and refeeding, mice were fasted for 24 h and then fed a high sucrose/low fat diet for 12 h.

Isolation and Culture of Hepatocytes—Primary hepatocytes were isolated form male Sprague-Dawley rats (200–300 g) (CLEA Japan). Cell were resuspended in Dulbecco's modified Eagle's medium containing penicillin and streptomycin supplemented with 10% fetal bovine serum before being seeded on 100-mm collagen-coated dishes at 2×10^6 cells/dish. After incubation for 4 h to allow attachment, medium was replaced, and experiments were performed.

Northern Blotting—Total RNA from mouse liver and rat primary hepatocytes was isolated as described previously (29). The cDNA probes for mouse SREBP-1, phosphoenolpyruvate carboxykinase, acidic ribosomal phosphoprotein PO (36B4), ABCA1, and LXRα were prepared as previously described (8, 29, 30).

Plasmids—A series of mouse SREBP-1c promoter linked to pGL2 basic were previously described (7). LXRE-enhancer construct linked to pGL2 promoter vector was previously described (8). Human ABCA1 promoter (−919 to +239) linked to pGL3 basic were previously described (30). Expression vector for Gal4-DBD-LXRα ligand binding domain (LBD) fusion protein and Gal4 RE Luc vector were previously described (8). Expression vectors for mouse LXRα (wild type, 195A 196A and 290A 291A) were constructed in pcDNA3.1(+). Expression vector for PKA c subunit was constructed in pFA. Expression vector for V5-tagged PKA regulatory subunit (dominant negative form) was constructed in pcDNA3.1(−) according to a previous report for the mutated form of the regulatory type 1 subunit of PKA (31). The expression vector for HA-tagged human LXRα was constructed in pcDNA3. Expression vectors for FLAG-tagged wild type human RXR and FLAG-tagged mutant RXR in which the serine 27 residue was substituted to alanine were constructed in pcDNA3.1(+). Expression vectors for histidine-tagged recombinant mouse LXRα (full-length, amino acids 1–163, 162–326, and 325–445) were constructed in pET28a(+). The expression vector for glutathione *S*-transferase fusion protein was constructed in pGEX4T-2. For the mammalian two-hybrid system, the mouse LXRα-coding region was ligated into pACT. Mouse SRC-1 nuclear receptor interaction domain (amino acids 568–779) and mouse NcoR1 nuclear receptor interaction domain (amino acids 1944–2453) were ligated into pM. For *in vitro* translation, SRC-1 nuclear receptor interaction domain (NID; amino acids 576–779) was ligated into pcDNA3.1(+). The expression vector for FLAG-tagged CBP was previously described. The expression vector for HA-tagged mouse CREB was constructed in pcDNA3.

Transfections and Luciferase Assay—293 cells and 293T cells were grown in Dulbecco's modified Eagle's medium containing penicillin and streptomycin supplemented with 10% fetal bovine serum at 37 °C in 24-well plate overnight before transfection. Cells were transfected with reporter vector, expression vector using FuGENE 6. Total amounts of DNA were adjusted to 0.5 μg/well using empty vector. After 24 h of incubation, the amounts of firefly luciferase activity in transfectants were measured. Firefly luciferase activity was normalized by the amounts of Renilla luciferase activity expressed from CMV or SV40 promoter linked-Renilla luciferase unless otherwise indicated.

In Vitro Kinase Assay—Histidine-tagged recombinant mouse LXRα proteins were expressed in *Escherichia coli* (BL21, DE3) and purified using standard techniques and purification kit Mag Extractor (TOYOBO) according to the manufacturer's protocol. Briefly, bacterial culture containing kanamycin were grown at 37 °C. After induction of recombinant proteins for 2–3 h, cells were resuspended in lysis buffer (6 M guanidine hydrochloride, 5 M NaCl (pH 8.0)) and lysed by sonication. The centrifuged supernatant was mixed for 30 min at room temperature on a rotator with magnetic nickel beads. Protein-absorbed beads were washed with lysis buffer, and recombinant LXRα proteins were eluted with elution buffer. After dialysis to exclude guanidine, the concentration and size of histidine-tagged proteins were estimated by SDS-PAGE followed by Coomassie Blue staining and immunoblotting using a known quantity of molecular weight standards. The purified proteins were stored at −80 °C until experiments were performed.

Bovine purified PKA catalytic subunit was purchased from Promega. PKA c subunit, histidine-tagged LXRα, and [γ-^{32}P]ATP were mixed in PKA buffer (50 mM Tris-HCl (pH 7.5), 10 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT) and incubated for 45 min at 30 °C. Samples were analyzed by SDS-PAGE, and phosphorylation was visualized by autoradiography. Quantity of histidine-tagged protein was confirmed by immunoblotting using anti-His 5 antibody. Histone H1 protein (Calbiochem) was used as positive control in kinase assays.

In Vivo Kinase Assay—For *in vivo* kinase assay, COS7 cells prepared in 100-mm collagen-coated dishes at 1×10^6 cells/dish were transfected with control or expression vector for HA-tagged human LXRα vectors. After transfection, the cells were starved in phosphate-deficient medium for 12 h and then incubated for 2 h in the same medium containing 500 μCi/ml [^{32}P]orthophosphate. Cells were treated with PKA activators (forskolin 10 μM, dibutyryl-cAMP 1 mM, isobutylmethylxanthine 1 mM) for 30 min before harvesting.

At the end of the labeling period the cells were washed ice-cold phosphate-buffered saline, harvested, and lysed on ice. Protein samples were extracted in lysis buffer mentioned elsewhere and centrifuged at 15,000 rpm 4 °C for 10 min. Supernatants were subjected to immunoprecipitation assay used with anti HA antibody (Roche Applied Science) as previously described. The immunocomplex were eluted in sample buffer, resolved by SDS-PAGE (8% gel), visualized with autoradiography, and immunoblotted.

Electrophoretic Mobility Shift Assay—Nuclear extracts were prepared from livers as previously described (32). RXR protein was generated from expression vector using a coupled *in vitro* reticulocyte transcription/translation system (Promega). Double-stranded oligonucleotides of LXR response element in SREBP-1c promoter using in EMSA were prepared as previously described. *In vitro* synthetic protein lysate or nuclear extracts (~1 μg) were incubated. DNA-protein complex were resolved on a 4% polyacrylamide gel.

Chromatin Immunoprecipitation (ChIP) Assay—The ChIP assay was conducted with a chromatin immunoprecipitation kit (Upstate Biotechnology, Lake Placid, NY). After H2.35 cells were treated with PKA activators (10 μM forskolin, 100 μM dibutyryl-cAMP) or vehicle under T0901317 (10 μM) for 1 h at 37 °C, H2.35 cells were cross-linked for 10 min by adding formaldehyde directly to culture medium to a final concentration of 1%. Cross-linked cells were washed with ice-cold phosphate-buffered saline containing protease inhibitors, scraped, pelleted, resuspended in 200 μl of SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl (pH 8.0)) per 1×10^6 cells and incubated for 10 min on ice. The lysates were then sonicated for 30 s each by sonicator (Sonifer 250, Branson) with 20% duty cycle and 10% output power. The samples were on ice for 1 min between the cycles. After sonication the samples were centrifuged, and supernatant was diluted 10-fold in ChIP dilution buffer containing protease inhibitors. 2-ml aliquots were precleared with 75 μl of a 50% slurry of salmon sperm DNA/protein A-Sepharose for 30 min at 4 °C and then incubated overnight with 3 μg of anti-RXR antibody or control IgG. Antibody-protein-DNA complexes were immunoprecipitated with 60 μl of protein A. After intensive washing, pellets were eluted by freshly prepared elution buffer (1% SDS, 0.1 M $NaHCO_3$). Formaldehyde cross-linking was reversed by overnight incubation at 65 °C after adding 20 μl of 5 M NaCl. Samples were purified with phenol:chloroform:isoamyl alcohol and precipitated with ethanol. DNA pellets were dissolved with Tris-EDTA buffer and used as templates in PCR. Eight μl of 20 μl of DNA solution was used for PCR amplification (1st step of 95 °C for 3 min (one cycle), 2nd step of 94 °C for 1 min, 62.1 °C for 30 s, 72 °C for 1 min (25 cycles)). ChIP primers were 5′-GAACCAGCGGTGGGAACACAGAGC-3′ and 5′-GACGGCGGCAGCTCGGGTTTCTC.

Immunoblotting and Immunoprecipitation—COS7 cells were seeded on 100-mm dishes and transfected with the expression vectors. After incubation for 48 h, dbcAMP (100 μM), forskolin (10 μM), and isobutylmethylxanthine (100 μM) were added into medium for 30 min, and then cells were harvested. Protein lysate was extracted in lysis buffer (25 mM HEPES (pH 7.9), 50 mM KCl, 6% glycerol, 5 mM $MgCl_2$, 0.5% Triton X-100, 1 mM DTT, 50 mM NaF, 40 mM β-glycerophosphate, 25 mM sodium pyrophosphate, protease inhibitor mixture (Roche Applied Science)) on ice 20 min and centrifuge at 15,000 rpm at 4 °C for 10 min. Supernatants were subjected to immunoprecipitation assay as previously described (29).

GST Pulldown Assay—GST and GST-LXRα proteins were prepared using standard techniques according to the manufacturer's protocol. Briefly, *E. coli* (BL21, DE3) transformed with the GST or GST-LXRα expression vector were incubated before induction at room temperature for 4 h with 0.4 mM isopropyl 1-thio-β-D-galactopyranoside. The cells were harvested by centrifugation and suspended in buffer A (25 mM HEPES (pH 7.9), 50 mM KCl, 6% glycerol, 5 mM EDTA, 5 mM $MgCl_2$, 0.5% Triton X-100, protease inhibitor mixture, 1 mM DTT). The cells were lysed by sonication, and cellular debris was removed by centrifugation at 15,000 rpm at 4 °C for 10 min. Recombinant GST or GST-LXRα proteins were purified by glutathione-Sepharose beads according to the manufacturer's protocol (Amersham Biosciences). GST fusion proteins were eluted (50 mM Tris HCl (pH 8.0), 50 mM NaCl, 10 mM glutathione, protease inhibitor mixture, 1 mM DTT). Amounts and sizes of GST or GST-LXRα proteins were estimated by SDS-PAGE followed by Coomassie Blue staining and immunoblotting using anti-LXR antibody. GST or GST-LXRα and the PKA c subunit or equal amounts of bovine serum albumin were mixed in PKA buffer (50 mM Tris HCl (pH 7.5), 10 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, 50 mM NaF, 20 mM β-glycerophosphate) and incubated for 45 min at 30 °C. After adding glutathione beads and ^{35}S-radiolabeled SRC-1 nuclear receptor interaction domain (amino acids 576–779) synthesized *in vitro* to samples and incubation for 2 h at 4 °C, beads were washed 3 times with ice-cold phosphate-buffered saline containing 0.1% Triton X-100. Proteins bound to beads were eluted with elution buffer (50 mM Tris-HCl (pH 8.0), 50 mM glutathione), resolved by SDS-PAGE, and visualized autoradiography.

RESULTS

PKA Represses Expression of SREBP-1 in Vivo—Expression of SREBP-1c in the liver is highly regulated by nutrition; it is suppressed at fasting and induced in a refed state as previously

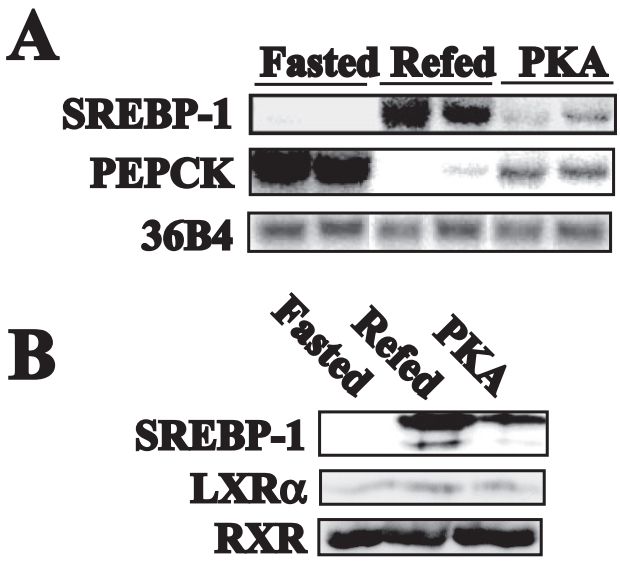

FIGURE 1. **Effects of PKA activation on expression of mouse hepatic SREBP-1.** C57BL6/J mice fasted for 24 h (*Fasted*) followed by refeeding with a high sucrose high fat diet for 12 h (*Refed*) were intravenously treated with vehicle or a combination of PKA activators, dbcAMP (35 mg/kg) and theophylline (30 mg/kg) (*PKA*). Two hours post-injection mice were sacrificed, and total liver RNA and nuclear extracts were prepared. Total RNA (10 μg) and nuclear extracts (40 μg of protein) were subjected to Northern (*panel A*) and immunoblotting (*panel B*), respectively.

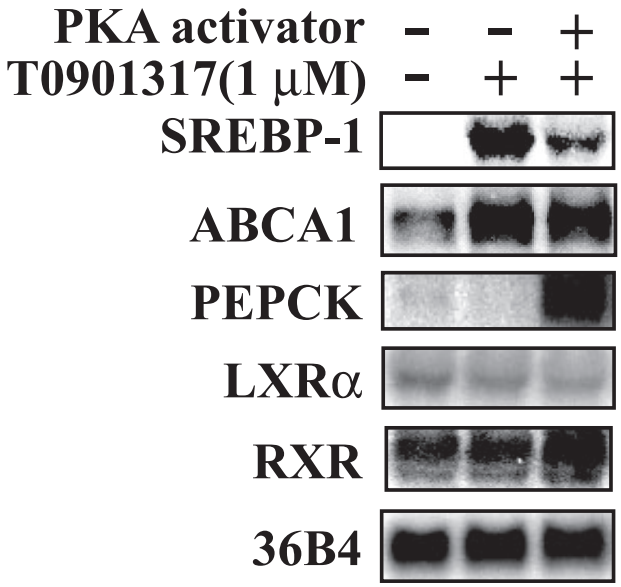

FIGURE 2. **Effects of PKA on mRNA level of SREBP-1 induced by the synthetic LXR ligand, T0901317.** Rat primary hepatocytes were incubated in medium containing vehicle or T0901317 (1 μM) in the presence or absence of dbcAMP (30 μM) for 24 h. Cells were harvested for extraction of total RNA followed by northern blotting as described under "Experimental Procedures." *PEPCK*, phosphoenolpyruvate carboxykinase.

reported (6). One potential mechanism of this suppression at fasting could be mediated through the cAMP/PKA system that mediates the glucagon signal. C57BL/6J mice in a refed state were administrated with dibutyryl-cAMP and theophylline for activation of PKA to mimic fasting signal. The SREBP-1c mRNA that was highly induced by refeeding was robustly repressed by cAMP/theophylline administration (Fig. 1*A*). Reciprocally, phosphoenolpyruvate carboxykinase, a gene that controls gluconeogenesis and a well known target of the glucagon/cAMP/PKA signal, was suppressed by refeeding but restored by these PKA activators. The reduction of SREBP-1 mRNA level by PKA activation was associated with a robust decrease in active SREBP-1c protein in hepatic nuclear extracts (Fig. 1*B*). SREBP-1c expression is dominantly controlled by LXR/RXR that binds directly to the two LXREs in the SREBP-1c promoter (8, 9). As shown in Fig. 1*B*, the expression of LXR/RXR was not affected by the PKA activation. Therefore, SREBP-1c suppression by PKA was not due to reduction in the amounts of LXR/RXR.

PKA Activation Represses LXR-mediated SREBP-1 Induction—The effect of PKA on LXR activation of SREBP-1c was estimated. In rat primary hepatocytes, the SREBP-1c mRNA level was essentially undetectable but was highly induced by T0901317, an LXR agonist. This induction was substantially suppressed by the addition of dibutyryl-cAMP whereas expression of LXR and RXR was not increased at the mRNA level (Fig. 2). These data suggest that PKA repression of SREBP-1c was mediated not through changes in LXR expression but through modulating the LXR activity. Meanwhile, LXR agonist-induced expression of ABCA1 (ATP binding cassette transporter A1), an LXR target gene, was only slightly affected by PKA activation.

LXRE Response Element Is Responsible for PKA Inhibition of SREBP-1c Promoter—To further investigate the PKA effect on SREBP-1 transcription, *in vitro* reporter assays for SREBP-1c promoter were performed. Luciferase reporters linked to different sizes of SREBP-1c promoter region around the LXR-binding sites (LXREs) and expression vectors of LXRα and the PKA catalytic subunit for PKA activation were transfected into HepG2 cells (Fig. 3*A*). PKA expression did not affect the basal level of SREPB-1c promoter activity in HepG2 cells. Meanwhile, PKA co-expression strongly suppressed LXRα-mediated activation of SREBP-1c promoter. This was only observed in LXRE-containing promoters (−2.6 kilobase pairs and −550 bp). The shorter construct lacking LXREs (90 bp) did not show LXR activation or PKA inhibition. Thus, the inhibitory effect of PKA on SREBP-1c promoter was conceivably mediated through LXREs. This was supported by the LXRE enhancer construct assays, demonstrating that endogenous PKA activation by forskolin, an adenylate cyclase activator, dose-dependently inhibited LXRE enhancer activity induced by the LXR ligand, and this inhibitory effect was abolished by co-expression of the PKA regulatory subunit dominant negative form (Fig. 3*B*). The mechanism against the PKA suppression of LXR activity was further explored the using Gal4 DNA binding domain (DBD) protein fused to LXRα LBD in HepG2 (Fig. 3*C*). LXR-LBD was highly activated in this system by the LXR synthetic ligand as previously reported. This activation was completely abolished by co-transfection of PKA, suggesting that PKA activation inhibits ligand-mediated activation of LXR. PKA-mediated inhibition of LXR transcriptional activity was supported by luciferase assays using the promoter of ABCA1, another LXR target gene. Mouse ABCA1 promoter activity was also dose-dependently inhibited by PKA expression in both basal and LXR-induced conditions (Fig. 3*D*).

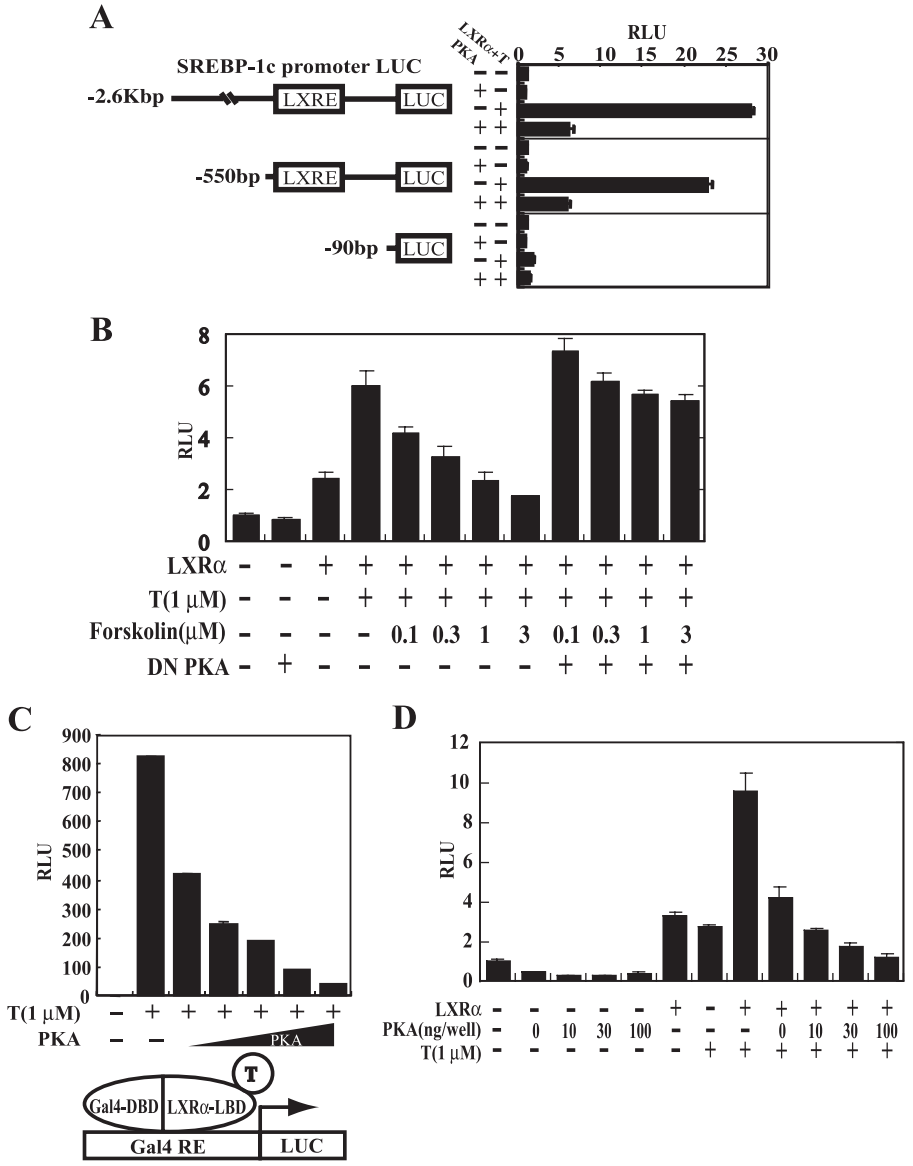

FIGURE 3. **Effects of PKA on SREBP-1c promoter activity induced by LXRα.** *A*, HepG2 cells were transfected with SREBP-1c promoter Luc vector (300 ng/well), CMV Renilla luciferase vector as an internal reference (2.5 ng/well), expression vectors for LXRα (100 ng/well), and PKA catalytic subunit (100 ng/well). After transfection, the cells were further incubated with the medium containing vehicle or T0901317 (*T*, 1 μM) for 20 h. *kbp*, kilobase pairs. *RLU*, relative light units. *B*, HepG2 cells were transfected with LXRE enhancer Luc vector (200 ng/well), CMV Renilla luciferase vector (2.5 ng/well), and expression vectors for LXRα (100 ng/well) and PKA regulatory subunit dominant negative form (*DN PKA*) (300 ng/well). After transfection cells were incubated in medium containing vehicle or T0901317 (1 μM) and forskolin (0, 0.1, 0.3, 1, 3 μM) for 20 h. Assays were performed as described under "Experimental Procedures." *C*, HepG2 cells were transfected with expression vectors for Gal4-DBD-LXRα ligand binding domain (150 ng/well), PKA Gal4 RE LUC vector (150 ng/well), PKA (3, 10, 30, 100, 200 ng/well), and CMV Renilla luciferase vector (2.5 ng/well). After each transfection cells were incubated in medium containing vehicle or T0901317 (1 μM)) for 20 h and subjected luciferase and Renilla reporter assays as in described under "Experimental Procedures." *D*, 293 cells were transfected with human ABCA1 promoter Luc vector (300 ng/well), expression vector for LXRα (100 ng/well), PKA (0, 3, 10, 30, 100 ng/well), and CMV Renilla luciferase vector for internal reference (2.5 ng/well). After each transfection cells were incubated in medium containing vehicle or T0901317 (1 μM) for 20 h.

LXRα Is Phosphorylated by PKA in Vitro—These data led us to speculate that PKA modulates LXR activity, most likely through direct phosphorylation of LXRα. To test this hypothesis, *in vitro* kinase assays of LXRα were performed. Purified recombinant protein of histidine-tagged LXRα was incubated with the PKA catalytic subunit. LXRα protein was phosphorylated by PKA as well as PKA autophosphorylation and histone H1 phosphorylation as positive controls of PKA target protein (Fig. 4*A*). Functional domains of the LXRα protein were also tested using this *in vitro* kinase assay (Fig. 4*B*). Every domain of LXRα was significantly phosphorylated. In the amino acid sequence of PKA, there are two potential PKA target sites (serines 195 and 196; threonine 290 and serine 291) in the LBD/heterodimerization domain. Introduction of mutation in which these serine residues were replaced by alanine caused resistance to PKA inhibition of SREBP-1c promoter activation (Fig. 4*C*). To assess whether LXRα is phosphorylated by PKA *in vivo*, COS7 cells were transfected with expression vector for HA-tagged LXRα and subsequently labeled with [^{32}P]orthophosphate. After treatment with PKA activators, the phosphorylation state of LXRα was examined by autoradiography. As evident from autoradiography and immunoblots (Fig. 4*D*), PKA stimulation induced the phosphorylation state of LXRα. These results indicate that PKA directly phosphorylates LXR protein, which is crucial for PKA inhibition of LXR activity.

DNA Binding Activity of LXR/RXR Heterodimer Was Decreased by PKA Phosphorylation of LXRα—The effect of LXRα phosphorylation by PKA on DNA binding was tested by EMSA assays (Fig. 5*A*). Recombinant LXRα protein requires RXR for its binding to LXRE. The signal of LXR/RXR bound to LXRE was partially decreased by PKA treatment of LXR. This effect required the incubation of LXR with PKA at 30 °C, indicating the PKA inhibition was mediated through its kinase activity. To confirm this effect *in vivo*, nuclear extracts of livers from dibutyryl-cAMP/theophylline-administrated mice were prepared and subjected to EMSA assay (Fig. 5*B*). PKA activation in these nuclear extracts was confirmed by phosphorylation of CREB, a well known PKA target in mouse livers, as shown by immunoblot analysis. EMSAs of these nuclear extracts demonstrated that the signal of LXRE binding mainly from LXR/RXR, as confirmed by supershift with LXR and RXR antibodies, was dose-dependently diminished by PKA administration. Next, to determine whether PKA activation reduced DNA binding activity of LXR/RXR heterodimer, ChIP assay analysis on

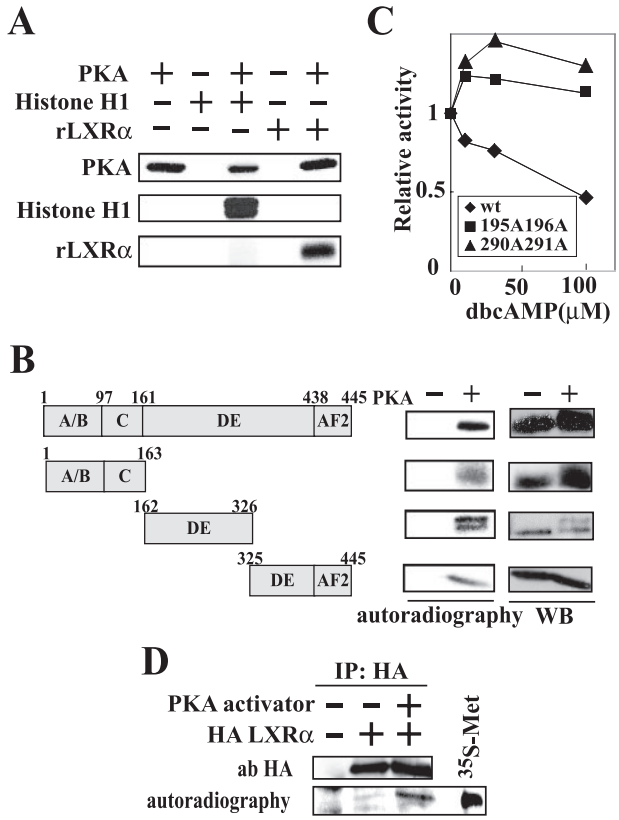

FIGURE 4. **Direct phosphorylation of recombinant LXRα (*rLXRα*) protein at the serine or threonine residues in the ligand binding domain is crucial for inhibition of LXR signaling by dbcAMP.** *A*, *in vitro* kinase assay of full-length LXRα. Histone H1 or recombinant histidine-tagged LXRα were incubated with PKA and [^{32}P]ATP and resolved by SDS-PAGE (8% gel). Phosphorylated protein was visualized by autoradiography. Phosphorylation of histone H1 and PKA itself was shown as a positive control of PKA target protein. *B*, *in vitro* kinase assay of several fragments of LXRα. Amino acids 1–163, 162–326, and 325–445, corresponding amino acids sequences of LXRα protein, respectively, were incubated with PKA. Samples were resolved by SDS-PAGE (12% gel) and electrophoretically transferred to PVDF membranes. Phosphorylated proteins were visualized by autoradiography. LXRα proteins were estimated by immunoblotting with anti-histidine antibody. *WB*, Western blot. *C*, substitution of serine or threonine amino acid residues for alanine in LXRα abolished the PKA-dependent repression of a luciferase reporter derived by LXRE. Expression vectors for wild type LXRα (*wt LXRα*) or an LXRα mutant with S195A and S196A (195A196A) or with T290 and S290A (290A 291A) (100 ng/well) and CMV Renilla luciferase vector (2.5 ng/well) were cotransfected with LXRE enhancer reporter plasmid (100 ng/well) into 293 cells. After transfection dbcAMP was added into the medium followed by incubation for 20 h. Luciferase activity was measured. Relative activity compared with each control was plotted. *wt*, wild type. *D*, PKA activation increases phosphorylation of LXRα *in vivo*. COS7 cells transfected with HA-tagged LXRα were metabolically labeled with [^{32}P]orthophosphate and incubated for 30 min with PKA activators (10 μM, forskolin, 1 mM dibutyryl-cAMP, 1 mM isobutylmethylxanthine) before harvesting. Protein samples were immunoprecipitated (*IP*) with anti-HA antibody (*ab*). Labeled proteins resolved by SDS-PAGE were autoradiographed and immunoblotted. [^{35}S]Met is methionine-labeled LXRα, used for the positive control.

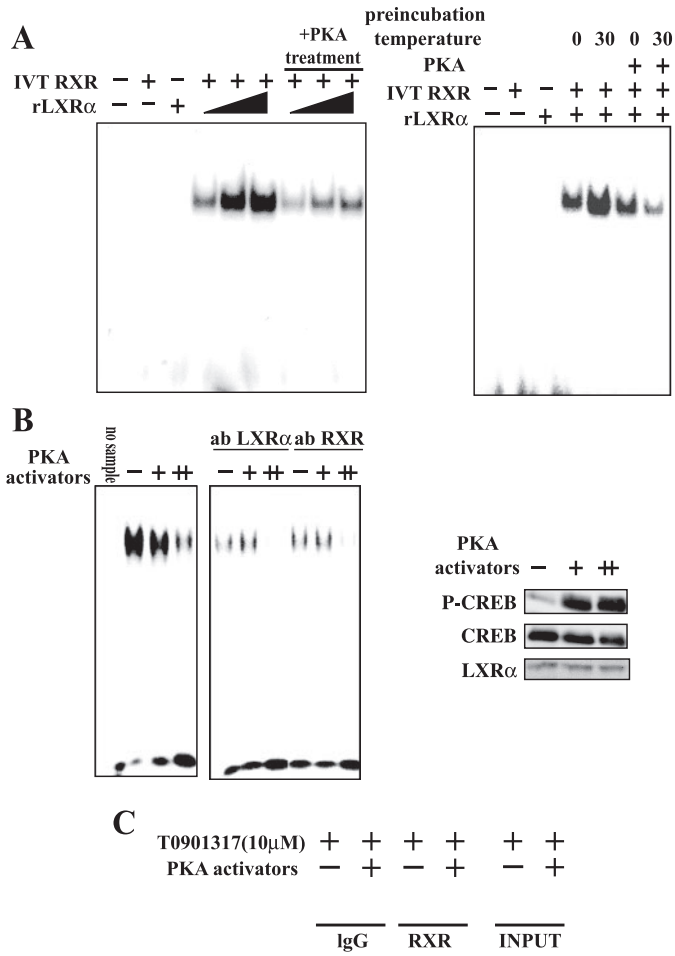

FIGURE 5. **Phosphorylation of LXRα by PKA decreases binding of LXRα/RXR to LXRE.** *A*, decreased binding of LXRα/RXR to LXRE by PKA treatment with LXRα in electrophoretic mobility shift assays. Increasing amounts of recombinant LXRα (*rLXRα*, *left panel*) were incubated with bovine serum albumin (used as nonspecific protein control) or PKA catalytic subunit and then incubated with radiolabeled LXRE probe and *in vitro* synthesized RXR for EMSA as described under "Experimental Procedures." In the *right panel*, recombinant LXRα was preincubated with bovine serum albumin or PKA at 30 or 0 °C (on ice) (*right panel*) and then incubated with radiolabeled LXRE probe and *in vitro* synthesized RXR for EMSA as described under "Experimental Procedures." *IVT*, *in vitro* translated. *B*, *in vivo* PKA activation decreased the DNA binding activity of LXR/RXR complex in EMSA (*left panel*). The various doses of PKA activators (vehicle, 35 mg/kg dbcAMP + 30 mg/kg theophylline, 105 mg/kg dbcAMP + 30 mg/kg theophylline) were administrated to refed C57BL6/J mice. Nuclear protein extracts prepared from livers were incubated with the ^{32}P-labeled LXRE probe. The signal from LXR/RXR was confirmed by supershift assays after the addition of LXR and RXR antibodies (*ab*) to the incubations. Nuclear protein (30 μg/lane) was resolved by SDS-PAGE (10% gel) and subjected to immunoblotting with anti phospho (*P*)-CREB, anti-CREB, and anti-LXRα antibodies (*right panel*). *C*, PKA activation decreases DNA binding of LXR/RXR complex to LXRE on SREBP-1c promoter *in vivo*. H2.35 cells were treated with PKA activators (10 μM forskolin, 100 μM dibutyryl-cAMP) or vehicle for 60 min. Because LXR antibody suitable for immunoprecipitation is not currently available (43), ChIP analysis was performed with anti-RXR antibody or control IgG. Primers specific to the LXRE region of mouse SREBP-1c promoter were used for PCR analysis. As a positive control (3% *INPUT*), the assay was performed with the same primers on the genomic DNA on which the ChIP assays were performed.

extracts from H2.35 cells, a mouse hepatocyte-derived cell line, was conducted. Anti-RXR antibody for immunoprecipitation of LXR/RXR heterodimer, and primers to detect LXRE on SREBP-1c promoter for PCR were used. As shown in Fig. 5*C*, the interaction of LXR/RXR heterodimer with LXRE was decreased upon PKA activation *in vivo*. These data suggest that the modification of LXR activity by PKA is attributed to reduction of DNA binding activity of LXR/RXR heterodimer via PKA phosphorylation of LXRα.

Coactivator or Corepressor Recruitments of LXRα Are Reciprocally Modified by LXRα Phosphorylation by PKA—It was reported that in LXRα or β-mediated transcription of SREBP-1c; SRC-1 works as a coactivator, and NcoR1 works as a corepressor (33). To test the possibility of involvement of these cofactors in PKA inhibition of LXR activity, mammalian two-

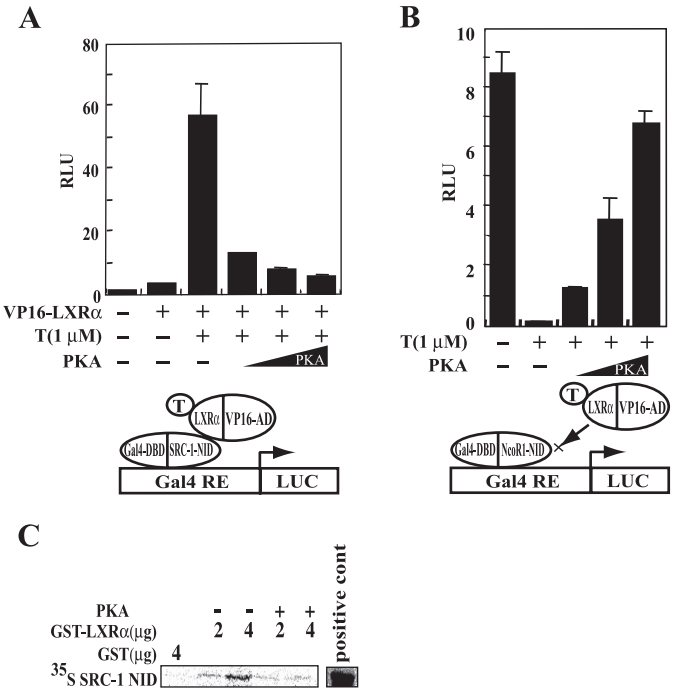

FIGURE 6. **PKA modulates recruitment of coactivator and corepressor for LXRα.** *A*, inhibition of recruitment of SRC-1 to LXRα by PKA in the mammalian two-hybrid system. 293 cells were transfected with Gal4 RE Luc vector, CMV Renilla luciferase vector (2.5 ng/well), and expression vectors for VP-16-LXRα (amino acids 1–445). *Gal4-DBD-SRC-1*, DBD of Gal4 fused to NID of SRC-1 and PKA. Four hours after transfection the cells were further incubated with medium containing vehicle or T0901317 (1 μM) for 20 h. *AD*, activation domain. *B*, activation of recruitment of NcoR-1 to LXRα by PKA. 293 cells were transfected with Gal4 RE Luc vector, SV40 Renilla luciferase vector (25 ng/well), and expression vectors for VP-16 LXRα ((amino acid 1–445), Gal4-DBD-NcoR1, and PKA. 4 h after transfection the cells were further incubated with medium containing vehicle or T0901317 (1 μM) for 20 h. *C*, PKA treatment inhibited the interaction of LXRα with SRC-1 *in vitro*. Recombinant GST-LXRα fusion protein was treated with PKA or bovine serum albumin (used as nonspecific control protein) at 30 °C and then incubated with ^{35}S-radiolabeled SRC-1-NID synthesized *in vitro* and glutathione-Sepharose beads. Pulled-down proteins were resolved by SDS-PAGE (12% gel) as described under "Experimental Procedures."

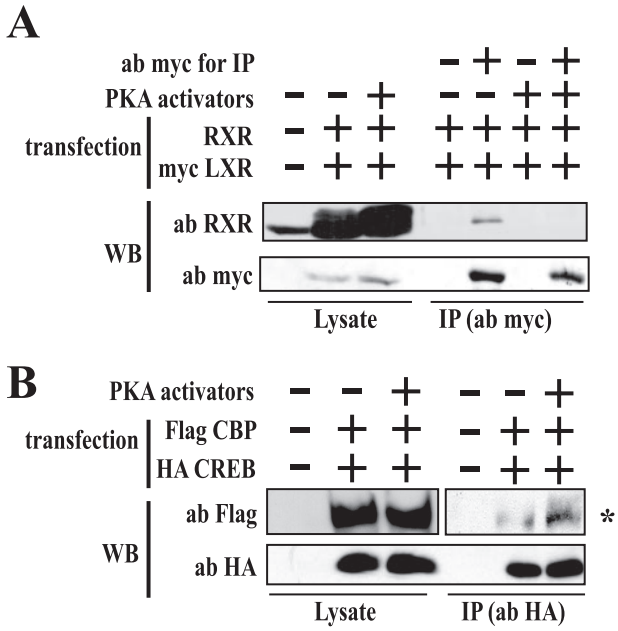

FIGURE 7. **PKA prevents interaction between LXRα and RXR *in vivo*.** *A*, PKA inhibition of co-immunoprecipitation of LXRα and RXR. *B*, PKA activation of co-immunoprecipitation of CREB and CBP. Cos7 cells were transfected with expression vector for Myc-tagged LXRα and RXR (*A*) or for FLAG-tagged CBP and HA-tagged CREB (*B*). After incubation for 48 h, cells were treated with medium containing PKA activators (100 μM dbcAMP, 10 μM forskolin, 100 μM isobutylmethylxanthine) for 30 min and harvested. Immunoprecipitation (*IP*) and immunoblotting (*WB*) with the indicated antibodies (*ab*) were performed as described under "Experimental Procedures." Proteins were extracted by lysis buffer and resolved by SDS-PAGE (8% gel for LXR and RXR, 4% gel for CBP, and 10% gel for CREB). There is different exposure time between lysate and immunoprecipitation samples for detection of FLAG-tagged CBP because intensity of protein amount of FLAG-tagged CBP in lysate was too strong.

hybrid assays were preformed. The system detects the binding of VP16-AD-LXRα fusion protein and Gal4-DBD-SRC-1-NID (a functional region that interacts with nuclear receptors (34)) in 293 cells. The addition of the LXR ligand markedly activated the signal, as evidenced by recruitment of SRC-1 to LXR. PKA co-expression markedly and dose-dependently inhibited the recruitment of SRC-1 (Fig. 6*A*). Inversely, PKA co-expression increased the recruitment of NcoR1-NID to AD-LXRα (Fig. 6*B*). Inhibition of the binding between LXRα and SRC-1 NID by PKA was directly confirmed in pulldown assays. The labeled SRC-1-NID was pulled down by GST-LXRα, and the signal was reduced by PKA phosphorylation (Fig. 6*C*). These data suggest that PKA activation modifies LXR and enhances dissociation of coactivator and recruitment of corepressor, leading to repression of LXR transcriptional activity.

LXRα/RXR Interaction Is Impaired by PKA—Finally, the effect of PKA on heterodimerization of LXR/RXR was estimated by co-immunoprecipitation *in vivo*. Myc-tagged LXRα and RXR were co-expressed in COS7 with or without PKA activation (Fig. 7*A*). RXR was co-immunoprecipitated with the tagged LXRα. This binding between LXRα and RXR was nearly eliminated by PKA activation. In contrast, PKA increased the co-immunoprecipitation of FLAG-tagged CBP and HA-tagged CREB, well characterized targets of PKA (Fig. 7*B*). These data demonstrated that the interaction of LXRα and RXR is impaired by PKA activation, explaining at least partly PKA inhibition of LXR transactivation.

Contribution of RXR to PKA Inhibition of LXR/RXR Effect on LXRE in SREBP-1c Promoter—In a previous report, RXR is shown as a substrate of PKA *in vitro*, and serine 27 is shown as essential for cAMP-mediated down-regulation of RXR transcriptional activation in COS cells by assessed in luciferase assay (35). Therefore, the effect of PKA on RXR activity to regulate activity of SREBP-1c promoter was tested. HepG2 cells were transfected with wild type RXR or mutant RXR (S27A). As a result (Fig. 8), both wild type and mutant RXRs activate the LXRE-luciferase reporter in response to LG268, a synthetic RXR agonist, with a only slight (~25%) decrease in both basal and ligand-stimulated response by the RXR mutation. PKA activation dose-dependently inhibited this RXR-mediated LXRE reporter. The RXR mutation modestly prevented this negative effect of PKA on RXR function especially at low dose dbcAMP(10, 30 μM) but less at high dose dbcAMP(100 μM). These results indicate that RXR phosphorylation is at least in part involved in suppression of SREBP-1c promoter activity by cAMP/PKA signals.

DISCUSSION

Our current studies demonstrate that PKA directly phosphorylates LXRα protein and inhibits its signaling, resulting in sup-

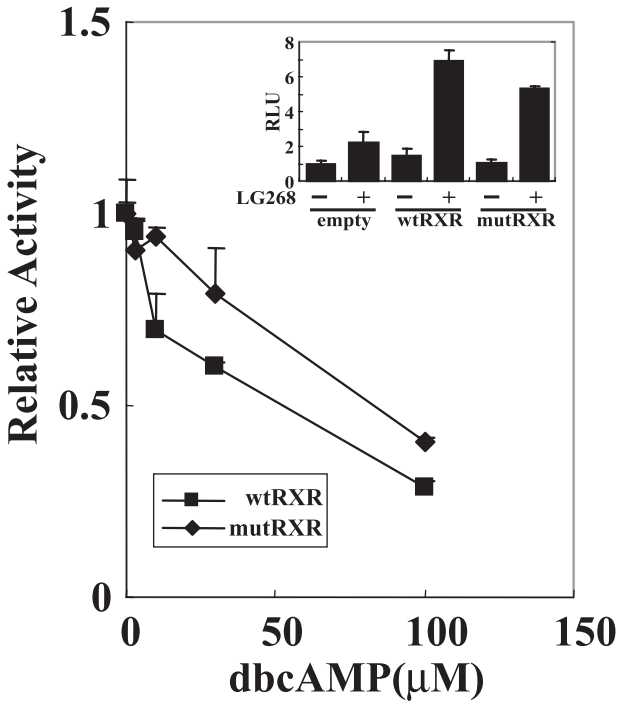

FIGURE 8. **PKA modifies RXR activity induced by RXR ligand on LXRE in SREBP-1c promoter.** 293T cells were transfected with LXRE enhancer Luc vector (300 ng/well), CMV Renilla luciferase vector (2.5 ng/well), and expression vector for wild type RXRα or mutant RXR (400 ng/well). After transfection cells were incubated for 20 h in medium containing vehicle or T0901317 (1 μM), LG268 (30 nM), or dbcAMP (0, 3, 10, 30, 100 μM) and subjected to luciferase and Renilla reporter assays. *RLU*, relative light units.

pression of SREBP-1c transcription both *in vitro* and *in vivo*. Phosphorylation of LXRα, presumably through its conformational change, causes two consequences; suppression of RXR dimerization leading to decreased binding to LXRE and suppression of the ligand activation followed by decreased recruitment of coactivator SRC-1 and increased recruitment of corepressor NcoR1. Both events lead to repression of LXR transactivation for SREBP-1c. PKA inhibition was more prominent for LBD activation of LXR than for its binding to LXRE.

Post-translational modification of transcription factors by phosphorylation provides a rapid cellular response to environmental changes. We now show that the ligand-induced transactivation of LXR can be regulated *in vivo*, *ex vivo*, and *in vitro* by PKA-dependent phosphorylation. The catalytic subunit of PKA has been identified in the nucleus and phosphorylates numerous transcription factors, modulating their transcriptional activities positively such as hepatocyte nuclear factor-4α and negatively such as L-type PK (28). Now, LXR can be added to the list of PKA-modulating factors. Although PKA-dependent phosphorylation sites (RX_{0-2}(S/T)) lie within all domains of LXR, the 195–196 serines and 290–291 threonine and serine residues might be crucial for ligand-induced conformational change since these sites in human LXRα completely match the most preferable consensus sequence (R(R/K)X(S/T)). It is also possible that phosphorylation states of these critical sites control other potential phosphorylation sites. LXR/RXR also transactivate other genes such as the ABCA1 (36) and the ABCG family (37, 38). The LXRα-induced activity of the ABCA1 promoter containing LXRE was also repressed by PKA similarly to the SREBP-1c promoter. However, the inhibitory effect of PKA on the ABCA1 mRNA level was minimal in our sets of experiments in livers and hepatic cells. Conversely, expression of ABCA1 in murine macrophage cell line RAW 264 cells was reported to be up-regulated by dbcAMP (39, 40), although its responsible regulatory motif in the ABCA1 promoter has not been identified. This undetermined cAMP-dependent activation mechanism might unmask LXR-mediated suppression of ABCA1 promoter by PKA in the liver.

It has been reported that the glucagon/cAMP signal suppresses the expression of SREBP-1c in rat primary hepatocytes (24). Our current data clarify its molecular mechanism. The hypothesis that PKA suppresses SREBP-1c through LXR signal is supported by the following observations. 1) LXR/RXR is a dominant activator for SREBP-1c. 2) PKA inhibition of SREBP-1c promoter activity was more prominent in its activation by the LXR agonist than in the basal level. 3) Deletion studies with SREBP-1c promoter luciferase constructs revealed that LXRE is responsible for PKA inhibition. Based upon our findings the glucagon/cAMP/PKA signal could at least partially explain fasting suppression of SREBP-1c through LXR/RXR and LXRE. In support, it was reported that the effect of insulin on SREBP-1c expression could be mediated by LXR and LXRE (41). Further studies are needed to fully determine the extent to which the glucagon/cAMP/PKA signal contributes to fasting regulation of hepatic metabolic genes including SREBP-1c.

In a recent report it is observed that LXRα and -β are phosphorylated in HEK293 cells by an unknown kinase(s) (42). In their data basal and ligand-stimulated LXRα activity to induce ABCA1 promoter were not altered by substitution of phosphorylation residue to alanine. However, the response of mutant LXRα to potential kinases remains to be tested. Our study for the first time suggests the possibility that LXR mediates a nutritional signal via phosphorylation by PKA. Further study is needed to more precisely identify the physiological function in LXR phosphorylation.

In summary, transcriptional activity of LXRα on SREBP-1c promoter was decreased by PKA. Direct phosphorylation of LXRα by PKA resulted in a decrease of DNA binding and coactivator recruitment of LXR. This first demonstration of modification of LXR activity by phosphorylation suggests that reduction of mRNA level of SREBP-1c in fasting conditions might be mechanistically at least in part through LXR phosphorylation.

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