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Sterol regulatory element binding protein 1 trans-activates 25-hydroxy vitamin D_3 24-hydroxylase gene expression in renal proximal tubular cells

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ABSTRACT

The physiological activity of the steroid derived hormone vitamin D is regulated by several enzymatic steps. Both 25-hydroxy vitamin D_3 1α-hydroxylase (CYP27B1) and 25-hydroxyvitamin D_3 24-hydroxylase (CYP24A1) modulate blood levels of 1,25-dihydroxyvitamin D_3, an activated form of vitamin D. We previously demonstrated that *CYP27B1* expression was trans-activated by sterol regulatory element binding protein 1 (SREBP1), although whether SREBP1 also regulates *CYP24A1* transcription was unclear. Here we investigated the ability of SREBP1 to affect *CYP24A1* transcription. In a luciferase reporter assay, mouse and human *CYP24A1* promoter activity was strongly activated by SREBP1 in opossum kidney proximal tubular cells (OK-P). Three putative SREs (pSREs) were found in the mouse *Cyp24a1* gene promoter and the SREBP1 protein showed specific binding to the pSRE1 element in EMSAs. Site-directed mutagenesis of the pSRE1 element strongly decreased SREBP1-mediated *Cyp24a1* gene transcription. Moreover, siRNA-mediated *SREBP1* knock-down repressed *CYP24A1* expression in human renal proximal tubular epithelial cells (HKC-8). In animal studies, mice given various doses of thyroid hormone (T_3) showed dose-dependent reductions in renal *Srebp1c* and *Cyp24a1* mRNA levels. Taken together, our results suggest that SREBP1 trans-activates *CYP24A1* expression through SREBP binding elements present in the promoter.

1. Introduction

Vitamin D regulates various biological actions such as bone metabolism, phosphate and calcium metabolism, cellular differentiation and immune systems. Most of these functions are thought to be mediated by the vitamin D receptor (VDR), which belongs to the nuclear receptor superfamily. 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), the activated form of vitamin D, is produced through a two-step hydroxylation. In the liver, vitamin D is first converted to 25-hydroxyvitamin D and is subsequently modified in the kidney by 1a-hydroxylase (CYP27B1) to form 1,25-dihydroxyvitamin D_3. To avoid effects of excess $1,25(OH)_2D_3$, inactivation of vitamin D modifying systems is critical. 25-hydroxyvitamin D_3 24-hydroxylase (CYP24A1) catalyzes the synthesis of 1,24,25-trihydroxyvitamin D_3 and 24,25-dihydroxyvitamin D_3, which are inactive forms of vitamin D. *CYP24A1* is expressed in the kidney, osteoblastic cells and many other tissues to mediate systemic and local vitamin D inactivation [1,2]. The expression of both *CYP27B1* and *CYP24A1* are regulated by parathyroid hormone (PTH), $1,25(OH)_2D_3$ and fibroblast growth factor-23 (FGF-23) [3,4].

We previously revealed the involvement of thyroid hormone in

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vitamin D metabolism. The thyroid hormone triiodothyronine (T_3) regulates 1,25$(OH)_2$D homeostasis through negative transcriptional regulation of *CYP27B1* expression in renal proximal tubular cells. Moreover, a sterol regulatory element (1α-SRE) that is known to the responsive element for SRE binding protein (SREBP) was identified in the *CYP27B1* promoter. SREBP1c and SREBP1a both activate *CYP27B1* transcription [5].

SREBPs are transcriptional factors that play an important role in lipid metabolism as master transcriptional regulators. The mammalian genome encodes three SREBP isoforms: SREBP1c, SREBP1a and SREBP2. Both SREBP1c and 1a are expressed from a single gene through the use of alternative transcription start sites that produce alternate forms of exon 1, whereas SREBP2 is encoded by a different gene. SREBP1c preferentially enhances transcription of genes required for fatty acid synthesis and SREBP2 preferentially activates cholesterol synthesis. SREBP1a activates both fatty acid and cholesterol synthesis [6,7].

Although SREBP1 is known to activate *CYP27B1* transcription [5], whether SREBP1 regulates *CYP24A1* expression is unclear. Here we investigated regulation of *CYP24A1* expression by SREBP1 and identified a novel SREBP binding element in the *CYP24A1* promoter.

2. Materials and methods

2.1. Cell culture

OK-P (opossum kidney proximal tubule), HepG2 (human hepatocellular carcinoma), HCT116 (human colorectal carcinoma) and Saos-2 (human osteosarcoma) cells were cultured in DMEM (Dulbecco's modified Eagle's medium; Life Technologies, Grand Island, NY, USA). HKC-8 (human kidney proximal tubule) cells were cultured in DMEM/Ham's F-12 (Wako, Osaka, Japan). Cell cultures were maintained at 37 °C under an atmosphere containing 5% CO_2. Growth media were supplemented with 10% or 5% fetal bovine serum (FBS) (Life Technologies, Grand Island, NY, USA), 100 units/ml penicillin, and 0.1 mg/ml streptomycin (Sigma-Aldrich Japan, Tokyo, Japan).

2.2. Plasmid construction

The luciferase reporter plasmid rat *Fasn* (Fatty acid synthase)-Luc was previously described [8]. Plasmids carrying the human and mouse *CYP24A1* promoter (ph24A-1.49k, pm24A-960, pm24A-217, pm24A-122, and pm24A-69) were constructed by PCR amplification using gene-specific primers (Table 1) and cloned into the pGL4.12-basic vector (Promega KK, Tokyo, Japan) digested with BglII. Human *SREBP1c* (amino acids 1–449) and human *SREBP1a* (amino acids 1–473) expression plasmids in a pcDNA3.1/Myc-His(+) vector were constructed as previously described [8]. The human vitamin D receptor (VDR) expression vector pSG5-VDR and the mouse retinoid X receptor (RXR) alpha expression vector pSG5-RXRα were previously described [9]. The plasmid pm24A-217 encoding mutated SRE1 was constructed using the QuikChange site-directed mutagenesis kit (Agilent Technologies, Palo Alto, USA) with the oligonucleotides shown in Table 3. Each plasmid was purified using the Pure Yield Plasmid Midiprep System (Promega KK, Tokyo, Japan).

Table 1
Oligonucleotides used in reporter plasmid construction.

Name	Sequence (5′ to 3′)
pmCyp24A-960-Bgl-S	GAAGATCTATCCAGATACAAAAGCAAG
pmCyp24A-217-Bgl-S	GAAGATCTCCGGGGTGGAGTC
pmCyp24A-122-Bgl-S	GAAGATCTTCCCACACCCGCCCCCCG
pmCyp24A-69-Bgl-S	GAAGATCTCAGCGTGCTCATTGGCCAC
pmCyp24A+211-Hind-AS	CCCAAGCTTGGAAGAGGCAGATGCCCAG
phCyp24A-1.49k-S	GGAGATCTACATACTGTATGCAATC
phCyp24A+80-Bgl-AS	GGAGATCTAGGGTCTGGCTGGAGCCAC

2.3. Transfection and luciferase reporter assays

Cell transfections were performed using the Lipofectamine 2000 reagent (Life Technologies, Grand Island, NY, USA) as previously described [5]. OK-P cells were transfected with 0.4 μg luciferase reporter plasmid and 0.2 μg expression vector. The DNA/Lipofectamine mixtures were removed after 4 h, and the cells were grown in DMEM containing 10% FBS and treated with 1,25$(OH)_2D_3$ (Solvay Pharmaceuticals, Marietta, GA, USA) or ethanol vehicle for an additional 18 h. Normalization of luciferase activity for transfection efficiency was achieved by co-transfection with 0.2 μg pCMV-β (Agilent Technologies, Palo Alto, CA, USA), a *β-gal* (β-galactosidase) expression vector. Cells were harvested in cell lysis buffer (Promega KK, Tokyo, Japan) and the lysates were assayed for luciferase activity and *β-gal* activity.

2.4. Coupled transcription/translation assays

The human SREBP1c (amino acids 1–449) and human SREBP1a (amino acids 1–473) proteins were each separately synthesized using the TNT Quick Coupled Transcription/Translation System (Promega KK, Tokyo, Japan) at 30 °C for 90 min in the presence of 20 μM methionine. The proteins were then used in electrophoretic mobility-shift assays (EMSAs).

2.5. EMSAs

EMSAs were performed as previously described [5]. Double-stranded oligonucleotides for mouse *CYP24A1*-putative SRE1 (pSRE1), pSRE2, pSRE3, pSRE1-mutant, AP-1 consensus, human *LDLR* (Low density lipoprotein receptor)-SRE and rat *Fasn*-SRE were synthesized (Table 2) and purified electrophoretically on 1% agarose gels. The purified DNA fragments were end-labeled using [γ-^{32}P] ATP (110 TBq/mmol; ICN Pharmaceuticals, Costa Mesa, CA, USA) and T4 polynucleotide kinase (Takara Bio Inc., Shiga, Japan). Gel shift assays were performed with 2 μl of the appropriate *in vitro*-translated protein. Proteins were incubated on ice for 30 min in binding buffer (20 mM HEPES-KOH pH 7.9, 1 mM EDTA pH 8.0, 50 mM KCl, 5% glycerol, 10 mM DTT, 0.5 mM PMSF) in a total volume of 20 μl before addition of labeled probes and incubation for 30 min at room temperature. Binding reaction specificity was determined using 100-fold molar excess of the relevant unlabeled competitor oligonucleotide. SREBP1 components of protein–DNA complexes were analyzed using antibodies specific for SREBP1 and c-fos (sc-8984x and sc-253x, respectively; Santa Cruz Biotechnology, CA, USA). The incubated samples were then electrophoresed on 6% (w/v) polyacrylamide gels in 0.25× TBE (22.5 mM Tris, 22.5 mM boric acid, 0.5 mM EDTA, pH 8.0) running buffer for 2 h at 150 V. The gel was dried and analyzed with a Fluorescent Image Analyzer FLA-9000 equipped with Multi-Gauge Version 3.0 software (Fujifilm, Tokyo, Japan).

2.6. RNA interference

HKC-8 cells were cultured in 35 mm culture plates at 37 °C under an atmosphere containing 5% CO_2. Upon reaching 50% confluence, cells were transfected. RNA mixtures containing control siRNA (AAGUCACGACUAGAUAUUGACUUUG, CAAAGUCAAUAUCUAGUCGUGACUU) or 100 pmol SREBP1 siRNA (AAGACAGCAGAUUUAUUCAGCUUUG, CAAAGCUGAAUAAAUCUGCUGUCUU) and

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