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SHARP-2/Stra13/DEC1 as a potential repressor of phosphoenolpyruvate carboxykinase gene expression

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Abstract The influence of the enhancer of split- and hairy-related protein-2 (SHARP-2) transcriptional repressor on the expression of rat *phosphoenolpyruvate carboxykinase* (*PEPCK*) gene was examined. When H4IIE cells were treated with epigallocatechin gallate, a green tea constituent, an increase in SHARP-2 mRNA levels and a decrease in PEPCK mRNA levels were observed. The adenovirus-mediated overexpression of SHARP-2 in H4IIE cells and primary cultured rat hepatocytes led to a decrease in the levels of PEPCK mRNA. Finally, when a SHARP-2 expression plasmid was transiently transfected with various reporter plasmids into MH_1C_1 cells, the promoter activity of a PEPCK reporter plasmid was specifically decreased. Based on these findings, we conclude that SHARP-2 is a potential repressor of *PEPCK* gene expression.
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Keywords: SHARP-2/Stra13/DEC1; Basic helix–loop–helix transcriptional repressor; Phosphoenolpyruvate carboxykinase; Transcriptional regulation; Insulin

1. Introduction

Rat enhancer of split- and hairy-related protein-2 (SHARP-2, also referred to as the Stra13 or DEC1) is a basic helix–loop–helix transcriptional repressor [1–6]. While SHARP-2 mRNA is expressed ubiquitously, its expression is regulated in a cell-type specific manner [1–3]. We previously reported that the levels of hepatic SHARP-2 mRNA are increased when a high-carbohydrate diet is fed to normal rats or when insulin is administered to diabetic rats [7]. Under these conditions, both glycolysis and lipogenesis are induced and gluconeogenesis is repressed. In addition, the increase in SHARP-2 mRNA levels by insulin is mediated by a phosphoinositide 3-kinase (PI 3-K) pathway in primary cultured rat hepatocytes, and insulin stimulates the transcription of the rat *SHARP-2* gene in the liver [7]. Insulin also induces the expression of the *SHARP-2* gene in both 3T3-L1 adipocytes and L6 myotubes [8]. In 3T3-L1 adipocytes, the induction of *SHARP-2* gene expression by insulin is also mediated by a PI 3-K pathway [8]. These findings raise the possibility that an insulin-inducible transcriptional repressor, SHARP-2, mediates the insulin-dependent transcriptional repression of gluconeogenic enzyme genes. The *phosphoenolpyruvate carboxykinase* (*PEPCK*) gene is a good candidate for a SHARP-2-target gene: Its expression is stimulated by fasting signals, such as glucagon (via cyclic AMP) and dexamethasone (Dex), and is inhibited by insulin; a PI 3-K pathway is involved in insulin action, and insulin inhibits transcription of the rat *PEPCK* gene [9,10].

In the present study, we evaluated the role of SHARP-2 on *PEPCK* gene expression. An elevation in the level of SHARP-2 mRNA by (–)-epigallocatechin gallate (EGCG), the adenovirus-mediated overexpression of SHARP-2, and the co-transfection of a SHARP-2 expression vector with a PEPCK gene promoter linked to the firefly luciferase reporter plasmid led to a decrease in both PEPCK mRNA levels and PEPCK gene promoter activity.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM), Williams' medium E, Dex, and EGCG were purchased from Sigma Chemical Co. (Saint Louis, MO). Collagenase was purchased from Yakult (Tokyo, Japan). Type I collagen-coated dishes were purchased from Asahi Techno Glass (Chiba, Japan). The TRIZOL reagent, RNase inhibitor, Superscript III, and Lipofectamine PLUS reagent were purchased from Invitrogen (Groningen, The Netherlands). The ExTaq DNA polymerase and BcaBest DNA labeling kit were obtained from Takara BIOMEDICALS (Kyoto, Japan). The pCI-neo, pGEM-T Easy, pGL3-Basic, pRL-CMV, and dual luciferase reporter assay system were obtained from Promega (Madison, WI). The Big Dye terminator cycle sequencing kit FS was purchased from Applied Biosystems Japan (Tokyo, Japan). The Adeno-X expression system, Adeno-X rapid titer kit, ExpressHyb hybridization solution, pβgal-Basic, rat genome walker kit, and Advantage-GC Genomic polymerase chain reaction (PCR) kit were purchased from Clontech (Palo Alto, CA). The Virakit Adeno4 was obtained from Virapur LLC (San Diego, CA). [α-^{32}P] dCTP (110 TBq/mmol) was purchased from Amersham Biosciences (Cleveland, OH). The Invisorb plasmid kit was purchased from Invitek (Berlin, Germany).

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Abbreviations: 36B4, ribosomal protein 36B4; SHARP-2, enhancer of split- and hairy-related protein-2; Stra13, stimulation of retinoic acid 13; PI 3-K, phosphoinositide 3-kinase; PEPCK, phosphoenolpyruvate carboxykinase; Dex, dexamethasone; EGCG, (–)-epigallocatechin gallate; DMEM, Dulbecco's modified Eagle's medium; PCR, polymerase chain reaction; m.o.i., multiplicity of infection; GK, glucokinase; IRS, insulin response sequence; PKB, protein kinase B; SREBP-1c, sterol regulatory element-binding protein-1c

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2.2. Cells, cell culture, and animals

Rat H4IIE hepatoma cells were a generous gift from Dr. Daryl K. Granner (Vanderbilt University, USA). Rat MH_1C_1 hepatoma cells were purchased from the American Type Culture Collection (Manassas, VA). These cells were grown in DMEM supplemented with 10% fetal bovine serum and antibiotics at 37 °C in a 5% CO_2 incubator. Hepatocytes were freshly isolated from a male Sprague-Dawley rat liver (6 weeks of age, 170–190 g body weight) using a collagenase perfusion method [11]. Cells (10^5 cells/cm^2) were plated on type I collagen-coated dishes and cultured in Williams' medium E supplemented with 10% fetal bovine serum, 100 units/ml penicillin G, 100 μg/ml streptomycin, and 1 μM Dex at a 37 °C, 5% CO_2 incubator. After 4 h, the medium was replaced with serum-free DMEM supplemented with antibiotics and 1 μM Dex. After 24 h, the medium was replaced with fresh medium and the cells were infected with adenovirus as described below.

2.3. Preparation of adenovirus

Fragments of SHARP-2 cDNA were prepared using the reverse transcription-PCR [12]. One μg of rat liver total RNA was employed as the starting material. Combinations of oligonucleotides, EcoMetSHARP-2, 5′-CCGGGAATTCCCATGGAGCGGATCCCCAGC-3′, and MluSHARP-2AS, 5′-GGTGGGGGGCTCTTCAGATTC-3′, and SHARP-2-1112, 5′-CACGGACGCAGGTTCACCGTGG-3′, and SHARP-2-1555AS, 5′-CCGGTCTAGATTAGTCTTTGGTTTCTAAGTTTAAAGG-3′, were used as primers. The former product was digested with *Eco*RI and *Mlu*I, and the latter with *Mlu*I and *Xba*I. These fragments were subcloned into the *Eco*RI/*Xba*I sites of the pCI-neo to give pCI-neo/SHARP-2. After confirmation of the nucleotide sequence of the insert, a *Nhe*I/*Not*I fragment of the resulting plasmid was subcloned into the *Nhe*I/*Not*I sites of the pShuttle to obtain pShuttle/SHARP-2. The production of an adenovirus expressing SHARP-2 was preformed according to the manufacturer's recommended protocol. The AdCMV-β Gal, an adenovirus expressing *Escherichia coli* β-galactosidase, was a generous gift of Dr. Donald K. Scott (Louisiana State University Health Sciences Center) [13]. The preparation and titration of the adenovirus were carried out using Virakit Adeno4 and Adeno-X rapid titer kits, respectively.

2.4. Preparation of total RNA and Northern blot analysis

A 10-cm dish was seeded with 2×10^6 H4IIE cells. After 24 h, the medium was replaced with serum-free DMEM supplemented with or without 1 μM Dex and then cultured for another 24 h. To analyze the effects of EGCG on SHARP-2 and PEPCK mRNA levels, the cells were treated with the indicated concentrations of EGCG for various times. In the case of adenovirus infection, each adenovirus was infected with the indicated multiplicity of infection (m.o.i.) and cultured for an additional 48 h.

Total RNA was prepared from these cells using the TRIZOL reagent. Total RNA (10 μg/lane) was subjected to 0.8% denaturating agarose gel electrophoresis, then transferred to a Biodyne membrane (ICN Biomedicals, Inc., Glen Cove, NY), and UV-crosslinked for fixation. The ExpressHyb hybridization solution was used for prehybridization and hybridization at 68 °C. After washing twice at 50 °C for 30 min with 0.1× SSC and 0.1% SDS, the filter was exposed to a FUJIX imaging plate. Hybridization signals were detected with the FUJIX BAS-2000 imaging analyzing system.

The intensity of bands corresponding to SHARP-2, PEPCK, LacZ, and ribosomal protein 36B4 (36B4) mRNAs was quantified. The mean and standard error of the ratio of the intensities between SHARP-2 and 36B4 and between PEPCK and 36B4, respectively, was calculated. Statistical differences in mRNA levels were determined by an ANOVA analysis.

2.5. Probe DNAs

The probes for SHARP-2, PEPCK, and 36B4 have been described previously [7]. For the preparation of the LacZ probe, oligonucleotides, 5′-ATGTCGTTTACTTTGACCAACAAG-3′ and 5′-CGCGTAAAAATGCGCTCAGGTC-3′, were used as primers. The pβgal-Basic plasmid was employed as a template. The PCR product was subcloned into the pGEM-T Easy vector to give pGEM-T Easy LacZ. After confirmation of the nucleotide sequence, an approximately 0.6-kb *Eco*RI fragment was used as the probe. These probe DNAs were labeled with [α-^{32}P] dCTP using the BcaBest DNA labeling kit.

2.6. Construction of reporter plasmids

Oligonucleotides, 5′-CCGGACGCGTGAATTCCCTTCTCATGACCTTT-3′ and 5′-AGATCTCAGACCGTCTCGCC-3′, which correspond to the promoter region of the rat *PEPCK* gene, and 5′-GGATCCCCCACTATTCACAAG-3′ and 5′-CCGGAAGCTTAAGGACTTCCGCACTAACGG-3′, which correspond to the promoter region of the rat *glucokinase* (*GK*) gene, were synthesized [14]. PCR was performed using a rat genome walker kit and the above combinations of primers. The PCR conditions have been described previously [15]. Each PCR product was digested with *Mlu*I and *Bgl*II or *Bam*HI and *Hin*dIII, then a 536-bp *Mlu*I/*Bgl*II fragment and a 326-bp *Bam*HI/*Hin*dIII fragment were subcloned into the *Mlu*I/*Bgl*II or *Bgl*II/*Hin*dIII sites of the pGL3-Basic to give prPEPCK/Luc and prGK/Luc, respectively. The pMPK287/Luc and pmZHX1/Luc59 plasmids have been described previously [12,16].

The nucleotide sequences of all inserts were confirmed.

2.7. Transient DNA transfections and luciferase reporter assays

MH_1C_1 cells were co-transfected with 200 ng of reporter plasmid, 100 ng of pRL-CMV and 100 ng of cytomegalovirus enhancer/promoter-directed expression vectors. All plasmids used for transfection were prepared using the Invisorb plasmid kit, followed by CsCl density gradient ultracentrifugation. The transfection conditions and luciferase reporter assays have been described previously [16,17].

3. Results

3.1. Effects of EGCG on expression of SHARP-2 and PEPCK mRNAs

In the rat liver and primary cultured rat hepatocytes, the time course for the increase in SHARP-2 mRNA levels following treatment with insulin coincided with that for the decrease in PEPCK mRNA levels [7]. It has recently been reported that EGCG decreases the level of PEPCK mRNA in H4IIE cells [18]. To examine the possible role of SHARP-2 in regulation of *PEPCK* gene expression, we initially examined the effect of EGCG on the expression of the *SHARP-2* gene by Northern blot analysis. H4IIE cells were treated with various concentrations of EGCG for 4 h. The level of SHARP-2 mRNA increased in a dose-dependent manner (Fig. 1A), reaching a plateau at 25 μM. The mRNA level was 3.2-fold higher than that in the absence of EGCG (Fig. 1A). In contrast, the level of PEPCK mRNA was decreased in a dose-dependent manner, as described previously [18], and the level of 36B4 mRNA remained essentially unchanged (Fig. 1A). The time course for the increase in SHARP-2 mRNA levels at 25 μM of EGCG was then analyzed (Fig. 1B). The level of SHARP-2 mRNA gradually increased, reaching a maximum level at 2 h, and this level was maintained for periods of up to 6 h. In contrast, the level of PEPCK mRNA was decreased in a time-dependent manner and the level of 36B4 mRNA remained essentially unchanged (Fig. 1B).

EGCG thus increases the level of SHARP-2 mRNA and decreases PEPCK mRNA levels.

3.2. Overexpression of SHARP-2 mRNA decreases the level of PEPCK mRNA

We then examined the effect of the overexpression of SHARP-2 on *PEPCK* gene expression. When H4IIE cells were treated with Dex, the level of PEPCK mRNA increased by 11.3-fold (Fig. 2A). In contrast, the level of SHARP-2 mRNA was not altered. An adenovirus expressing SHARP-2 or β-galactosidase was infected into the cells in the presence of Dex. As expected, the size of the SHARP-2 mRNA derived

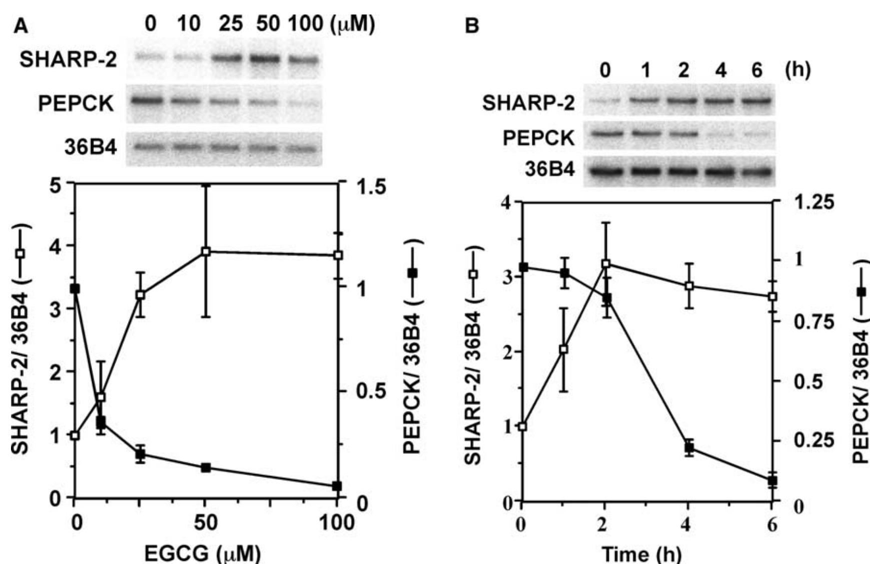

Fig. 1. Effects of EGCG on SHARP-2 and PEPCK mRNA levels. Total RNAs (10 μg) were analyzed. The probes used are shown on the *left*. Each experiment was carried out at least three times. Photos of representative data are shown. The mean and standard error of the ratio of the mRNA levels of SHARP-2 or PEPCK and 36B4 mRNAs (ribosomal protein 36B4, used as a loading control) is plotted on the *bottom*. The value of the ratio in the absence of EGCG was set to 1. (A) H4IIE cells were treated for 4 h with the concentrations of EGCG indicated on the *top*. (B) Time course for alterations in the levels of SHARP-2 and PEPCK mRNAs by EGCG. Cells were cultured in the presence of 25 μM EGCG for the times indicated on the *top*.

from the adenovirus was smaller than that of the endogenous SHARP-2 mRNA (because of differing 5′- and 3′-untranslated regions), and its expression was dependent on infection by the adenovirus SHARP-2. When the exogenous SHARP-2 mRNA was overexpressed, the level of PEPCK mRNA was decreased in a dose-dependent manner. At m.o.i. 5, the level of PEPCK mRNA was decreased by 41.4%. In contrast, when the cells were infected with AdCMV-Gal expressing β-galactosidase, the level of PEPCK mRNA was not altered. Under all conditions, the levels of 36B4 mRNA remained unchanged. Basically the same results were obtained when primary cultured rat hepatocytes were infected by the viruses (Fig. 2B).

These results indicate that the overexpression of SHARP-2 decreases the level of PEPCK mRNA.

3.3. SHARP-2 represses transcription from the rat PEPCK gene promoter

We finally examined effects of SHARP-2 on the *PEPCK* gene promoter. MH_1C_1 cells were employed for the transient transfection experiments. A cytomegalovirus enhancer/promoter-directed SHARP-2 expression plasmid was co-transfected with several reporter plasmids into MH_1C_1 cells. Nucleotide sequences between −467 and +69 of the rat *PEPCK* gene, between −309 and +17 of the rat *GK* gene, between −287 and +17 of the rat *pyruvate kinase M* gene, and between −59 and +50 of the mouse *zinc-fingers and homeoboxes 1* gene were inserted into a luciferase reporter plasmid to give plasmids prPEPCK/Luc, prGK/Luc, pMPK287/Luc, and pmZHX1/Luc59 respectively. When the pCI-neo plasmid, an empty vector for the SHARP-2 expression plasmid, was transfected with the reporter plasmid, the relative luciferase activity was set to 1. As shown in Fig. 3, when prPEPCK/Luc, which contains all the elements required for hepatic expression and hormonal regulation, was co-transfected with pCI-neo/SHARP-2, the luciferase activity was decreased by 46%. In contrast, when other reporter plasmids were co-transfected with the pCI-neo/SHARP-2, the luciferase activities remained unchanged. SHARP-2 therefore specifically affects the transcription of the *PEPCK* gene promoter.

4. Discussion

The potential role of SHARP-2 in the regulation of the rat *PEPCK* gene was investigated. The level of SHARP-2 mRNA rapidly increased when H4IIE cells were treated with EGCG (Fig. 1). The expression of SHARP-2 mRNA is regulated in a cell-type specific manner by a variety of stimuli, including insulin, cyclic AMP, gonadotropins, serum starvation, transforming growth factor-β, and hypoxia [2,3,5–7,19,20]. Thus, EGCG can be also added to the list. It remains to be determined whether EGCG increase transcription of the rat *SHARP-2* gene, or instead act at a post-transcriptional level to affect mRNA stability. Since we previously cloned the rat *SHARP-2* gene [21], a detailed analysis of the regulatory elements of the gene will be required to address this question.

The dose-dependency and time-course of the increase in SHARP-2 mRNA levels were reciprocal to those for the decrease in PEPCK mRNA levels (Fig. 1B). In addition, the adenovirus-mediated overexpression of SHARP-2 decreased the level of PEPCK mRNA in both H4IIE cells and primary cultured rat hepatocytes (Fig. 2). The levels of SHARP-2 mRNA induced by the virus infection were much higher than those seen after treatment with EGCG, yet the effect on PEPCK expression was less. Presumably higher levels

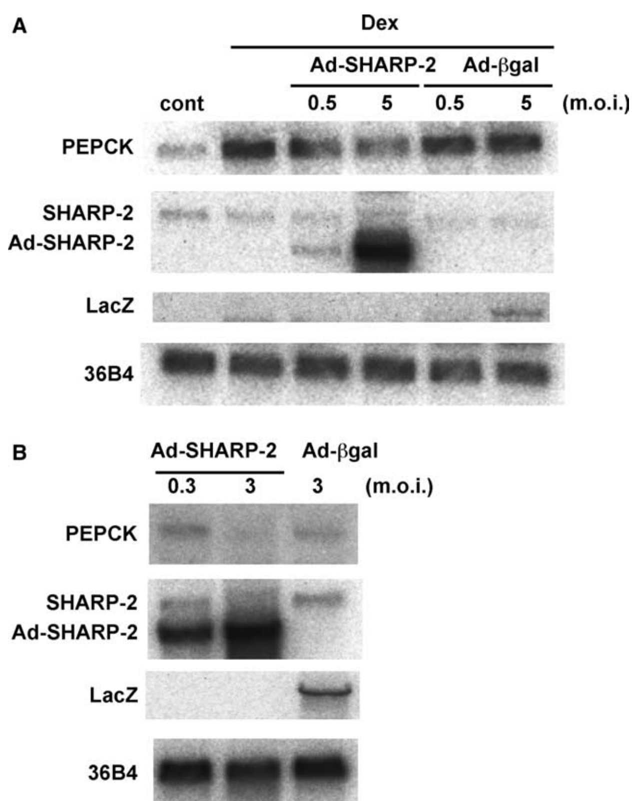

Fig. 2. Adenovirus-mediated overexpression of SHARP-2 mRNA in H4IIE cells and primary cultured rat hepatocytes inhibits Dex induction of PEPCK mRNA. The procedures and abbreviations are the same as those shown in the legend for Fig. 1. Cells were infected with adenoviruses (Ad-SHARP-2 and Ad-βgal) at the m.o.i. indicated on the *top*, and then cultured for another 48 h. LacZ, *Escherichia coli* β-galactosidase. For panel (A), H4IIE cells were cultured in serum-free DMEM without or with 1 μM Dex as indicated. Each experiment was carried out three times. For (B), hepatocytes were cultured with serum-free DMEM and 1 μM Dex. Each experiment was carried out twice.

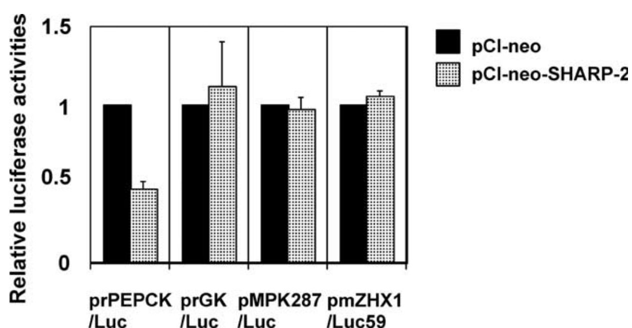

Fig. 3. Transcriptional regulation by SHARP-2. MH_1C_1 cells were co-transfected with a luciferase reporter plasmid, an expression vector for SHARP-2 or control, and the pRL-CMV plasmid as control for transfection efficiency. The prPEPCK/Luc, prGK/Luc, pMPK287/Luc, and pmZHX1/Luc59 plasmids were used as luciferase reporters. Plasmid pCI-neo/SHARP-2 encodes a full-length SHARP-2 and the pCI-neo plasmid is a control empty vector. A value of 1 was assigned to the promoter activity of each reporter plasmid in the presence of pCI-neo. Each column and bar represents the mean and standard error of three transfection experiments.

are required to overcome the strong positive effect of the Dex treatment of the virus-infected cells. The level of PEPCK mRNA, but not of SHARP-2 mRNA, was increased by Dex in H4IIE cells, indicating that a decrease in the level of SHARP-2 mRNA is not required to increase PEPCK mRNA. These findings, therefore, suggest that only when the level of SHARP-2 mRNA increases can the expression of the *PEPCK* gene be regulated.

SHARP-2 specifically repressed the transcription of the rat *PEPCK* gene promoter, a well-known promoter that is repressed by insulin (Fig. 3) [22]. The rat *PEPCK* gene promoter consists of at least two insulin response sequences (IRSs) as negative regulatory elements. One is a distal IRS, 5′-TGGTGTTTTG-3′ and the other is a proximal IRS, which has not been precisely characterized [23]. It has been reported that FOXO1 or a related protein mediates the insulin regulation of the transcription from the distal IRS of the *PEPCK* gene [24]. Although FOXO1 or a related protein bind to and activate the distal IRS of the *PEPCK* gene promoter, protein kinase B (PKB)/Akt activated by insulin stimuli phosphorylates these proteins and leads to transcriptional inactivation and exclusion of the proteins from the nucleus [25–27]. As a result, *PEPCK* gene transcription is repressed. This mechanism would appear to account for the insulin regulation of *PEPCK* gene transcription. However, there are no FOXO1-binding sites in the proximal IRS of the *PEPCK* gene promoter, indicating that one or more other proteins must be involved in the insulin regulation of *PEPCK* gene transcription [28]. To fully understand the physiological regulation of *PEPCK* gene expression by insulin, the identification and analysis of transcription factors other than FOXO1 or a related protein will be required. It has recently been reported that the sterol regulatory element-binding protein-1c (SREBP-1c) mimics the insulin regulation of *PEPCK* gene transcription [29,30]. SREBP-1c inhibits *PEPCK* gene transcription via two elements which are located between nucleotide sequences −590 and −581, and between −322 and −313 which is accessory element 3 of the glucocorticoid response unit of the rat *PEPCK* gene [30]. SREBP-1c is also an insulin-inducible bHLH transcription factor [31]. Although a PI 3-K/PKB/Akt pathway is involved in the increase of SREBP-1c and SHARP-2 mRNA caused by insulin treatment, the time-course for the increase in SREBP-1c mRNA in rat hepatocytes is slower than that of SHARP-2 mRNA [7,31,32]. This suggests that SHARP-2 may be a more important factor in the rapid transcriptional repression of the *PEPCK* gene.

SHARP-2 functions as an E box-binding protein [6,33]. Accessory element 3 of the glucocorticoid response unit of the rat *PEPCK* gene contains an E box sequence that overlaps an SREBP-1c-binding sequence [30,34]. In addition, two imperfect E boxes in the proximal IRS region are also present just downstream from the transcription initiation sites of the gene [35]. The issue of whether SHARP-2 directly binds to these *cis*-acing elements of the rat *PEPCK* gene promoter and represses its transcription, or whether a function of SHARP-2 as a repressor is directly linked to insulin action on the *PEPCK* gene promoter, remains to be determined. In addition to the characterization of the effects of SHARP-2 on *PEPCK* gene regulation, a modification of SHARP-2 or an alteration of its transcriptional activity by insulin or EGCG

can also be envisaged. The precise identification of the SHARP-2-responsive element on the rat *PEPCK* gene promoter by co-transfection experiments with the proximal IRSs of the *PEPCK* gene, as well as development of effective antibody, will be required to address these questions.

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