GATA4 inhibits expression of the tryptophan oxygenase gene by binding to the TATA box in fetal hepatocytes

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Abstract The glucocorticoid receptor regulates liver-specific expression of the tryptophan oxygenase gene through glucocorticoid responsive elements located −0.45 and −1.2 kb from the transcription start site. However, the hormone-mediated induction is restricted to adult hepatocytes, and fetal hepatocytes are unable to express the gene even in the presence of the receptor and glucocorticoid hormone. The difference in sensitivity to the hormone between adult and fetal hepatocytes has not been well understood. In this study, we analyzed the structure of the tryptophan oxygenase gene's promoter. The promoter has two TATA boxes, and transcription starts from the downstream TATA box. We found that a transcription factor GATA4 bound to the downstream TATA box and may inhibit the binding of TATA-binding protein, resulting in transcriptional repression even in the presence of glucocorticoid in fetal hepatocytes.

Keywords Tryptophan oxygenase ·
Glucocorticoid · Glucocorticoid receptor ·
GATA · Liver-specific expression

Introduction

Steroid hormones such as glucocorticoids, estrogen, androgen, and vitamin D play important roles in the response of organisms to stimuli (Kumar and Tindall 1998), but during development, they prepare various organs for metabolic adaptations allowing autonomous life. Tryptophan 2, 3-dioxygenase (TO) is a liver-specific enzyme, found only in mature liver cells, and is induced to express by glucocorticoids (Danesch et al. 1987; Nakamura et al. 1987). The enzyme appears 2 weeks after birth and reaches the adult level after 4–5 weeks in the rat liver (Nakamura et al. 1987). This induction takes place only in terminally differentiated hepatocytes, although the levels of the glucocorticoid receptor (GR) in adult and fetal hepatocytes are almost the same. It is also well established that pancreatic hormones such as glucagon induce, whereas insulin suppresses, production of the enzyme (Nakamura et al. 1980). However, the molecular mechanism controlling *TO* gene expression is largely unknown, although it is understood to involve glucocorticoid.

Expression of the *TO* gene is controlled by glucocorticoid and its receptor through glucocorticoid responsive elements (GREs) located −1.2 kb and −0.45 kb from the major transcription start site (Danesch et al. 1987). Furthermore, this gene has two TATA-like sequences, which are close together, just 180 bp apart, in the promoter region (Schmid et al. 1982). However, the function of the tandem

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TATA sequences in the regulation of the *TO* gene has not yet been clear. Several previous reports indicated that major transcription starts from the proximal site of the downstream TATA sequence.

The GATA family of transcription factors is known to bind to a consensus DNA sequence containing GATA. The family has six members (GATA1–6), which seem to play important roles in the early stages of development (Patient and McGhee 2002; Bresnick et al. 2005). GATA1–3 are required for hematopoiesis. An examination of *gata4*^{−/−} ES cell-derived embryos found GATA4 to be necessary for expansion of the primary hepatic rudiment and for the onset of ventral pancreatic development (Watt et al. 2007).

In this study, we confirmed that RNA polymerase II (polII) binds in the downstream TATA box in both adult and fetal hepatocytes. Furthermore, we found that a transcription factor, GATA4, may play pivotal roles in the repression of the *TO* gene in fetal hepatocytes by binding to the downstream TATA box.

Materials and methods

Cell culture and media

Adult rat hepatocytes were prepared from male Sprague-Dawley rats (6–8 weeks old) by the collagenase perfusion method. The fetal hepatocytes were prepared from fetuses (embryonic day 17). Cells were cultured as described previously (Dohda et al. 2004). C33A (human cervical carcinoma) cells were cultured in DMEM (Invitrogen, Carlsbad, CA, USA) with 10% FBS.

Plasmids and gene transfection

The full-length cDNAs of GATA1–6 were obtained by reverse transcription-PCR (RT-PCR) and subcloned into pcDNA4/TO/myc-His (Invitrogen). Primers for PCR were designed based on the following cDNA sequences: GATA1 (NCBI accession number NM012764), GATA2 (NM033442), GATA3 (NM133293), GATA4 (NM144730), GATA5 (NM001024316), and GATA6 (NM019185). For the luciferase reporter assay, we cloned the proximal region of the rat *TO* gene's promoter (up to −0.5 kb, which contains one GRE sequence) using the following primers: 5'-CTACTCGAGAAGAT

TGGTACAGGGTGATG-3' and 5'-CTAGTCGACC CAGATATTTTCGTGCTTGC-3', and introduced it into a luciferase reporter plasmid, pGL3 (Promega, Madison, WI, USA).

Luciferase reporter assay

C33A cells seeded in 24-well plates were transfected with 50 ng of the luciferase reporter plasmid and 4 ng of *Renilla* luciferase expression plasmid containing the promoter region of the elongation factor *I* α gene with or without 100 ng of the expression plasmid for GR and 400 ng of the expression plasmids for GATA1–6. The amount of transfected DNA was kept constant by adding empty vector. Luciferase activity was measured with a dual luciferase assay kit (Promega) according to the manufacturer's instructions using a luminometer (luminescencer-JNR II; Atto, Tokyo, Japan).

Gel shift assay

DNA fragment of the downstream TATA sequence (5'-CTGGTAATCAGGATACATAAAAGGCAAG CAC-3') were synthesized and labeled by [γ -³²P]ATP. The DNA-binding reaction was carried out in a final volume of 20 μ l containing ³²P-labeled DNA fragments (5 $\times$ 10⁴ cpm), 1 μ g of poly (dI-dC), DNA binding buffer (100 mM Tris-HCl, pH 7.5, 1 mM EDTA, 50 mM KCl, 4% glycerol, and 1 mM 2-mercaptoethanol), and purified GST-fused proteins, purified TBP protein (# sc-4000, Santa Cruz, CA, USA), or nuclear extracts of fetal hepatocytes. After a 30 min incubation at room temperature, the samples were analyzed on a 5% polyacrylamide gel in 22.2 mM Tris, 22.2 mM borate, 0.5 mM EDTA buffer, pH 8.0.

Chromatin immunoprecipitation (ChIP) assay

Hepatocytes (1 $\times$ 10⁷ cells) were fixed with a final concentration of 1% formaldehyde for 10 min at 37 °C. ChIP assays were performed by using salmon sperm DNA-coated protein A agarose (# 16-157, Upstate, Lake Placid, NY, USA) or protein G agarose (# 16-201, Upstate) according to the manufacturer's instructions. Anti-GATA4 (# sc-1237, Santa Cruz), anti-polII (# sc-899, Santa Cruz) and anti-TATA box-binding protein

(TBP) (# 06-241, Upstate) antibodies were used for immunoprecipitation. The immunoprecipitated DNA and input DNA were analyzed by PCR using 5'-TATGTTAGATATATAAAAGCTCAACT TG-3' and 5'-GCTTGCCTTTTATGTATCCTGATT AC-3' for the upstream TATA sequence and 5'-GTA ATCAG GATACATAAAAGGCAAGC-3' and 5'-GC TTGCC TTTTATGTATCCTGATTAC-3' for the downstream TATA sequence. As a negative control, the coding region of the *TO* gene was also amplified. The average size of DNA fragments after sonication was about 200 bp.

siRNAs

For the knockdown of GATA4, siRNAs (siGATA4-1, 5'-UUCAGGAGUUGUUGUCCCCACACAGAGG-3', siGATA4-2, 5'-UUCACCUUAAGACAAUGUU AACGGG-3') were purchased from Invitrogen. As a negative control, scramble siRNA was used. Cells cultured to semiconfluence were transfected with siRNA (50 pmol/well in a 24-well plate) by using LipofectamineTM2000 (Invitrogen). Cells were washed with phosphate-buffered saline (PBS) at 6 h post-transfection and grown in fresh medium for an additional 42 h. After the harvesting of cells, total RNA was prepared and reverse transcribed for detection of GATA4 and *TO* expression. GATA4 cDNA was amplified by using 5'-CAGTCCTGCAC AGCTACCTG-3' and 5'-GGCCGGTTGATACCA TTCATC-3' as primers. *TO* cDNA was amplified using 5'-AGACGGAGCTCAAAGTGGTG-3' and 5'-TTTTCTAGCAGCCGGAAGT-3'.

Results

The downstream TATA box of the promoter region is occupied by polII

In the promoter region of the *TO* gene, there are two adjacent TATA-like sequences (Danesch et al. 1987), which reside within a distance of 180 bp. We investigated the location of polII in the promoter region by conducting a ChIP assay using cells isolated from rat liver without cultivation. PolII was detected in the downstream TATA box region in both adult and fetal hepatocytes and the anti-polII antibody precipitated

Fig. 1 PolII mainly exists in the downstream TATA region. ChIP assays were performed using anti-polII antibody with primary fetal and adult hepatocytes. The *TO* promoter regions containing upstream and downstream TATA boxes were detected by PCR. Normal IgG was used for a negative control

only a small amount of the DNA fragment corresponding to the upstream TATA box-like region, suggesting that the upstream TATA-like sequence did not function substantially as a promoter (Fig. 1). The localization of polII of adult hepatocytes is consistent with the results of Northern blotting showing that the downstream TATA box is more important for transcription (Schmid et al. 1982). From the ratio of the precipitated/input DNA (Fig. 1), it is evident that only a part of the downstream TATA box was occupied by polII in fetal hepatocytes that did not express the gene.

GATA4 can repress transcription of the *TO* gene

From computer-aided analyses, we found potential binding sites for two transcription factors overlapping with the downstream TATA box (Fig. 2). We therefore studied the ability of these transcription factors to bind to the sequence by conducting a gel shift assay using nuclear extracts of fetal hepatocytes and typical competitor sequences of these transcription factors. In this assay, only a DNA fragment containing the GATA-binding site consensus sequence competitively inhibited the binding of a protein in the crude extract to the probe (Fig. 2). This result suggested that GATA as well as polII binds to the TATA sequence. It is well established that there are six GATAs in mammalian cells. Therefore, we

Fig. 2 The downstream promoter contains a GATA-binding site. Consensus sequences for Oct1 (5'-TTCTAGTGATTTGC ATTGCACA-3') and GATAs (5'-CACTTGATACAGAAA GTGATAACTCT-3') were used as competitors

investigated effects of GATA1-6 on the transactivation of the *TO* promoter by GR using the luciferase reporter assay (Fig. 3). GATA1 showed stimulatory activity, possibly due to the existence of three putative GATA1-binding sites that were predicted by TFSEARCH (<http://www.cbrc.jp/research/db/TFSEARCHJ.html>) in the promoter proximal region. On the other hand, only GATA4 diminished the promoter activity. It is thus possible that GATA4 represses the *TO* gene by binding to the TATA sequence in fetal hepatocytes.

GATA4 inhibits TBP from binding the downstream TATA sequence

We confirmed the binding of GATA4 to the downstream TATA box by conducting ChIP assay using primary cultured hepatocytes. As shown in Fig. 4, the binding of GATA4 was observed in fetal hepatocytes, but the transcription factor was not detected in adult hepatocytes. TBP and polII bound to the promoter in both fetal and adult hepatocytes but the binding seemed weak in fetal hepatocytes judging from the precipitated/input ratio of PCR-amplified DNA of ChIP. In the negative control amplifying coding

Fig. 3 GATA4 represses the *TO* promoter. C33A cells were transfected with the pGL3-*TO* promoter plasmid together with the pGL3-EF-renilla, GR, and GATA expression plasmids. Luciferase activity was measured 48 h post-transfection. Values represent the average of three independent experiments ($\pm$ SE)

region, weak band of polII was detected. This seemed to be caused by transcriptional elongation in adult hepatocytes.

To confirm whether TBP and GATA4 bind in a competitive manner to the downstream TATA box in vitro, we performed a gel shift assay using purified GST-fused GATA4 and DNA fragments containing the downstream TATA box sequence. As shown in Fig. 5, the binding of GATA4 and TBP was observed and competitors inhibited the bandshift. Furthermore, as expected by the ChIP assay, an increase in the amount of GATA4 inhibited TBP from binding to the TATA sequence. Similarly, an increase in the amount of TBP hampered the binding of GATA4. These results suggest that TBP and GATA4 bind at overlapping sites in the downstream promoter sequence in a mutually exclusive manner.

Inhibition of GATA4 expression by siRNA

If GATA4 is involved in the repression of the *TO* gene in fetal hepatocytes, inhibition of its expression may cause up-regulation of the *TO* gene. To achieve

Fig. 4 GATA4 exists in the downstream TATA region in fetal hepatocytes. A ChIP assay was performed with fetal and adult hepatocytes. As a negative control, rabbit normal IgG was used. The ratio of precipitated/input DNA is shown below the result of ChIP

Fig. 5 The binding of GATA4 and TBP to the downstream TATA sequence. A gel shift assay was performed with purified GST-fused GATA4 (+: 1 μ g, ++: 2 μ g) and TBP (+: 1 μ g, ++: 2 μ g). Arrows indicate the specific shifted bands. As a negative control, purified GST protein was used. Non-labeled oligonucleotide was added at a 100-fold molar excess as a competitor

this, we performed a siRNA experiment using fetal hepatocytes. In this assay, we used two kinds of siRNAs for inhibiting the expression of GATA4. As shown in Fig. 6, both siRNAs repressed the expression of GATA4 in fetal hepatocytes. The results of RT-PCR showed that the expression of the *TO* gene was significantly enhanced by inhibition of GATA4 expression. This result again suggested that GATA4 plays important roles in repression of the *TO* gene in fetal hepatocytes.

Discussion

TO is a typical enzyme specific for liver cells. However, the molecular mechanism responsible for the tissue-specific expression of the *TO* gene has remained unclear although it is known to involve the GRE located in the promoter's proximal region. Previously, we found that an ATP-dependent chromatin remodeling factor, SWI/SNF, binds to GR and down-regulates the expression of the *TO* gene (Inayoshi et al. 2005). In the present study, we found that GATA4 also down-regulated this gene's expression in fetal hepatocytes. GATA4 seemed to be replaced with TBP in adult hepatocytes. However, the

Fig. 6 GATA4 is required for repression of the *TO* gene in fetal hepatocytes. siRNAs for rat GATA4 were transfected to E17 fetal hepatocytes at 50 nM. RT-PCR assays were carried out with RNAs extracted from hepatocytes 48 h post-transfection. As a negative control, scrambled siRNA was used

mechanism, which causes the difference in the pattern of binding of GATA4 and TBP to the downstream TATA box between fetal and adult hepatocytes, remains elusive, since the level of GATA4 is constant throughout the liver's development (Ito unpublished result).

The present results suggest that GATA4 inhibits the formation of a transcription initiation complex at the downstream TATA sequence of the *TO* gene in fetal hepatocytes. Alternatively, GATA4 may recruit histone deacetylases and/or a C-terminal-binding protein to repress the gene expression as reported for several genes (Svensson et al. 2000; Chen et al. 2006). Furthermore, global changes of chromatin structure in this region may play a role. For example, GR is known to change the chromatin structure of the promoter-enhancer region containing GRE together with the SWI/SNF chromatin remodeling complex (Hebbar and Archer 2003; Inayoshi et al. 2005). Therefore, it is possible that GR gives rise to a different chromatin structure, which influences the binding of TBP and GATA4 to the TATA box in adult and fetal hepatocytes. However, the mechanism determining the behavior of TBP and GATA4 is largely unknown and requires further study.

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