Regulation of Gelatin-Binding Protein 28 (GBP28) Gene Expression by C/EBP

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We have previously reported the isolation of human gelatin-binding protein 28 (GBP28) gene which is specifically expressed in adipose tissue. The transcriptional activity of the flanking region of the GBP28 gene was examined by the transient transfection of promoter-luciferase reporter constructs into 3T3 adipocytes and electrophoretic mobility shift assay. This revealed the existence of a protein which binds to the 5’-flanking region of the GBP28 gene in nuclear extracts from human adipose tissue, but not in nuclear extracts from mouse liver. The C/EBP sites contained in this region are thought to take part in the regulation of GBP28 gene expression.

Key words: gelatin-binding protein (GBP28), C/EBP, adipose tissue

Previously, we purified a novel plasma gelatin-binding protein of 28 kDa from human plasma by affinity chromatography with gelatin-Cellulose.13 We named this protein gelatin-binding protein 28 (GBP28). GBP28 has been found to be a translated product of the apM1 mRNA, which is specific to adipose tissue.27 Although its biological function is presently unknown, its adipose-specific expression suggests that GBP28 may function as an endogenous factor involved in lipid catabolism and storage. To elucidate the function of this protein and the mechanism of adipose-specific expression, we isolated the gene, which encodes the human GBP28.33 The overall homology of the amino acid and nucleotide sequences of GBP28 with those of Leptin was 35.7% and 48.0%, respectively. However, the structure of the GBP28 gene was very similar to that of Leptin gene.42 Both genes are composed of three exons, and have a very long first intron and Alu-like sequences in the 3’-untranslated region. In addition to structural similarities, both genes are specifically expressed in adipose tissues. To understand GBP28’s transcriptional regulation, we isolated the GBP28 promoter and report here its detailed characterization.

MATERIALS AND METHODS

Reagents Restriction endonucleases, T4 phage DNA ligase, recombinant Taq DNA polymerase, and pUC119 vector were purchased from Takara Shuzo (Japan). [α-32P]dCTP (deoxyctydine 5’-triphosphate; 400 Ci/mmole), [γ-32P]ATP (adenosine 5’-triphosphate; 6000 Ci/mmole) and nylon filters were obtained from Amersham Pharmacia Biotech. A random-primer labeling kit was obtained from Du Pont-New England Nuclear. Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), Lipofectamine and OptiMEM were obtained from Gibco BRL.

Cell Culture The immortalized mouse preadipocyte cell line, 3T3-L1, was maintained at 37°C in an atmosphere of 5% CO2 in DMEM supplemented with 10% FBS.

Northern Blot Analysis Total RNA was isolated using Isogen (Wako, Japan) according to the manufacturer’s directions. Ten micrograms of total RNA was fractionated on a 2.2 M formaldehyde–1% agarose denaturing gel and transferred onto Hybond N+ (Amerham Pharmacia Biotech) in 10×SSC.63 Blots were hybridized overnight with a 35S-labeled probe, in 50% formamide, at 42°C. The random primed probe used to detect GBP28 transcripts was generated from a Bam HI-Eco RI fragment of GBP28 cDNA. The β-actin cDNA was also labeled with [32P]dCTP and used as an internal control. The blot was exposed to X-ray film overnight.

Plasmid Construction To test the transcriptional activity of the 5’-flanking region of human GBP28 gene, several reporter constructs were generated. The human GBP28 gene 5’-flanking region (base −548 to −1) was amplified by PCR7 using BAC 604H12 clone DNA3 as a template with sense primer 5’-CCGAGCCTCGGTACTGTTG-3’ (inserted Sac I site underlined), and antisense primer 5’-CCAACTTGGAAACCACAGAG-3’ (inserted Hind III site underlined). The PCR product was digested with Sac I and Hind III, and inserted into the corresponding sites of pGL3 Basic plasmid vector (Promega). This construct was named p(-548/-1)GBP28-Luc. A similar strategy was employed to generate another constructs, p(-128/-1)GBP-Luc, p(-548/+18)GBP-Luc and p(-128/+18)GBP-Luc (Fig. 4).

Transient Transfection For transient transfection, DNA concentrations were independently determined by UV spectrophotometry and analytical gel electrophoresis. 3T3-L1 cells were differentiated into adipocytes by culturing the cells with DMEM containing 10% calf serum, 0.5 mM 3-isobutyl-1-methylxanthine, 10−6 M insulin and 0.25 mM dexamethasone for 6 d before transfection. All transient transfections were carried out using the Lipofection reagent, Lipofectamine (Gibco BRL). The differentiated cells on 60-mm dishes were transiently transfectanted with 10 μg plasmid DNA in 1.6 ml of OptiMEM (Gibco BRL) containing 30 μl Lipofectamine for 3 h. After transfection, 10 ml fresh DMEM containing 10% calf serum was added to the dishes, and the cells were incubated for another 48 h. To correct for variations in transfection efficiency among different samples, pRL-TK vector was co-transfected as an internal control.

Luciferase Assay Cells were harvested with 300 μl lysis...
buffer 48 h after transfection. The cell lysates were centrifuged at 10000 × g for 2 min to pellet residual cellular debris and stored at −80 °C. The activity of luciferase in cell lysates was measured using a Dual-Luciferase Reporter System (Promega). Measurements of light units were integrated over a 10-s interval. Data in raw luciferase units (RLU) were normalized for the nonspecific background of mock-transfected cells, which represented 0.5% of most of the experimental luciferase activities. Intra- and inter-assay coefficients of variation averaged 7% and 9%, respectively.

Preparation of Nuclear Extract Nuclear extracts were prepared from human adipose tissue and mouse liver by a modification of the method of Dingam et al.30 The tissue was minced with scissors, and washed with several volumes of cold buffer A (10 mM HEPES–NaOH, pH 7.9, 10 mM KCl, 1.5 mM MgCl2, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 0.5 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonily fluoride (PMSF), 10 µg/ml aprotinin, 10 µg/ml pepstatin, 10 µg/ml leupeptin, 0.35 mM sucrose) to remove erythrocytes. The minced tissue was brought up to four times the volume of tissue with buffer A and homogenized with a motor-driven Potter homogenizer (10—15 strokes, 1400 rpm) until cell lysis reached about 90%, as shown by microscopy. The homogenate was filtered through cheesecloth, and the filtrate was centrifuged at 4000 rpm for 20 min. The combined nuclear pellets were resuspended in buffer A, using a Teflon-glass Potter homogenizer (1 cycle, 800 rpm), layered over a cushion of buffer B (buffer A containing 0.5 mM sucrose), and centrifuged as before. The washed nuclei were resuspended in buffer C (buffer A without sucrose) and centrifuged at 15000 × g for 15 min. The packed nuclei were resuspended in buffer D (20 mM HEPES–NaOH, pH 7.9, 420 mM NaCl, 1.5 mM MgCl2, 0.1 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 14 µg/ml aprotinin, 10% glycerol) using an all-glass Dounce homogenizer (10 strokes with an A pestle), gently stirred with a magnetic stirring bar for 45 min, and centrifuged at 12000 × g for 15 min. After addition of NP-40 to give a concentration of 0.1%, the clear supernatant was diluted for two 2-h-periods against 50 vol. buffer E (20 mM HEPES–NaOH, pH 7.9, 0.2 mM EDTA, 0.5 mM DTT, 10% glycerol, 0.1% NP-40) containing 300 mM KCl. The dialysate was centrifuged at 10000 rpm for 15 min to eliminate the precipitate formed during the dialysis. The protein concentration of the supernatant, designated as the crude nuclear extract, was determined by the bicinonic acid (BCA; Pierce) method.

Electrophoretic Mobility Shift Assay (EMSA) For the EMSA reaction, 5 µg nuclear extract was incubated for 10 min on ice in 20 µl binding buffer (10 mM HEPES–NaOH, pH 8.0, 50 mM KCl, 2.5 mM MgCl2, 10% glycerol, 0.1 mM DTT, 50 µg/ml bovine serum albumin, 2 µg polydoiyoxyinosinic-polydeoxyyridylic acid (poly(dI)-(dC)) (Amersham Pharmacia Biotech, mean chain length = 500 bp)). Five pico moles of double-stranded probes was labeled using T4 polynucleotide kinase and [γ-32P]ATP. Five femto moles of labeled probe was added to the binding reaction mixture and incubated at room temperature for 30 min. Binding reaction mixtures were size-fractionated on a non-denaturing 4.5% polyacrylamide gel in 0.25×tris–acetate–EDTA (TAE) buffer. The gel was subsequently dried and autoradiographed at −80 °C with an intensifying screen.

DNA Sequence Analysis The nucleotide sequence of the 5′-flanking region of GBP28 gene was determined by the dideoxynucleotide chain termination method31 with DNA sequencer model DSQ-1000 (Shimadzu, Kyoto).

RESULTS

The nucleotide sequence of the 5′-flanking region of the GBP28 gene contained in the BAC 604H12 clone was determined, and several kinds of cis-elements were found in this region (about 600 bp). Figure 1 shows the sites of these elements schematically. Three C/EBP sites, two Sp 1 sites and six AP 1 sites were found in this region. The nucleotide numbers corresponding to these elements are summarized in Table 1. The first intron of the GBP28 gene which is very long, estimated to be 12 kb by PCR,30 was also analyzed for about 3 kb of the 3′-flanking region of the exon 1. Four C/EBP sites were found upstream of the 1st intron (Fig. 2), and an Alu-like sequence was also found more downstream of the 1st intron (data not shown).

The expression of GBP28 was examined using 3T3-L1 cells, which were differentiated into adipocytes by insulin, dexamethasone and 3-isobutyl-1-methylxanthine. Figure 3 shows the Northern blot analysis using mouse GBP28 cDNA as a probe. GBP28 transcripts appeared in the cells on the 5th day after differentiation.

To analyze the mechanisms of transcriptional regulation which are important in the human GBP28 gene and to define the functionally important cis-DNA elements in the 5′-flanking

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**Table 1. The Position and Nucleotide Sequence of Elements Contained in the GBP28 Promoter Region**

<table>
<thead>
<tr>
<th>Element</th>
<th>Sequence</th>
<th>Position</th>
<th>Consensus sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>AP-1</td>
<td>ttggaccagtg</td>
<td>530−539</td>
<td>(T/U)GA⋯⋯(T/U)(A/C)A</td>
</tr>
<tr>
<td>C/EBP</td>
<td>gggagcgccagggg</td>
<td>542−547</td>
<td>G/C⋯⋯G/C(T)/G</td>
</tr>
<tr>
<td>Sp-1</td>
<td>gggagctgaggagggg</td>
<td>496−505</td>
<td>G/G⋯⋯G/G/T</td>
</tr>
<tr>
<td>Sp-1</td>
<td>gggagctgaggagggg</td>
<td>496−505</td>
<td>G/G⋯⋯G/G/T</td>
</tr>
<tr>
<td>C/EBP</td>
<td>gggagctgaggagggg</td>
<td>496−505</td>
<td>G/G⋯⋯G/G/T</td>
</tr>
</tbody>
</table>

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The nucleotide sequence of the first exon is in outline type. The nucleotide sequences similar to the regulatory element are underlined. The numbering uses the transcription start site upstream of exon 1 as the reference point.

![DNA Sequence](image)

**DISCUSSION**

Firstly, GBP28 production is tissue-specific, suggesting a tissue-selective transcription factor. Secondly, it is believed that the GBP28 mRNA content in each cell reflects its adiposity. This suggests that a cell-autonomous mechanism(s) exists for sensing adiposity and converting the signal to regulation of GBP28 mRNA levels. To examine these mechanisms, it is necessary to understand the GBP28 promoter.

Four transcriptional factors functionally implicated in the regulation of Leptin gene expression are C/EBPa, PPARγ, Sp-1, and a novel factor that binds an LP1 motif. The nucleotide sequence of the 5'-flanking region of GBP28 gene was compared with that of the Leptin gene, but the sequence corresponding to LP1 or PPARγ was not found in the GBP28 promoter. Six AP1 sites, three C/EBP sites and two Sp-1 sites were identified in GBP28 promoter (Fig. 1 and Table 1).

Northern blot analysis revealed that the concentration of GBP28 mRNA reached a detectable level on the 5th day after differentiation in 3T3-L1 cells. This is consistent with the adipose-specific expression of this protein.

Studies of fat cell gene expression have been helped by the identification of two specific classes of transcription factors: the CCAAT/enhancer binding proteins (C/EBPs), members of the b-ZIP (basic DNA binding domain and a leucine zipper domain required for dimerization) family and PPARγ, a member of the peroxisomal proliferator activated receptor family of nuclear hormone receptors. The C/EBP isoforms
are expressed at high levels in adipocytes and are induced during adipogenesis.\(^{17}\) Furthermore, C/EBP\(\alpha\) has been demonstrated to play an important role in the differentiation of preadipocytes to adipocytes\(^{18-20}\) and can convert fibroblasts into adipocytes.\(^{21}\) C/EBP\(\beta\) can also induce adipocyte differentiation,\(^{22}\) possibly by inducing PPAR\(\gamma\), which contains C/EBP sites in its promoter.\(^{23}\) PPAR\(\gamma\) isoforms are also potent triggers of the adipocyte differentiation cascade\(^{24}\) and can synergize with C/EBP\(\alpha\) to promote adipocyte differentiation\(^{25}\) or the differentiation of myoblasts into adipocytes.\(^{26}\) In addition, C/EBP\(\alpha\) and PPAR\(\gamma\) can bind to the promoter and activate adipose-specific genes such as aP2\(^{16,25,27,28}\) and PEPCK.\(^{29,30}\)

To look for the adiposity-mediated regulation of GBP28 expression, the promoter activity of the 5'-flanking region of the GBP28 gene was examined by transient transfection of GBP28 promoter-Luciferase reporter constructs into 3T3-L1 adipocytes. Deletion of the C/EBP element in the GBP28 promoter results in a loss of transactivation in 3T3-L1 adipocytes. This C/EBP element appears to be critical for transactivation of the GBP28 promoter in transiently transfected adipocytes (Fig. 4). However, the decrease in transcriptional activity following deletion of the C/EBP element was suppressed by addition of 18 bases derived from the 1st exon to the same constructs (Fig. 4). The mechanism of up-regulation produced by the addition of these 18 bases is unknown. In addition, the existence of C/EBP in the 1st intron (Fig. 2) suggests that the expression of this protein is controlled not only by the elements contained in the 5'-flanking region but also by the elements contained in the intron or further upstream. Although it has been reported that introns can be involved in the regulation of the expression of some
genes, it can not be excluded that other elements regulate the expression of GBP28. However, it is likely that C/EBP takes part in the regulation of the expression of this protein.

EMSA revealed the existence of the protein which binds to the 5′-flanking region of the GBP28 gene in nuclear extracts from human adipose tissue, but not in nuclear extracts from mouse liver. The probe used in this experiment contained C/EBP elements, but the deleted probe lacked this C/EBP element. The deleted probe did not form the complex, suggesting that the deleted region contained the essential sequence for formation of the complex. Although the protein bound to the probe was not identified, it may well be C/EBP.

Based on the results of EMSA and transfection of truncated GBP28 promoter-Luciferase reporter constructs into 3T3 adipocytes, C/EBP is thought to take part in the regulation of GBP28 gene expression.

During the preparation of this manuscript, Schaffer et al. reported the identification and characterization of human adipocyte aPM-1 promoter. We have used the 5′-flanking region of the cloned GBP28 gene to assay promoter activity; however, they amplified the promoter region by PCR and analyzed its activity using promoter-Luciferase reporter constructs. Although the nucleotide sequence reported by them was identical to that of the 5′-flanking region of the GBP28 gene we determined, the regions used for the determination of promoter activity were different. While there are some discrepancies between the results obtained by the two groups, C/EBP seems to be critical for the expression of GBP28 gene.

The elucidation of the functional DNA elements of the GBP28 promoter and their cognate transcription factor presented here is a significant step toward a detailed understanding of the transcriptional regulation of the GBP28 gene.

REFERENCES