CCAAT/Enhancer Binding Protein \( \beta 2 \) Is Involved in Growth Hormone-Regulated Insulin-Like Growth Factor-II Gene Expression in the Liver of Rainbow Trout (\textit{Oncorhynchus mykiss})

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Previously, we showed that levels of different CCAAT/enhancer binding protein (C/EBP) mRNAs in the liver of rainbow trout were modulated by GH and suggested that C/EBPs might be involved in GH-induced IGF-II gene expression. As a step toward further investigation, we have developed monoclonal antibodies to detect rainbow trout C/EBP\( \alpha \)-, \( \beta 1 \)-, \( \beta 2 \)-, and \( \alpha 2 \) isoform proteins. Injection of GH into adult rainbow trout resulted in a significant increase of C/EBP\( \beta 1 \), C/EBP\( \beta 2 \), and C/EBP\( \alpha 2 \) proteins in the liver. Chromatin immunoprecipitation analysis revealed that C/EBP\( \beta 2 \) binds to multiple sites at the 5’ promoter/regulatory region, introns, and the 3’ untranslated region of the IGF-II gene. GH treatment reduced C/EBP\( \beta 2 \) binding to several of these regions at 6 h after injection. The decreased occupancy of C/EBP\( \beta 2 \) coincided well with an increase of histone H4 acetylation at the proximal promoter and elevation of the IGF-II mRNA level. Immunoblotting analysis showed that C/EBP\( \beta 2 \) existed predominantly as a truncated form in the liver, and cotransfection analysis further showed that the truncated C/EBP\( \beta 2 \) acted as a negative regulator on IGF-II proximal promoter. GH treatment caused deacetylation of C/EBP\( \beta 2 \) in the liver. In addition, we observed a GH-dependent interaction of C/EBP\( \beta 2 \) with a complex involving histone H1. All together, these results suggest that C/EBP\( \beta 2 \) was regulated at multiple levels by GH, and C/EBP\( \beta 2 \) may play a suppressive role in mediating GH-induced IGF-II expression in the liver of rainbow trout. (\textit{Endocrinology} 151: 2128–2139, 2010)

GH is a pleiotropic hormone produced and released from the pituitary gland. GH exerts its pleiotropic functions by targeting the GH receptor at multiple peripheral tissues and initiates signal transduction cascades leading to the regulation of gene expression and physiological responses (1–3). Liver is considered as the main target organ of GH action and is responsible for production of IGF-I, which is known to mediate the growth-promoting effects of GH (4). In the rat liver, GH stimulates IGF-I gene expression via activating the signal transducer and activator of transcription 5b (STAT5b), which once phosphorylated, dimerizes and translocates into nuclei and binds directly to multiple regions across 170 kb of the IGF-I gene leading to activation of gene transcription (5–7). Despite the well-established role of GH regulation on IGF-I gene expression, very little is known about GH dependency of IGF-II gene expression in vertebrates. Humans are the only example of GH-dependent hepatic IGF-II gene expression in mammals (8). Four promoter/regulatory regions were found in the human IGF-II gene, and these four regions drive the expression of various lengths of IGF-II transcripts in tissue- and developmental-specific manners (9, 10). These promoter/regulatory regions also respond differently to GH treatments; whereas acute GH treatment increases P2- but not P1- and P4-derived transcripts, short-term GH treatment increases P4-derived but not P1- and P2-derived transcripts (8).

In teleost fish, GH-induced IGF-II gene expression was first reported in the rainbow trout liver by Shamblott et al. (11), and since then, GH dependency of IGF-II gene ex-
pression has been shown in many other fish species (12–14). Unlike humans, teleost IGF-II genes characterized to date possess only one promoter (15). Using EMSA, Shambrot et al. (16) showed that a stronger binding signal to the presumptive CCAAT/enhancer binding protein (C/EBP) binding site located in the proximal promoter region of IGFI was observed in liver nuclear extracts from GH-treated fish than that from the mock-treated controls. Recently, we have cloned the cDNAs of five C/EBP isoforms from the liver of rainbow trout and showed that the mRNA levels of some C/EBP isoforms were modulated by GH (17).

In the current study, we determined the change of C/EBP isoform protein levels in response to GH treatment in the trout liver and confirmed that C/EBPβ2 acts as a regulator in mediating the GH-induced IGF-II gene transcription. Truncated C/EBPβ2 was observed as the major form existing in the trout liver, and it likely played a negative regulatory role in the IGF-II proximal promoter. In addition, a GH-dependent deacetylation and interaction with the histone H1 complex of C/EBPβ2 was also observed.

Materials and Methods

Antibodies (Abs)

Affinity-purified polyclonal Abs against rainbow trout C/EBP isoforms were developed by the Complete Peptide Polyclonal Ab Service (SC-1015; GenScript, Piscataway, NJ) in rabbit against a keyhole limpet hemocyanin-conjugated synthetic peptide with the following sequences: C/EBPα, DRRDRTDKGKSRR; C/EBPβ1, SGERDESQEPARM; C/EBPβ2, Ab 1, SGGGKGKKRLKEDS; C/EBPβ2 Ab 2, SPGTPATPGKGRS; and C/EBPβ2, SPRK-VGKEKGGKNF. The other Abs used in the study include rabbit polyclonal anti-β-actin Ab (A2066; Sigma Chemical Co., St. Louis, MO), rabbit polyclonal anti-actin-α-lysine 12 histone H4 antiserum (ab1761; Abcam, Cambridge, MA), mouse monoclonal anti-acetyl-lysine Ab (05-515; Millipore, Temecula, CA), rabbit polyclonal anti-c-Myc polyclonal Ab, (A00172; GenScript), alkaline phosphatase (AP)-conjugated goat antirabbit secondary Ab (111-055-003; Jackson ImmunoResearch, West Grove, PA), AP-conjugated goat antimouse secondary Ab (115-055-003; Jackson ImmunoResearch), and monoclonal AP-conjugated mouse antirabbit light chain specific secondary Ab (211-052-171; Jackson ImmunoResearch).

Animal acclimation and GH treatment

Ethical approval for the use of animals was granted by Institutional Animal Care and Use Committee, and animals were reared and handled according to the guidelines of institutional policies. One-year-old adult fish with body weights of 180–300 g were maintained and treated with bovine GH (USDA-bGH-B-1) or carrier solution for various periods of time as described previously (17).

Protein extraction

Frozen liver tissues were homogenized in 10 ml tissue homogenizing buffer [10 mM HEPES-KOH (pH 7.5), 25 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 1 mM EDTA, 2 mM sucrose, 10% glycerol, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 1× protease inhibitor cocktail (P2714; Sigma)] with a Polytron tissue homogenizer. The homogenate was layered over 3 ml of the tissue homogenization buffer and centrifuged at 39000 × g for 30 min at 4°C. For immunoblotting, EMSA, and DNA affinity pull-down analysis (DAPA), the liver nuclear protein was extracted from the nuclei pellet in nuclear extraction buffer [10 mM HEPES (pH 7.5), 1.5 mM MgCl₂, 0.5 mM dithiothreitol (DTT), 2.5% glycerol, 0.5 mM PMSF, 1× protease inhibitor cocktail, 400 mM NaCl]. For immunoprecipitation (IP), the nuclear pellet was extracted in sodium dodecyl sulfate (SDS) lysis buffer [50 mM Tris (pH 8.0), 10 mM EDTA, 0.5% SDS, 1 mM PMSF, 1× protease inhibitor cocktail]. Total liver proteins were prepared by polyonowering liver tissues in SDS lysis buffer and sonicated to reduce lysate viscosity.

Preparation of total cellular protein from sodium butyrate-treated rainbow trout hepatoma (RTH) cells

RTH cells (RTH 1B1A) (18) were plated on 100-mm cell culture dishes overnight in a CO₂-independent medium (Life Technologies, Inc.-BRL Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS). The cells were subsequently treated with 0–50 mM sodium butyrate for 24 h, trypsinized from the dishes, washed twice with PBS, and lysed in 200 μl SDS lysis buffer.

Immunoblotting

Protein samples were resolved by SDS-PAGE in 15% gels along with prestained protein ladder (BenchMark, 10478-010; Invitrogen) and transferred to Immobilon-P (Millipore, Bedford, MA). The blot was blocked for 1 h at room temperature in Tris-buffered saline (TBS) [10 mM Tris-HCl (pH 8.0), 150 mM NaCl] containing 10% nonfat dry milk. After blocking, the blot was incubated with the first Ab (1:4000) in TBS with 0.05% Tween 20 for 2 h at room temperature. The membrane was washed twice with TBS with 0.05% Tween 20 and reacted with the appropriate AP-conjugated secondary Ab (1:10,000). The blot was developed using 5-bromo-4-chloro-3-indolyl phosphate (R-8503; Sigma) and nitroblue tetrazolium (N-6876; Sigma) as substrates in AP buffer [100 mM Tris (pH 9.5), 100 mM NaCl, 5 mM MgCl₂].

Chromatin IP (ChIP), IP, and co-IP

Frozen trout liver tissues were homogenized in 9 ml tissue homogenizing buffer containing 0.8% formamide to cross-link the chromatin. After 10 min incubation at room temperature, 1 ml 1.25 M glycine was added into the homogenate for 5 min to quench the reaction. The nuclei were pelleted under the same conditions as described above and washed with PBS. Chromatin was released by resuspending the pelleted nuclei in SDS lysis buffer and sheared with Sonifier 450 (90% duty cycle, output setting 3, and 10 cycles of 40 pulses; Branson Ultrasonics Corp., Danbury, CT). Under the specified conditions, chromatin was consistently sheared to sizes ranging from 100–600 bp (Supplemental Fig. 1 published on The Endocrine Society’s Journals Online web site at http://endo.endojournals.org). One volume of the fixed amount of chromatin DNA was diluted with 9 vol of the ChIP dilution buffer [1% Triton X-100, 2 mM EDTA, 20 mM
Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM PMSF, 1× protease inhibitor cocktail) and immunoprecipitated with 4°C overnight with anti-C/EBPα Ab (5 μg Ab/20 μg chromatin), anti-C/EBPβ Ab 1 (8 μg/20 μg chromatin), anti-C/EBPβ2 Ab 2 (6 μg/20 μg chromatin) or anti-acetyl-lysine 12 histone H4 antiserum (3 μl/10 μg chromatin). Protein A/G resin (L00209, L00210; GenScript) was preblocked with 500 μg/ml BSA and 400 μg/ml fragmented calf thymus DNA (2618; Calbiochem, La Jolla, CA) in ChIP dilution buffer at 4°C overnight. The antibody/chromatin complex was collected by adding the blocked protein A/G resin (15 μl bed volume each) into each sample and incubated for 2 h at 4°C with rotation. The samples were washed five times with 500 μl ChIP dilution buffer and eluted with 400 μl ChIP elution buffer (1% SDS, 0.1 M NaHCO₃). The cross-linked DNA was incubated overnight at 65°C. Each sample was further treated with protease K and purified by phenol-chloroform extraction. The precipitated DNA fragments were dissolved in 80 μl Tris-EDTA buffer, and 2 μl was used for each PCR using primers described in Table 1. The PCR-amplified products were resolved in 2% agarose gels, stained with ethidium bromide, and quantitated by Image J (National Institutes of Health, Bethesda, MD). The enrichment cutoff was arbitrarily set at a 1.5-fold increment based on densitometric analysis of immunoprecipitated signals vs. background.

For IP analysis, 80 μg RTH total cellular protein or trout liver nuclear protein extracted in SDS lysis buffer was diluted in a ratio of 1:9 with ChIP dilution buffer. The IP was performed under the same conditions as ChIP analysis except with antirabbit IgG resin (sc-45043; Santa Cruz Biotechnology, Santa Cruz, CA) to collect the immune complex. The pulled-down complex was eluted by boiling for 10 min in 30 μl SDS-PAGE loading buffer [1% SDS, 5% glycerol, 10 mM DTT, 5% β-mercaptoethanol, 125 mM Tris-HCl (pH 6.8), 0.01% bromophenol blue]. Fifteen microliters of each sample was resolved in duplicate SDS-polyacrylamide gels and immunoblotted with anti-C/EBPβ2 or anti-acetyl-lysine Abs.

For co-IP analysis, 8 μg anti-C/EBPβ2 Ab was added directly into 250 μl liver nuclear protein in 500 μl nuclear extraction buffer and incubated at 4°C overnight with rotation. Protein A resin was reconstituted with co-IP wash buffer [20 mM Na₂HPO₄ (pH 7.0), 150 mM NaCl] and 50 μl bed volume of resin was added into each sample for 2 h at 4°C with rotation. The samples were washed three times with co-IP wash buffer and eluted by boiling in SDS-PAGE loading buffer. The eluates were resolved in duplicate SDS-polyacrylamide gels and stained with Coomassie blue or Silver Stain Plus (161-0449; Bio-Rad, Hercules, CA).

**Transfection analysis**

Truncated cDNA sequences of C/EBPβ1 and C/EBPβ2 were PCR cloned into HindIII and BamHI sites of pcDNA 3.1(+) expression vector (Invitrogen) with an optimal Kozak sequence using the following primers: C/EBPβ1 forward primer, tcaaaagcttgccac-catggaacagctgcaact; C/EBPβ1 reverse primer, gtggagatctctaacc-ctcgtggctttgaagaacc-

tgcaattggcagtaatct-

**TABLE 1. Oligonucleotides used in ChIP analysis**

<table>
<thead>
<tr>
<th>Site designation</th>
<th>Sequence (5’–3’)</th>
<th>C/EBP binding site position</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1</td>
<td>Forward Reverse</td>
<td>acacacccgctgtctcttc</td>
<td>1389-1403, 5’ upstream&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>S7</td>
<td>Forward Reverse</td>
<td>cggtcgggttcagacatgccccg</td>
<td>9003-9016, proximal promoter&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>S10 and S11&lt;sup&gt;e&lt;/sup&gt;</td>
<td>Forward Reverse</td>
<td>gagccgtgtttgtaaggttccag</td>
<td>S10, 9838-9852, first intron</td>
</tr>
<tr>
<td>S12</td>
<td>Forward Reverse</td>
<td>tggaggggtctgttatgacaa</td>
<td>S11, 9974-9988, first intron&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>S13</td>
<td>Forward Reverse</td>
<td>acctgtgagagagagaacata</td>
<td>10117-10131, first intron</td>
</tr>
<tr>
<td>S14 and S15&lt;sup&gt;e&lt;/sup&gt;</td>
<td>Forward Reverse</td>
<td>tacccctccatctatatcgcgg</td>
<td>10459-10470, second intron</td>
</tr>
<tr>
<td>S16 and S17&lt;sup&gt;e&lt;/sup&gt;</td>
<td>Forward Reverse</td>
<td>gcagcttccaaatccttta</td>
<td>S14, 13076-13090, 3rd intron&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>S19&lt;sup&gt;g&lt;/sup&gt;</td>
<td>Forward Reverse</td>
<td>tctggagctttgtaaagacc</td>
<td>3’ UTR</td>
</tr>
</tbody>
</table>

<sup>a</sup> The numerical annotations correspond to the positions on the rainbow trout genomic DNA sequence with GenBank accession no. FJ817293.

<sup>b</sup> PCRs were carried out under standard conditions with 54°C annealing temperature and 35 amplification cycles.

<sup>c</sup> The C/EBP binding site was located on the antisense strand.

<sup>d</sup> The C/EBP binding site was reported in the literature (28).

<sup>e</sup> The PCR primer pairs flank both predicted C/EBP binding sites.

<sup>f</sup> The PCRs were carried out with 40 amplification cycles.

<sup>g</sup> This C/EBP binding site was identified on position 788–802 of the expressed sequence tag BX881430, which together with CX139957 constituted the extended IGF-II 3’ untranslated region (UTR) overlapping with 3’ end of trout genomic DNA sequence FJ817293.
gaccggt; C/EBPβ2 forward primer, tcaaaagtgcaccattggcaca-
aacggtct; and C/EBPβ2 reverse primer, cattagatcttaaact-
cacggt.

The 300-bp fragment (from −1 to −298; relative to ATG translation start codon, +1) of rainbow trout IGF-II proximal promoter was cloned into SacI and XmaI sites of the pG3L-Basic reporter vector (Promega, Madison, WI) using the following primers: forward primer, caccagctgcagccacttcttctgctc, and reverse primer, gggtccgggctaacgctggatcacttt.

Three single-stranded oligonucleotides were synthesized for EMSA including sense strand C/EBP binding site oligomer (CG-TGCAGTCCAGACATGGCGG), antisense strand C/EBP binding site oligomer (CCGGCATTGTCTGAAACCGACCG), and a palindromic oligonucleotide harboring an optimal C/EBP binding site oligomer (CCGGCATGTCGCAATCTGCA). The sense strand C/EBP binding site oligomer was radiolabeled with γ-32P-ATP and annealed with the antisense oligomer. Two micrograms of liver nuclear extracts in an EMSA binding buffer were incubated with 2.5 pmol biotin-labeled probe. DNA-protein complexes were resolved in a 5% non-denaturing polyacrylamide gel and stained with ProteoSilver Plus (PROTSIL2; Sigma). The protein band was excised from the gel, destained, and analyzed by mass spectrometry conducted by a protein identification service (Pick’n Post, Alphalyse, Palo Alto, CA).

Statistics

Data in this study were evaluated for statistical significance by an unpaired two-tailed Student’s t test using Statistica 7.0 (StatSoft, Tulsa, OK).

Results

GH-dependent protein levels of C/EBP isoforms in the liver of rainbow trout

The specificity of each C/EBP isoform protein Ab was determined by immunoblotting recombinant C/EBP proteins expressed in Escherichia coli or in CHO-K1 cells. Except anti-C/EBPβ2 Ab 1, which cross-reacted slightly with the recombinant C/EBPβ1, each anti-C/EBP isoform Ab specifically recognizes the corresponding C/EBP isoform (Supplemental Fig. 2). It was known that two forms of C/EBPα (P42 and P30) and three forms of C/EBPβ proteins (LAP*, LAP, and LIP) were present in humans and mice (20–22). These different lengths of C/EBP proteins were produced from alternative translational initiation (20–23) or from proteolytic cleavage of full-length polypeptides (24). To determine whether multiple forms of C/EBPα and C/EBPβ are also present in the liver of rainbow trout, the total liver extract and the liver nuclear extract were analyzed by immunoblotting (Fig. 1A). A major band with an apparent molecular mass close to the estimated size of a full-length C/EBPα (36.5 kDa) was detected in the total liver extract and to a lesser extent in the nuclear extract. A few protein bands with apparent molecular masses close to the estimated size of the potential truncated C/EBPα [23 kDa, starting from amino acid (aa) 125 of the full-length peptide] were also detected in the total liver extract but not in the nuclear extract. On the blot probed with anti-C/EBPβ2 Ab, two groups of protein bands, a strong-reacting band of 23 kDa accompanied by a few minor protein bands with apparent molecular masses ranging from 37–45 kDa, were observed (Fig. 1A).

Because the estimated molecular masses of different species of C/EBPβ2 starting from different methionines positioned at aa 1, 38, and 159 are 33, 29, and 15 kDa, respectively, the two groups of bands likely represent the full-length and the truncated forms starting from aa 1 and 159, respectively. In mammals, C/EBPα and C/EBPβ proteins are subjected to several posttranslational modifications (25, 26); therefore, it’s conceivable that several species of C/EBPβ2 deviated from the estimated sizes on the immunoblot could be derived from the different extent of posttranslational modifications.

Protein identification by mass spectrometer analysis

The proteins eluted from DAPA were resolved in a 22-cm 12% polyacrylamide gel and stained with ProteoSilver Plus (PROTSIL2; Pick’n Post, Alphalyse, Palo Alto, CA). The protein band was excised from the gel, destained, and analyzed by mass spectrometry conducted by a protein identification service (Pick’n Post, Alphalyse, Palo Alto, CA).
We have shown previously that mRNA levels of C/EBP isoforms and IGF-II in the trout liver were modulated by GH treatment (11, 17). Results of injecting adult fish with bovine GH or carrier solution for shorter (0.5 and 1.5 h) or longer (9 and 12 h) periods of time further corroborated our previous observations (Supplemental Fig. 3). C/EBPβ1, -β2, and -β2 mRNA levels increased after GH treatment, whereas the C/EBPα mRNA level started to decrease at 6 h after GH injection. To determine whether the GH-dependent changes of C/EBP
mRNA levels can be translated into changes at the protein levels, liver nuclear extracts prepared from samples at 1.5, 3, 6, and 9 h after injection were immunoblotted with the respective anti-C/EBP isoform Abs (Fig. 1B). Although GH treatment caused a decrease of C/EBP/H9251/H9251 protein at 9 h after injection, C/EBP/H9252/H9252 protein level started to increase at 3 h and maintained its increase throughout 9 h after GH injection. Both the full-length (43 kDa) and the truncated (23 kDa) forms of C/EBP/H9252/H9252 showed obvious increase at 3 h and 9 h after GH injection. An increased level of C/EBP/H9254/H9254 protein was detected at 1.5, 3, and 9 h after GH injection. The restoration of GH/control mRNA ratios for C/EBP/H9252/H9252 and C/EBP/H9254/H9254 at 6 h after injection was also reflected at the protein levels.

Identifying the GH-dependent occupancy of C/EBPs on IGF-II gene by ChIP

Previously, Shamblott et al. (16) isolated a 14.6-kb DNA fragment (accession no. FJ817293) containing the entire coding region of the IGF-II gene and a 9-kb 5′ upstream region. This DNA fragment together with two expressed sequence tag sequences (accession nos. CX139957 and BX881430) overlapping with the 3′ end of the 14.6-kb fragment were subjected to prediction for the C/EBP binding site by using MatInspector (library version 7.1) (27). Including those reported in the literature (16, 28), a total of 19 putative binding sites were predicted (Fig. 2A). To determine which binding sites and what C/EBP isoforms were involved in the binding, we performed ChIP analysis with the anti-C/EBP isoform Abs. The suitability of each Ab for ChIP analysis was initially tested by IP analysis. Under the optimized conditions, significant immunoprecipitates were obtained only for anti-C/EBP/H9251/H9251 and both anti-C/EBP/H9252/H9252 Abs (Supplemental Fig. 4), suggesting that these Abs might be suitable for ChIP analysis.

To avoid the potential signal variations resulting from the changes in protein levels, we performed ChIP analysis on trout liver chromatin samples at 6 h after GH treatment, when the GH/control ratio of IGF-II mRNA peaked (Supplemental Fig. 2), and protein levels of C/EBP/H9251/H9251 and C/EBP/H9252/H9252 were unaffected (Fig. 1B). For C/EBP/H9251/H9251, no significant DNA enrichment was observed for all the predicted C/EBP binding sites (Ab/agarose background <1.5, data not shown). But for C/EBP/H9252/H9252, significant DNA enrichments were observed at several regions including the 5′ distal region (S1), proximal promoter (S7), first intron (S10/S11 and S12), second intron (S13), third intron (S14/S15 and S16/S17), and 3′ untranslated region (S19) (Fig.
Interestingly, significant reductions of DNA enrichments at the proximal promoter region (S7), the end of the first intron (S12), and the third intron (S16/S17) were observed in GH-treated samples compared with mock-treated samples (Fig. 2B). These results suggest a GH-dependent interaction of C/EBPβ2 with IGF-II regulatory regions in the trout liver. Because IGF-II mRNA level was 2.5-fold higher in the trout liver at 6 h after GH injection (Supplemental Fig. 3), we performed ChIP analysis on the same chromatin samples using an anti-acetyl-lysine 12 histone H4 antiserum (GenBank accession no. P62797, 100% identity with human histone H4) and analyzed the regions that were enriched by anti-C/EBPβ2 Abs. Of these regions, significant DNA enrichments were detected at S7 and S10/S11, and a significant increase of DNA enrichment was observed only at the proximal promoter region (S7) in the GH-treated samples.

**Negative regulatory effect of truncated C/EBPβ1 and -β2 on the IGF-II proximal promoter**

Palamarchuk et al. (28) reported previously that human C/EBPα and C/EBPβ activated the chum salmon IGF-II proximal promoter. Because the 300-bp proximal promoter of trout IGF-II gene shares about 98% sequence identity with that of chum salmon and because the functional C/EBP binding sites identified in chum salmon also reside within this 300-bp region, we questioned whether truncated trout C/EBPβ1 and -β2 may play any regulatory function in this region. To answer this question, CHO-K1 or Hep3B cells were cotransfected with a trout IGF-II promoter-luciferase construct and a truncated C/EBPβ1 or -β2 expression vector (expressing truncated C/EBPβ1 and C/EBPβ2 starting from aa 159). As expected, the truncated rainbow trout C/EBPβ1 and -β2 exhibited negative regulation on IGF-II proximal promoter, and the truncated C/EBPβ2 appeared to have a stronger suppression activity than that of truncated C/EBPβ1 in both CHO-K1 and Hep3B cells (Fig. 3).

**GH caused deacetylation of C/EBPβ2 in the trout liver**

A study by Cesena et al. (29) suggested that GH treatment could cause acetylation of C/EBPβ in 293T cells, and acetylation of transcription factors was known to modulate their DNA-binding affinities (30). Because we observed a decreased occupancy of C/EBPβ2 at multiple regions of the IGF-II gene in GH-treated samples, we questioned whether GH might modulate the acetylation status of C/EBPβ2 in the trout liver. To determine whether trout C/EBPβ2 protein is modified via acetylation, we treated rainbow trout hepatoma cells (RTH 1B1A) with sodium butyrate, a nonspecific histone deacetylase (HDAC) inhibitor and performed IP-immunoblot analysis using anti-C/EBPβ2 and anti-acetyl-lysine Abs. Unexpectedly, treating RTH cells with sodium butyrate resulted in a decrease of multiple forms of C/EBPβ2 (Supplemental Fig. 5). Of the immunoprecipitated C/EBPβ2 proteins, three forms were detected by the anti-acetyl-lysine Ab with apparent molecular masses of 15, 18, and 37 kDa (Fig. 4A). All three bands showed stronger intensities in the butyrate-treated samples than controls, suggesting that trout C/EBPβ2 can be modified by histone acetyltransferases or HDACs.

When performing the same experiments with trout liver nuclear protein, we observed that GH treatment caused a reduction in the amount of acetylated C/EBPβ2, and the decrease was observed as early as 1.5 h after GH injection (Fig. 4B).
GH treatment resulted in the dissociation of C/EBPβ2 from a complex involving histone H1

Because GH treatment led to reduced C/EBPβ2 occupancies at the IGF-II proximal promoter (S7), and Palamarchuk et al. (28) showed in chum salmon that the C/EBP binding site (S7) was functionally essential for IGF-II basal promoter activity, oligonucleotide duplex with sequence corresponding to S7 was used as a probe for EMSA. As shown in Fig. 5A, the signal of a low-mobility binding complex was much more pronounced in the mock-treated sample. To characterize this binding complex, the same oligonucleotide duplex was tagged with biotin and used for DAPA. As shown in Fig. 5B, a protein band of an apparent molecular mass around 30 kDa with a stronger intensity was observed in the mock-treated sample. The identity of this protein band was determined by matrix-assisted laser desorption/ionization–time-of-flight mass spectrometer/time-of-flight mass spectrometer mass spectrometry, and results showed peptide sequences of KAGPSVGELIVK and SGVSLAALKK, corresponding to rainbow trout histone H1 (GenBank accession no. P06350). To determine whether C/EBPβ2 and histone H1 might be involved in the same complex in the trout liver nuclei, we performed co-IP analysis with anti-C/EBPβ2 Ab. The protein with apparent molecular mass around 30 kDa was again observed in the precipitated products and showed higher abundance in mock-treated than GH-treated samples (Fig. 5C). Because histone proteins are extremely abundant in the nuclei and because histone H1 is the largest subunit that can be removed from chromatin by salt extraction (31), the result showing the most pronounced band in nuclear extracts that ran to the same size class of 30 kDa with the immunoprecipitates suggested that C/EBPβ2 may interact with histone H1 in the trout liver.

Discussion

In mammals, C/EBPα and C/EBPβ mRNAs can give rise to different lengths of polypeptides by alternative use of translation initiation codons (20–23). In the current study, although we have detected minor protein bands with apparent molecular masses close to the presumptive truncated C/EBPα (23 kDa) in the total liver extract samples, these bands were not observed in the liver nuclear extracts and the immunoprecipitates of the RTH total cellular extracts. In addition, we did not detect a decrease of these protein bands at 9 h after GH injection. Together, these results suggest that only the full-length C/EBPα is encoded in the trout liver and the minor species, around 23...
kDa, is probably due to nonspecific reactions of Ab. However, failure to detect the presence of the truncated C/EBPα in the trout liver may not exclude entirely the possibility of the presence of the truncated C/EBPα in other tissues because multiple size classes of C/EBPα were detected in CHO-K1 cells transfected with a full-length trout C/EBPα cDNA expression construct (Supplemental Fig. 2B). In addition, multiple forms of zebrafish C/EBPα had also been detected by in vitro translation (32).

Multiple species of C/EBPβ2 were detected in the extracts of the trout liver or the liver nuclei in immunoblots, and these different species of C/EBPβ2 likely represent the full-length polypeptides and the truncated polypeptides starting from aa 159 with various extents of posttranslational modifications. This conclusion was supported by the observation that lysates of CHO-K1 cells transfected with the full-length C/EBPβ2 cDNA expression construct contained polypeptides with similar apparent molecular masses of the full-length (37–45 kDa) and the truncated (15–23 kDa, Supplemental Fig. 2B) C/EBPβ2. Because anti-C/EBPβ1 Ab was raised against a peptide sequence in front of aa 159 of C/EBPβ1 (first methionine residue for the potential truncated C/EBPβ1), it will detect only full-length C/EBPβ1. When expressing the C-terminal c-Myc-tagged C/EBPβ1 in CHO-K1 cells, similar to that of C/EBPβ2, a protein band with apparent molecular mass corresponding to the presumptive truncated C/EBPβ1 (starting from aa 159) was detected by anti-c-Myc Ab (Supplemental Fig. 2C), implying the possible existence of a truncated C/EBPβ1 in the trout liver. It is interesting to note that both C/EBPβ isoform mRNAs of rainbow trout retained a conserved upstream open reading frame (uORF) residing in the IGF-II gene promoter region, an increase of histone H4 acetylation in multiple regions of the IGF-II gene, and GH treatment resulted in dissociation of C/EBPβ from a complex involving histone H1. A, EMSA of liver nuclear extracts. Liver nuclear extracts prepared from 3-h post-injected fish and radiolabeled oligonucleotides encompassing C/EBP binding site (S7, Fig. 2) were used in EMSA. The unlabeled oligonucleotides harboring an optimal C/EBP binding site were used for competition to determine binding specificity. C, Fish injected with carrier solution; GH, fish injected with GH. B, DAPA of GH-treated and mock-treated liver nuclear extracts. The same oligonucleotides used in EMSA were tagged with biotin and used for protein pull-down. The pulled-down proteins were excised from the gel and analyzed by mass spectrometry. A control sample (w/o protein) was processed as A, EMSA of liver nuclear extracts. Liver nuclear extracts from 3-h post-injected fish were immunoprecipitated (IP) with anti-C/EBPβ2 Ab under conditions of co-IP. Equal volumes of the immunoprecipitated eluates were resolved in duplicate 10% SDS-polyacrylamide gels and stained with Coomassie blue or silver stain. Ab, Direct loading of 8 μg anti-C/EBPβ2 Ab; C, fish injected with carrier solution; GH, fish injected with GH; 1/60 NE, direct loading of 1/60 of liver nuclear extract used in this co-IP assay.
Homodimer or heterodimer of the truncated C/EBPβ2 may bind to the IGF-II proximal promoter to suppress gene transcription, and GH treatment results in the dissociation of the truncated C/EBPβ2 from the region to allow active IGF-II gene transcription. In mammals, it has been reported that truncated C/EBPβ displayed a negative regulatory effect on the albumin and c-fos promoters (21, 36).

Histone H1 is known as a linker histone involved in the formation of higher chromatin structure by sealing two rounds of DNA at its entry/exit site on the surface of nucleosome core particles. Interaction of chromatin with histone H1 limits the mobility of nucleosomes and the accessibility of chromosomal DNA for chromatin remodeling (37–39). In addition to the architectural role in the assembly of chromatin fibers, recent studies have indicated that histone H1 also interacted with other regulatory factors for its action as a repressor of specific genes (40–42). Our detection of histone H1 protein pulled down together with C/EBPβ2 coupled with the observation of the formation of a low-mobility binding complex in EMSA suggests that C/EBPβ2 may be in complex not only with histone H1 but also with other factors. Because this low-mobility complex can be competed out by the optimal C/EBP binding site oligomer duplex, it’s unlikely that the complex derives from direct binding of histone H1 to the probe. When scaling up DAPA and immunoblotting, the pulled-down proteins with anti-C/EBPβ2 Ab, lower amounts of C/EBPβ2 were observed in GH-treated than mock-treated samples (Supplemental Fig. 6), particularly the 23-kDa truncated form, providing another line of evidence supporting the fact that GH treatment may lead to a decreased association of C/EBPβ2 and other proteins to the C/EBP binding site. These observations are similar to that reported by Kim et al. (40) showing that histone H1.2 served as an anchoring protein for other negative regulatory factors that prevented p53-dependent acetylation of chromatin by p300 in HeLa cells. At present, it is uncertain whether C/EBPβ2 engages directly with histone H1 or indirectly through other factors in the formation of the low-mobility complex. More studies will be required to delineate their relationship.

In mammals, C/EBP family proteins are regulated by various types of posttranslational modifications including phosphorylation, acetylation, sumoylation, and ubiquitylation (25, 26). The conservation of several modification sites found in rainbow trout C/EBP family proteins suggests that these proteins may be subjected to similar modifications as found in mammals (17). The observation of hyperacetylated C/EBPβ2 in butyrate-treated RTH cells supported the hypothesis that the C/EBPβ2 may be modified by histone acetyltransferases/HDACs in the trout liver. It was shown that C/EBPβ was present in multiple forms with different degrees of phosphorylation in 3T3-F442A fibroblast cells, and GH treatment promoted phosphorylation and dephosphorylation on different residues of C/EBPβ causing changes of DNA-binding affinity and transactivational capacity (43). Recent studies have also shown that lysine acetylation could cross talk with other posttranslational modifications (44, 45). Therefore, it is possible that deacetylation of C/EBPβ2 in response to GH treatment indirectly causes modulation of C/EBPβ2 phosphorylation status, which leads to alteration on its DNA-binding affinity and protein interaction capability.

There are two possible scenarios that could be used to explain the potential role of C/EBPβ2 in mediating the GH-regulated IGF-II gene transcription. In scenario 1, in the absence of GH, truncated C/EBPβ2 may bind via the C/EBP binding sites to the proximal promoter or other regions of the IGF-II gene and recruit histone H1 complex to suppress IGF-II transcription. In the presence of GH, the truncated C/EBPβ2 reduces its binding to the C/EBP binding sites and dissociates from histone H1 complex due to posttranslational modification, thus allowing transcription of IGF-II gene to occur. In scenario 2, truncated C/EBPβ2 may interact with the IGF-II gene via linker histone H1 and provide steric hindrance that limits the access of activators or chromatin remodeling complexes to activate IGF-II transcription. GH treatment causes dissociation of C/EBPβ2 from linker histone H1, which increases the accessibility for transcription activator binding and allows chromatin remodeling to occur. To date, 11 histone H1 subtypes have been identified in humans (39); however, there are only two subtypes of trout histone H1 reported (46, 47). Because human histone H1.1, H1.2, H1.3, H1.4, and H1.5 are highly similar in overall structure, it is difficult to assign the phylogenetic counterpart for the trout histone H1 described in this study without isolating all the subtypes. However, sequence alignments of the trout histone H1 with all human histone H1 subtypes revealed that it has a high degree of sequence identity with human H1.1–1.5 (H1.1, 57.5%; H1.2, 60.8%; H1.3, 59.9%; H1.4, 62.8%; and H1.5, 62.3%), indicating a potential ortholog of one of the five human histone H1 subtypes.

Although more molecular characterization is required, results of our study suggest that GH may regulate C/EBPβ2 at multiple levels, and the truncated C/EBPβ2 may play a negative regulatory role in mediating GH-induced IGF-II gene transcription in the liver of rainbow trout.

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