Cloning, Expression, and Regulation of Bovine Cellular Retinoic Acid-binding Protein-II (CRABP-II) during Adipogenesis*

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ABSTRACT: The mammalian cellular retinoic acid-binding proteins, CRABP-I and CRABP-II, bind retinoic acid which acts as an inducer of differentiation in several biological systems. To investigate a possible role for CRABP-II in bovine adipogenesis, we have cloned bovine CRABP-II cDNA and the coding region for CRABP-I. The predicted amino acid sequences of CRABP-II were highly conserved among several animal species (human, mouse, and rat at 97%, 93%, and 93%, respectively). The expression pattern of bovine CRABP-II was examined in greater details by applying RT-PCR to various bovine tissues. CRABP-II mRNA was expressed in most adipose-containing tissues. Moreover, the expression of CRABP-I and -II mRNA dramatically increased during the differentiation of adipocytes from bovine intramuscular fibroblast-like cells. The effects of retinoic acid on adipocyte differentiation of bovine intramuscular fibroblast-like cells were concentration-dependent. Retinoic acid activated the formation of lipid droplets at a level of 1 nM, whereas inhibition was observed at a level of 1 µM. CRABP-I gene was up-regulated and CRABP-II gene down-regulated by retinoic acid during adipocyte differentiation. These results suggest that CRABPs may play an important role in the regulation of intracellular retinoic acid concentrations during adipogenesis. (Key Words: Bovine Adipocyte Differentiation, Lipid-binding Protein, Retinoic Acid, Retinoic Acid Receptor, Retinoid X Receptor)

INTRODUCTION

Retinoic acid plays an important role in mammalian development and cellular growth and differentiation. Retinoic acid receptors (RARs) and the retinoid X receptor (RXR), which forms RAR-RXR heterodimers, regulate transcription following binding at specific DNA target sequences (retinoic acid response element) in the promoter/enhancer regions of retinoic acid-responsive genes (Chambon, 1996). Retinoic acid (RA) is bound to two intracellular proteins, the cellular retinoic acid-binding proteins type I and II (CRABP-I and CRABP-II) that are highly conserved among several animal species (Li and Norris, 1996; Miano and Berk, 2000). CRABPs are believed to be involved in modulating intracellular RA concentrations (Ong et al., 1993). CRABP-I is expressed ubiquitously in the tissues of adult animals, whereas CRABP-II appears to be limited to the skin (Ong et al., 1993); uterus and ovary (Wardlaw et al., 1997; Zheng and Ong, 1998).

Adipocyte differentiation (adipogenesis) is a complicated process in which the expression of many adipocyte-specific genes is regulated by the complex interplay of several transcription factors and hormones (Cowherd et al., 1999). Adipose tissues have long been recognized as potential sites for RA action, and where retinoid receptors of two different subfamilies (RAR and RXR) are expressed (Haq and Chyluk, 1991; Kamei et al., 1993). The differentiation of 3T3-L1 preadipocytes was inhibited at an early stage by high RA doses (Kawada et al., 1990; Xue et al., 1996). The retinoids inhibited the adipogenesis induced by the PPARy ligand in primary culture of bovine stromal-vascular cells (Ohyama et al., 1998). However, RA behaves as a positive effector of adipocyte differentiation of both Ob1771 cells and rat...
preadipocytes when applied at physiological concentrations (0.1-10.0 nM) (Safonova et al., 1994). It was previously reported that low levels of serum vitamin A were associated with increased intramuscular fat deposition in Japanese black cattle (Oka et al., 1998). Although the genes for both CRABP isoforms from various species have been cloned and characterized, neither the exact functions of these proteins in the bovine nor the distinct roles of the two isoforms are completely understood. In addition, the mechanisms of RA action, especially the role of CRABPs in adipogenic differentiation, remain poorly understood in the bovine.

In this study, we have cloned the bovine CRABPs and determined CRABPs expression in normal tissues and regulation of CRABPs during adipogenesis using intramuscular fibroblast-like cells to evaluate the importance of CRABPs on adipocyte differentiation in the bovine.

MATERIALS AND METHODS

Cloning of bovine CRABP-I and -II genes by reverse transcriptase-PCR (RT-PCR)

Bovine (Bos taurus) tissues were collected from stockbred at the National Livestock Research Institute (Korea). Total RNA was extracted from the sirloin and adipose tissues using Trizol reagent™ (Gibco BRL Co., USA) according to the manufacturer's instructions. Reverse transcription was performed in a final concentration of 1×transcription buffer (50 mM Tris-HCl, 8 mM MgCl₂, 50 mM KCl, 2 mM of each dNTP, 10 mM dithiorthreitol), 10 pmol of oligo (dT)₁₇ primer, 5 μg of total RNA and 1 U of reverse transcriptase (Promega, USA). The reaction mixture was incubated at 42°C for 2 h and later used as a template in PCR. To clone bovine CRABP-II, 1 μl of synthesized cDNA from bovine sirloin tissue was added to a sterile 0.2-ml tube containing 5 μl of 10×PCR buffer, 4 μl of 10 mM dNTP mixture, 1 μl of forward primer (20 pmol/μl, TaKaRa, Korea), 1 μl of reverse primer (20 pmol/μl, TaKaRa, Korea) and 0.5 μl of EX Taq DNA polymerase (5 U/μl, TaKaRa, Japan) and the volume was adjusted to 50 μl with distilled water. PCR was performed with forward (5'-YGGYGCAC GACGATGGCAACCTTCTTGCA-3', TaKaRa, Korea) and reverse primers (5'-CGCCTTCTCCATCCGACCTGA GACCCCTTGCTGCA-3', TaKaRa, Korea) designed as degenerative primers using an amino-acid sequence specific for homologs of the human and mouse CRABP-II (GenBank accession no. M35522, and BC001109). The PCR conditions were as follows: 94°C for 30 s, 68°C for 30 s and 72°C for 1 min, repeated for 33 cycles. Moreover, bovine CRABP-I cDNA was amplified from adipose tissue using RT-PCR with a specific primer for bovine CRABP-I (forward, 5'-TGCCACATGCCCAACTT-3' and reverse, 5'-CGCCTTCTCCATCCGACCTGA GACCCCTTGCTGCA-3'). PCR conditions for CRABP-I were 94°C for 30 s, 50°C for 30 s and 72°C for 1 min. The amplified cDNA fragment was subcloned into a pGEM-T-easy vector (Promega, USA) for DNA sequencing determination.

Tissue specific expression of bovine CRABP-II mRNA

To determine the tissue expression of bovine CRABP-II mRNA, total RNA was extracted from heart, liver, lung, colon, small intestine, spleen, rib meat, sirloin and adipose tissues of a 12-month-old bull. First-strand cDNA was synthesized using reverse transcriptase-Superscript II (Gibco BRL, USA) with 5 μg of total RNA from each sample as template. The cDNA generated was used as a template in PCR with sense (5'-CGCGGCAGGACTATGCCTA AATCGTATTGATTTGCAAA-3') and antisense (5'-CGC TCACTTCCAGCTGATCCCTGTGCA-3') primers. A PCR reaction with total RNA was used as a negative control in the RT-PCR experiment (RT-). The PCR conditions involved initial denaturation at 94°C for 2 min, followed by 30 cycles of 94°C for 30 s, 68°C for 30 s, 72°C for 1 min and a final extension at 72°C for 15 min. Then 15 μl of each PCR product was size-fractionated using 1.2% (w/v) agarose gel electrophoresis.

Adipocyte differentiation of bovine intramuscular fibroblast-like cells

Isolation of primary intramuscular fibroblast-like cells was performed using a modification of the method described by Aso et al. (1995). Sirloin tissues were obtained from 12-month-old Korean cattle bulls by dissection. The sirloin tissues (0.1 mg/ml) were cut into pieces and suspended in Dulbecco's modified Eagle medium (DMEM, Gibco BRL, USA) containing 2 mg/ml collagenase (Sigma, USA). 10% fetal bovine serum (FBS; Hyclon, USA), 100 U/ml penicillin (Sigma, USA), 100 μg/ml streptomycin (Sigma, USA) and 0.1% bovine serum albumin (BSA; Sigma, USA). Tissue digestion was performed at 37°C for 90 min with gentle stirring. The digested tissues were filtered through a stainless steel mesh (40-μm pores) to remove any larger undigested tissues, including some mature adipocytes. The remaining filtrate (cell suspension) was centrifuged at 800 rpm for 5 min at 4°C. The supernatant was removed by aspiration. The collected cells were washed with culture medium (DMEM containing 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin). The intramuscular fibroblast-like cells were plated into 60-mm dishes (Falcon, USA) at a density of 1-10⁵ cells/ml in culture medium. The medium was changed every 4 days until the cells were used for experiments.

For adipocyte differentiation of the intramuscular
fibroblast-like cells, cells were plated into 60-mm dishes at a density of 4×10⁴ cells/ml in culture medium at 37°C under a 5% CO₂ atmosphere. Induction of 2-day post-confluent cells was carried out using differentiation medium (DMEM supplemented with 10% FBS, 33 mM bismuth (Sigma, USA), 200 mM ascorbic acid (Sigma, USA), 17 mM pantethein (Sigma, USA), 1 mM caprylic acid (Sigma, USA), 100 U/ml penicillin, 100 μg/ml streptomycin, 10 μg/ml insulin (bovine pancreas, Sigma, USA), 5 mM dexamethasone (Sigma, USA), 0.5 mM 1-methyl-3-isobutylxanthine (Sigma, USA), and 10 mM acetic acid (Sigma, USA)) for 18 days. The medium was changed every other day. During these periods, confluent cells underwent an adipose conversion. To identify the effects of RA on adipocyte differentiation of the bovine intramuscular fibroblast-like cells, the cells were induced by the addition of differentiation medium in the absence or presence of 1 mM and 1 μM all-trans RA. The differentiated cells were harvested for total RNA isolation and the cells were stained with Oil Red O at the times indicated to show triglyceride droplets. For the triglyceride assay, cultured cells on 60-mm dishes were washed twice with phosphate buffered saline (PBS), gently scraped into 0.4 ml of 25 mM Tris–HCl (pH 7.5) containing 1 mM EDTA, and then homogenized using an ultrasonic homogenizer (SMT, Japan). Triglycerides (TG) in cell lysates were quantified enzymatically using a Triglyceride G Test Kit (Wako, Japan). All experiments were performed in triplicate.

**Real-time PCR analysis**

Comparative real-time PCR was performed using a Roter Gene 2000 system (Corbett Research, Australia) and a Quantitect SYBR Green RT-PCR kit (Qiagen, Germany) according to the manufacturers' instructions. With the following primer pairs: CRABP-I sense, 5′-AAGGCTTTGAGGAGGAGACC-3′; CRABP-I antisense, 5′-TCAGGATGATTGCTGTTG-3′; CRABP-II sense, 5′-AACAGGAGGGGACACTTT-3′; CRABP-II antisense, 5′-CCCACTTACAGGCTCTTA-3′; 18S rRNA sense, 5′-CCCAGCGTTTTACTTGGAA-3′; and 18S rRNA antisense, 5′-CCCTCTTAATCATGGCCTCA-3′. Reactions were performed in triplicate and threshold cycle (Ct) values were normalized to 18S rRNA.

**RESULTS**

Cloning and characterization of bovine CRABP-II cDNA

A cDNA encoding a bovine CRABP-II was isolated from bovine sirloin tissue by RT-PCR using degenerative primers. Figure 1 shows the nucleotide and deduced amino acid sequences of bovine CRABP-II cDNA, which had an open reading frame of 414 bp corresponding to 138 amino acids. The deduced protein was approximately 15.7 kDa in size and similar to that of other retinoic acid-binding protein family members. The amino-acid sequence comparison presented in Figure 2A reveals an overall degree of identity between bovine CRABP-I and -II of approximately 75%. Alignment of the deduced bovine CRABP-II amino-acid sequence to CRABP-II sequences from human, mouse, and rat revealed approximately 97% identity to human CRABP-II, suggesting that the cDNA clone codes for bovine
CRABP-II. Bovine CRABP-II also showed approximately 93% identity to rodent (mouse and rat) CRABP-II (Figure 2B). These results represent the first reported cloning and sequencing of a bovine CRABP-II gene.

**Tissue-specific expression of bovine CRABP-II mRNA**

The mRNA expression levels of CRABP-II were undetectable by Northern blot analysis using various bovine tissues (data not shown). Thus, we next examined the expression pattern of bovine CRABP-II transcript in various bovine tissues by RT-PCR (Figure 3). Bovine CRABP-II transcripts were expressed in a wide range of tissue, with the highest level in rib meat, relatively high levels in sirloin, spleen, and colon, and lower levels in adipose tissue, small intestine, lung, liver, and heart. A negative control (RT-) in adipose tissue and sirloin, lacking cDNA, did not generate any RT-PCR product. We found that the mRNA distribution of bovine CRABP-II was more extensive than that reported for mouse and human CRABP-II.

**Up-regulation of bovine CRABPs during adipocyte differentiation of intramuscular fibroblast-like cells**

To analyze the function of bovine CRABPs during adipocyte differentiation of intramuscular fibroblast-like cells, we isolated bovine intramuscular fibroblast-like cells from bovine sirloin tissues and differentiated to adipocytes by exposure to differentiation medium for 18 days. To identify the success of adipocyte differentiation, Oil Red O staining and TG assays were performed. The results indicated accumulation of lipid droplets, suggesting that fibroblast-like cells were induced to form adipocytes by day 18 (Figure 4A). Based on the TG assay results, a significant increase in TG levels was evident by 18 days, supporting the result that cells at 18 days were induced to become adipocytes (Figure 4B).
Figure 4. Adipocyte differentiation of bovine intramuscular fibroblast-like cell and expression of bovine CRABPs during adipogenesis. (A) Bovine intramuscular fibroblast-like cells were grown under adipogenesis conditions as described in Materials and Methods. The cells were fixed and stained with Oil Red O, visualized, and photographed. The images shown are representative of two experiments performed in duplicate. (B) Comparison of TG results between days 0 and 18. Values are the least squares mean±SE for three replicate cultures. (C) Real-time RT-PCR was performed to determine the relative steady-state levels of bovine CRABPs mRNA during adipogenesis. Data are presented as the mean±SE of three individual mRNA levels for each sample.

To determine the expression of bovine CRABPs mRNA during adipocyte differentiation, we performed real-time RT-PCR analysis using total RNA over the period day 0-day 18. Bovine CRABP-I transcripts were undetectable in undifferentiated intramuscular fibroblast-like cells, but were markedly induced during differentiation. In contrast, bovine CRABP-II transcripts were detected in undifferentiated intramuscular fibroblast-like cells and their levels on day 15 had been induced approximately seven-fold compared to day 0 (Figure 4C).

Effects of RA during adipocyte differentiation of bovine intramuscular fibroblast-like cells

To identify the effects of RA on adipocyte differentiation of bovine intramuscular fibroblast-like cells, cell differentiation was induced by the addition of differentiation medium in the absence or presence of 1 nM and 1 µM all-trans RA (Figure 5). Cell differentiation was assessed by staining with Oil Red O. Undifferentiated cells which were cultured for the same time periods and stained with Oil Red O are shown for comparison. Cells which were cultured in the differentiation medium lacking RA accumulated lipid droplets and adopted the morphological characteristics of adipocytes. When the cells were differentiated with 1 nM all-trans RA, a positive effect on the formation of lipid droplets was observed compared with cultures lacking RA. In contrast, when cultured in differentiation medium with 1 µM all-trans RA, the accumulation of lipid droplets was inhibited compared with cultures lacking RA and those with 1 nM RA.

To examine the correlation of CRAB expression and RA during adipocyte differentiation of bovine intramuscular fibroblast-like cells, we next determined the expression of CRABPs mRNA in these cells. The level of CRABP-I transcripts increased by more than two-fold after RA treatment at low or high concentrations (Figure 5C); however, the expression of CRABP-II mRNA decreased by more than four-fold compared with cultures lacking RA (Figure 5D).

DISCUSSION

In this study, we report for the first time the cloning and characterization of bovine full-length CRABP-II cDNA. Multiple sequence alignment revealed that the predicted amino-acid sequence was 97% identical to the previously identified human CRABP-II isoform and 93% identical to the published mouse and rat CRABP-II, suggesting that the cDNA clone codes for bovine CRABP-II (Giguere et al., 1990; Astrom et al., 1991; Bucco et al., 1995). The primary
Moreover, we cloned CRABP-II in adipose tissue, so perhaps CRABP-I is involved in the regulation of bovine adipogenesis. Adipose tissue is a known target for RA, and retinoid receptors belonging to the two different subfamilies (RAR and RXR) are expressed in rat adipose tissue and mouse 3T3-L1 adipose cells (Haq and Chytíl, 1991; Kamei et al., 1993). Furthermore, RA metabolism is also thought to be regulated by the CRABPs, which may be involved in the cellular transport of RA to RARs and RXRs (Buocco et al., 1995; Dong et al., 1999). These results suggest the possibility that CRABPs are expressed in adipose tissue and therefore may have an important function in adipose tissue.

We next determined the level of CRABP-I and -II mRNA during adipocyte differentiation of bovine intramuscular fibroblast-like cells. Bovine CRABP-I transcripts were undetectable in undifferentiated intramuscular fibroblast-like cells and were markedly induced during the entire period of differentiation. However, expression of bovine CRABP-II was induced in the late stage of differentiation. These differences may reflect the biological roles of these genes during adipogenesis. In mice, CRABP-I was induced during adipogenesis and the downstream targets of peroxisomal proliferator-activated receptor γ (PPARγ) in adipocyte differentiation (Okuno et al., 2002). The nuclear receptor PPARγ plays a central role in adipocyte differentiation, whereby it forms a DNA-binding complex with its heterodimeric partner RXRα that regulates the transcription of adipocyte-specific genes (Tontonoz et al., 1994). In addition, little is known about the CRABP-II mechanisms in adipogenesis. Based on the expression of RAR and RXR in adipocytes (Haq and Chytíl, 1991; Kamei et al., 1993), we suggest that CRABP-I and -II may control the intracellular concentration of RA during adipocyte differentiation of bovine intramuscular fibroblast-like cells.

To elucidate the mechanism of RA action during adipogenesis, we analyzed the effects of RA during adipocyte differentiation of bovine intramuscular fibroblast-like cells. A high concentration of RA during adipocyte differentiation of bovine intramuscular fibroblast-like cells inhibited adipogenesis, but a lower RA concentration induced adipogenesis. Our results are in agreement with previous investigations demonstrating that high RA concentrations (micromolar range) inhibit adipose conversion, whereas lower RA concentrations (nanomolar range) stimulate adipocyte differentiation in Ob1771 cells (Safoñova et al., 1994). However, terminal differentiation of human liposarcoma cells could be induced by ligands for PPARγ and RXR (Tontonoz et al., 1997) and it is notable that RA treatment induces early commitment of embryonic stem cells into the adipocyte lineage (Dani et al., 1997).

On the other hand, RA is known to be an effective inhibitor of adipocyte differentiation in mouse 3T3-L1, pig
preadipocytes, bovine stromal-vascular cells and intramuscular adipose tissue (Kamei et al. 1994; Suryawan and Hu, 1997; Ohyama et al., 1998; Lee et al. 2000). However, feeding low vitamin A diets to cattle increased intramuscular fat deposition and marbling (Oka et al. 1998; Gorocica-Buenfil et al., 2007a; 2007b, 2007c). Apparent discrepancies between our results and earlier studies may be due to differences of RA concentration used in the experiments. The reason for this difference is not known. However, it may be related to the concentration of intracellular RA.

CRABP-I and -II are thought to regulate the availability of RA to the receptors by either enhancing RA metabolism or sequestering RA in the cytoplasm, distant from the receptors (Napoli, 1996). However, the expression of CRABP-I and -II differs in various cells after RA treatment. The CRABP-I gene is up-regulated in P19 teratocarcinoma cells and J1 embryonic stem cells and down-regulated in the murine teratocarcinoma cell line (Means et al., 2000). In addition, CRABP-II transcripts are rapidly induced (within 2-6 h) in cultured human skin fibroblasts by RA (Astrom et al., 1992). These results indicate that expression of CRABP-I and -II is regulated in various cells by RA. CRABP-II gene is an RA-target gene and expression of CRABP-II gene by RA treatment is regulated with phosphorylation of RXRγ gene (Bastien et al., 2002; Bruck et al., 2005). In this study, our results showed that CRABP-I gene is up-regulated and CRABP-II gene is down-regulated during adipocyte differentiation by RA. However, when intramuscular fibroblast-like cells were differentiated into adipocytes by medium in which RA was not included, expression of bovine CRABP-II mRNA was up-regulated. The reason for this difference in CRABP-I and -II expression by RA is not known, but it may be related to the phosphorylation of RXRγ gene during adipocyte differentiation. These results represent the first report of CRABP expression during adipocyte differentiation of bovine intramuscular fibroblast-like cells by RA and it appears that CRABPs are differentially expressed during adipogenesis and modulated by RA concentrations.

In conclusion, we successfully cloned bovine CRABP-II and determined its expression patterns in various tissues and during adipocyte differentiation. These results, therefore, suggest that CRABPs are involved in adipogenesis, and it is conceivable that CRABPs are important modulators of the regulation of intracellular RA concentrations during adipogenesis. Further research is required to understand the capacities of retinoid-dependent pathways in the regulation of adipogenesis.

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