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Cloning and characterization of a novel zinc finger protein (rZFP96) in the rat corpus luteum

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Abstract

The corpus luteum (CL) is a temporary organ involved in the maintenance of pregnancy. In the course of its life-cycle, the CL undergoes two distinct and consecutive processes for its inevitable removal through apoptosis: functional and structural luteolysis. We isolated a gene encoding for a novel rat zinc finger protein (ZFP), named rat ZFP96 (rZFP96) from an ovarian lambda cDNA library. Sequence analysis revealed close sequence and structural similarity to mouse ZFP96 and human zinc finger protein 305 (ZNF305). Quantitative reverse transcription-polymerase chain reaction analysis revealed a positive correlation with the end of pregnancy, that is, the onset of structural luteolysis of the CL. Messenger RNA levels increased 3-fold ($P<0.01$) between days 13 and 22 of pregnancy and 8-fold ($P<0.01$) between day 13 of pregnancy and day 1 post-partum. In addition, we detected rZFP96 expression in mammary, placenta, heart, kidney and skeletal muscle. Sequence analysis predicted that rZFP96 has a high probability of localizing to the nuclear compartment. The presence of both a perfect consensus TGEKP linker sequence between zinc fingers 2 and 3 as well as several similar sequences between the other zinc fingers suggests physical interaction with DNA. Speculatively, rZFP96 may therefore function as a transcription factor, switching-off pro-survival genes and/or upregulating pro-apoptotic genes and thereby contributing to the demise of the CL.

Keywords: Zinc finger protein; rZFP96; Apoptosis; Corpus luteum; Structural luteolysis

1. Introduction

Many of the molecular components of the apoptotic pathways have been elucidated and show remarkable homology across species and cell types [1]. However, the fine regulators that trigger or inhibit apoptosis in response to physiological or pathological triggers are yet to be elucidated for many tissues and organs. The corpus luteum (CL) is a temporary organ that develops in the ovary and secretes progesterone in preparation for the conceptus and maintenance of pregnancy [2]. Once its function is no longer required, that is, through failure of fertilization or at the end of pregnancy (in rodents), it undergoes both functional and structural regression. During the rat pregnancy, the peak of progesterone production is day 16, following which the CL undergoes functional luteolysis [3]. However, cellular integrity is maintained and structural luteolysis commences after parturition [4]. In fact, the weight of the CL remains relatively constant until parturition [5], which occurs at day 23 of pregnancy in the rat. The initial reduction in weight is attributed to the death of luteal cells by apoptosis while the endothelial cells of the CL appear to be more resistant to apoptosis in the rat and the rabbit [4,6]. There is an accumulating body of evidence that implicates apoptosis as the underlying physiological process responsible for structural regression of the CL [6–12]. However, this process is limited, as the CL is not completely removed after its

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primary cycle, but regresses over several estrus cycles [13,14] and may be subject to growth and increase in blood flow due to pregnancy [4]. Furthermore, in a previous study on a rat inhibitor of apoptosis protein (rIAP), a known broad-spectrum inhibitor of the apoptotic cascade [15], we have shown that between 1 day before parturition and 1 day after the mRNA expression levels of rIAP3 in the rat CL decrease 2-fold. In contrast, 24 h in vivo treatment with gonadotropic releasing hormone (GnRH)-agonist resulted in widespread death of luteal cells of the CL and a 9-fold reduction in rIAP3 mRNA expression levels [16].

Zinc finger proteins (ZFPs) are one of the largest family of regulatory proteins in mammals and have been shown to have diverse functions: DNA-binding transcription factors [17], RNA binding proteins [18,19] and protein–protein interactions [20]. It is estimated that 1% of the human genome consists of genes encoding zinc finger motifs of the type cysteine2–histidine2 (C_2H_2). Zinc finger proteins may contain multiple copies of the zinc finger motif, which is a simple structure consisting of 25–30 amino acid residues including two β-pleated sheets in the amino-terminus half and an α-helix in the *carboxyl*-terminus half, held together at the base by a zinc atom. These motifs have been shown to fit in the major groove of DNA [17]. Herein, we present the sequence for a novel rat gene encoding a putative protein containing zinc finger motifs, called rat ZFP96 (rZFP96) that was cloned from the pregnant rat CL. In addition, we present mRNA expression data showing its positive correlation with regression of the rat CL.

2. Materials and methods

2.1. Animals and sample collection

Sexually mature (10 to 12 weeks old) female Wistar rats were used in this study. They were housed at 21 °C with 55% humidity in a 12 h light/12 h dark cycle. Their feed was autoclaved normal cubes and acidified water, both provided ad libitum. Rats were placed overnight for mating and the next morning, rats positive for spermatozoa in vaginal smears were designated day 1 of gestation. Litters were born on day 23 of pregnancy. In order to collect various tissues, rats were anaesthetized with and mixture of 3–5% halothane, 0.8 L/min nitrous oxide and 0.2 L/m oxygen. Tissues were immediately snap-frozen in liquid nitrogen and stored at −80 °C. All protocols were approved by The University of Western Australia Animal Experimentation Ethics Committee.

2.2. Full-length cloning of rZFP96

The full-length rZFP96 clone was isolated from a rat ovarian complimentary DNA (cDNA) library (Lambda *Zap*II/*Eco*RI library; Stratagene, La Jolla, CA, USA) at day 15 of pregnancy. The clone was coincidentally cloned while isolating rat inhibitor of apoptosis proteins [16]. Briefly, three rounds of screening were performed using a mixture of two probes generated with the polymerase chain reaction (PCR) employing digoxigenin-11-deoxy-uridine triphosphate (Roche Diagnostics, NSW, Australia) label (for details see [16]).

Automated sequencing was performed with the ABI PRISM BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, CA, USA) on the ABI 373 DNA Sequencer according to the manufacturer's protocol (for details see [16]). In addition to the standard M13F (−20) and M13R sequencing primers, the sequencing primers used in the forward direction were 5′-ACT TGG ACT ATA TCC TTA, 5′-GTA GAA GCT CTC TAT TCT G, 5′-CAG AGA CTT CAC TCG GGA and in the reverse direction were 5′-CAG GTA TTG AGA CTG GAA, 5′-GCC ACA AAA CAT TAC AAG.

2.3. Sequence analysis

The homology search was performed with BLAST v 2.1 [21]. Protein motif identification was performed with InterProScan [22] and zinc finger location was according to Pfam [23]. Sequencing primers were designed with Web Primer [24]. Sub-cellular localization was conducted with PSORT II [25]. Multiple sequence alignment was performed with CLUSTAL W [26].

2.4. RNA extraction

Total RNA was isolated from snap-frozen CL by homogenization and extraction with RNAzol B (Tel-Test Inc., Texas, USA) as described by [12,16]. RNA samples were resuspended in RNase-free water and stored at −80 °C until used. RNA quantitation and integrity were determined by spectrophotometry and agarose gel electrophoresis.

2.5. Reverse transcription (RT)-PCR

Complementary DNA was generated with 500 ng of Oligo $(dT)_{15}$ primer (Promega Corporation, VIC, Australia) and 200 units of SuperScript II RNase H^- Reverse Transcriptase (Gibco-BRL; VIC, Australia) from a total of 5 μg of RNA (for details, see [16]). For both the qualitative and quantitative RT-PCRs for rZFP96 and the ribosomal protein L19, a single PCR primer set was used, respectively. All reactions were performed in 20 μl volumes in thin-walled 200 μl tubes. The PCR primer set for the rZFP96 was based on the sequence isolated in this study (GenBank accession number AY129561): forward 5′-TTG CTA CCC ATC AGG AAA CC and reverse 5′-CCC ACC CTC AGC ACA TTT AT. They generated a predicted PCR product of 197 bp. Two μl of cDNA were amplified with 1.25 units of recombinant Platinum® *Taq* DNA Polymerase (Invitrogen, VIC, Australia) containing 1× *Taq* DNA polymerase buffer, 3.75 mM magnesium chloride ($MgCl_2$), 0.38 mM of each of the 4 deoxyribonucleic acids and 0.2 μM of each primer. The thermal-cycling program consisted of 94 °C for 5 m, followed by 35 cycles of 94 °C for 20 s (s), 52 °C for 20 s and 72 °C for 20 s. The RT-PCR primer set for the constitutively expressed L19 gene was based on previously published rat primers by Orly et al. [27]. The RT-PCR reaction component concentrations and conditions were as for the rZFP96 RT-PCR assay with the following differences: optimal $MgCl_2$ concentration was 3.0 mM and the annealing temperature was 56 °C, and produced a PCR product of 194 bp. The real-time quantitation of rZFP96 and L19 mRNAs was preformed on the RotorGene 3000 (Corbett Research, QLD, Australia) as described previously by Lareu et al. [16]. The same optimized reaction component concentrations and conditions were employed for the rZFP96 and L19 assays as in their qualitative RT-PCRs with the addition of SYBR Green I (Fisher Biotech, WA, Australia).

2.6. Statistical analysis

One-way analysis of variance (ANOVA) with least significant difference was used to determine statistical significance between treatment groups.

3. Results

3.1. Cloning and sequence analysis of rZFP96

Nucleotide sequencing for rZFP96 revealed a 2249 nucleotide cDNA clone (GenBank accession number AY129561). Sequence analysis revealed a single long open-reading frame which coded for a putative protein of 298 amino acids starting at nucleotide position 539 and ending at nucleotide position 1435 (Fig. 1). Further sequence analysis classified this clone as a novel rat ZFP, coding for 8 zinc finger motifs which span most of the sequence.