Molecular and Cellular Endocrinology 307 (2009) 118–124

Contents lists available at ScienceDirect

Molecular and Cellular Endocrinology

journal homepage: www.elsevier.com/locate/mce

Specificity protein-1 and -3 trans-activate the ovine placental lactogen gene promoter

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ARTICLE INFO

Article history:
Received 23 October 2008
Received in revised form 10 April 2009
Accepted 15 April 2009

Keywords:
Sheep
Placenta
OPL
Transcription
Sp1
Sp3
CEBP

ABSTRACT

The proximal promoter (−383/+16) of the ovine placental lactogen (oPL) gene provides trophoblast-specific expression *in vitro*. Footprint 6 (FP6; −319/−349) lies within this region, and transfection of two-base pair mutations across FP6 into BeWo cells identified potential binding sites for CCAAT-enhancer binding protein (CEBP) and specificity proteins (Sp). Transfection of CEBP dominant negative or over-expression constructs did not impact transactivation of the proximal promoter. However, Sp1 and Sp3 over-expression constructs increased ($p \leq 0.05$) transactivation. Additionally, Sp1 and Sp3 short-hairpin RNA constructs reduced ($p \leq 0.01$) transactivation of the proximal promoter. In EMSA supershift assays, Sp1 and Sp3 antibodies were able to inhibit migration of the complexes formed with nuclear extracts from BeWo cells and ovine chorionic binucleate cells (oBNC). Furthermore, Southwestern analysis of oBNC nuclear extracts identified a nuclear protein corresponding with Sp3, identified by Western analysis. In conclusion, these results indicate that Sp1 and Sp3 are capable of interacting with FP6 of the oPL gene proximal promoter and function to enhance its transactivation.

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1. Introduction

The placenta is a transitory organ, with the sole purpose of providing an optimal environment for the growth and development of the fetus. The endocrine functions of the placenta influence fetal and maternal tissues in order to re-direct the flow of nutrients towards the fetus. This is accomplished through the production of a plethora of hormones and growth factors, including the placental lactogens (PL). This hormone, a member of the growth hormone/prolactin gene family, is found across a wide range of species, including ruminants, primates, and rodents, and it is believed to alter maternal metabolism such that the fetal nutrient pool increases, providing sufficient reserves for fetal growth (Anthony et al., 1995).

Although, the human placenta is quite different from that of the sheep from a strict anatomical perspective, from a functional standpoint, they are quite similar. The multi-villous nature of the maternal-fetal vasculature in the sheep and human is more similar than either the sheep and rodent or the human and rodent (Steven, 1975). Thus, the sheep provides a useful model for investigating the hormones involved in the regulation and maintenance of fetal development. Functional placental insufficiency leads to intrauterine growth restriction, a disease affecting ≈8% of all pregnancies, which in severe cases requires early delivery of the fetus in order to avoid fetal mortality (Brar and Rutherford, 1988; Pollack and Divon, 1992). Intrauterine growth restriction is caused by a variety of factors, including aberrations in hormones and growth factors, decreased maternal nutrient intake and restricted nutrient flow to the fetus (Anthony et al., 2003). Placental lactogen is one hormone found to be decreased in growth restricted pregnancies, providing support for its role in maintaining or providing nutrients to the developing fetus throughout gestation (Regnault et al., 1999).

The transcriptional regulation of the sheep PL (oPL) gene has been studied through investigation of 4.5 kb of the 5′-flanking sequence, relative to the transcriptional start site (Liang et al., 1999). Trophoblast-specific activation of the reporter gene occurred with 1.1 kb of the 5′-flanking sequence and within this region 19 protein protected regions (footprints) were identified through DNase I digestion analysis (Liang et al., 1999). Maximal transcriptional activation in trophoblast-derived cell lines was found to lie within the proximal −383 bp of the oPL gene and six of the footprints are found within this region (Liang et al., 1999). The minimal promoter region from −124/+16 bp provides trophoblast-specific transactivation and includes an AP-2 element, two GATA elements and a Puro element (Liang et al., 1999; Limesand and Anthony, 2001; Limesand et al., 2004).

The region encompassing −383/−217 bp of the oPL promoter appears to enhance activation of the minimal promoter (Liang et al., 1999) to convey maximal trophoblast-specific transactivation. Within this region two footprints were identified, footprint 5

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doi:10.1016/j.mce.2009.04.006

Table 1
Primers used in generating two-base pair transversion mutations in FP6.

Mutation #	Forward primers	Reverse primers
MUT 1	cctgagtagg CT agacccctgg	ccaggggtct AG cctactcagg
MUT 2	tgagtaggga TC acccctggag	ctccaggggt GA tccctactca
MUT 3	agtagggaag TG ccctggagga	tcctccaggg CA cttccctact
MUT 4	tagggaagac GG ctggaggagg	cctcctccag CC gtcttcccta
MUT 5	gggaagaccc GA ggaggagggc	gccctcctcc TC gggtcttccc
MUT 6	gaagacccct CC aggagggcat	atgccctcct GG aggggtcttc
MUT 7	agacccctgg TC gagggcatgg	ccatgccctc GA ccaggggtct
MUT 8	acccctggag CT gggcatggca	tgccatgccc AG ctccaggggt
MUT 9	ccctggagga CC gcatggcaac	gttgccatgc GG tcctccaggg
MUT 10	ctggaggagg CG atggcaaccc	gggttgccat CG cctcctccag
MUT 11	ggaggagggc TA ggcaacccat	atgggttgcc TA gccctcctcc
MUT 12	aggagggcat CC caacccattc	gaatgggttg GG atgccctcct
MUT 13	gagggcatgg GT acccattcca	tggaatgggt AC ccatgccctc
MUT 14	gggcatggca TG ccattccagc	gctggaatgg CA tgccatgccc
MUT 15	gcatggcaac GG attccagcat	atgctggaat CC gttgccatgc
MUT 16	atggcaaccc TA tccagcattc	gaatgctgga TA gggttgccat

(−286/−246) and footprint 6 (−349/−319). Although, no previously defined *cis* elements were identified within these regions, a direct repeat of the GAGGAG sequence, located within these footprints, was found to be functional through mutation analysis (Liang et al., 1999). However, the *trans*-acting factors binding to FP6 have yet to be identified. Therefore, the objective of this research was to identify and analyze the transcription factors interacting with FP6 of the oPL proximal promoter.

2. Materials and methods

2.1. FP6 block mutation constructs

Block mutations encompassing the GAGGAG sequence of FP6 (−319/−349) in the oPL gene were generated using site directed mutagenesis as previously described by this laboratory (Liang et al., 1999; Limesand and Anthony, 2001). The following overlapping, synthetic oligonucleotides were used in dual PCR: FP6Δ1F, 5′-GCA GCG GCC GCC ATT CCA GCA TTC TTG-3′; FP6Δ1R, 5′-ATG CGG GCC GCT GCC CTC CTC CA-3′; FP6Δ2F, 5′-CTG GCG GCC GCC ATG GCA ACC CAT-3′; FP6Δ2R, 5′-ATG CGG GCC GCC AGG GGT CTT CCC T-3′; FP6Δ3F, 5′-GGA GCG GCC GCG GAG GAG GGC AT-3′; and FP6Δ3R, 5′-TCC GCG GCC GCT CCC TAC TCA GGG AT-3′. After digestion with restriction endonucleases *Kpn*I and *Hind*III (New England Biolabs, Inc., Beverly, MA), the mutated FP6 promoter products were ligated into pGL3 Basic vector (Promega, Madsion, WI). The FP6 block mutations Δ1, Δ2 and Δ3 were confirmed using Southern analysis and nucleotide sequencing. An alkaline-lysis procedure, followed by CsCl equilibrium centrifugation, was used to obtain covalently closed circular DNA (Liang et al., 1999; Limesand and Anthony, 2001).

2.2. Construction of two-base pair mutation constructs

Two-base pair transversion mutations, encompassing FP6 (−349/−318), were generated using dual PCR amplification. Each primer contained a two-base pair transversion mutation flanked by 10 base pairs on each side of the wild type (WT) −383 oPL promoter sequence (Liang et al., 1999). Sixteen forward and sixteen reverse primers containing the mutations were generated starting at −349 and ending at −318 (Table 1). PCR, using *Taq* DNA Polymerase, was employed using the linearized WT −383 pGL3 (Liang et al., 1999) plasmid as the template with a separate reaction for the forward and the reverse primers. The resulting products were then agarose gel purified, annealed together and used as the template, with the pGL3 Basic primers (RV3 and GL2), for the second PCR. After restriction endonuclease digestion (*Kpn*I and *Hind*III) the product was ligated into the pGL3 plasmid using a 1:3 molar ratio of plasmid to PCR insert. Competent DH5α cells were transformed with the ligation mixture and plasmid DNA was isolated from resulting colonies, digested and separated to confirm the size of the insert. The sequences were subsequently compared to the WT −383 pGL3 sequence using BLAST analysis to confirm that the mutation was correct. Once the mutations were correctly obtained the plasmids were amplified and isolated using a CsCl centrifugation gradient.

2.3. Cell culture and transient transfections

BeWo cells (a human choriocarcinoma cell line) were obtained from American Type Culture Collection (Rockville, MD) and maintained as previously described by Limesand et al. (2004). Cell passage was kept below 10 after thawing cells for the transient transfection analysis to ensure optimal results. Transient transfections were performed in 6-well dishes at a density of 0.2×10^6 cells/well. Cells were additionally treated for 48 h with 80 μM forskolin (Sigma Chemical Co., St. Louis, MO) in complete F12K medium (10% heat-inactivated fetal bovine serum (Gemini Bio-Products, Inc., Calabasas, CA), 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma Chemical Co., St. Louis, MO), 3 ml per well, to cause them to differentiate into syncytiotrophoblasts, producing a more homogenous population (Kudo and Boyd, 2002; Wice et al., 1990).

Transient transfections were performed using Polyfect (Qiagen, Valencia, CA) transfection reagent. All transfection experiments were repeated three times, with each experiment using separate plasmid DNA isolations, and three replicate wells/construct. For each reaction, 5 μg of pGL3 plasmid DNA (either mutated or WT −383 pGL3), 12 μl Polyfect, 0.25 μg of control β-galactosidase expression plasmid (RSV promoter and enhancer, ClonTech Laboratories, Inc., Palo Alto, CA) and F12K culture medium without serum or antibiotics in a total volume of 100 μl. The polycationic lipid/DNA complexes were allowed to form at room temperature for 15 min, at which time the cells were washed twice with 3 ml serum- and anitibiotic-free medium. After the 15 min incubation, 900 μl of complete F12K medium was added to the Polyfect-DNA mixture and added to the differentiated BeWo cells. The cells were incubated at 37 °C for 48 h and then harvested by lysing. Luciferase and β-galactosidase activities were measured using a Luciferase Assay System (Promega, Madison, WI) and a Galacto-Light Plus Assay kit (Applied Biosystems, Bedford, Massachusetts).

For transient co-transfections with the CEBP-α, -β, and -δ over-expression vectors (Liu et al., 2001), kindly provided by Dr. Norman Curthoys (Colorado State University), 5 μg of WT −383 pGL3 was added to the cells with either 5 μg of control plasmid (pGL2), CEBP-α, -β, or -δ plasmids. The dominant negative (DN) co-transfections were performed in the same manner, with the WT −383 pGL3 construct being added to the cells in addition to either control plasmid (pcDNA3.1), A-CEBP (Moitra et al., 1998), generously donated by Dr. Charles Vinson (National Cancer Institute, NIH), or DN- CEBP-α (Pabst et al., 2001), a kind gift from Dr. Daniel Tenen (Harvard Medical School).

For transient co-transfections using the Sp1 and Sp3 over-expression vectors, the Sp1 construct (Naar et al., 1998) was provided by Dr. Robert Tjian, (University of California, Berkeley), and the Sp3 construct (Kennett et al., 1997) was obtained from Dr. Jon Horowitz (North Carolina State University). Over-expression co-transfections were performed using WT −383 pGL3 with either the control plasmid (pcDNA3.1) or the Sp1 or Sp3 expression constructs. The co-transfections were also performed with a construct containing the FP6 (−319/−349; FP6/PRL) sequence in front of the minimal rat prolactin promoter construct (Duval et al., 1999) and the Sp1 or Sp3 expression plasmids. All DNA concentrations remained constant at 5 μg/reaction.

For Sp RNA interference (RNAi), Sp1 and Sp3 siRNA constructs were purchased from Panomics (Redwood City, CA). The concentration of DNA used in the RNAi experiments was 2.5 μg/reaction. The WT −383 pGL3 construct was co-transfected with either the pU6 + 27 control plasmid (the backbone vector for the siRNA plasmids), the Sp1 or the Sp3 siRNA plasmids.

All transfection experiments were repeated three times, with each experiment using separate plasmid DNA isolations, and three replicate wells/construct. The luciferase activity for each construct was normalized for transfection efficiency (β-galactosidase activity). The activity obtained for each construct was statistically compared to the control construct (WT −383 pGL3) by least square analysis of variance followed by paired Dunnett tests (Statistical Analysis Systems, Cary, NC). In this analysis, "construct" was the independent variable, blocked by "replicate experiment", and "normalized activity" was the dependent variable. Data are presented as the mean "normalized activity" ± SEM.

2.4. Nuclear protein isolation and separation

Mature ewes were bred at behavioral estrus (day 0) and at 100 days post coitus (dpc), fetal cotyledonary tissue was removed from the placenta and the chorionic binucleate cells (oBNC) were isolated as described previously by our laboratory (Liang et al., 1999; Limesand and Anthony, 2001). BeWo cells were expanded in culture and the nuclear protein isolated as described by Limesand et al. (2004). The nuclear protein from these cells was subsequently extracted using the procedure of Dignam et al. (1983).

2.5. Electrophoretic mobility supershift assays

Electrophoretic mobility shift assays (EMSA) were performed as previously described by this laboratory (Liang et al., 1999; Limesand et al., 2004). The synthetic oligonucleotide used for EMSA was FP6 (−345/−325) (5′- ACC CCT GGA GGA GGG CAT GGC -3′; sense strand represented). For supershift analysis, the antibodies (2 μl of rabbit polyclonal crude serum) to Sp1, Sp3 or CEBP-α (Active Motif, Carlsbad, CA) were added to the reaction mixture containing the nuclear protein (20 μg) and the buffer, and allowed to incubate overnight at 4 °C. Following pre-incubation with the antiserum, the unlabeled competitors (100-fold molar excess) were then added to the reaction mixture and incubated at room temperature for 5 min, after which the labeled oligonucleotides (10,000 cpm/fmol) were added and incubated at room temperature for an additional 20 min before electrophoresis. The nuclear protein mixture was electrophoresed through a 5% TBE polyacrylamide non-denaturing gel for 3 h. The gel was subsequently dried onto Whatman paper and exposed to X-ray film for 24 h at −80 °C.

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