Original

The P2 Promoter of the CREM Gene is Responsive to cAMP-PKA Signaling Pathway in Osteoblastic MC3T3-E1 Cells

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Abstract : The expression of inducible cAMP early repressor (ICER) has been demonstrated in cultured osteoblastic MC3T3-E1 cells, calvarial cultures and *in vivo*, mainly via the cAMP-PKA signaling pathway. To determine the molecular mechanism(s) for this action, a fragment of the CREM P2 promoter from -238 bp to +14 bp was linked to a luciferase reporter (CREMP2-Luc238). This region contains 2 clusters of cAMP-responsive element (CRE). The 5' cluster contains CRE1 and CRE2 while the 3' cluster contains CRE3 and CRE4 and the two clusters are separated by 12 bp. In order to study if any or all CREs are responsive to cAMP induction, osteoblastic MC3T3-E1 cells were stably transfected with constructs having serial deletions of each CRE or mutations in each cluster of the CREMP2-Luc construct. Agonists that stimulate the cAMP-PKA pathway induced CREMP2-Luc238 activity, and neither PKC nor Ca⁺⁺ pathways did. The deletion of CRE1 and CRE2 did not significantly abolish the cAMP inducibility of CREMP2-Luc238 activity. Electrophoretic mobility shift assay showed many DNA/protein complexes when probes from either CRE1-2 or CRE3-4 were incubated with FSK-treated nuclear extract from MC3T3-E1 cells. However, only a small portion of the complexes was competed by a consensus CRE oligonucleotide. Interestingly, a major DNA/protein complex seen in the binding of CRE3-4 was not competed by the consensus CRE oligonucleotide, but by a mutated CRE oligonucleotide suggesting that other transcription factor(s) may be involved in the binding of CRE3-4. The CRE binding DNA/protein complexes were supershifted mainly by antibodies against ICER and CREB, mildly by C/EBP β suggesting these transcription factors may be involved in the FSK induction of the P2 promoter of the CREM gene.

Key words: CREM, CRE, Transfection, EMSA, cAMP

Introduction

The CREM gene, cAMP responsive element modulator, encodes multiple isoforms of transcription factors depending on the promoters driving the expression. The inhibitory isoforms, including α , β and γ contain phosphorylation P-box region, DNA and dimerization domains. On the other hand, the activators, the δ isoforms, contain additional glutamine-rich transactivation domain. All these isoforms of the CREM gene are transcribed from an upstream GC-rich promoter (P1) and constitutively expressed in a tissue specific and developmentally-regulated pattern¹⁻³⁾. The hallmark of CREM biology is the switchover from inhibitory to stimulatory isoforms in the testis upon the onset of spermatogenesis, a process that is due to a change in tissue specific alternative splicing and directed by follicle stimulating hormone. It is not surprising, therefore, that CREM male knockout mice

completely lack mature sperm and are sterile.

Another isoforms have been isolated recently, $\theta 1$ and $\theta 2$, which are inducible by cAMP signaling pathway and are transcribed from an intronic promoters, the P3 or P4 promoters, respectively³⁾. The functions of CREM $\theta 1$ and $\theta 2$ have been demonstrated to be stimulatory. On the other hand, the inducible cAMP early repressor (ICER), encodes a 15 kDa protein and is a cAMP inducible member of the CREM transcription factor superfamily²⁾. It is transcribed from another alternative intronic promoter, the P2 promoter. Therefore, ICER transcripts only contain basic region for DNA binding and leucine-zipper dimerization domain. ICER was originally discovered in pituitary gland and was studied on different *in vitro* system. Most of these studies showed that ICER delivers different functions in different system. For example, in neuroendocrine tumor cells, ICER is anti-proliferative⁴⁾, while it is anti-apoptotic in T cells^{5,6)}. In our laboratory, we have reported that ICER mRNA and protein are induced by PTH in cultured osteoblastic cells and mouse calvarial cultures and *in*

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Figure 1 Schematic diagram of the CREM P2 promoter-luciferase construct

vivo mainly via a cAMP-PKA signaling pathway ^{7,8}). Since PTH shows both anabolic and catabolic effects on bone depending on the mode of administration, which requires regulation of gene expression mainly through cAMP-PKA signaling pathway, it becomes very important to study the regulation mechanism of ICER by PTH to understand the mechanisms involved in PTH functions.

The first aim of this study was to demonstrate the signaling pathway involved in the transcriptional regulation of ICER by PTH. The second aim was to characterize the responsive elements and transcription factors involved in the cAMP-dependent transcription of the CREM P2 promoter.

Materials and Methods

Reagents

PTH(1-34) was purchased from Sigma Chemical Inc, and was diluted in a vehicle containing bovine serum albumin in a slightly acidic condition. Forskolin (FSK), phorbol myristate acid (PMA) and ionomycin(iono) were obtained from Biomol Co, and were constituted with absolute ethanol. 8-bromo-cAMP (8-Br-cAMP) was purchased from Sigma Chemicals Inc, and was constituted with culture medium. H89 was from Biomol and was diluted in absolute ethanol.

Constructs

A fragment of the CREM P2 promoter was kindly provided by Dr. C. Molina (UNJMD, Newark, NJ) which is from -238 bp to +14 bp. The promoter fragment was cloned into a pGL3 vector

containing a luciferase reporter (CREMP2-Luc238). Within the promoter fragment, there are 4 CRE-like binding sites positioned in 2 clusters with each CRE within the cluster spaced by 3 bp (Figure 1).

To generate promoter constructs with 5' serial deletion, site-specific primers were synthesized and the deletion constructs were generated by PCR. The PCR products were eventually cloned to the pGL3 vector containing the luciferase reporter. CREMP2-Luc139 lacks the most 5' CRE (CRE1), while CREMP2-Luc129 had additional removal of CRE2. CRE1 to CRE3 were removed in CREMP2-Luc107 leaving only an intact CRE4. Constructs with serial deletion were all sequence confirmed (Figure 1).

Mutagenesis of each CRE cluster was performed with a site-specific mutagenesis kit (Invitrogen) following the manufacturer's instruction. In brief, oligonucleotides containing specific mutations were synthesized and PCR was performed using CREMP2-Luc238 circular plasmid as a template. The PCR products were digested with Dpn I and the constructs were purified with Qiagen plasmid preparation (Qiagen, Inc., Valencia, CA, USA). CREMP2-Luc CRE1-2 and CREMP2-Luc CRE3-4 were obtained representing mutations at CRE1-2 and CRE3-4, respectively (Figure 1). Both constructs were sequenced to verify the containing of desired mutations.

Stable transfection

All constructs were stably transfected into osteoblastic MC3T3-E1 cells ⁹). In brief, MC3T3-E1 cells were plated at 200,000 cells/well in a 35-mm plate overnight. Stable transfection was carried

Figure 2 Time course of PTH and FSK treatment

out by using lipoFECTAMINE reagent (Invitrogen). After 6 weeks selection with G418, two separate populations were obtained with each population containing multiple clones of transfectants.

Luciferase assay

Stably transfected cells were plated at 5000 cells/well in a 35-mm plate in DMEM supplemented with 10% heat inactivated fetal bovine serum, 1000U/ml penicillin, 500U/ml streptomycin, and 1:1000 G418. Confluent cells were treated with 10nM PTH(1-34) or signaling molecules. The induced CREMP2 expression was measured as luciferase activity normalized to total protein content estimated by the indicator bicinchoninic acid¹⁰. The assay was performed as triplicate samples for each treatment group and all constructs were studied at least 4 times.

Nuclear extract preparation

Nuclear extracts were prepared as previously described¹¹ with some modifications. Briefly, MC3T3-E1 cells were treated with FSK for up to 2 h, washed with cold PBS, scraped and resuspended in 2 ml cold PBS. Cells were pelleted by centrifugation at 500 X g at 4°C and resuspended in buffer A (10 mM HEPES-KOH, pH 7.9, 0.75 mM spermidine, 0.15 mM spermine, 0.1 mM EDTA, 10 mM KCl and freshly added 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 1 µg/ml leupeptin and 1 µg/ml pepstatin). Cells were re-pelleted, resuspended in buffer A and homogenized. Homogenates were centrifuged for 10 min at 16000 X g at 4°C and pellets were resuspended in 0.5 ml of buffer B (20 mM HEPES-KOH, pH 7.9, 20% v/v glycerol, 0.42 mM NaCl, 0.75 mM spermidine, 0.15 mM spermine, 0.2 mM EDTA and freshly added 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 1 µg/ml leupeptin and 1 µg/ml pepstatin) and homogenized. Homogenates were left at 4°C for 30 min and centrifuged for 30 min at 25,000 X g at 4°C. Supernatants were collected, dialyzed for 6 h against 250 ml of buffer C containing 20 mM HEPES-KOH pH 7.9, 20% glycerol (v/v), 0.1 mM NaCl, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.5

mM phenylmethylsulfonyl fluoride, 1 µg/ml leupeptin and 1 µg/ml pepstatin. Nuclear proteins were quantified by BCA assay¹⁰ and stored at -70°C.

Electrophoretic mobility shift assay

Ten picomole of each complementary deoxyoligonucleotides were separately incubated with 5X kinase forward reaction buffer, [γ-³²P]ATP (3000 Ci/mmol, New England Nuclear Research Products, DuPont Co, Wilmington, DC) and T₄ DNA kinase (GIBCO, Grand Island, NY) for 1 h at 37°C. The two reactions were mixed, boiled, and left at room temperature to allow proper annealing. The resulting double-stranded oligonucleotide probes were extracted with phenol/chloroform, and precipitated with ethanol in the presence of 2.5 M ammonium acetate. After pelleting, the probes were dissolved in 50 ml H₂O. Nuclear extract or purified CREB-bZip DNA binding domain (Santa Cruz Biotechnology, Santa Cruz, CA) was used in a 20-ml binding reaction in the presence of 20 mM HEPES pH 7.9, 50 mM KCl, 6.25 mM MgCl₂, 0.5 mM EDTA, 10 % glycerol¹², 1mM DTT and 0.1 mg/ml poly(dI-dC)·poly(dI-dC) (Pharmacia Biotech, Piscataway, NJ), and 10⁵cpm deoxyoligonucleotide probes at room temperature for 30 min. In the competition experiments, the CRE and C/EBP consensus oligonucleotides as well as a mutated CRE oligonucleotide (Santa Cruz Biotechnology, Santa Cruz, CA) were incubated with nuclear extract for 15 min followed by the addition of radiolabeled probes for 15 min. In the supershift experiments, probe and nuclear extract were incubated at room temperature for 30 min followed by the addition of specific antibodies against various transcription factors. All antibodies were obtained from Santa Cruz Biotechnology, Santa Cruz, CA. The reactions were fractionated on a 5% polyacrylamide gel electrophoresis in 0.5 X TBE buffer for 90 min at 150 V. Gels were placed on a piece of dry 3 M blotting paper, fixed in 10% acetic acid/10% methanol for 30 min, dried at 60°C for 75 min, and exposed to radiographic films at room temperature.

Results

CREMP2-Luc238 was induced by PTH mainly through cAMP-PKA signaling pathway

To determine if PTH induces CREMP2-Luc238 expression in a time-dependent manner, MC3T3-E1 cells stably transfected with CREMP2-Luc238 were grown to confluence and treated with PTH (10nM) or FSK (10µM) for up to 8 h in the presence of heat-inactivated fetal bovine serum. CREMP2-Luc238 expression was measured as luciferase activity, which was normalized to total protein content. CREMP2-Luc238 activity was highly induced by PTH or FSK in a time-dependent manner, which peaked at 4 h. Thus, CREMP2-Luc238 was treated with signaling effectors in all experiments for 4 h (Figure 2).

To determine the signaling pathways involved in the induction of CREMP2-Luc238 expression, cells stably transfected with

Figure 3A The activity of the CREMP2-Luc238 by various signaling molecules at 4-h treatment

Figure 3B The activity of CREMP2-Luc238 by either PTH(1-34) or PTH(3-34)

Figure 4 The cAMP inducibility of CREMP2-Luc238 by H89 pretreatment

Figure 5 The cAMP-inducibility of various CREMP2-Luc constructs

CREMP2-Luc238 were treated with various pharmacological effectors for 4 h and the promoter activity was measured as luciferase activity. CREMP2-Luc238 expression was significantly induced by 10nM PTH, 10μM FSK, and 3mM 8-bromo-cAMP (8-Br) to 205±35%, 494±68% and 369±81% of control, respectively, whereas 100nM PMA, 1μM ionomycin (Iono) (Figure 3A) and 100nM PTH(3-34) did not (Figure 3B).

To further demonstrate that CREMP2-Luc238 activity was induced by PTH mainly via the cAMP-PKA signaling pathway, cells stably transfected with CREMP2-Luc238 were pre-treated with 30μM H89, a specific PKA inhibitor, and 1 h before subsequent treatment with PTH for 4 h. The results showed that PTH inducibility of the CREMP2-Luc238 was significantly abolished by H89 pretreatment (Figure 4, H89/PTH).

The data described above suggested that the PTH inducibility of the CREMP2-Luc238 was mediated mainly via the cAMP-PKA signaling pathway. In order to determine the involvement of each CRE-like responsive element in the PTH induction, deletions and mutations of the CREM P2 promoter fragment were

generated as described in the Materials and Methods. All constructs were stably transfected into MC3T3-E1 cells. After induction with 10μM FSK, deletion of the CRE1 (CREMP2-Luc139) did not reduce but significantly enhanced cAMP induction of the promoter activity (Figure 5). Further deletion of the CRE2 (CREMP2-Luc129) did not show any alteration in the inducibility of the FSK compared to CREMP2-Luc238 (Figure 5). However, additional removal of the CRE3 (CREMP2-Luc107) completely reduced the FSK inducibility (Figure 5). Mutations at CRE1/2 (CREMP2-Luc238 CRE1/2) did not alter significantly the FSK inducibility of the promoter activity. However, mutations at CRE3/4 (CREMP2-Luc238 CRE3/4) significantly reduced FSK induction of the promoter activity (Figure 5). The data suggested that CRE3/4 plays a more responsive role in cAMP induction of the CREMP2-Luc238 activity.

The EMSA of the nuclear extracts from FSM-induced MC3T3-E1 cell formed at least 4 DNA/protein complexes with radiolabeled CRE1/2 probe (Figure 6). Complex I was competed by a

Figure 6 EMSA with various oligo fragments

consensus CRE oligonucleotide (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and not by either a mutated CRE or C/EBP oligonucleotides (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). None of the remaining DNA/protein complexes (complexes II, III and IV) were competed by these and other consensus oligonucleotides, such as AP-1 and NF- κ B (data not shown). Similarly, the FSK-induced nuclear extract of MC3T3-E1 cells formed at least 5 different DNA/protein complexes with radiolabeled CRE3/4 and only complex V was competed by the consensus CRE oligonucleotide. Although the DNA/protein binding patterns were similar using CRE1/2 and CRE3/4 probes, CRE3/4 formed additional complexes with the nuclear extracts as shown in Figure 6, complexes IIIIV and IX. The intensity of complex IX suggested that there was a significant protein binding with the CRE3/4 and it was competed by the mutated CRE oligonucleotide (Figure 6).

Discussion

This minimal fragment of the CREM P2 promoter contains only 2 clusters of CREs. The results of this study were in agreement with others^{2,7)} that this promoter does not respond to other stimuli, including PKC and Ca^{++} ionophore, but only to cAMP-PKA signaling pathway. Therefore, this promoter fragment could be a good model to study cAMP-PKA pathway.

The responsiveness of the CREs in the P2 promoter of the CREM gene has been briefly studied²⁾. In that study, CRE1-2 and CRE3-4 are separately cloned upstream of the thymidine kinase minimal promoter in pBLCAT2 linked to a CAT reporter and show strong inducibility by cAMP, with CRE3-4 showing more responsiveness than CRE1-2. Our results were in agreement with their report that the CREM P2 promoter fragment was exclusively responsive to cAMP-PKA signaling pathway, in

addition that CRE1-2 and CRE3-4 are both responsive to cAMP induction. Our study further characterized the involvement of each CRE in cAMP inducibility by serial deletion of the P2 promoter fragment and mutations of either CRE cluster. The data showed that CRE3 was the most significant responsive element in mediating cAMP induction of the CREMP2-Luc activity since deletion of CRE1-2 (CREMP2-Luc129) did not lose any cAMP inducibility while additional removal of CRE3 (CREMP2-Luc107) significantly abolished the promoter activity, both baseline as well as cAMP inducibility. Interesting to note was that when only CRE1 was deleted, cAMP inducibility was significantly increased suggesting that one or more transcription suppressors may be involved in the binding of CRE1 or the region between -238 bp and -139 bp. Brief computer search of this region did not reveal any consensus DNA sequences for known transcription factors. EMSA using different probes, CRE1-2 and CRE3-4, along with FSK-treated nuclear extract demonstrated different DNA/protein binding patterns suggesting that there may be different groups of transcription factors involved in the binding of CRE1-2 and CRE3-4. CRE3-4 probe always generated a more intense binding pattern which may reflect that CRE3-4 plays a more direct effect on the response of the CREM P2 promoter activity. Many DNA/protein complexes were observed and only 2 of them were competed by a consensus CRE oligonucleotide. Interestingly, a major DNA/protein complex (complex IX in Figure 6) was observed mainly with the CRE3-4 probe, and was competed by a commercially available mutated CRE oligonucleotide (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). The EMSA analysis was repeated at least 4 times using different preparations of nuclear extracts from FSK-treated MC3T3-E1 cells with similar results. Preliminary searching the transcription factors involved in the binding of the mutant CRE oligonucleotide did not yield any

results. This suggested that an unidentified transcription factor is involved in the binding of CRE3-4 which, based on the intensity of the complex, may play a major role in the expression of the CREMP2-Luc activity. Further study to characterize the protein is necessary.

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