Characterization of Human Icer Promoter and Comparative Studies of Its Activity in Human Prostate Cancer Cells

Dipika Bhagvanji Patel

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CHARACTERIZATION OF HUMAN ICER PROMOTER AND COMPARATIVE
STUDIES OF ITS ACTIVITY IN HUMAN PROSTATE CANCER CELLS

A THESIS

Submitted in partial fulfillment of the requirements
For the degree of M.S. Molecular Biology

by

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Montclair State University
Montclair, NJ
2012
The Inducible cAMP Early Repressor (ICER) is a dominant transcriptional repressor that binds to cAMP responsive elements (CRE) located in the promoter of a target gene to repress cAMP-mediated gene transcription. The promoter of ICER contains four regulatory CRE sequences, termed cAMP autoregulatory response elements (CAREs) that are highly inducible by cAMP signaling. In a cell, cAMP pathway induces the expression of ICER, however, when ICER protein level increases above normal level, it can repress its own production by binding to these CARE sites located in its promoter region. ICER is normally present in neuroendocrine cells, however, it is not present in tumor cells. In addition, the growths of these tumor cells have been known to be hampered when ICER was reintroduced. Therefore, it is hypothesized that ICER, putative tumor suppressor, manipulation could potentially be used as a new treatment for cancers. Until now, the activity of ICER promoter has been studied in organisms such as rat and mouse; however, human form of ICER promoter has not been characterized yet. Thus, the goal of this research was to characterize the human ICER promoter and to do the comparative studies of the promoter activity in human prostate cancer cells, LNCaP. In order to achieve our goal, the human ICER promoter, composed of four putative CAREs, was analyzed in vitro as well as in LNCaP cells. Our research show that human ICER promoter is composed of three CAREs, namely CARE1, CARE3 and CARE4. Result of our in vivo analysis show that the hICER promoter activity is inducible by cAMP/PKA pathway and repressible by ICER in LNCaP cells. We also show that the induction and repression of ICER promoter activity was specifically due to the presence
of three CAREs in hICER promoter. Comparative studies of hICER promoter activity show that CARE1, CARE3 and CARE4 function in a cooperative manner to regulate ICER gene expression in a very fine tune manner.
ACKNOWLEDGEMENT

Conducting research is my passion and my time at Montclair University as a graduate student has been an unforgettable experience. I was able to successfully complete my thesis project and contribute my finding to science. All this would not have been possible without the GOD’s blessing and the help and contributions of numerous people along the way. It my pleasure to express my deepest gratitude to people who have made this research possible.

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INTRODUCTION

Inducible cAMP Early Repressor, an Isoform of CREM Gene

Cyclic adenosine monophosphate (cAMP) signaling pathway plays a key role in regulation of numerous physiological processes in most organisms. The second messenger cAMP regulates the gene expression mediated by activation of protein kinase A (PKA) and in turn activation of large family of nuclear transcription factors. Two of the well-studied transcription factors of this kind are the cAMP responsive element (CRE) binding protein (CREB) and cAMP responsive element modulator (CREM) (Reviewed in Montminy, 1997; Masquilier et al., 1993; Mayr and Montminy, 2001). Particularly, CREM, a multiexonic gene, is of a greater interest to us since it encodes a variety of activators and antagonists of cAMP-inducible transcriptions. Through alternative splicing, CREM gene encodes multiple activators, such as CREMt and antagonists such as CREM-α, -β, and -γ, of cAMP-induced transcriptions. Nevertheless, these activators and repressors are ubiquitously found in most tissues and are mainly regulated by phosphorylation. In addition, none of these isoforms of CREM are known to be inducible by cAMP (Masquilier et al., 1993; Foulkes and Sassone-corsi, 1992; Mayr and Montminy, 2001).

A novel and unique inducible isoform of the CREM gene, termed Inducible cAMP Early Repressor (ICER), was discovered by Molina et al. in the year 1993. ICER expression is tissue specific and it has been found to be predominantly expressed in neuroendocrine tissues such as adrenal and pituitary glands (Molina et al., 1993; Stehle et al., 1993). Since its discovery, ICER has been found to play an important role in a wide range of physiological processes. The role of ICER has been associated with regulation
of circadian rhythms in pineal gland (Stehle et al., 1993). The expression of ICER has been also documented to play a vital role in ovulation and reproduction in female (Mukherjee, 1998; Muniz et al., 2006). Furthermore, studies show the association of ICER with regulation of neuronal plasticity, learning, memory and programmed cell death (Borlikova and Endo, 2009; Mioduszewska et al., 2003). Importantly, expression of ICER is known to play a pivotal role in human choriocarcinoma cells (Razavi et al., 1998), human prostate cancers (Yehia et al., 2001; Memin et al., 2002), and in leukemia (Pigazzi et al., 2008).

Transcriptional and Structural Organization of ICER

ICER is a small transcription factor that belongs to CREB/CREM/ATF (activator transcription factors) family of transcription factors. It is transcribed from an internal promoter, P2, located at 3’ portion of the CREM gene. Unlike other CREM isoforms (e.g. CREM-α, -β, -γ and -τ) that are transcribed from the major P1 promoter of the CREM gene, ICER is unique in a sense that it is highly inducible by cAMP signaling. In addition, ICER also lacks N-terminal domains, specifically the P box domain known for phosphorylation sites, that is found in all other CRE-binding factors and CREM isoforms described (de Groot et al., 1993). Therefore, activity of ICER is predominantly determined by the intracellular concentration rather than the degree of phosphorylation (Molina et al., 1993; Stehle et al., 1993).

ICER essentially consists of a DNA binding domain (DBD) and the presence (or absence) of a gamma domain. There are four isoforms of ICER resulting from an alternative splicing, namely ICER I, ICER Iγ, ICER II and ICER IIγ. ICER I and ICER II
utilize and differ only by a DBD (DBD I or DBD II), whereas ICER Iγ and ICER IIγ also lacks the gamma domain (Molina et al., 1993). ICER transcript encodes small proteins of about 108 amino acids in absence of γ exon or 120 amino acids in presence of γ exon. Furthermore, ICER belongs to the family of basic leucine zipper transcription factors and hence contains highly conserved DBD composed of basic and leucine zipper (bZIP) domains. Therefore, ICER can form homodimers with another ICER molecule or heterodimers with other family members, including the CREB, CREM, and AFT-1. After dimerization, ICER binds to CRE sequences in the promoter of target genes to represses the gene expressions. Interestingly, ICER is a transcription factor that lacks a transactivation domain and consists primarily of only a DBD, thus, ICER has been characterized as a dominant transcriptional repressor of a cAMP-mediated gene expression (Schumacher et al., 2000; Masquilier et al., 1993; Laoide et al., 1993; Molina et al., 1993).

The Promoter of ICER Consisting of cAMP Autoregulatory Elements

An exclusive feature of ICER and CREB/CREM/AFT family of transcription factors is their ability to bind to canonical consensus CRE (TGACGTCA) or CRE-like elements. These nuclear factors regulate gene expression via CREs located in the promoter of a target gene (Zhang et al, 2005; Mayr and Montminy, 2001; Molina et al., 1993). Interestingly, the promoter of ICER contains a cluster of four CRE like sequences, termed cAMP autoregulatory elements (CAREs) arranged in tandems. ICER gene including these CAREs, namely CARE1, CARE2, CARE3 and CARE4 is highly conserved among various organisms. In mouse, where CAREs were first described,
CARE3 and CARE4 are highly similar to consensus palindromic CRE (TGACGTCA) sequence; whereas, CARE1 and CARE2 are about 60 percent similar to the consensus CRE (Molina et al., 1993, Mao et al., 1998). CARE1 and CARE2 are separated by only 3 base pairs (bp) and likewise for CARE3 and CARE4. In addition, CARE1-CARE2 and CARE3-CARE4 segments are separated by 12 bp (Molina et al., 1993). Similar to most CREs, these CAREs are highly inducible by cAMP signaling and are also recognized by various CRE binding proteins, including ICER. Hence CAREs play a pivotal role in ICER transcriptional regulation in response to cAMP signaling (Molina et al., 1993; Stehle et al., 1993).

ICER in Regulating Gene Expressions, Including Autoregulation, in Response to cAMP/PKA mediated pathway.

In a mammalian cell, when a ligand such as hormone or growth factor binds to transmembrane receptors, it stimulates adenylyl cyclase enzyme via the interactions with G-protein. Adenylyl cyclase then converts ATP to cAMP, which in turn activates the protein kinase A. Upon activation, the catalytic subunits of PKA disassociate from the regulatory subunits and translocate into the nucleus where it phosphorylates various transcription factors, including CREB. This results in stimulation of transcriptional activators binding to the CREs and in turn in induction of transcription from the promoter of cAMP responsive genes (Reviewed in Zhang et al, 2005; Mayr and Montminy, 2001; Molina et al., 1993). Since ICER contains four CRE like elements, CAREs, ICER gene transcription is also induced in response to the increase in cAMP. Therefore, cAMP
signaling pathway ultimately results in induction of ICER due to the presence of CAREs in the promoter region (Molina et al., 1993; Stehle et al., 1993).

Once ICER is transcribed and translated, it acts as a dominant transcriptional repressor and binds to CREs in promoter of target genes. Consequently, this phenomenon results in repression of transcription of cAMP responsive genes. However, when ICER protein accumulates in the cells above normal level, it can repress and control its own production by binding to four CAREs located in its own promoter region. This fascinating autoregulatory negative feedback loop cause repression of ICER transcription which ultimately leads to a new cycle of transcriptional induction by cAMP (Molina et al., 1993). In summary, due to CAREs located in the promoter of ICER gene, ICER is highly inducible by cAMP signaling, but ultimately also leads to inhibition of its own transcription. Therefore, in this research, we focus on characterizing the ICER promoter, specifically the region containing the four autoregulatory elements, CARE1-CARE4.

*Role of ICER in Prostate Cancers*

In humans, prostate is a small gland that secretes fluid that nourishes and protects sperms; hence, it is an essential part of a male’s reproductive system (reviewed in Abate-Shen and Shen, 2000). According to the cancer statistic of 2010, prostate cancer is the second leading cause of cancer related mortality among men in the United States. It is estimated that about 1 in every 6 male will develop prostate cancer at some point in their lifetime (Jemal et al., 2010). Among many available common cancer treatments such as radiation therapy, chemotherapy and surgery, the androgen deprivation therapies are
commonly used for advanced stages of prostate cancers. Unfortunately, many tumors eventually become androgen insensitive and ultimately result in more aggressive metastatic prostate cancers (Huggins and Hodges 1941; Abate-Shen and Shen 2000). Strikingly, abnormal ICER expression has been found in human prostate cancers compared to normal prostate tissues. ICER protein expression has been shown to be undetectable and uninducible in human prostate cancer cell line, LNCaP (Yehia et al., 2001). Importantly, research shows that growth of LNCaP cells was hampered when ICER was reintroduced into these tumor cells, strongly suggesting a possible role of ICER as a tumor suppressor in prostate cancers (Yehia et al., 2001). Research also shows that ICER reverses the tumorigenesis of highly metastatic and androgen-insensitive rat prostate cancer cell line AT6.3, suggesting manipulation of ICER could potentially be used as a treatment for metastatic and androgen-insensitive tumors (Memin et al., 2002). These evidences indicate that deregulation of ICER expression is associated to carcinogenesis of prostate tissues. Therefore, it is strongly believed that manipulation of ICER could potentially be used as a prostate cancer treatment and possibly a variety of other cancers.

The Goal of This Research

CAREs are vital elements of ICER promoter that regulate ICER expression by inducing transcription in response to cAMP signaling. They also play a central role in suppressing ICER expression in autoregulatory feedback loop. The promoter of ICER has been describe and studied well in mouse (Molina et al., 1993), however, the human form of ICER promoter has never been presented or characterized. Because of the
importance of CAREs in transcriptional regulation of ICER and consequently due to the importance of ICER expression in wide range of physiological processes, we have studied and characterized the human ICER promoter (hICER) primarily consisting of regulatory elements, CAREs. Based on the previous studies and knowledge of mouse ICER promoter, we hypothesized that the human ICER promoter is likely to be composed of four CAREs (CARE1-CARE4). Assuming hICER promoter contains these autoregulatory elements, we also speculated that hICER promoter activity is inducible and repressible by cAMP signaling pathway. In this study, we found that unlike mouse ICER promoter, human ICER promoter is composed of only three CAREs, namely CARE1, CARE3, and CARE4. Analysis of hICER promoter activity in vivo show that wild-type hICER promoter activity is inducible by cAMP signaling and repressible by ICER due to the presence of three CAREs. In this research, we also show that multiple CAREs of hICER promoter function in a cooperative manner in controlling ICER gene expression. Thus, presence of all three CAREs is necessary for maximum effect in controlling ICER gene expression.

MATERIALS & METHODS

In-Silico Analysis of ICER Promoter

A portion, 52bp, of human ICER promoter (Fig.1A) containing four putative CAREs was used to search for homologous sequences in other organism using basic local alignment search tool BLASTN (NCBI). Organisms showing high degree of
homology were selected and subsequently, the resulting sequences were compared and aligned using ClustalW2 (EMBL-EBI).

Electrophoretic Mobility Shift Assay (EMSA)

Electrophoretic mobility shift assay or gel shift assay was performed using Odyssey Infrared EMSA reagent kit from LI-COR Biosciences. The five prime of the wild-type and mutant CARE (supplementary table 1) sense strand DNA oligonucleotides were end-labeled with IRD®700 infrared dye (Integrated DNA Technologies, IA). Complementary strand of each CARE oligonucleotides were synthesized without end-labeled IRDye (Integrated DNA technologies, IA). Each oligonucleotide was reconstituted to 100μM in TE buffer (10mM Tris-HCl, 1mM EDTA; pH6.8). After that, IRD®700 end-labeled sense and non-labeled complementary oligonucleotides were annealed to form a double-stranded DNA fragment by first heating the oligonucleotides at 100°C for 3 minutes, then slowly cooling at room temperature for 3 hours. Annealed duplex oligonucleotides were diluted to 200nM (for single CAREs oligos) or 50nM (for multiple CAREs oligos) concentration. CARE oligonucleotides with or without bacterially purified ICERIIγ protein in binding reaction was performed using EMSA reagent kit components (LI-COR Biosciences, NE). Binding reaction mixture contained 2μL of 10X Binding Buffer (100 mM Tris, 500 mM KCl, 10 mM DTT; pH 7.5), 1μL of Poly dl.dC (1 μg/μl in 10 mM Tris, 1 mM EDTA; pH 7.5), 1μL of 100 mM MgCl2, 1μL of CARE oligonucleotide and ICER protein in concentrations of 1μg, 2μg, and 4μg. Water was added to bring total volume reaction to 20μL. Binding reaction was incubated at room temperature for 20 minutes and 2μL of 10X Orange Loading Dye ((LI-COR
Biosciences) was added prior to electrophoresis. The separation of protein-DNA complex from unbound oligonucleotides was done by loading the 20μL binding reaction in 5% TBE, 10-well, precast native polyacrylamide gel (BioRad, CA) and performing electrophoresis at 100V for 45 minutes at room temperature. Gel image was done using Odyssey® infrared imaging system (LI-COR Biosciences, NE).

**Development of Mutant CARE Constructs by Site-Directed Mutagenesis**

Various combination of mutant CARE hICER promoter constructs (Figure 5) were created by introducing mutations at each CARE site using QuikChange Lightning Site-Directed Mutagenesis kit (to introduce mutation at a single site) and QuickChange Lightning Mutli Site-Directed Mutagenesis Kit (to introduce mutations at multiple sites) (Stratagene, Agilent Technologies) by following manufacturer's recommended protocol. A pGL3 basic-0.5Kb hICER WT promoter construct containing 518bp of wild-type hICER promoter upstream of luciferase reporter gene of pGL3 basic vector (Promega) was then used as a template DNA for site-directed mutagenesis polymerase chain reaction (PCR). PCR primers containing TAGTGTATA sequence, in place of WT CARE, was used to introduce mutations at each of the putative wild-type CARE sites. PCR and cells transformations were performed according to the manufacturer's recommended protocol. After that, plasmid containing desired mutant CARE constructs were purified using the QIAprep spin miniprep kit (Qiagen) according to manufacturer’s protocol. Each mutant constructs were then sequenced (Genewiz, NJ) to confirm mutations. Furthermore, to achieve high concentrated mutant CAREs DNA constructs, we purified large volume of plasmid using Qiagen Plasmid Midi and Maxi Kit.
Transfection and Dual-Luciferase Reporter Assay

LNCaP cells (LNCaP clone FGC; CRL-1740) were obtained from American Type Culture Collection (ATCC). Cells were cultured in RPMI 1640,1X with L-glutamin & 25mM HEPES (Cellgro, Mediatech ) complete medium supplemented with 10% fetal bovine serum and 1% Penicillin & Streptomycin. DNA transfection was done using ESCORT™ transfection reagent (Sigma) and according to provided manufacturer’s protocol (scaled down for 12-well dishes).

For all DNA transfection experiments, a total of 2.5ng of DNA mixture containing 1.4μg of pGL3 basic-0.5Kb hICER promoter construct, 0.5μg of MT-CEV catalytic subunit PKA, 0.1μg of pCMV Renilla and 0.5μg of WT pSG ICERIIγ was prepared. When only hICER promoter construct alone or hICER promoter construct with only cPKA was tested, empty pGL3 basic vector was added to make a total of 2.5ng DNA mixture. Each experiment was done in triplicates. After DNA transfection, cells were incubated in 37°C (5% CO2) cell culture incubator for 12-16 hours. Following incubation, cell medium was removed and fresh medium was added. Cells were then incubated for additional 35-40 hours in 37°C (5% CO2) cell culture incubator. Prior to the luciferase reporter assay, cells were briefly washed with 1.0ml of Dulbecco’s phosphate saline buffer with calcium and magnesium (DPBS, 1X) (Cellgro, Mediatech) and lysed by adding 250μL of Passive Lysis Buffer (Promega, Madison, WI), followed by 15 minutes incubation at room temperature. Cells extract was collected and dual-luciferase reporter assay was performed using Dual-Luciferase Reporter Assay kit according to manufacturer’s manual (Promega, Madison, WI). Firefly luciferase activity and renilla luciferase activity was measured using recommended TD-20/20 Luminometer.
(Turner BioSystems, Sunnyvale, CA).

**Statistical Analysis**

Measured firefly luciferase activity was normalized with measured renilla luciferase activity and presented in graphs as the normalized relative light units (e.g. normalized luciferase activity, RLU). In addition, the differences between treatments were analyzed for significance using Student t-test.

**RESULTS**

*cAMP Autoregulatory Elements (CAREs) of ICER Promoter are Highly Conserved*

ICER promoter sequence containing four CAREs was first identified and described in mouse (Molina et al., 1993). Human ICER promoter containing all four putative CAREs (Fig. 1A) was identified in our laboratory by finding homology to a known mouse ICER promoter sequence. Then, 1.5 kilobase pairs sequence of the promoter was isolated from LNCaP cells. A portion of human ICER promoter containing CAREs was used to search for homologous sequences in other organisms using basic local alignment search tool (BLASTN). Organisms showing high degree of homology were selected and resulting homologous sequences were then aligned and compared (Fig. 1B) using ClustalW2 (EMBL-EBI). Interestingly, we found that ICER promoter containing CAREs, specifically the CARE3 and CARE4, is highly conserved in mouse, *(Mus musculus)*, Japanese quail, *(Coturnix Japonica)*, chicken *(Gallus gallus)*, European seabass *(Dicentrarchus labrax)*, and frog *(Xenopus silurana)*. In addition to
sequence conservation, we found that the relative spacing between CARE clusters are also highly conserved. In concordance with mouse CAREs (Molina et al., 1993), human putative CARE2, CARE3 and CARE4 are all 8 nucleotides elements, whereas CARE1 is 10 nucleotides element. Remarkably, the sequences of CARE3 (TGATGTCA) and CARE4 (TGATGTCA) are identical to one another. Result of this in-silico analysis shows 100% homology for CARE4 and about 90% homology for CARE3 among all six organisms compared. Additionally, putative CARE1 (TGACAAAGCA) and CARE2 (TGATGGGCA) showed about 60% homology between human, mouse, Japanese quail and chicken. We also compared each of the CAREs to consensus palindromic CRE (TGACGTCA) sequence and found that CARE3 and CARE4 differ only by 1bp; whereas, CARE2 and CARE1 differ by two or more nucleotides. ICER is known to regulate gene expression by virtue of these CAREs, hence the variations observed in homology of CARE1 and CARE2 compared to CARE3 and CARE4 may reflect their binding affinity to ICER and in turn ICER gene regulation in different organisms. Therefore, based on our data and previous knowledge, we hypothesized that since CARE3 and CARE4 are highly conserved in various organisms, they are likely to play a significant role in transcriptional regulation of ICER in humans. In contrast, since CARE1 and CARE2 are not well conserved, they may play a weak role, if at all, in controlling transcriptional regulation of ICER.

Figure 1

A.

TGACAAAGCA AAT TGATGGGCA GTGATAGGCT AGTGATGTCA TTGTGATGTCA
CARE1 CARE2 CARE3 CARE4
B.

**CLUSTAL 2.1 multiple sequence alignment**

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Figure 1. In-silico analysis of putative CAREs of ICER promoter. A) DNA sequence of a portion of the human ICER promoter containing four putative CAREs. B) DNA sequence alignment of putative CAREs among selected organisms. Human ICER promoter, containing all four putative CAREs was searched against DNA nucleotide database using BLASTN (NCBI) and subsequently compared and aligned using ClustalW2 (EMBL-EBI).

**ICER Interacts and Binds to CARE1, CARE3, and CARE4 but Not to CARE2 of hICER Promoter in Vitro.**

One of the fascinating facts about ICER is that it can regulate its production by binding to CAREs located in its own promoter in an autoregulatory negative feedback loop (Molina et al., 1993). Since the binding of ICER to CAREs is essential for ICER transcriptional regulation, we first assessed the interactions and binding of ICER to four putative CAREs of hICER promoter in vitro by electromobility shift assay (EMSA). Short, 27bp, DNA nucleotides containing only a single wild-type CARE site was end labeled with infrared dye. The binding reaction was performed by incubating the oligonucleotides with or without bacterially purified ICER protein. Figure 2 shows that ICER strongly binds to putative CARE3 and CARE4 (lanes 1-4); however, ICER binding to putative CARE1 was much weaker (lanes 7 and 8). Furthermore, no shifted band was observed in lane 6, where putative CARE2 and ICER were added. This lack of shifted band indicates that ICER does not bind to CARE2 (lanes 5 and 6). Overall, this data
suggested that ICER binds to putative CARE1, CARE3 and CARE4 but not to the CARE2.

Figure 2

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Figure 2: ICER binding to single CARE sites of hICER promoter in vitro. EMSA was performed by incubating 1μg of purified ICER-IIγ protein with 50nM of IRDye® 700 end labeled wild-type CARE oligonucleotides (lanes 2, 4, 6, and 8). Negative control (lanes 1, 3, 5, and 7) contains only the CAREs oligonucleotides, without ICER. Image is a representative and combinations of two separate experiments.

To further strengthen our preliminary findings shown in previous figure (Fig. 2) and to show specificity, we analyzed the ICER interaction with putative CARE1 CARE2, CARE3 and CARE4 individually. EMSA was performed using three different concentrations of ICER protein with wild-type as well as mutant labeled CARE oligonucleotides. Additionally, excess amount of ICER (1μg, 2μg and 4μg) was used to eliminate any concerns regarding concentrations of ICER required in binding of CAREs.
Figure 3A clearly shows that ICER binds to wild-type CARE1 (lanes 1-4) but not to mutant CARE1 (lanes 5-8) oligonucleotides. The ICER-CARE complex (shifted band) showed dose dependent increase in signal intensities in response to increasing amount of ICER added. Surprisingly, ICER does not bind to wild-type CARE2 (Fig. 3B, lanes 1-4) even in excess amount (4μg) of ICER. These results are in concordance with the preliminary observations made in figure 2. Furthermore, we observed that the ICER strongly binds to wild-type CARE3 (Fig. 3C, lanes 1-4) and wild-type CARE4 (Fig. 3D, lanes 1-4) with a dose dependent increase in signal intensities of the ICER-CARE complex in response to increasing amount of ICER added. Also, the binding of ICER to CAREs seen here was specific because ICER binding was not observed when mutant CARE3 (Fig.3C lanes 5-8) or mutant CARE4 (Fig.3D, lanes 5-8) oligonucleotides were tested. To further show that binding of ICER was specific to CARE sites and not due to any other sequence present in the promoter, a non-CRE oligonucleotides containing 8bp non-CRE like sequence of the same ICER promoter was analyzed. No binding of ICER to wild-type or mutant non-CRE oligonucleotides was observed (Fig. 3E) which proves the ICER specificity in binding to the CAREs. Together, these data suggest that ICER specifically interacts and binds to CARE1, CARE3 and CARE4, but not to the CARE2 of hICER promoter. Based on these data, it was concluded that CARE2 is not a true CARE of hICER promoter and therefore is insignificant in controlling ICER gene expression in humans.
Figure 3

A. WT CARE-1 Oligo  
   ICER-IIγ protein  
   Mut CARE-1 Oligo  

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ICER-CARE complex

Free Oligos

B. WT CARE-2 Oligo  
   ICER-IIγ protein  
   Mut CARE-2 Oligo  

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Free Oligos
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**ICER-CARE complex** →

**Free Oligos** →

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**ICER-CARE complex** →

**Free Oligos** →
E.

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Figure 3: Interactions of ICER with four putative CAREs of human ICER promoter in vitro. ICER binding to wild-type and mutant CARE1 (A); CARE2 (B); CARE3 (C); CARE4 (D); and non-CRE (E) of hICER promoter was assessed. EMSA was performed by incubating ICER-II\(\gamma\) protein, in concentrations of 1\(\mu\)g (lanes 2 and 6), 2\(\mu\)g (lanes 3 and 7), and 4\(\mu\)g (lanes 4 and 8), with 200nM of IRDye® 700 end-labeled wild-type (lanes 1 to 4) or mutant (lanes 5 to 8) CARE oligonucleotides. Negative controls (lanes 1 and 5) contain only the CARE oligonucleotides, without ICER.

**Cooperative Binding of ICER With Multiple CAREs In Vitro**

We proved that ICER binds to CARE1, CARE3 and CARE4 individually, however all three CAREs are present together as a cluster in a wild-type hICER promoter. Hence, we next wanted to explore how ICER binding affinity is affected in presence of two or more CAREs together. So, in hope to study cooperative interactions of ICER with CAREs, we performed EMSA using wild-type hICER oligonucleotide
containing CARE1, CARE3 and CARE4 together, as well as with oligonucleotide composed of various combinations of two different CAREs. DNA oligonucleotides, 64bp, containing two or more wild-type CAREs, end-labeled with infrared dye, was incubated with or without bacterially purified ICER protein. Figure 4A shows strong binding of ICER to wild-type CARE oligonucleotide (CARE1,3,4) where all three CAREs were present together (lanes 1-2). Then, we analyzed the binding of ICER when only two of the three wild-type CAREs were present. We found that ICER binding is much stronger when CARE3&4 were present together (lanes 7 and 8) compared to when CARE1&3 were present together (lanes 3 and 4). Furthermore, a much weaker binding was seen when CARE1&4 were present together (lanes 5 and 6). Quantitative analysis comparing relative strength of ICER binding to two or more CAREs by measuring the signal intensities of CARE-ICER complex is shown in figure 4B. Data indicates that the presence of CARE3&4 results in ICER binding almost as strong as ICER binding to wild-type oligonucleotide containing CARE1,3,4 together. The ICER binding to CARE1&3 was weaker compared to wild-type CAREs (CARE1,3,4) or CARE3&4, but it was stronger compared to CARE1&4 which showed the weakest ICER binding. Overall, these results show that the binding of ICER is stronger when CARE3 and CARE4 were present together compared to when CARE1 and CARE3 were present together which, in turn is stronger than when CARE1 and CARE4 were present together. However, the ICER binding affinity is strongest when all three CAREs were present together. Ultimately, our data indicates that that ICER may require presence of all CAREs together for maximum effect in regulating gene expression in a negative feedback loop.
Figure 4

A. ICER-IIγ protein (1μg) was incubated with 50nM of IRDye® 700 end labeled wild-type CAREs oligonucleotides to analyze cooperative binding to two or more CAREs together. EMSA was performed using WT CARE Oligos with WT CARE-1,3,4, WT CARE-1&3, WT CARE-1&4, and WT CARE-3&4.

B. Comparison of multiple CAREs binding to ICER with signal intensity.

Figure 4: Cooperative binding of ICER with multiple CAREs of hICER promoter in vitro. A) ICER binding to two or more CAREs together was analyzed. EMSA was performed by incubating 1μg of purified ICER-IIγ protein with 50nM of IRDye® 700 end labeled wild-type CAREs oligonucleotides (lanes 2, 4, 6, and 8). Negative controls
(lanes 1, 3, 5, and 7) contain only the CAREs oligonucleotides, without ICER. B) Quantitative analysis of EMSA results measuring the signal intensities of CARE-ICER complex (lanes 2, 4, 6, and 8 of Fig 4A).

Various Mutant CAREs Constructs Created by Site-Directed Mutagenesis

Next, we wanted to study the activity of hICER promoter containing CARE1, CARE3 and CARE4 in vivo. In order to study this, we first generated various luciferase reporter constructs consisting of different combination of CAREs (Fig. 5). A 518bp hICER promoter composed of all wild-type CAREs was clone into pGL3 basic luciferase reporter vector. Using this pGL3 basic-0.5Kb hICER WT promoter construct, several combination of mutant CAREs were generated by introducing TAGTGTGA sequence in place of wild-type CAREs. We first generated the mutant CO (pGL3 basic-0.5Kb hICER C0) reporter construct containing mutation at all three CAREs of hICER promoter. Then, to study the cooperative regulation of hICER in presence of only one wild-type CARE at a time, we created C1 (pGL3 basic-0.5Kb hICER C1), C3 (pGL3 basic-0.5Kb hICER C3), and C4 (pGL3 basic-0.5Kb hICER C4) reporter constructs consisting of wild-type CARE1, CARE3 or CARE4 only, respectively. Then to study cooperative regulation of hICER in presence of two CAREs, we created C13 (pGL3 basic-0.5Kb hICER C13), C14 (pGL3 basic-0.5Kb hICER C14), and C34 (pGL3 basic-0.5Kb hICER C34) reporter constructs consisting of only two wild-type CARE at a time. Each of the constructs was sequenced to confirm for mutations and functional wild-type CARE sequence.
### Wild-Type hICER Promoter is Inducible and Repressible in LNCaP Cells

The expression of ICER is known to be inducible by cAMP/PKA signaling and repressible by ICER protein in normal neuroendocrine cells (Molina et al., 1993; Stehel, 1993). Data presented thus far of in vitro analysis suggests possibility of regulating hICER expression in vivo via CARE1, CARE3 and CARE4 located in the promoter region of hICER. Therefore, we next studied the ability of hICER to be induced and repressed by cAMP/PKA signaling in the human prostate cancer cell line, LNCaP. We conducted experiments to study the activity of hICER promoter containing the regulatory elements, CAREs, that account for the inducibility and repressibility of ICER transcription.
First, we tested a luciferase reporter construct containing Cyclin D2 promoter as a positive control, since it is expressed widely in all types of cells and it has been shown to be inducible by PKA signaling and repressible by ICER in granulosa cells (Muniz et al., 2006). We transfected Cyclin D2 reporter construct into LNCaP cells alone (control), as well as cotransfected with plasmid expressing catalytic subunit of PKA (cPKA) or cPKA with a plasmid containing ICER1IIγ (ICER). Following the transfection, luciferase assay was performed to test the promoter activity. As expected, results of Cyclin D2 promoter (Fig. 6A) showed significantly (P<0.005) higher promoter activity when cells were cotransfected with cPKA compared to the control where cell were transfected with promoter only. In addition, significant repression in promoter activity was also observed when cells were cotransfected with cPKA and ICER together compared to the cells cotransfected with cPKA only. These data confirm and further prove that cPKA can induce activity of a promoter containing CRE sites. This data also confirms that ICER can repress transcription of a gene containing CRE sites in the promoter of targeted genes in LNCaP cells.

Since hICER promoter contains three CAREs in its promoter, we hypothesized that hICER promoter is likely to be inducible by cAMP/PKA pathway in LNCaP cells. We also hypothesized that PKA-induced activity of hICER promoter should be repressed when ICER is added. To test our hypothesis, we studied the activity of wild-type hICER promoter (Fig.5) and assessed the inducibility of hICER promoter by cAMP/PKA signaling as well as assessed the repressibility of PKA-induced activity of the promoter by ICER. We transfected the wild-type hICER promoter (WT IP) construct in LNCaP cells alone (control), as well as cotransfected with plasmid expressing cPKA or cPKA
with a plasmid containing ICERIIγ. Interestingly, results of luciferase assay of hICER promoter (Fig. 6B) showed significantly higher ($P<0.005$) promoter activity in cells cotransfected with cPKA compared to the control (WT hICER promoter only). Also, the hICER promoter activity was observed to be greatly ($P<0.005$) suppressed in cells cotransfected with cPKA and ICER together compared to promoter activity of cells cotransfected with cPKA only.

After that, to determine and prove that induction and repression observed in wild-type hICER promoter activity was due to the presence of three CAREs, we generated and tested a mutant hICER promoter construct (C0) where all three CAREs were mutated (Fig. 5). The C0 promoter reporter construct was transfected in LNCaP cells by the same transfection strategies described earlier. When cells were cotransfected with mutant hICER promoter and cPKA (Fig. 6C), no increase in the promoter activity was observed; instead, we observed about three fold suppression compared to controls where cells were transfected with promoter only. Together, we demonstrated that activity of wild-type hICER promoter is highly inducible by PKA/cAMP signaling pathway in LNCaP cells, specifically due to the presence of CARE1, CARE3 and CARE4. Results also show that the PKA-induced activity of wild-type hICER promoter is repressible by ICER due to the ICER binding with CARE1, CARE3 and CARE4 in LNCaP cells.
Figure 6

A.

Cyclin D2 Promoter

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B.

WT hICER Promoter

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C.

CO (All mutCAREs)

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Figure 6: Induction and repression of hICER promoter activity by cAMP pathway in-vivo. A plasmid containing the wild-type Cyclin D2 promoter (A), wild-type hICER promoter (B) and mutant hICER promoter (C) were transfected into LNCaP cells alone (control), as well as cotransfected with plasmid expressing catalytic subunit of PKA or cPKA with plasmid containing ICERIIy. Luciferase activity was measured after about 48 hours and the firefly luciferase data was normalized with renilla luciferase. Data are presented as the mean ± SEM of three trials per sample. Bars with (*) differs significantly from the control and bars with (**) differs significantly from cells transfected with PKA ($P<0.005$).

**hICER Gene Expression is Regulated by CAREs in a Cooperative Manner in Vivo**

We discovered that wild-type hICER promoter is inducible by cAMP/PKA signaling and repressible by ICER in vivo due to the presence of three CAREs. Normally, ICER is able repress cAMP-induced transcription of target genes containing even a single CRE sequence in the promoter region. Nonetheless, ICER promoter contains three CAREs, indicating the importance of these multiple CAREs in regulating ICER expression in a very fine tune and orderly fashion. Therefore, we speculated that all three CAREs must be necessary for regulation of ICER expression. Hence, we conducted in vivo experiments to see whether these CAREs function independently or in a cooperative manner in controlling ICER gene expression. First, we tested C13, C14 and C34 promoter constructs (Fig. 5) consisting of combinations of only two of the three wild-type CAREs together, CARE1&3, CARE1&4 and CARE3&4 respectively. We transfected each of constructs in LNCaP cells by transfection strategies explained in the previous section and performed the luciferase assay. We found a significant ($P<0.05$) increase in the promoter activity for CARE1&3 construct (Fig. 7A) in cells cotransfected with cPKA compared to the control where cells were transfected with C13 promoter only. We also found a noticeable decrease in promoter activity in cells cotransfected with cPKA and ICER compared to cells cotransfected with cPKA only. However, when the
C14 promoter (Fig. 7B) construct was tested, there was no significant increase or decrease observed in the promoter activity in cells cotransfected with cPKA or in cells cotransfected with cPKA and ICER. Next, we assessed the promoter activity of the C34 promoter construct consisting of only CARE3&4 together (Fig. 7C). Strikingly, we found a significant ($P<0.05$) increase in the promoter activity in cells cotransfected with cPKA compared to the control where cells were transfected with C34 promoter only. We also found a significant decrease in the promoter activity in cells cotransfected with cPKA and ICER compared to the cells cotransfected with cPKA only. The fold increase and decrease in the promoter activity seen in C34 promoter construct was greater than in C13 promoter. Altogether, these data show that CARE3 and CARE4 together are essential and relatively more important in regulating ICER gene expression than CARE1 and CARE3 together, which in turn is more important than CARE1 and CARE4 together. These data are also in concordance with the observation made in figure 4 when cooperative binding of ICER to multiple CAREs was assessed in vitro.

**Figure 7**

A.

![Bar chart showing CARE 1&3](image)
B.

Figure 7: Regulation of hICER promoter activity in presence of only two of the three CAREs. A plasmid containing hICER CARE1&3 (A), hICER CARE1&4 (B) and hICER CARE3&4 (C) were transfected into LNCaP cells alone (control), as well as cotransfected with plasmid expressing catalytic subunit of PKA or cPKA with plasmid containing ICERIIy. Luciferase activity was measured after about 48 hours and the firefly luciferase data was normalized with renilla luciferase. Data are presented as the mean ± SEM of three trials per sample. Bars with (*) differs significantly from control and bars with (**) differs significantly from cells transfected with PKA ($P<0.05$).
Presence of Only One of the Three Wild-Type CAREs in hICER Promoter is Unable to Regulate ICER Gene Expression in Vivo.

To further study the cooperative regulation of hICER promoter activity in presence of only one CARE, we tested the C1, C3 and C4 promoter constructs consisting of only a single wild-type CARE (Fig. 5). We analyzed each of the three CAREs independently by performing cell transfection followed by luciferase assay as described in the previous sections. Results of these experiments did not show any significant increase in the promoter activity when constructs composed of CARE1 (Fig.8A), CARE3 (Fig.8B) or CARE4 (Fig.8C) were cotransfected in LNCaP cells with cPKA compared to the controls where cells were transfected with promoter only. However, there was a noticeable decrease observed in the promoter activity in cells transfected with C4 promoter and cPKA. Furthermore, we did not observe any significant suppression in cPKA-induced promoter activity when cells were cotransfected with cPKA and ICER compared to the cell cotransfected with cPKA without ICER. Based on these data, it was concluded that a single CARE present independently in the hICER promoter is insufficient in either inducing or repressing the hICER promoter activity.

Figure 8
Figure 8: Regulation of hICER promoter activity in presence of only single CARE. A plasmid containing hICER CARE 1 (A), hICER CARE 3 (B) and hICER CARE4 (C) were transfected into LNCaP cells alone (control), as well as cotransfected with plasmid expressing catalytic subunit of PKA or cPKA with plasmid containing ICERIIγ. Luciferease activity was measured after about 48 hours and the firefly luciferase data was normalized with renilla luciferase. Data are presented as the mean ± SEM of three trials per sample. Bars with (*) differs significantly from control ($P<0.05$).

**DISCUSSION**

CREM is an evolutionary highly conserved multiexonic gene that encodes variety of activators and repressors of cAMP-induced gene transcription (Masquillier et al., 1993;
Schumacher et al., 2000). Interestingly, ICER is an only isoform of a CREM gene that is known to be highly regulated by cAMP/PKA signaling due to the presence of multiple CRE sequences (CAREs) in the promoter region of ICER (Molina et al., 1993; Stehle et al., 1993). The promoter of ICER has been characterized in mouse; however, the human form of ICER promoter has never been characterized until now. We first performed an in-silico analysis using human ICER promoter containing four putative CAREs. Results clearly showed that the CARE3 and CARE4 are identical to one another and are highly conserved in various organisms such as mouse, chicken, frogs, and fish. However, CARE1 and CARE2 showed only about 60 percent homology in four different organisms.

Experiments were conducted to study interactions of ICER with the four CAREs of hICER promoter. Results of our in vitro analysis showed that ICER specifically binds to CARE1, CARE3 and CARE4. Even though CARE1 showed low homology among different organism compared, a weak binding of ICER to CARE1 was observed. However, data show that ICER does not bind to CARE2. Therefore, it was concluded that CARE2 is not a true CARE of hICER promoter and thus not essential in regulating ICER gene expression. Based on these data, it was concluded that human ICER promoter is composed of only three CAREs, namely CARE1, CARE3 and CARE4. Furthermore, even though CARE3 and CARE4 are identical in sequence, we observed different binding affinity of ICER to these CAREs in presence of CARE1. We observed that ICER binding is stronger when CARE1 and CARE3 were present together compared to when CARE1 and CARE4 were presents. These observations lead us to theorize that in
addition to the importance of sequence homology of CAREs, their relative position on the promoter also plays an important role in ICER binding affinity.

To further characterize the hICER promoter and study its activity, we conducted experiments in vivo using human prostate cancer cell line, LNCaP. Our data clearly show that the activity of wild-type hICER promoter is highly inducible by cAMP/PKA pathway in LNCaP cells. Moreover, results also show that ICER negatively regulates the PKA-induced activity of wild-type hICER promoter. The induction and repression observed in the ICER promoter activity was specifically due to the presence of CAREs in ICER promoter. ICER has been characterized as a dominant negative suppressor and it is known to repress cAMP-induced transcription of target genes containing even a single CRE in the promoter region (Razavi et al., 1998; Molina et al., 1993; Stehle et al., 1993). Nonetheless, ICER promoter contains three CAREs, emphasizing the necessity of multiple CAREs to regulate gene expression in a tight and orderly fashion. Therefore, we conducted experiments to see whether these CAREs function independently or in a cooperative manner to regulate ICER expression. We discovered that the presence of a single CARE alone in hICER promoter is not sufficient to induce promoter activity by cAMP signaling or repress the promoter activity by ICER. Our results also show that the presence of CARE1&3 and CARE3&4 together in hICER promoter can induce promoter activity by cAMP and repress the promoter activity by ICER in LNCaP cells. However, the induction and repression observed was lowered compared to wild-type hICER promoter composed of all three CAREs. Collectively, data from our in vitro as well in vivo analysis suggest that the multiple CAREs of hICER function cooperatively in regulating ICER expression. In addition, these results indicate CARE3 and CARE4 are
more significant in controlling gene expression compared to CARE1. One unexpected result observed was the significantly decreased activity of mutant hICER promoter (C0 constructs) as well as C4 construct (CARE4 only) upon addition of cPKA. This phenomenon is likely to be independent of CAREs since effect was observed in C0 constructs. The 8bp sequence used to mutate wild-type CARE site has been proven to be in effective in response to cAMP, however, the exact function of this element is not known. Therefore, it is possible that presence of this mutant CRE is contributing to the inhibition of the promoter activity.

Researches show abnormal expression of ICER in human prostate cancer tissues and in turn suggest the role of ICER in tumorigenesis of prostate cancers (Yehia et al., 2001; Memin et al., 2002). A study shows that ICER expression is altered in LNCaP cells and it is not inducible by 8-Br-cAMP. This study also showed that the lack of ICER expression was due to the lack of ICER RNAs and hence, it was speculated that abnormal regulation is likely to be at the level of gene expression (Yehia et al., 2001). In contrast, our results clearly prove that the activity of hICER promoter is inducible by cAMP/PKA signaling and it is also repressible by the ICER in LNCaP Cells specifically due to the presence of three CAREs. Therefore, we speculated that the lack of ICER expression inducibility observed in previous studies in human prostate cancers is not a result of ICER promoter region that is composed of these regulatory elements, CAREs. Instead, the phenomenon is possibly due to the unstable ICER mRNA via alternative polyadenylation sites, abnormal regulation of miRNA involved in RNA interference or some other molecular mechanisms. In this study, we tested only a portion of hICER promoter; therefore, another possibility that can explain this adverse phenomenon is the
regulation of ICER promoter by DNA methylation, histone acetylation, presence of additional regulatory elements, or interactions of other transcription factors in the full ICER promoter region.

ICER has been characterized a tumor suppressor protein and due to its role in various cancers, studying the regulation of its expression in a cell is critical. Here, we characterized the human ICER promoter containing the cAMP autoregulatory elements and studied its activity in human prostate cell line, LNCaP. We clearly prove and present for the first time that the hICER promoter is composed of three CAREs and the promoter activity is inducible by cAMP/PKA pathway and negatively regulated by ICER in LNCaP cells. We also show that the induction and repression observed in the ICER promoter activity is specifically due to the presence of three CAREs in hICER promoter. Based on our data, we suggest that CARE1, CARE3 and CARE4 function in a cooperative manner in regulating ICER gene expression. ICER is a tumor suppressor protein and hence, its expression must be tightly regulated in a cell for normal growth control. These are essential initial finding that will provide us valuable information in understating the abnormal ICER expression seen in human prostate cancers. In future, we would like to investigate the full region of hICER promoter and study its activity in response to cAMP/PKA pathway. We will also expand our focus and study the transcriptional regulation of ICER in various other cancer cell lines. Our long term goal is to fully understand the regulation of ICER at transcriptional level and eventually manipulate the expression of ICER in hope to provide alternative treatment for cancers.
REFERENCES


