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CLONING, CHARACTERIZATION AND ACTIVITY ANALYSIS OF HUMAN CHOLINE KINASE PROMOTERS

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Introduction

Choline kinase (CK) is the first enzyme in the CDP-choline pathway, a *de novo* biosynthetic pathway for major phospholipid in the membrane of eukaryotic cells i.e phosphatidylcholine (Lykidis *et al.*, 2001). This enzyme catalyzes the phosphorylation of choline by ATP to form phosphocholine. In mammalian cells, choline kinase exists as three isoforms that are encoded by two separate genes named *ckα* and *ckβ*. *ckβ* codes for a single protein (CKβ) while *ckα* undergoes alternative splicing to produce CKα1 and CKα2 isoforms (Malito *et al.*, 2006).

Increased activity of CK and its product, phosphocholine, have been implicated in human carcinogenesis. Elevated phosphocholine level is a common feature in cell lines derived from human tumors and this parameter seems to be able to distinguish malignant cell lines from normal cell lines irrespective of their proliferation rates (Bhakoo *et al.*, 1996; Aboagye and Bhujwalla, 1999). Overexpression of CK has been reported in a variety of human cancers such as lung, colorectal as well as prostate adenocarcinomas (Nakagami *et al.*, 1999, Ramirez de Molina *et al.*, 2002a, Ramirez de Molina *et al.*, 2002b). In addition, studies had demonstrated the increased of CK activity upon induction of the H-*ras* oncogene in mouse fibroblast cell lines. Inhibition of CK has been proposed to be a potential antitumor strategy (Rodriguez-Gonzalez *et al.*, 2004). Rodriguez-Gonzalez *et al.* (2004) demonstrated that CK inhibitors could become potent antitumor drugs both *in vitro* and *in vivo*. Recently, CKα protein levels have been found to be drastically increased in both human tumors and cell lines derived from human tumor, when compared to normal tissues or appropriate human primary cells, respectively (Aoyama *et al.*, 2004). Increased levels of CKα mRNA but not CKβ in tumor-derived cell lines was also reported (Gallego-Ortega *et al.*, 2009).

A promoter by definition is a DNA sequence, generally directly upstream to the coding sequence, required for basal and/or regulated transcription of a gene. Gene transcription is regulated by transcription factors, binding mostly and specifically to the promoter regions

(Xuan *et al.*, 2005). Transcription factors are specific regulatory proteins that regulate the transcription of a gene either positively or negatively (Latchman, 1997). Transcription factors are essential for the regulation of gene expression and consequently are found in all living organisms. Findings indicate that the interaction of different factors with a common target site does not necessarily result in equivalent transcriptional responses. While some factors activate transcription, others that bind to the same site repress this process (Karin, 1990).

Previously, Aoyama *et al.* (2004) have identified the possible regulatory elements in mouse *ckα* and *ckβ* promoters. The proximal region of *ck-α* promoter could be divided into two sub-regions (from –162 bp to –119 bp and from –93 bp to –46 bp). Two CCAAT boxes, two SP-1 sites and one CREB site could be found in those regions. The distal region of *ckα* promoter was located between –875 bp and –867 bp, which was identified as a 9 bp AP-1 binding element. Interestingly, a xenobiotic responsive element was found to be located just downstream of the putative AP-1 site, indicating that this distal promoter region could be involved in the induction of *ckα* gene expression in the liver of mice when treated with either CCl_4 or polycyclic aromatic hyrocarbons (PAHs). In contrast, the *ckβ* gene does not have any distal promoter region and its expression seems to be regulated by only the proximal promoter region. This region contains several common transcription factor-binding sites such as two CCAAT boxes, one each of SP-1 and CREB site (Aoyama *et al.*, 2004).

Despite the importance of CK in phospholipid biosynthesis and its implication in cancer pathogenesis, the promoters of human *ckα* and *β* genes have never been studied. It is essential to look at how the expression of both isoforms are regulated by their promoter sequences to understand their differential expression in normal and cancer cell lines, i.e only CKα but not CKβ was overexpressed in tumor derived cell lines (Gallego-Ortega *et al.*, 2009). This study aims to identify the important regulatory regions of human *ck-α* and *β* promoters and the cis-acting elements on the promoters. The binding of important

transcription factors identified in this study was confirmed by electromobility shift assay and their effect was modulated by phorbol 12-myristate 13-acetate (PMA).

Materials and Methods

Prediction of *cis*-acting elements on human *ckα* and *β* promoter regions

The promoter sequences of *ckα* and *β* genes were identified from human genome sequence by using BLAT search. About 2000 bp of the promoter sequences was subjected to analysis with free online softwares for identification of transcription factor binding sites including "Transcription factor search" at http://www.cbrc.jp/research/db/TFSEARCH.html and MatInspector 8.0 (Quandt *et al.*, 1995).

PCR cloning of the human *ckα* and *β* promoter regions

A two kilobases 5' flanking regions of human *ckα* and *ckβ* genes was amplified from human genomic DNA (Roche, Germany) using primers containing specific restriction enzyme sites as shown in table 1.

The PCR reaction mix consisted of 10 µl 5x Green GoTaq Flexi buffer (Promega), 5 µl $MgCl_2$ solution (25 mM), 1.5 µl dNTP mix (10 mM), 2.5 µl forward primer (10 mM), 2.5 µl reverse primer (10 mM), 500 ng human genomic DNA, 0.5 µl GoTaq DNA polymerase (5 units/µl) and distilled water to a total volume of 50 µl.

The PCR was run for one cycle of 2 min at 95°C, followed by 35 cycles of 1 min at 95°C, 2 min at specific annealing temperature (table 1), and 2 min at 72°C. One cycle final extension of 7 min at 72°C was introduced in the end of the PCR. The PCR products were run on 1% agarose gel electrophoresis and purified for cloning into the *NheI/HindIII* (for *ckα*) and *XhoI/HindIII* (for *ckβ*) sites of promoterless pGL4.10 [luc2] vector (Promega), upstream of the firefly luciferase gene. For ligation, 0.3 µl of vector DNA, 8.2 µl insert DNA, 1 µl 10X T4 DNA

ligase buffer, 0.5 µl T4 DNA ligase and 0.2 µl 70% 10x PEG (polyethylene glycol) were added. The reaction mixture was then incubated overnight at 4°C.

Table 1: Primers for the amplification of *ckα* and *β* promoters

Primers	Primer sequence (5' to 3')	Annealing temperature (°C)
ckα-Forward	CTAGCTAGCGTGGGACAAAGGGCTTGT	54
ckα-Reverse	CCCAAGCTTGCCCGACAGGCGGCCGA	60
ckβ-Forward	CCGCTCGAGATGATGCTTCAGGGCTCC	54
ckβ-Reverse	CCCAAGCTTGGCGCGGGCTCGACCGGG	66

Cell lines and culture condition

MCF-7 (breast carcinoma) cell was used as host for promoter activity assay in this study. The cell was cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco) with high glucose, 10% fetal bovine serum (FBS) and 10000 U/mL penicillin-streptomycin and maintained in a humidified incubator at 37°C with 5% CO_2. Cells were passaged at pre-confluent densities every 2 to 3 days.

Transfection and quantitation of promoter activity by Dual-Glo luciferase assay

Twenty-four hours prior to transient transfection, 0.5 – 2.0 x 10^5 MCF-7 cells were seeded into each well of a 24-well dish containing 500 µl of DMEM growth medium without antibiotics. Cells were then transfected using Lipofectamine 2000 (Invitrogen) transfection reagent either with firefly luciferase or *Renilla* luciferase vector. Briefly, the pGL4.10(*luc2*) firefly (80 ng) and pGL4.73 (*hRluc*/SV40) *Renilla* luciferase (40 ng) vectors were diluted in 50 µl of Opti-MEM I medium without serum. Then, Lipofectamine 2000 was diluted in 50 µl of Opti-MEM I medium and incubated at room temperature for 5 minutes. The diluted firefly and *Renilla* luciferase vectors were combined with the diluted Lipofectamine 2000 and incubated at room temperature for 20 minutes. The 100 µl complexes were added to each well

containing cells and medium as described above. The cells were then incubated at 37°C with 5% CO_2 for 48 hours following transfection.

The transfected cells were assayed for both firefly and control *Renilla* luciferase activities with Dual-Glo luciferase assay system (Promega) according to the manufacturer's instructions. The promoterless pGL4.10-firefly luciferase vector was used as the experimental reporter, while the pGL4.73-renilla luciferase vector which is regulated by SV40 promoter was used as the control vector for each transfection assay. Promoter activity was measured in the GloMax 20/20 luminometer and was expressed as relative firefly luciferase activity normalized to *Renilla* luciferase activity. The GloMax 20/20 luminometer is an ultrasensitive instrument for measuring luminescent materials including the measurement of firefly and *Renilla* luciferase for genetic reporter studies.

Construction and functional analysis of 5' deletion constructs of *ckα* and *β* promoters

To delineate sequences that drive the expression of the human CKs in MCF-7 cell, various length of 5' deletion mutants were created and cloned into pGL4.10 vector. The 5' truncated constructs were made from the original 2 kb promoter sequences by PCR mutagenesis. The relative transcriptional activities of the truncated promoter fragments were determined by the luciferase activity assay as described above. Recombinant plasmids were verified by sequencing.

PMA and PKC inhibitor treatment

The day before transfection, MCF-7 cells were plated into 96-well plate at a density of 1.5 x 10^4 cells per well. After 24 hours, the cells of each well were transfected with 200 ng of pGL4.10-*ckα*-(1275/+9) or pGL4.10-*ckβ*(-2000/-1) and 2.5 ng of pGL4.73[*hRluc/SV40*]. Six hours after transfection, the medium was replaced with 1% (v/v) serum starvation medium for 20 hours before adding the desired drug. The cells transfected with pGL4.10-*ckα*-(1275/+9) plasmid were treated with 10, 20 and 30 ng/mL of PMA in a fresh medium for 6 or 24 hours. The cells transfected with pGL4.10-*ckβ*(-2000/-1) plasmid were treated with 10, 20 and 30

ng/mL of PMA in a fresh medium for 6 hours. The effect of PMA treatment duration on *ckα* and *ckβ* promoters was studied by treating the MCF-7 cells with 20 ng/mL and 30 ng/mL PMA, respectively for 6, 12 and 24 hours. For control sample, DMSO was added to the cells instead of PMA. Luciferase activities were measured using Dual-Glo luciferase assay.

To investigate the combination effects of PMA and PKC inhibitors treatment on *ckα* promoter activity, the cells were treated with 30 ng/mL of PMA together with 2 mM of PKC412 or 1 mM of Go 6983 for 24 hours. For *ckβ* promoter, MCF-7 cells were treated with 20 ng/mL of PMA together with 1 mM of PKC412 or 0.1 mM of Go 6983 for 6 hours. Luciferase activities were then measured using Dual-Glo luciferase assay.

Electrophoretic mobility-shift assay (EMSA)

Nuclear extracts were prepared from MCF-7 cells using NE-PER® Nuclear and Cytoplasmic Extraction kit (Thermo Fisher Scientific, USA) according to the manufacturer's protocol. DNA probe (1 μM) was labeled with biotin using 3' End DNA Labeling Kit (Thermo Fisher Scientific, USA). Complementary probes were mixed at a 1:1 molar ratio allowed to anneal in a thermal cycler using a four-step protocol with the initial denaturation at 95°C for 5 minutes, followed by 1°C decreased per minute until the temperature reached the probe annealing temperature. The probes were further incubated at the annealing temperature for 30 minutes and the annealing process was ended by decreasing the temperature to 25°C (1°C decreased/min). Binding reaction of nuclear extract to the specific biotin-labeled DNA oligonucleotide was carried out in accordance with the protocol of LightShift Chemiluminescent EMSA kit (Pierce) with slight modifications.

For electrophoresis of DNA-protein complexes, non-denaturing gel was prepared using the Bio-Rad Mini PROTEAN III system (Bio-Rad, USA). Six percent (w/v) non-denaturing gel was used throughout the EMSA experiments. The gel was pre-electrophoresed in 0.5× TBE for 60 minutes with constant voltage of 100 V at 4°C. The wells of the gel were flushed and 25 μL of the binding reaction mix were loaded into the non-denaturing gel. Electrophoresis

was carried out at 100 V at 4°C until the bromophenol blue dye has migrated approximately three-fourths of the distance from the bottom of the wells. The protein DNA-protein complexes were transferred to Biodyne B nylon membrane by layering the filter sponge at the bottom of a Trans-blot® SD Semi-dry Electrophoretic Transfer cell (Bio-Rad, USA), followed by nylon membrane, the gel and another filter sponge on top. The transfer was performed at 12 V for 50 minutes. When the transfer was complete, the membrane was placed with the bromophenol blue side up on a dry paper towel and immediately subjected to DNA-membrane crosslinking by exposure to UV light at 312 nm for 15 minutes. The biotin-labeled DNA on the membrane was detected by LightShift Chemiluminescent EMSA kit according to the manufacturer's protocol.

Results

Cloning of human *ckα* and *β* promoters

A region of about 2000 bp located upstream of human *ckα* and *β* translation start sites was PCR amplified and cloned into pGL4.10[*luc2*] luciferase vector as shown in figure 1. The *ckα* and *β* full length promoter constructs were designated as pGL4.10-CKα(-2000/+9) and pGL4.10-CKβ(-2000/-1), respectively. These constructs were used for subsequent 5'-end truncation experiments and promoter activity assays.

Identification of important regulatory region in *ckα* and *β* promoters

The 5'-ends of *ckα* and *β* promoters were systematically deleted by PCR to identify important regulatory regions in these promoters. As shown in figure 2, the first round of *ckα* promoter 5'-end deletion showed that the most significant effect was observed within the region between -1970/-818. Another round of truncation of this region narrowed the important regulatory region to between -1354/-1217. Further deletion of this region produced the most significant effect when the region between -1275/-1252 was removed. It was also clear that this region contained repressive regulatory element.

Similarly, figure 3 shows the effect of 5'-end truncation of *ckβ* promoter on the promoter activity. In the first round of 5'-end truncations, all three mutations resulted in significant effect on *ckβ* promoter activity. However, the region between -2000/-1477 was identified as having the strongest effect on promoter activity. Further truncations were made in this region and the results showed that there were significant negative regulation in regions between -2000/-1886 and -1886/-1651. Significant positive regulation was present in the region between -1651/-1477.

Identification of transcription factors for the regulation of *ckα* and *β* promoters

The region between -1275/-1252 of *ckα* promoter was predicted to contain a single Ets transcription factor binding element (figure 4). Mutation of this element resulted in almost 100% increase of promoter activity compared to the wild-type full length *ckα* promoter. The results show that Ets element located at -1270 acts as negative regulatory element of *ckα* promoter activity.

Figure 5 shows the predicted transcription factors binding sites between -2000/-1900 on *ckβ* promoter. There were two overlapping Ets and GATA elements in this region. Mutation of either one of these elements produced significantly higher promoter activities compared to wild-type full length *ckβ* promoter. GATA element at -1959 was a stronger repressive element compared to Ets element at -1966.

Effect of PKC activator and inhibitor on *ckα* and *β* promoters

Phorbol 12-myristate 13-acetate (PMA) is a direct protein kinase C (PKC) activator, it has been shown to stimulate the uptake and incorporation of choline into phosphatidylcholine. As shown in figures 6 and 7, PMA treatment decreased the *ckα* and *β* promoter activities. The maximum effect on *ckα* promoter was observed at 24-hour with 30 ng/ml PMA concentration. *Ckβ* promoter activity was suppressed most significantly at 6 hour treatment with 20 ng/ml PMA. Longer exposure to PMA treatment eliminated the repressive effect on *ckβ* promoter.

In order to determine which PKC isozyme was involved in the down-regulation of *ckα* and *β* promoters by PMA, PKC inhibitors (PKC412 and GO6983) were included in the PMA treatment. Figure 6 (lower panel) shows that the effect of PMA on *ckα* promoter was abrogated by both PKC412 and GO6983. On the contrary, only PKC412 abolished the effect of PMA treatment on *ckβ* promoter (figure 7, lower panel). These results show that PKCζ is likely the PKC isozyme that mediates the effect of PMA treatment on *ckα* promoter activity while PKCε and η are the likely isozymes that affect the *ckβ* promoter.