Cloning and Activity Analysis of Human Choline Kinase Beta Promoter

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ABSTRACT: Choline kinase catalyzes the phosphorylation of choline yielding phosphocholine in the presence of ATP and Mg^{2+}. This is the first enzymatic step committing choline to the *de novo* (CDP-choline) pathway in all animal tissues leading to the biosynthesis of phosphatidylcholine. In human, this enzyme exists in at least three isoforms known as CKα1, CKα2 and CKβ which are encoded by two separate genes termed *CKα* and *CKβ*. Despite the importance of CK in phospholipid synthesis and its implication in multiple cancers, the promoter of the human CK gene has not been analyzed. Only the mouse CK promoter has been characterized and its CKα promoter was found to be regulated by a AP-1 element. The main objective of this study is to clone and establish a reporter system for evaluating the promoter activity of human CKβ gene. 2000 bp of the human CKβ promoter was cloned into a firefly luciferase vector. The firefly luciferase reporter vector, pGL4.10-CKβ and *Renilla* luciferase internal control vector, pGL4.73 were co-transfected into MCF-7 cells. Normalizing the activity of the experimental reporter to the activity of the internal control was done to minimize experimental variability caused by the differences in cell viability or transfection efficiency. Our results showed that the 2000 bp human CKβ promoter was able to drive the luciferase expression in MCF-7 cells.

Keywords: Choline kinase, luciferase expression, MCF-7

Introduction

Choline kinase (CK) was first discovered by Wittenberg and Kornberg in 1953 (Wittenberg & Kornberg 1953). It is important for the generation of two major membrane phospholipids, which are phosphatidylcholine and sphingomyelin, and subsequently for the cell division (Janardhan et al. 2006). It is now generally appears that choline kinase of mammalian cells have at least three isoforms: CKα1, CKα2 and CKβ. Each isoform is not active in its monomeric form. The active enzyme exists as either homo- or hetero-dimeric (or oligodimeric) forms (Aoyama et al. 2004). CK is responsible for the phosphorylation of choline to phosphocholine as the first step of the CDP-choline pathway for the biosynthesis of phosphatidylcholine. It has been recognized as a new target for anticancer therapy. Studies showed that there was an increased of CK enzymatic activity, and consequently the increased levels of phosphatidylcholine in *ras* transformed cells (Malito et al. 2006). CK has been proposed to play a relevant role in the onset of human cancer, because it is found to be overexpressed in breast, lung, colon and prostate tumors. Nuclear magnetic resonance techniques have shown elevated levels of phosphatidylcholine in human tumoral tissues compared to their normal counterparts (Malito et al. 2006; Ramírez et al. 2005). CK is also activated by several growth factors and oncogenes (Ramírez et al. 2005). Despite the importance of CK in phospholipid synthesis and its implication in multiple cancers, the promoter of the human CK gene has not been analyzed. Only the mouse CK promoter has been characterized and its CKα promoter was found to be regulated by the AP-1 element. In this work, the 2000 bp promoter of CKβ was amplified from human genomic DNA and was ligated into a pGL4.10, the experimental vector. The pGL4.10-CKβ construct and pGL4.73, *Renilla* luciferase internal control vector, were co-transfected into MCF-7 cells. The results showed that the pGL4.10-CKβ construct was able to drive the luciferase expression in MCF-7 cells.

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Materials and Methods

Materials

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), Triple Express, penicillin-streptomycin, Lipofectamine 2000 and Opti-MEM I Reduced Serum Medium without serum were obtained from Invitrogen (Carlsbad, California). The restriction enzymes were from

New England Biolabs (USA). 10X ligation buffer for T4 DNA ligase, T4 DNA ligase enzyme, 1 kb DNA ladder and blue dye were from Fermentas (Lithuania). The luciferase vectors, pGL4.10 and pGL4.73, Dual-Glo luciferase assay system were from Promega (Madison, WI). KOD Hot Start DNA polymerase was from Novagen (USA). The primers were from 1st Base oligo (Malaysia). Human genomic DNA was from Roche (Switzerland). QIAquick Gel Extraction Kit was from Qiagen (Germany).

Cell lines and culture condition

The human breast cancer cell line, MCF-7, was kindly provided by Dr. Khoo B.Y. (INFORMM, USM) and was cultured in Dulbecco's modified Eagle's medium (DMEM) with high glucose and supplemented with 10% fetal bovine serum (FBS) and 10000 U/mL penicillin-streptomycin in a 5% CO_2 humidified incubator at 37°C.

Construction of pGL4.10 with human CKβ promoter

The promoter sequence of CKβ was identified from human genome sequence using the BLAT Search Genome. CKβ promoter fragment, -2000/-1, was amplified by PCR with forward primer 5'-CCGCTCGAGATGATGCTTCAGGGCTCC-3', containing an *XhoI* site (underlined) and the reverse primer 5'-CCCAAGCTTGGCGCGGGCTCGACCGGG-3', containing a *HindIII* site (underlined). The promoter fragment was cloned by PCR amplification in a 50 µl reaction mixture using the 1X Buffer for KOD Hot Start DNA Polymerase with 1.5 mM $MgSO_4$, 0.25 mM of each dNTPs, 0.3 mM of each forward and reverse primer, 0.02 U/µl KOD Hot Start DNA Polymerase and 100 ng genomic DNA. PCR amplification conditions were as follows: one cycle of preamplification denaturation at 95°C for 2 min followed by 28 cycles of denaturation at 95°C for 20 s and 10 s of annealing at 58°C and extension at 70°C for 40 s. It was followed by a final extension of 7 min at 70°C. The annealing temperature and/or extension time was changed based on the primer specification and size of the amplification products whenever applicable. Negative control (non-template control) was run together with the sample. The amplified 2000 bp fragment was analysed using 1% agarose gel electrophoresis before the amplicon was extracted and purified using QIAquick Gel Extraction Kit. The purified PCR product was digested using *XhoI* and *HindIII*, and ligated into the upstream of the luciferase gene in the pGL4.10 vector. Both of the double digested pGL4.10 vector and CKβ promoter (DNA insert) were ligated using T4 DNA ligase in a 10.3 µl reaction consisting of 0.8 µl of plasmid, 8 µl of DNA insert, 1 µl of T4 DNA buffer and 0.5 µl of T4 DNA ligase. The reaction was set up in a PCR tube and gently mixed before briefly centrifuged to collect the content. The reaction was incubated overnight at 4°C. The recombinant plasmid, pGL4.10-CKβ was chemically transformed into the *E. coli* XL1-Blue competent cells for the purpose of propagation and verification. The integrity of the clone was verified by sequencing reaction which was carried out by the Centre for Chemical Biology, USM (CCB).

Luciferase assays

The MCF-7 cells were seeded on a 96 well plate in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) in a 5% CO_2 humidified incubator at 37°C. MCF-7 cells were co-transfected with the pGL4.10-CKβ construct (200 ng) and the hRluc/SV40 (20 ng) as an internal control by using 0.5 µl Lipofectamine 2000. 48 hours after transfection, cells were harvested and luciferase assays were performed using the Dual-Glo luciferase assay by using Glomax 20/20 Luminometry system according to the manufacturer's manual (Promega). Firefly luciferase activity was normalized to *Renilla* luciferase activity.

Statistical analysis

Data were analysed statistically using the SPSS Version 15.0 (SPSS Inc., Chicago, IL, USA). The Mann-Whitney U test was employed to determine the statistical difference between sample medians, with the level of significance set at $P < 0.05$. All data were presented as median $\pm$ interquartile range values of four replicates.

Results

Construction of pGL4.10 with human CKβ promoter

A 2000 bp fragment upstream of the CKβ was amplified from human genomic DNA by PCR using the cycling condition as described in the method section. The PCR product was analyzed with1% agarose gel electrophoresis (**FIG. 1**). A band was detected at lane 1 which was located at 2000 bp corresponding to the expected size of the CKβ promoter PCR amplicon. The negative control at lane 2 did not produce any distinct band which ruled out the possibility of a false positive amplification reaction. The clone was verified via restriction enzymes analysis before subjected to sequencing reaction. The gel picture (**FIG. 2**) showed that two bands were detected. The first band located at about 4000 bp was the vector of

pGL4.10 while the second band located at 2000 bp was the DNA insert which was released from the recombinant plasmid upon double digestion.

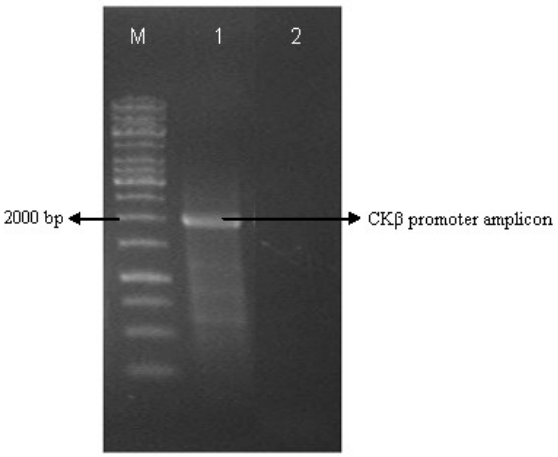

FIG. 1- PCR product visualized by EtBr staining on a 1% TAE agarose gel.
(M: GeneRuler 1 kb DNA ladder; Lane 1: PCR product; Lane 2: negative control)

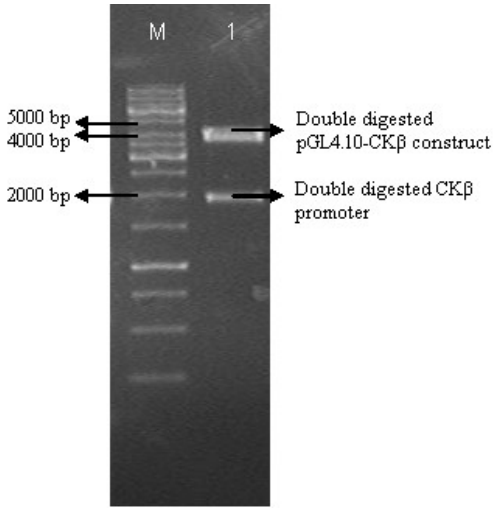

FIG. 2- Double digestion of pGL4.10-CKβ construct visualized by EtBr staining on a 1% TAE agarose gel.
(M: GeneRuler 1 kb DNA ladder; Lane 1: Double digestion reaction)

Promoter activity analysis

The pGL4.10-CKβ construct was transiently transfected into the MCF-7 cell line (human breast cancer cells). Normalizing the activity of the experimental reporter to the activity of the internal control was done to minimize the experimental variability caused by the differences in cell viability or transfection efficiency. Promoter activities presented in **FIG. 3** was the ratio of the construct promoter activity (RLU1) divided by the *Renilla* activity (RLU2). The 2000 bp human CKβ promoter was shown to be able to drive the luciferase expression in MCF-7 cell (Fig. 3) as indicated by the approximately 40 folds higher activity compared to the promoterless negative control vector (P<0.05).

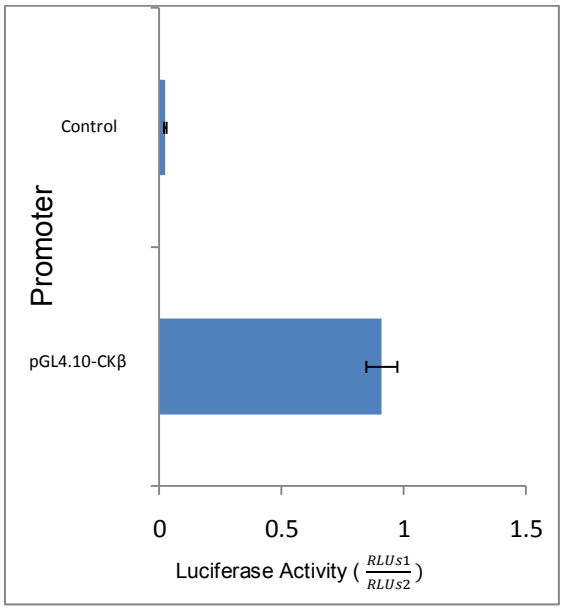

FIG. 3- Promoter activity analysis of human CKβ promoter in MCF-7 cells. pGL4.10-CKβ construct (200 ng) was co-transfected with *Renilla* luciferase (hRluc/SV40, 20 ng) as an internal control into MCF-7 cells. Forty-eight hours after transfection, cells were harvested and assayed for luciferase activity. The data represented the median ± interquartile range. There was a significant difference in terms of luciferase activity between the pGL4.10-CKβ construct and promoterless negative control vector (P<0.05). The significance of the difference was tested using Mann-Whitney U test.

Discussion

Recently, CK has been implicated in cell proliferation by playing an important role in mitogenic signal transduction pathways (de Molina et al. 2004). Elevated phosphocholine level is a common characteristic feature in cell lines derived from human tumors and this parameter seems to be able to distinguish the malignant cell lines from the normal cell lines irrespective of their proliferation rates (Bhakoo et al. 1996; Aboagye & Bhujwalla 1999). This fact indicates that the activation of CK in these malignant cells does not co-operate with the net phosphatidylcholine biosynthesis, but that it leads to the accumulation of intracellular phosphocholine. Although the elevated levels of phosphocholine have been investigated in a variety of different types of human tumors (breast, lung, colon, colectal and prostate) as well as in diverse murine tumors (Liu et al. 2002), the exact role of phosphocholine in these maglinant phenotypes still

remains to be clarified. Recently, CK 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 (Janardhan et al. 2006).

The results from our study showed that the promoter of CKβ was successfully cloned into the upstream of firefly luciferase reporter vector, pGL4.10, to generate the pGL4.10-CKβ construct. Transfection studies showed that the 2000 bp length of CKβ promoter sequence was found to be active, and was able to drive the luciferase expression in MCF-7 cells as indicated by the approximately 40 folds higher activity compared to the promoterless negative control vector ($P<0.05$).

Promoters are the best-characterized transcriptional regulatory sequences in complex genomes because of their predictable location immediately upstream of the transcription start site (TSS). They are often described as having two separate segments: the core and the extended regions (Cooper et al. 2006). Transcription of a eukaryotic protein-coding gene is preceded by multiple events; these include decondensation of the locus, nucleosome remodeling, histone modifications, binding of the transcriptional activators and coactivators to enhancers and promoters, and recruitment of the basal transcription machinery to the core promoter (Smale & Kadonaga 2003). However, only a few nucleotides within a promoter are absolutely necessary for its function (Baliga 2001). The transcriptional of a gene is regulated by numerous trans-acting protein factors that interact with specific cis-acting elements. Changes in these interactions occur during the transition from an inactive to an active state and are often accompanied by alterations in cytosine methylation (Schwemmle et al 1997). Eukaryotic transcription factors can be classified into several families base on the conserved sequences among their DNA-binding domains. Because of such structural conservation, several different trans-acting factors can often interact with a common binding site. Recent 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). Hence, identification and characterization of these elements are vital to understand the complex network of human gene regulation.

In conclusion, the 2000 bp CKβ promoter was found to be active in MCF-7 cells when compared to the promoterless negative control vector ($P<0.05$). 5' deletion analysis of CKβ promoter is yet to be determined but is currently under investigation. 5' deletion analysis will be performed by varying the lengths of the CKβ promoter (500 bp, 1000 bp and 1500 bp) to elucidate the regulatory regions within the promoter.

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References

1. Aboagye, E.O. and Bhujwalla, Z.M. (1999). Malignant transformation alters membrane choline phospholipid metabolism of human mammary epithelial cells. *Cancer Res.* 59: 80-84.
2. Aoyama, C., Liao, H. and Ishidate, K. (2004). Structure and function of choline kinase isoforms in mammalian cells. *Progress in Lipid Research*, 43: 266-281.
3. Baliga, N.S. (2001). Promoter analysis by saturation mutagenesis. *Biol Proced Online,* 3: 64-69.
4. Bhakoo, K.K., Williams, S.R., Florian, C.L., Land, H. and Noble, M.D. (1996). Immortalization and transformation are associated with specific alterations in choline metabolism. *Cancer Res*., 56: 4630-4635.
5. Cooper, S.J., Trinklein, N.D., Anton, E.D., Nguyen, L., Myers, R.M. (2006). Comprehensive analysis of transcriptional promoter structure and function in 1% of the human genome. *Genome Res*., 16(1): 1-10.
6. de Molina, A.R., Báñez-Coronel, M., Gutiérrez, R., Rodríguez-González, A., Olmeda, D., Megías, D. and Lacal, J.C. (2004). Choline kinase activation is a critical requirement for the proliferation of primary human mammary epithelial cells and breast tumor progression. *Cancer Research*, 64: 6732-6739.
7. Janardhan, S., Srivani, P. and Sastry, G.N. (2006). Choline kinase: an important target for cancer. *Curr. Med. Chem.* 13: 1169-1186.
8. Karin, M. (1990). *Too* many transcription factors: positive and negative interactions. *New Biol*, 2(2): 126-31.
9. Liu, D., Hutchinson, O.C., Osman, S., Price, P., Workman, P. and Aboagye, E.O. (2002) .Use of radiolabelled choline as a pharmacodynamic marker for the signal transduction inhibitor geldanamycin. *Br. J Cancer*, 87: 783-789.

10. Malito, E., Sekulic, N., See Too, W.C., Konrad, M. and Arnon, L. (2006). Elucidation of human choline kinase crystal structures in complex with the products ADP or phosphocholine. *J Mol Biol*, 364: 136-151.
11. Ramírez, A.M., Gallega, D.O., Sarmentero, J., Bañez, M.C., Martín, Y.C. and Carlos, J.L. (2005). Choline kinase is a novel oncogene that potentiates RhoA-induced carcinogenesis. *Cancer Res*, 65: 5647-53.
12. Schwemmle, S., de Graaff, E., Deissler, H., Glaser, D., Wohrle, D., Kennerknecht, I., Just, W., Oostra, B.A., Doerfler, W., Vogel, W., and Steinbach, P. (1997). Characterization of FMR1 promoter elements by in vivo-footprinting analysis. *Am J Hum Genet*, 60(6): 1354-62.
13. Smale, S.T. and Kadonaga, J.T. (2003). The RNA polymerase II core promoter. *Annu Rev Biochem*, 72: 449-79.
14. Wittenberg, J. and Kornberg, A. (1953). Choline phosphokinase, *J. Biol. Chem.* 20 (2): 431-444.