

UNIVERSITI SAINS MALAYSIA UNIVERSITI SAINS MALAYSIA UNIVERSITI PENYELIDIKAN UNIVERSITI PENYELIDIKAN LAPORAN AKHIR

EFFECTS OF PROTEIN KINASE A PHOSPHORYLATION ON THE BIOCHEMICAL PROPERTIES AND SUBCELLULAR LOCATION OF HUMAN CHOLINE KINASE BETA

PENYELIDIK

ASSOC. PROF. DR. SEE TOO WEI CUN

PENYELIDIK BERSAMA

PROFESOR SHAHARUM SHAMSUDDIN ASSOC. PROF. DR. FEW LING LING

2016

RU GRANT FINAL REPORT CHECKLIST

RUJUKAN

Please use this checklist to self-assess your report before submitting to RCMO. Checklist should accompany the report.

		PLE	PLEASE CHECK (~)		
NO.	ITEM	PI	JKPTJ	RCMO	
1	Completed Final Report Form	~	~	\checkmark	
2	Project Financial Account Statement (e-Statement)	~	~	\checkmark	
3	Asset/Inventory Return Form (Borang Penyerahan Aset/Inventori)	~	Hera	~	
4	A copy of the publications/proceedings listed in Section D(ii) (Research Output)	~	~	\checkmark	
5	Comprehensive Technical Report	~	~	\checkmark	
6	Other supporting documents, if any	~	. /	\checkmark	
7	Project Leader's Signature	~	1	\checkmark	
8	Endorsement of PTJ's Evaluation Committee		1	\checkmark	
9	Endorsement of Dean/ Director of PTJ's		1	\checkmark	

AMOANTAT

menghasilkan bukti yang menyokong kemungkinan pengawalaturan CKβ di dalam sel oleh PKA dan agen pengaktifannya, cAMP. Kesan pemfosforilan PKA terhadap aktiviti dan perencatan enzim juga penting untuk strategi antikanser yang menyasarkan keluarga enzim CK.

English

Choline kinase (CK) is the first enzyme in CDP-choline pathway for the synthesis of phosphatidylcholine. In mammals, CK exists as CK α 1, α 2 and β isoforms. Increased activity of CK has been implicated in carcinogenesis and CK β knockout mice developed severe muscular dystrophy. Despite the importance of CK in phospholipid biosynthesis and its involvement in cancer and animal development, regulation of human CK by post-translational modifications including phosphorylation have received very little attention. Earlier, we discovered that CK β is phosphorylated by PKA at serine residues 39 and 40. This study aims to investigate the effect the phosphorylation on the catalytic properties, subcellular location and tertiary structure of CK β . The results showed that phosphorylated CK β had higher catalytic efficiency with choline and ATP as substrates. The phosphorylation also increased the sensitivity of CK β to inhibition by hemicholinium-3. PKA phosphorylation did not alter the oligomeric structure and subcellular location of CK β . This study provides the evidence for possible regulation of CK β by PKA and its activator cAMP in the cell. The effect of PKA phosphorylation on enzyme activity and inhibition is also important for anticancer strategy that targets CK family of enzymes.

С	BUDGET & EXPENDITURE		na Ser and September (Provident)
i	Total Approved Budget	: RM 162	127.87
		Yearly E	Budget Distributed
		Year 1	: RM 58376.62
		Year 2	: RM 50949.63
		Year 3	: RM 52801.62
	Total Expenditure	: RM 1533	300.51
	Balance	: RM 8827	7.36
	Percentage of Amount Spent (%)	: 95%	

Please attach final account statement (eStatement) to indicate the project expenditure

ii | Equipment Purchased Under Vot 35000

No.	Name of Equipment	Amount (RM)	Location	Status
1	Canon Printer LBP 7200CD	2050	School of Health Sciences	Operational (In-use)

Please attach the Asset/Inventory Return Form (Borang Penyerahan Aset/Inventori) – Appendix 1

ject C	bjectives (as stated/approved in the project proposal)	
No.	Project Objectives	Achievement
1	To determine the effects of PKA phosphorylation on the activity, substrate selectivity and inhibition of $CK\beta$	100%
2	To determine the subcellular locations of wild-type and PKA -phosphorylation-mutant $CK\beta$	100%
3	To investigate the effect of PKA phosphorylation on the interaction between $CK\beta$ and $CK\alpha$	100%
4	To determine the effect of PKA phosphorylation on the oligomeric state of $CK\beta$	100%
5		
6		

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i Research Output

a) Publications in ISI Web of Science/Scopus

No.	Publication (authors,title,journal,year,volume,pages,etc.)	Status of Publication (published/accepted/ under review)
7	Chee Sian Kuan, Yoke Hiang Yee, Wei Cun See Too and Ling Ling Few (2015). Ets and GATA transcription factors play a critical role in PMA-mediated repression of the $ck\beta$ promoter via the protein kinase C signaling pathway. PLOS ONE.	Published (impact factor: 3.234)
3	Chee Sian Kuan, Wei Cun See Too and Ling Ling Few (2016). Sp1 and Sp3 are the transcription activators of human <i>ek1</i> promoter in TSA-treated human colon carcinoma cells, HCT116. PLOS ONE.	Published (impact factor: 3.234)
3	Chua Siang Ling, Khoo Boon Yin, See Too Wei Cun and Few Ling Ling (2015). Expression profiling of choline and ethanolamine kinases in MCF-7, HCT-116 and HepG2 cells, and the transcriptional regulation by epigenetic modification. Molecular Medicine Reports. 11: 611-618.	Published (impact factor: 1.554)

b) Publications in Other Journals

No.	Publication (authors,title,journal,year,volume,pages,etc.)	Status of Publication (published/accepted/ under review)

c) Other Publications

(book, chapters in book, monograph, magazine, etc.)

No.	Publication (authors,title,journal,year,volume,pages,etc.)	Status of Publication (published/accepted/ under review)

	No.	Conference (conference name,date,place)	Title of Abstract/Article	Level (International/National)				
	1	UMT 12th International Annual Symposium	Prediction of miRNAs that bind human choline and ethanolamine kinase mRNAs	International				
	2	UMT 12th International Annual Symposium	Expression, purification and homology modeling of <i>Entamoeba</i> <i>histolytica</i> choline kinase	International				
# Please attach a full copy of the publication/proceeding listed above								
Other Research Ouput/Impact From This Project (patent, products, awards, copyright, external grant, networking, etc.)								
On	e exter	nal grant from FRGS has been o	btained by Assoc. Prof. Dr. Few Ling	Ling (co-researcher)				
Clo	Close collaboration with Dr. Manfred Konrad from Max Planck Institute for Biophysical Chemistry, Goettinger Germany has been established.							
Ge								

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a)	Graduated Human Capital					
	Chudent	Nation	ality (No.)	Nama		
	Student	National	International	Name		
	PhD	1		1. Chang Ching Ching 2.		
	MSc	1		1. Nur Farah Hani Azemi 2.		
	Undergraduate	2		1. Lai Siew Ing 2. Simon Hoi Theng Pang		

b) On-going Human Capital

Student	Nationality (No.)		Namo	
Student	National	International	Name	
PhD			1. 2.	
MSc	1		1. Sharzehan binti Mohamad Ayub Khan 2.	
Undergraduate			1. 2.	

c) Others Human Capital Nationality (No.) Name Student International National 1. Post Doctoral Fellow 2. 1. **Research Officer** 2. 1. Tan Lii Lian 2 **Research Assistant** 2. Kuan Chee Sian 1. Others (.....) 2. **COMPREHENSIVE TECHNICAL REPORT** Applicants are required to prepare a comprehensive technical report explaining the project. The following format should be used (this report must be attached separately): Introduction . Objectives . Methods .

- Results
- Discussion
- Conclusion and Suggestion
- Acknowledgements
- References

G	PROBLEMS/CONSTRAINTS/CHALLENGES IF ANY		
-	(Please provide issues arising from the project and how they were resolved)		
	Nil		
Ĥ	RECOMMENDATION		
1.4	(Please provide recommendations that can be used to improve the delivery of information, grant management, guidelines and policy, etc.)		
	Nil		





PUSAT PENGAJIAN SAINS KESIHATAN SCHOOL OF HEALTH SCIENCES

No. Rujukan : PPSK(R&D/LG(bil. 268)

Tarikh : 24 Mac 2016

Profesor Dr. Lee Keat Teong Pengarah Pejabat Pengurusan & Kreativiti Penyelidikan (RCMO) Bahagian Penyelidikan & Inovasi Tingkat 2, Bangunan Canselori 2 Bangunan E42 Universiti Sains Malaysia 11800 Pulau Pinang (U/P En. Muhamad Nizam – Pegawai Sains)

Y.Bhg. Profesor Dr.,

LAPORAN PROJEK GERAN RUI

Dengan segala hormatnya saya merujuk kepada perkara di atas.

2. Bersama-sama ini saya sertakan 01 salinan laporan bagi projek penyelidikan yang berikut.:-

Bil.	Nama	Tajuk	Catatan
1.	Profesor Madya	Effects of protein kinase A phosphorylation on the	Laporan akhir
	Dr. See Too Wei	biochemical properties and subcellular location of	
	Cun	human choline kinase beta	
		(1001/PPSK/815101)	

3. Diharapkan ianya mendapat perhatian untuk rekod pejabat Y.Bhg. Profesor Dr.

Sekian. Terima kasih.

BERKHIDMAT UNTUK NEGARA

Memastikan Kelestarian Hari Esok

Yang benar, SURIATI WAN WAN

Pegawai Sains Kanan Unit Penyelidikan

s.k. Profesor Madya Dr. See Too Wei Cun

Ph. Wins Utte securation dan tindakan laijut. TE. Aunt 1 4 16



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RESEARCH ARTICLE

Ets and GATA Transcription Factors Play a Critical Role in PMA-Mediated Repression of the $ck\beta$ Promoter via the Protein Kinase C Signaling Pathway

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Abstract

Background: Choline kinase is the most upstream enzyme in the CDP-choline pathway. It catalyzes the phosphorylation of choline to phosphorylcholine in the presence of ATP and Mg²⁺ during the biosynthesis of phosphatidylcholine, the major phospholipid in eukaryotic cell membranes. In humans, choline kinase (CK) is encoded by two separate genes, $ck\alpha$ and $ck\beta$, which produce three isoforms, CK α 1, CK α 2, and CK β . Previous studies have associated $ck\beta$ with muscle development; however, the molecular mechanism underlying the transcriptional regulation of $ck\beta$ has never been elucidated.

Methodology/Principal Findings: In this report, the distal promoter region of the $ck\beta$ gene was characterized. Mutational analysis of the promoter sequence and electrophoretic mobility shift assays (EMSA) showed that Ets and GATA transcription factors were essential for the repression of $ck\beta$ promoter activity. Supershift and chromatin immunoprecipitation (ChIP) assays further identified that GATA3 but not GATA2 was bound to the GATA site of $ck\beta$ promoter. In addition, phorbol-12-myristate-13-acetate (PMA) decreased $ck\beta$ promoter activity through Ets and GATA elements. PMA also decreased the $ck\beta$ mRNA and protein levels about 12 hours after the promoter activity was down-regulated. EMSA further revealed that PMA treatment increased the binding of both Ets and GATA transcription factors to their respective DNA elements. The PMA-mediated repressive effect was abolished by chronic PMA treatment and by treatment with the PKC inhibitor PKC412, but not the PKC inhibitor Go 6983, suggesting PKCε or PKC η as the PKC isozyme involved in the PMA-mediated repression of $ck\beta$ promoter. Further confirmation by using PKC isozyme specific inhibitors identified PKC ε as the isozyme that mediated the PMA repression of $ck\beta$ promoter.



OPEN ACCESS

Citation: Kuan CS, Yee YH, See Too WC, Few LL (2014) Ets and GATA Transcription Factors Play a Critical Role in PMA-Mediated Repression of the $ck\beta$ Promoter via the Protein Kinase C Signaling Pathway. PLoS ONE 9(12): e113485. doi:10.1371/ journal.pone.0113485

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Competing Interests: The authors have declared that no competing interests exist.

Conclusion/Significance: These results demonstrate the participation of the PKC signaling pathway in the regulation of $ck\beta$ gene transcription by Ets and GATA transcription factors.

Introduction

Choline kinase (CK) (EC 2.7.1.32) uses Mg^{2+} as a cofactor to catalyze the ATPdependent phosphorylation of choline, yielding phosphocholine (PCho) and ADP. CK commits choline to the Kennedy pathway for the *de novo* biosynthesis of phosphatidylcholine (PC) [<u>1</u>]. PC, also known as lecithin, is the most abundant membrane phospholipid (40–60%) in eukaryotic cells [<u>1</u>]. Apart from being a component of the membrane architecture, PC has also been linked to mitogenic signaling by serving as a substrate for the production of mitogenic signaling molecules, such as diacylglycerol (DAG) and phosphatidic acid (PA), which are generated from the hydrolysis of PC by phospholipase C and phospholipase D, respectively [<u>2</u>, <u>3</u>].

CK was first discovered as a cytosolic enzyme in brewer's yeast [4]. It was later found in other organisms, ranging from yeast to mammals [5, 6]. In humans, CKs are encoded by two separate genes, $ck\alpha$ and $ck\beta$. Based on their high sequence homology, $ck\alpha$ and $ck\beta$ might have appeared after genetic duplication from a common ancestor [7]. The expression of these two genes results in three different proteins, CK α 1 (439 amino acids; NCBI accession number NP_997634), CK α 2 (457 amino acids; NCBI accession number NP_001268), and CK β (395 amino acids; NCBI accession number NP_005189) [8]. CK α 1 and CK α 2 are splice variants derived from the primary $ck\alpha$ mRNA, while CK β is a separate product of the $ck\beta$ gene [8].

Apart from its traditional role in phospholipid biosynthesis, CK β is also involved in muscle development [9, 10]. Deletion of the murine $ck\beta$ gene causes rostrocaudal muscular dystrophy (RMD), which is characterized by neonatal forelimb bone deformity and progressive muscle wasting [9, 10]. RMD mice display a high abundance of abnormally enlarged mitochondria at the periphery of cells [11]. In $ck\beta$ knockout mice, the decreased levels of PC, along with the decreased ATP synthesis caused by deficiencies in complex III of the electron transport chain, result in mitochondrial dysfunction and subsequent significant mitochondrial loss through mitophagy [11]. In humans, genetic mutation of the $ck\beta$ gene and consequent altered PC biosynthesis is the primary cause of congenital muscular dystrophy (CMD). CMD patients have reduced levels of PC and CK activity due to a genetic defect in the $ck\beta$ gene [12].

Despite the importance of $ck\beta$ in PC synthesis, mitochondrial function, and muscular dystrophy, literature describing the transcriptional regulation of the $ck\beta$ gene is still lacking. Important *cis*-acting regulatory elements of the $ck\beta$ gene promoter have yet to be identified. The molecular mechanisms that regulate $ck\beta$ expression can be elucidated by characterization of the $ck\beta$ promoter. Previous studies in various mammalian cells showed that phorbol esters stimulate the incorporation of choline into PC [13, 14]. Phorbol 12-myristate 13-acetate (PMA) is a direct activator of protein kinase C (PKC), and stimulates both the cellular uptake of radiolabeled choline and its incorporation into PC [15, 16]. Previously, we isolated a 2000 bp human $ck\beta$ promoter that was repressed by PMA treatment [17]. In this report, we localized the repressive effect of PMA to the -2000/-1886region upstream of the ATG translation start site, which is bound by Ets and GATA transcription factors. We also demonstrate that PMA exerts its effect on the $ck\beta$ promoter through a PKC-dependent pathway.

Materials and Methods

In silico analysis of the $ck\beta$ promoter region

The 2000 bp upstream region of the $ck\beta$ gene (transcript NM_005198) was analyzed using MatInspector 8.0 [18] and TFSEARCH [19] to identify putative transcription factor binding sites. CpG islands within the $ck\beta$ promoter (the 2000 bp region upstream of the $ck\beta$ gene) were identified using CpGplot [20] and CpGIS [21]. CpGplot defines a CpG island as a DNA region with an observed/ expected ratio >0.60, a length >200 bp, and GC content >50% [20, 22]. CpGIS defines a CpG island as a sequence having an observed/expected ratio >0.65, a length >500 bp, and GC content >55% [21].

Cell culture

The human liver carcinoma cell line HepG2 (ATCC No. HB-8065), the human colorectal carcinoma cell line, HCT116 (ATCC no. CCL-247) and the human breast adenocarcinoma cell line MCF-7 (ATCC No. HTB-22) were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μ g/mL streptomycin. Cells were maintained at 37 °C in a humidified atmosphere of 5% (v/v) CO₂.

Plasmid constructs

The $ck\beta$ promoter fragment -2000/-1 was amplified by PCR from human genomic DNA (Roche, Germany) using a forward primer [$ck\beta$ -2000-5'] and reverse primer ($ck\beta$ -3') that incorporate XhoI and HindIII restriction sites, respectively. The PCR product was cloned in-frame at the 5' XhoI/HindIII site of the promoterless firefly luciferase reporter vector pGL4.10[*luc2*] (Promega, USA), yielding pGL4.10- $ck\beta(-2000/-1)$. The 5'-end deletion constructs of pGL4.10 $ck\beta(-1886/-1)$, pGL4.10- $ck\beta(-1770/-1)$, pGL4.10- $ck\beta(-1651/-1)$, pGL4.10 $ck\beta(-1477/-1)$, pGL4.10- $ck\beta(-914/-1)$, and pGL4.10- $ck\beta(-519/-1)$ were generated by PCR using pGL4.10- $ck\beta(-2000/-1)$ as the template and subcloned into the XhoI/HindIII site of the pGL4.10[*luc2*] vector. The primers used are listed

PLOS ONE

Name	Sequence 5' to 3'	Orientation		
Promoter-luciferase constructs				
ckβ-2000-5'	CCGCTCGAGATGATGCTTCAGGGCTCC	Forward		
<i>ckβ</i> -1886-5′	CCGCTCGAGGCTGTTCAGACAGTCTTGCTG	Forward		
ckβ-1770-5'	CCGCTCGAGGCTGGAACCACAGGCACCTG	Forward		
ckβ-1651-5'	CCGCTCGAGTCGGCTTCCCAAAGTGCAGG	Forward		
ckβ-1477-5'	CCGCTCGAGCGTGTAGCTGGGATTACAGGC	Forward		
ckβ-914-5′	CCGCTCGAGCTCAATGGGAGGTGCGCAGG	Forward		
<i>ckβ</i> -519-5′	CCGCTCGAGTGGACGGCTCTTCCTTGTCGG	Forward		
ckβ-3'	CCCAAGCTTGGCGCGGGCTCGACCGGG	Reverse		
Site-directed mutagenesis				
mut-Ets	CCGCTCGAGATGATGCTTCAGGGCTCCTGGAAACAGTGTCAGCTCgtAcgCTGTATCAGCCCTTC	Forward		
mut-GATA	CCGCTCGAGATGATGCTTCAGGGCTCCTGGAAACAGTGTCAGCTCAGATCCTGTgcCAttCCTTCAAAGACCTAG	Forward		
mut-Ets/GATA	CCGCTCGAGATGATGCTTCAGGGCTCCTGGAAACAGTGTCAGCTCgtAcgCTGTgcCAttCCTTCAAAGACCTAG	Forward		
ckβ-3'	CCCAAGCTTGGCGCGGGCTCGACCGGG	Reverse		
Real-time PCR				
ckβ-Fwd	ATGTTCGCCATACTTGCGGA	Forward		
ckβ-Rvr	AATTGCGCCATCTTCGTGG	Reverse		
UBC-Fwd	TTCTTGATCCCCAATGCTTC	Forward		
UBC-Rvr	AGTTAAGGGCCAGACCCAGT	Reverse		
YWHAZ-Fwd	CTGATCAGCAGAGGTTGATCTT	Forward		
YWHAZ-Rvr	GTCTTGCCAGTGAGTGTCTT	Reverse		
ChIP				
ckβ-2000-5'	CCGCTCGAGATGATGCTTCAGGGCTCC	Forward		
ckβ-Distal-3'	CCAGCCTGAGCAACACAGC	Reverse		

Table 1. Primers used for cloning of promoter-luciferase constructs, PCR-based site-directed mutagenesis, real-time PCR and ChIP.

The mutations introduced into the binding sites are italicized and in lowercase.

doi:10.1371/journal.pone.0113485.t001

in <u>Table 1</u>. All deletion mutant constructs generated by PCR were verified by DNA sequencing.

Site-directed mutagenesis

Mutation of the Ets and GATA consensus binding sites was carried out by onestep PCR mutagenesis using Platinum Pfx DNA polymerase (Invitrogen, USA). Primers used to introduce the mutations are shown in <u>Table 1</u>. The pGL4.10mut(Ets), pGL4.10-mut(GATA), and pGL4.10-mut(Ets/GATA) mutant constructs were verified by DNA sequencing.

Transient transfection and luciferase assay

Transfection was performed using Lipofectamine 2000 (Invitrogen/Life Technologies) according to the manufacturer's instructions. Briefly, MCF-7 cells were plated in 100 μ L of medium/well on a 96-well plate at a density of 1.5×10^4

PMA-Mediated Repression of ckβ Promoter

cells/well for 24 hr. The cells in each well were transfected with 200 ng of pGL4.10[luc2] or the various promoter-luciferase constructs and 2.5 ng of the pGL4.73[hRluc/SV40] (Promega) vector, as the control for transfection efficiency. Forty-eight hours after transfection, cells were harvested and assayed using the Dual-Glo Luciferase Reporter Assay System (Promega). The luminescent signals were measured on a GloMax 20/20 luminometer (Promega, USA). The luciferase assays were performed in triplicate in three independent experiments.

PMA, PKC412, Go 6983 and PKC isozyme specific inhibitors treatments

MCF-7 cells were seeded in 96-well plates overnight and then transfected with pGL4.10- $ck\beta(-2000/-1)$ and pGL4.73[hRluc/SV40]. Six hours after transfection, the culture medium was replaced with 1% (v/v) serum-free DMEM for 20 hr before stimulation. The cells were treated with 10, 20, or 30 ng/mL PMA (EMD Chemicals, USA) for 6 hr, except for the time-course study, in which cells were treated with 20 ng/mL PMA for 6 to 48 hr. For controls, DMSO was added to the cells instead of PMA. For the identification of PKC isozyme involved in the PMAmediated repression of the $ck\beta$ promoter, MCF-7 cells were individually or cotreated with PMA (20 ng/mL) and a PKC inhibitor [1 mM PKC412 (Tocris Bioscience, USA), 0.1 mM Go 6983 (Tocris Bioscience, USA), 10 µM PKCE inhibitor peptide (Santa Cruz, USA) or 10 µM PKCŋ pseudo-substrate inhibitor (Santa Cruz, USA)] for 6 hr. After the treatments, luciferase activities were measured using the Dual-Glo luciferase assay. To determine whether the PMA response element is localized to the Ets and GATA binding sites, pGL4.10mut(Ets), pGL4.10-mut(GATA), and pGL4.10-mut(Ets/GATA) mutant constructs were individually transfected into MCF-7 cells, followed by incubation with 20 ng/mL PMA for 6 hr before luciferase activity was determined.

Quantitative real-time PCR of $ck\beta$

Total RNA from PMA treated (20 ng/mL for 6 hr) or untreated (DMSO) cells were extracted using the RNeasy Mini Kit (Qiagen, USA) according to the manufacturer's instructions. One microgram of RNA was reverse transcribed by using the RevertAid H Minus First Strand cDNA synthesis kit (MBI Fermentas, USA). The mRNA level of $ck\beta$ was measured by quantitative real-time PCR (qPCR) performed with an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, USA). The intron-spanning primers used to amplify $ck\beta$ and the primers for the reference genes (UBC and YWHAZ) are listed in <u>Table 1</u>. The PCR efficiencies for all the primers were higher than 90%. Each reaction was performed in a 25 µL volume containing 12.5 µL Power SYBR Green I Master Mix (Applied Biosystems, USA), 300 nM of each primer and 1 µL of 1:2 diluted cDNA as template. The cycling program was the default settings of the ABI PRISM 7000 SDS software as follows: 2 min at 50 °C, 10 min at 95 °C, followed by 40 cycles of 10 sec at 95 °C and 1 min at 60 °C. Melting curve analysis was carried out immediately after the amplification with temperatures ranging from 60 to 95 °C in 0.1 °C increments to verify the PCR specificity. The obtained Ct value was normalized with the geometric mean of UBC and YWHAZ reference genes [23]. The expressions of $ck\beta$ gene in PMA treated cells relative to control (DMSO treated) cells were calculated by the $\Delta\Delta Ct$ method whereby $\Delta\Delta Ct = (Ct_{target gene, test sample} - Ct_{endogenous control, test sample}) - (Ct_{target gene, calibrator sample} - Ct_{endogenous control, test sample}) - (24].$

Western detection of CKB

Thirty micrograms of cell lysate were separated on 10% SDS-PAGE and electroblotted at 13 volts for 2 hr onto nitrocellulose membrane. After the transfer step, the nitrocellulose membrane was immersed in blocking buffer (10 mM Tris-HCl, pH 7.5 containing 5% (w/v) skim milk, 150 mM NaCl and 0.1% (v/v) Tween 20) for 1 hr with gentle agitation at room temperature. Next, the membrane was incubated with 1:1000 diluted CK β antibody [2] in the blocking buffer at 4 °C for overnight. The membrane was washed 3 times with Tris-buffered saline for 1 hr before incubation with 1:5000 diluted HRP-conjugated rabbit IgG secondary antibody for 1 hr at room temperature. Subsequently, the membrane was washed 3 times for 30 min with Tris-buffered saline and the signal was detected with SuperSignal West Pico chemiluminescent substrate (Thermo Scientific, USA) and Fusion FX luminescence detector system (Vilber Lourmat, France). For loading control, the same membrane was stripped with stripping buffer (200 mM glycine, pH 2.5 containing 0.1% (w/v) SDS and 1% (v/v) Tween 20) and re-probed with 1:1000 diluted β -actin antibody (Abcam, USA). Signal intensities on the blots were analyzed by using ImageJ version 1.49b software (downloaded from http://imagej.nih.gov/ij/). The integrated densities of bands were measured in triplicate and the average values were then normalized to the corresponding integrated densities of β -actin signals.

Electrophoretic mobility shift assays (EMSAs)

Nuclear extract from MCF-7 cells was prepared using the NE-PER nuclear and cytoplasmic extraction kit (Pierce, USA) according to the manufacturer's protocol. All DNA probes used in EMSAs were synthesized by 1st Base (Malaysia) and labeled with biotin using a biotin 3' end DNA labeling kit (Pierce). Complementary DNA probes were annealed with each other at a 1:1 molar ratio by heating at 95 °C for 5 min, followed by a gradual decrease to room temperature using a thermocycler set to decrease 1 °C per cycle. EMSAs were performed using the LightShift chemiluminescent EMSA kit (Pierce). The binding reaction mixture contained 200 fmol biotin-labeled probe, 10 μ g of nuclear extract, 1 × binding buffer, 2.5% glycerol, 5 mM MgCl₂, 1 μ g poly(dI–dC), 2 μ g BSA, and 0.05% NP-40. For the competition assay, 100-fold unlabeled double-stranded DNA was added into the reaction mixture. DNA probes used in EMSAs are listed in Table 2. For the supershift assay, 5 μ g of GATA2 (Abcam, United Kingdom) or GATA3

(Millipore, USA) antibody was added into the reaction mixture and incubated on ice for 30 min prior the addition of the biotinylated probe. DNA-protein complexes were resolved by electrophoresis on a 6% (w/v) non-denaturing polyacrylamide gel in $0.5 \times$ Tris-borate-EDTA (TBE) at 4°C. The gel was run until the bromophenol blue dye has reached 3/4 of the length of the gel for EMSA or until the dye has completely migrated out of the gel for supershift assay (to increase the possibility of detecting slower-migrating complexes). The biotinlabeled DNA was electro-blotted onto a Biodyne B nylon membrane (Pierce) and the membrane was cross-linked by a UV transilluminator (Spectronics, USA) at 312 nm for 15 min. The signal was then developed with a chemiluminescent nucleic acid detection module (Pierce). The images were developed by exposing the signal to X-ray film for 2–5 min or detected with Fusion FX luminescence detector system (Vilber Lourmat, France).

Chromatin immunoprecipitation (ChIP)

Chromatin immunoprecipitation (ChIP) assay was performed using Pierce Agarose ChIP Kit (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. MCF-7 cells were treated with 1% (v/v) formaldehyde (Sigma-Aldrich, USA) for 10 min to induce protein-DNA cross-linking. Glycine solution $(1 \times)$ was added to the mixture, incubated for 5 min and the cells were washed with PBS twice. The cells were harvested by scraping, then pelleted and resuspended in 400 µL of lysis buffer. Samples were centrifuged and the nuclear pellet was mixed with 400 µL of micrococcal nuclease (MNase) solution at 37 °C for 15 min. The reaction was stopped by adding 40 µL of MNase Stop Solution and the samples were centrifuged to prepare the supernatants (digested chromatin) for immunoprecipitation. The DNA-protein complexes were immunoprecipitated at 4°C for overnight with 10 µg of GATA2 or GATA3 antibody. Immunoprecipitation with 2 μ L of pre-immune normal rabbit IgG was used as control. DNA recovered from the immunoprecipitated samples was PCR amplified by using $ck\beta$ -2000-5' and $ck\beta$ -Distal-3' primers (Table 1) followed by agarose gel electrophoresis. The positive PCR product was verified by sequencing.

Table 2. DNA probes used in EMSA. The Ets and GATA core sequences are underlined.

Name	Sequence 5' to 3'	References
Biotin-ckβ-Ets/GATA	TGTCAGCTCAGATCCTGTATCAGCCCTTCAAAG	This study
Biotin-ckβ-mut-GATA	TGTCAGCTCAGATCCTGTGCCATTCCTTCAAAG	This study
Biotin- <i>ckβ</i> -mut-Ets	TGTCAGCTCGTACGCTGTATCAGCCCTTCAAAG	This study
Biotin-ckβ-mut-Ets/GATA	TGTCAGCTCGTACGCTGTGCCATTCCTTCAAAG	This study
Ets consensus probe	GGCCAAGCC <u>GGAA</u> GTGAGTGC	[25]
Ets/GATA consensus probe	GATCTCCGGCAACTGATAAGGATTCCCCTG	[26, 27, 28]

loi:10.1371/journal.pone.0113485.t002

Statistical analysis

Data were analyzed using Student's *t*-test or one-way ANOVA, followed by the Tukey Honestly Significant Differences (HSD) *post-hoc* test. The analysis was performed using PASW Statistics 18, Release Version 18.0.0 (SPSS, Inc., 2001, USA). Data are presented as the mean \pm SEM of at least three independent experiments.

Results

The human $ck\beta$ promoter is a TATA-less, CpG island-containing promoter

A 2000 bp region 5' of the human $ck\beta$ gene was first analyzed in silico. For convenient description hereafter, the nucleotide A at the ATG translational start site of the $ck\beta$ gene was designated +1, and nucleotides upstream of +1 were assigned negative numbers. The putative promoter sequence was analyzed for potential transcription factor binding sites using MatInspector 8.0 and TFSEARCH (Fig. 1). The promoter region contains several potential binding sites for Sp1, Ets, GATA factors, SREBP, MZF1, NF-κB, AP-1, and E2F (Fig. 1). The analysis showed that the $ck\beta$ promoter contains neither a CAAT box nor a recognizable consensus TATA box in close proximity to the transcription start site, which is typical of GC-rich promoters. The presence of numerous Sp1 binding sites indicates that the $ck\beta$ promoter contains a high GC content. Thus, CpGPlot and CpGIS were used to identify potential CpG island(s) in the $ck\beta$ promoter. Two potential CpG islands were identified by CpGPlot (Fig. 2), spanning the region between -989 and -713 (percent GC content: 59.21% and observed/expected ratio: 0.73) and the region between -666 and -56 (percent GC content: 71.52% and observed/expected ratio: 0.94). CpGIS analysis identified one potential CpG island covering both the putative CpG islands that were predicted by CpGPlot. This CpG island is 1085 bp long, stretches from -1085 to -1, and has a GC percentage of 65.6% and an observed/expected ratio of 0.864.

Repressive regions are identified at the $ck\beta$ promoter

To identify the important *cis*-acting regulatory elements in the $ck\beta$ promoter, various fragments of the $ck\beta$ promoter were cloned upstream of the firefly luciferase reporter vector. An initial deletion analysis of the $ck\beta$ promoter was performed with four $ck\beta$ promoter constructs [pGL4.10- $ck\beta(-2000/-1)$, pGL4.10- $ck\beta(-1477/-1)$, pGL4.10- $ck\beta(-914/-1)$, and pGL4.10- $ck\beta(-519/-1)$] that contain promoter fragments of different lengths. Two repressive regions were identified in the first deletion analysis (Fig. 3A). Deletion of the 5' sequence from the parental promoter activity compared to that of the full-length 2000 bp promoter. Deletion of the region between -519 to -914 increased the promoter activity by 66%. This result showed that there is at least one inhibitory element

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TGATGCTTCAGGGCTCCTGGAAACAGTGTCAGCTCAGATCCTGTATCAGCCCTTCAAAG	-1941
	-1881
	-1821
	-1761
GATA	1,01
CAGGCACCTGCCACCACGCCCAGCTAATCTTTTAATTTTTGTAAAGACAGGTTCTTGCTA	-1701
Snl	
CTTGCCCAGGCTGGTCTCAAACTCCTGGGCTCAAACGATCCTCCTACTTCGGCTTCCCA	-1641
GATA C/EBP	
	-1581
	-1521
	-1461
	-1401
	-1341
	1341
	_1291
	-1201
	1001
JTAGGTCCCTTTAGGGAAGGATTGGAGGAATATTTCTTGGTATATGCTCAGATGCTGGTA	-1221
TTGTAAGATCCTTCCTGAGAGGAACCAAGCCTCCTTGTCAATTAATGGGCTCTAGGTCTC	-1101
	1101
AAAACTGACTCAGATTTTAAACAGAATGATCATGATTTTCCACTGCAGCAAAAGTGACATT	-1101
AP-I SRY HSF1/2 CRE-BP	1041
AGCCTTGTGATCACCAATTTTTTGATTTCTTTCTGGTTATCAAACAATACTCTAGTCAGAT	-1041
SKY C/E-BP SKY	0.01
<u>3CCCCCAAAGAACACCATGGTTCGACAGACATTGCCACAGAT</u> CTCTGTGGGTCCAGCGTTC	-981
E2F YY1 C/EBP	
TGGTCGCCGCTCTAGCCTGGGGGCAGAAGCAGAAATGCAGGAGGCACGCGTTTAAATCAG	-921
JACGCCCTCAATGGGAGGTGCGCAGGCCGAGGAGGGGGGCTGCGGCAAGCTG <u>CGAGTCTTC</u>	-861
HSF2	
ACTTTCTTCGGCTCTTGGGTTTGACTCTGTAATTGGTTCTAGGCCTGGAGGAGACGAGGA	-801
3GATAGGGGGAGAGGGGAATGAATGAAGACGCGCATCTCAAGCGGGGCAGCAAAAGACTC	-741
GATA MZFI	
CTCATTCAGAGGTGCGGCTCTACCGGCCA <u>GGGAGTCCCC</u> GTGTAGACTGGG <u>CATTTATGC</u>	-681
NF-kap USF C/EBPb	
AACTGGAGTCATCGGAACTGTCCCAAGGGTCACATTCCTTCAGCAAATGTTTACTGCGTA	-621
CCACCGGGTGCCCGCATCCCGCACTCTGCCCGCGCTGTGCGGCCCTGCCTAGCGCGCGC	-561
GATA	
CCTCCTCCAGGCCTCACCCACGCTCAGGACGGACGCGCGCG	-501
JGAGCGCCCCAGGGGTCGGGGAAGAGGGCCCGGCAAGGGAGCCCTCGCGCCGGAGCTGCA	-441
MZF1	
CTGCAGCCGCCGCCGCCGCCGCCGGCTCCCACGGGGCAGAGACGCAGCTCCTCTC	-381
Spl	
CGTCTTCCCGTACGCTACCGCGCCCGGGCAGTTCCTCGCCCGCGCACGCCGCCGCCCCGC	-321
CAACTGATTGGCCTCCGGCGCCTCGGATTGGCCCAGGCCGTCCAACAGCAGCCCCGCCCA	-261
GATA Fin GATA Spl	201
AGAGAGACCATTGGTCCTTGCCCATAGGGGCGGGGCCCGGCAGAGATCTGCGGAATTCG	-201
Sn1	201
	-141
GAATAGAGCCGGAGAGAGCCCGAGTATGACCGGAGAGAGCCCGCGCGCCGCAGCCGCGAGCGCGCGAGCGCGGAGAGAGCCCGGAGAGAGCCGGAGAGAGCCGGAGAGAGCCGGAGAGAGCGCGGAGAGAGCGCGGAGAGAGCGCGGAGAGGCGGC	-81
	-01
File Translation start and a	-21
	140
CCCCGGICGAGCCCGCCGAGGCCGAGGCCGAGGCCGAGGCGGGGGGG	740

PMA-Mediated Repression of ckβ Promoter

Figure 1. Sequence analysis of the *ckβ* 5' flanking region. The predicted transcription factor binding sites are underlined. The translation start codon (ATG) is indicated in boldface.

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located within the region of -2000/-1477 and another within -914/-519. The deletion analysis also showed that the basal promoter activity of $ck\beta$ was dependent on the promoter region between -519 and -1. Subsequently, another series of deletion mutants were constructed to narrow down the region between -2000 and -1477 that contained the inhibitory element(s). As shown in Fig. 3B, deletion of the sequence between -2000 and -1886 increased the promoter activity by 800%, demonstrating that this region significantly repressed the transcription of the $ck\beta$ promoter. Our subsequent experiments focused on the Ets (-1954/-1966) and GATA (-1950/-1959) binding elements located in this



Figure 2. Identification of CpG island in the promoter region of $ck\beta$ gene by using CpGPlot. Regions with \geq 200 bp of nucleotides, observed/expected ratio>0.6 (\downarrow) and GC% >50% (∇) were identified as putative CpG islands.

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Figure 3. Deletion analysis of the $ck\beta$ promoter in MCF-7 cells. (A) Results from the first round of luciferase assays with 5'-truncated $ck\beta$ promoter constructs (ranging from -2000 to -519). (B) Results from the second round of luciferase assays with 5'-truncated $ck\beta$ promoter constructs (ranging from -2000 to -1477). The schematic structures of the step-wise 5' deletion constructs are shown on the left. Ets and GATA binding sites investigated in this study are indicated by an open circle and open square, respectively. Each bar represents the mean \pm SEM from three independent experiments performed in triplicate (*p<0.05; **p<0.01; and ***p<0.001).

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region, which are simply referred to as "Ets" and "GATA" in all subsequent parts of this study.

Ets and GATA elements are responsible for the repression of $ck\beta$ promoter activity

Bioinformatic analysis revealed that a canonical Ets binding site and a GATA transcription factor binding site were located within the -2000/-1886 region (Fig. 1). Therefore, point mutations were introduced in these binding sites to investigate the importance of specific sequences in the repression of the $ck\beta$ promoter. Mutation of the Ets element increased $ck\beta$ promoter activity to 513% of the wild-type promoter activity, while disruption of the core sequence of the GATA binding site markedly increased the promoter activity to 703% of the wild type (Fig. 4). Results from these experiments were consistent with the deletion analysis, in which the removal of both the Ets and GATA binding sites (5' deletion

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from -2000 to -1886) caused a significant loss of $ck\beta$ promoter repression. Our data suggest that both the GATA and Ets sites located between -2000 and -1886 are negative regulatory elements and these two factors may interact to repress $ck\beta$ promoter activity.

Ets and GATA3 transcription factors bind to the $ck\beta$ distal promoter

EMSAs with a biotin-labeled DNA probe were used to assess the binding of the Ets and GATA repressive elements (between -2000 and -1886 in the $ck\beta$ promoter) by transcription factors from nuclear extracts from MCF-7 cells. Fig. 5A shows that the biotin-labeled DNA probe produced two slowly migrating shifted complexes, indicating two different nuclear proteins were bound to the probe. Mutations of either Ets, GATA or both elements in the promoter sequence markedly reduced the formation of the upper shifted complex, suggesting that the protein component of the complex was Ets- and GATA-related.

The binding specificity of Ets and GATA in the shifted complexes was verified by competition binding assays with DNA probes containing consensus binding sequences for Ets [25] and Ets/GATA [26]. The core motif, (A/T)GATA(A/G) in the Ets/GATA probe is recognized by six members (GATA1 to GATA6) of GATAfamily transcription factors [27, 28]. The competition assay presented in Fig. 5B showed that the upper shifted complex was nearly eliminated by a 100-fold molar excess of unlabeled Ets consensus probe, while both shifted complexes were significantly reduced when the unlabeled Ets/GATA consensus probe was included in the binding reaction. These results demonstrated that the upper shifted complex was due to the Ets transcription factor and that both Ets and GATA transcription factors were bound to the repressive elements on the $ck\beta$ distal promoter.

Next, GATA2 and GATA3 antibodies were used in a supershift assay to determine if any of these two GATA transcription factors was involved in the formation of complex with the $ck\beta$ promoter. As shown in Fig. 5C, GATA2 antibody did not change the mobility and intensity of the DNA-protein complexes. The addition of GATA3 antibody into the EMSA did not produce other slower-migrating complex. However, it reduced the intensity of both the original upper and lower shifted complexes possibly by disrupting the interaction of GATA3 with $ck\beta$ promoter. The results showed that GATA3 but not GATA2 was bound to the $ck\beta$ distal promoter. Unbound probes were not observed in Fig. 5C because the gel was run for extended duration in order to increase the possibility of detecting other slower-migrating complexes. Similar results (reduced intensity of shifted complexes) were also obtained when the gel was not over-run and the free probes were still visible in an earlier supershift experiment with GATA3 antibody (S1 Figure).

Chromatin immunoprecipitation assay was performed to confirm the intracellular binding of GATA3 transcription factor to the $ck\beta$ distal promoter. Protein-DNA complexes were immunoprecipitated with GATA2 or GATA3





Figure 4. Effect of Ets and GATA binding site mutations on $ck\beta$ promoter activity. Ets and GATA binding sites are indicated with a circle and square, respectively. Mutated sites are indicated with closed circles and squares. The mutations introduced into the binding sites (underlined) are in lowercase. Each bar represents the mean \pm SEM from three independent experiments performed in triplicate [***p<0.001 vs. pGL4.10- $ck\beta(-2000/-1)$].

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antibodies, followed by PCR amplification using the primers flanking the GATA binding site of the $ck\beta$ promoter. Fig. 5D showed that immunoprecipitate obtained with GATA3 antibody yielded a prominent PCR product at the expected size of 150 bp, whereas immunoprecipitates obtained with pre-immune rabbit IgG and GATA2 antibody produced much lower amount of amplified products. Sequencing of the PCR product obtained with GATA3 immunoprecipitate confirmed the specificity of the reaction. These results further support the binding of GATA3 to the GATA binding site in $ck\beta$ distal promoter.

PMA represses $ck\beta$ promoter activity

PMA regulates certain Ets and GATA family transcription factors by activating the PKC-mediated mitogen-activated protein kinase (MAPK) pathway [29, 30]. This prompted us to examine the effect of PMA on $ck\beta$ promoter activity. The effects of PMA on PKCs are dependent on the PMA concentration and the duration of treatment. Short-term treatment with a low concentration of PMA activates PKCs, while long-term exposure to a high concentration has an inhibitory effect on PKCs [31, 32]. In this study, MCF-7 cells were treated with different concentrations of PMA (10, 20, and 30 ng/mL) for 6 hr after transfection with the wild-type pGL4.10- $ck\beta(-2000/-1)$ reporter plasmid. PMA repressed $ck\beta$ promoter activity in MCF-7 cells starting at 10 ng/mL and reaching a maximal effect at 20 ng/mL (Fig. 6A). However, a 6 hr exposure to 30 ng/mL PMA attenuated the repression of $ck\beta$ promoter activity. The effect of PMA treatment duration on $ck\beta$ promoter activity was examined in the next series of experiments, ranging from 6 to 72 hr with a PMA concentration of 20 ng/mL. As shown in Fig. 6B, the maximum repressive effect of PMA occurred at 6 hr after treatment (approximately 38% of the DMSO control). Promoter activity began to increase and was almost equal to that of the DMSO control treatment after 12 hr of PMA treatment. Overall, chronic or extended PMA treatment eliminated the PMA





Figure 5. Characterization of Ets and GATA binding to the *ckβ* promoter by EMSA and ChIP. (A) EMSA was performed by using biotinylated *ckβ*-Ets/ GATA probes with or without mutations at the Ets and GATA binding sequence. The blot shown is representative of two independent experiments that produced similar results. (B) Competition assay was performed by using unlabeled Ets and Ets/GATA consensus probes as competitors in the EMSA. The

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lot shown is representative of three independent experiments that produced similar results. (C) Supershift assay was performed by using GATA2 or GATA3 antibody in the EMSA. The blot shown is representative of two independent experiments that produced similar results. (D) ChIP was performed with GATA2 or GATA3 antibody and pre-immune normal rabbit IgG as control. Lane M: GeneRuler DNA Ladder Mix, Lane T: total input positive control (unprocessed hromatin), Lane N: pre-immune normal rabbit IgG (negative control).

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effect on the $ck\beta$ promoter activity, suggesting that PMA-sensitive PKC isozymes are involved in the PMA-mediated repression of the $ck\beta$ promoter. This result also ruled out the involvement of PKC ζ , as this isozyme is known to be resistant to chronic PMA treatment [31, 33].

The effect of PMA treatment on endogenous $ck\beta$ gene expression was also investigated. Real-time PCR quantifications of $ck\beta$ mRNA and Western detection of CK β protein were performed on DMSO (control) and PMA treated (20 ng/mL for 6 to 24 hr) MCF-7 cells. Based on the results shown in Fig. 6C, PMA treatment resulted in significant reduction of $ck\beta$ mRNA (by 20%) and protein (by 30%) expressions compared to controls after 12 hr. There was no significant difference for mRNA and protein levels between PMA treated samples and controls for the other treatment durations.

PKC412 and PKC ε inhibitor peptide abolished the repressive effect of PMA on $ck\beta$ promoter activity

PKC412 (midostaurin, or CGP 41251), an inhibitor of PKC α , $-\beta$, $-\gamma$, $-\delta$, $-\varepsilon$, $-\eta$, and $-\zeta$ [<u>34</u>], completely abolished the PMA-induced down-regulation of the $ck\beta$ promoter, whereas Go 6983, an inhibitor of PKC α , $-\beta$, $-\gamma$, $-\delta$, and $-\zeta$ [<u>32</u>, <u>35</u>], did not show the same effect (Fig. <u>7A</u>). The $ck\beta$ promoter activity was also increased by the individual treatment with PKC412 but not Go 6983. These findings, along with the resistance of PKC ζ to chronic PMA treatment, suggest that PKC ε or PKC η is most likely the PKC isozyme that mediates the repressive effect of PMA in this system. Subsequently, PKC ε and PKC η specific inhibitors were used to identify the isozyme involved. The results in Fig. <u>7B</u> show that PKC ε inhibition abolished the PMA repression of $ck\beta$ promoter. PKC η inhibition did not affect the down-regulation of $ck\beta$ promoter by PMA treatment. The promoter activity of treatment with PMA and PKC η inhibitor was also significantly lower than treatment with PKC η alone. This further excludes the involvement of PKC η isozyme in the PMA repression of $ck\beta$ promoter.

Ets and GATA binding is required for PMA-mediated repression of the $ck\beta$ promoter

To investigate the role of the Ets and GATA binding sites in the PMA-mediated repression of the $ck\beta$ promoter, the wild-type promoter, pGL4.10- $ck\beta(-2000/-1)$, and constructs in which the Ets and GATA binding sites were mutated were transfected into MCF-7 cells followed by treatment with or without PMA. Fig. 8 shows that PMA treatment decreased the promoter activities of pGL4.10-





Figure 6. Effects of PMA concentration and treatment duration on $ck\beta$ promoter activity. (A) MCF-7 cells were treated with 10, 20, or 30 ng/mL PMA for 6 hr. Each bar represents the mean \pm SEM of triplicate samples from three independent experiments. (*p<0.05 vs. DMSO control; #p<0.05, within the PMA treatment group). (B) MCF-7 cells were treated with 20 ng/mL of PMA for 6 to 72 hr. The values shown are $ck\beta$ promoter activities relative to their respective controls (control value is set at 1.0, *p<0.05 vs. DMSO control). Each bar represents the mean \pm SEM of triplicate samples from three independent experiments. (C) Effect of PMA treatment on the $ck\beta$ mRNA and protein levels in MCF-7 cells. Cells were treated with 20 ng/mL of PMA or DMSO for 6 to 24 hr. Left panel: The levels of mRNA and proteins are relative to their respective controls (control value is set at 1.0, *p<0.05 vs. DMSO control). Each bar represents the mean \pm SEM of triplicate samples from three independent experiments. (C) Effect of PMA treatment on the $ck\beta$ mRNA and protein levels in MCF-7 cells. Cells were treated with 20 ng/mL of PMA or DMSO for 6 to 24 hr. Left panel: The levels of mRNA and proteins are relative to their respective controls (control value is set at 1.0, *p<0.05 vs. DMSO control). Each bar represents the mean \pm SEM of triplicate samples from three independent experiments. Right panel: The blot shown is representative of three independent experiments that produced similar results. M: SuperSignal Molecular Weight protein ladders (Thermo Scientific, USA), C: control, T: PMA treated, P: purified CK β protein (45 kDa) as reference.

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Figure 7. Effect of PKC inhibitors (PKC412 and Go 6983) on the PMA-mediated repression of $ck\beta$ promoter activity. (A) MCF-7 cells were individually or co-treated with 20 ng/mL PMA, 1 mM PKC412 and 0.1 mM Go 6983 for 6 hr. Each bar represents the mean \pm SEM of triplicate samples from three independent experiments. (*p<0.05 and *p<0.01 vs. DMSO control). (B) MCF-7 cells were individually or co-treated with 20 ng/mL PMA, 10 μ M PKC ϵ inhibitor peptide and 10 μ M PKC η pseudo-substrate inhibitor fo 6 hr. Each bar represents the mean \pm SEM of triplicate samples from three independent experiments. (*p<0.05 and *p<0.05 between the two indicated treatments).

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 $ck\beta(-2000/-1)$, pGL4.10-mut(Ets), and pGL4.10-mut(GATA) to 38%, 57%, and 68%, respectively, as compared to the control (DMSO treatment). This result indicated the participation of both Ets and GATA in the $ck\beta$ promoter repression by PMA. The involvement of other transcription factors in PMA-mediated downregulation of $ck\beta$ promoter activity cannot be ruled out because the mutation of both the Ets and GATA sites did not completely abolish the effect of PMA treatment (Fig. 8).