PHOSPHORYLATION AND REGULATION OF HUMAN CHOLINE KINASE BETA BY PROTEIN KINASE A

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PHOSPHORYLATION AND REGULATION OF HUMAN CHOLINE KINASE BETA BY PROTEIN KINASE A

by

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ADP	Adenosine diphosphate
AKAPs	A-kinase-anchoring proteins
AKT	Serine/threonine kinase
Ala	Alanine
ANS	1-anilino-8-naphthalene sulfonate
APS	Ammonium persulfate
Arg	Arginine
Asn	Asparagine
Asp	Aspartate
ATM	Ataxia-telangiectasia mutated kinase
ATP	Adenosine triphosphate
[γ^{-32}] ATP	ATP labeled with gamma ^{32}P
bp	Base pair
8-Br-cAMP	8-bromo-cAMP
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CBP	CREB-binding protein
CCl_4	Carbon tetrachloride
CCT	CTP-phosphocholine cytidylyltransferase
CCTα	CTP-phosphocholine cytidylyltransferase alpha
CCTβ	CTP-phosphocholine cytidylyltransferase beta
cdK1	Cyclin dependent kinase I
cdK5	Cyclin dependent kinase 5
cDNA	Complementary deoxyribonucleic acid
CFTR	Cystic fibrosis transmembrane conductance regulator
$C_2H_3NaO_2$	Sodium acetate
CK	Choline kinase
CKα	Choline kinase alpha
CKA-2	*Caenorhabditis elegans* CK from family A
CKβ	Choline kinase beta
CNG	Cyclic nucleotide-gated ion channel
CO_2	Carbon dioxide
CPT	CDP-choline phosphoryltransferase
CREB	cAMP response element-binding protein
c-Src	Proto-oncogene tyrosine protein kinase Src
D_{50}	Half maximal inhibitory concentration
DAPI	4',6-Diamidino-2-phenyl indole
DGK	Diacylglycerol kinase
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNAPK	DNA dependent protein kinase
dNTP	Deoxynucleotide triphosphate
DTT	Dithiothreitol
EBP50	Ezrin-Radixin-Moesin (ERM) binding phosphoprotein 50
E. coli	*Escherichia coli*
ECT	Phosphoethanolamine cytidylyltransferase
EDTA	Ethylenediaminetetraacetic acid

EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EGTA	Ethyleneglycoltetraacetic acid
EK	Ethanolamine kinase
Epacs	Exchange proteins activated by cAMP
EPT	Ethanolamine phosphotransferase
EtBr	Ethidium bromide
ETS	E26 transformation specific
FBS	Fetal bovine albumin
FRET	Fluorescence resonance energy transfer
GATA	Transcription factor that binds to the DNA sequence of 'GATA'
GFP	Green fluorescent protein
Gln	Glutamine
Glu	Glutamic acid
GPCRs	G protein-coupled receptors
GSH	Glutathione
GSK3β	Glycogen synthase kinase 3 β
GST	Glutathione S-transferase
GTPase	Guanosine triphosphate (GTP) hydrolyzing enzyme
HC-3	Hemicholinium 3
HCl	Hydrochloric acid
HCT-116	Human colorectal carcinoma
HEK293	Human embryo epithelial kidney fibroblasts
HeLa	Human cervical cancer cell line
HEPES	4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid
HepG2	Human hepatocarcinoma cell line
HIF-1α	Hypoxia-inducible factor alpha
His	Histidine
HMEC	Human primary mammary epithelial cell line
HREs	Hypoxia responsive elements
HRP	Horse radish peroxidase
IBMX	3-Isobutyl-1-methylxanthine
IC_{50}	Half maximal inhibitory concentration
IPTG	Isopropyl-β-D-thiogalactopyranoside
IRS	Insulin receptor substrate
k_{cat}	Enzyme turnover rate
k_{cat}/K_m	Enzyme catalytic efficiency
KCl	Potassium chloride
kDa	Kilo dalton
KH_2PO_4	Potassium phosphate
K_m	Michaelis constant
LB	Luria-Bertani
LDH	Lactate dehydrogenase
Leu	Leucine
Lys	Lysine
MCF-7	Human breast adenocarcinoma cell line
M-CPTI	Muscle type carnitine palmitoyltransferase I
mCRY2	Muscle cryptochrome 2
MDCK	Madin-Darby canine kidney cell line

Mg^{2+}	Magnesium ion
$MgCl_2$	Magnesium chloride
$MgSO_4$	Magnesium sulfate
mRNA	Messenger RNA
NaCl	Sodium chloride
NADH	Nicotinamide adenine dinucleotide
NaF	Sodium fluoride
Na_2HPO_4	Disodium phosphate
NaOH	Sodium hydroxide
$Na_4P_2O_7$	Sodium pyrophosphate
NIH 3T3	Mouse embryonic fibroblast cells line
NLS	Nuclear localization signal
NMR	Nuclear magnetic resonance
NP-40	Nonidet P-40
OH	Hydroxyl
PBS	Phosphate buffered saline
PC	Phosphatidylcholine
PCho	Phosphocholine
PDEs	Phosphodiesterases
PDPK1	3-phosphoinositide-dependent protein kinase-1
PCR	Polymerase chain reaction
PE	Phosphatidylethanolamine
PEG	Polyethylene glycol
PEMT	Phosphoethanolamine methyltransferase
PEtn	Phosphoethanolamine
PEP	Phosphoenolpyruvic acid mono-potassium salt
phosphoPKAS	Phospho-(Ser/Thr) PKA substrate
PI3K	Phosphoinositide 3-kinase
PKA	Protein kinase A
PKC	Protein kinase C
PKG	cGMP dependent protein kinase
PKI	PKA specific peptide inhibitor
PK-LDH	Pyruvate kinase-lactate dehydrogenase
PMSF	Phenylmethyl-sulfonyl fluoride
PO_4^{3-}	Phosphate
Prdx6	Peroxiredoxin 6 protein
Pro	Proline
pSer	Phosphoserine
PTK	Protein tyrosine kinase
Pto	Serine/threonine protein kinase, ‘Pto’ named from resistance to *Pseudomonas syringae* pathovar tomato
RAS	Rat sarcoma, described the small GTPase protein
RFU	Relative fluorescence unit
RhoA	Ras homolog gene family, member A
RSK	Ribosomal S6 kinase
SDS	Sodium dodecylsulfate
SDS-PAGE	Sodium dodecylsulfate polyacrylamide gel electrophoresis
Ser	Serine
SEM	Standard error of mean
siRNA	Small interfering RNA

SPSS	Statistical package for the social sciences
StarD10	START protein of the domain 10
SUS	Sucrose synthase
TAE	Tris acetate-EDTA buffer
TBS	Tris buffered saline
TEMED	Tetramethylethylenediamine
TGF-β	Transforming growth factor beta
Thr	Threonine
Tyr	Tyrosine
UV	Ultraviolet
V	Volt
V_{max}	Maximum reaction velocity
v/v	Volume to volume
w/v	Weight to volume
$\times g$	Fold gravity
XBP-I(S)	X-box-binding protein I spliced form

PEMFOSFORILAN DAN PENGAWALATURAN KOLINA KINASE BETA MANUSIA OLEH PROTEIN KINASE A

ABSTRAK

Kolina kinase (CK) adalah enzim pertama yang terlibat dalam laluan CDP-kolina untuk proses biosintesis fosfatidilkolina yang merupakan komponen utama fosfolipid membran. CK terdiri daripada tiga isoform iaitu CKα1, CKα2 dan CKβ. Pengawalaturan enzim ini adalah penting dari segi fisiologi. Perubahan metabolik CKα telah dikaitkan dengan pembentukan tumor, manakala mutasi atau pemadaman gen *chkβ* boleh menyebabkan distrofi otot. Dalam kajian antikanser, perencatan aktiviti CK telah diteroka sebagai strategi terapeutik yang berpotensi. Pengubahsuaian pasca translasi merupakan salah satu mekanisme untuk mengawal fungsi CK. Semakin banyak bukti menunjukkan bahawa fungsi CK dalam yis dan CKα dalam manusia dikawalatur oleh pemfosforilan. Namum begitu, pemfosforilan CKβ tidak pernah dilaporkan. Dalam kajian ini, protein kinase A (PKA) telah dikenalpasti sebagai protein kinase yang bertanggungjawab dalam pemfosforilan CKβ melalui analisis in-gel kinase. Pemfosforilan oleh PKA telah disahkan melalui teknik analisis perencat PKA dan blot Western. Analisis *in vitro* dengan menggunakan PKA komersil juga membuktikan bahawa CKβ merupakan substrat untuk pemfosforilan PKA. Pemfosforilan ini berlaku pada terminal-N CKβ iaitu asid amino serine 39 dan 40. Pemfosforilan CKβ telah dilihat dalam sel embrio ginjal manusia (HEK293) dan sel karsinoma hati manusia (HepG2). Rawatan forskolin dan 3-isobutil-1-metilxantin meningkatkan tahap pemfosforilan pada CKβ manakala kesan tersebut direncatkan oleh perencat PKA (H-89). Tahap pemfosforilan CKβ juga ditingkatkan oleh rawatan faktor pertumbuhan epidermis. Seterusnya, kesan

pemfosforilan terhadap ciri-ciri biokimia CKβ juga dikaji. Pemfosforilan PKA telah meningkatkan aktiviti pemangkinan CKβ terhadap kolina, etanolamina dan ATP. Nilai V_{max} untuk kolina, etanolamina dan ATP telah masing-masing meningkat sebanyak 47.1%, 81.8% dan 50.8%. Pemfosforilan PKA juga telah meningkatkan tarikan CKβ terhadap substrat kolina dan ATP, tetapi pemfosforilan menurunkan tarikan CKβ terhadap substrat etanolamina. Kecekapan pemangkinan CKβ untuk kolina dan ATP telah meningkat sebanyak 121.0% dan 97.5% masing-masing. Kesan pemfosforilan PKA terhadap ciri-ciri biokimia CKβ telah ditiru oleh mutasi berganda pada serine yang difosforilasi dengan penukaran kepada aspartat. Pemfosforilan juga meningkatkan sensitiviti CKβ terhadap perencatan oleh hemicholinium-3 (HC-3) yang merupakan perencat CK yang kuat. Nilai IC_{50} CKβ terfosforilasi (50 μM) adalah 29 kali ganda lebih rendah daripada enzim tidak terfosforilasi (1.45 mM). Selain itu, pemfosforilan juga mengurangkan kestabilan CKβ terhadap penyahaslian urea. Sebaliknya, pemfosforilan tidak menjejaskan pH optima, lokasi subsel dan status oligomer CKβ. Kajian ini melaporkan fosforilasi dan pengawalaturan CKβ oleh PKA untuk kali pertama. Pengetahuan ini memberikan pandangan baru terhadap pengawalaturan intrasel ciri-ciri pemangkinan CKβ yang mungkin merupakan mekanisme penting untuk mengawal metabolisme lipid and pertumbuhan sel.

PHOSPHORYLATION AND REGULATION OF

HUMAN CHOLINE KINASE BETA BY PROTEIN KINASE A

ABSTRACT

Choline kinase (CK) is the first enzyme involved in CDP-choline pathway for the biosynthesis of phosphatidylcholine, the major component of membrane phospholipid. CK exists as three isoforms, which are CKα1, CKα2 and CKβ. The regulation of these enzymes is physiologically important. Metabolic alterations of CKα are associated with tumorigenesis, while mutation or deletion of *chkβ* gene leads to the development of muscular dystrophy. In anticancer research, inhibition of CK activity has been explored as a potential therapeutic strategy. Post-translational modification is one of the mechanisms to regulate the function of CK. Growing evidences support that yeast and human CKα are regulated by phosphorylation but the phosphorylation of CKβ has never been reported. In this study, protein kinase A (PKA) was identified as the protein kinase responsible for the phosphorylation of CKβ by in-gel kinase assay. PKA phosphorylation was confirmed with specific PKA inhibitor and Western blotting. *In vitro* assay with commercial PKA further supported CKβ as the substrate for PKA phosphorylation. The phosphorylation occurred at serine 39 and 40 residues in the N-terminal region of CKβ. Phosphorylation of CKβ was observed in human embryonic kidney cells (HEK293) and liver hepatocellular carcinoma cells (HepG2). Forskolin and 3-isobutyl-1-methylxanthine treatment increased the phosphorylation level of CKβ, while the phosphorylation was inhibited by PKA inhibitor (H-89). The phosphorylation level of CKβ was also increased by epidermal growth factor. The effects of PKA

phosphorylation on the biochemical properties of CKβ were subsequently examined. PKA phosphorylation increased the catalytic activities of CKβ with choline, ethanolamine and ATP as substrates. The V_{max} values for choline, ethanolamine and ATP were increased by 47.1%, 81.8% and 50.8%, respectively. PKA phosphorylation improved the affinity of CKβ for choline and ATP, but decreased the affinity of CKβ for ethanolamine. Consequently, the catalytic efficiencies of CKβ for choline and ATP were increased by 121.0% and 97.5%, respectively. The same effects of PKA phosphorylation on the biochemical properties of CKβ were mimicked by double mutation of the phosphorylated serines to aspartates. PKA phosphorylation also dramatically increased the sensitivity of CKβ to hemicholinium-3 (HC-3), a potent inhibitor of CK. The IC_{50} value for phosphorylated CKβ (50 μM) was 29 times lower than the unphosphorylated enzyme (1.45 mM). In addition, PKA phosphorylation also decreased the stability of CKβ protein against urea denaturation. On the contrary, phosphorylation did not affect the optimum pH, subcellular location and oligomeric state of CKβ. This study reports the phosphorylation and regulation of CKβ by PKA for the first time. The knowledge provides new insight into the intracellular regulation of CKβ catalytic properties by phosphorylation that might be an important mechanism to modulate lipid metabolism and cell growth.

CHAPTER ONE

INTRODUCTION

1.1 Kennedy pathway

Kennedy pathway (Figure 1.1) which consists of CDP-choline and CDP-ethanolamine pathways decribes the *de novo* biosynthesis of major phospholipid components of the cell (Kennedy and Weiss, 1956). In CDP-choline pathway, CK catalyzes the phosphorylation of choline to phosphocholine (PCho) using adenosine triphosphate (ATP) and magnesium (Mg^{2+}) as substrate and cofactor, respectively (Ishidate, 1997). The second enzyme in this pathway is CTP-phosphocholine cytidylyltransferase (CCT) which converts the PCho into CDP-choline. CDP-choline phosphoryltransferase (CPT) catalyzes the final condensation of CDP-choline to form PC (Kent, 1990, Gibellini and Smith, 2010). For CDP-ethanolamine pathway, ethanolamine is converted into phosphoethanolamine (PEtn) by EK and followed the similar steps as the CDP-choline pathway (Gibellini and Smith, 2010). In liver, the end product of CDP-ethanolamine pathway, the PE, can be converted into PC by phosphoethanolamine methyltransferase (PEMT) (Li and Vance, 2008).

1.2 Choline kinase

Human CK is composed of CKα1, CKα2 and CKβ isoforms. CKα and CKβ are encoded by two separate genes, *chkα* (NCBI Gene ID: 1119) and *chkβ* (NCBI Gene ID: 1120) in chromosomes 11q13.2 and 23q13.33, respectively. CKα undergoes alternative splicing to form CKα1 (NCBI reference sequence: NP_005189) and CKα2 (NCBI reference sequence: NP_001268) with the calculated molecular size of

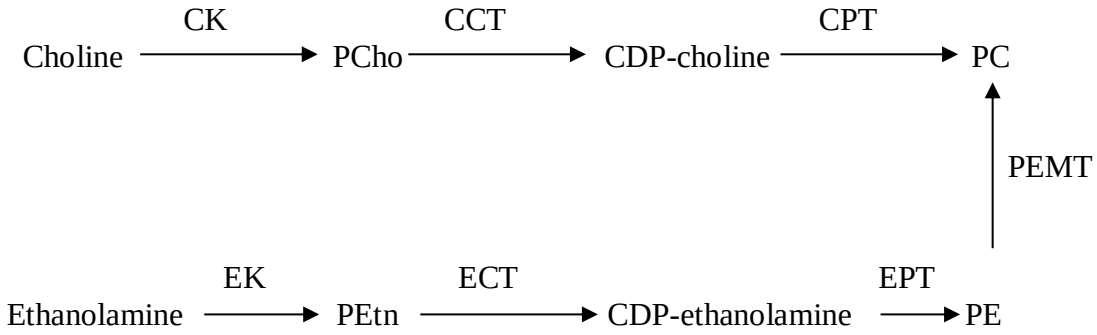

Figure 1.1: Kennedy pathway for the biosynthesis of phosphatidylcholine (PC) and phosphatidylethanolamine (PE). Choline kinase (CKα or CKβ) phosphorylates choline to form phosphocholine (PCho). PCho is converted into CDP-choline by CTP-phosphocholine cytidylyltransferase (CCT). CDP-choline is condensed into PC by CDP-choline phosphoryltransferase (CPT). Ethanolamine kinase (EK) phosphorylates ethanolamine to form phosphoethanolamine (PEtn). PEtn is converted into CDP-ethanolamine by phosphoethanolamine cytidylyltransferase (ECT). CDP-ethanolamine is condensed into PE by ethanolamine phosphotransferase (EPT). PE can be converted into PC by phosphoethanolamine methyltransferase (PEMT). Figure is adapted from Aoyama *et al*. (2004).

50 and 52 kDa, respectively (Aoyama *et al.*, 2004); whereas, CKβ (NCBI reference sequence: NP_005189) is encoded by a separated gene with the calculated molecular size of 45 kDa.

The enzyme activity of CK was first described in brewer's yeast by Wittenberg and Kornberg (1953). Due to the difficulty in purifying yeast CK, the understanding of CK progressed slowly until the highly homogeneous CK was obtained from rat tissues in 1984 (Ishidate *et al.*, 1984, Porter and Kent, 1990, Uchida and Yamashita, 1990). The cDNA of rat and human CKα1 was first isolated from rat liver and human glioblastoma (Hosaka *et al.*, 1992, Uchida and Yamashita, 1992a). Amino acid sequence comparison between rat and human CKα1 shows 84.9% identity. Later, the second transcript of rat CKα1, termed CKα2 was isolated and characterized (Uchida, 1994). CKα2 and CKα1 are differed by an extra stretch of 18 amino acids on the CKα2 isoenzymes with a discrepancy of 2 kDa. The insertion of extra amino acids on the CKα2 significantly increased its substrate affinity for choline (Malito *et al*., 2006). The additional stretch of amino acid was proposed to be the important region to facilitate the conformational change of the enzyme upon substrate binding (Malito *et al*., 2006).

Another isoenzyme of CK, named CKβ was first cloned from rat liver and characterized by Aoyama *et al*. (1998). The amino acid sequence of rat CKβ shows 57-59% identity with the amino acid sequence of rat CKα1 and CKα2. The human homolog of rat CKβ was identified later by Yamazaki *et al*. (1997). The cDNA sequence of human *chkβ* was cloned in a large-scale cDNA sequence project and the sequence was deposited in NCBI with GenBank accession number: BC082263,

BC101488, BC113521(Strausberg *et al.*, 2002). CKβ consists of 395 amino acids. CKβ is less studied as compared to the CKα isoenzyme. CKβ is not as active as CKα. Most importantly, CKβ is not involved in tumorigenesis as compared to CKα, which is the main factor for most of the studies were focused on CKα isoenzyme (Gallego-Ortega *et al.*, 2009). CKβ started to attract attention when the association between CKβ and muscular dystrophy was reported (Wu *et al.*, 2009).

CK from *Caenorhabditis elegans* has been extensively studied also. The second isoenzyme of *C. elegans* CK from family A (CKA-2) shows 48% identity with the human CKα2. Due to its high similarity in gene sequence and biochemical properties to human CKα2, CKA-2 has been used as the model for structure function study for better understanding of CK function (Gee and Kent, 2003).

1.3 Structure of choline kinase

CKs consist of two clusters of highly conserved motif, Brenner's and CK/EK motif (Figure 1.2). Brenner's motif with consensus sequence of $hxHxDhx_3N$ (h refers to large hydrophobic residue and x refers to an unknown amino acid residue) is found in most of the protein kinases that catalyze the transfer of phosphoryl groups (Brenner, 1987), whereas, CK/EK motif ($hxhhDhEx_4Nx_3hDhx_2HhxE$) is conserved among the CK from different organisms (Aoyama *et al.*, 2000).

CKA-2 from *C. elegans* was the first crystal structure to be solved (Peisach *et al.*, 2003). The crystal structure of CKA-2 revealed the enzyme as a homodimeric protein. The structure of CKA-2 is similar with those eukaryotic protein kinases (ePK) and aminoglycoside phosphotransferases (AP) although their amino acid

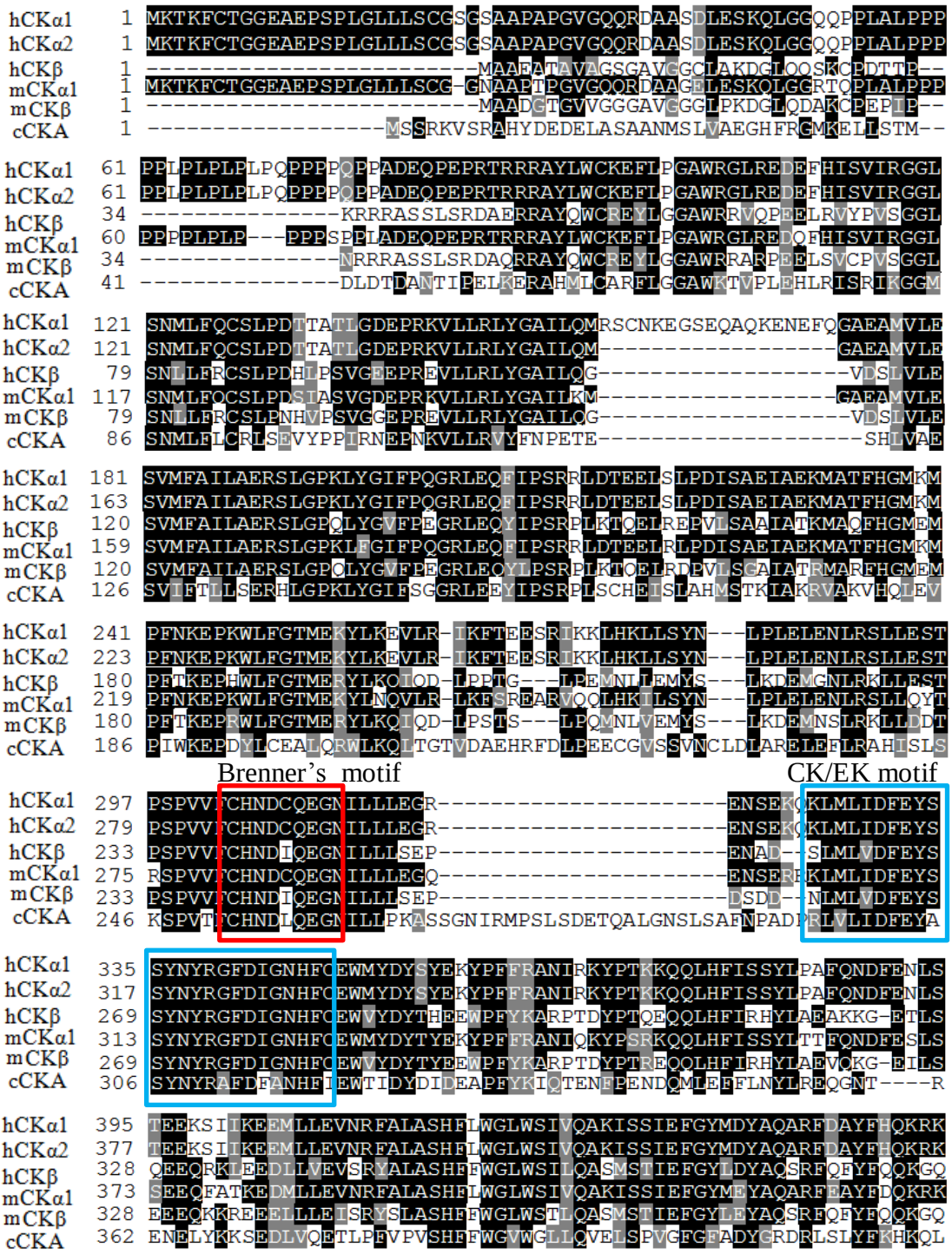

Figure 1.2: Sequence alignment of CK from human, mouse and *C. elegans*. The NCBI accession numbers for human CKs; hCKα1, hCKα2 and hCKβ are NP_997634, NP_001268, and NP_005189. The NCBI accession numbers for mouse CKs; mCKα and mCKβ are NP_038518 and NP_031718. The NCBI accession number for *C. elegans*; CKA-2 is NP_001024480. Figure is adapted from Malito *et al*. (2006).

sequences are different. Structural comparison of CKA-2 with the catalytic subunit of protein kinase A (PKA) and aminoglycoside 3'-phosphotransferase [APH(3')-IIIa] shows conserved structural cores of a N and C-terminal domains. The conserved structure of the N-terminal region consists of five strands of β sheet (first five strands) and one helix (second helix), whereas the C-terminal domain consists of three helices (third to fifth helices) and four strands of β sheet (ninth to twelfth strands). The smaller N-terminal domain is connected to the large C-terminal domain by a short linker. The crystal structure of CKA-2 also showed the location of Brenner's and the CK/EK motif at the C-terminal domain. There was no bound substrate, so the ATP site was predicted based on the existing structure of highly similar proteins ePK and AP. The choline binding site was proposed to be near to the ATP binding pocket which was formed by several structurally flexible loops (Peisach *et al.*, 2003).

Later, Malito *et al*. (2006) solved the crystal structure of human CKα2 protein with bound ADP and PCho to reveal the molecular details of ATP and the choline binding sites on CKα2. The ribbon diagram of the CKα2 crystal structure is shown in Figure 1.3. CKα2 was also crystalized as homodimeric form. The structure of CKα2 is very similar with the structure of CKA-2. As compared to the CKA-2, a small difference is found on the fifth helix of CKα2. However, this region is not part of the catalytic region.

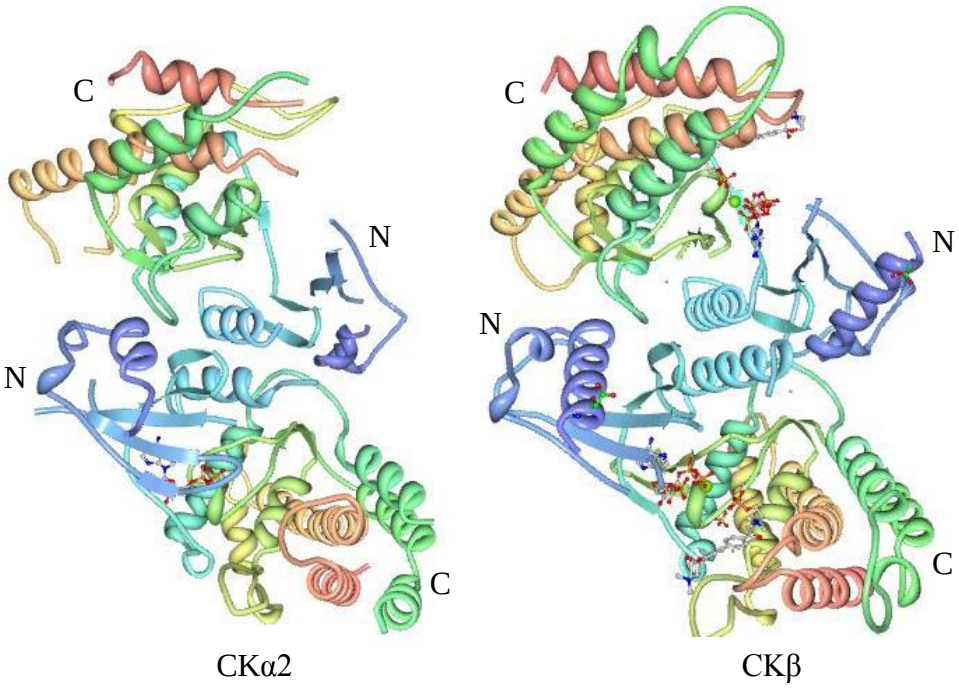

Figure 1.3: Ribbon diagrams of human CKα2 and CKβ. CKα2 (PDB 3G15) and CKβ were bound with the hemicholinium (HC-3) (PDB 3FEG). C and N indicate C and N-terminal regions. The dimeric structure shown of CKβ was the suggested biological model based on the asymmetric unit of CKβ (Hong *et al*., 2010).

CKα2 overexpressed in *E. coli* BL21(DE3) was the N-terminal truncation mutant without the first 49 amino acids (Malito *et al*., 2006). The truncated version of the CKα2 shows very similar biochemical properties as the full length protein and this suggests that the N-terminus of CKα2 is not important for enzyme catalysis. On the crystal structure, the first visible residue is on the Pro 85 which indicates that the first 30 amino acids on the crystal structure are disordered. In this structure, the choline binding pocket is described as a deep hydrophobic groove with a rim of negatively charged residues. ATP is bound within a cleft between two domains (the N and C-terminal domain) of the enzyme. Residues from both N and C-terminal lobes contribute to the formation of large pocket for ATP binding. Upon binding of choline, it undergoes conformational changes affecting the N-terminal domain and the ATP-binding loop.

Subsequently, Hong *et al*. (2010) solved the crystal structure of another CK isoenzyme, CKβ in complex with the potent CK inhibitor, hemicholinium (HC-3). HC-3 molecule was bound onto the choline binding pocket. The structure of CKβ exhibited the same bilobal architecture as CKα2 with the major difference being the C-terminal lobe (Figure 1.3) (Hong *et al.*, 2010). This difference lowers the sensitivity of CKβ towards HC-3 inhibition (Hong *et al.*, 2010). Besides, the N-terminal truncated (35 amino acids) CKβ was shown as a monomer rather than dimer like the CKα2 and CKA-2. The first 35 amino acid might be important for the oligomeric formation.

Mutagenesis study was performed on the conserved Brenner's and CK/EK motif based on information obtained from the crystal structural of CK and other ePK with similar protein structure. Aspartate residue on Brenner's motif was responsible for the removal of the proton charge from the hydroxyl group of ATP (Zheng *et al.*, 1993). Thus, mutation on the Asp 255 residue of CKA-2 and Asp 306 residues of CKα2 caused the total loss of CK activity (Malito *et al.*, 2006, Yuan and Kent, 2004). On the other hand, mutation on the CK/EK motif also impaired the catalytic activity of CK. These residues were shown to coordinate the enzyme co-factor, Mg^{2+} ion by two carboxyl oxygen atoms. Mutation on Asn 260 and Asp 301 on CK/EK motif of CKA-2 resulted in the loss of the enzyme activity (Peisach *et al.*, 2003; Yuan and Kent, 2004). However, mutation on the Asn 330 on the CK/EK motif of CKα2 did not alter the catalytic activity although the crystal structure of CKα2 showed a direct contact of this residue with Mg^{2+} ion (Malito *et al.*, 2006). Besides, Ser 121 in the ATP loop is also important for the activity of CK. Mutation of this residue decreased the catalytic efficiency of the enzyme. Hydroxyl group on this position is essential for a full activity of the CKα2 protein.

1.4 CK oligomeric structures

CK exists as dimer, tetramer or higher oligomer. Homo or hetero-oligomer formation of CK isoforms had been reported. According to Aoyama *et al.* (2002), the most active form of CK in mouse is the homo-oligomer of α/α, followed by hetero-oligomer of α/β and the least active CK is homo-oligomer of β/β. The mRNA abundance of CKα isoform was the highest in testis, while the expression of CKβ isoform was relatively high in the heart and liver. In liver tissue, hetero-oligomer contributed 60% of the total activity, while the homo-oligomer contributed 20%

each to the remaining activity. In contrast, the enzyme obtained from heart tissue showed 70% of activity from homo-oligomer of β/β, 25% from hetero-oligomer of α/β, and <5% was contributed by the homo-oligomer of α/α (Aoyama *et al.*, 2002). These observations indicate that the expression, distribution and the combination of the CK oligomer were tissue type-dependent.

The dimeric structure of CKα2 protein is stabilized by the dimer interface formed at the second α-helix (Glu 175−Arg 190) of each monomer (Malito *et al*., 2006). For CKA-2, another dimer interface is identified at the first helix (Pro 50−Leu 64) and the S-shaped loop [formed by the fourth (Ala 167−His 174) and fifth (Leu 194−Thr 208) helices] (Peisach *et al.*, 2003). The extra dimer interface on CKA-2 is absent from the CKα2 protein as the structure at this region is disordered (Malito *et al*., 2006). In mouse CK, the important regions for oligomer formation were also identified (Liao *et al*., 2006). The amino acids between first (Pro 73−Arg 85) and ninth helices (Gln 424−Lys 430) as well as single amino acid on seventh helix, Asp 320 are critical for oligomer formation of CKα. The region between first (Arg 35−Arg 62) and tenth helices (Gln 379−Lys 385) is important for oligomer formation of CKβ (Liao *et al.*, 2006).

1.5 CK subcellular location

In early studies, CK had been reported as a cytosolic protein. The CK activity was detected in the cytosol fraction of the cells (Uchida and Yamashita, 1990; Aoyama *et al*., 2002). In addition, Miyake and Parsons (2011) also showed CKα as a cytoplasmic protein when overexpressed in breast cancer cell line. CKα was translocated from cytoplasm to the membrane of the cell when co-expressed with its

interacting partner, the epidermal growth factor receptor (EGFR) (Miyake and Parsons, 2011). Besides, there was also a report on nucleus translocation of CKα at the mitotic phase of the cell cycle (Gruber *et al.*, 2012). These observations provide the evidences for CKα translocation into different cell compartments. However, no information is available for the subcellular location of CKβ.

1.6 Biochemical properties of CK

Extensive biochemical characterizations of CK from rat, *S. cerevisiae* and *C. elegans* had been performed in the earlier studies (Gee and Kent, 2003, Ishidate *et al.*, 1984, Kim *et al.*, 1998, Porter and Kent, 1990, Uchida and Yamashita, 1990, Ishidate *et al.*, 1985). The details were summarized in Table 1.1 (Aoyama *et al.*, 2004). Among rat CK, CKα2 possessed the highest specific activity, followed by CKα1 and the least active form of CK is CKβ (Ishidate *et al*., 1984; Ishidate *et al*., 1985; Porter and Kent, 1990; Uchida and Yamashita, 1990). As compared to rat CK, the similar characteristic was reported for human CK (Hong *et al*., 2010). Yeast CK (*S. cerevisiae*) was almost as active as the rat CKα2 with lower affinity towards choline (Kim *et al*., 1998). In contrast, *C. elegans* CK was less active than the yeast CK. However, its affinity toward choline was higher than the yeast CK (Gee and Kent, 2003).

All the purified CKs possess EK activity (Aoyama *et al*., 2004; Hong *et al*., 2010). Therefore, the nomenclature of CK becomes choline/ethanolamine kinase in the early nineties. The subsequent discovery of *Drosophila* EK established the existence of a separate gene (*ek*) encoding an ethanolamine specific kinase (Uchida, 1997). In human, the cDNA of the *ek1* gene was isolated and characterized by Lykidis *et al*.

Table 1.1 The catalytic activity of CK from rat, *S. cerevisiae and C. elegans*. The table is adapted from Aoyama *et al*. (2004).

Source	**Oligomeric form**	**S.A(mmol /min/mg)**	**K_m Choline (μM)**	**K_m ATP (mM)**	**Corresponding protein**
Rat kidney[a,b]	dimer	3.3	100	1.5	CKβ
Rat liver[c]	tetramer	143	13	0.04	CKα2
Rat brain[d]	dimer	40	14	1.0	CKα1
S. cerevisiae[e] (Recombinant)	dimer	128	270	0.09	CKI
C. elegans[f] (Recombinant)	Dimer (oligomer)	43	1.6 mM	2.4	CKA-2
		24	13 mM	0.72	CKB-2

[a] Ishidate *et al*. (1984)
[b] Ishidate *et al*. (1985)
[c]Porter and Kent (1990)
[d] Uchida and Yamashita (1990)
[e] Kim *et al*. (1998)
[f] Gee and Kent (2003)

S.A: specific activity.

(2001). It possessed high EK activity with negligible CK activity. In general, CK prefers choline rather than ethanolamine as substrate. The affinity of CK for choline is higher than ethanolamine (Porter and Kent, 1990). Gallego-Ortega *et al*. (2009) reported that both human CKα and CKβ isoforms showed higher affinity toward choline rather than ethanolamine. However, human CKβ showed a higher ethanolamine kinase activity than CK activity in the cells (Gallego-Ortega *et al*., 2009). The overexpression of CKα in human derived cell line increased the production of both PCho and PEtn. However, CKβ overexpression increased PEtn production, but not PCho (Gallego-Ortega *et al*., 2009), which showed that CKβ catalyzed the phosphorylation of ethanolamine rather than choline when both substrates are present in the cell.

In terms of the substrate affinity, CKα purified from bacterial expression system possesses a higher affinity for both choline and ethanolamine than the CKβ (See Too, 2006). CKα overexpressed from human derived cell line (crude cell lysate) also gave similar results (Gallego-Ortega *et al*., 2009). However, Hong *et al*. (2010) showed a contradicting result. Their purified CKβ from bacterial expression system had a higher substrate affinity for choline than CKα although the catalytic efficiency of CKα remained higher than the CKβ isoenzyme.

1.7 The roles of choline kinase

1.7.1 Cell proliferation

CK is involved in cell proliferation. The product of CK, PCho was shown to induce mitogenesis. In human primary mammary epithelial cells (HMEC) and mouse embryonic fibroblast cells (NIH 3T3), PCho production was increased by growth factor, insulin and hydrocortisone treatments, which were the effectors for normal cell proliferation (Ramirez de Molina *et al.*, 2004, Kiss and Chung, 1996). The treatment also increased the DNA synthesis of HMEC cells and promotes G1 to S phase transition of the cell cycle (Ramirez de Molina *et al.*, 2004). CK overexpression was found to alter the expression of 31 genes and promote cell proliferation (Ramirez de Molina *et al.*, 2008). The expression of transforming growth factor beta (TGF-β), one of the important proteins in G1 cell cycle arrest was down-regulated by the overexpression of CK (Ramirez de Molina *et al.*, 2008). The role of CK in down regulating cell arrest was further confirmed by specific CK inhibitor, MN58b. MN58b was shown to reverse the TGF-β mediated transcriptional activation which was activated by CK overexpression (Ramirez de Molina *et al.*, 2008). In addition, Yamashita and Hosaka (1997) showed that CK mRNA and protein levels were elevated during the exponential phase of tumor cell growth but decreased in the stationary phase. This leads to the accumulation of PC which is the end product of CK. The accumulation of PC resulted from an increased level of CK protein at the enterance of S phase was also found to be essential for cell division (Lykidis and Jackowski, 2001).

1.7.2 Tumorigenesis

CK is overexpressed in both tumorous tissue and tumor derived cell lines (Ramirez de Molina *et al.*, 2002a, Ramirez de Molina *et al.*, 2002b, Ramirez de Molina *et al.*, 2007, Hernando *et al.*, 2009). The overexpression of CK was detected at both mRNA and protein levels (Eliyahu *et al.*, 2007). Furthermore, the levels of the choline metabolites were also elevated in cancerous cells (Katz-Brull *et al.*, 2002, Iorio *et al.*, 2005, Eliyahu *et al.*, 2007). These observations raise the question of whether CK acts as an oncogene or as a byproduct of the physiological alteration associated with oncogene expression.

Earlier study showed that overexpression of CK was the consequence of tumorigenic transformation. Bhakoo *et al.* (1996) showed the elevation of PCho in *ras* oncogene transformed cell. The activity of CK was up-regulated by ras protein through the direct effectors of Ral-GDS and phosphoinositide 3-kinase (PI3K), two of the important mediators for tumorigenesis (Ramirez de Molina *et al.*, 2002a). Treatment with the PI3K inhibitor (PI-103) was shown to suppress the expression level of CK and in turn decrease the level of PCho production and total choline metabolites in the cells (Al-Saffar *et al.*, 2010). In addition, the activity of serine/threonine kinase (AKT), one of the protein kinase in PI3K pathway was also regulated by CK (Chua *et al.*, 2009). Besides, the breakdown product of PC, phosphatidic acid was found as the key activator for the PI3K pathway (Yalcin *et al.*, 2010). Knockdown of the phospholipase D, the enzyme to hydrolyze PC was shown to attenuate the activation of AKT (Toschi *et al.*, 2009).

Oncogenic property of CK in tumor transformation was reported by Ramirez de Molina *et al*. (2005). Overexpression of CK induced oncogenic transformation of human embryo epithelial kidney fibroblasts (HEK293) and Madin-Darby canine kidney cells (MDCK). Co-expression of CK with RhoA from GTPases family further potentiates anchorage independent growth and tumorigenesis. This suggested that CK plays role in Rho-mediated tumor transformation. The role of CK in cell transformation was further confirmed by the specific CK inhibitor, MN58b, which inhibited the CK mediated tumorigenesis (Ramirez de Molina *et al.*, 2005).

Later, Gallego-Ortega *et al.* (2009) showed that the CKα isoform was oncogenic and able to induce cell transformation, but not CKβ isoform. The overexpression of CKβ did not induce tumor growth. In addition, the study also showed the CKα but not CKβ mRNA was elevated in a panel of mammary cancer cell line as compared to the non-tumorogenic mammary cell lines (Gallego-Ortega *et al.*, 2009).

CKα is an important enzyme in cancer cell survival. CKα knockdown in cervical cancer cell line (HeLa) using small interfering RNA (siRNA) resulted in cell death (Glunde *et al.*, 2005, Falcon *et al.*, 2013). However, the inhibition of CKα activity with specific inhibitor, MN58b is not sufficient to induce cell death. This result indicates that the non-catalytic role of CKα is important for the cancer cell survival (Falcon *et al.*, 2013). Cells with single CKβ or double CKα/CKβ knockdown have no aberrant phenotype compared to the single knockdown of CKα (Gruber *et al.*, 2012). In this case, the balance of the CKα and CKβ isoforms also important for cancer cell survival and simultaneous knockdown of CKβ reduced or abolished the cell-killing effect of single CKα knockdown (Gruber *et al.*, 2012).

1.7.3 Differential role of CKα and CKβ

CKα and CKβ knockout mice generated a different phenotype. Early embryonic lethality was observed on CKα knockout mice (Wu *et al.*, 2008) while CKβ knockout mice developed muscular dystrophy (Sher *et al.*, 2006). Thus, CKα plays important role in early development of mouse embryo while CKβ is involved in the later part of mouse development. Heterozygous CKα knockout mice ($ck\alpha^{+/-}$) have a normal early embryonic development and the biosynthesis of PC was unaffected although the PC synthesis was decreased by 30% (Wu *et al.*, 2008). No significant compensation was found from CKβ in homo and heterozygous CKα knockout mice because the mRNA and protein levels of CKβ were not increased in both of the CKα knockout mice (Wu *et al.*, 2008). The evidence supported that CKα and CKβ have different roles in maintaining the PC homeostasis.

Sher *et al*. (2006) reported that CKβ knockout mice developed hindlimbs muscular dystrophy and neonatal forelimb bone deformity. Total CK activity was generally decreased in all tissues, however muscle dystrophy was only observed in skeletal muscle of hindlimbs (Sher *et al.*, 2006). CKβ was involved in PC metabolism of hindlimb muscle, while CKα was responsible for PC synthesis in forelimb muscle as muscular dystrophy did not develop in forelimbs due to CKα abundance and stable PC homeostasis (Wu *et al.*, 2010). CKα was not overexpressed in the CKβ knockout mice to compensate for the loss of CKβ activity (Wu *et al.*, 2009).

Mitochondria abnormalities were observed on the skeletal muscle of CKβ knockout mice whereby mitochondria were absent on the center of muscle fibers and large mitochondria was found at the peripheral fiber (Mitsuhashi *et al.*, 2011a, Wu *et al.*, 2009). The PC level was low in the isolated mitochondria. The activity of the respiratory chain enzyme (complex I-IV) and the ATP production of the defected mitochondria in CKβ knockout mice also decreased. In addition, the molecular markers of mitophagy were found in the defected mitochondria suggested that the loss of *ckβ* gene resulted in mitochondria dysfunction and led to the development of muscular dystrophy (Mitsuhashi *et al.*, 2011a).

In human, heterozygous mutation of *chkβ* was detected in 15 patients with congenital muscular dystrophy from Japan, Turkey and Britain (Mitsuhashi *et al.*, 2011b). CK activity was not detected in the muscle tissue of the patients and the PC content of the frozen biopsied muscle tissues was lower than normal individual. A total of 11 mutations were identified and these mutations mostly truncated the protein or eliminated the conserved region of CKβ protein (Mitsuhashi *et al.*, 2011b). Besides muscular dystrophy, patients with *chkβ* gene mutation also have severe mental retardation (Mitsuhashi *et al.*, 2011b). Previously, the decreased CKβ expression has been linked with narcolepsy, a sleep disorder (Miyagawa *et al.*, 2008). The findings supported the involvement of CKβ in the maintenance of normal brain function in humans.

Gutierrez Rios *et al*. (2012) also reported the *chkβ* gene mutation in an American patient with congenital muscle dystrophy. The mutation also truncated the protein by introducing a stop codon at Gln 292. Giant mitochondria containing densely packed

and whorled cristae were observed in the tissue biopsy. The authors concluded that CKβ was involved in mitochondria-associated membrane phospholipid metabolism (Gutierrez Rios *et al.,* 2012). They also postulated that CKβ gene defect could consequently affect the production of active human muscle type carnitine palmitoyltransferase I (M-CPTI) protein, a key lipid transport enzyme in the outer membrane of mitochondrial. The transcription of *chkβ* and *cpt1β* genes were bicistronic (Yamazaki *et al.,* 2000). In consequence, the mitochondria dysfunction might also be due to the defect of *cpt1β* gene expression which affect the activity of mitochondrial respiratory chain.

1.8 Regulation of choline kinase

1.8.1 Transcriptional level

Several studies on the promoter regions have shed light on the transcription regulation of CK genes by transcription factors. Aoyama *et al*. (2007) reported an up-regulation of CKα expression in mouse liver after treatment with carbon tetrachloride (CCl_4). The overexpression of CK was contributed by the binding of c-jun transcription factor to an AP-1 element (at −866 bp upstream of translational start site) upon treatment with CCl_4 (Aoyama *et al.*, 2007).

In human, the putative promoter region upstream of *ckα* gene (− 2.3 kb region upstream of translational start site) was isolated by Glunde *et al*. (2008). Their study showed that the expression of CKα was regulated by hypoxic condition. Eight hypoxia responsive elements (HREs) sites were predicted by promoter sequence analysis. The responsive elements composed of two non-overlapping regions which up-regulated (− 1068/− 851) and down-regulated (− 670/+1) the CKα expression

during hypoxia (Glunde *et al.*, 2008). Highly repressive element was found at the position −225/−222 bp upstream of translational start site (Bansal *et al.*, 2012). The binding of hypoxia-inducible factor (HIF-1α) on the respective HRE sites was shown to suppress the mRNA expression of CKα (Glunde *et al.*, 2008; Bansal *et al.*, 2012).

Recently, Yee (2012) reported the isolation of promoter region of human c*kβ* gene (−2 kb region upstream of translational start site). GATA and Ets were identified as the important transcription factors that suppressed the expression of CKβ expression (Yee, 2012).

1.8.2 Translational level

To date, translational regulation of CK had not been reported. The translational regulation of CTP-phosphocholine cytidyltransferase (CCT), a second enzyme in CDP-choline pathway had been postulated in X-box-binding protein (XBP-I(S)) transducted fibroblasts (Sriburi *et al.*, 2007). The expression of the XBP-I(S) was shown to increase the assembled (80S) ribosomes which enhanced the protein synthesis of CCT. However, the detailed mechanism of XBP-I(S) in enhancing the translation of CCT needs further investigation.

1.8.3 Post-translational level

Post-translational regulation of CK by phosphorylation was first described in yeast CK. Yeast CK is phosphorylated by protein kinase A (PKA) and protein kinase C (PKC) (Kim and Carman, 1999, Yu *et al.*, 2002, Choi *et al.*, 2005). Phosphorylation of yeast CK with PKA and PKC increased the catalytic activity by 1.9 and 1.6 folds,

respectively (Kim and Carman, 1999, Choi *et al.*, 2005). Furthermore, phosphorylation of yeast CK was shown to increase the production of PCho and PC. Ser 30 and Ser 85 residues were identified as the PKA phosphorylation sites (Yu *et al.*, 2002), whereas Ser 25 and Ser 30 residues were identified as the PKC phosphorylation sites (Choi *et al.*, 2005). PKA and PKC were found to phosphorylate yeast CK at the same residue of Ser 30 (Choi *et al.*, 2005).

In human, regulation of CKα by phosphorylation had been reported by Miyake and Parsons (2011). This proto-oncogene tyrosine protein kinase Src (c-Src) dependent phosphorylation occurred at Tyr 197 and Tyr 333. Phosphorylation of CKα was important for its interaction with EGFR complex. The phosphorylation level of CKα was increased by c-Src co-expression. The phosphorylation increased the catalytic activity of CKα by 2.5 folds (Miyake and Parsons, 2011). In addition, the subcellular location of CKα is also affected by phosphorylation. Co-expression of c-Src and EGFR translocated the CKα protein from cytosol to the cell membrane (Miyake and Parsons, 2011).

CTP-phosphocholine cytidylyltransferase (CCT), the second enzyme in CDP-choline pathway is also regulated by phosphorylation (Dennis *et al.*, 2011). The phosphorylated form of CCT is inactive and retained in cytosol, while the unphosphorylated form of CCT is active and being translocated to the cell membrane for PC biosynthesis (Hatch *et al.*, 1992). The isoforms of CCTα and CCTβ possess a divergent N-terminal and C-terminal phosphorylation region. Generally, the binding affinity of CCTβ towards anion membrane was weaker than the CCTα isoforms. However, after phosphorylation, CCTβ showed a higher binding

affinity to the anion membrane than the phosphorylated CCTα isoforms (Dennis *et al.*, 2011).

1.9 Phosphorylation

About one third of the human proteins are reported as phosphoproteins (Cohen, 2000). The modification occurs rapidly and less energy is required since it does not involve re-synthesis or degradation of the existing protein. Furthermore, the process of phosphorylation is reversible (Fischer and Krebs, 1955). Protein kinase is responsible for protein phosphorylation by transferring the phosphate (PO_4^{3-}) group from ATP, while protein phosphatase removes the phosphate group from the protein substrate. In eukaryotes, the hydroxyl groups (OH) of serine, threonine and tyrosine amino acid side chains are the common targets of protein phosphorylation (Manning *et al.*, 2002).

The process of phosphorylation was first described by Burnett and Kennedy (1954). They showed that a liver enzyme catalyzed the phosphorylation of casein. A year later, Fischer and Krebs (1955) discovered a process known as reversible phosphorylation of an enzyme involved in glycogenolysis. They won the Nobel Prize in medicine in 1992 by showing the phosphorylation of glycogen phosphorylase. The process involved the phosphorylation of phosphorylase b (unphosphorylated form) into phosphorylase a (phosphorylated form) in the presence of metal ion and ATP. Later, the key enzyme called protein kinase involved in phosphate transfer was discovered (Fischer and Krebs, 1955). Extensive investigation was then focused on protein phosphorylation after Linn *et al*. (1969) suggested that phosphorylation played a key role in the regulation of metabolic

pathway. This led to the discovery of important protein kinases in different metabolic pathways.

Phosphates are negatively charged, thus the addition of a phosphate group onto a protein will certainly change its characteristic (Kitchen *et al.*, 2008). These changes had been reported to regulate the function of enzyme, cellular movement, protein-protein interaction and protein stability (Johnson and Barford, 1993, Nishi *et al.*, 2011). Tight regulation of protein phosphorylation is important and phosphorylation is generally regulated by the balance between the activity of protein kinases and phosphatases.

1.10 Protein kinase A

PKA holoenzyme is a heterotetramer protein composed of two regulatory (R) subunits and two catalytic (C) subunits (Corbin *et al.*, 1973, Corbin and Keely, 1977). Two classes of PKA holoenzymes have been identified; type I and type II with the difference in the R subunit with RI for type I and RII for type II. Both R and C subunits exist as multiple isoforms; RIα, RIβ, RIIα, RIIβ, Cα, Cβ, and Cγ. Type I PKA is mostly found in the cytosol of the cell, while the type II is anchored in a specific compartment. Generally, the type I holoenzyme has a higher affinity towards cAMP than the type II holoenzyme (Tasken and Aandahl, 2004). RI subunit is expressed predominantly in brain and nervous system, while the RII subunit is generally detected in heart, liver and fat tissues (Cummings *et al.*, 1996). Cα subunit is expressed ubiquitously, while the other isoforms are detected in specific tissues. For example the Cγ is mostly found in testis (Beebe *et al.*, 1990).

PKA holoenzyme is inactive. There are two cAMP binding sites on each R subunit of PKA, known as A and B sites. Only B site is exposed and available for binding of the cAMP. When the B site is occupied, it enhances the binding of cAMP to the A site by an intramolecular steric change. Bindings of four cAMP molecules on both R subunits cause a conformational change and the holoenzyme is dissociated into dimer R subunit and monomer of C subunits (Kopperud *et al.*, 2002). The dissociated C subunits are active and able to phosphorylate the nearby substrate. Figure 1.4 shows the schematic diagram of the PKA activation. PKA phosphorylates the serine and threonine residues in specific consensus sequences of RRXS/T, AKXS/T, and KKXS/T, X represents any residue (Shabb, 2001, Songyang *et al.*, 1994).

1.10.1 Regulation of Protein kinase A

PKA is regulated by the cAMP-signaling pathway. The pathway is triggered extracellularly via binding of a specific ligand to the G protein-coupled receptor (GPCRs). The ligands are small molecules such as catecholamines, lipids, neurotransmitters or a large protein for example hormones. Upon binding of the ligand on the receptor, GPCRs will undergo conformational change and activate the heterotrimeric G protein. This leads to the dissociation of α subunit of G protein from β and γ subunits and activates the adenylyl cyclases. The activated adenylyl cyclases are responsible to increase the intracellular level of cAMP by converting ATP into cAMP (Taylor *et al.*, 1990, Pidoux and Tasken, 2010). The cAMP-signaling pathway is shown in Figure 1.5. Conversely, phosphodiesterases (PDEs) are responsible to control the local pool of adenylyl cyclase-generated cAMP by