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Role of Protein Kinase C-iota in Glioblastoma

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Role of Protein kinase C-iota in Glioblastoma

by

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A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
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DEDICATION

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LIST OF ABBREVIATIONS

GBM	Glioblastoma
CNS	Central nervous system
PKC	Protein kinase C
aPKC	atypical protein kinase C
PKC- ι	Protein kinase C-iota
PKC- ζ	Protein kinase C-zeta
PRK	Protein kinase related kinases
LOH	Loss of Heterozygosity
EGFR	Epidermal growth factor receptor
TP52	Tumor protein 53
PTEN	Phosphatase and tensin homology
PKA	Protein kinase A
PKB	Protein kinase B
PI (3)-kinase	Phosphatidylinositol (3)-kinase
PDK-1	Phosphoinositide-dependent kinase -1
RACKS	Receptors for activated C- kinase
STICKS	Substrates that interact with C-kinase
PS	Phosphatidylserine
DAG	Diacylglycerol
FFA	Free fatty acid
Bcl-2	B cell lymphoma
Bcl-XL	B cell lymphoma extra large
Bad	Bcl-2 associated death promoter
IL3	Interleukin 3
CDK	Cyclin dependent kinase
Cdk7	Cyclin dependent kinase 7
Cdk2	Cyclin dependent kinase 2
CAK	Cyclin dependent kinase activating kinase
CKI	CDK inhibitory family
RNA PolII	RNA polymerase II
CTD	C-terminal domain
MAT1	menage a trois-1
G phase	Gap phase
S phase	DNA synthesis phase
M phase	Mitosis phase
IgG	Immunoglobulin G
IP	Immunoprecipitation
kDa	Kilo Dalton
MAP	Mitogen-activated protein kinase
NSCLC	Non-small Cell Lung Cancer

PARP	Poly (ADP-ribose) polymerase
Rb	Retinoblastoma
SCF	Stem cell factor
Skp2	S-phase kinase-associated protein 2
CBP	CREB binding protein
NPAT	Nuclear protein, ataxia-telangiectasia
Cdc6	Cell division control protein 6
MCM6	Minichromosome maintenance deficient 6
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
siRNA	Short interfering RNA
µg	Microgram
NNK	Nitrosoamine 4-(methylnitrosomino)-1-(3-pyridyl)-1butanone
Ser	Serine
Thr	Threonine
Lys	Lysine
Caspase	Cysteine dependent aspartate-specific proteases
IAP	Inhibitor of apoptosis protein
Smac	Second mitochondria-derived activator of caspases
Diablo	Direct IAP-binding protein with low pI
APAF1	Apoptotic protease activating factor 1

ABSTRACT

The focus of this research was to investigate the role of protein kinase C- ι (PKC- ι) in the regulation of Bad function, a pro-apoptotic member of the Bcl-2 family and Cdk7 function, a master cell cycle regulator in glioblastoma.

The results were obtained from the human glial tumor derived cell lines, T98G and U87MG. In these cells, PKC- ι co-localized and directly associated with Bad as shown by immunofluorescence, immunoprecipitation, and Western blotting. Furthermore, *in-vitro* kinase activity assay showed that PKC- ι directly phosphorylated Bad at phospho specific residues, S112, S136 and S155 which in turn induced inactivation of Bad and disruption of the Bad/Bcl-XL dimer. Knockdown of PKC- ι by siRNA exhibited a corresponding reduction in Bad phosphorylation suggesting that PKC- ι may be a Bad kinase. Since, PKC- ι is an essential downstream mediator of the PI (3)-kinase, we hypothesize that glioma cell survival is mediated via a PI (3)-kinase/PDK1/PKC- ι /Bad pathway. Treatment with PI(3)-kinase inhibitors Wortmannin and LY294002, as well as PDK1 siRNA, inhibited PKC- ι activity and subsequent phosphorylation of Bad suggesting that PKC- ι regulates the activity of Bad in a PI (3)-kinase dependent manner.

Robust expression of PKC- ι is a hallmark of human glioma and benign and malignant meningiomas, however, little is understood about its role in glioma cell proliferation. The cyclin dependent kinase activating kinase complex (CAK), comprises of cyclin dependent kinase 7 (Cdk7), cyclin H and MAT1, is the master cell regulator.

Cdk7 phosphorylates its downstream cyclin dependent kinases (cdks) and promotes cell proliferation. Results show that PKC- ι directly associated and phosphorylated Cdk7 at T170. Furthermore, Cdk7 phosphorylated its downstream target, cyclin dependent kinase 2 (cdk2) at T160. Purified PKC- ι was also observed to phosphorylate endogenous as well as exogenous Cdk7. PKC- ι knockdown with siRNA, PDK1 siRNA and (PI) 3-kinase inhibitors, Wortmannin and LY294002 treatment exhibited corresponding reduction in phosphorylation of Cdk7 and subsequently cdk2. In addition, PKC- ι knockdown reduced cell proliferation; led to cell cycle arrest and also induced apoptosis. Thus, these findings suggest the presence of a novel PI (3)-kinase/PKC- ι /BAD mediated cell survival and PI (3)-kinase/PKC- ι /Cdk7 mediated cell proliferation pathway.

CHAPTER 1

INTRODUCTION

1.1 Project hypothesis

An activated PKC- ι dependent anti-apoptotic pathway drives the cell cycle proliferation and survival of glioma cells.

1.1.1 *Main objective.* PKC- ι is shown to be involved in tumor proliferation, angiogenesis, invasion, cell survival as well as chemoresistance in several types of cancers [1-5]. Although, PKC- ι is implicated to be involved in these cellular events, its mechanism has not been completely studied. We initiated a study to examine the effect of PKC- ι overexpression and/or aberration in glioma cell survival and proliferation. T98G (p53 mutated) and U87MG (PTEN null) are Grade IV metastatic human tumor derived glioma cells. Mutations of similar nature are commonly observed characteristics in glioblastoma[6]. Therefore, understanding the signaling mechanism by which such uncontrolled cell progression and proliferation occur may help diagnose the disease earlier and aid in improving long term survival of glioblastoma patients. In the current study, our main aim was to determine whether PKC- ι was highly activated and whether it is crucial for glioma cell proliferation and survival.

1.2 Glioma

1.2.1 Overview. Brain tumors are the biggest challenges of all cancers since they destroy the most important part in the body. Brain tumors are known to be the second most common cause of cancer-related death in people in their old age. Each year 1 of every 5,000 people in the age group 35- 60 years develops a brain tumor. Brain tumors are the most common form of solid tumor in children [7]. The incidence of brain tumor is higher in men than in women. The American Cancer Society estimates that there are more than 18,000 new diagnoses of brain and nervous system cancers causing more than 12,000 deaths each year in the United States [8]. Among all age groups, survival of younger patients was significantly longer with a range from a median of 8.8 months (<50 years) to 1.6 months (>80 years) [6, 9].

1.2.2 Types of brain tumors. Brain tumor, in common term has long been understood as a tumor of brain as a whole. However, it is interesting to note that according World Health Organization, there are about 126 different types of brain and spinal cord tumors. [10]. Each and every part of brain and spinal cord are susceptible to tumor formation (Fig 1). There are two main categories of brain tumor – (i) Primary tumors are of two types (a) Benign (b) Malignant and (ii) Secondary (metastatic) brain tumors - originate from the malignant tumors of other body parts. They can be classified according to cell type, by grade and by location (Table 1).

1.2.3 *Symptoms.* Glioma symptoms depend on which part of the central nervous system is affected. Glioma of the brain can cause headaches, nausea and vomiting, memory loss, language deficit, seizures and cranial nerve disorders due to aggravated intracranial pressure. Optic nerve glioma can cause visual loss. Spinal cords gliomas can cause pain, weakness, or numbness in the extremities [8].

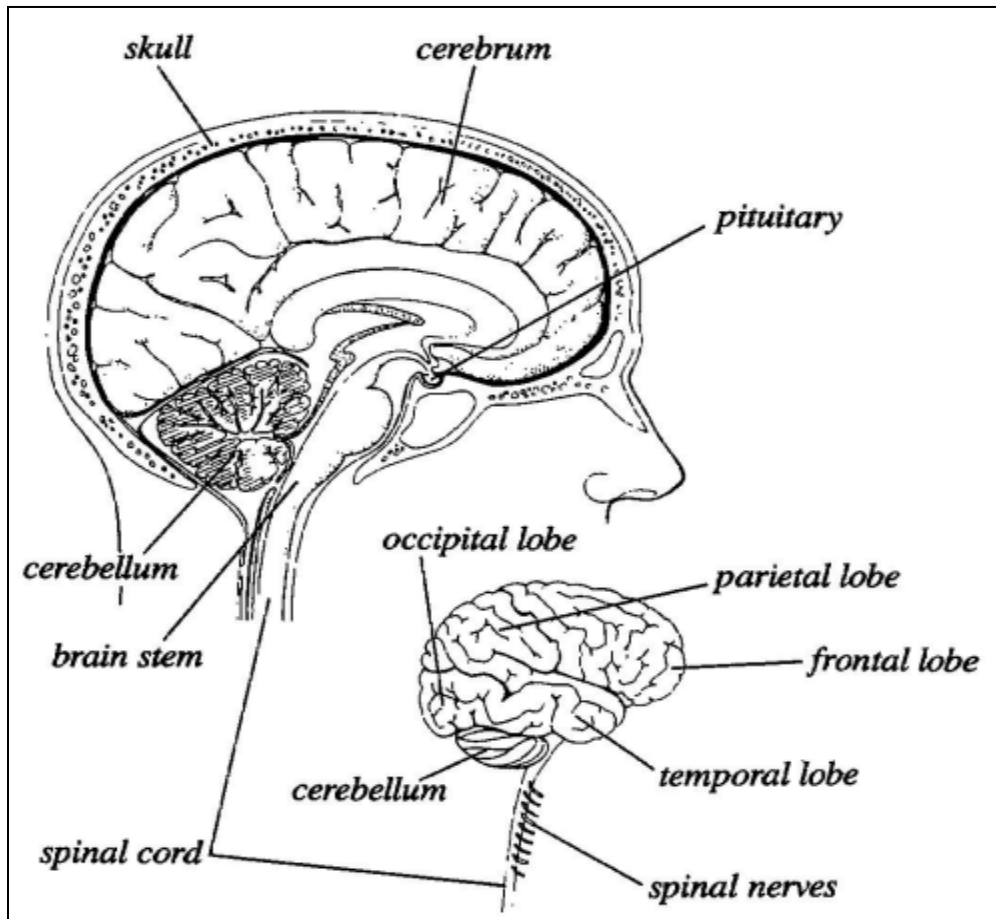


Figure 1 Parts of the brain and spinal cord.

Table 1 WHO Classification of Primary brain tumors.

Tumors	Grade	Type
Neuroepithelial tumors		
1.	<i>Astrocytic tumors</i>	
a	Pilocytic astrocytoma	I Benign
b	Subependymal giant cell astrocytoma	I Benign
c	Diffuse astrocytoma	II Benign
d	Pleomorphic xanthoastrocytoma	II Benign
e	Anaplastic astrocytoma	III Malignant
f	Glioblastoma	IV Malignant
2.	<i>Oligodendroglial tumors</i>	
a	Oligodendroglioma	II Benign
b	Anaplastic oligodendroglioma	III Malignant
3.	<i>Oligoastrocytic tumors</i>	
a	Oligoastrocytoma	II Benign
b	Anaplastic oligoastrocytoma	III Malignant
4.	<i>Other neuroepithelial tumors</i>	
a	Angiogenic glioma	I Benign
b	Chordoid glioma of the third ventricle	II Benign
5.	<i>Neuronal and mixed neuronal-glioma tumors</i>	I-III Low-high
6.	<i>Pineal tumors</i>	I-IV Low-high
7.	<i>Embryonal tumors</i>	IV Malignant
Tumors of cranial and paraspinal nerves		
1.	<i>Schwannoma</i>	I Benign
2.	<i>Neurofibroma</i>	I Benign
3.	<i>Perineurioma</i>	I-IV Low-high
4.	<i>Malignant peripheral nerve sheath tumor</i>	II-IV Low-high
Tumors of the meninges		
1.	<i>Meningioma (benign)</i>	I Benign
2.	<i>Atypical meningioma</i>	II Benign
3.	<i>Anaplastic meningioma</i>	III Malignant
Lymphomas and hematopoietic neoplasms		
Tumors of the sellar region		
		I Benign

Information from reference [11]

1.2.4 Treatment strategy. Every part of brain plays a very delicate yet highly complex function which makes surgical removal of this organ impossible. Brain tumors are found in the tissue inside the skull that leaves very little room for tumor growth without compressing or damaging the normal brain. This can disrupt the blood-brain barrier which then presses onto the sensitive tissue, blocking the flow of blood and other fluid causing pain and inflammation leading to necrosis thereby ruining the neurological functions of the brain. Thus, treatment of gliomas depends on the cell type, location and grade of malignancy (histopathology) [12].

Often, treatment is a combinatorial approach using surgery, radiation therapy and chemotherapy. (A) Surgery - Even though it is difficult to perform surgery on the brain tissue, surgery still is usually the first treatment in brain tumor. It is generally a treatment of choice for primary brain tumor if the subject is a candidate for complete resection. Surgery is often curative for intracranial tumors that occur outside the brain, such as pituitary adenomas, schwannomas, and meningiomas [13, 14]. (B) Radiation therapy - Radiation therapy post-surgical resection is a standard therapy for high-grade gliomas. Radiation can be delivered by external or internal methods. External beam radiation can be conventional or stereotactic radiosurgery [15]. The dosage of treatment depends on the location, grade, histology and extent of resection. Interstitial radiotherapy (brachytherapy) is also prescribed however it involves surgically implantation of the radioactive material directly inside the tumor. (C) Chemotherapy - Recent studies in signaling mechanisms have shown new therapeutic targets and development of chemotherapy against these targets have slightly improved the management of primary

brain tumors. Identifying and targeting these important down-stream mediators and combining chemotherapy with radiation may improve survival in high grade gliomas [16, 17]. Temozolomide in combination with radiation has shown a positive response against high-grade gliomas [18]. Agents like irinotecan (Camptosar) and targeted agents such as bevacizumab (Avastin), which targets the vascular endothelial growth factor; and gefitinib (Iressa), erlotinib (Tarceva), and imatinib (Gleevec), which target the epidermal and platelet-derived growth factor receptors, have shown some promise in the treatment of recurrent malignant gliomas [19].

1.2.5 Overview of Glioblastoma. Glioblastoma Multiforme is the most lethal of brain tumors as it infiltrates the entire central nervous system (CNS) and may develop *de novo* (primary glioblastoma) or by progression from low-grade astrocytoma (secondary glioblastoma) (Table 2). Glioblastoma Multiforme is labeled as a Grade IV Astrocytoma. It comprises of more than 25% of all primary brain tumors [7, 20]. Adults are known to be more prone to brain tumor in comparison to children. Its infiltrative nature makes it highly incurable. Glioblastoma patients typically show Loss of Heterozygosity (LOH) on chromosome 10q, genetic alterations such as epidermal growth factor receptor (EGFR) amplification, tumor protein 53 (TP53) mutation, p16^{INK4} deletions and phosphatase and tensin homology (PTEN) mutation [21]. Surgical removal of the tumor in most cases seems to increase survival time but generally the tumor spreads throughout CNS which makes its recurrence very obvious. Radiation therapy has induced more toxic effects on the brain and its surrounding tissue instead of curing it [15]. Glioblastoma is found to be highly resistant to most chemotherapeutic treatment as well, although, drugs like

Temozolomide have been shown to increase survival in patients with high grade glioblastoma [18].

Table 2 Genetic pathways leading to primary and secondary glioblastomas

Type of glioblastoma	Type of mutation	% prevalence	WHO grade
Low-grade astrocytoma	TP53 mutation	59	II
Anaplastic astrocytoma	TP53 mutation	53	III
Primary glioblastoma (<i>de novo</i>)	LOH 10q	70	IV
	EGFR amplification	36	
	p16 ^{INK4a} deletion	31	
	TP53 mutation	28	
	PTEN mutation	25	
Secondary glioblastoma	LOH 10q	63	IV
	EGFR amplification	8	
	p16 ^{INK4a} deletion	19	
	TP53 mutation	65	
	PTEN mutation	4	

Information from reference [6].

1.3 Protein kinase C

1.3.1 Overview. Protein kinases are main controllers of cell function which by adding phosphate groups to the downstream molecules regulate the signaling cascade. Protein kinases can be classified into (i) serine/threonine protein kinases - which phosphorylate serine or threonine residues, (ii) protein tyrosine kinases - which phosphorylate tyrosine residues. Protein kinase C (PKC) is one of the primary protein kinases discovered. PKC is a family of 12 or more related serine/threonine kinases which are important targeted mediators in signal transduction mechanism [22-25].

1.3.2 PKC isozymes. PKC can be divided into three main groups of isozymes depending on their protein structures as well as their requirement of specific activators and co-factors (i) the conventional PKC isozymes -alpha (α), beta I (β I), beta II (β II) and gamma (γ) are dependent on calcium, diacylglycerol (DAG) and phospholipids, (ii) the novel PKC isozymes - delta (δ), epsilon (ϵ), eta (η) and theta (θ) are calcium independent but dependent on DAG and phospholipids, (iii) the atypical PKC isozymes - PKC zeta (ζ) and PKC iota (ι) in humans/ lambda (λ) in mouse are insensitive to calcium and DAG. An additional family member has been introduced into the PKC family, Protein Kinase D, mu (μ) [26]. Another, distant related family member includes PKC-related kinases (PRK 1-3), known as PKN [27] (Table 3).

1.3.3 Structure of Protein kinase C. Protein kinase C family members are made up of a single polypeptide chain (Fig 2). Primary structure of PKC consists mainly of regulatory domain (NH₂ terminal, approximately 20 - 40 kDa) involved in the binding of phorbol esters and Ca²⁺ [28] and a catalytic domain (COOH terminal, approximately 45 kDa) involved in ATP and substrate binding. Conventional PKCs and Novel PKCs have two tandem repeats of these motifs called C1A and C1B involved in the binding of second messenger DAG and phorbol esters as well as membrane lipids [29, 30]. C2 domain is the Ca²⁺ binding domain involved in the activation of conventional PKCs [31, 32]. Novel PKCs have a C2-like domain but are not regulated by Ca²⁺ and thus utilize protein-protein interactions for activation [33]. Atypical PKCs lack the residues for Ca²⁺ binding C2 domain but comprise of an additional PB1 domain which is known to function in protein-protein interaction [34].

The catalytic domain (45kDa) is structurally similar amongst all the PKC isozymes made up of two lobes. It is also 40% identical to the kinase domain of protein kinase A (PKA) and protein kinase B (AKT). Phosphorylation at kinase domain and release of pseudosubstrate from the regulatory domain are required for activation of PKC which opens the frame for substrate binding at the catalytic site [35].

1.3.4 Protein kinase C regulation. Protein kinase C becomes catalytically competent as well as localized intracellularly by a cascade of three orchestrated phosphorylations [36] (Fig 3). The first step in the maturation of PKC is phosphorylation at the entrance of the kinase core called activation loop. Phosphoinositide-dependent kinase -1 (PDK-1) has been shown to act upstream of PKC and triggers this step [37]. This initiates autophosphorylation at the tip of the kinase domain turn known as the turn motif. It is important for the maturation of PKC; for protein-protein interaction and for initiating the mechanism of docking the enzyme to the membrane. PKC is then localized into the cytosol and upon extracellular stimulation, phospholipase C mediated breakdown of phosphatidylinositol into phosphatidylinositol (3)- kinase (PI(3)-kinase) and in the presence of co-factors, a conformational change is triggered resulting in the protein releasing the auto-inhibitory pseudosubstrate [38]. Subsequently, PKC undergoes autophosphorylation at hydrophobic motif [39]. The primary role of this phosphorylation site is to stabilize the protein from proteolytic degradation [40].

PKC function is dependent upon its correct distribution and localization in the cells. PKC interacts with certain anchoring proteins such as RACKS (receptors for activated C- kinase) or STICKs (substrates that interact with C-kinase) whose functions are (i) bring PKC isozymes in close proximity with their regulators and substrates, (ii)

help in translocation of newly synthesized PKCs to the plasma membrane and (iii) help in binding the PKC cofactors [41-44]. Most PKCs are ubiquitously present in most tissues but each PKC differs in their substrate specificity and co-factor activation. There are several activators of PKCs known till date, some of which are mentioned in Table 4.

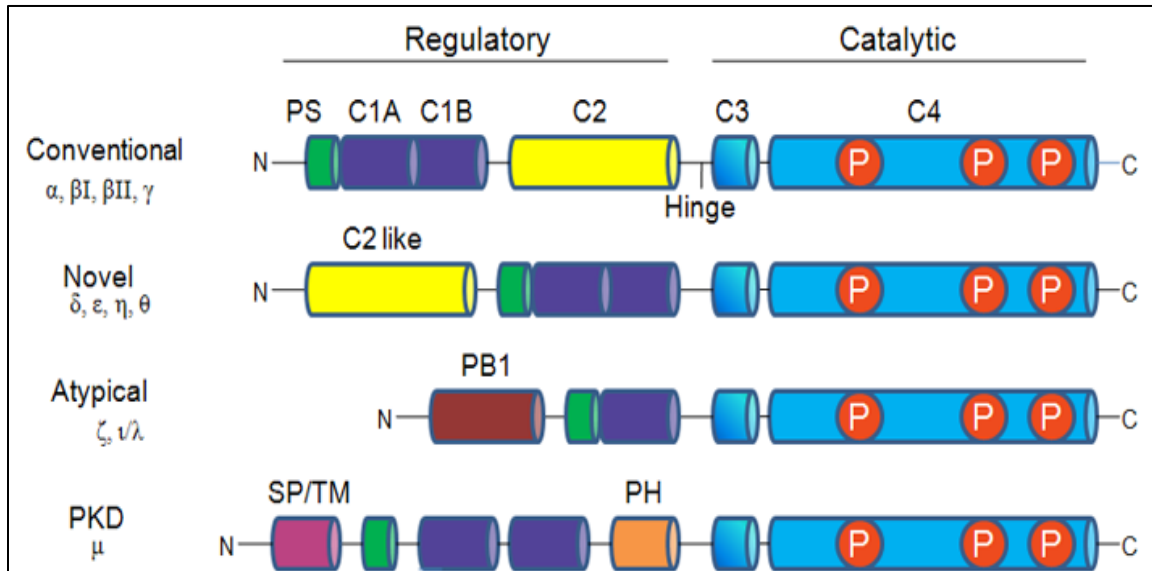


Figure 2 Primary Structure of Protein Kinase C and its members. Depicted in the figure are pseudosubstrate (PS, green), cysteine-rich domain (C1A and C1B, purple), calcium-binding domain (C2, yellow), protein:protein interaction motif (PB1, dark red), transmembrane domain (SP/TM, pink), pleckstrin homology domain (PH, orange), kinase domains (C3, C4, light blue) consists of 3 phospho residues for each PKC isozyme (Adapted from [45]).

Table 3 PKC isozymes and their size distribution

PKC isozymes	M _w (kDa)	Amino Acids	Source	Tissue distribution
Conventional PKC isozymes				
alpha (α)	76.8	672	human	Ubiquitous
beta I (βI)	76.9	673	human	omnipresent in most tissues
beta II (βII)	76.8	671	human	omnipresent in most tissues
Novel PKC isozymes				
gamma (γ)	78.4	697	human	neural
delta (δ)	77.5	673	rat	ubiquitous
epsilon (ε)	83.5	737	rat	ubiquitous
eta (η)	77.6	680	human	neural, epithelium
theta (θ)	82.0	706	Human	ovary, skeletal muscle, platelets
Atypical PKC isozymes				
zeta (ζ)	67.7	592	Rat	omnipresent in most tissues
iota (ι)/ lamda (λ)	67.2	586	human/ mouse	ubiquitous
Protein Kinase D				
mu (μ)	115	912	mouse	Unknown

Adapted from [46].

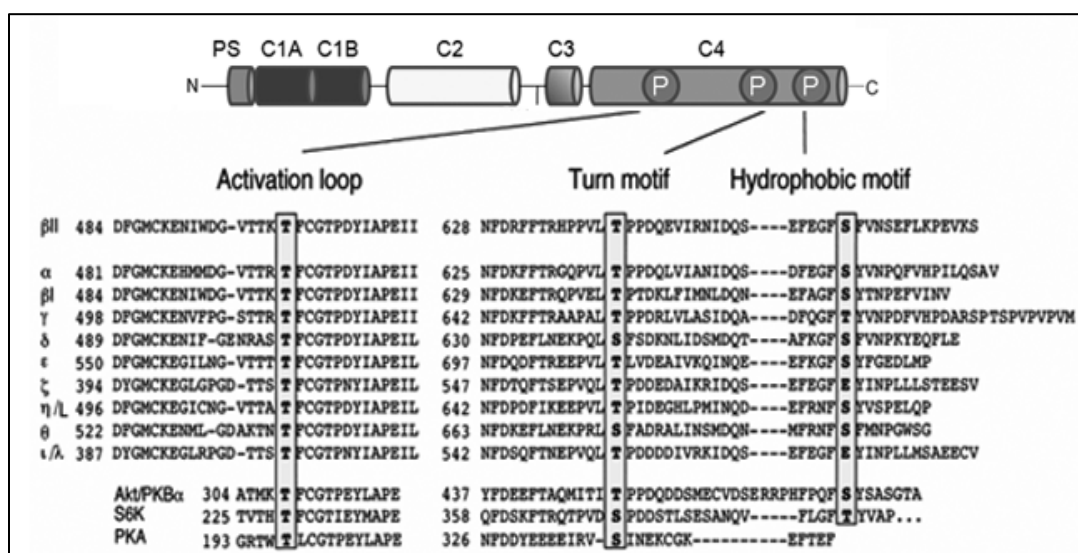


Figure 3 PKC activation motifs.(adapted from [24])

Table 4 PKC activators

	Conventional PKCs				Novel PKCs					Atypical PKCs	
	α	βI	βII	γ	δ	ε	η	θ	ζ	ι	
PS	+	+	+	+	+	+	+	+	+	+	
DAG	+	+	+	+	+	+	+	+	-	-	
Ca ²⁺	+	+	+	+	-	-	-	-	-	-	
FFA	+	+	+	+	+	+	+	?	+	+	
LysoPC	+	+	+	+	+	+	+	?	+	+	

Adapted from [47-51].

1.4 Phosphatidylinositol (3)-kinase

Atypical PKC- ι is activated by phosphatidylinositol (3)-kinase (PI (3)-kinase) through phosphorylation and activation of phosphatidylinositol dependent kinase (PDK-1)

1.4.1 Overview. Phosphatidylinositol 3-kinases are a family of enzymes that play a pivotal role in regulating important cellular mechanisms. PI (3)-kinases are capable of generating lipid second messengers by phosphorylating the 3' hydroxyl group of phosphoinositide lipids (PIs). PI (3)-kinases are at the heart of major intracellular signal transduction pathways. The signals transduced by PI (3)-kinase are known to influence numerous biological processes including cell growth, differentiation, survival, proliferation, migration, glucose metabolism, cytoskeletal organization amongst few. Dysregulation of these pathways can lead to cancer [52]. Evidence suggests that many steps in PI (3)-kinase signaling cascade are known to be mutated, upregulated, overexpressed or hyperactivated in human cancers [53-56]

1.4.2 Classes of PI (3)-kinase. The PI3K family is divided into three classes termed I, II, and III. They are divided on the basis of their structural similarities, regulation and substrate specificity.

All human class I members are heterodimers consisting of a catalytic subunit (MW approx. 110 kDa) and a non-catalytic subunit (MW 50, 55, 85, 87 or 101 kDa). Class I members are further subdivided into class IA and IB PI (3)-kinases. Class IA comprises three isoforms (p110 α , p110 β and p110 δ) whereas the only class IB member is termed p110 γ . [57, 58]. Class I catalyze the production of phosphotidylinositol 3-

phosphate (PI (3) P), phosphatidylinositol 3, 4-bisphosphate (PI (3, 4) P₂), phosphatidylinositol 3, 4, 5-trisphosphate (PI (3, 4, 5) P₃) from phosphatidylinositol (PI).

Class II consists of three catalytic isoforms (C2 α , C2 β , and C2 γ), but, unlike Classes I and III, there are no regulatory proteins. Class II catalyze the production of PI (3) P and PI (3, 4) P₂ from PI. The C-terminal C2 domain of PI 3-kinases lacks critical Asp residues to coordinate binding of Ca²⁺, which suggests class II PI3Ks bind lipids in a Ca²⁺-independent manner.

Class III proteins catalyzes only PI (3) P from PI; however, it is more similar to Class I in structure. They exist as heterodimers of a catalytic (Vps34) and a regulatory domain (Vps15/p150)

There are a more distantly related group of enzymes that are often referred to as class IV PI 3-kinases. The class IV PI 3-kinases family contains ataxia telangiectasia mutated (ATM), ataxia telangiectasia and Rad3 related (ATR), mammalian target of rapamycin (mTOR) and DNA-dependent protein kinase (DNA-PK) [59].

1.4.3 Mechanism of action of PI (3)-kinase and its implication in cancer. Kinases such as AKT, PDK1, PKC contain a pleckstrin homology (PH) domain which enables them to bind to (PI (3, 4, 5) P₃) and (PI (3, 4) P₂) which are produced by activated PI 3-kinase. This translocates the kinase to the plasma membrane. The co-localization of activated PDK1 and AKT (or PKC) allows AKT to be phosphorylated at T308 leading to partial activation of AKT. Full activation of AKT occurs upon phosphorylation at S473 by other upstream kinases (Fig. 4). Several other downstream kinases are activated by a similar PI (3)-kinase mechanism.

The PI (3)-kinase/AKT signaling mechanism had been shown to be required for various cellular activities such as cellular proliferation and survival. The lipid phosphatase, PTEN, antagonizes this process by dephosphorylating (PI (3, 4, 5) P₃) to inhibit activation of AKT (Fig. 4). PTEN in the cell is frequently observed to be altered, mutated or deleted in cancers. PI (3)-kinase activation is tightly regulated by various receptor tyrosine kinases (RTKs) and thus a slight modulation in the receptor activity can multiply PI (3)-kinase activity. EGFR is an upstream activator of PI (3)-kinase and is frequently upregulated in cancer [60]. HER2 is another member of the EGFR family (activator of PI 3-kinase), is often over-expressed due to gene amplification or transcriptional deregulation in breast and ovarian carcinomas [61]. Mutations in multiple AKT isoforms have been reported in several cancer models [54].

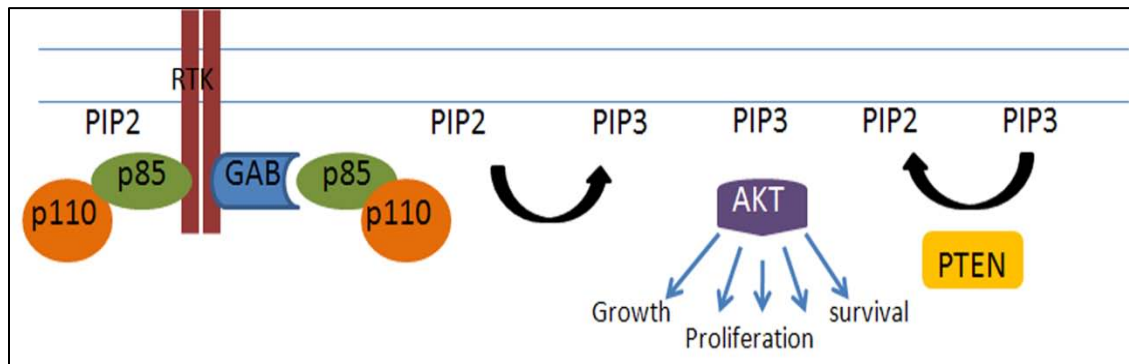


Figure 4 PI3-kinase signaling mechanism RTK recruits PI3-kinase directly or through the binding of protein GAB, catalyzes the conversion of (PI (3, 4) P₂) to (PI (3, 4, 5) P₃) which activates AKT to induce growth, proliferation and survival. PTEN negatively regulates AKT activation by converting (PI (3, 4, 5) P₃) to (PI (3, 4) P₂). All major proteins involved in this cascade are observed to be altered in cancer. [60]

1.4.4 *Inhibitors of PI (3)-kinase.* Some of the inhibitors of PI (3)-kinase are mentioned in Table 1. Inhibitors have also been developed against downstream substrates to PI (3)-kinase [62] (Table 5).

Table 5 Some inhibitors of PI (3)-kinase

Inhibitor	Type of drug	Inhibition profile	IC₅₀ concentration
Wortmannin	- Cell permeable - Potent and selective - Irreversible	- PI (3)-kinase	5nM
LY294002	- Cell permeable - Potent and selective	- PI (3)-kinase	1.4μM
Quercetin	- Flavinoid - Potent	- Mitochondrial ATPase - Phosphodiesterase - PI (3)-kinase	3.8μM
Myricetin	- Flavinoid (differs from Quercetin) - Potent	- α-glucosidase - glyoxalase I - xanthine oxidase - PI (3)-kinase	1.8μM
Staurosporine	- Cell permeable - potent	- Protein Kinase C - Protein Kinase A - CaM kinase - myosin light chain kinase - PI (3)-kinase	9μM

Adapted from reference [63-65].

1.5 Bcl-2 family

PKC- ι phosphorylates and inactivates the function of Bad, a pro-apoptotic member of the Bcl-2 family to promote glioma cell survival.

1.5.1 Overview. A variety of death signals due to physiological and pathological cellular abuse, trigger the genetically programmed apoptotic pathway [66]. Apoptosis induces two major execution programs downstream of the death signal: the caspase activation and organelle dysfunction, of which mitochondrial dysfunction is the best described [67, 68]. BCL-2 family members are primarily involved in death response and play a pivotal role in deciding whether a cell will live or die, manifesting far reaching implication in tumor biology. The Bcl-2 proto-oncogene, the first anti-death gene was discovered at the chromosomal breakpoint of t(14;18) in follicular lymphomas [69].

1.5.2 Members of the Bcl-2 family. The members of the Bcl-2 family can be divided into three sub-families- (i) Bcl-2 subfamily – Bcl-2, Bcl-XL, Bcl-w and Mcl-1 are anti-apoptotic, (ii) Bax subfamily – Bax, Bak and Bok and (iii) BH3 subfamily – Bad, Bid, Bik, Hrk, BNIP3, PUMA, NOXA and BimL (Fig. 5). Upon stimulation by extracellular death signal, all BH3 only proteins interact with Bcl-2/Bcl-XL molecules and modulate their survival function [70-73] (Fig. 5)

1.5.3 Basic features and regulation of Bcl-2 family. The Bcl-2 (B cell lymphoma-2) family proteins are vital regulators of the mitochondrial cell death machinery. Bcl-2 gene codes for a 25kDa protein which resides on the cytoplasmic face on the mitochondrial membrane, the nuclear envelope and endoplasmic reticulum. Bcl-2 family

of proteins has expanded substantially and consists of both pro and anti-apoptotic molecules. The ratio between these two subsets is the deciding factor, in part, the susceptibility of cells to death signals. The members form homo and/or heterodimers to execute their action as well as to compete and titrate one another's function [67].

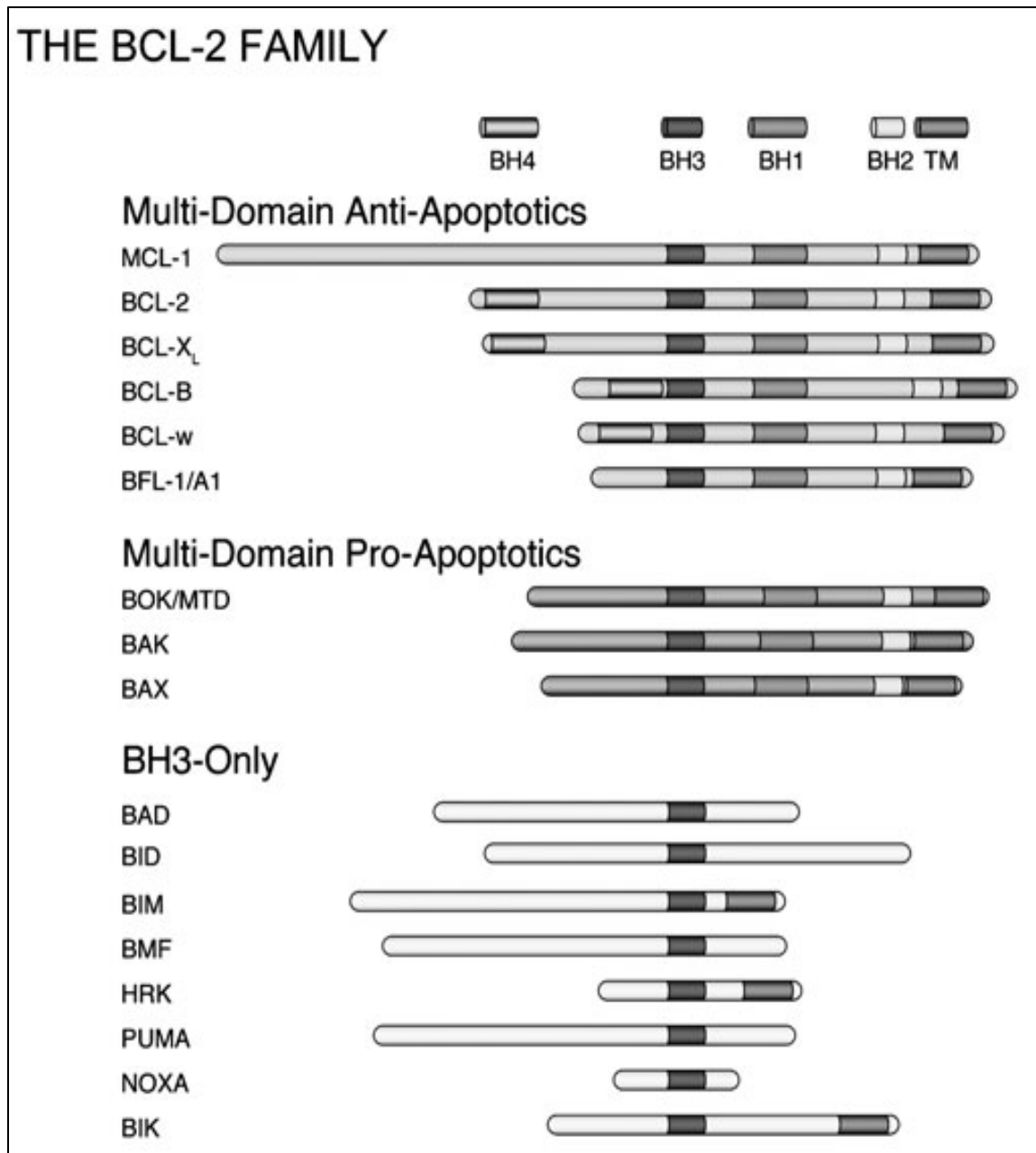


Figure 5 Summary of anti-apoptotic and pro-apoptotic Bcl-2 family members. Adapted from Reference [74].

The members of the Bcl-2 family share one or more Bcl-2 homology (BH) domains (named BH1, BH2, BH3 and BH4). These BH domains are critical as they define the characteristic function of a particular family member. Many anti-apoptotic members have conserved sequence homology in all four BH domains whereas pro-apoptotic are less conserved and differ in their sequence homology. Characterization of BH domains amongst Bcl-2 family members is shown in Table 6. Several BCL-2 family members contain a carboxy-terminal hydrophobic helix, which is essential for docking to membranes such as the mitochondrial outer membrane. The Bcl-2 gene members are regulated on the transcriptional level upon the cell receiving several survival signals from cytokines. Furthermore, the function of Bax is modulated by p53. Bcl-2 family members are also regulated by post-translational modifications such as phosphorylation and ubiquitination [73].

1.5.4 Overview of Bad and its mechanism of action. Bad, (Bcl-2 associated death promoter) is the pro-apoptotic molecule of the BH3 only molecule of the Bcl-2 family involved in initiation of apoptosis. Unlike other Bcl-2 family BH3 only proteins, Bad lacks the hydrophobic C-terminal domain required for mitochondrial outer membrane and nuclear envelop targeting. Bad activity is dependent on three prime residues – Ser112, Ser136 and Ser155 [75]. Upon external death signals (eg., IL3 deprivation), these residues remain dephosphorylated which enables Bad to associate with Bcl-2/Bcl-XL and quench their anti-apoptotic function [72].

To date, several survival kinases such as Akt (PKB), PKC, PKA, Ras, p70S6K and PAK are known to phosphorylate these residues deactivating and disrupting Bad/Bcl-XL dimerization [76]. Dissociated Bad further binds to 14-3-3 scaffold protein and is subsequently degraded by proteosomal ubiquitination [77]. Thus, phosphorylation and dephosphorylation can alternate the binding target of Bad, modulating its pro-apoptotic function. An imbalance among the Bcl-2 family of proteins with more dependency on the anti-apoptotic members is often observed to occur in cancer cells [78, 79] (Fig. 6).

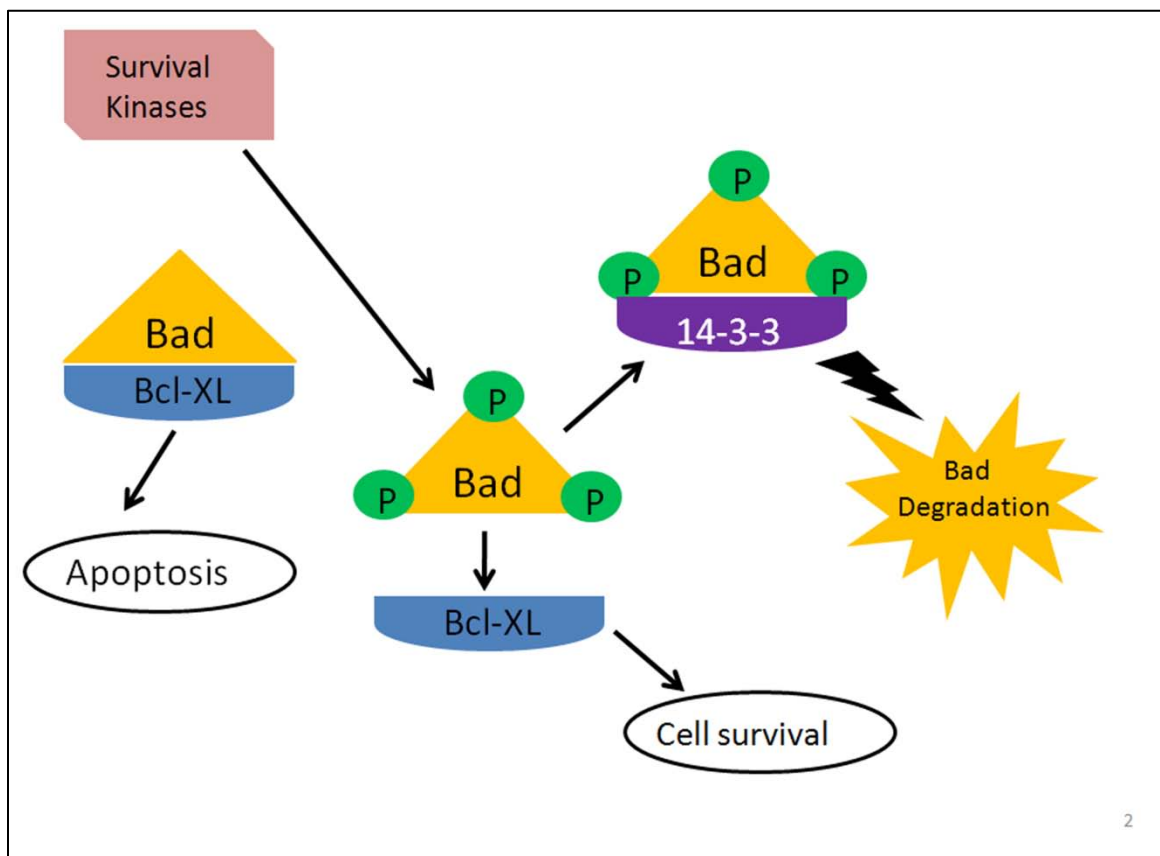


Figure 6 Mechanism of action of Bad, a pro-apoptotic molecule of Bcl-2 family protein.

Table 6 Bcl-2 family of proteins and their domains

Bcl-2 family and their domains					
	BH1	BH2	BH3	BH4	Transmembrane (TM)
<i>Bcl-2 subfamily (anti-apoptotic)</i>					
Bcl-2	+	+	+	+	+
Bcl-XL	+	+	+	+	+
Bcl-w	+	+	+	+	+
Mcl-1	+	+	+	-	+
<i>Bax subfamily (multi-domain pro-apoptotic)</i>					
Bax	+	+	+	-	+
Bak	+	+	+	-	+
Bok	+	+	+	-	+
<i>BH3 subfamily (pro-apoptotic)</i>					
Bad	-	-	+	-	-
Bid	-	-	+	-	-
Bim	-	-	+	-	+
Hrk	-	-	+	-	+
PUMA	-	-	+	-	-
NOXA	-	-	+	-	-
Bik	-	-	+	-	+

Adapted from [74]

1.6 Cyclin-dependent kinases

PKC- α associates, phosphorylates and activates cyclin dependent kinase (CDK) activity thereby regulating glioma cell cycle progression and proliferation.

1.6.1 Overview. Cyclin-dependent kinases (CDKs) are a family of protein serine/threonine kinases that play an important role in the molecular machinery that regulates the cell cycle [80]. CDK activity is tightly regulated throughout the cell cycle by complicated mechanisms including the binding of regulatory proteins and the phosphorylation/dephosphorylation of CDKs which controls the completion of one phase before progressing to the next phase [81]. In addition to the control of cell division and transcription, CDKs also regulate cell differentiation and apoptosis. CDKs also play an essential part in neuronal events of the CNS leading to differentiation, migration, degeneration and apoptosis [82, 83]. CDKs are present in all eukaryotic cells and are evolutionarily conserved.

1.6.2 Types of CDKs. CDKs are relatively small proteins, approximately 35-42kDa. They are regulated by proteins called cyclins without which CDKs have finite kinase activity. The consensus sequence for the amino acid sequence in a CDK substrate is [S/T*]PX[K/R], where S/T* is the serine or threonine phosphorylation residue, P is proline, X is any amino acid, K is lysine and R is arginine [84]. The primary role of these Cdk-cyclin complexes is to regulate the progression through the cell cycle. There are approximately nine mammalian CDKs (Table 7), of which, four Cdk, Cdk1, 2, 3 and 4 are directly involved in cell cycle regulation and progression (Fig. 7 and Table 8).

Table 7 Types of CDKs and their cyclin partners and functions

CDKs	Cyclin partner	Function
Cdk1	Cyclin B	M phase
Cdk2	Cyclin E	G1/S transition
Cdk2	Cyclin A	S phase, G2 phase
Cdk3	Cyclin C	G1 phase, ?
Cdk4	Cyclin D	G1 phase
Cdk5	p53	Transcription
Cdk6	Cyclin D	G1 phase
Cdk7	Cyclin H	CDK-activating kinase, transcription
Cdk8	Cyclin C	Transcription
Cdk11	Cyclin L	?
?	Cyclin F	?
?	Cyclin G	?

Adapted from reference [85]

1.6.3 Regulation of CDKs. CDK levels are significantly constant throughout the cell cycle however their activity is regulated by various mechanisms. CDKs are regulated by four main mechanisms, binding of cyclins, CAK phosphorylation, inhibitory phosphorylations and binding of either of the two major CDK inhibitory family (CKIs) – INK4 family or Cip/Kip family [81].

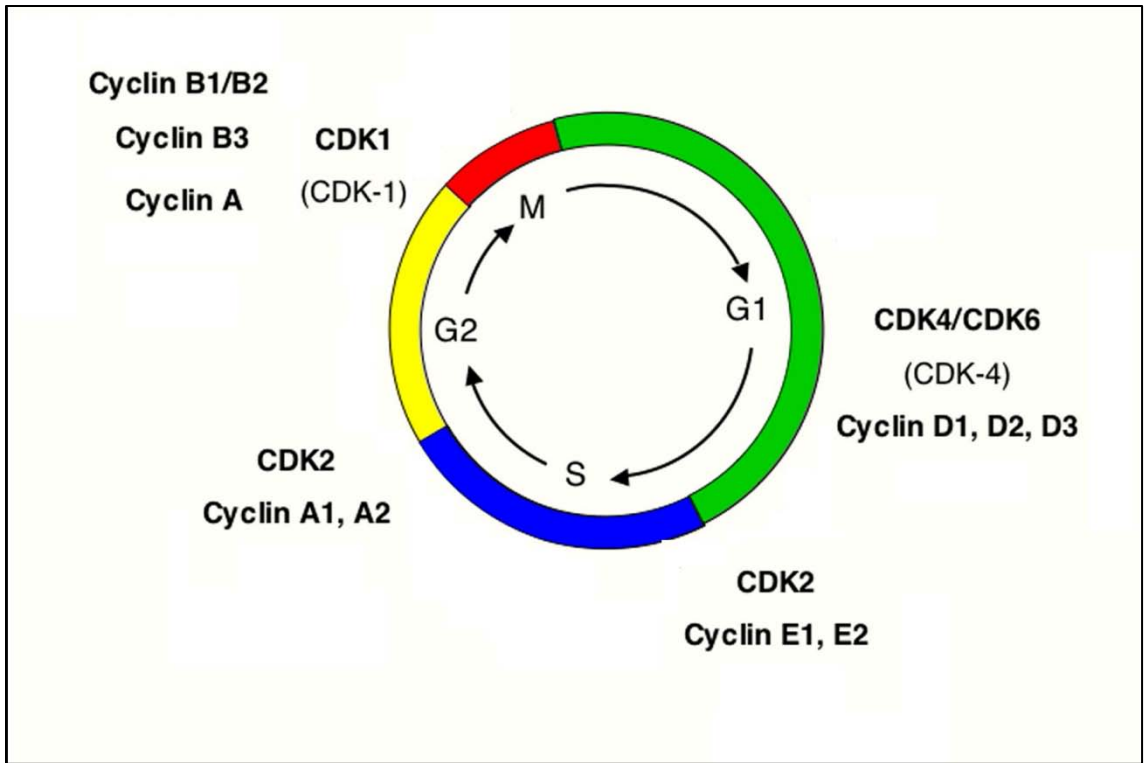


Figure 7 CDKs, their cyclin partners in different cell cycle phases.

Table 8 CDKs and their cyclin partners in cell cycle phases

CDKs	Cyclin partners	Cell cycle phases
Cdk3	C	G0
Cdk4, Cdk2, Cdk6	D, E	G1
Cdk2	A, E	G2
Cdk2, Cdk1	A	S
Cdk1	B	M

Adapted from reference [84, 85]

1.6.4 Role of Cdk7. Amongst all Cdks, Cdk7 is the master cell cycle regulator as it has the ability to regulate the activities of other Cdks by phosphorylating their activation loop thus initiating the mammalian cell cycle [86]. It is part of a trimeric CAK (cyclin dependent kinase activating kinase) complex (comprising of Cdk7, cyclin H and MAT1) which stabilizes and activates Cdk7. Cdk7 is the only eukaryotic CAK known till date [87]. This trimeric complex subsequently phosphorylates downstream cdks on their activation segment inducing their activity [88]. Studies have shown that Cdk7 levels are low in cells and are predominantly located in the nucleus [89]. Cdk7 also helps in the regulation of transcription as part of the transcription factor TFIIH complex by phosphorylating RNA polymerase II (RNA pol II) large subunit C-terminal domain (CTD) [90]. Cdk7 particularly phosphorylate Ser-5 and this phosphorylation on the CTD facilitates promoter recognition, initiation of transcription and RNA processing enzymes [91]. Cdk7 has two phosphorylation sites. The primary phosphorylation site at the conserved T-170 on the T-loop induces stable dimerization with cyclin H. The secondary phosphorylation at Ser164 on the activation segment further stabilizes the complex [92]. The trimeric complex is active in the absence of Cdk7 phosphorylation however phosphorylation stabilizes the complex leading to its further activation [93]. One key residue Lys-166 is conserved amongst most Cdks, cdk2, cdk1, cdk4, cdk5 and cdk6 and may be a main requirement for these cdks to be a substrate for Cdk7. Structural and biochemical studies have proved that cdk2 is an *in-vitro* substrate to Cdk7 [87].

1.6.5 Role of cdk2. Cdk2 is a prime regulator of G1 to S phase transition in cells. Cyclin E/cdk2 have important roles in not only regulating the cell cycle phase but also in centrosome duplication [94]. Research shows that cdk2 exerts its biological activities by phosphorylating its substrates probably at the *RXL* motif coinciding with cyclin binding [95]. A number for cdk2 substrates are identified which regulate cell division and few of them are mentioned below:

- Cdk2 mediated phosphorylation of Rb, inactivates Rb by derepressing E2F transcription factor [96].
- Cdk2 induces phosphorylation of p27 which stimulates its degradation by SCF-Skp2 ubiquitin ligase [97].
- Cdk2 phosphorylates NPAT which promotes histone transcription (cells without NPAT cease to enter S phase from quiescence) [98].
- Cdk2 phosphorylates CBP/p300 at G1/S transition to activate its histone acetyltransferase activity which also functions as a co-factor for many transcription factors such as E2F [98].
- Cdk2 co-operates with cdc6 to allow formation of pre-replication complexes during G1/S transition [99].
- Cdk2 also phosphorylates MCM family members, MCM2 and MCM3 that play essential roles in eukaryotic DNA replication [100].

1.6.6 Implications of CDKs in cancer. Abnormal activation or loss of function of CDKs and their regulators have great impact in human cancers [101]. Such adverse transformation or molecular changes in cells can modulate the tightly regulated progression of the cell cycle their checkpoints at the G1-S and G2-M transitions [80, 102]. As shown in Fig 6. CDKs and their cyclin partners are predominantly involved in cell cycle phase transition and any insults on this cascade can have undesirable repercussions on the cell machinery. Its unfavorable effects have been seen in many cancer models leading to uncontrolled cell proliferation, cell survival, migration and angiogenesis.

CHAPTER 2

PKC- ι IS OVEREXPRESSED IN GLIOBLASTOMA

2.1 Overview

Inherent over expression of protein kinase Cs (PKC) [103, 104] is a characteristic of many cancers. Several studies show that PKC hyperactivity is concordant with the malignant growth rates in gliomas. The atypical PKCs- ι and ζ - are stimulated by protein-protein interaction and by phospholipids that are involved in mechanistic pathways that control cellular responses such as growth, proliferation, survival and apoptosis [105]. PKC- ι and ζ are targeted mediators in the PI (3)-kinase signal transduction repertoire [106]. PKC- ι is the most common genomic amplicon as identified by comparative genomic hybridization and has been recognized as an oncogene [107-109]. It stimulates cell survival and prevents apoptosis in non small cell lung cancer (NSCLC), prostate cancer and gastric carcinoma [2, 4, 110]. PKC- ι hyper-activation is also responsible for chemoresistance in chronic myelogenous leukemia cells and glioblastoma [1, 3].

Although, several studies have shown that PKC- ι (an atypical isoform of PKC family) is a key regulator of invasion, metastasis and chemoresistance in glioblastoma [3, 111, 112], the endogenous expression of PKC- ι in glioblastoma remains obscure and thus, we initiated a study to investigate the role of PKC- ι in glioblastoma. In the current study, T98G and U138MG cells were used to explore the expression and importance of PKC- ι in glioblastoma. T98G (CRL-1690), U138 (HTB-16) and U87MG (HTB-14) cells

were obtained from the American Tissue Culture Collection (Rockville, MD, USA). The T98G cell line was isolated by *Stein, 1979* [113] as a spontaneous variant of the parental T98G cells which were derived from a glioblastoma multiforme tumor of a 61-year-old Caucasian male. The cells have a hyperpentaploid chromosome count and display immortality but are not tumorigenic in nude mice. The doubling time for T98G cells is approximately 18 hours. The trait that distinguishes T98G cells from fully transformed cells is that they behave similarly to normal cells and can become arrested and stationary in G1phase [114]. U-138MG cells were originally isolated and established into a stable cell line by *Ponten & MacIntyre, 1968* [114], having been derived from an astrocytoma-glioblastoma (grade III) of a 47-year-old Caucasian male.

2.2 Results

2.2.1 Expression of PKC- ι in brain tissue. The relationship between absence of PKC- ι in normal brain tissue and its robust presence in either benign/malignant meningiomas or gliomas is shown in Fig. 8. Western blots probed for PKC- ι in 12 normal brain biopsies, 15 benign meningiomas and 6 malignant tumours revealed complete absence ($N = 9$) or very low detection ($N = 3$) of PKC- ι in normal brain tissue. By comparison, PKC- ι was very clearly evident in the majority of benign meningiomas ($N = 14$) but was only weakly present in one. Similarly, PKC- ι was abundant in all malignant meningiomas ($N = 3$) and gliomas ($N = 3$). PKC- ι immunoreactivity in glioma, benign and malignant meningiomas was 38-46 fold higher when compared to normal brain tissue. PKC- β II did not show a pattern of expression specific to either normal brain tissue, or benign or malignant brain tumors (data not shown).

2.2.2 Over-expression of PKC-ι in glioma cells. Actively proliferating cells (50% confluent) and 100% confluent serum starved cells were harvested and samples were prepared for cell cycle analysis or Western blotting. T98G cells and U-138MG glioma cells that were 100% confluent had 94% and 74% of the cells in quiescence/Gap 1 (G0/G1), respectively (Fig. 9A, C and Table 9). In contrast, 64% of rapidly dividing 50% confluent T98G cells accumulated in G0/G1 phase and 54% of U-138MG glioma cells were in G0/G1 phase (Fig. 9B, D). Western blotting for PKC-ι in these cell populations depicted robust quantities of PKC-ι in proliferating 50% confluent T98G and U138MG glioma cells. In contrast, 100% confluent cells had either 71% (T98G) or 47% (U-138MG) less PKC-ι than 50% confluent cells (Fig. 9E, $P < 0.05$).

To determine a distribution pattern of different PKCs in glioma cells, we performed Western blots for PKC-α, PKC-β1, PKC-δ and PKC-ε in 100% confluent (plus serum starved) and proliferating cells. PKC-β1 is known to play a role in angiogenesis and tumorigenesis [115]. Similarly, PKC-δ [116] and PKC-ε [117] are involved in glioma cell proliferation. Invariant levels of PKC-α were observed between confluent versus proliferating cells (Fig. 9E). Interestingly, Western blots for PKC-δ did not detect PKC-δ in T98G glioma cells and invariant levels were detected in U-138MG cells. Decreased levels of PKC-β1 (35% and 53% reduction) were seen in 100% confluent T98G and U-138MG cultures compared to rapidly proliferating 50% confluent cells, respectively (Fig. 9E).

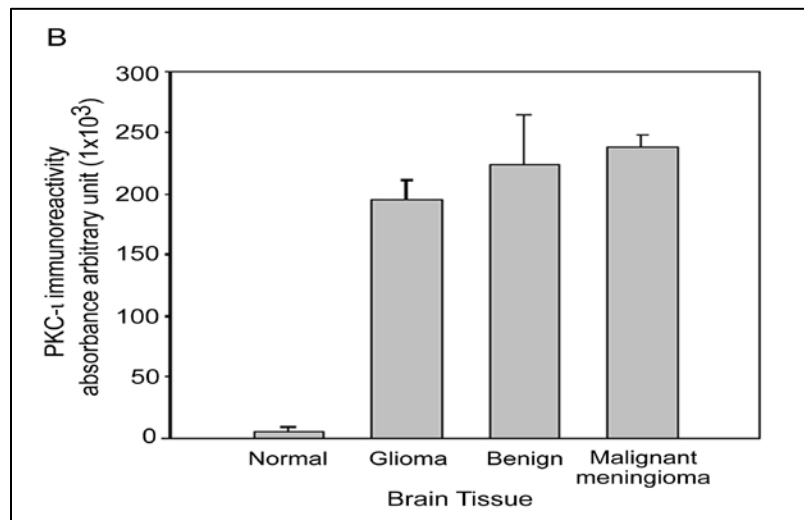
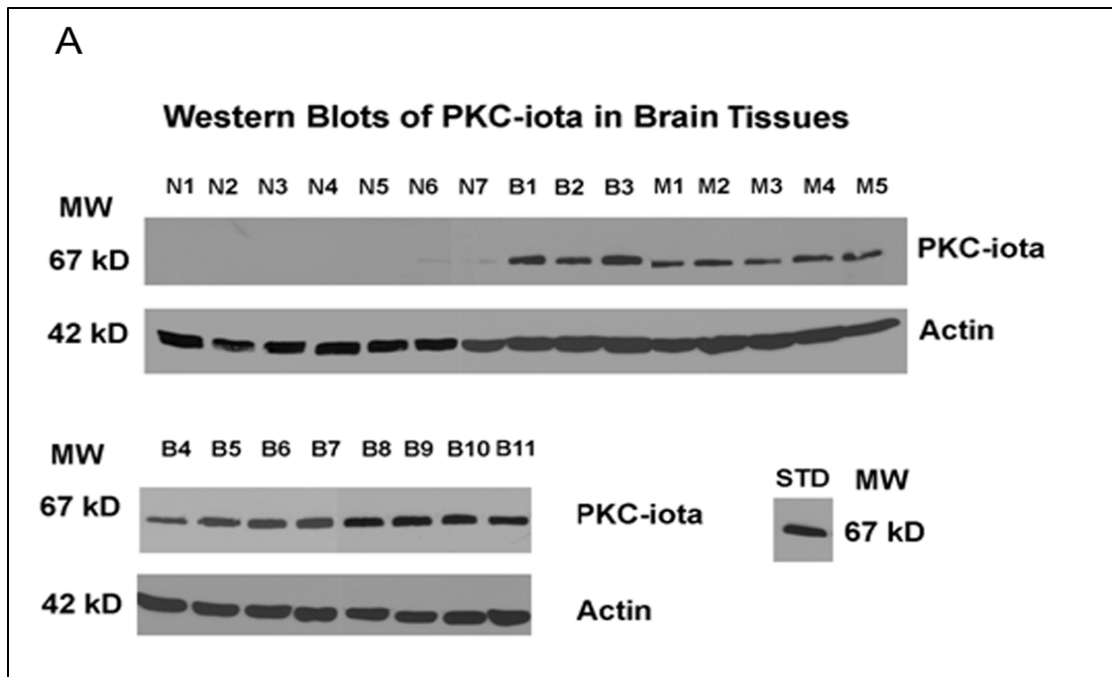


Figure 8 PKC-iota is present in benign and malignant meningiomas, gliomas but not normal brain tissue. (A) Human autopsy-derived normal brain tissue (N1, frontal lobe; N2, cortex; N3 and N4, unspecified brain; N5, cortex; N6, cerebellum), benign tumour tissue (B1, B4, B7, B9 and B10, [WHO grade 1] meningotheial meningioma; B5 and B8, meningioma; B6, fibroblastic meningioma; B2, B3 and B11, fibrous meningioma [WHO grade 1], and malignant tumour tissue; M1 and M2, [WHO grade IV] glioblastoma multiforme; M3, right frontal lobe meningioma; M4, atypical meningioma [WHO grade II]; M5, Astrocytoma [WHO grade IV]; M6, anaplastic meningioma [WHO grade III]).Specimens were obtained from the Cooperative Human Tissue Network. (B) Band density for PKC- ι was measured by densitometry.[118]

Differences between PKC- β 1 protein content in 100% confluent and 50% confluent rapidly proliferating cells were significant at ($P < 0.05$; $n = 3$) for both cell lines. In contrast, PKC- ϵ was not detected in either T98G or U-138MG cells. Western blots of PKC- α , PKC- β 1, PKC- δ and PKC- ϵ , suggest that the presence or absence of these PKC isozymes may be cell type specific. To insure that 48 h serum deprivation did not lead to cell death or alteration in intracellular cascades, we evaluated Caspase-7 activation (Fig. 9E). Results demonstrated that PKC- ι is highly overexpressed in glioma cells suggesting that PKC- ι may be required for cell cycle progression, proliferating and survival of glioma cells. Although, PKC- β 1 and PKC- δ may have some role in glioma cell proliferation it is evident from the data that it is dependent on cell type. For subsequent studies, we focused on the role of PKC- ι in glioblastoma.

Table 9. Summary of 50% and 100% confluent cell cycle phases

Summary of cell cycle phases			
Cell Type	G0/G1	S	G2M
100% T98G	94 +/- 1	4 +/- 4	2
50% T98G	64 +/- 3	33 +/- 3	4 +/- 4
100% U138MG	74 +/- 11	20 +/- 10	2 +/- 3
50% U138MG	54 +/- 17	39 +/- 18	7 +/- 1

N=3 experiments per cell line

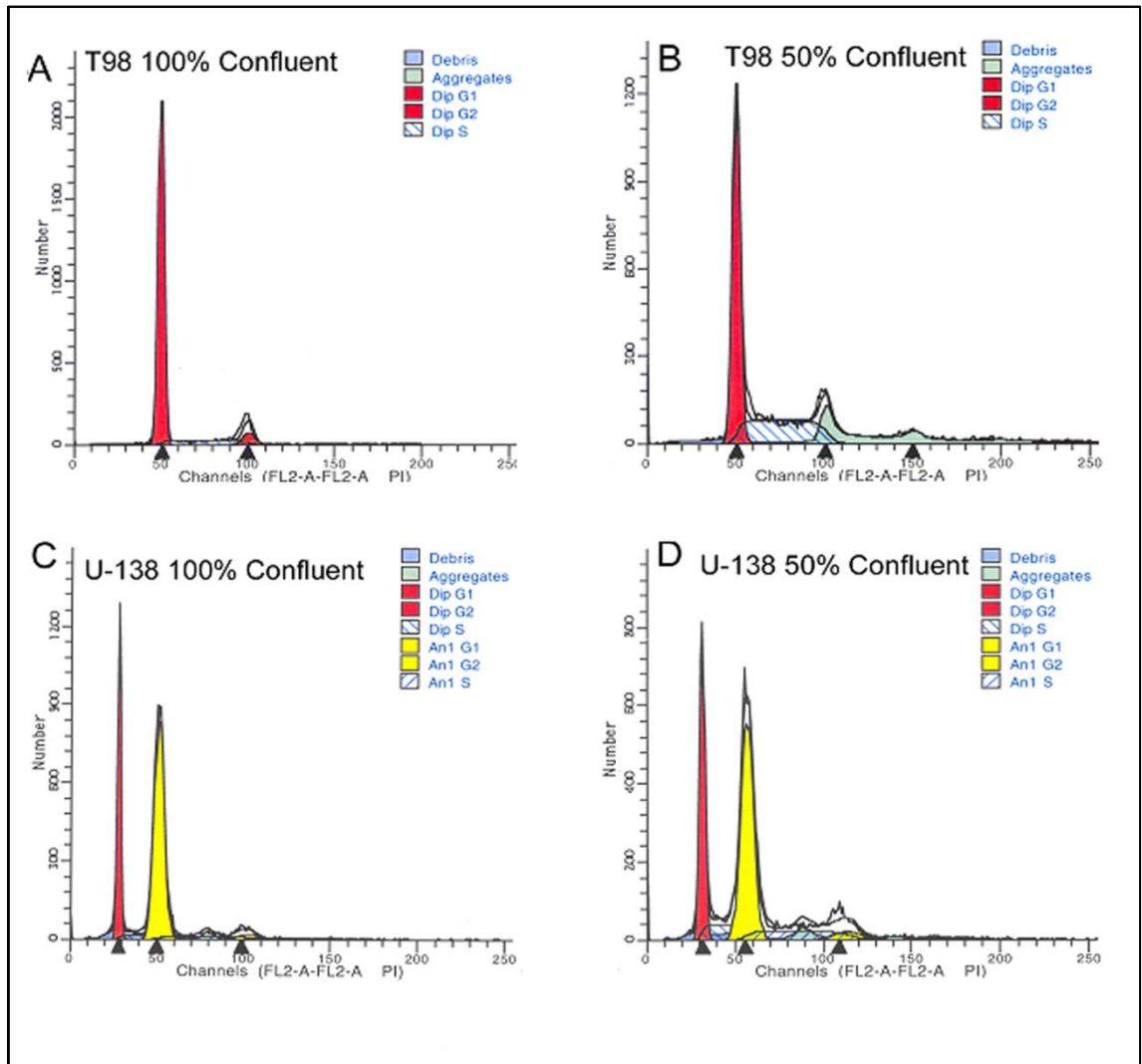


Figure 9A-D Cell cycle analysis and expression of PKC- ι in T98G and U-138MG glioma cells. FACS analysis of DNA content in 100% confluent (A) T98G or (C) U-138MG cells and 50% confluent (B) T98G or (D) U-138MG cells. U-138 glioma cells are aneuploid; U-138MG DNA histograms are from one representative experiment and illustrate two cycling populations with a G0/G1 peak at 2 N (first red shaded peak) and another at 4 N (second yellow unshaded peak). Total DNA content for G0/G1, DNA synthetic phase (S), gap 2 and mitosis (G2/M) was quantified by addition of each of the phases in both populations. Forty thousand events were collected per time point and treatment group.

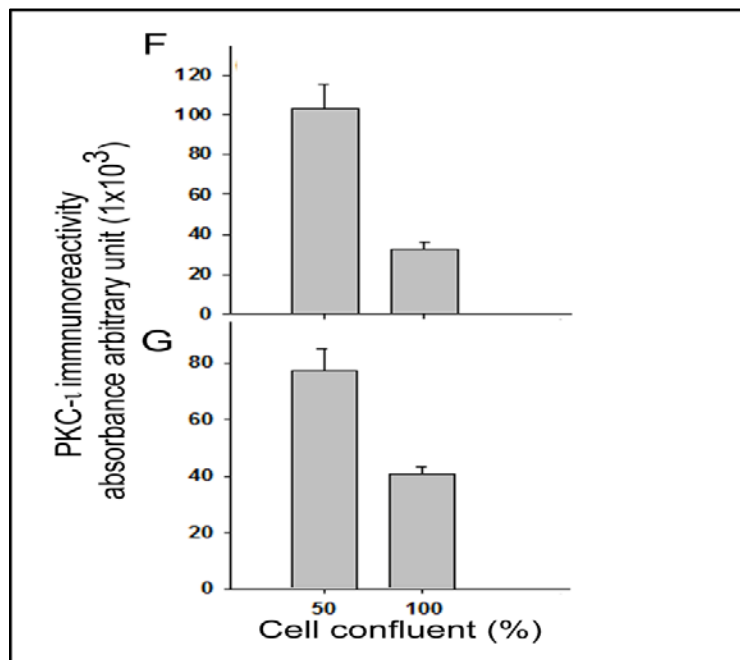
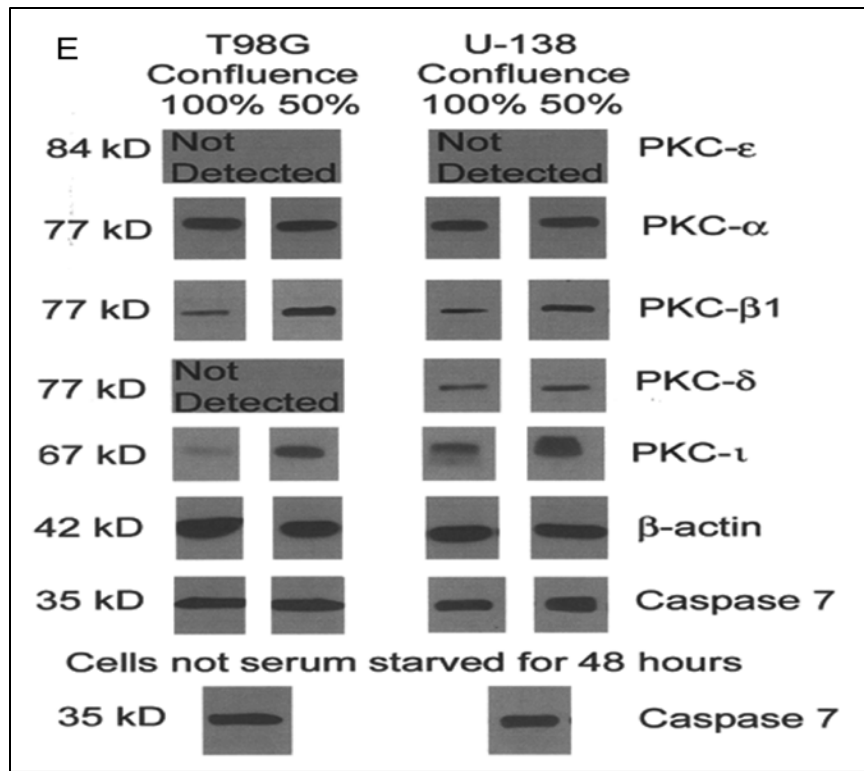


Figure 9E Cell cycle analysis and expression of PKC-ι in T98G and U-138MG glioma cells (E) Western blots of PKC-α, PKC-β1, PKC-δ, PKC-ε, PKC-ι, and Caspase 7 present in T98G or U138MG cells that were at different stages of confluence. Band intensity of PKC-ι from the Western blots in T98G (F) and U-138 MG (G) was quantified by densitometry.

2.3 Discussion

Despite rigorous therapy, median survival is less than 1 year for patients with high-grade tumors. While post-operative radiation therapy clearly delays tumor re-growth and prolongs survival, total tumor control is rarely achieved. Rapid glioma growth has been attributed to inherently high levels of PKCs. PKC also appears to be involved in growth regulation of low-passage number human meningioma cells *in vitro*, as judged by decreased proliferation of cells following treatment with the PKC inhibitor, Staurosporine.

In this study, we observed significant overexpression of PKC- ι in transformed phenotypes of human glioma and benign and malignant meningioma [118]. According to our understanding this is a first report on PKC- ι overexpression in glioma tissues. Notably, PKC- ι expression was also robust in glioma cell lines (T98G and U138MG). In fact, when the cells were grown to 100% confluency and serum starved for 48h, expression of PKC- ι was significantly inhibited correlating with corresponding reduction in cell proliferation in comparison to actively proliferation (50% confluent) cells. Interestingly, such a reduction in cell proliferation did not drastically affect the expression of other PKC family members (PKC- α , δ , β I) suggesting that these isoforms may not be required for cell proliferation in these cell lines.

Collectively, these data suggest a tissue specific role of different PKC family members. Results also indicate that PKC- ι is highly activated and overexpressed in glioma cells and its presence may be essential for glioma cell proliferation and survival.

CHAPTER 3

PKC- ι INDUCES CELL SURVIVAL IN GLIOMA CELLS

3.1 Overview

Glioblastoma multiforme is the most lethal form of all primary brain tumors with median survival time being less than a year. Fast growth, aggressive proliferation and susceptibility to invasion and metastasis result in a poor prognosis in glioblastoma. Progress in glioblastoma therapy has remained stagnant despite rigorous therapeutic methods such as surgery, radiation and chemotherapy [18, 119]. Thus, there is a pressing need to identify novel molecular targets that might help improve survival of glioblastoma patients.

PI(3)-kinase is frequently augmented in glioblastoma due to activating mutations or amplifications in EGFR (36% of the cases) or loss of function/deletion of PTEN (25% of the cases) [6, 17]. The atypical PKC family member, PKC- ι is a targeted mediator in the PI (3)-kinase signal transduction pathway [107]. The importance of PKC- ι as a signaling mediator is attributed to the fact that it can function as an oncogene [108, 109].

Bcl-2 (B cell lymphoma-2) family members are vital regulators of the mitochondrial cell death machinery and are comprised of 15 members [69]. Among the BH3 only protein, Bad lacks the hydrophobic C-terminal domain which prevents its direct mitochondrial interaction [120]. Upon external survival signals, Bad is

phosphorylated, deactivating and disrupting Bad/Bcl-XL dimerization [76]. Dissociated Bad further binds to 14-3-3 scaffold protein and is subsequently degraded [77]. Such imbalance in Bcl-2 family with more dependency on anti-apoptotic members is often observed to in cancer cells [78].

We have previously reported that PKC- ι is highly expressed glioblastoma tissues [118]; however, little is understood about its role in glioma cell survival. PKC- ι has been shown to inhibit the pro-apoptotic function of Bad in non-small cell lung cancer (NSCLC) cells when activated by nitrosoamine 4-(methylnitrosomaino)-1-(3-pyridyl)-1butanone (NNK) [121].

In this study we provide evidence that PKC- ι inhibited the pro-apoptotic function of Bad to promote glioma cell survival in T98G and U87MG glioma cells. The U87MG is classified as a grade IV astrocytoma and was isolated from highly malignant glioblastoma of a 44-year-old Caucasian female. The cells have a hypodiploid chromosome count, display epithelial-like morphology and are tumorigenic in nude mice. The doubling time of U-87MG cells is approximately 32 h. PI (3)-kinase inhibition, PDK1 inhibition and knockdown of PKC- ι activity upon siRNA treatment exhibited corresponding reduction in Bad phosphorylation hampering glioma cell survival. Thus, our data suggests the presence of a novel PI (3)-kinase/PDK1/PKC- ι /Bad signaling cascade. Targeting this pathway may be a possible glioblastoma targeted therapy.

3.2 Results

3.2.1 PKC- ι , Bad and Bcl-XL expression in glioma cells. In 50% confluent T98G cells (rapidly proliferating cells), there was increase in the expression of PKC- ι (90-fold, $P=0.001$) and Bcl-XL (66-fold, $P=0.006$) when compared with 100% confluent plus serum starved cells (contact inhibited) (Fig. 10). Similarly, in U87MG cells, an increase in the levels of PKC- ι (50-fold, $P=0.008$) and Bcl-XL (20-fold, $P=0.01$) was observed in actively proliferating cells compared to serum starved cells (Fig. 10). In contrast, the expression of Bad was enhanced in contact inhibited cells versus proliferating cells (44-fold in T98G, $P=0.004$ and 52-fold in U87MG, $P=0.002$). Caspase-3 was constitutively expressed in 50% confluent and 100% serum starved cells (Fig. 10). Caspase-3 expression levels were analyzed to ensure that the decrease in the expression of PKC- ι was not an outcome of cells undergoing apoptosis. β -actin was used as the loading control. Therefore, our data suggests that presence of PKC- ι may be required for glioma cell survival and is inversely correlated with the regulation of mitochondrial Bad.

3.2.2 PKC- ι co-localizes and directly associates with Bad. The cellular distribution of Bad and Bcl-XL in T98G cells was determined by double immunofluorescence (IF). Negative controls for FITC green, Texas red and their merged image exhibited minimal background immunofluorescence (IF) (Fig. 11A, top panel). PKC- ι had some nuclear staining but was stronger in the cytosolic region (FITC green) whereas Bad stained only in cytosolic fraction of the cells (Texas red) (Fig. 11A, bottom panel). Merging of the two individual frames showed that PKC- ι predominantly co-localized with Bad in the cytosolic region of the cells as displayed by yellow coloration

(Fig. 11A, bottom panel). Small crescent shaped structures were observed near the perinuclear region of the cells, as indicated by the arrows. The identity of this structure requires further investigation. Subcellular fractionation of both T98G and U87MG cells showed that PKC- ι was present in both cytosolic as well as nuclear fractions ($P < 0.01$ for T98G and U87MG). Bad was present only in the cytosolic fraction of the cells ($P = 0.002$). β -actin was used as loading control. Histone H1 was used as a purity control for nuclear fraction (Fig. 11B).

To further demonstrate that PKC- ι directly associates with Bad, PKC- ι was immunoprecipitated. In T98G cells, PKC- ι was constitutively expressed at all time points and directly associated with Bad. The association, however, was transient as observed at T0, T6, T14, T16 hours (Fig. 11C). Although PKC- ι associated with Bad in U87MG cells at most time points (T0-T36 hours), distinct variation in the Bad expression was observed (Fig. 11D). We speculate that Bad may be involved in glioma cell cycle regulation however further investigation is required to confirm this hypothesis.

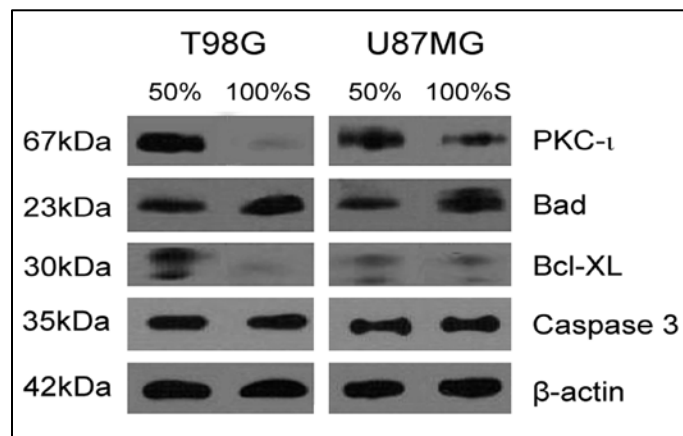


Figure 10 Expression profile of PKC- ι , Bad and Bcl-XL in T98G and U87MG cells. PKC- ι , Bad, Bcl-XL and Caspase-3 expression levels in actively proliferating and contact inhibited plus serum starved T98G and U-87MG cells were determined by Western blot analysis. β -actin indicates equal loading of the total lysate. Western Blot is representative of $N=3$ independent experiments.

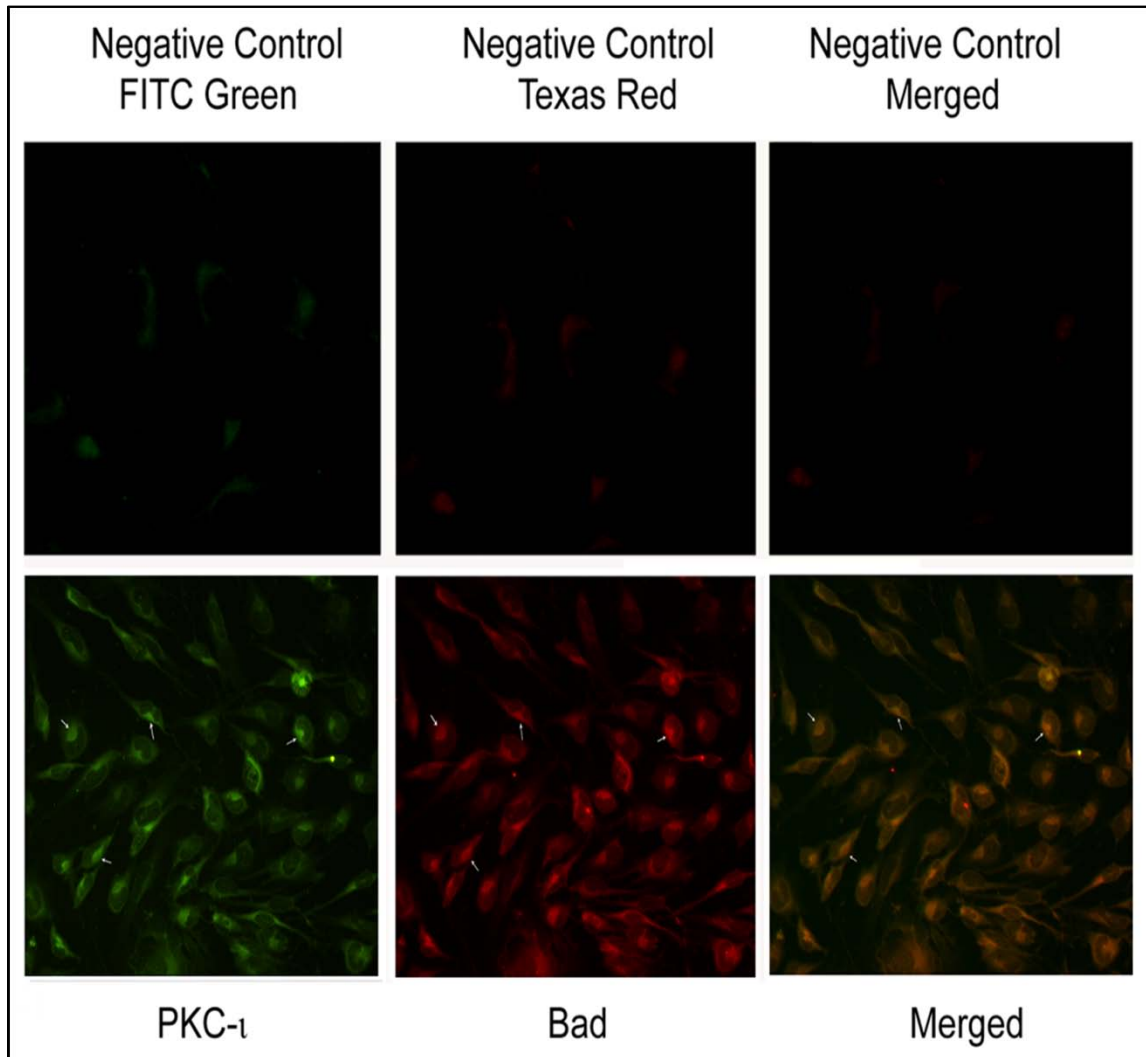


Figure 11A PKC-ι co-localizes and directly associates with Bad. (A, top panel) displays IF controls incubated without primary antibody. (A, bottom panel) represents the fluorescence from FITC green used to detect PKC-ι and the fluorescence from Texas Red to detect Bad, the merged image (yellow color) depicts co-localization of PKC-ι and Bad in the cytosol. IF data represented is that of three repetitions.

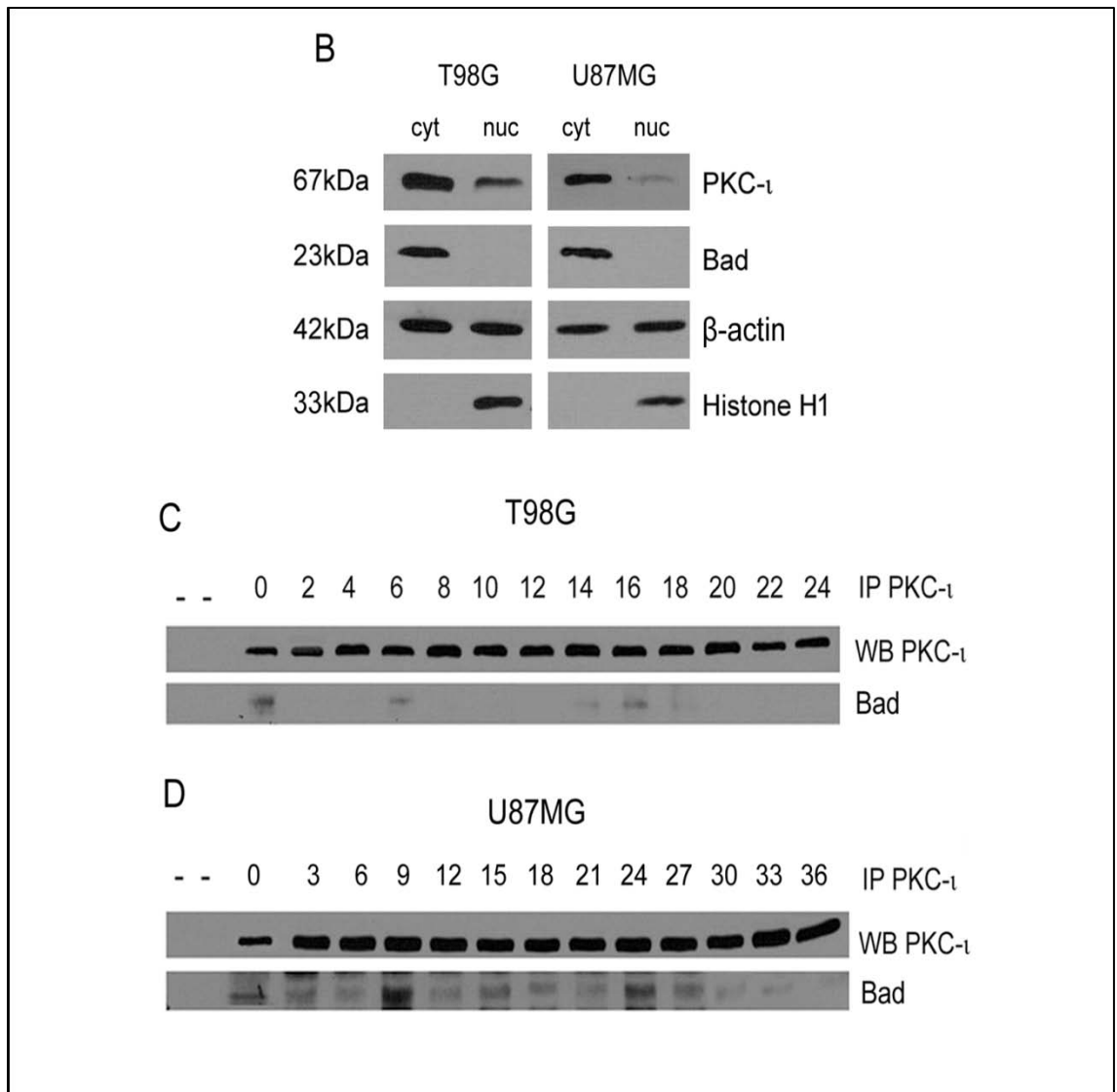


Figure 11B-D PKC- ι co-localizes and directly associates with Bad (B) Both T98G and U87MG cells were fractionated and Western blot analysis showed that PKC- ι is highly expressed in the cytosolic fraction and nuclear fraction of both cells, whereas Bad is expressed only in the cytosolic fraction. β -actin was used as loading control. Histone H1 was used as the purity control for the nuclear fraction. (C-D), Whole cell extracts (1mg) from each time point (every 2h for T98G and 3h for U87MG) were IP with PKC- ι antibody. The 1st (-) contains whole cell extract plus rabbit IgG whole molecule (50 μ l of 1:1v/v) and the 2nd (-) contains whole cell extract plus rabbit IgG whole molecule (50 μ l) and normal rabbit IgG serum (5 μ g). (C) PKC- ι transiently associates with Bad in T98G cells. (D) PKC- ι in U87MG cells associates with Bad at most time points compared to T98G though its expression varies throughout the cell cycle (36h). Data represents $N=3$ independent experiments.

3.2.3 PKC- ι directly phosphorylates Bad at all three serine residues *in-vitro*.

Since, our data showed that PKC- ι directly associates with Bad in both T98G and U87MG cells (Fig. 11C, D), we examined whether PKC- ι is a potential kinase to Bad in glioblastoma. Significant increase in the levels of pBad at Ser-112, Ser-136 and Ser-155 in co-IP (PKC- ι and Bad) samples compared to individual IP samples (Fig. 12A) suggests that Bad might be a direct downstream substrate to PKC- ι in T98G and U87MG cells. Further, when purified, active PKC- ι was incubated with IP Bad in an *in-vitro* kinase assay, active PKC- ι directly phosphorylated Bad at all three serine residues (Fig. 12B, $P < 0.05$ for all the residues). As reported by Jin *et al.* [121], our data also showed that active PKC- ι phosphorylated recombinant Bad at all three residues ($P < 0.01$ for all residues) (Fig. 12C, upper panel). In contrast, when, another atypical PKC, active PKC- ζ was incubated with recombinant Bad in an *in-vitro* kinase assay, PKC- ζ phosphorylated Bad at only Ser-112 residue ($P = 0.02$) (Fig. 12C, upper panel) suggesting that it could also regulate Bad but would require other kinases in order to completely abrogate the pro-apoptotic function of Bad. To confirm that the activity of PKC- ζ was not compromised, PKC- ι and PKC- ζ were subjected to an *in-vitro* kinase activity assay using a common substrate, myelin basic protein (MBP). Results showed that both, PKC- ι and PKC- ζ induced equivalent phosphorylation of MBP at T98, demonstrating equal activity ($P < 0.01$) (Fig. 12C, bottom panel).

Furthermore, when, Bad-associated Bcl-XL (bound Bcl-XL) was IP and incubated with purified, active PKC- ι (0.5 μ g) in an *in-vitro* kinase assay, we found lower amounts of bound Bcl-XL and increased levels of unbound Bcl-XL (Fig. 12D) in both

T98G ($P=0.013$) and U87MG cells ($P=0.002$). Additionally, when, Bad associated 14-3-3 (bound 14-3-3) was IP and incubated with purified, active PKC- ι (0.5 μ g), we found higher amounts of 14-3-3 in the samples incubated with PKC- ι , suggesting that increased phosphorylation of Bad (Fig. 12B) as well as Bad/Bcl-XL dissociation (Fig. 12D) led to increased Bad/14-3-3 dimerization (Fig. 12E) in both T98G ($P=0.03$) and U87MG cells ($P=0.023$). 14-3-3 is a proteosomal scaffold protein involved in Bad inactivation and degradation in the cytosolic fraction of the cells. Thus dimerization of Bad with 14-3-3 suggests that Bad will be subsequently sequestered by ubiquitin proteosomal pathway. These findings indicated that PKC- ι is a potential upstream kinase to Bad.

3.2.4 Inhibition of PKC- ι activity leads to corresponding reduction in Bad phosphorylation. Previous studies showed that pharmacological inhibition of PI (3)-kinase blocked PKC- ι activity, suggesting that PI (3)-kinase is an upstream regulator of PKC- ι in glioblastoma [111]. Similarly, our results indicated that treatment of both T98G and U87MG cells with LY294002 (50 μ M) and Wortmannin (0.1 μ M) for 2 hours blocked PKC- ι activity in the form of diminished phosphorylation at T555 residue (53% with LY294002 and 68% with Wortmannin in T98G cells), (69% with LY294002 and 83% with Wortmannin in U87MG cells). Marked reduction in the amount of Bad phosphorylation at Ser-112, Ser-136 and Ser-155 was also observed (Fig. 13A).

PDK1 is a downstream mediator of PI (3)-kinase and previous studies have shown that PDK1 interacts and regulates the activity of atypical PKCs [106]. Thus, we investigated the effect of PDK1 knockdown on PKC- ι activity in glioma cells. Our data showed that PDK1 knockdown inhibited the phosphorylation of PKC- ι at T555.

Subsequent reduction in the levels of Bad phosphorylation at Ser-112, Ser-136 and Ser-155 was also observed (Fig. 13B). Additionally, it has also been shown that in embryonic stem cells, where PDK1 is knocked out, expression of atypical PKC isoforms was significantly reduced [122]. Thus, we investigated the expression levels of PKC- ι and results showed marked reduction in expression of PKC- ι in PDK1 knockdown cells suggesting that PDK1 could also regulate PKC- ι expression, however further investigation may be required to explore this possibility.

3.2.5 PKC- ι knockdown diminishes Bad phosphorylation; augments Bad/Bcl-XL association and inhibits Bad/14-3-3 dimerization. When T98G and U87MG cells were treated with PKC- ι siRNA (100nM for 24h), Bad phosphorylation at Ser-112, Ser-136 and Ser-155 was significantly inhibited in both the cell lines (Fig. 14A). Furthermore, incubation with purified, active PKC- ι (0.5 μ g) restored the amount of Bad phosphorylation in PKC- ι siRNA treated cells (Fig. 14A). Also, Bad-associated Bcl-XL (bound Bcl-XI) increased ($P=0.02$) and Bad-associated 14-3-3 decreased ($P=0.004$) in PKC- ι siRNA treated cells (Fig. 14B). In addition, when T98G and U87MG cells were treated with PKC- ζ siRNA, Bad phosphorylation at Ser-112 ($P<0.05$) was inhibited while phosphorylation at S136 and S155 remained constitutive (Fig. 14C). These results indicated that PKC- ζ does not have the ability to completely inhibit Bad function.

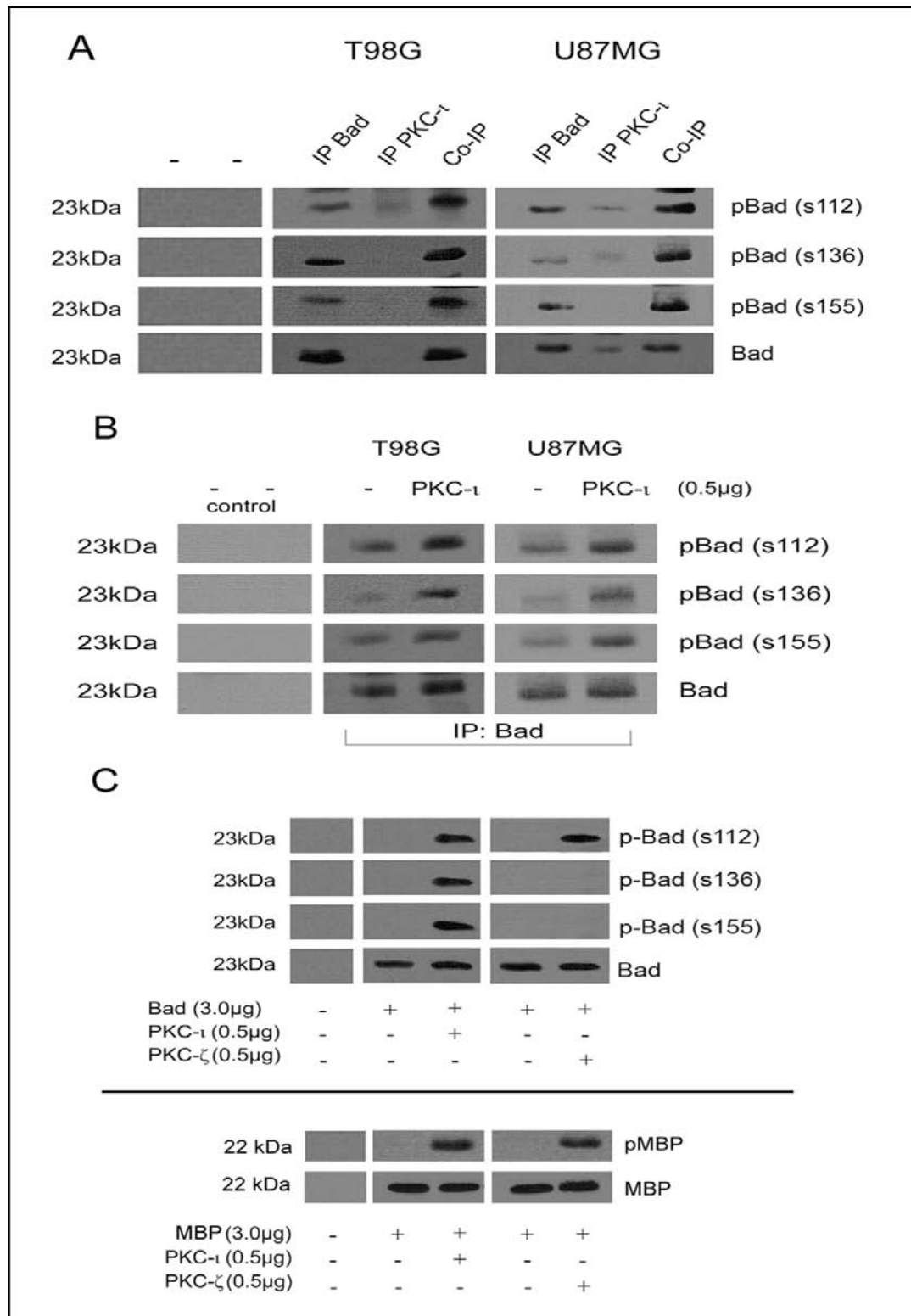


Figure 12A-C PKC- ι induces direct phosphorylation of Bad.

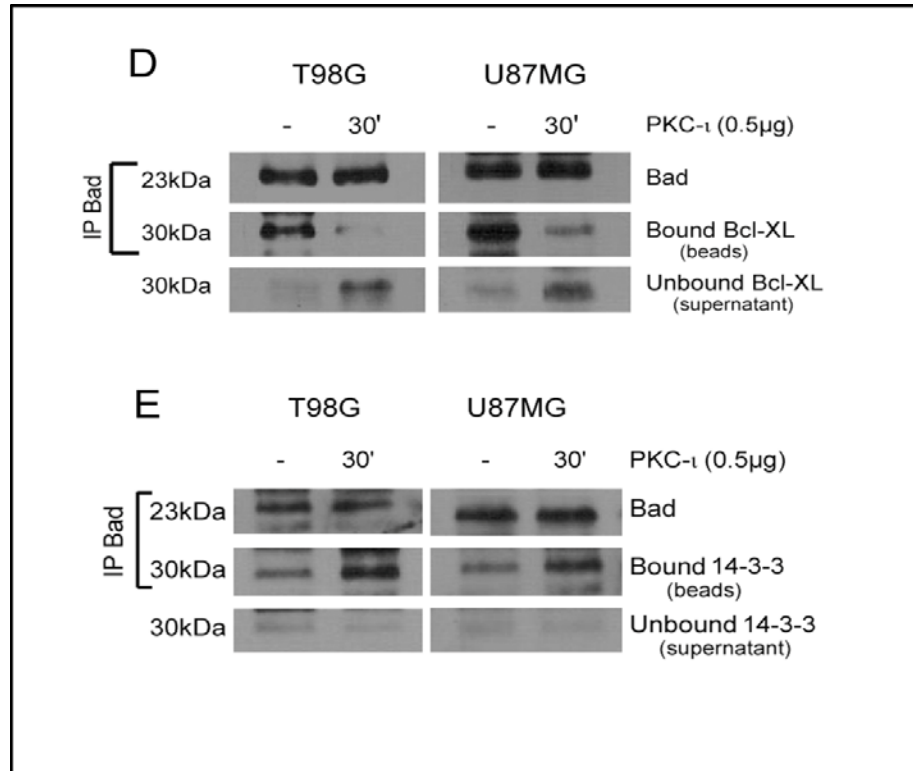


Figure 12D-E PKC- ι induces direct phosphorylation of Bad. The first negative control (-) contains whole cell extract plus rabbit IgG whole molecule (50 μ l of 1:1v/v) and the second negative control (-) contains whole cell extract plus rabbit IgG whole molecule (50 μ l) and normal rabbit IgG serum (5 μ g). **(A)** IP Bad, IP PKC- ι and co-IP from T98G and U87MG cells were subjected to kinase activity assay. Phosphorylation of Bad at Ser-112, Ser-136, Ser-155 and Pan Bad were quantified using Western blot analysis. **(B)** IP Bad from both T98G and U87MG cells was incubated with active PKC- ι (0.5 μ g) in an *in-vitro* kinase activity assay as explained in “Methods”. Western blot analysis was performed to determine phosphorylation of Bad at Ser112, Ser136 and S155 and Pan Bad. **(C, upper panel)** Recombinant Bad (3.0 μ g) was incubated with purified, active PKC- ι and PKC- ζ (0.5 μ g) for 30 mins in an *in-vitro* kinase assay. Western blot analysis was performed to determine pBad at Ser-112, Ser-136, Ser-155 and Pan Bad. **(C, lower panel)** Recombinant MBP (3.0 μ g) was incubated with purified, active PKC- ι and PKC- ζ (0.5 μ g) for 30 mins in an *in-vitro* kinase assay followed by Western blot analysis to determine pMBP at Thr-98. **(D)** Bad/Bcl-XL complex was IP from T98G and U87MG cells and subsequently incubated with purified active PKC- ι (0.5 μ g) for 30 mins in an *in-vitro* kinase activity assay. The samples were centrifuged at 14000g for 15mins. Western blot analysis was performed on the resulting supernatant and the beads to determine the amount of bound Bcl-XL, unbound Bcl-XL and Pan Bad. **(E)** Bad/14-3-3 complex was IP from both the cell lines and subsequently incubated with purified active PKC- ι (0.5 μ g) for 30 min in an *in-vitro* kinase activity assay. The samples were centrifuged at 16000g for 15 min. Western blot analysis was performed on the resulting supernatant and the beads to determine the amount of bound 14-3-3, Pan Bad. Western blots are representative of three independent experiments.

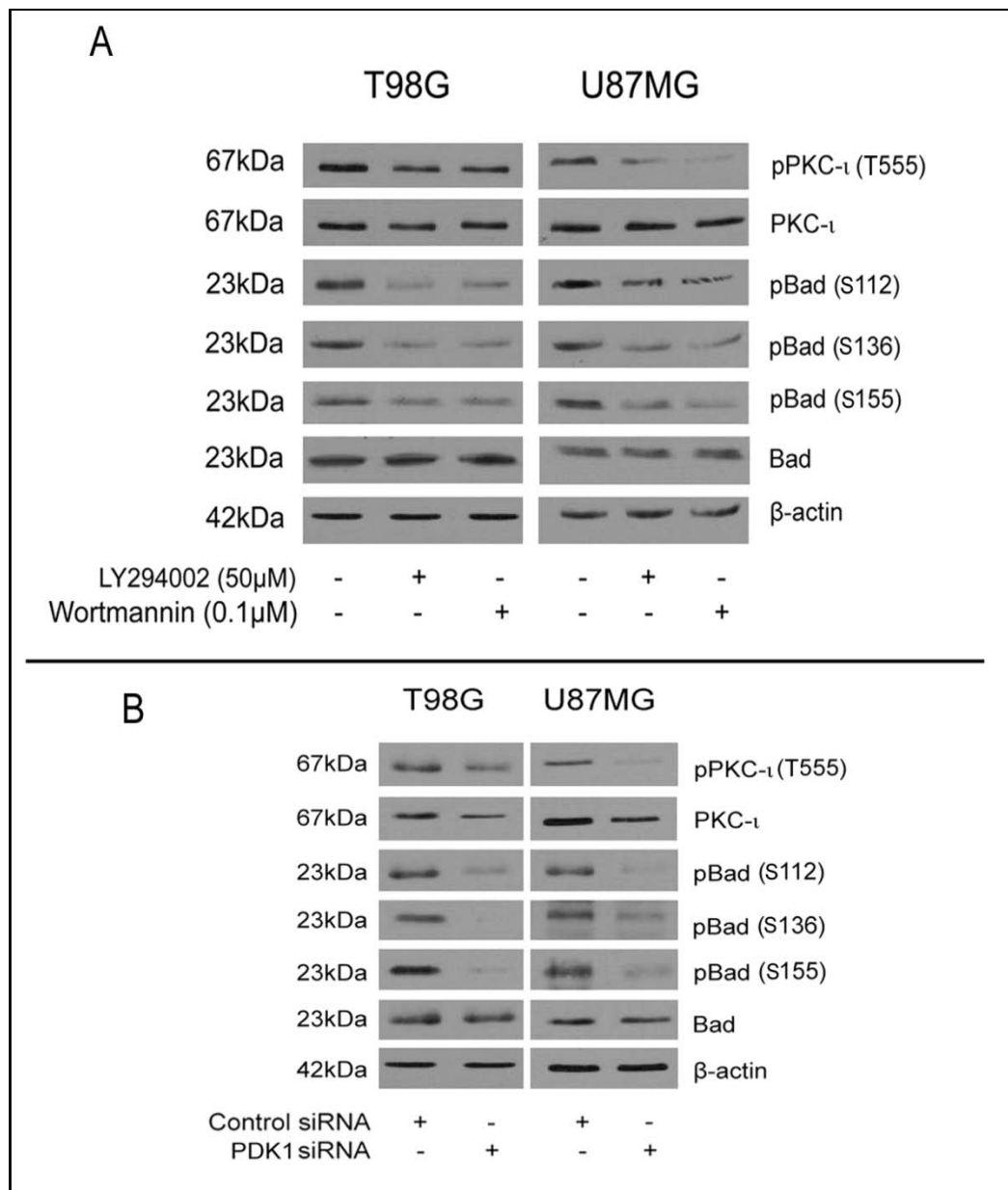


Figure 13 Effect of PI (3)-kinase inhibition and PDK1 knockdown in T98G and U87MG cells. (A) Western blot analysis of T98G and U87MG cells for phospho-PKC-ι (T555), Pan PKC-ι, phospho-Bad (S112), (S136), (S155) and Pan Bad after individual treatment for 2 h with LY294002 (50μM) and Wortmannin (0.1μM). (B) Both the cells were treated with PDK1 siRNA (100nM for 24h) followed by Western blot analysis to detect phospho-PKC-ι (Thr-555), phospho-Bad (Ser-112), (Ser-136), (Ser-155) and Pan PKC-ι and Bad. Data is representative of three independent experiments.

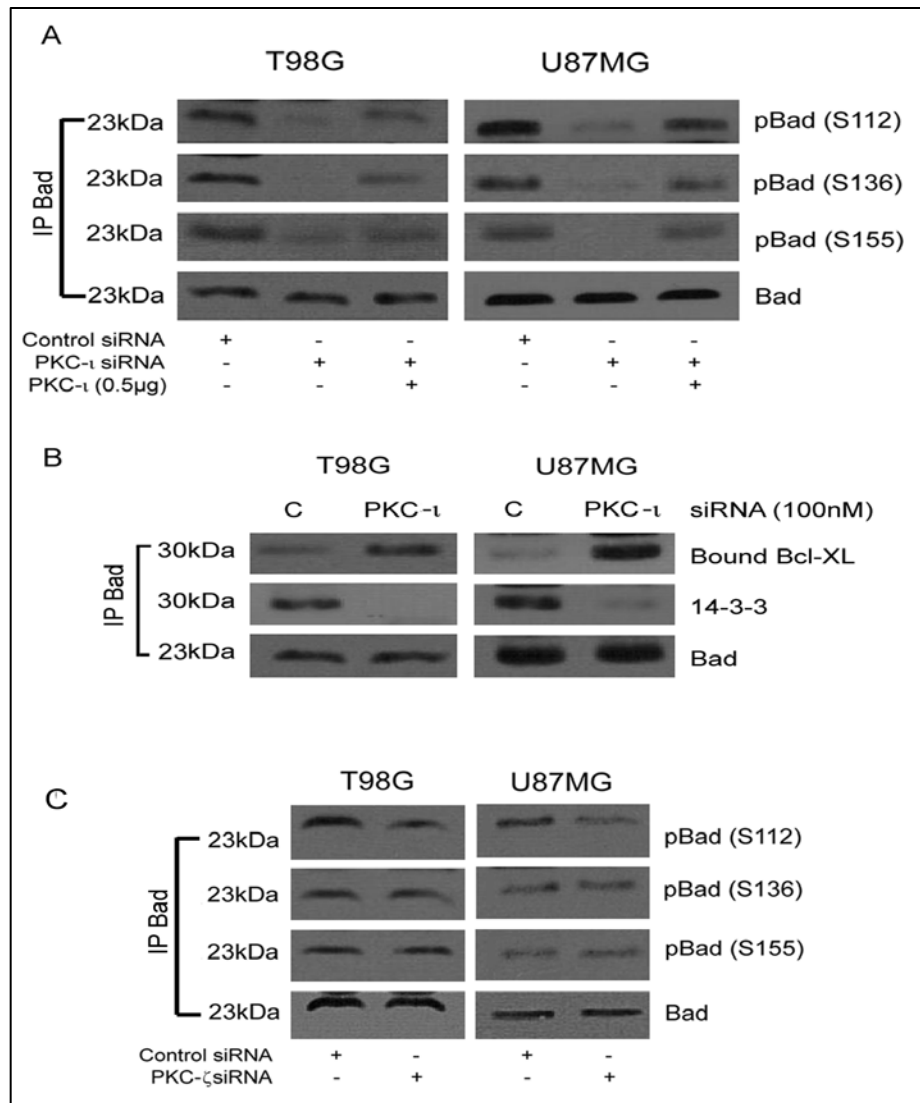


Figure 14 Knockdown of PKC-1 exhibits corresponding reduction in Bad phosphorylation and increase in Bad/Bcl-XL interaction. (A) Both T98G and U87MG cells were treated with either control siRNA or PKC-1 siRNA (100nM) for 24 hours. Subsequently, Bad was IP and subjected to kinase activity assay followed by Western blot to detect pBad at Ser-112, Ser-136 and Ser-155 and Pan Bad. Another set of IP Bad was incubated with purified, active PKC-1 in an *in-vitro* kinase assay to detect its effect on the levels of pBad in PKC-1 siRNA treated samples. (B) Bad was IP from both T98G and U87MG cells treated with either control or PKC-1 siRNA. Bad associated Bcl-XL (bound Bcl-XL), Bad associated 14-3-3 (bound 14-3-3) and Pan Bad was analyzed using Bcl-XL, Pan 14-3-3 and Bad antibodies respectively. (C) Both T98G and U87MG cells were treated with either control siRNA or PKC-ζ siRNA (100nM) for 24 hours. Subsequently, Bad was IP and subjected to kinase activity assay followed by Western blot to detect pBad at Ser-112, Ser-136 and Ser-155 and Pan Bad. Data is representative of three independent experiments.

3.3 Discussion

We and others recognize PKC- ι as a potential molecular target because it is involved in cell proliferation, cell invasion, metastasis and chemoresistance in glioblastoma [111, 118]. In the current study, we observed that PKC- ι also mediates cell survival in glioblastoma. In order to elucidate the signaling mechanism by which PKC- ι mediates its survival effect in glioblastoma, we investigated the role of Bcl-2 family protein, Bad and Bcl-XL [123].

Our present data showed an inverse relation between the expression levels of PKC- ι and Bad in glioma cells, indicating a potential correlation between these two proteins. In actively proliferating cells, PKC- ι is highly expressed, complementing our previous findings [118], whereas the levels of Bad were found to be diminished. However, under serum starved conditions, where the cells were quiescent [118], the expression of PKC- ι was significantly decreased while that of Bad was higher. Recent findings of Fernando *et.al* have shown that overexpression of Bad in G1phase may not necessarily promote apoptosis, but it could inhibit cell proliferation [124]. Additionally, Bad has been shown to arrest G1-S progression of MCF7 breast cancer cells and this function depends on the phosphorylation state of Bad [124]. Thus, we hypothesized that, in glioma cells, PKC- ι might be regulating the activity of Bad by phosphorylation. In order to test our hypothesis, we analyzed the association between PKC- ι and Bad in these cells. Since, we observed transient association between PKC- ι and Bad, we speculated that there could be a cell cycle dependent involvement of the two proteins. As predicted, there was transient but direct association of PKC- ι and Bad at different time points in the

cell cycle. Further investigation may be required to determine the mechanism which induces such variable Bad expression in glioma cells. Since, the function of Bad is dictated by its three phosphorylation sites [72] and as PKC- ι directly associated with Bad, we hypothesize that PKC- ι might be an upstream kinase to Bad in glioblastoma.

Our results showed that endogenous PKC- ι not only co-localized and associated with Bad but also directly phosphorylated Bad at Ser-112, Ser-136 and Ser-155. Additionally, purified, active PKC- ι induced *in-vitro* phosphorylation of recombinant Bad, demonstrating that it is a potential Bad kinase. This inactivation of Bad promoted its dissociation from Bcl-XL and inhibited its ability to quench the survival function of Bcl-XL.

siRNA knockdown of PKC- ι reduced Bad phosphorylation, increased Bad/Bcl-XL interaction and decreased Bad/14-3-3 dimerization. Furthermore, active PKC- ι re-stimulated the phosphorylation of Bad in these cells confirming that PKC- ι is a potent regulator of Bad function. Such a cell survival mechanism has been previously shown in NSCLC; however, the activation of PKC- ι in these cells was NNK dependent [121]. In contrast, our results demonstrated that endogenous PKC- ι is highly activated in glioblastoma and it promotes survival of cells independent of any external stimulation.

PI (3)-kinase has been shown to induce activation of PDK-1 which subsequently phosphorylates and activates PKC- ι [106, 107, 111]. PI (3)-kinase and PDK1 inhibition not only blocked PKC- ι activity but also inhibited Bad phosphorylation. This suggests that glioma cell survival occurs through a PI (3)-kinase signaling pathway. Intriguingly, PDK1 knockdown also significantly inhibited the endogenous expression of PKC- ι in our

cells. Previous studies have shown that in embryonic stem cells, where PDK1 was knocked out, there was marked reduction in the expression of atypical PKC isoforms (PKC- ζ , PRK1 and PRK2) [122], however, regulation of PKC- ι expression by PDK1 has never been shown before. Understanding the mechanism by which PDK1 regulates PKC- ι expression will require further investigation.

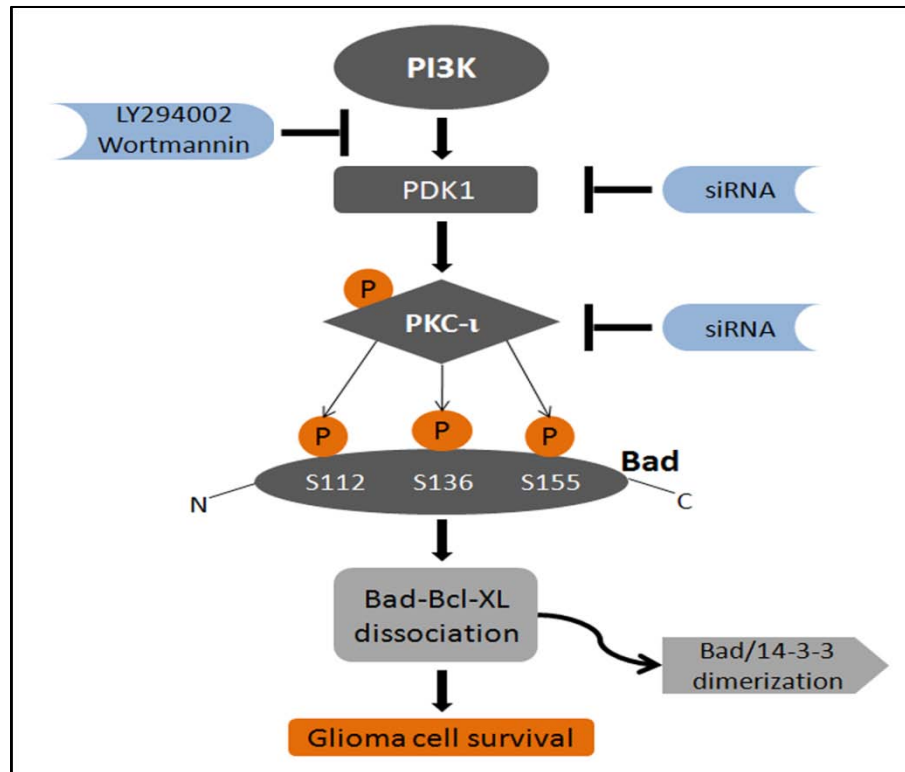


Figure 15 Graphical representation of PI (3)-kinase/PDK1/PKC- ι /BAD cell survival pathway

Collectively, our data shows that PKC- ι promotes glioblastoma cell survival by regulating the pro-apoptotic function of Bad through PI (3)-kinase in absence of any external stimulation. Thus, we show that PKC- ι expression may be used as a prognostic marker for the identification of glioblastoma patients that may benefit from anti-PKC- ι therapy for personalized medicine.

CHAPTER 4

PKC- ι INDUCES CELL CYCLE PROGRESSION AND PROLIFERATION IN GLIOMA CELLS

4.1. Overview

Glioblastoma multiforme is a highly fatal primary brain tumor. Its high propensity of invading the surrounding healthy brain tissue has prevented good prognosis in glioblastoma patients [12]. Rapid proliferation, fast infiltration and late diagnosis are hallmarks of glioblastoma that have rendered glioblastoma incurable.

PKC is frequently observed to be altered and/or overexpressed in glioblastoma [103, 104]. Several studies have shown that PKC hyperactivity is correlated with the malignant growth rates in gliomas. Frequent mutations/deletion in the PTEN gene or amplifications in EGFR occur in glioblastoma [9, 10] which constitutively augment PI (3)-kinase activity. PKC- ι , a downstream mediator of PI (3)-kinase is highly expressed in glioma tissues [118]; however, its role in glioma cell cycle regulation and proliferation has not yet been completely defined. Studies have also demonstrated a crosstalk between PKC- ι and Cdk7 in glioma, prostate cancer and neuroblastoma cells [125-127]. Cdk7, a member of the cyclin dependent protein kinase family, is a master cell cycle regulator [23] and mediates by phosphorylating and activating downstream cdks [24]. Among, all the cdks activated by Cdk7, cdk2 co-ordinates the transition from G1 phase to S phase of the cells [26]. In the current study we provide evidence that PKC- ι directly associated

and phosphorylated Cdk7 in a cell cycle dependent manner. We also demonstrate that PI (3)-kinase and PDK1 inhibition suppressed PKC- ι activity and exhibited corresponding reduction in Cdk7 and cdk2 phosphorylation. Direct PKC- ι knockdown inhibited Cdk7, cdk2 phosphorylation and abrogated glioma cell proliferation. Thus, our data suggests that glioma cells may be proliferating through a novel PI (3)-kinase /PKC- ι /Cdk7 signaling cascade that can be targeted for glioblastoma therapy.

4.2 Results

4.2.1 PKC- ι , Cdk7 and Cdk2 expression in glioma cells. The expression of PKC- ι , Cdk7 and cdk2 were higher in 50% confluent T98G cells (rapidly proliferating cells) than in 100% confluent plus serum starved cells (contact inhibited) (Fig. 16A). A similar but less robust decrease in the levels of PKC- ι , Cdk7 and cdk2 in 100% confluent cells occurred in U87MG cells (Fig. 16A). Constitutive expression of Caspase 3 showed that the decrease in the expression of PKC- ι was not due to cells undergoing apoptosis. β -actin was used as the loading control. The cytoplasmic and nuclear expression of these proteins was higher in actively proliferating glioma cells (Fig. 16B). In T98G cells, expression of PKC- ι , cdk2 was significantly depleted in 100% confluent plus serum starved cells (contact inhibited) versus proliferating cells. Whereas, Cdk7 expression was reduced only by 2-fold in serum starved T98G cells. The overall difference in the levels of PKC- ι and cdk2 was not especially robust in the sub-cellular fractions of U87MG cells (actively proliferating versus serum starved) in comparison to T98G cells (Fig. 16B). Thus, presence of PKC- ι in nucleus suggests a potential role of PKC- ι in glioma cell cycle progression and proliferation.

4.2.2 PKC- ι regulates cell cycle progression and cell proliferation. To determine cell cycle progression, we analyzed the DNA content of both T98G and U87MG cells at time points over a 36h period. Initially the cells were populated (87% in T98G and 65% in U87MG) in the G1 phase. There was progression into S phase from 18 until 24 h followed by increase in G2 phase by 30h, suggesting that the cells were progressing into mitosis (Fig. 17A, E). Western blot analysis of whole cell lysates at the indicated time points showed that expression of PKC- ι , Cdk7 and cdk2 was correlated with the cell cycle phases in both T98G (Fig. 17B) and U87MG cells (Fig. 17F). When a majority of the cells were synchronized in the G1 phase, the levels of PKC- ι , Cdk7 and cdk2 was lower and expression of all three proteins increased as the cells progressed to S phase (Fig. 17B, F). Such corresponding variation in the expression suggests an involvement of these proteins in T98G cell cycle progression. Furthermore, PKC- ι was directly associated with Cdk7 and the levels of PKC- ι at the indicated time points coincided with the amount of pCdk7 and total Cdk7 (Fig. 17C, G). Data from sub-cellular fractionation demonstrated that at the time points when the cells were transitioning from G1 the amount of PKC- ι and Cdk2 increased in the nuclear fraction (Fig. 17D, G). The majority of Cdk7 was present in the nuclear fraction of both cell lines with slightly increased levels at later time points suggesting that the cells were proliferating. Overall, these relationships were less evident in U87MG compared to T98G cells because of the innate asynchronous nature of U87MG cells. A robust unidentified band was observed below the phosphorylated form of Cdk7 (Fig. 17C, G). Determining the identity of this band requires further investigation.

4.2.3 *PKC- ι is an upstream Cdk7 kinase.* We recently demonstrated that PKC- ι is an *in-vitro* kinase to Cdk7 [33]. Here, we investigated whether PKC- ι /Cdk7 mediated signaling may also be occurring in glioma cells. We measured a significant increase in pCdk7 at T170 in co-IP (PKC- ι and Cdk7) samples compared to individual IP samples (Fig. 18A). In contrast, pcdk2 at T160 was observed only in IP Cdk7 samples and not in IP PKC- ι samples suggesting that PKC- ι may not be a cdk2 kinase. Moreover, a three - fold increase in pcdk2 at T160 was observed in co-IP samples (PKC- ι and Cdk7) suggesting that the presence of PKC- ι probably increased Cdk7 activity which may have promoted a more robust downstream phosphorylation and activation of cdk2. In addition, when purified, active PKC- ι was incubated with IP Cdk7 in an *in-vitro* kinase assay, direct phosphorylation of Cdk7 at T170 was observed (Fig. 18B, $P < 0.05$). These results suggest that PKC- ι is an upstream Cdk7 kinase and that glioma cells may be progressing through PKC- ι /Cdk7/cdk2 mediated signaling.

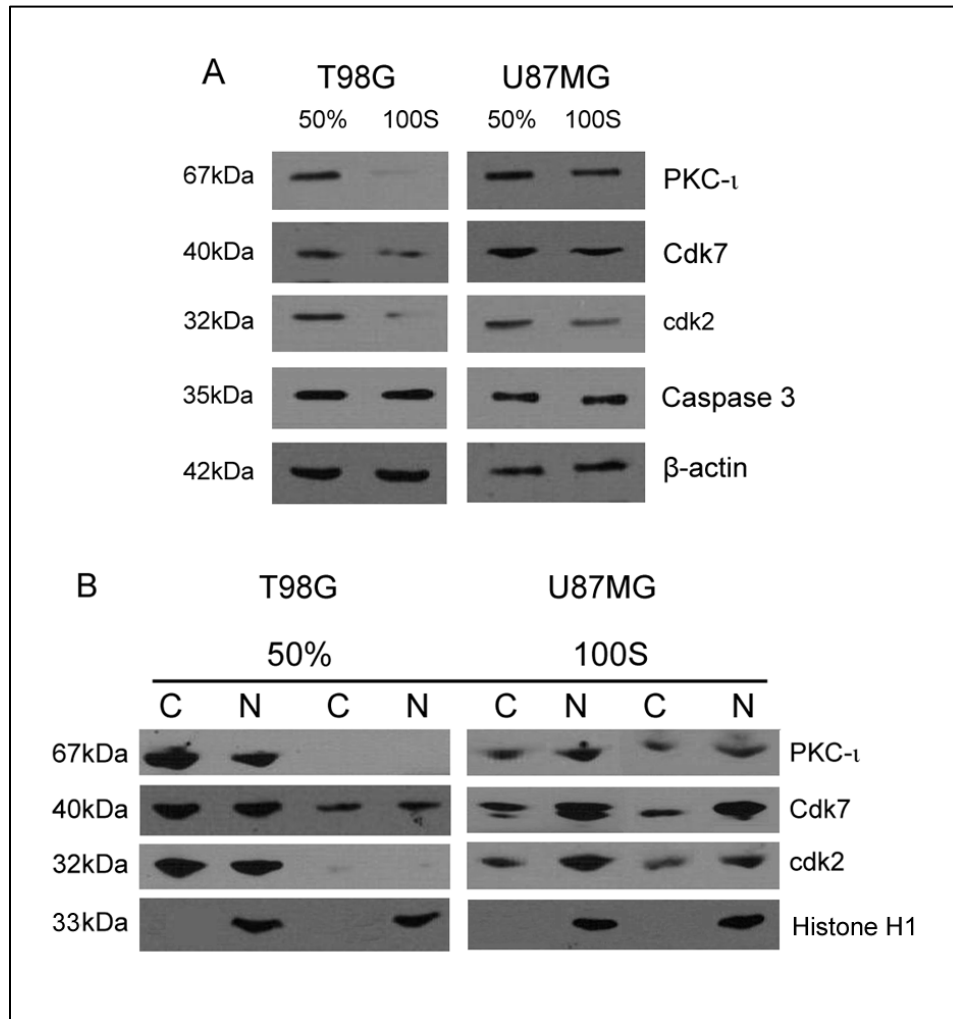


Figure 16 Expression profile of PKC-ι, Cdk7 and Cdk2 in T98G and U87MG cells. **(A)** PKC-ι, Cdk7, cdk2 and Caspase 3 expression levels in actively proliferating as well as contact inhibited plus serum starved T98G and U-87MG cells were determined by Western blot analysis. β-actin was used as loading control. **(B)** Actively proliferating and contact inhibited plus serum starved T98G and U-87MG cells were fractionated into cytosolic and nuclear fraction. Western blot analysis was performed to determine the expression of PKC-ι, Cdk7 and cdk2. Histone H1 was used as the purity control for the nuclear fraction. Data is representative of N=3 independent experiments.

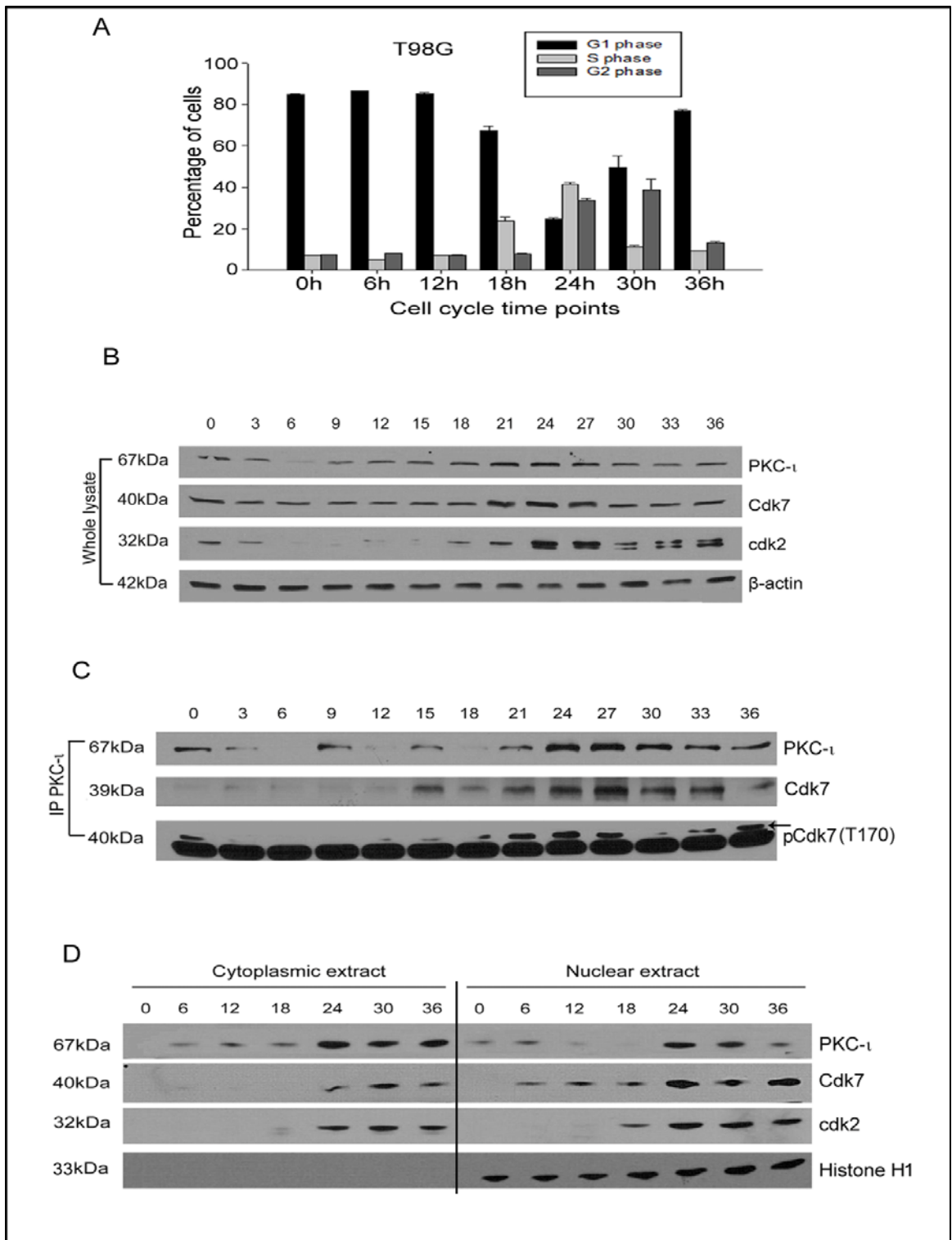


Figure 17A-D. Cell cycle progression and PKC- ι , Cdk7, cdk2 protein expression in T98G and U87MG cells

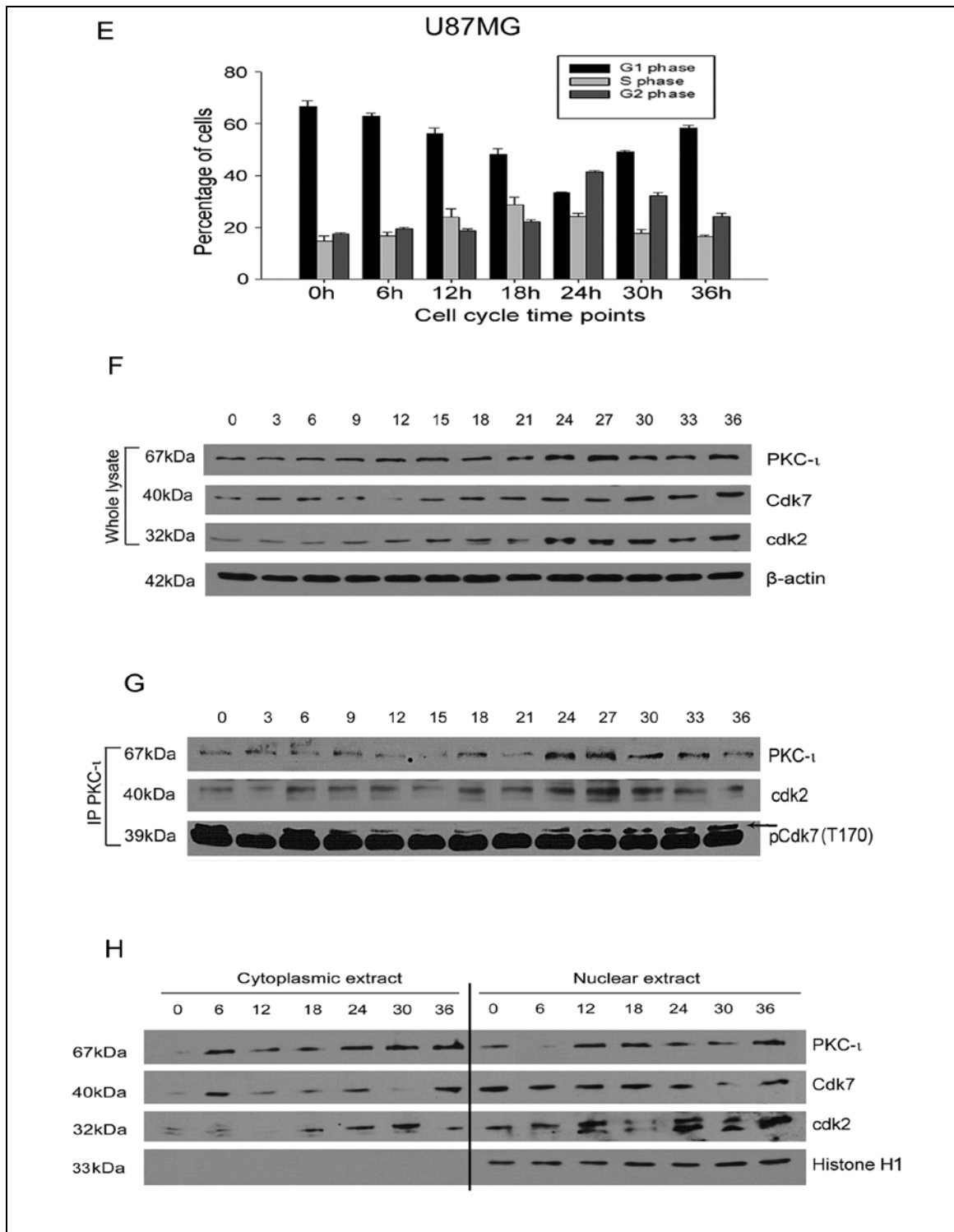


Figure 17E-H. Cell cycle progression and PKC- ι , Cdk7, cdk2 protein expression in T98G and U87MG cells. Briefly, T98G and U87MG cells were grown to 60-70% confluency followed by serum starvation for 48 hours and subsequently serum stimulated for 36h. Cells were harvested every 3h and prepared total Western blot. (A, E) Total expression of PKC- ι , Cdk7 and cdk2 were analyzed by Western blotting of

whole cell lysates from the indicated time points. β -actin was probed as a loading control. **(B, F)** Duplicate time point samples were prepared for cell cycle analysis. The histograms display the total DNA content in G1 phase (black bar), S phase (light grey bar) and G2 phase (Dark grey bar). **(C, G)** PKC- ι was IP and its associated proteins were determined by Western blot analysis by probing for pCdk7 (T170), total Cdk7 and PKC- ι . The first negative control (-) contains whole cell extract plus rabbit IgG whole molecule (50 μ l of 1:1v/v) and the second negative control (-) contains whole cell extract plus rabbit IgG whole molecule (50 μ l) and normal rabbit IgG serum (5 μ g). **(D, H)** Sub-fractionated cell lysate (cytosolic and nuclear fraction) were subjected to Western blot analysis to determine the expression of PKC- ι , Cdk7 and cdk2 at each time point.

4.2.4 PKC- ι knockdown leads to reduction in expression and activity of Cdk7 and cdk2. To further prove the involvement of a PKC- ι /Cdk7/cdk2 proliferation pathway in T98G and U87MG cells, cells were treated with PKC- ι siRNA (100nM for 24h). PKC- ι knockdown was determined by Western blot analysis of whole cell lysate (data not shown). The Cdk7 phosphorylation at T170 and cdk2 phosphorylation at T160 was significantly inhibited in both cell lines treated with PKC- ι siRNA (Fig. 18A, $P < 0.005$). In addition, a reduction in the expression of total Cdk7 and cdk2 was also seen. This result supports our earlier data (Fig. 16, and 17) [34] that showed that expression of PKC- ι , Cdk7 and cdk2 is crucial for glioma cell proliferation. Furthermore, the reduction in PKC- ι induced Caspase-9 cleavage (Fig. 18B, $P < 0.05$) implies that the cells are undergoing apoptosis via the intrinsic mitochondrial pathway. PKC- ι knockdown also decreased the phosphorylation of the retinoblastoma (Rb), a tumor suppressor gene (Fig. 18B, $P < 0.05$) and increased the amount of p27^{kip1}, a known Cdk inhibitor (Fig. 18B, $P < 0.05$). These results suggest that PKC- ι is involved in regulating Rb and p27^{kip1} which play essential roles in cell cycle machinery.

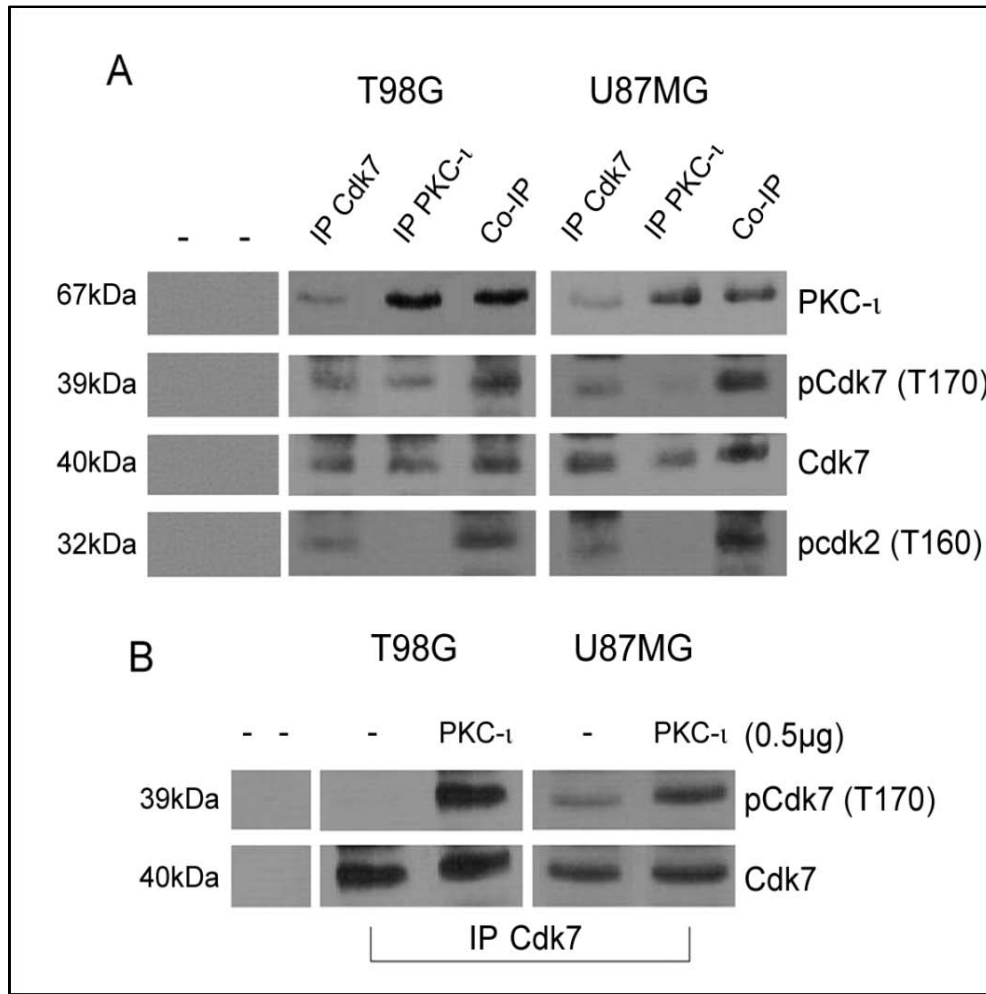


Figure 18 PKC- ι induces direct phosphorylation of Cdk7. **(A, B)** the first negative control (-) contains whole cell extract plus rabbit IgG whole molecule (50 μ l of 1:1v/v) and the second negative control (-) contains whole cell extract plus rabbit IgG whole molecule (50 μ l) and normal rabbit IgG serum (5 μ g). **(A)** IP Cdk7, IP PKC- ι and co-IP from T98G and U87MG cells were subjected to kinase activity assay. Phosphorylation of Cdk7 at Thr-170, pcdk2 at Thr-160 and Pan Cdk7 were quantified using Western blot analysis. **(B)** IP Cdk7 from both T98G and U87MG cells was incubated with active PKC- ι (0.5 μ g) in an *in-vitro* kinase activity assay. Western blot analysis was performed to determine phosphorylation of Cdk7 at Thr-170, pcdk2 at Thr-160, PKC- ι and Pan Cdk7.

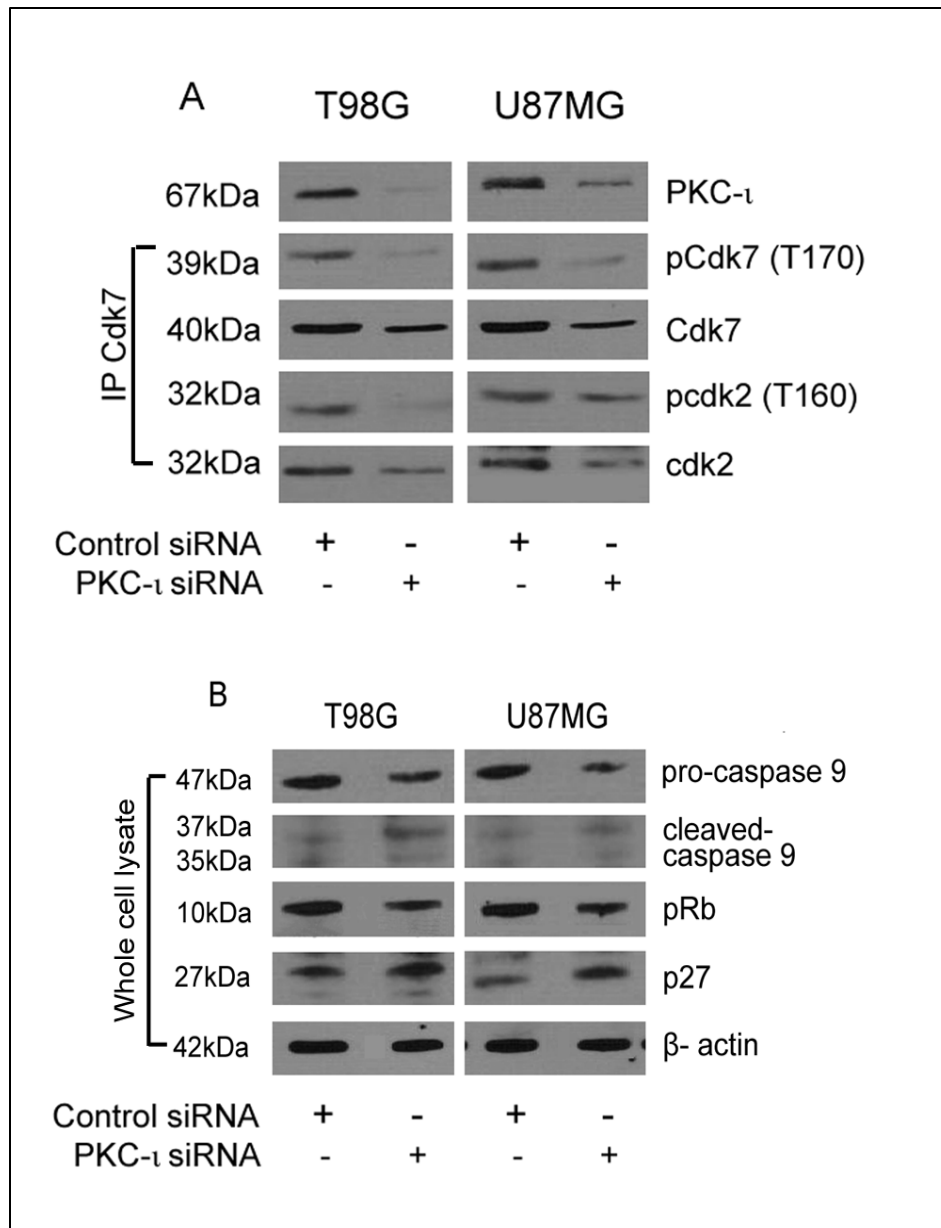


Figure 19 PKC-ι knockdown diminishes Cdk7 and cdk2 phosphorylation as well as cdk2 activity. **(A)** Both T98G and U87MG cells were treated with either control siRNA or PKC-ι siRNA (100nM) for 24 hours. Subsequently, Cdk7 was IP and subjected to kinase activity assay followed by Western blot to detect pCdk7 at T170, cdk2 at T160, total Cdk7 and total cdk2. **(B)** Whole cell lysate were from siRNA treated cells and subjected to Western blot analysis to detect Caspase 9, cleaved Caspase 9, pRb and p27^{kip1}.

4.2.5 Inhibition of PKC- ι activity precedes a reduction in Cdk7/cdk2

phosphorylation. Treatment with LY294002 (50 μ M) or Wortmannin (0.1 μ M) for 2 h blocked PKC- ι activity by diminishing its phosphorylation at the T555 residue in both T98G cells and U87MG cells (Fig. 20A, $P < 0.05$). Marked reduction in the amount of Cdk7 phosphorylation at T170 and cdk2 phosphorylation at T160 was also observed (Fig. 20A, $P < 0.05$), suggesting that PKC- ι regulates the expression of these proteins as previously observed (Fig. 17, 18 and 19). β -actin was used as a loading control. Inhibition of PDK1 by siRNA (100nM) also diminished the endogenous amount of PKC- ι which subsequently inhibited the activity of PKC- ι (pPKC- ι at T555) (Fig. 20B, $P < 0.05$). Correspondingly, a reduction in the levels of Cdk7 phosphorylation at T170 and cdk2 at T160 was observed (Fig. 20B, $P < 0.05$). A modest reduction in the Cdk7 and cdk2 level was also observed further supporting our data shown in Fig. 16, 17 and 18. These results indicate that the PI (3)-kinase/PDK1 regulate the activity and expression of PKC- ι in these cells.

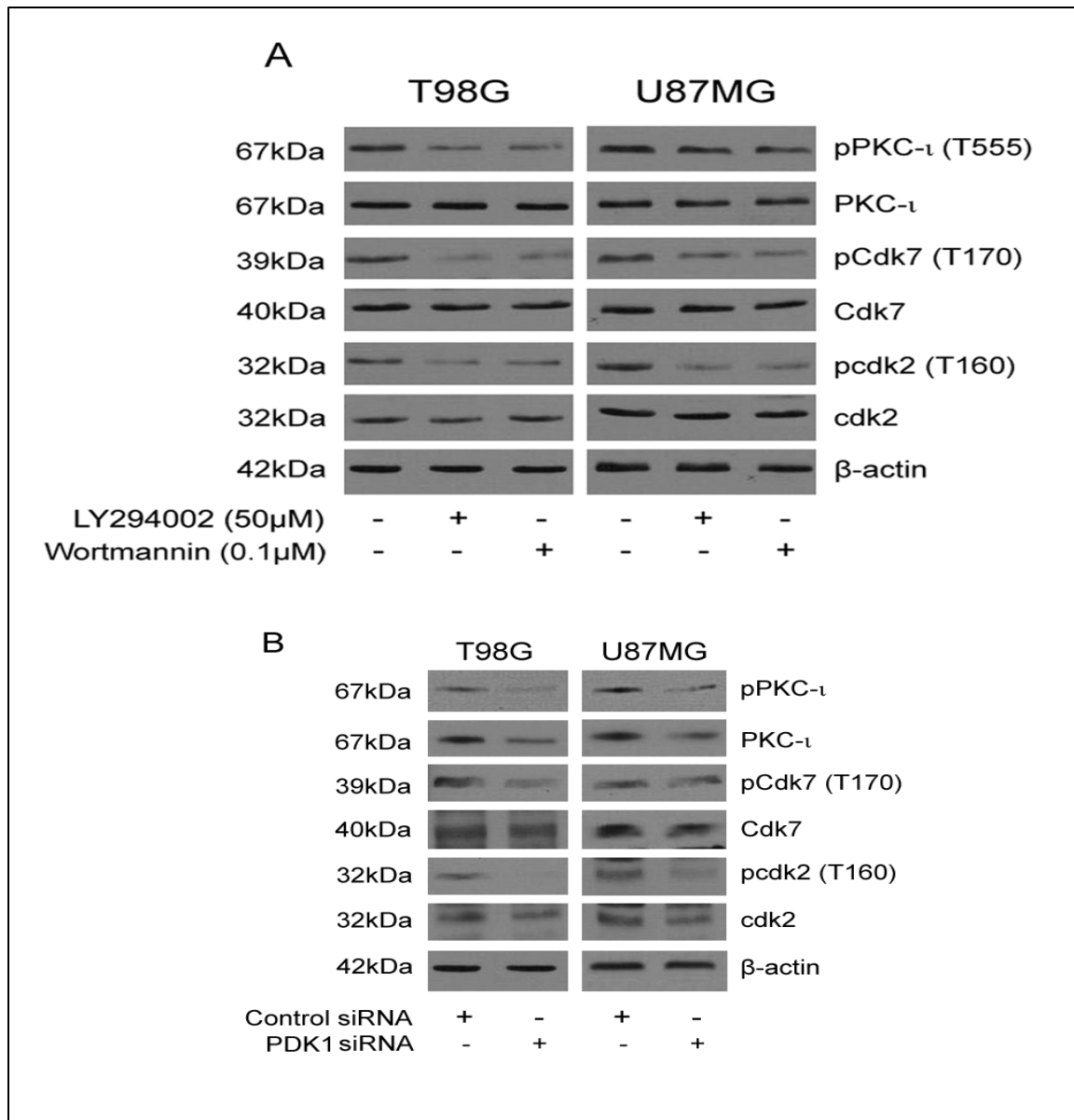


Figure 20 Inhibition of PKC- ι activity precedes a reduction in Cdk7 and cdk2 phosphorylation in T98G and U87MG cells. (A) Western blot analysis of T98G and U87MG cells for phospho-PKC- ι (Thr-555), phospho-Cdk7 (Thr-170), phospho-cdk2 (Thr-160), Pan Cdk7, Pan cdk2 and PKC- ι following individual treatments for 2 h with LY294002 (50 μ M) and Wortmannin (0.1 μ M). (B) Both cell lines were treated with PDK1 siRNA (100nM for 24h) followed by Western blot analysis to detect phospho-PKC- ι (Thr-555), total PKC- ι , phospho-Cdk7 (Thr-170), phospho-cdk2 (Thr-160), Pan Cdk7, Pan cdk2. Data is representative of three independent experiments.

4.3 Discussion

PKC- ι , an atypical isoform of PKC family, is a key regulator of cell survival, invasion and chemoresistance in glioblastoma [3, 111, 112]. Previous data from our lab had demonstrated role of PKC- ι in glioma cell proliferation and involvement in malignant phenotype of glioblastoma. However, the mechanistic signaling remained unclear [118].

PKC isotype profiling in previous studies demonstrated that PKC- ι was highly expressed in actively proliferating neuroblastoma, glioblastoma and prostate cells. However, under serum starved conditions, where the cells were quiescent, PKC- ι was significantly inhibited [118, 126, 127]. Our current studies also displayed similar overexpression of PKC- ι in proliferating glioma cells (T98G and U87MG) and reduced levels in serum starved cells. Cdk7 and cdk2 level was also reduced in the serum starved conditions. In addition, the analyses of the cytoplasmic and nuclear expression of these proteins (Fig. 16B) revealed that even though Cdk7 was constitutively present in both cell fractions, there was reduced cdk2 expression (in both T98G and U87MG cells) in the absence of PKC- ι , indicating that PKC- ι may be essential for regulating the expression of cdk2 (in presence of Cdk7) thereby triggering the cell proliferation. Thus, the data suggest the importance of crosstalk between these proteins for glioma cell growth and proliferation. This putative mechanism of regulation of cdk2 expression in glioma cells has never been reported before, however, further experimentation may be required to confirm this data.

Cdk7 regulates the eukaryotic cell cycle and is an *in-vitro* upstream kinase to cdk2 [87, 128]. Phosphorylation and activation of cdk2 controls the transition of cells from G1-S phase. Our results showed that PKC- ι , Cdk7 and cdk2 levels increased continuously as cells progressed to S and subsequently G2 phase. This suggests a potential correlation between these proteins in a cell cycle dependent manner. Thus, we hypothesized that PKC- ι might be regulating the expression and activity of Cdk7, a cell cycle regulator. To test this hypothesis, we analyzed the association between PKC- ι and Cdk7 in these cells. The majority of PKC- ι associated with and phosphorylated Cdk7 at T18-T30 h suggesting that PKC- ι regulates cell cycle progression of glioma cells. There was also a cell cycle dependent increase and translocation of PKC- ι , Cdk7 and cdk2 into the nucleus further supporting this hypothesis. Although, an overall correlation was observed in both cell lines, U87MG did not show a similar pattern to T98G because U87MG cells are highly asynchronous in nature.

Recent findings by *Pillai et.al* have shown that PKC- ι is an *in-vitro* upstream kinase to Cdk7 [127]. In the current studies, we found that PKC- ι not only associated with Cdk7 but also directly phosphorylated Cdk7 at T170 endogenously as well as exogenously suggesting that PKC- ι may also be an upstream Cdk7 kinase in glioblastoma.

PKC- ι knockdown by siRNA treatment has been reported to inhibit glioma cell survival by suppressing the pro-apoptotic function of Bad [129]. Moreover, in this study, we observed that PKC- ι knockdown reduced Cdk7 and cdk2 phosphorylation suggesting that PKC- ι is not only required for glioma cell survival but may also be crucial for glioma

cell proliferation. Such a mechanism has been previously shown in prostate cancer; however, PKC- ι did not directly associate with Cdk7 in DU-145 cells and only transiently associated with Cdk7 in RWPE-1 cells implying that PKC- ι may not be a direct upstream kinase to Cdk7 and that other kinases may be required to completely inhibit proliferation in these cells [126]. PKC- ι knockdown also regulated the expression of known cdk2 substrates, Rb [96] and p27^{kip1} [130], implying that PKC- ι inhibition may lead to cell cycle arrest. PKC- ι depletion also triggered Caspase-9 cleavage suggesting induction of the intrinsic pathway mediated apoptosis in glioma cells, complementing our recently published data [129].

PKC- ι silenced glioma cells as well as U.V. exposed glioma cells generated different distribution patterns of cell death. To our knowledge, these findings are the first evidence that PKC- ι inhibition generates early apoptosis and late apoptosis. However, a conventional method of apoptosis induction, U.V. irradiation, produced mostly late apoptosis. The CFSE/7-AAD analysis for the first time revealed the activity of the T98G cells that resisted cell death following PKC- ι silencing. These resistant T98G cells were viable but experienced hampered cell division due to G₂/M arrest. A similar distribution pattern was also observed in the cell cycle analysis data wherein, a two-fold increase in G₂/M cell population suggested that the cells might be going from G₂/M arrest directly to apoptosis following RNA silencing.

PI (3)-kinase, an upstream kinase of PDK-1 phosphorylates and activates atypical PKCs [106, 107, 111]. Pharmacological inhibition of PI (3)-kinase and PDK1, blocked PKC- ι activity and also inhibited Cdk7, cdk2 phosphorylation. These results suggest that

glioma cell proliferation occurred through a PI (3)-kinase mediated signaling pathway. Intriguingly, PDK1 knockdown also significantly inhibited the endogenous expression of PKC- ι and slightly that of Cdk7 and cdk2 in our cells. Previous studies in embryonic cells showed that PDK1 knockout markedly reduced the expression of atypical PKC isoforms (PKC- ζ , PRK1 and PRK2) [106, 122]; however, regulation of PKC- ι by PDK1 was never explored. Thus, our findings are the first to postulate a role of PDK1 in the regulation of PKC- ι . Further investigation is required to confirm this theory.

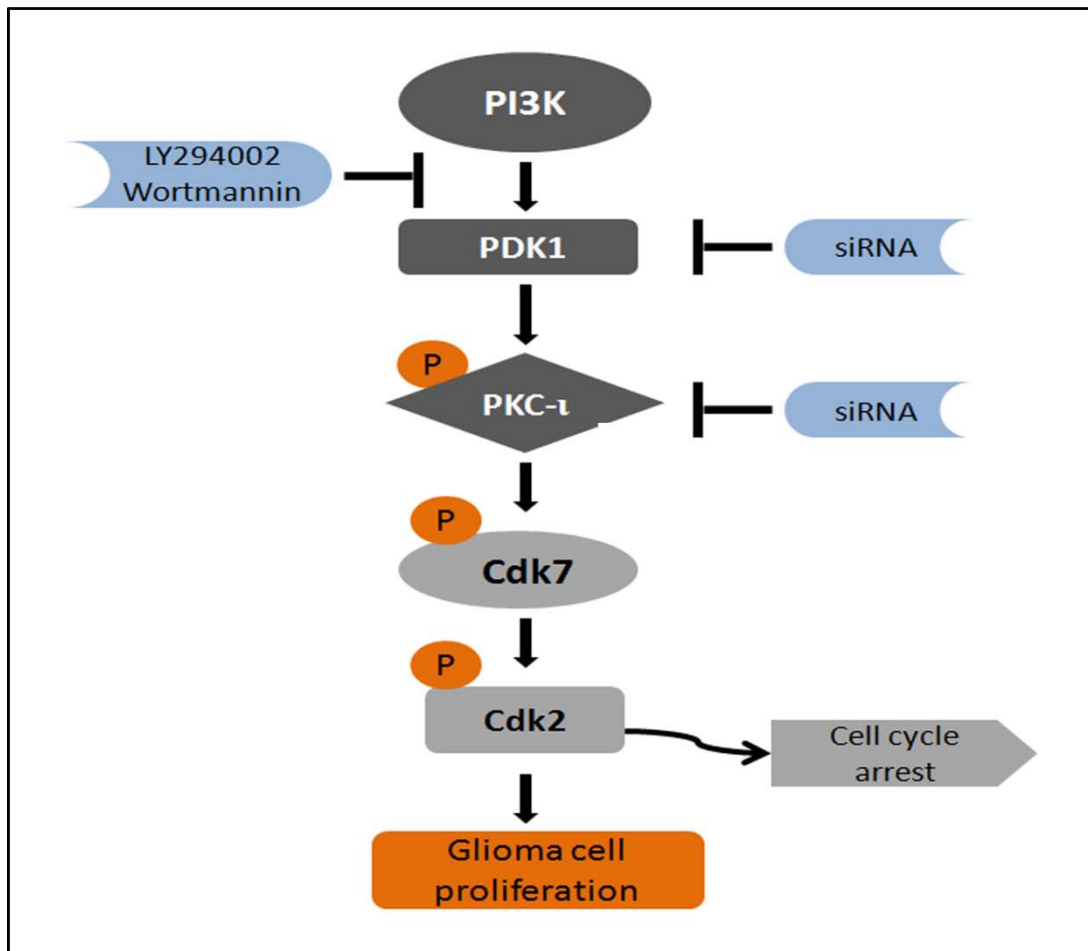


Figure 21 Graphical representation of PI (3)-kinase/PKC- ι /Cdk7/cdk2 cell proliferation pathway

In conclusion, these data suggest a role of PKC- ι in glioblastoma cell cycle progression and proliferation. We show a novel mechanism indicating that PKC- ι is highly activated and overexpressed in glioblastoma. PKC- ι induces uncontrolled glioma cell cycle progression and proliferation by modulating Cdk7/cdk2 activity in a PI (3)-kinase dependent manner. Furthermore, PKC- ι silencing studies demonstrated that glioma cells are highly resilient to conventional modalities suggesting that a combinatorial therapy may be required in the future to combat the apoptosis-resistant glioblastoma cells following PKC- ι downregulation. Thus, these results suggest that identification of PKC- ι and employing anti-PKC- ι therapy may help improve patient outcome in glioblastoma.

CHAPTER 5

PKC- ι KNOCKDOWN INHIBITS CELL PROLIFERATION AND INDUCES APOPTOSIS IN GLIOMA CELLS

5.1 Overview

Glioblastoma is a deadly disease with a median survival time of 8-12 months following prognosis. Due to its extremely infiltrative and rapidly growing behavior, glioblastoma is a tremendously drug resistant type of cancer [18, 119]. Therefore, a better understanding of glioblastoma cells, proliferative and survival mechanisms and methods to achieve its inhibition are appropriate strategies to improve glioblastoma treatment.

Rapid glioma growth and survival has been attributed to inherently high levels of protein kinase Cs (PKC) [103]. In this study, we tested the effects of PKC- ι (an atypical PKC family member) silencing on the proliferative and survival mechanisms in glioblastoma. The pathways involved in glioma cell survival and proliferation have been studied and detailed in the previous chapters (Chapter 3 and 4 respectively). We observed that there was corresponding reduction in cell proliferation and induction of apoptosis with cell cycle arrest in glioma cells.

Apoptosis or programmed cell death is broadly characterized by cellular morphological changes and energy dependent biochemical mechanism [131, 132].

Apoptosis is an essential component required for maintenance of cellular turnover, development and functioning of the immune system, aging and embryonic development [133]. Any alteration in this tightly regulated mechanism can lead to human conditions such as neurodegenerative disorders, autoimmune diseases and cancer [134]. Apoptosis plays a vital role in regulating the homeostasis to maintain various mechanisms in tissues [135].

During early apoptosis, morphological changes in the cell occur, displaying cell shrinkage, chromatin condensation involving single cells or small clusters of cells, followed by extensive plasma membrane blebbing and budding [136, 137]. Such morphological observations were seen following PKC- ι silencing in this study. There are two main mechanisms of apoptosis-extrinsic and intrinsic [138]. The molecular execution of apoptosis occurs via activation on cysteine dependent aspartate-specific proteases (Caspases) [139]. The intrinsic mode of apoptosis is dependent on a non-receptor mediated mitochondrial-array of events involving the pro-apoptotic members of the Bcl-2 family [140]. The extrinsic apoptosis involves cell surface “death receptors” along with their ligands.

Necrosis and autophagy are other types of cell death. Necrosis is the premature death of cells or living tissue. Necrosis is caused by external factors to the cell including infection, toxins or trauma. It is in contrast to apoptosis which is a programmed cell death. Cells that die due to necrosis do not send the same signals to the immune system as apoptosis preventing phagocytes from locating and engulfing the dead cells leading to dead cell build up. Such a build up of necrotic tissue has to be removed surgically.

Autophagy or autophagocytosis also known as the type II programmed cell death, is a catabolic process involving the degradation of cell's own components through the lysosomal system. A tightly regulated process involved in cell growth, development and homeostasis, maintains a balance between synthesis, degradation and recycling of cellular products. It is a main mechanism by which starved cells reallocates nutrient from unnecessary process to a more necessary one.

In the previous study (Chapter 3), we observed a corresponding reduction in Bad (pro-apoptotic Bcl2- family member) phosphorylation following PKC- ϵ silencing. Such activation of Bad promotes an apoptotic response that triggers energy dependent biochemical changes in the cells [132]. This causes loss of inner mitochondrial membrane that results in opening of the mitochondrial permeability transition (MPT) [141], loss of mitochondrial transmembrane potential [142] and release of cytochrome C and Smac/Diablo [143, 144]. This subsequently binds APAF1 [145] and pro-Caspase 9 and activates Caspase 9 thereby triggering apoptosis by inhibiting the inhibitors of apoptosis proteins (IAPs) [146]. This further activates executioner Caspases (3, 6, 7) which degrade various nuclear and cytoplasmic proteins including PARP, cytokeratins and others [147]. Another marker apoptosis is reduction in Survivin protein which plays a vital role during the entire process of mitosis. An additional apoptotic marker is the detection of Annexin V mediated binding of phosphatidylserine which is exposed on the outer leaflet of apoptotic cells [148].

5.2 Results

5.2.1 PKC- ι knockdown reduces cell viability. A dose response treatment of T98G cells with PKC- ι siRNA (25nM-125nM) over a 48 h time course was performed using MTS assay according to manufacturer's instructions. The absorbance data showed that PKC- ι siRNA significantly inhibited cell proliferation of T98G cells with 71.4% at 24 h using a concentration of 100nM ($P < 0.001$) (Fig. 22A). We also determined the amount of cell viability in PKC- ι siRNA treated cells when compared to control cells. The average percentage of cell death was analyzed by Trypan blue exclusion assay. Results showed that PKC- ι siRNA (100nM) significantly inhibited cell viability of T98G (74% at 24h and 63% at 48h) and U87MG (85% at 24h and 72% at 48h) cells (Fig. 22B). These results suggest that PKC- ι silencing inhibited cell viability and proliferation rate of glioma cells.

5.2.2 PKC- ι siRNA treatment specifically inhibits PKC- ι expression. Western blot analysis illustrated that PKC- ι siRNA treated cells showed diminished expression of PKC- ι in T98G (77% at 24h and 61% at 48h) and U87MG (80% at 24h and 69% at 48h) cells compared to control siRNA treated cells (Fig. 23). The silencing effect of PKC- ι was highly specific as no cross reactivity was observed when the membrane was immunoblotted for PKC- ζ (Fig. 23). This suggests that the siRNA sequence is highly specific for PKC- ι and hence is potent in silencing the translation of PKC- ι in T98G and U87MG cells.

5.2.3 PKC- ι knockdown potently inhibits cell proliferation in glioma cells. The distribution pattern of cell cycle phases following PKC- ι silencing in T98G cells demonstrated significant changes (Fig. 24B, D). A significant (2-fold) increase in G2 phase was seen following 48h-post siRNA transfection in comparison to control cells (Fig. 24C, D). In addition, a significant increase in cell death was observed suggesting that the cells might be going from G2/M arrest directly into apoptosis after 48h PKC- ι siRNA treatment (Fig. 24D).

Additionally, it was important to gain further understanding of the extent to which PKC- ι modulates the mitotic phase of the cell cycle (Fig. 24E). Therefore, the proliferation rate was measured by the CFSE dilution assay (i.e., a shift demonstrating a decrease in fluorescence intensity). By 48h, the proliferation rates for the no treatment group and control siRNA group (also fixed CFSE positive control) were significantly greater than the proliferation rate for PKC- ι silenced T98G cells (Fig. 24F, G). By simultaneously measuring the actively proliferating cells and nonviable cells, it was confirmed that many of the cells were going from G₂/M arrest directly into apoptosis after PKC- ι inhibition (Fig. 24E, G).

5.2.4 PKC- ι silencing induces cellular changes indicating apoptosis in glioma cells. PKC- ι siRNA (100nM) treated cells displayed distinct morphological changes such as loss of cell membrane symmetry, cell shrinkage, as well as detachment of cells was observed indicating that cells might be undergoing apoptosis (Fig. 25A). Several biochemical changes were also observed following siRNA treatment upon Western blot analysis on whole cell lysates. Primary marker of the apoptosis process is activation of effector Caspases. Current data showed that there was robust decrease in the amount of

pro-Caspase 3 upon PKC- ι siRNA treatment. A downstream effect of the Caspase activation is release of cytochrome C from the mitochondria which was notably seen following PKC- ι siRNA treatment in both cell lines. Expression of Survivin was also observed to be significantly inhibited in PKC- ι siRNA treated cells (Fig. 25B). These data suggest that PKC- ι silencing led to apoptosis induction in glioma cells.

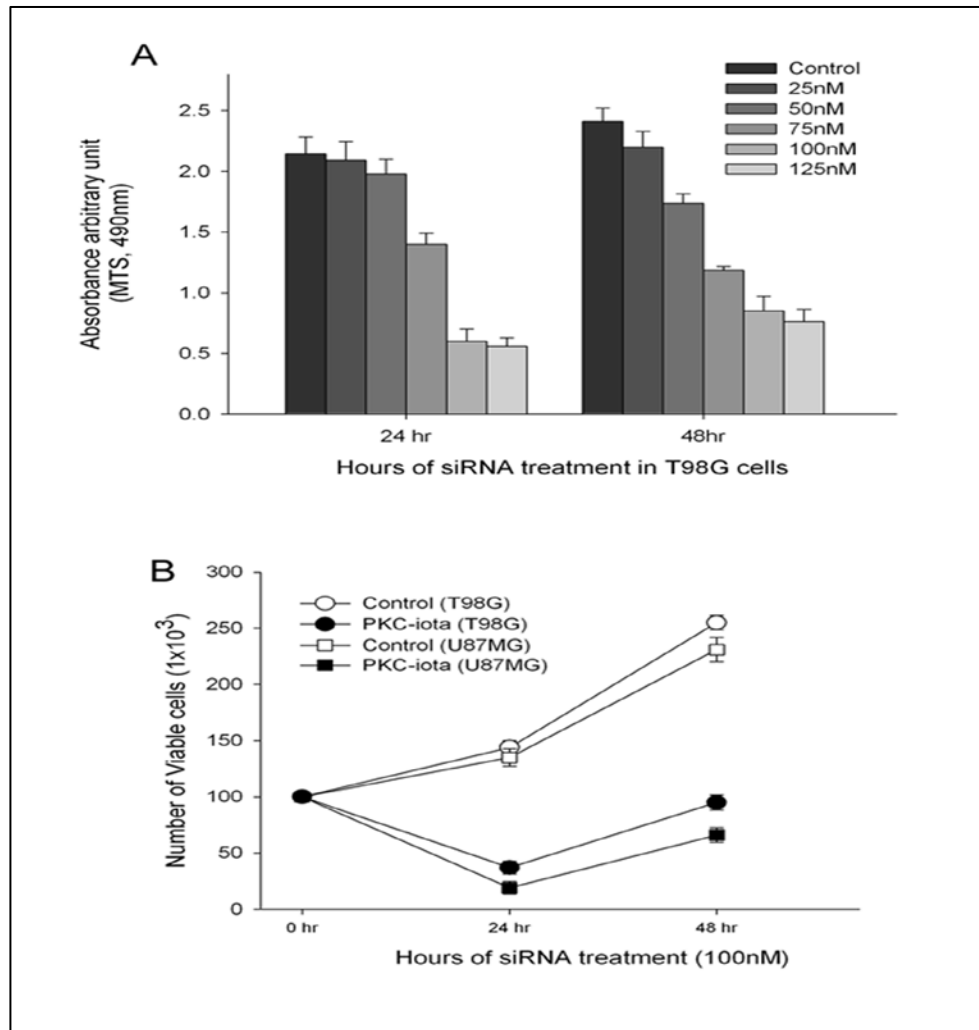


Figure 22 PKC- ι knockdown reduces cell viability (A) T98G cells were treated with increasing concentration of either control siRNA or PKC- ι siRNA (25nM-125nM) for 24 and 48 hours. At each time point, cell proliferation was detected using MTS assay. Data represents $N=3$ independent studies. (B) Both T98G and U87MG cells were treated either with control siRNA or PKC- ι siRNA (100nM) for 24-48 h. At the indicated time points, cell viability was quantified using Trypan blue exclusion assay. Data represents three independent studies.

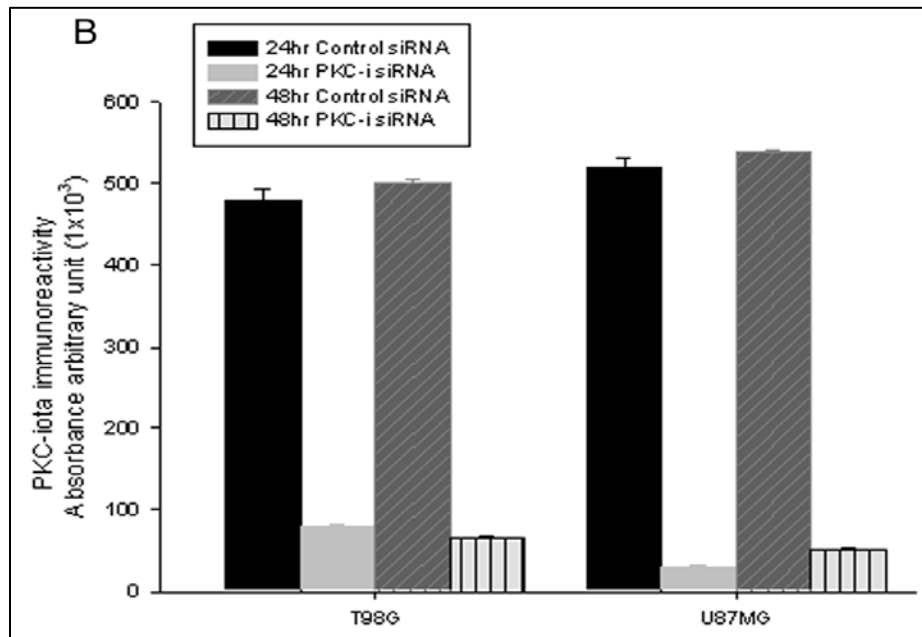
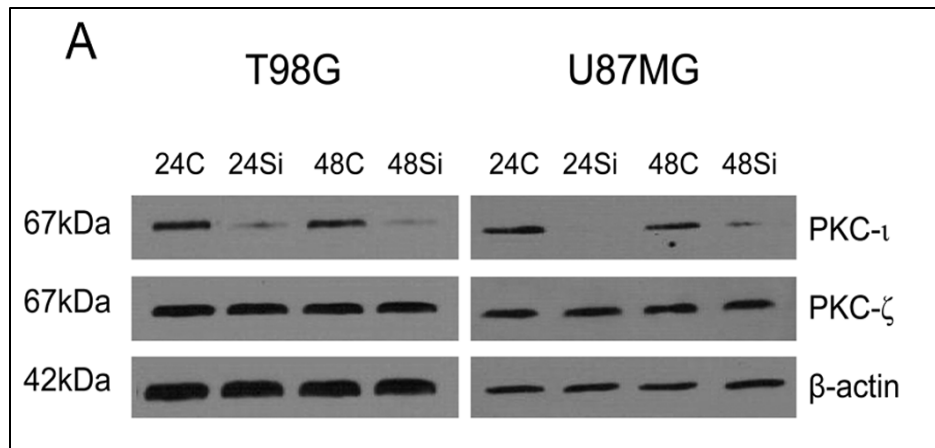


Figure 23 PKC-ι expression is highly diminished upon siRNA treatment. (A) Western blot analysis in total cell extracts (30μg) was performed to determine the expression of PKC-ι and PKC-ζ. β-actin displays equal loading of protein. *(B)* Western blots represent three independent studies. PKC-ι immunoreactivity absorbance is shown by densitometry.

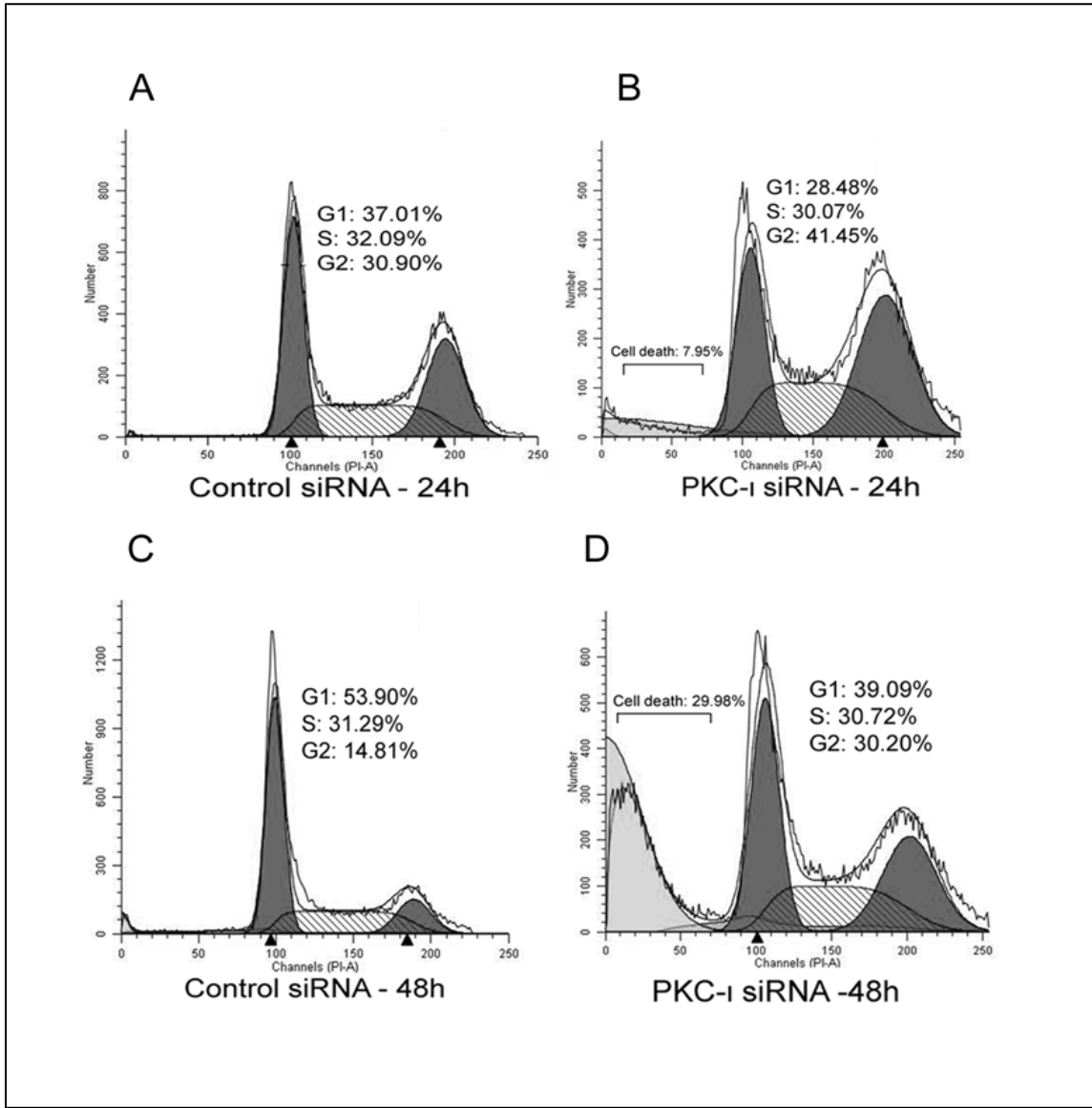


Figure 24A-D PKC-1 inhibits cell proliferation in glioma cells The distribution pattern of cell cycle phases in PKC-1 knockdown cells is compared with control cells (A) 24h-Control siRNA treated cells, (B) 24h-PKC-1 siRNA treated cells, (C) 48h-control siRNA treated cells, (D) 48h-PKC-1 siRNA treated cells.

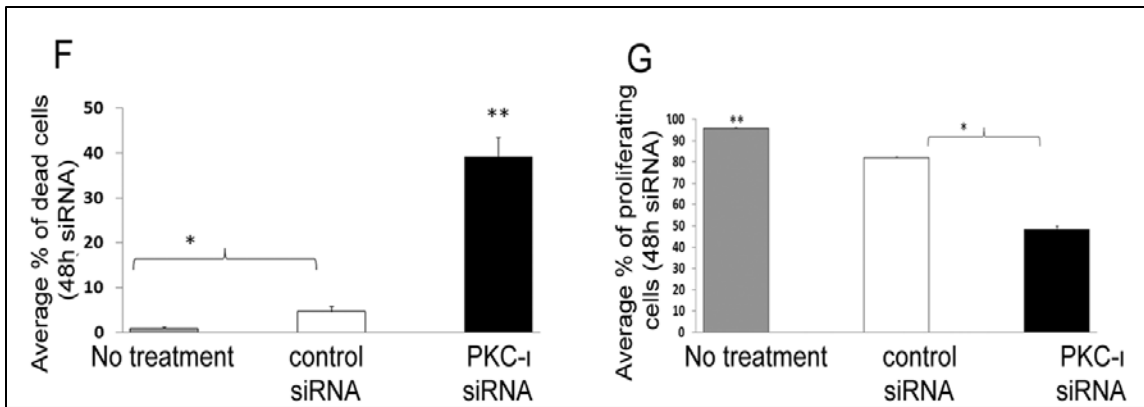
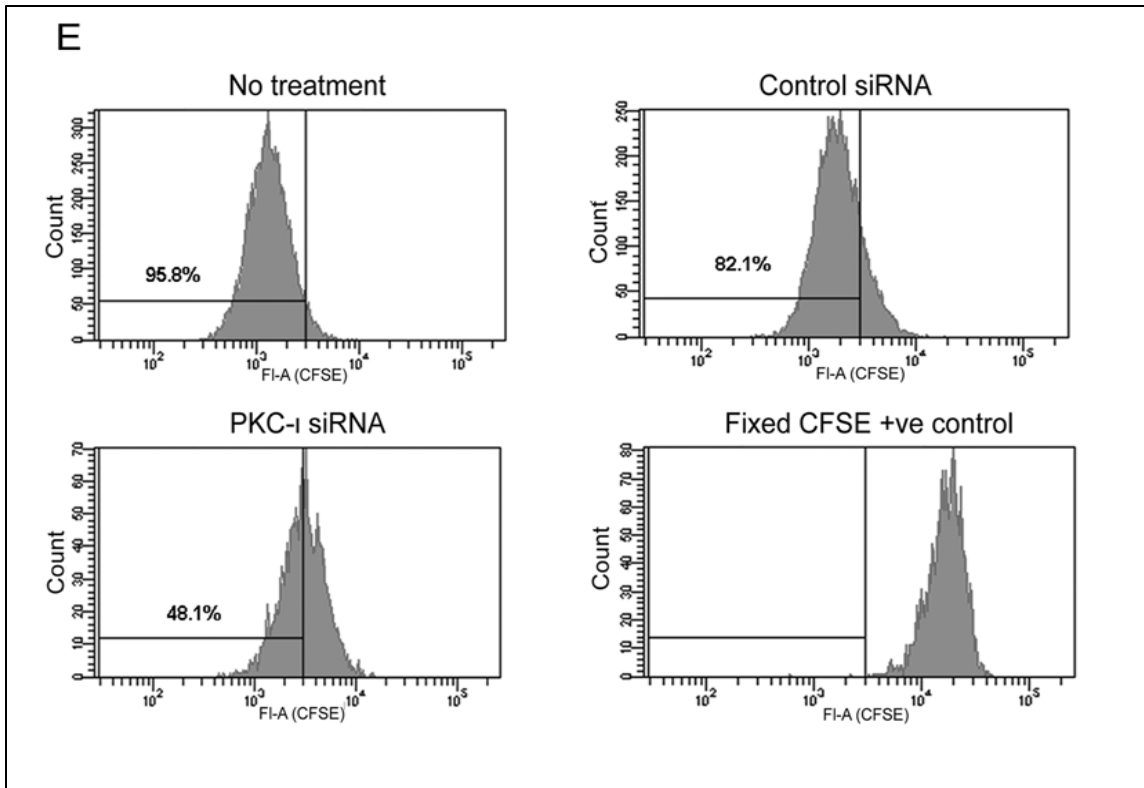


Figure 24E-G PKC-1 inhibits cell proliferation in glioma cells. (E) Proliferation inhibition after PKC-1 silencing in comparison to control cells was assessed by the CFSE dilution assay with live gate analysis. (F) PKC-1 silenced cells and control cells were analyzed by 7-AAD staining to determine the average percentage of dead cells. (G) Average percentage of proliferating cells was measured by CFSE dilution (examined from the Live Gate) from PKC-1 knockdown and control cells. The experiment was repeated twice with a total $N=4$. * $p<0.05$, student's t-test for specified treatment groups, ** $p<0.05$, student's t-test for all treatment groups.

5.2.5 *PKC- ι* silencing induces apoptosis in T98G glioma cells. *PKC- ι*

downregulation along with the subsequent inhibition of cell proliferation and increase in cell death, lead us to examine the specific assessment of cell death rates after siRNA transfection in T98G cells. The average death rate obtained with the Trypan blue method demonstrated similar cell death rates as the Annexin V-FITC/PI assay for each treatment group under investigation (Fig. 26A). *PKC- ι* siRNA treatment or both U.V. exposures generated significantly higher percentages of overall cell death in comparison to the no treatment group and the control siRNA group (Fig. 26A). Nevertheless, the 25 min U.V. exposure produced significantly more overall cell death than any of the treatment groups (Fig. 26A). Further analysis of Annexin V-FITC/PI data provided an additional perspective on the change in the cell death distribution patterns following the downregulation of *PKC- ι* in T98G cells (Fig. 26B). The T98G cells were so resilient that U.V. irradiation for less than 20 min did not induce much cell death (data not shown). *PKC- ι* siRNA exposure or 25 min exposure to U.V. irradiation induced a 34% overall apoptotic rate which was significantly more than that observed in untreated cells or the control siRNA treated cells (Table 10). Notably, it was found that 20 min was the threshold for the T98G cell's capacity to hold its membrane integrity intact after direct, continuous exposure to U.V. irradiation. Both U.V. exposure times induced significantly more late apoptosis than *PKC- ι* siRNA treated T98G cells where as *PKC- ι* silencing produced the most significant level of early apoptosis in comparison to all treatments (Fig. 26B and Table 10). Furthermore, both exposure times to U.V. irradiation led to significantly more necrotic T98G cells than any other treatments (Fig. 26B and Table 10).

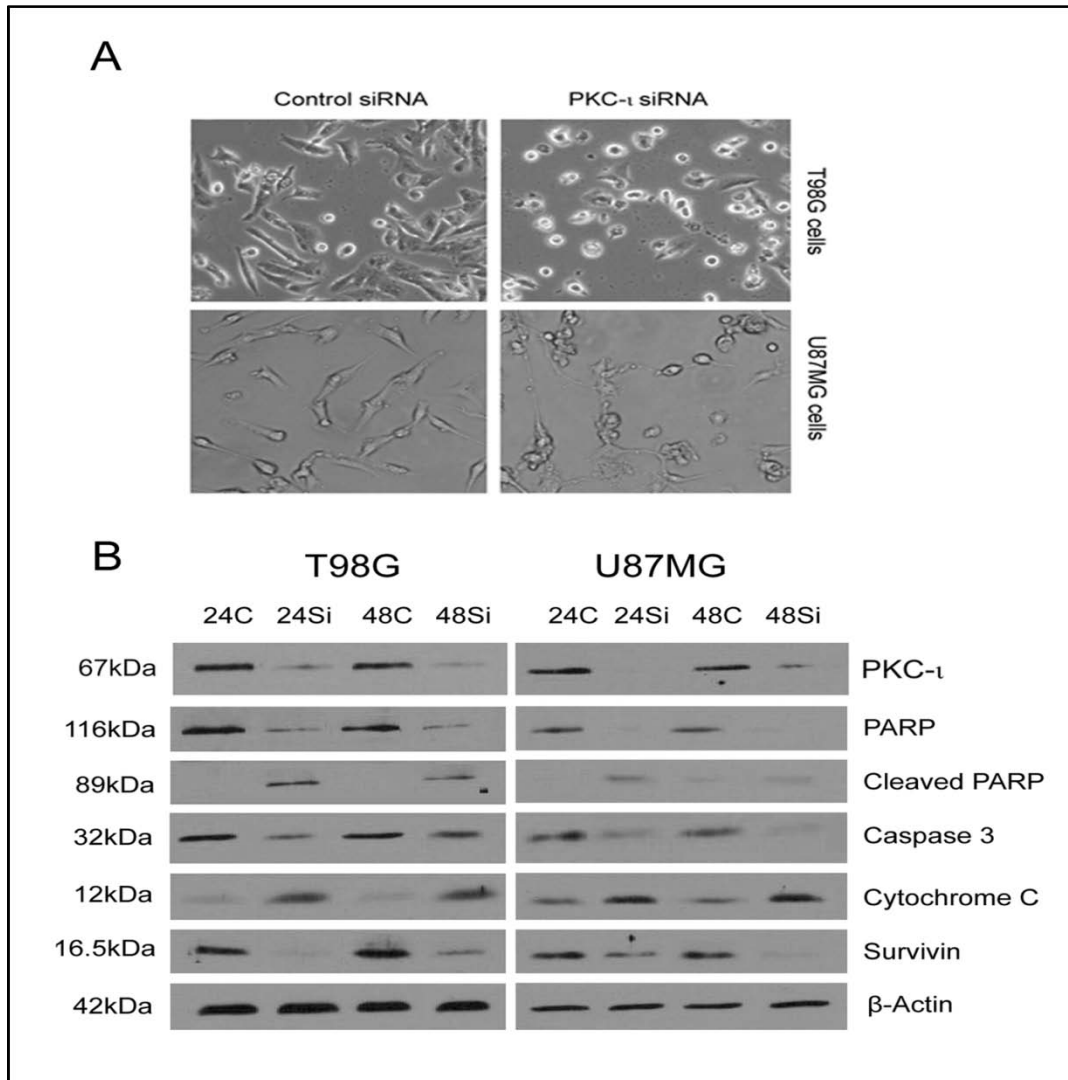


Figure 25 PKC- ι knockdown induces morphological and biochemical changes. (A) Brightfield pictures following PKC- ι siRNA and control siRNA treatment depicting morphological changes in T98G and U87MG cells **(B)** Control siRNA or PKC- ι siRNA (100nM for 24 h and 48 h) treated T98G and U87MG cells were harvested and total protein (100 μ g) was analyzed by Western blot to detect several biochemical changes such as PARP, PARP cleavage, Caspase 3, cytochrome C and Survivin. β -actin displays equal protein loading. Data represents $N=3$ independent experiments.

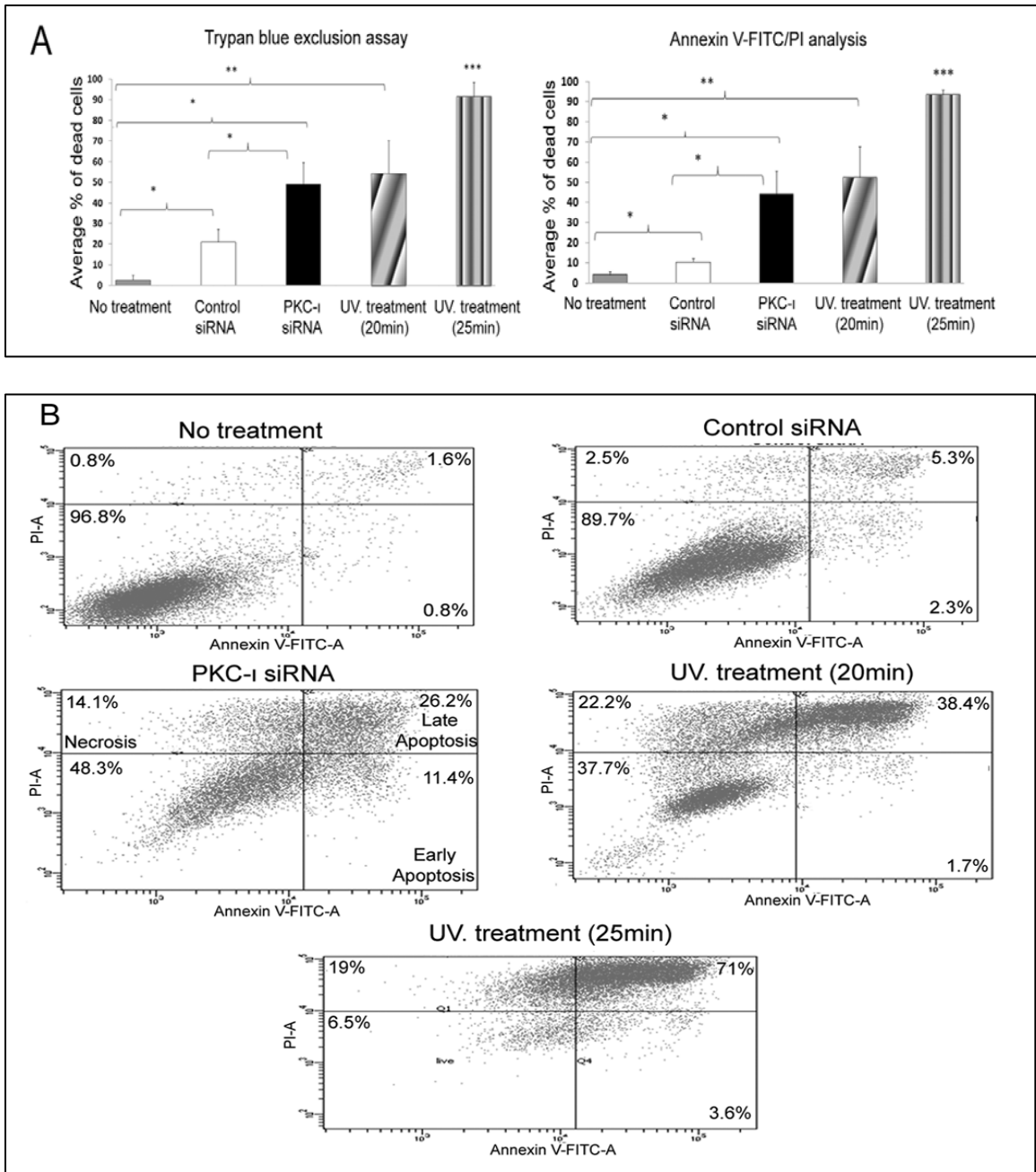


Figure 26 PKC-1 silencing induces apoptosis in T98G glioma cells (A) Cell death induced by PKC-1 inhibition was detected using the Trypan blue exclusion method (left panel) and the Annexin V-FITC/PI assay (right panel). U.V. treatment was used as positive control for apoptosis detection. (B) The distribution pattern on cell death by PKC-1 knockdown was compared with control siRNA treated and untreated cells. U.V. treatment was used as positive control for apoptosis detection. Representative Dot plots of the Annexin V/PI analysis for overall apoptotic rate, late apoptotic rate, early apoptotic rate and necrotic rate. Experiments were repeated four times with a total $N=8$ for all treatment groups. U.V. treatment groups had $N= 3-5$. The significance is explained in Table I for this data.

Table 10 Distribution pattern of cell death after PKC- ι silencing

Treatment Groups	Overall % Apoptotic Cells	% Late Apoptotic (Annexin V+PI+) Cells	% Early Apoptotic (Annexin V+ only) Cells	% Necrotic (PI+ only) Cells
	Average \pm S.D.	Average \pm S.D.	Average \pm S.D.	Average \pm S.D.
No Treatment	2.89 \pm 0.70	2.26 \pm 0.63	0.63 \pm 0.21	1.21 \pm 0.83
Control siRNA	7.43 \pm 1.06 *	5.01 \pm 0.95 *	2.41 \pm 0.39 *	2.89 \pm 0.77 *
PKC- ι siRNA	33.69 \pm 12.00 **	16.19 \pm 8.59 **	17.50 \pm 9.93 ****	10.46 \pm 4.26 **
UV treatment (20 min.)	33.82 \pm 12.08 **	30.54 \pm 14.05 ***	3.28 \pm 2.13 *	18.84 \pm 4.17 ***
UV treatment (25 min.)	75.77 \pm 3.02 ****	72.30 \pm 3.06 ****	3.47 \pm 0.12 **	17.90 \pm 1.15 ***

* $p < 0.05$, Student's t-test compared to untreated groups; ** $p < 0.05$, Student's t-test for all control groups; *** $p < 0.05$, Student's t-test for all treatment groups except for alternate U.V treatment group; **** $p < 0.05$, Student's t-test for all treatment groups.

5.3 Discussion

PKC- ι , an atypical PKC isoform is known to be hyperactivated and overexpressed in glioblastoma and our previous data has shown that PKC- ι is required for glioma cell proliferation and survival [118, 129]. However, the effects of PKC- ι inhibition in glioma cells have remained unclear.

In this study, data showed that PKC- ι expression was significantly downregulated following siRNA transfection in both T98G and U87MG cells. PKC-knockdown not only inhibited the endogenous expression of PKC- ι but also triggered alterations in the cells indicating apoptosis. Several morphological changes and biochemical changes confirmed the activation of intrinsic mode of apoptosis in both cell lines.

Additionally, PKC- ι silenced glioma cells as well as U.V. exposed glioma cells generated different distribution patterns of cell death. To our knowledge, these findings are the first evidence that PKC- ι inhibition generates early apoptosis and late apoptosis. However, a conventional method of apoptosis induction, U.V. irradiation, produced mostly late apoptosis. The CFSE/7-AAD analysis for the first time revealed the activity of the T98G cells that resisted cell death following PKC- ι silencing. These resistant T98G cells were viable but experienced hampered cell division which reflected G₂/M arrest.

A similar distribution pattern was also observed in the cell cycle analysis data wherein, a two-fold increase in G₂/M cell population suggested that the cells might be going from G₂/M arrest directly to apoptosis following siRNA silencing. Such effects were observed in cells grown in tissue culture (also demonstrated by *Baldwin et.al*) [149], contradicting the observations in other cancer models where such an effect was seen only in anchorage independent cells signifying a tissue specific role of PKC- ι [1, 150].

CHAPTER 6

BROAD CONCLUSION AND FUTURE DIRECTIONS

6.1 Conclusion

This research demonstrated that PKC- ι is hyper activated and over-expressed in glioma cells. PKC- ι expression is also significantly robust in transformed phenotypes of human glioma and benign and malignant meningioma. This study showed that PKC- ι is multifunctional and its expression is concordant with malignant growth rates in gliomas.

Our data showed that PKC- ι is an *in-vitro* kinase to Bad, a pro-apoptotic molecule of Bcl-2 family as well as Cdk7, a master cell cycle regulator. In glioma cells, PKC- ι directly associated and inactivated Bad function to promote uncontrolled cell survival via Bad/Bcl-XL dissociation. PKC- ι also triggered cell cycle progression and cell proliferation in glioma cells by regulating the activity of Cdk7. Results also indicated that PKC- ι is activated by PI (3)-kinase and PDK1 mediated signaling. PI (3)-kinase downregulation and PDK1 knockdown abrogated PKC- ι activity resulting in inhibition of PKC- ι downstream substrate function. Additionally, direct PKC- ι silencing inhibited Bad phosphorylation subsequently inducing apoptosis in glioma cells. This suggests that apoptosis occurs via Bad mediated mitochondrial pathway. Furthermore, PKC- ι downregulation inhibited Cdk7 activity and subsequently reduced cdk2 function. Silencing the PKC- ι /Cdk7/cdk2 pathway inhibited cell proliferation, induced G2/M cell cycle arrest as well as triggered intrinsic mode of apoptosis in glioma cells.

In conclusion, these results show that PKC- ι executes cell survival, cell cycle progression and cell proliferation mechanisms in glioblastoma. Collectively, this study suggests that PKC- ι is a potential predictive biomarker for the identification of glioblastoma patients that may benefit from anti-PKC- ι therapy for personalized medicine to improve the prognosis of glioblastoma patients.

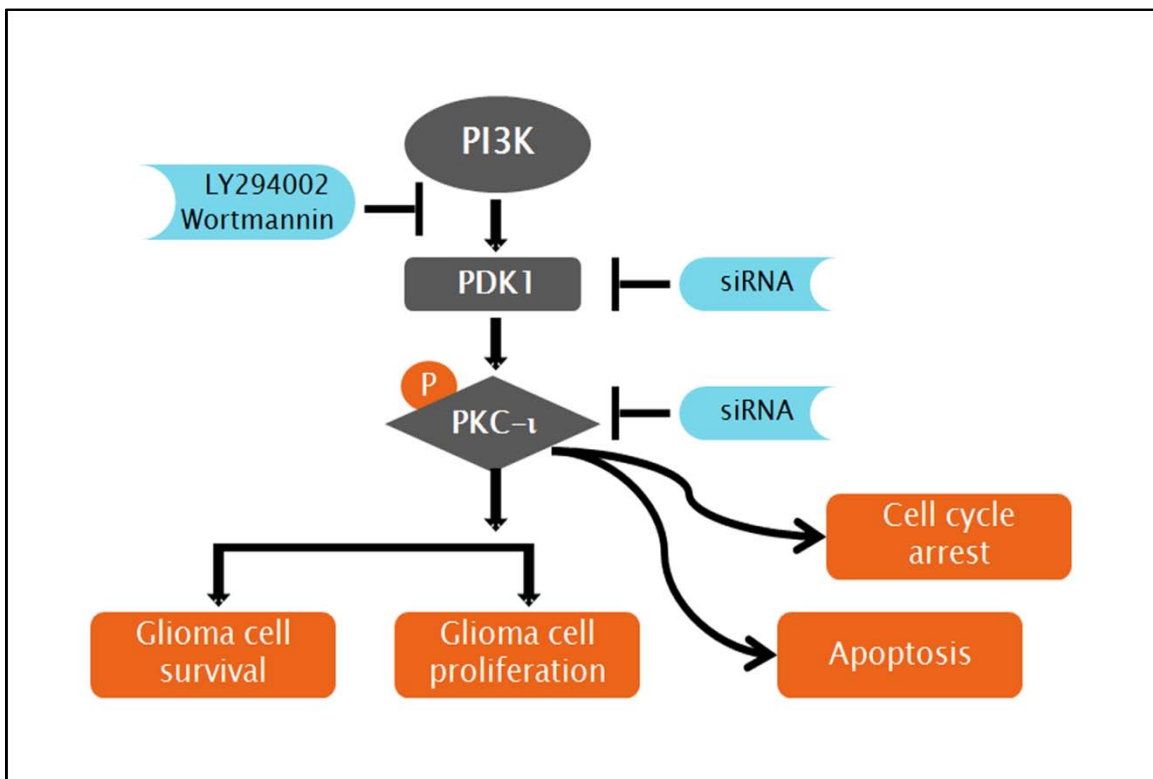


Figure 27. Graphical representation of PKC- ι signaling in glioblastoma

6.2 Future directions

This study has paved the way for future studies in glioblastoma as well as other cancer models.

1. Other Bcl-2 family proteins may also be involved in PKC- ι mediated survival mechanism. Studying these possible pathways may help identifying new therapeutic targets in glioblastoma.
2. Using novel chemotherapeutic agents to inhibit the PI (3)-kinase/PKC- ι /Bad cell survival cascade may be strong strategy to fight glioblastoma.
3. Verify the effect of PKC- ι overexpression of cell cycle progression and proliferation in glioblastoma.
4. Target PI (3)-kinase/PKC- ι /Cdk7 cdk2 pathway to prevent uncontrolled cell proliferation in glioblastoma.
5. Identify and investigate whether PKC- ι may be a potential biomarker in other cancer models

CHAPTER 7

MATERIALS AND METHODS

7.1 Materials.

Recombinant active PKC- ι (14-505), PKC- ζ (14-525), soluble Bad (14-357) and primary antibodies for PKC- ζ (07-264), myelin basic protein (MBP, 05-675), phospho MBP (05-429) and 14-3-3 (AB9748) were purchased from Millipore (Temecula, CA). PKC- ι primary antibody was purchased from BD Biosciences (San Jose, CA). Bad (sc-7869), PKC- ι (sc-727), phospho Bad Ser-112 (sc-7998), phospho Bad Ser-136 (, phospho Bad Ser-155 (sc-101641), histone H1 (sc-8030) and β -actin (sc-1616) primary antibodies were purchased from SantaCruz Biotechnology (SantaCruz, CA). Bad (9292), Bcl-XL (2764) were purchased from Cell Signaling. Cdk7 (sc-529), cdk2 (sc-748), pRb, p21 (sc-187), p27 (sc-527), histone H1 (sc-8030) and β -actin (sc-1616) primary antibodies were purchased from SantaCruz Biotechnology (SantaCruz, CA). pCdk7 primary antibody was purchased from Abcam. pcdk2 (2561), Caspase 9 (9502) and cleaved Caspase 9 (9501), Poly adipose ribose polymerase (PARP, 9542), Caspase-3 (9662), cytochrome C (4272) and survivin primary antibodies were purchased from Cell Signaling Technology (Danvers, MA). Horseradish peroxidase (HRP) conjugated bovine anti-goat IgG (sc-2350) and HRP Goat anti-rabbit IgG (sc-2004), as well as secondary antibodies were purchased from SantaCruz Biotechnology (SantaCruz, CA). Goat anti-mouse IgG, HRP conjugate (12-349) and normal rabbit IgG (12-370) were purchased from Millipore

(Temecule, CA). Wortmannin (W1628), LY294002 (L9908) and anti-rabbit IgG (whole molecule)-conjugated with agarose beads (1:1 v/v) (A8914) were purchased from Sigma Aldrich (St. Louis, MO). PKC- ι silencing RNA (siRNA) (sc-44320), PKC- ζ siRNA (sc-29451) and PDK1 siRNA (sc-36203) was purchased from SantaCruz Biotechnology (SantaCruz, CA). Nuclear and cytoplasmic kit (78833) was purchased from Pierce (Rockford, MO), Apodetect Annexin V-FITC kit (33-1200) and Vybrant CFDA SE Cell kit (V12883) were bought from Invitrogen (Grand Island, NY)

Other chemicals such as HEPES (BP310-500), tris-glycine 10X (BP1306), SDS (BP166-500), tris-base (BP152-500), glycerol (BP229), triton x100 (BP151-500), methanol (A407), hydrochloric acid (A144) were purchased from Fisher Scientific, Norcross, GA. Ethylene glycol bis(β -aminoethyl ether)- N,N,N',N' , -tetraacetic acid EGTA (E4378), sodium fluoride (S6521), sodium orthovanadate (S6508), PMSF (P7626), leupeptin (62070), aprotinin (A6279) were purchased from Sigma Aldrich, St. Louis MO. Protein assay dye (500-0006), EDTA (161-0729), tris-buffered saline 10X (170-6435) were purchased from Bio-Rad, Richmond, CA. Bovine serum albumin (BSA) (23209) and SuperSignal West Pico chemiluminescent substrate (34080) were purchased from Pierce, Rockford, IL.

7.2 Cell Culture.

All cell lines were cultured as a monolayer in 75cm² flasks containing Eagles Minimum Essential Medium (MEM), 10% fetal bovine serum (FBS) and antibiotics (penicillin 10 U/ml and streptomycin 10 μ g/ml) at 37°C in a humidified atmosphere containing 5% CO₂.

7.3 Cell fractionation and Western blotting.

Cells (1×10^6) were placed on ice to terminate the incubation. Cell extracts were prepared by washing twice with 10 ml of ice cold 1X Dulbecco's phosphate buffered saline (DPBS). Monolayers were scraped at 4°C , resuspended and sonicated in homogenization buffer (50 mM HEPES, p.H. 7.5), 150 mM NaCl, 0.5% triton-X100, 1mM EDTA, and 2mM EGTA, 0.1 mM sodium orthovanadate, 1mM NaF, 2mM PMSF, 1mM DTT, 0.15 U/ml aprotinin. Cell suspensions were centrifuged at 16000g for 30 min to obtain cell extracts. Protein content was measured according to Bradford using bovine serum albumin (BSA) as standard. [151]. Protein samples were separated by 12% on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (1.5mm thick gel) [152] using Protean II xi 16cm gel loading system (BioRad) and electrophoresed at 160volts and subsequently transblotted [153] by electroblotting with transfer buffer 100 ml of 10 x tris glycine (0.25 M tris and 1.92 M glycine) and 20% methanol in 1 L of distilled water and electroblotted for one hour at 24 volts. For Western blot analysis, each blot was blocked for 1 hour with 5% (w/v) fat-free dry milk in tris-buffered saline with 0.05% tween-20 (TBST) solutions at room temperature. Protein bands were probed with primary antibody in 5% milk in blocking buffer at 4°C overnight. The primary antibodies dilutions were 1:1000-1:5000. Membranes were subsequently washed three times for 15 minutes with TBST. Secondary antibodies such as horseradish-peroxidase-conjugate anti-mouse, anti-rabbit or anti-goat were diluted 1:1000 in 5% milk TBST. The membranes were incubated with secondary antibody (1:5000) at room temperature

for 1 hour. Immunoreactive bands were visualized with SuperSignal West Pico Chemiluminescent substrate.

7.4 Cell-cycle time course and immunoprecipitation.

Cells were cultured as monolayers in 150 mm tissue culture plates until 50-60% confluent, followed by serum starvation for 48h. Subsequently, starvation media was removed and complete media was added to each plate to allow cells to complete the cell cycle. T98G were harvested every 2h and U87MG were harvested every 3h by placing the plates containing cells on ice and washing twice with ice cold 1x DPBS. Cells were subsequently scraped and re-suspended in homogenization buffer. Protein content was measured according to Bradford using BSA as standard. Cell lysates (1 mg) were pre-cleared for 30 min at 4°C with anti-rabbit IgG-agarose beads (1:1 v/v, 10µl) and incubated with 5µg of either anti-PKC- ι , anti-Bad rabbit polyclonal antibody or both, overnight at 4°C and additionally with anti-rabbit IgG-agarose beads (1:1 v/v, 50 µl). The protein associations were subsequently determined by Western blot analysis.

7.5 Brain Tissue.

Human autopsy-derived brain tissue and meningiomas were obtained from the Cooperative Human Tissue Network (Southern Division) at the University of Alabama (Birmingham, AL, USA). Tissue specimens were obtained from both males and females of varying ages (23 - 80 years of age). Normal brain tissue included specimens from the frontal lobe, brain cortex, cerebellum, hippocampus, pons, corpus collosum and basal ganglia. Tumors labeled 'benign' were meningiomas. Malignant tumors were either

meningiomas or gliomas. The brain tissues were stored in -80°C freezer. Tissue samples were lysed by a sonicator and processed by a lysis buffer and the lysates (50µg) were subjected to gel electrophoresis and Western blotting was performed using antibodies against PKC- ι and β -actin.

7.6 Cell-cycle analysis.

T98G and U87MG cells were cultured as monolayers in 150 mm tissue culture plates until 50-60% confluent, followed by serum starvation for 48h. Subsequently, complete media was added to each plate allowing the cells to complete the cell cycle. Cells were harvested every 3h and fixed in ice-cold 70% ethanol at 4°C overnight. Fixed cells were washed with 1x DPBS and re-suspended in PBTB (PBS, 0.2% triton X-100, 1% bovine serum albumin) with final concentration of 1×10^6 cells/ ml. Subsequently, 50µl RNase (1µg/µl) was added and the nuclei were stained with PI (1µg/µl). The distributions of nuclei were quantified using FAC STAR plus flow cytometry (Becton Dickinson, San Jose, CA) and ModFitLT Cell cycle analysis program, version 2.0 (Verity Software House, Topsham, ME).

7.7 Immunofluorescence.

Approximately 1×10^6 cells were plated and grown in 2-well culture slides (Collagen type I coated). Twenty-four hours post-plating, cells were washed with cold 1x DPBS and fixed with 1:1 concentration of methanol and acetone for 5 min at -20°C. Cells were incubated with 8% normal goat serum blocking buffer for 45 min followed by incubation with anti-mouse antibody against PKC- ι for 90 min and incubated with anti-

mouse secondary antibody for 30 min followed by staining with fluorescein isothiocyanate (FITC) dye diluted in its blocking buffer (Vector Laboratories) for 30 min. Cells were washed 3 times with 1x DPBS followed by blocking with 8% normal horse serum for 45 min. Subsequently, cells were incubated with primary anti-rabbit antibody against Bad for 90 min and then incubated with anti-rabbit secondary antibody for 30 min followed by staining with Texas red diluted in its blocking buffer. Cells were washed 3 times with 1x DPBS and cell nuclei were visualized with mounting medium containing DAPI (4', 6-diamidino-2-phenylindole) which stains blue. Cells were observed with a Nikon Eclipse TE2000-U microscope. Pictures were captured in NIS-Elements F Version 2.10. To illustrate subcellular regions of protein co-localization, individual red and green-stained images derived from the same field were merged in Image-Pro Express Version 5.1.

7.8 Subcellular fractionation.

Cells were washed twice with cold PBS and resuspended in hypotonic Buffer A [50 mM HEPES, pH 7.4), 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM PMSF, 2.5 µg/m leupeptin, 0.15 U/ml aprotinin. The lysates were centrifuged at 4000 g for 30 min at 4°C and the supernatant (cytoplasmic proteins) was collected. The pelleted nuclei were extracted with Buffer A containing 400 mM KCl and 0.5% triton X-100. After centrifugation at 14000 g for 30 min at 4°C, the supernatant containing nuclear extracts was collected. The expression of PKC-ι, Bad, Cdk7 and cdk2, histone H1 and β-actin were determined by Western blot analysis.

7.9 PKC activity assay.

PKC activity assay was performed by suspending recombinant active PKC- ι , recombinant active PKC- ζ (0.5 μ g each) individually in 200 μ l of PKC kinase buffer [154] to test the ability of each PKC to phosphorylate recombinant as well as endogenously immunoprecipitated Bad and recombinant MBP. The PKC kinase buffer consisted of 20mM Tris- HCl (pH 7.5), 6mM magnesium acetate, phosphatidylserine (5 μ g) and adenosine triphosphate ATP (0.96 μ g). The reaction was terminated after incubation for 30 min at 30°C by addition of sample loading buffer and by placing the samples on ice. Proteins were subsequently separated by SDS-PAGE and analyzed by Western blot analysis using PKC- ι , PKC- ζ , Bad, pBad at Ser-112, Ser-136 and Ser-155, MBP, pMBP at T-98 as well as Cdk7 and phospho-Cdk7 (T170).

7.10 Inhibition of gene expression.

PKC- ι siRNA was a pool of three combined RNA sequences for targeting PKC- ι .

Their mRNA sequences were

663: 5'-CAAGCCAAGCGUUUCAACA-3'

5'-UGUUGAAACGCUUGGCUUG-3'

729: 5'-GGAACGAUUGGGUUGUCAU-3'

5'-AUGACAACCCAAUCGUUCC-3'

2137: 5'-CCCAAUAUCUUCUCUUGUA-3'

5'-UACAAGAGAAGAUUUGGG-3'

and control siRNA contained a scrambled sequence which does not lead to specific degradation of any known cellular mRNA and whose sequence is proprietary information of Santa Cruz Biotechnology.

Approximately, 1×10^6 cells were grown as monolayers in 100mm tissue culture plates. Twenty-four hour post-plating, cells were transfected with either PKC- ι or control siRNA (100nM) using lipofectamine medium. Cells were harvested after 24 h treatment and Bad was IP (1mg) followed by kinase activity assay and Western blotted to investigate to amount of Bad phosphorylation and Bcl-XL expression in both T98G and U87MG cells. Another set of IP Bad was incubated with purified, active PKC- ι in *in-vitro* kinase assay to examine the level of Bad phosphorylation.

siRNA transfected cells were harvested every 24 h and cell viability was quantified using trypan blue exclusion assay.

siRNA transfected cells were harvested after 24 h treatment and Cdk7 was IP (1mg) followed by kinase activity assay and Western blotting to investigate to amount of Cdk7 and cdk2 phosphorylation in both T98G and U87MG cells.

Another set of cells were harvested 24h post-treatment and Western blot analysis with total protein (30 μ g) was performed to determine the expression of PKC- ι . Total protein (30-100 μ g) was also fractionated to detect Caspase 9, cleaved Caspase 9 and probed for several cdk2 substrates such as pRb, p21, p27.

Approximately, 4×10^3 T98G cells were grown in 96 well tissue culture plates. Twenty-four hour post-plating, cells were transfected with increasing concentration with

either PKC- ι or control siRNA (25nM-125nM) for 48h. Cell proliferation was assessed using MTS assay.

Another set of cells were harvested 24h post-treatment and Western blotting with total protein (30 μ g) was performed to determine the expression of PKC- ι and PKC- ζ . Total protein (30-100 μ g) was also separated by Western blot analysis to investigate the expression of several apoptotic markers such as anti-whole PARP, anti-PARP cleavage, anti-Caspase 3, anti-cytochrome C and anti-survivin antibodies.

7.11. (3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (MTS) assay.

Approximately, 4×10^3 T98G cells were grown in 96 well tissue culture plates. Twenty-four hours post-plating, cells were transfected with either PKC- ι or control siRNA (25nM-125nM) for 24-48 h. At the specific time points, cells were incubated for 3 h with MTS reagent according to manufactures instructions (Promega). Cell viability was quantified by measuring the absorbance at 490nm using a microtiter plate reader.

7.12 Trypan blue exclusion assay.

Approximately, 4×10^3 T98G cells were grown in 96 well tissue culture plates. Twenty-four hours post-plating, cells were transfected with either PKC- ι or control siRNA (25nM-125nM) for 24-48 h. At the indicated time, cells were trypsinized, pelleted, washed with 1ml of DPBS, resuspended in 0.4% Trypan Blue Solution, and counted using a hemacytometer. Live (dye excluded) cells were counted. The results

from three separate independent experiments were used to determine the mean viability and standard deviation for each time point.

7.13 Annexin V-FITC/PI apoptosis detection.

The cells were stained according to the manufacturer's instructions with Annexin V-FITC at room temperature for 10 minutes (Invitrogen). Following a wash with binding buffer, the cells were co-stained with propidium iodide and immediately analyzed via Canto II (BD Biosciences) and FACsDIVA software. The number of events collected for each analysis was 20000.

7.14 CFDA SE staining dilution assay.

One day prior to siRNA transfection experiments, T98G cells were resuspended in 1 μ M of CFDA SE solution which is commonly referred to as CFSE (Molecular Probes/Invitrogen) and incubated for 15 min at 37° C. Following an incubation in complete medium for 30 mins, the CFSE labeled cells were seeded into 6 well plates at 2×10^5 cells per well. T98G cells were harvested at 48h post-siRNA or mock siRNA transfections and stained with 7-AAD (BD Biosciences) for examination by two-color flow cytometry of cell division and exclusion of dead cells with CFSE and 7-AAD, respectively. The live gate was established by excluding the 7-AAD stained cells. The number of events collected for each analysis was 20000 via Canto II and FACsDIVA software.

7.15 U.V. Irradiation.

T98G cells were washed and resuspended in 1x PBS and seeded onto non-treated petri dishes for exposure to U.V. light from the U.V. transilluminator box for specified exposure times. Preliminary experiments were conducted to establish the exposure times that were required for cell death. After irradiation and harvesting, the cells were examined with Trypan blue with light microscopy and stained with Annexin V-FITC/PI for further analysis.

7.16 Densitometry.

The Intensity of each band was measured using the Quantity One, 1-D analysis software (BioRad Laboratories). In order to achieve the correct intensity of each band, the background intensity was subtracted from the intensity of each band. Mean absorbance of three independent studies were compared by means of Student's t-test. $p < 0.05$ was determined to be statistically significant.

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