Université d'Ottawa • University of Ottawa

Université d'Ottawa · University of Ottawa

FACULTÉ DES ÉTUDES SUPÉRIEURES ET POSTDOCTORALES

FACULTY OF GRADUATE AND POSTDOCTORAL STUDIES

Michelle GARRATT-LALONDE

AUTEUR DE LA THÈSE - AUTHOR OF THESIS

M. Sc. (Microbiology and Immunology)

GRADE - DEGREE

Department of Biochemistry, Microbiology and Immunology

FACULTÉ, ÉCOLE, DÉPARTEMENT - FACULTY, SCHOOL, DEPARTMENT

TITRE DE LA THÈSE - TITLE OF THE THESIS

The Role of Atypical PKC iota in Glioblastoma Multiforme

I. Lorimer

DIRECTEUR DE LA THÈSE - THESIS SUPERVISOR

CO-DIRECTEUR DE LA THÈSE - THESIS CO-SUPERVISOR

EXAMINATEURS DE LA THÈSE - THESIS EXAMINERS

P. Liston

J. Ngsee

J.-M. De Koninck, Ph.D.

LE DOYEN DE LA FACULTÉ DES ÉTUDES SUPÉRIEURES ET POSTDOCTORALES

SIGNATURE

DEAN OF THE FACULTY OF GRADUATE AND POSTDOCTORAL STUDIES

The role of atypical PKC iota in glioblastoma multiforme

Michelle Garratt-Lalonde

Thesis submitted to the Department of Biochemistry, Microbiology and Immunology in partial fulfillment of the requirements for the degree of Master's of Science

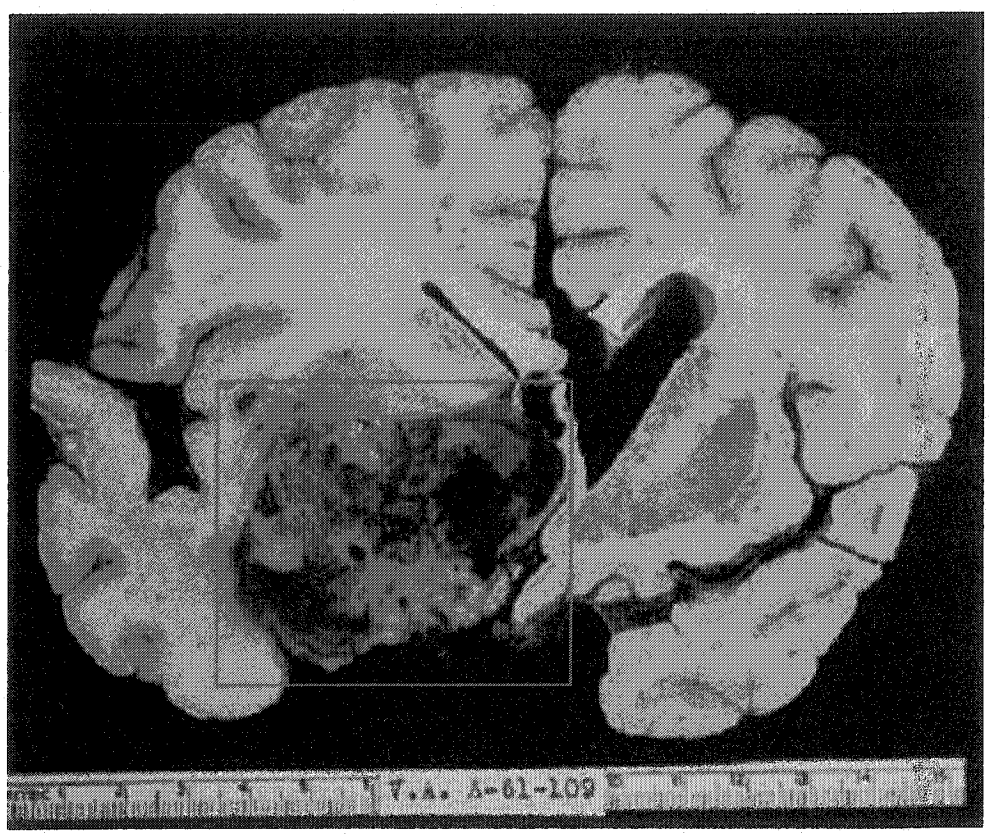
University of Ottawa

Ottawa, Ontario, Canada

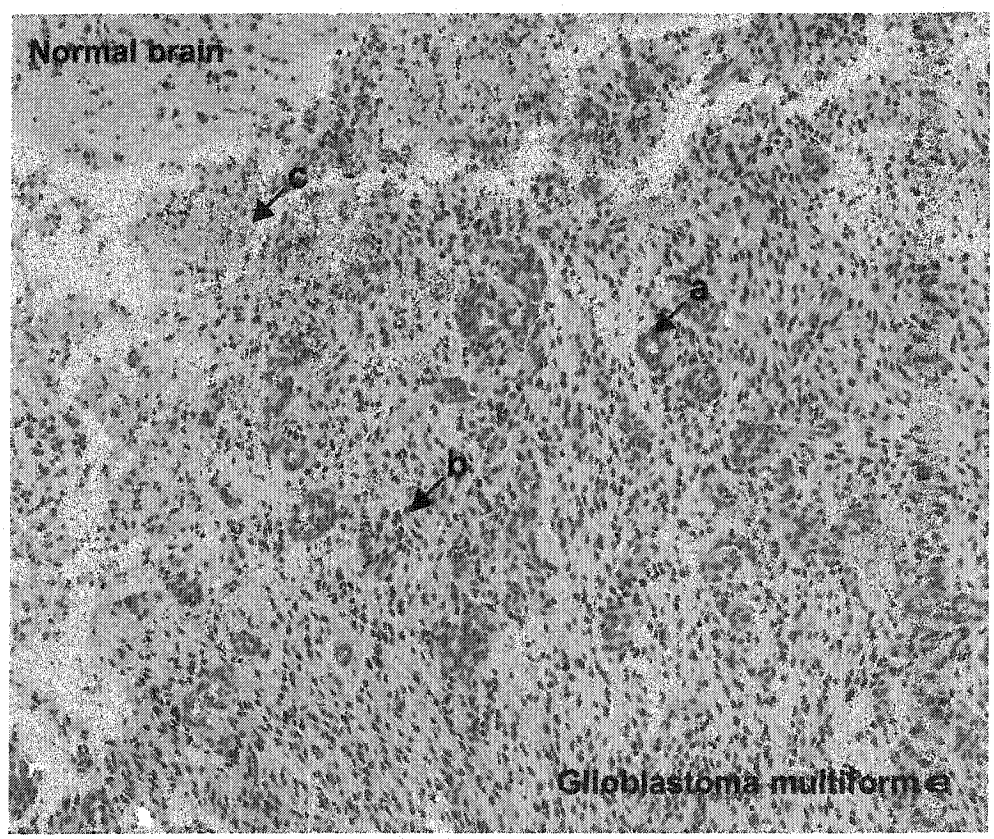
Your file Votre référence
ISBN: 0-612-90073-8
Our file Notre référence
ISBN: 0-612-90073-8

Figure 1.1

A



B



Thesis Abstract

Glioblastoma multiforme, a high grade malignant astrocytoma, is the most common and mortal intracranial tumor in adults. The median survival time for glioblastoma patients remains from 9-12 months despite aggressive treatment programs. These high-grade central nervous system tumors bear genetic and molecular aberrations that result in innate chemoresistance to commonly used chemotherapeutic agents.

The main focus of this thesis is on exploring signaling events downstream of phosphoinositide 3-kinase (PI3-K) in glioblastoma multiforme in attempts to discover molecular targets that may be highly specific and thus less toxic therapeutic targets. The PI3-K pathway is constitutively activated in glioblastoma. The Protein Kinase C family of serine/threonine kinases is activated downstream of PI3-K. Our focus is placed on a member of the atypical protein kinase C subfamily called, atypical PKC iota. Our lab has previously shown that inhibition of atypical PKC, with a myristolated pseudosubstrate inhibitor, induced rapid apoptosis in U87MG glioblastoma cells. RT-PCR work also indicated that atypical PKC iota to be the only atypical PKC isoform present in U87MG, which became our model cell line.

Using RNA interference, a highly specific post-translational gene silencing mechanism, we found that atypical PKC iota plays a role in U87MG cells. RNAi of atypical PKC iota in U87MG glioblastoma cells resulted in a decrease in proliferation with an increase of the cellular population in G0/G1. Atypical PKC iota RNAi concomitantly caused an increase in the expression and nuclear localization of the cdk inhibitor, $p27^{Kip1}$.

This is the first report of atypical PKC iota negatively mediating $p27^{Kip1}$ activity by altering its cellular localization. Atypical PKC iota RNAi also chemosensitized U87MG to the G1 acting drug cisplatin.

The Akt/PI3-K and ERK signaling pathways are constitutively activated in glioblastoma allowing for increased proliferation and resistance to apoptosis. Through western blot analysis of U87MG cells subject to atypical PKC iota RNAi, we showed that atypical PKC iota RNAi caused a transient decrease in the levels of phosphorylated Erk. However there was no modulation in the expression of total Erk. Akt is thought to increase proliferation in part by negatively regulating $p27^{Kip1}$ activity by causing its cytoplasmic sequestration. Interestingly, total Akt and phospho-Akt levels were also unchanged in these samples. These data further illustrate atypical PKC iota's role in U87MG proliferation.

30%-50% of glioblastomas exhibit oncogenic amplification and/or mutations of the epidermal growth factor receptor (EGFR) gene. The majority of EGFR gene amplification is also accompanied by rearrangements, the most common of which is the genomic deletion of exon 2-7 of the 26 exon gene, resulting in a mutated receptor with a truncated extracellular domain, designated as EGFRvIII. EGFRvIII has been shown to have a critical role in tumorigenicity, proliferation, cell cycle progression, growth and resistance to apoptosis. Expression of this mutant receptor however, is lost in tissue culture. Cell lines therefore must be stably transduced with EGFRvIII. In our studies, the presence of EGFRvIII in U87MG cell lines prevented both the decrease in proliferation and the

transient decrease in Erk phosphorylation due to atypical PKC iota RNAi. U87MGEGFRvIII cells were also resistant to cisplatin-induced toxicity.

We finally began examining the role of atypical PKC iota and invasion in U87MG glioblastoma cells using scratch/wound assays. We found no major difference in the migratory capacities between U87MG control cells and those with atypical PKC iota RNAi.

Our results indicate that atypical PKC iota plays a role in U87MG proliferation, apoptosis, and chemoresistance. Our data therefore support atypical PKC iota as a possible ideal therapeutic target in glioblastoma. Further studies are required to determine whether its inhibition is an effective means of sensitizing glioblastoma multiforme to chemotherapy.

Acknowledgements

I would like to take this opportunity to thank Dr. Ian Lorimer for his constant support and encouragement. Ian, I am truly in awe of your expertise and grateful for your mentorship and open demeanor. I would like to express my gratitude to the Lorimer girls (Ian's angels) who have made my graduate experience unforgettable. To Sylvie Brett, Manijeh Desmandesh, Doris Palorin, and Jennifer Hanson, be assured that your technical expertise and positive outlook on life have not gone unnoticed. I am privileged to have been a part of such a supportive team both on a professional and personal level.

I would also like to thank my parents for their constant love and support without whom I would not be where I am today. Lastly, and most importantly, I would like to thank my wonderful loving husband, Paul. Paul, I love you dearly, you are my rock. Thank you for supporting my dreams, no matter how they may change!

Table of Contents

List of tables

List of figures

Chapter 1:

General Introduction

1.1 Glioblastoma multiforme

Glioblastoma multiforme, a high grade malignant astrocytoma, is the most common and mortal intracranial tumor in adults, and is designated as a Grade IV astrocytoma by the World Health Organization (WHO) (Kleihues and Sobin 2000). On a worldwide scale, the incidence of glioblastoma multiforme is relatively similar. In Canada, approximately 2400 primary central nervous systems tumors are estimated to occur this year alone and approximately 1600 of these will result in death. 50-60% of these cases are glioblastoma multiforme; these are among the most aggressive and invasive of all cancers (Kleihues et al. 2002).

Despite advances in treatment for patients with glioblastoma, the survival rate is invariably poor. Upon diagnosis the median survival time is only 9-12 month and fewer than 5% of these patients will be alive 5 years after diagnosis (Markert 2003). Aggressive treatment programs exist consisting of a combination of neurosurgical resection and radiotherapy usually followed by adjuvant chemotherapy; however the majority of clinical responses to even the most current forms of treatment are only palliative. Unfortunately, the use of chemotherapy in the combined treatment programs only has a modest effect in prolonging survival, increasing the mean survival time to 51 weeks (Botturi and Fariselli 1998; Vives and Piepmeier 1999). The low response of tumors to most chemotherapeutic agents is partly due to the low penetrance of these drugs into the central nervous system. In addition, high-grade central nervous system tumors bear genetic and molecular aberrations that result in innate chemoresistance to commonly used chemotherapeutic agents (Nagane et al. 1999).

The etiology of glioblastoma is unknown. There are no known diagnostic markers that

can be used for early detection of glioblastoma multiforme. However, a number of genetic and molecular aberrations have been characterized in human gliomas that underlie the invasive, pathogenic and chemoresistant nature of glioblastoma. Multiple genes and proteins implicated in critical biological events such as signal transduction, cell growth, cell cycle control, proliferation, apoptosis and differentiation are activated during gliomagenesis (Benjamin et al. 2003). A goal of current research is to target these molecular aberrations to develop novel therapeutics that will have increased efficacy with decreased toxicity in glioblastoma patients.

As a gross specimen, glioblastoma multiforme tumors are of irregular shape, consisting of areas of solid tumor, hemorraghing, and necrosis (Gasparetto et al. 2003) **(Figure 1.1A)**. Histologically, glioblastoma multiforme is an anaplastic, highly cellular tumor with poorly differentiated, round, or pleomorphic cells. These tumors are characterized by high levels of microvascular proliferation. Glioblastoma multiforme cells are often multinucleated and characterized by nuclear atypia (Nagashima et al. 1999; Gasparetto et al. 2003) **(Figure 1.1B)**.

1.2 Genetic, molecular, and clinical background of glioblastoma multiforme

A number of signature genetic and molecular alterations have been characterized in glioblastoma that distinguish it from normal glial cells. The two fundamental cellular processes involved are signal transduction and cell cycle arrest control. Mutations affecting these two cellular processes cooperate to give rise to glioblastoma multiforme. Receptor tyrosine kinases like epidermal growth factor receptor (EGFR) (Strommer, Hamou et al. 1990; Wong, Ruppert et al. 1992) and platelet derived growth factor receptor (PDGFR) are among the signaling pathways most frequently modified (Guha et al. 1995; Di Rocco et al. 1998). Mutations

Figure 1.1 Glioblastoma multiforme

A. Gross specimen of glioblastoma multiforme tumor (white) displays the characteristic bleeding (black) and associated necrosis (brown). **B.** Histological representation of glioblastoma multiforme displaying the vascularization with the prominence of endothelial cells (a), hypernucleated and nuclear atypic tumour cells (b), and the red blood cells caused by the hypervascularization (c).

affecting cell cycle control involve the INK4A/CDK4/RB pathway and the ARF/MDM2/P53 pathway (Ivanchuk et al. 2001). Such genetic and molecular abnormalities result in abnormal activation of signal transduction pathways and disruption of cell cycle arrest pathways.

Receptor tyrosine kinases are often amplified or constitutively activated by mutations in glioblastoma multiforme. As a result of gene amplification and rearrangements, receptor tyrosine kinases can become constitutively active. Consequentially, multiple signal transduction pathways promoting cell survival, proliferation, growth and differentiation are constitutively stimulated (Benjamin et al. 2003). The phosphatidylinositol-3 kinase (PI3-K) pathway, RAS/mitogen-activated protein kinase (MAPK) pathway, protein kinase C pathway, JAK/STAT pathway, and the JNK pathway are among the most commonly stimulated pathways promoting tumor growth, survival, proliferation and invasion. For example, the epidermal growth factor receptor (EGFR) has been linked to oncogenicity in glioblastoma. EGFR is a typical receptor tyrosine kinase that is important in signal transduction cascades mediating growth, proliferation, survival, and differentiation. As a means to keeping signaling under control and preventing aberrant cell growth and survival, once activated through dimerization by ligand binding, EGFR signal downregulation occurs by receptor endosomal internalization. However, 30%-50% of glioblastomas exhibit oncogenic amplification and/or mutations of the EGFR gene (Strommer et al. 1990; Wong et al. 1992). Amplification of the EGFR gene can result in up to 100 copies per cell correlating to increased survival, invasion, and chemotherapeutic resistance (Okada et al. 2003). The majority of EGFR gene amplification is also accompanied by rearrangements, the most common of which is the genomic deletion of exon 2-7 of the 26 exon gene resulting in a mutated receptor with a truncated extracellular domain. This mutant receptor has been designated EGFRvIII. EGFRvIII has been found in approximately 50% of glioblastoma and

contributes to their pathogenicity. This tumor specific receptor is ligand independent with weak but constitutive unattenuated kinase activity remaining at the cell surface for up to 8 hours before signal downregulation (Lorimer 2002). EGFRvIII has been shown to have a critical role in tumorigenicity, proliferation, cell cycle progression, growth and resistance to apoptosis (Nishikawa et al. 1995; Nagane et al. 1998). Introduction of EGFRvIII into cell lines, such as the U87MG glioblastoma cell line, only has a mild effect on their growth in tissue culture. However, EGFRvIII significantly enhances their growth as xenografts (Nishikawa et al. 1994). In addition, tumorigenicity of MCF-7 breast cancer cells in nude mice is significantly enhanced by EGFRvIII (Tang et al. 2000). In humans, the incidence of EGFRvIII in tumors is high. EGFRvIII is also tumor specific, being undetectable in normal tissue (Lorimer 2002). EGFRvIII has been detected in numerous tumor types including: breast (Luo et al. 2003), ovarian (Moscatello et al. 1997), non-small cell lung (Okamoto et al. 2003), prostate cancer (Olapade-Olaopa et al. 2000), as well as glioblastoma multiforme.

EGFRvIII preferentially uses different signal transduction pathways than the wild type EGFR to induce cellular responses (Lorimer and Lavictoire 2001). EGFRvIII constitutively activates a myriad of survival responses involved in cell growth and differentiation, two of which are the PI3-K and RAS/MAPK pathways (Lorimer and Lavictoire 2001). Deletion of the tumor suppressor PTEN (phosphatase and tensin homologue on chromosome ten) is also a common event in glioblastoma multiforme. PTEN is an inhibitor of the PI3-K pathway. EGFRvIII activity and/or loss of PTEN activity result in the constitutive activation of phosphoinositide-dependent kinase 1 (PDK1) and PKB/Akt to contribute to the malignant phenotype (Stambolic et al. 1998) (**Figure 1.2**).

Figure 1.2

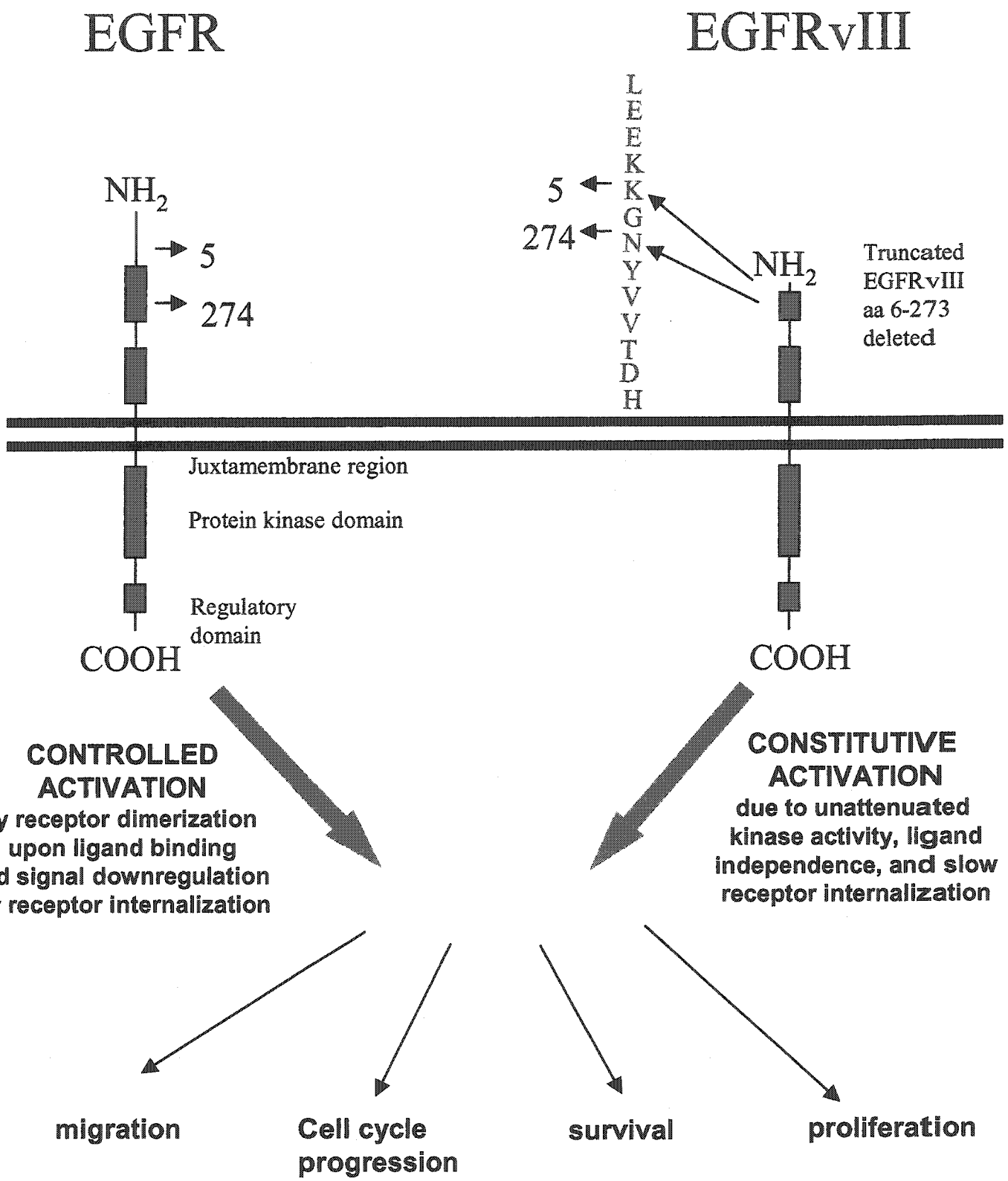

EGFR
EGFRvIII
NH2
5
274
L E E K K G N Y V V T D H
5
274
NH2
Truncated EGFRvIII aa 6-273 deleted
Juxtamembrane region
Protein kinase domain
Regulatory domain
COOH
COOH
CONTROLLED ACTIVATION
by receptor dimerization upon ligand binding and signal downregulation by receptor internalization
CONSTITUTIVE ACTIVATION
due to unattenuated kinase activity, ligand independence, and slow receptor internalization
migration
Cell cycle progression
survival
proliferation

Figure 1.2 Comparison of EGFR and EGFRvIII structure and activity

Disruption of cell cycle arrest is caused by the genetic and molecular alterations found in glioblastoma. The active cell cycle is divided into 4 phases: Gap1 (G1), synthesis (S), Gap2 (G2), and mitosis (M). G1 is when growth and preparation for DNA synthesis occurs. The S phase is when cells replicate their DNA. G2 is the preparatory stage for the cell division. Finally, M stands for mitosis and is when cell division occurs. Following division, daughter cells can immediately re-enter another round of growth and division or exit the active cell cycle from G1. Cells that exit the active cell cycle enter a state of quiescence termed the G0 phase of the cell cycle, where normal cellular functions are carried out. If required, a cell may leave G0 and re-enter the active cell cycle.

Normally, the complex system of events governing the cell cycle is highly regulated. Cell cycle progression is governed by cyclin dependent kinases (cdks) that are activated by cyclin binding and inhibited by cdk inhibitors. Cdks regulate biochemical pathways that integrate mitogenic and growth-inhibitory signals and coordinate cell-cycle transitions. Passage through G1 into S phase is regulated by cyclin D-, cyclin E-, and cyclin A-associated cdks. Transition from G2 into M is regulated by cyclin B-associated cdks. The cell cycle is negatively regulated by cdk inhibitors. Two families of cdk inhibitors can associate with CDK-cyclin complexes and inhibit their activities. The inhibitors of the cdk4 (INK4) family members that include p15INK4B, p16INK4A, p18 and p19ARF inhibit cyclin D association by directly binding cdk4 and cdk6. The kinase inhibitor protein (KIP) family include $p21^{CIP}$, $p27^{Kip1}$, and $p57^{Kip2}$, which bind and inhibit cyclin D,E,B, and A bound cdks (Slingerland and Pagano 2000) **(Figure 1.3)**.

Homozygous deletions of the p16/INK4A-ARF locus are common in glioblastoma. This mutation is found in greater than 50% of glioblastomas and in 80% of gliomas cell lines.

Figure 1.3

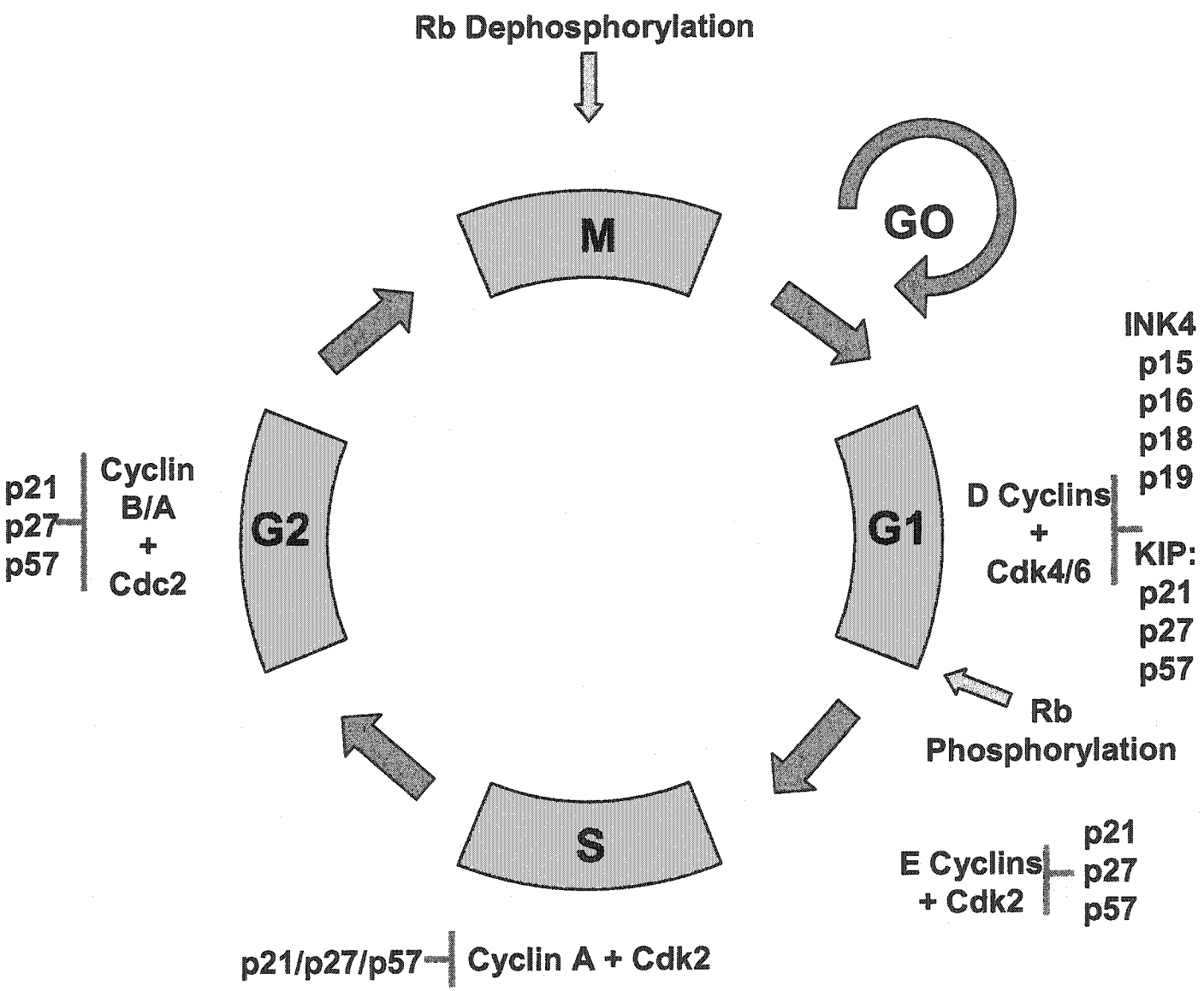

Rb Dephosphorylation
M
GO
INK4
p15
p16
p18
p19
D Cyclins
+
Cdk4/6
KIP:
p21
p27
p57
G1
Rb
Phosphorylation
E Cyclins
+ Cdk2
p21
p27
p57
S
p21/p27/p57
Cyclin A + Cdk2
G2
p21
p27
p57
Cyclin
B/A
+
Cdc2

Figure 1.3 The cell cycle

The cell cycle consists of transitions from G1 to S then G2 to M. The process is regulated by a family of cyclin-dependent kinases (cdks) whose activity is regulated by binding of the cyclins, by phosphorylation, and by negative regulators, the cdk inhibitors.

Alterations of p16/INK4A-ARF cause disruptions in the G1/S and G2/M checkpoints (Benjamin et al. 2003; Dai and Holland 2003). The INK4A-ARF locus on chromosome 9p21 encodes two separate proteins through differential splicing of alternative first exons to produce p16INK4a and p14ARF (Nishikawa et al. 1995; Newcomb et al. 2000). p16/INK4a is a tumor suppressor gene that encodes a specific inhibitor of cyclin D-dependent kinases, cdk4 and cdk-6, thereby preventing their association with cyclins. In turn, phosphorylation of the retinoblastoma tumor suppressor gene (pRB) is inhibited. This prevents the transition from G1 to S phase, causing G1 cell cycle arrest. The p14ARF gene product can complex with and cause nuclear sequestration of murine double minute 2 (mdm2), thereby preventing mdm2 from inhibiting p53 tumor suppressor activity by ubiquitination, nuclear export, and proteosomal degradation of p53 (Newcomb et al. 2000; Zheleva et al. 2003). Therefore, the high frequency of p16/INK4A-ARF loss and alterations in glioblastoma allows for uncontrolled proliferation. Restoration of p16/INK4a expression in p16 null glioblastoma cell lines induces growth suppression both *in vitro* and *in vivo* (Lee et al. 2000). In addition, deletions of the Rb gene, deletions or mutations of the p53 gene, and amplifications of CDK4, CDK6, Cyclin D1 and mdm2 can also disrupt cell cycle arrest and lead to uncontrolled cell cycle progression in glioblastoma multiforme. Among all the described mutations, the genetic loss of INK4A-ARF and p53 function by deletion or mutation are the most common mechanisms by which cell cycle arrest is prevented. Normal glial cell function requires these elements of signal transduction and cell cycle control; however tumorigenesis activates or inhibits these cellular responses to promote survival **(Figure 1.4)**.

Two distinct subsets of glioblastomas, primary and secondary, have been characterized based on clinical and genetic characterization of tumors (Lang et al. 1994; Ng and Lam 1998). Primary, or *de novo*, type tumors occur commonly in patients over 55 years of age. Primary

glioblastomas have no clinically evident precursor and harbour epidermal growth factor receptor (EGFR) gene amplification in 80% of the cases (Wong et al. 1992; Kleihues and Ohgaki 1999). EGFR amplification is also associated with genetic rearrangement, the most frequent of which produces the most common EGFR mutant, EGFRvIII, in 50% of the cases. Primary glioblastomas are further characterized by the overexpression of the mdm2 gene, loss of the INK4a/Arf locus, and mutations of PTEN and retinoblastoma (Rb) genes on chromosome 12, 9, 10, and 13, respectively. In *de novo* glioblastomas, cooperation between EGFRvIII expression and the homozygous deletion of the INK4A-ARF locus correlate with higher proliferation indexes (Jen et al. 1994; Schmidt et al. 1994; Ono et al. 1996; Hayashi et al. 1997). Alterations in PTEN occur in 30-40% of primary glioblastomas (Kraus et al. 2002).

The secondary type of glioblastoma develops from a low-grade astrocytoma. It is characterized by a sequential accumulation of various genetic aberrations that contribute to malignant progression. These tumors tend to have a p53 null background and overexpress PDGF ligands and receptors, two occurrences that play a pivotal role in the initial steps of the evolution (Kleihues and Ohgaki 1999). Tumor progression is then promoted by the loss of chromosomes 1, 9p, 13q, 19q, and then finally 10q (Wooten et al. 1999). The loss of chromosome 10q, where the PTEN gene is located, is the final genetic alteration required to transition from anaplastic astrocytoma to secondary glioblastoma (Tohma et al. 1998; Wooten et al. 1999). The evolution of another form of secondary glioblastoma results from the losses of chromosome 1p and 19q, by inactivation of PTEN and p16, and finally by EGFR gene amplification in pre-existing oligodendroglioma (Behin et al. 2003) **(Figure 1.4)**.

Although histologically similar, the two subtypes of glioblastoma multiforme may respond quite differently to therapeutic agents due to their specific molecular differences. These molecular characteristics may provide avenues for therapeutic intervention with highly specific novel treatments based on these defined molecular aberrations.

1.3 Glioblastoma multiforme mouse models

Genetically defined mouse models of glioblastoma multiforme have given useful insights into gliomagenesis. Introducing the genetic alterations found in human gliomas in mouse models produces tumors that are histiologically similar to human gliomas (Holland 2000). Animal model studies provide valuable information on the probable causes of glioma formation and on novel potential targets for therapy. These mice can also potentially be used to evaluate molecularly targeted therapeutics.

There are two general approaches used to develop glioma mouse models, based on germline-modification strategies and somatic cell gene-transfer. These techniques are used in transgenic mice and knockout mice. Transgenic mice express a gene of interest in all cells using a cell-type specific promoter to drive expression (Aguzzi et al. 1995). Knockout mice lose expression of a targeted gene in all cells that normally express it (Macleod and Jacks 1999). In many mouse tumour germline-modification models, the tissue with altered genetic expression is generally developmentally normal. The tumours that develop require additional unknown secondary genetic events. This method is informative

Figure 1.4

	Signal transduction	Cell Cycle Arrest
Glial Differentiation	Pdgf, Egf	p53, Rb, Ink-4a-Arf, p21, p27
Human Glial Malignancy	PDGF, EGF, NF1 PTEN, AKT, RAS	P53, RB, INK4A-ARF P27, CDK4, MDM2 CYCLIN D1
Causal Implication in Mouse Models	PDGF, EGF, Nf1, Pten, Akt Ras	p53, Ink4a-Arf, Rb

Figure 1.4 Summary of known major genetic alterations in glioblastoma tumorigenesis

Representation of the genetic alterations affecting signal transduction and cell cycle arrest in glial differentiation, malignancy, and mouse models. This table has been adapted (Dai and Holland 2003).

regarding the induction and progression of tumorigenesis (Holland 2001). Somatic-cell gene transfer involves the delivery of mutant genes postnatally by retroviral infection. This method allows for the analysis of multiple mutations. However since the number of cells used for infection is relatively small, secondary events for tumor initiation are less likely to occur. However, if there is tumor formation, the initial alteration is likely the causative factor behind tumor formation (Fisher et al. 1999).

Mouse models also highlight the cooperativity between aberrations in cell cycle arrest and signal transduction pathways in the induction of gliomagenesis. Aberration to cell cycle arrest or signal transduction pathways alone cannot induce gliomas. Rather these mutations together enhance oncogenicity and work in a cooperative manner to induce tumorigenesis. For example, the constitutive activation of the many survival and proliferative pathways by EGFRvIII, like PI3-K and Ras, alone cannot induce gliomagenesis. Rather, for EGFRvIII to induce glioma formation, it must cooperate with the disruption of G1 cell-cycle arrest pathways induced by deletions in the INK4A-ARF locus and/or the oncogenic amplification of cyclin-dependent kinase-4 (cdk-4) (Holland et al. 1998; Uhrbom et al. 2002). Neither an Ink4A-Arf deficient background nor the gene transfer of constitutively active EGFR alone causes gliomagenesis in mice. However, when transgenic mice bearing an Ink4A-Arf null background are infected with retroviral vectors to transfer a constitutively active mutant EGFR gene, these two components combined cause glioma-like lesions (Holland et al. 1998).

Another example of cooperative effects in glioblastoma is with homozygous deletions of p53 and Nf1 (neurofibrotosis type 1). Nf1 encodes a RAS-guanosine triphosphatase activating protein, which keeps Ras in its inactive state. As in the case of EGFRvIII and the

deletion of the Ink4A-Arf locus, homozygous deletion of p53 or Nf1 on their own do not induce gliomagenesis, as seen in mice (Donehower et al. 1992; Bajenaru et al. 2002). However, the double deletion of p53 and Nf1 can produce glioblastomas in mice (Reilly et al. 2000).

Animal models are required to distinguish the genetic and molecular alterations that are responsible for tumor formation and progression. Such models will allow for the identification of critital targets for therapy and the ability to test them *in vivo*.

1.4 Molecular targets developed into therapies

Since conventional treatments for glioblastoma multiforme only modestly increase mean survival, it is important to look for novel, more effective modes of treatment. Targeting glioblastoma's molecular aberrations offers the possibility of novel therapeutic modalities that could act as single agents or could make current therapies more effective. Advances into the understanding of the molecular characteristics of glioblastoma have already led to the development of novel and potentially more effective, specific, and less toxic treatment approaches such as immune and gene therapies.

Tumor suppressor genes like p53 (Ikeda et al. 2001) or Rb (Fueyo et al. 1998) can be restored by gene therapy and results in a marked reduction in cell proliferation by inducing a G(1)/S cell cycle block *in vivo*. In the case of p53, increasing wild-type p53 levels by adenoviral-mediated gene transfer caused apoptosis *in vitro* (Ikeda et al. 2001). Although not useful clinically, the restoration of Rb activity rendered the human glioma cells unable to form tumors in nude mice (Fueyo et al. 1998).

An example of progress in the development of molecular cancer therapeutics is the development of the novel antitumor drug ZD1839, commercially known as Iressa. Iressa is an orally active, selective EGFR tyrosine kinase inhibitor that prevents signal transduction pathways promoting cancer cell growth, survival, and proliferation and is currently in clinical trials (Villano et al. 2003). Aberrant EGFR has also been a tumor specific target for the delivery of antisense DNA sequences that can abolish its over-stimulation or constitutive activation (Lorimer 2002). In addition, vaccines derived from the fusion junction in EGFRvIII have shown potential in animal models as seen by the inhibition of tumor formation, the regression of existing EGFRvIII expressing tumors, and an antibody response (Moscatello et al. 1997). Another therapeutic approach is EGFRvIII-specific antibodies capable of recognizing a secondary structure exclusive to this mutant receptor (Lorimer 2002). These antibodies can be used to target toxins, radioisotopes, and viral vector gene therapy in EGFRvIII expressing cancer cells. These techniques have been examined both in tissue and animal models with modest promise (Lorimer 2002).

Other possible treatments are based on anti-angiogenic approaches (Zachary 2003) and inhibition of matrix metalloproteases that are being examined in tissue culture and animal models (Bello et al. 2001). Glioblastoma multiforme is one of the most highly vascularized tumors, and therefore may be particularly sensitive to anti-angiogenic approaches. For example, the development of vascular endothelial growth factor (VEGF)-specific or vascular-specific peptides are now being examined as therapeutic molecules targeted against angiogenesis (Zachary 2003). Glioblastomas are also highly invasive thus inhibition of matrix metalloproteases is being examined in animal models. Though such

research has shown that inhibition of MMPs efficiently decreases glioma invasion, angiogenesis, and tumor growth in xenografts (Bello et al. 2001; Lakka et al. 2003), MMP inhibition has not yet been clinically successful.

1.5 PI3-K pathway

One of the challenges in the area of glioblastoma research is developing effective treatments with low toxicity. Both cancerous and non-cancerous cells utilize common cellular and molecular mechanisms so as with most chemotherapeutic and radiation treatments, toxicity is a major concern. The potential side effects of targeting molecular abnormalities in glioblastoma must therefore be carefully examined. Many potential target genes and molecules play significant roles in normal cellular events. Nonetheless, targeting such genes involved in tumor initiation, progression and invasion offers the most promise for glioblastoma patients.

Some of the alterations that occur in glioblastoma, like EGFRvIII and the loss of the PTEN tumor suppressor, lead to the constitutive activation of the PI3-K pathway, which provides a target for therapy (Choe et al. 2003). The PI3-K pathway lies downstream of many receptor tyrosine kinases and plays a role in membrane trafficking, cytoskeletal organization, cell growth and survival **(Figure 1.5)**.

The PI3-K family consists of eight members divided into three classes. These classes are based in sequence homology and substrate preference (Fruman et al. 1998). The

Figure 1.5

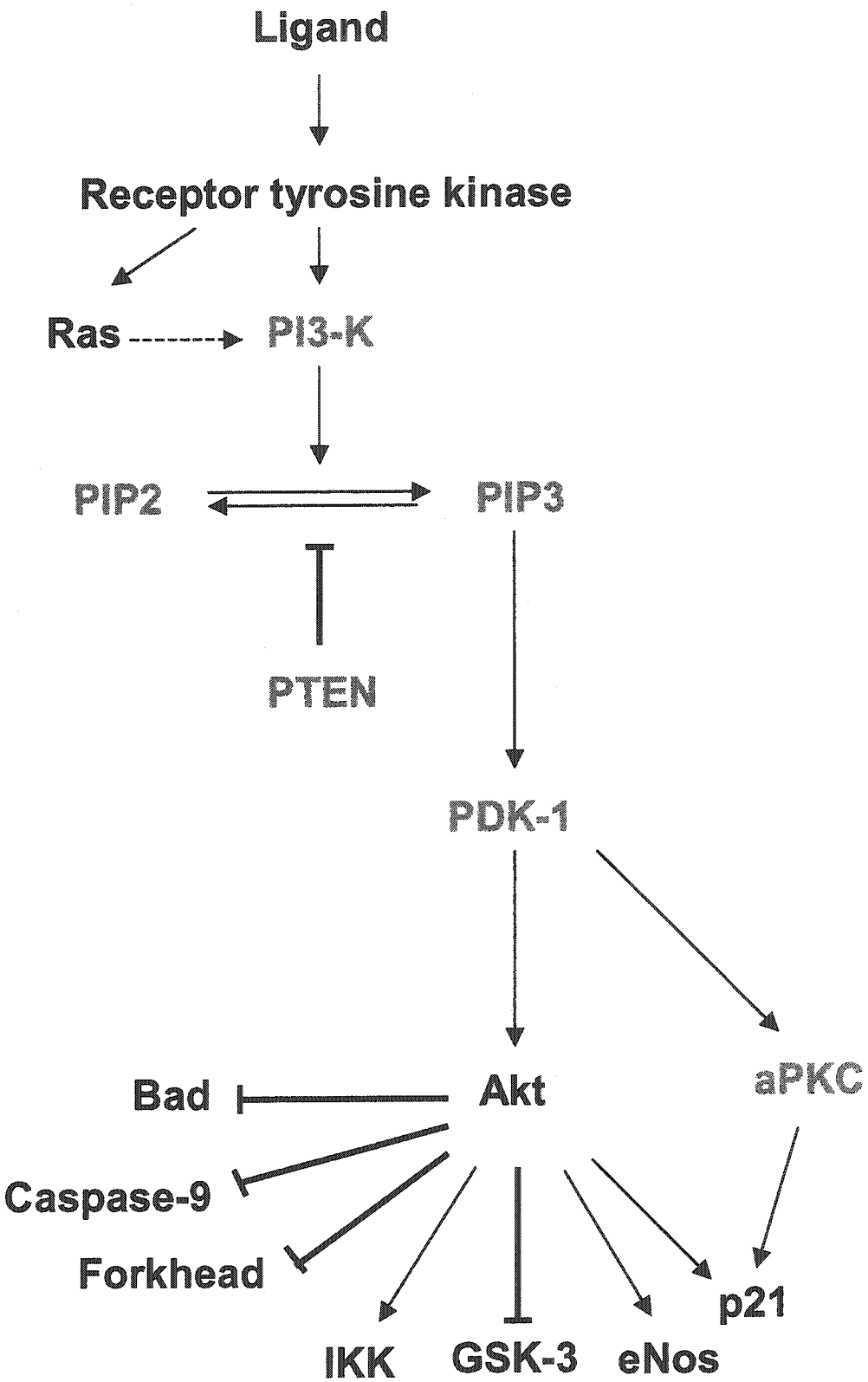

Ligand
Receptor tyrosine kinase
Ras
PI3-K
PIP2
PIP3
PTEN
PDK-1
Bad
Akt
aPKC
Caspase-9
Forkhead
p21
IKK
GSK-3
eNos

Figure 1.5 PI3-K pathway

Divergent signaling may pose a window of opportunity to achieve a highly specific, less toxic therapeutic target.

class I PI3-K family consists of four members that are subdivided based on their mechanism of activation. Class Ia consists of catalytic subunits p110 α, β, and γ that individually associate with a p85 regulatory subunit to form a heterodimeric complex. There are eight isoforms of p85 encoded by three genes, each containing two SH2 (Src homology) domains that interact with phosphotyrosine on activated receptor tyrosine kinases or with adaptor molecules. This results in recruitment of the PI3-K to the plasma membrane and activation of its enzymatic activity. In addition, activated GTP- bound Ras can activate class Ia kinases by direct association with the catalytic subunit (Downward 1998). Class Ib PI3-Ks consist of only a p110 γ catalytic subunit that is activated by the βγ subunits of the heterotrimeric G proteins. G proteins are released by transmembrane receptor activation (Paez and Sellers 2003).

The class II PI3-Ks are comprised of three members, PI3-KC2 α, β, and γ. These members lack a regulatory subunit. A carboxyl-terminal phospholipid-binding domain characterizes class II PI3-Ks. These enzymes are mainly membrane bound and are activated by receptor tyrosine kinases and integrins. Finally, the class III kinase VPS34p produces the majority of the cellular PtdIns-3-P and is involved in lysosomal protein trafficking (Paez and Sellers 2003).

In general the basic method of receptor tyrosine kinase activation of the PI3-K signaling cascade is simple (**Figure 1.5**). When ligand binding activates receptor tyrosine kinases, autophosphorylation on their tyrosine residues occurs. This leads to the recruitment of PI3-K to the plasma membrane by the SH2 domains of the p85 regulatory subunits. PI3-K is also activated by the interaction of its SH2 domains with phosphotyrosine on the receptor

tyrosine kinases. PI3K then phosphorylates phosphoinositol lipids on the D3 position of their inositol ring generating phosphatidyl-3-phosphates. PI3-K thereby transduces signals by catalyzing the conversion of phosphatidyl-3,4 bisphosphate (PIP2) to generate a secondary messenger phosphatidyl-3,4,5 trisphosphate (PIP3). These lipids recruit pleckstrin homology (PH) domain-containing proteins such as phosphoinosotide-dependent kinase 1 (PDK1) to the plasma membrane. Once PDK-1 is activated by binding to the plasma membrane, it can activate downstream proteins, like the serine-threonine kinase PKB/Akt, by phosphorylation. Akt and PDK-1 phosphorylate multiple target proteins thereby carrying out their role as critical regulators of cellular functions (Narita et al. 2002; Paez and Sellers 2003).

PTEN (phosphatase and tensin homolog deleted on chromosome 10) is a tumor suppressor gene localized to chromosome 10q23 (Myers et al. 1997). PTEN is a protein and lipid phosphatase. The lipid phosphatase activity allows PTEN to dephosphorylate the D3 position of the lipid products PIP2 and PIP3 produced from PI3-K activity (Maehama and Dixon 1998). PTEN therefore antagonizes PI3-K signaling. PTEN's C-terminal tail contains a PDZ protein-protein interaction module (Adey et al. 2000). Phosphorylation of Serine 380, Threonine 382 and Threonine 383 within this domain maintains PTEN in a closed conformation and functionally inhibited (Adey et al. 2000). Only in an unphosphorylated form can PTEN interacts with other PDZ containing proteins and thereby localize to the plasma membrane where it can exert its phospholipid phosphatase activity. Targeted disruption of PTEN in mice leads to embryonic lethality (Di Cristofano et al. 1998). $PTEN^{+/-}$ mice are cancer prone and develop breast, prostate, gastrointestinal, and thyroid cancers (Di Cristofano et al. 1998).

PDK-1 stands at a critical point in the PI3-K signal transduction cascade. Activated by plasma membrane localization (Paez and Sellers 2003), PDK-1 phosphorylates and activates several kinase substrates namely PKB/Akt, p70S6-kinase, and members of the classical, atypical and protein kinase C related (PKR) protein kinase C (PKC) family (Toker and Newton 2000). Two mechanisms exist by which PDK-1 activates its substrates, directly or indirectly. For PKB/Akt and the atypical PKCs, phosphorylation of their activation loop leads to catalytic activity (Dutil et al. 1998). PDK-1 indirectly activates the classical PKCs as phosphorylation of their activation loop only primes them for subsequent catalytic activation triggered by two C-terminal autophosphorylation events. Even then, the removal of the classical PKC autoinhibition and subsequent phosphorylation by a pseudosubstrate sequence must occur by binding to the plasma membrane and diacylglycerol (Dutil et al. 1998).

The serine-threonine protein kinase PKB/Akt mediates many downstream effects of the PI3-K signal transduction cascade. Three closely related PKB/Akt isoforms, Akt1 (PKBα), Akt2 (PKBβ) and Akt3 (PKBγ) exist encoded by three different genes. They are structurally similar, thought to be activated by a common mechanism, and likely have identical substrate specificities (Okano et al. 2000). The three isoforms are widely expressed though Akt3 expression is primarily expressed in brain and testis (Konishi et al. 1995). Activation of PKB/Akt involves both plasma membrane binding and phosphorylation. Upon PI3-K activation and production of PIP2 and PIP3, PKB/Akt is recruited to the plasma membrane via its PH domains where it binds to phosphoinositides (Franke et al. 1997). PKB/Akt activation then involves a conformational change and the phosphorylation of

threonine 308 in its activation loop by PDK-1(Biondi et al. 2001; Leslie et al. 2001). Phosphorylation of serine 473 by an unknown kinase is also required for PKB/Akt enzymatic activity. The phosphorylation of Ser 473 is thought to result from either PDK-1 activity (Balendran et al. 1999), integrin-linked kinase (Persad et al. 2001), or from Akt autophosphorylation (Paez and Sellers 2003).

Aberrations to the PI3-K pathway allow it to play a role in tumorigenesis and cell cycle progression (Narita et al. 2002). PI3-K signaling is one of the most commonly deregulated pathways in glioblastoma multiforme. This is evident by the inappropriate activation of PI3-K, PKB/Akt gene amplifications, PKB/Akt protein overexpression and loss of PTEN (Paez and Sellers 2003).

Much research has been focused on elucidating and targeting the upstream and downstream effectors of the PI3-K pathway for the development of possible novel therapeutic targets. However a problem associated with targeting PI3-K is toxicity. PI3-K inhibitors such as wortmannin and LY294002 disrupt the ATP binding pockets of PI3K and PI3-K like enzymes. Both have been widely studied in neurons and induce growth inhibition at concentrations that inhibit class Ia PI3-Ks (Paez and Sellers 2003). However, targetting PI3-K with these compounds at such concentrations induces neuronal apoptosis (Yao and Cooper 1995). The noted toxicity may be due to the broad tissue distribution of all the PI3-K isoforms regulating a multitude of normal cellular mechanisms (Paez and Sellers 2003). The class Ia p110 α catalytic subunit is mainly responsible for transmiting mitogenic signals. However, while p110α seems to be the most attractive target in cancer therapy, its germline deletion in mice results in embryonic lethality (Paez and Sellers 2003). Therefore, therapies

aimed at further downstream targets in the PI3-K pathway may prevent such side effects thereby providing more highly specific and less toxic clinically relevant therapeutics (Stambolic et al. 1999). For example, much research has been focused on the role of PKB/Akt in tumorigenesis. In this case, the viability and phenotypes of PKBα/Akt1 knockout mice have shown that reduced levels of PKBα/Akt1 activity *in vivo* can be well tolerated during development and in adult mice (Chen et al. 2001). However, Akt1/Akt2 double-knockout (DKO) mice exhibit severe growth deficiency and die shortly after birth (Peng et al. 2003). Given the fact that there are three PKB/Akt isoforms that are thought to be redundant in function, knock out models and inhibition may still pose a problem of undesirable toxicity and does not necessarily predict toxicity in adults.

PDK-1 is a key enzyme downstream of PI3-K that not only directly activates PKB/Akt but also activates other enzymes that have been implicated in gliomagenesis, such as the Protein Kinase C family of serine/threonine kinases (Vanhaesebroeck and Alessi 2000). PDK-1 is known to phosphorylate and activate atypical PKCs. In terms of glioblastoma, where the PI3-K pathway is constitutively activated in part by growth factor-mediated signaling, this divergent signaling may pose a window of opportunity to achieve a highly specific, less toxic therapeutic target (**Figure 1.5**).

1.6 Area of investigation

Glioblastoma responds poorly to standard chemotherapy. Research on the molecular basis for their resistance is of utmost importance. To date, research on the molecular

aberrations characterizing glioblastoma has provided invaluable information yet much still remains to be elucidated. Therefore, there is still a great need to continue research in this field in order to develop new therapies, based on currently known or new molecular aberrations aiming at more specific and clinically relevant therapeutic targets. We are interested in experimental therapeutic research involving novel molecular aberrations found in glioblastoma multiforme. Identifying and studying their physiological role in glioblastoma may allow for their exploitation as highly specific therapeutic targets. A better understanding of glioblastoma multiforme on the molecular level will lead to new and innovative insights into the cause and treatment of this horrible disease.

Our primary interests lie in exploiting PI3-K downstream signaling in glioblastoma in attempts to discover molecules that may pose as highly specific less toxic therapeutic targets. We have focused on a member of a subgroup of the protein kinase C (PKC) family called, atypical PKC iota. PKCs have been shown to be critically involved in cell proliferation, transformation, cancer cell survival and invasion, and modulation of the cell cycle (da Rocha et al. 2002). Atypical PKC iota itself has been shown to protect human leukemia cells against drug-induced apoptosis (Murray and Fields 1997). However, the specific role of atypical PKC iota in glioblastoma has never been examined. Chapter 2 therefore describes the role that atypical PKC iota plays in glioblastoma as a possible target for therapeutic intervention.

Chapter 2: Targetting atypical Protein Kinase C iota in Glioblastoma Multiforme

2.1 Introduction

2.1.1 Protein Kinase C family

The protein kinase C (PKC) family consists of a family of homologous serine/threonine kinases that are involved in a number of cellular functions such as proliferation, cytokine secretion, invasion, malignant transformation and multidrug resistance (Nishizuka 1988; Nishizuka 1989; Matsumoto et al. 1995; Brown 2000). PKCs are at the crossroads of several mitogenic signal transduction cascades. In terms of PKC involvement in PI3-K signaling, this is an area that is still poorly understood (Moscat and Diaz-Meco 2000).

The PKC family consists of 13 isoforms that are classified into four distinct subgroups based on their mode of activation. PKC signaling paradigms are conserved through evolution from yeast to humans, underscoring the importance of this family in cellular function. The classical PKCs (cPKCs) consists of α, βI, βII, and the γ isoforms which all require calcium and diacylglycerol (DAG) for activation. The novel PKCs (nPKCs), δ, ε, η and θ, only require DAG, not calcium, for their activation. The atypical PKCs (aPKCs) consists of the ζ and ι/λ (PKCλ is the mouse homologue of PKCι) isoforms and do not require calcium or DAG for their activation. Atypical PKCs are regulated by other lipid co-factors like phosphstidylinositol 3,4,5-P3 (PIP3) and ceramide (Nakanishi and Exton 1992) which are produced after cell activation by inflammatory cytokines and growth factors (Kolesnick and Kronke 1998). Finally the fourth and least characterized group is called the PKC-related kinases, consisting of PRKs 1, 2, and 3. This final group also does

not require calcium nor DAG for activation (Mellor and Parker 1998).

2.1.2 PKC structure and activity

The different activation requirements among the PKC subgroups are a result of their structural differences. Every PKC isoform is comprised of an NH_2-terminal regulatory and COOH-terminal catalytic domain with serine-threonine kinase activity. These domains are comprised of four conserved regions (C1 to C4) and five variable regions (V1 to V5). The C1 and C2 regions lie within the regulatory domain and the C3 and C4 regions lie within the catalytic domain **(Figure 2.1)**. Both cPKCs and nPKCs contain a C1 domain with two cysteine-rich zing fingers, aPKCs contain a C1 domain with only one zinc finger while the PRKs do not possess one at all. The two cysteine-rich zinc fingers in the conventional and novel PKCs are involved in direct binding with DAG and phorbol esters. The function of the sole zinc finger in the aPKCs is still unknown. Activation of all PKC family members involves translocation to the plasma membrane where they bind to an activated membrane receptor (Hurley et al. 1997; da Rocha et al. 2002).

cPKCs, nPKCs, and aPKCs also contain a pseudosubstrate domain that can interact with the catalytic region maintaining these enzymes in their inactive state (Hurley et al. 1997). The affinity of the pseudosubstrate for the catalytic region must decrease in order for conversion into the active state. For the classical and novel PKCs, this occurs in response to co-factor DAG binding to the C1 domain and calcium and membrane-bound phosphatidylserine binding to C2 regions of the enzymes (Ponting and Parker 1996). The mechanism of the decrease in affinity of the pseudosubstrate for the catalytic region in the

Figure 2.1

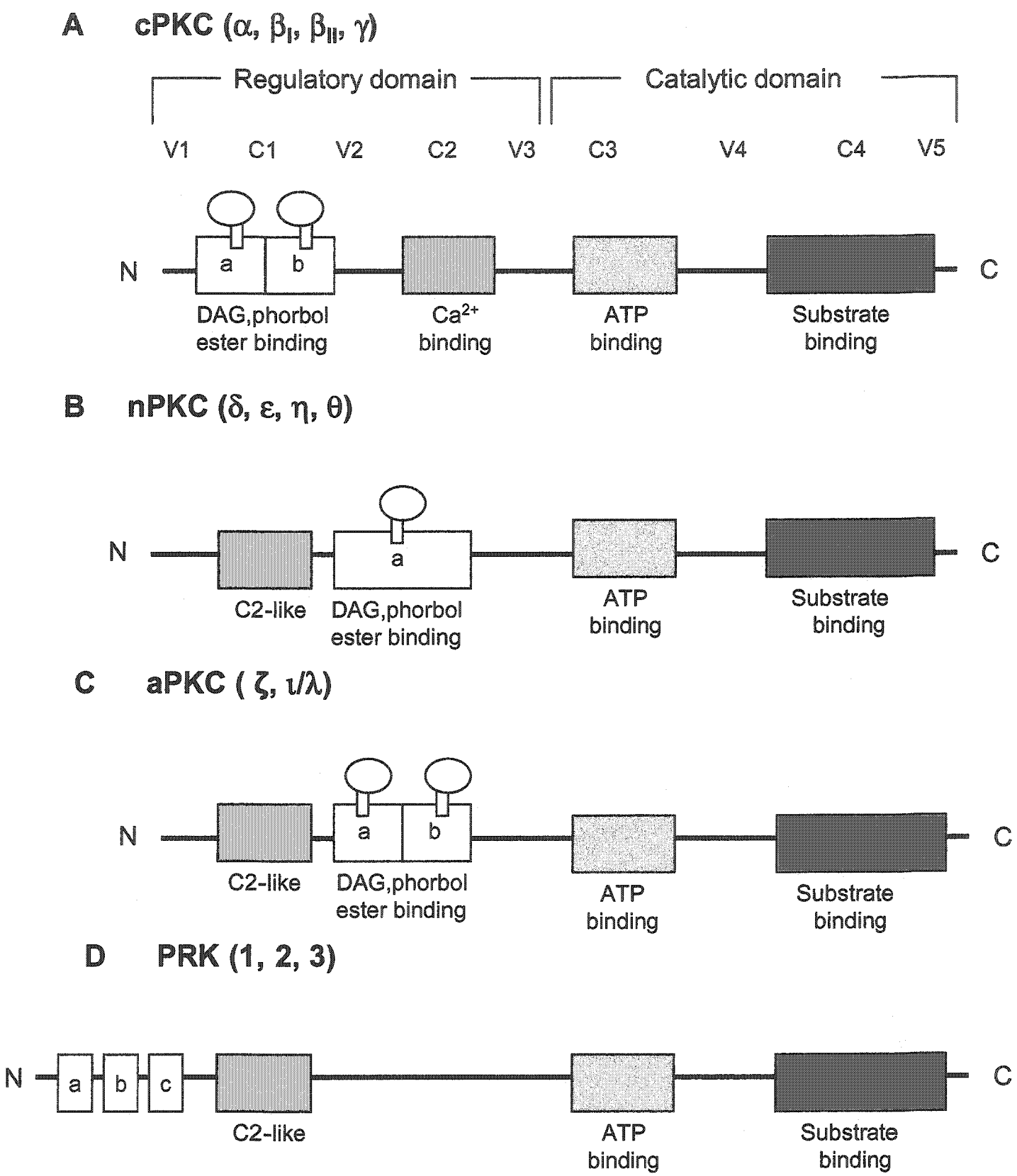

A cPKC (α, βI, βII, γ)
Regulatory domain
Catalytic domain
V1
C1
V2
C2
V3
C3
V4
C4
V5
N
a
b
C
DAG,phorbol ester binding
Ca2+ binding
ATP binding
Substrate binding
B nPKC (δ, ε, η, θ)
N
a
C
C2-like
DAG,phorbol ester binding
ATP binding
Substrate binding
C aPKC (ζ, ι/λ)
N
a
b
C
C2-like
DAG,phorbol ester binding
ATP binding
Substrate binding
D PRK (1, 2, 3)
N
a
b
c
C
C2-like
ATP binding
Substrate binding

Figure 2.1 **Comparison of the protein architecture of the 4 different subgroups of the PKC family**

aPKCs is still unknown (Moscat and Diaz-Meco 2000).

2.1.3 Atypical PKC ζ and ι/λ

Atypical PKCs are activated through a phosphatidylinositol 3-kinase (PI3-K)/ PDK-1 dependent mechanism **(Figure 2.2 D)**. In addition, atypical PKC activity and function are modulated by specific scaffolding and adaptor proteins that can bind to and modulate atypical PKC function (Moscat and Diaz-Meco 2000). A number of proteins contain an atypical PKC interaction-domain (AID) that allows for the direct interaction with atypical PKCs. These protein complexes possibly make aPKCs accessible to their substrates by inducing conformational changes (Moscat and Diaz-Meco 2000). However, the relationship between these various mechanisms for atypical PKC activation is unclear.

The VI region of aPKCs is a domain to which three different adaptor/effectors can bind: p62, Mek-5, and Par-6 (Moscat and Diaz-Meco 2000). Therefore specific interactions can allow aPKCs to participate in distinct signaling complexes where they can interact, directly or indirectly, with effectors. Such a quality allows specific atypical PKC isoforms to have unique functions depending on the context of stimulation.

The AID domain of the protein p62 binds to the V1 region of atypical PKCs. p62 also contains a cysteine-rich sequence that forms an atypical zinc finger (ZZ) (Sanchez et al. 1998). p62 provides a scaffold that links atypical PKCs to TNFalpha and IL-1 receptor signaling through its interactions with RIP and TRAF6, respectively. These interactions occur through a p62 zinc finger. Atypical PKCs activated in such a manner can directly interact with and activate IKKβ by phosphorylation (Lallena et al. 1999). Such activity leads

to IκB degradation and the release of NFκB from its inactive state in the cytosol, permitting its nuclear translocation (Karin 1999). Therefore p62 functions in cytokine signaling by linking aPKCs to the NFκB signaling pathway **(Figure 2.2 A and B)**.

The mitogenic cell growth regulator MEK-5, a member of the Map Kinase family and an upstream regulator of BMK1/ERK5, also contains an AID region (English et al. 1998). Atypical PKCs can interact with the AID site of MEK5 through their V1 region in an EGF dependent manner. The V1 domain of atypical PKCs also interact with partitioning-defective-6 (Par-6), a scaffolding protein controlling cell polarity (Watts et al. 1996). Par-6 itself interacts with CDC42, Rac, and Par-3. Par-3 can interact with the catalytic domain of aPKCs (Izumi et al. 1998). Par-6 responds to CDC42 signaling by linking the aPKCs with the actin cytoskeleton structure. This interaction between Par-6 and CDC42 implicates the role of atypical PKCs in both Ras and CDC42 induced cell transformation, since Ras is known to activate CDC42 (Qiu et al. 2000) **(Figure 2.2 C)**.

Atypical PKCs also have two zinc finger domain binding partners: LIP (lambda-interacting protein) (Diaz-Meco et al. 1996) and Par-4 (prostate androgen response-4) (Diaz-Meco et al. 1996). LIP is an activator of aPKC activity (Diaz-Meco et al. 1996) while Par-4 is an inhibitor (Diaz-Meco et al. 1996). Little is known about LIP's cellular role. Par-4 is a gene induced in prostate cancer cells undergoing apoptosis (Sells et al. 1994). Par-4 levels have also been shown to increase during neuronal cell death due to pro-apoptotic signals associated with the pathogenesis of Alzheimer disease (Guo et al. 1998). Overexpression of Par-4 induces apoptosis however in a manner that depends on its ability to inhibit aPKC enzymatic activity. Par-4 levels are also downregulated in Ras-transformed cells (Barradas

et al. 1999). Restoration of Par-4 to normal physiological levels in Ras-transformed cells makes them sensitive to apoptosis and chemotherapeutic agents (Barradas et al. 1999) **(Figure 2.2 C)**.

In brief, atypical PKCs are important regulators of the NF-κB signaling pathway. P62 is the adapter protein linking atypical PKCs to cytokine signaling. Unidentified scaffold proteins likely exist that place atypical PKCs at the crossroads of other cell growth and proliferative signaling pathways. Par-4 inhibits atypical PKC activity and is subject to down-regulation by oncogenic transformation and upregulation by pro-apoptotic signals suggesting that therapies aimed at modulating atypical PKC activity, their interaction with p62, or Par-4 levels could be the basis for novel therapies for cancer (Moscat et al. 2001).

2.1.4 Classical and novel PKCs in gliomagenesis

Some PKC isoforms likely play an essential role in the CNS, as suggested by the high levels of PKC expression and activity in neuronal tissue (Bredel and Pollack 1997). PKC α and δ have been shown to regulate astrocyte growth, differentiation of oligodendrocytes, neuron potentiation, axon outgrowth and neurotransmitter release (Yong et al. 1994; O'Driscoll et al. 1995).

Glioblastoma multiforme is characterized by high proliferation rates due to a number of molecular aberrations involved in signaling pathways for mitogenesis and apoptotic

Figure 2.2

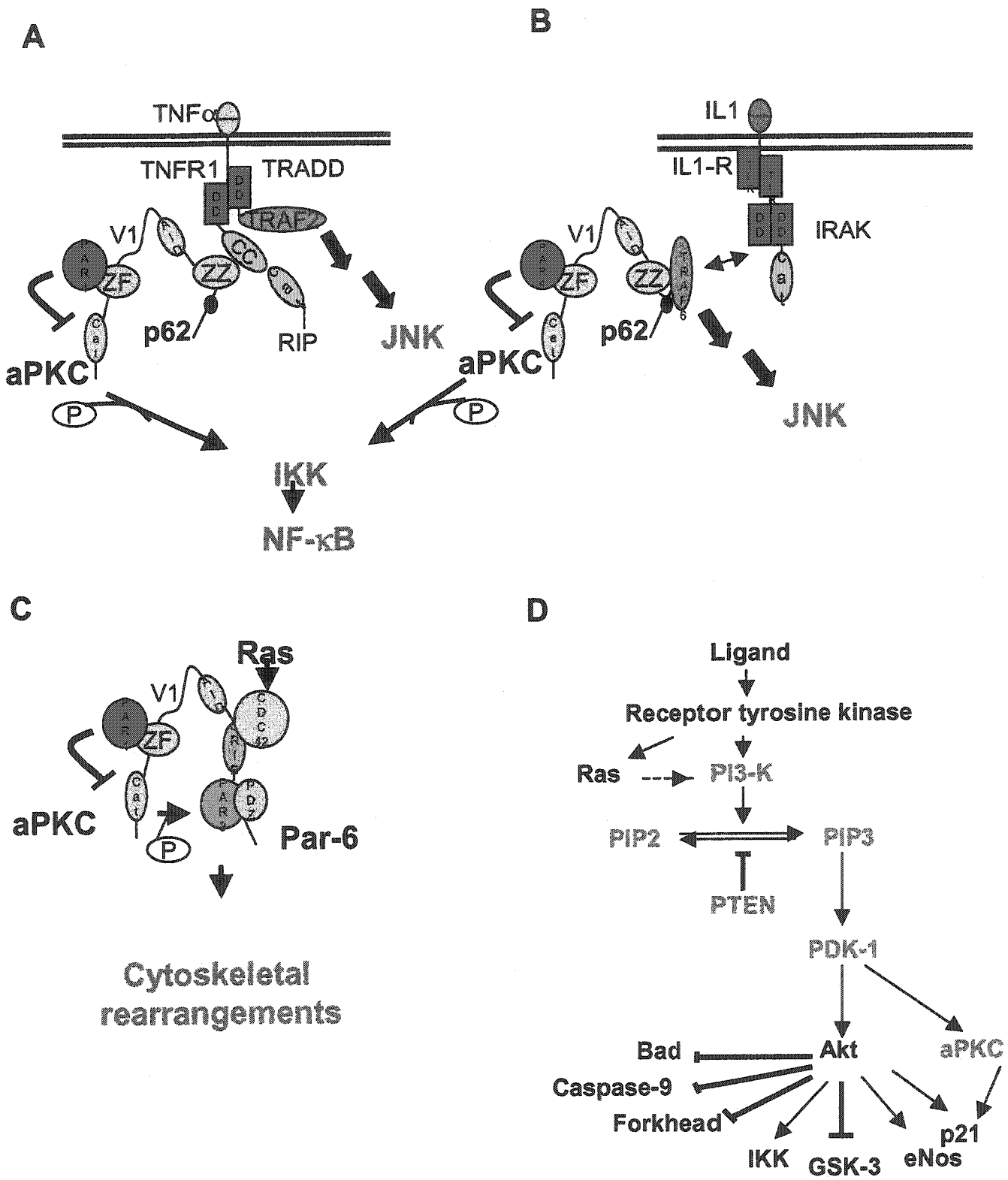

A
TNFα
TNFR1
TRADD
TRAF2
V1
ZF
ZZ
CC
p62
RIP
JNK
aPKC
P
IKK
NF-κB
B
IL1
IL1-R
IRAK
V1
ZF
ZZ
p62
aPKC
P
JNK
C
Ras
V1
ZF
aPKC
P
Par-6
Cytoskeletal rearrangements
D
Ligand
Receptor tyrosine kinase
Ras
PI3-K
PIP2
PIP3
PTEN
PDK-1
Bad
Akt
aPKC
Caspase-9
Forkhead
IKK
GSK-3
eNos
p21

Figure 2.2 Regulation of atypical PKC activity

A. In response to TNFα receptor stimulation, the death domain (DD) of the receptor binds to the adapter TRADD. TRADD recruits RIP. The coiled-coil (CC) domain of RIP interacts with atypical zinc finger of p62. The AID sequence of p62 interacts with the V1 domain of atypical PKC ι and . and recruits them to the signaling complex. The mechanism of activation of the atypical PKCs is unknown. Par-4, the inhibitory protein, can target the zinc finger (ZF) of the atypical PKCs thereby inducing apoptosis. **B.** Atypical PKCs are linked to IL-1 signaling by TRAF6 also interacting with p62. IRAK, subsequently recruited to the IL-1 signaling complex, interacts with the adapter MyD88 which binds to the IL-1 receptor through its TIR domain. IRAK then becomes hyperphosphorylated and interacts with TRAF6. IKK can be recruited to these complexes through RIP or the TRAF6 where the β subunit can be phosphorylated and activated by the atypical PKCs. **C.** Cdc42 or Rac can stimulate Par-6 through its AID site. Par-6 forms a complex with the atypical PKCs (V1 site) close to Par-3. Par-3 interacts with the PDZ domain of Par-6. Thus, Par-3 is phosphorylated by the atypical PKCs leading to cytoskeletal rearrangements. **D.** Atypical PKC PI3-Kinase activation mechanism is PDK-1 dependent as previously described in detail. (This figure has been adapted (Moscat and Diaz-Meco 2000))

regulation. PKC plays an important role in these pathways and aberrant classical and novel PKC activity, in particular PKC α and ε, have been directly shown to play a role in the development of glioblastomas (Baltuch and Yong 1996). Such PKC activity levels have been shown to be upregulated in malignant gliomas in vitro and in astroglial brain tumors (Couldwell et al. 1991; Baltuch et al. 1995; Bredel and Pollack 1997; Sharif and Sharif 1999). Studies have shown that classical and novel PKC overexpression in glioblastomas promotes increased proliferation and provides protection from apoptosis while its inhibition restricts tumor growth and increases the susceptibility to apoptosis under cytotoxic stimuli (Couldwell et al. 1994; Ikemoto et al. 1995). The function of specific PKC isoforms in glioma cell proliferation and invasion is an important area of research.

Differential expression of specific PKC isozymes has been reported in gliomas when compared to normal astrocytes (Xiao et al. 1994). Research has shown the classical and novel PKC isoforms, PKC α and ε, to play a particularly significant role in these areas. PKC α and ε activity has been shown to be increased in gliomas and glioma cell lines. In addition, PKC α and ε inhibitors drastically reduce glioma cell proliferation (Couldwell et al. 1992; Hussaini et al. 2000).

PKC α has been shown to be essential in glial cell proliferation whereby a decrease in its expression and activity using a specific antisense oligonucleotide caused a significant decrease in cell cycle progression and proliferation and an increase in the cdk inhibitor $p21^{WAF1/CIP1}$ (Besson and Yong 2000). In addition, the degree of malignancy in C6 rat glioma cells correlated with the degree of PKC α overexpression (Baltuch et al. 1995). *In*

vivo studies also showed that the use of PKC α -specific antisense oligonucleotides significantly reduced the proliferation and invasion of implanted tumors in laboratory animals (Philip and Zonder 1999). PKC α has also been shown to enhance cell motility (Ng et al. 1999) and the loss of cell-cell and cell-matrix adhesions(Carey et al. 1999), and to increase expression of bcl-2 and bcl-x_L (Dooley et al. 1998). However, Phase I-III clinical trails using classical PKC inhibitor like bryostatin, UCN-01 and ISIS 3521 have had limited results (Swannie and Kaye 2002).

In terms of protein kinase C ε in glioma cell proliferation and invasion, its high expression and activity levels in primary malignant gliomas correlates to the degree of tumor malignancy. As in the case of PKCα, this isoform has increased expression in gliomas and glioma cells (Sharif and Sharif 1999). This PKC isoform is highly oncogenic even at moderate overexpression levels in rat colonic epithelial cells (Perletti et al. 1996). In addition it promotes malignancy by the overproduction of autocrine growth factors like EGF and TGF alpha (Ueffing et al. 1997; Cacace et al. 1998).

Recently Rocha *et al.* provided additional evidence for the significant role of PKC α and ε in glioma cell proliferation (da Rocha et al. 2000). This group showed that the inhibition of growth of the human glioma cell lines, U87MG, U138MG, and U373, *in vitro* by tamoxifen, a triphenylethylene antiestrogen, led to a decrease in PKC α and ε activity. In addition, inhibition of PKC α and ε by specific antisense oligonucleotides eliminated the stimulatory effects of EGF or PMA on the expression of the ODC (ornithine decarboxylase) gene that plays a role in glioma cell proliferation and invasion. Alterations in the activity of

PKC and in the expression of specific PKC isoforms have been reported in gliomas. Various studies therefore reported gliomas and glioma cell lines to exhibit elevated PKC activity, in particular increased PKC α and ε activity (Bredel and Pollack 1997; Zellner et al. 1998; Sharif and Sharif 1999).

Mandil *et al.* have examined the role of PKCδ in the proliferation and apoptosis of glioma cells (Mandil et al. 2001). Their results showed that the expression of PKCδ is decreased in malignant gliomas when compared to normal astrocytes and with low-grade astrocytomas. When they overexpressed PKCδ in U87MG cells, this caused a drastic decrease in cell proliferation and sensitized these cells to etoposide induced apoptosis.

Studies to date emphasizing the differential role of various PKC isoforms highlight the importance of pursuing this area of research. The research points towards the idea that deregulation of various PKC isozymes may be involved in the tumorigenesis of gliomas. Therefore, elucidating the role of each specific PKC isoform may allow for the development of novel therapeutics.

2.1.5 Atypical protein kinase C in proliferation

In terms of participating in signal transduction cascades involved in survival and proliferation, atypical PKC has been shown to negatively regulate PKB/Akt signaling and induce NFKB transcriptional activity (Sajan et al. 1999; Wen et al. 2003).

Donson *et al.* screened expression of PKC isoforms in four human glioblastoma cell lines both when proliferating and in a quiescent state. PKC α, β and ζ were expressed in all

the cell lines examined while PKCε was expressed in all but one. Using PKC isoform specific inhibitors bisindolylmaleimide-I (most potent against PKCζ) and G06976 (most potent against PKC α and β), only inhibition of PKCζ blocked glioblastoma proliferation. Therefore, only PKC ζ was suggested to be required for glioblastoma proliferation and not PKC α and ε as previously reported (Baltuch et al. 1995; Yazaki et al. 1996; Donson et al. 2000). However, the PKC family consists of 13 closely related isoforms; a limitiation to this study is the possible low selectivity of the kinase inhibitors used. In addition, atypical PKCζ has also been shown to regulate the transcription of the matrix metalloproteinase-9 gene in glioma cells, thereby further implicating the role of atypical PKCs in tumor invasion (Esteve et al. 2002).

Atypical PKCι has been shown to constitutively associate with cyclin dependent kinase activating kinase (CAK), cdk7, in glioma cells. Cdk7 was shown to acts as a substrate for PKCι by *in vitro* phosphorylation studies, co-immunoprecipitation, and autoradiography. CAKS phosphorylate and activate cyclin-dependent kinases (cdks). This study implicates PKCι in the glioma cell cycle phase transition (Acevedo-Duncan et al. 2002). Given such a role, overexpression of PKCι can likely lead to aberrant cell cycle progression and uncontrolled glioma proliferation.

2.1.6 Atypical Protein Kinase C in chemosensitization

Previous studies have shown atypical PKCζ to be a target for etoposide and cisplatin chemosensitization in human leukemic cells and colon cancer cells, respectively (Filomenko

et al. 2002). Inhibition of PKCζ by stable transfection of a kinase-dead dominant negative PKCζ mutant into U937 leukemic cells decreased bcl-2 expression, increased bax and procaspase expression, prevented etoposide induced nuclear translocation of nuclear factor-κB (NF-κB), and prevented the accumulation of X-linked inhibitor of apoptosis protein (XIAP). This also occurred *in vivo* where PKCζ inhibition also sensitized tumor cells grown in nude mice to etoposide. Clonogenic assays of the HT-29 colon cancer cells line transfected with kinase-defective PKCζ exposed to cisplatin caused a significant decrease in colony number in the PKCζ mutant-transfected cells. Thus expression of the kinase-defective PKCζ sensitized HT-29 colon cancer cells to cisplatin induced cytotoxicity (Filomenko et al. 2002). PKCζ is additionally suggested to be a protective signal activated in response to cytotoxic agents in human leukemia cell lines in a study that showed atypical PKC iota to provide protection against okadaic acid and taxol induced apoptosis (Murray and Fields 1997). In addition, atypical PKCs activity has been shown to play a role in cell-cell contacts, establishing cell polarity, directing cell migration and morphogenesis, and mediating cell division by forming complexes with Par-3 and Par-6 partitioning proteins (Etienne-Manneville and Hall 2003). This complex localizes at the edges of migrating cells thus possibly implicating atypical PKC in invasion (Etienne-Manneville and Hall 2001; Suzuki et al. 2001; Yamanaka et al. 2001).

Our lab has previously shown that a myristolated atypical protein kinase C pseudosubstrate peptide inhibitor induced rapid apoptosis in glioblastoma cell lines. The presence of EGFRvIII sensitized these cells to apoptosis induced by this inhibition (Lorimer

et al. 2002). EGFRvIII constitutively activates Ras. This oncogenic Ras in turn, constitutively activates the NF-κB pathway. However, oncogenic ras can initiate p53-independent apoptosis that is suppressed by the NF-κB pathway (Lorimer et al. 2002). It is thought that atypical PKCs allow for the activation of NF-κB (Dominguez et al. 1993; Wooten 1999). One possible explanation is that inhibition of atypical PKCs could cause the inactivation of NF-κB, thereby allowing for the apoptotic inducing oncogenic ras activity in the glioblastoma cell line used.

2.1.7 RNA interference (RNAi) and its application to the study of PKC isoforms

RNA interference is a sequence-specific post-transcriptional gene silencing mechanism that is gaining much attention for its use in studying mammalian gene function. The basic process involves either endogenous double stranded RNA or artificially introduced double stranded RNA, which targets the degradation of mRNAs homologous in sequence to the dsRNA (Elbashir et al. 2002). RNAi has previously been observed in eukaryotes such as plants; however, it has only recently become possible to silence human genes in cultured somatic cells (Elbashir et al. 2001), RNAi was initially difficult to observe in mammalian cells as dsRNA triggers a global shutdown of protein synthesis through an interferon response. The interferon response activates PKR, a dsRNA-dependent protein kinase that stalls translation by phosphorylating the eIF2a initiation factor and causes the activation of 2',5'-oligoadenylate synthetases that activate of ribonuclease Rnase L. RnaseL degrades mRNA in a non-specific manner (Hunter et al. 1975; Elbashir et al. 2001). This response

however is not triggered by dsRNA 30 bp or less in length (Hunter et al. 1975; Bass 2001).

Post-transcriptional gene silencing mediated by dsRNA is an evolutionary conserved defense mechanism for the control of foreign genes in eukaryotes (Elbashir et al. 2002). Random integration of transposons or viral infection causes the production of dsRNA. The dsRNA leads to sequence specific degradation of homologous single stranded mRNA thereby silencing genetic expression of the foreign genes (Elbashir et al. 2001). In mammals, this process is referred to as RNA interference (RNAi). The mechanism of RNAi involves the processing of long dsRNA into duplexes of 21-25 nucleotides (Fire et al. 1998). This processing reaction was first observed *in vitro* in extracts from *D.melanogaster* embryos (Zamore et al. 2000). In the embryo lysate, it was seen that the target mRNA was cleaved into ~21 nt intervals by a ribonuclease III Dicer enzyme. When the synthetic 21 and 22 nt RNA duplexes were added to the lysate, they could also guide efficient sequence-specific mRNA degradation. However, duplexes of 30 bp dsRNA were inactive (Elbashir et al. 2001). The 21 nt RNA products were named small interfering RNAs (siRNAs).

The process of RNAi involves the recruitment of siRNAs by the Dicer enzyme forming a siRNA-bound endonuclease complex referred to as a RNA-induced silencing complex (RISC). In mammalian cells, Dicer localizes to the cytoplasm which suggests that RNAi is mainly a cytoplasmic process (Elbashir et al. 2002). Argonaute 2, normally a nucleoprotein essential for gene silencing in *Caenorhabditis elgans*, *Neurospora* and *Arabidopsos*, is also present cytoplasmically in the RISC (Hammond et al. 2001). It is the RISC that destroys mRNA homologous to the silencing trigger. Dicer and Argonaute 2 possibly aid in the siRNA incorporation into the RISC (Hammond et al. 2001).

The siRNA endonuclease complex cleaves a single stranded target RNA in the middle of the region complementary to the 21nt siRNA duplex (Elbashir et al. 2001). The RNA cleavage products are then rapidly degraded as they lack a poly(A) tail or stabilizing cap. The most efficient siRNA duplexes are 21 nucleotides in length consisting of a 19 nt base-paired sequence with 2 nt 3'-deoxynucleotide (dTdT) overhangs that are more nuclease resistant than ribonucleotides. Symmetric TT overhangs also ensure that the RISC is formed with an equal ratio of sense to antisense target RNA cleaving complexes (Elbashir et al. 2001). The 2 nt overhangs contribute to the specificity of target recognition. The 5' end of the complimentary (antisense) siRNA strand defines the position of target RNA cleavage (Elbashir et al. 2001) **(Figure 2.3)**.

RNAi has many advantages over other gene-silencing techniques. The dsRNA activates a normal cellular process and can be used in most eukaryotic cells; it may also be useful in animals (Caplen 2003). This method leads to a highly specific RNA degradation with a 90-95% knockdown efficiency. Even a single nucleotide mismatch between the siRNA duplex and the target mRNA abolishes interference. One can also achieve a cell-to-cell spreading of gene silencing using RNAi called a *systemic response*. This effect has been reported in several RNAi models as exemplified in experiments performed on mice where a gene silencing effect occurred in most tissue analyzed (Shuey et al. 2002). In

Figure 2.3

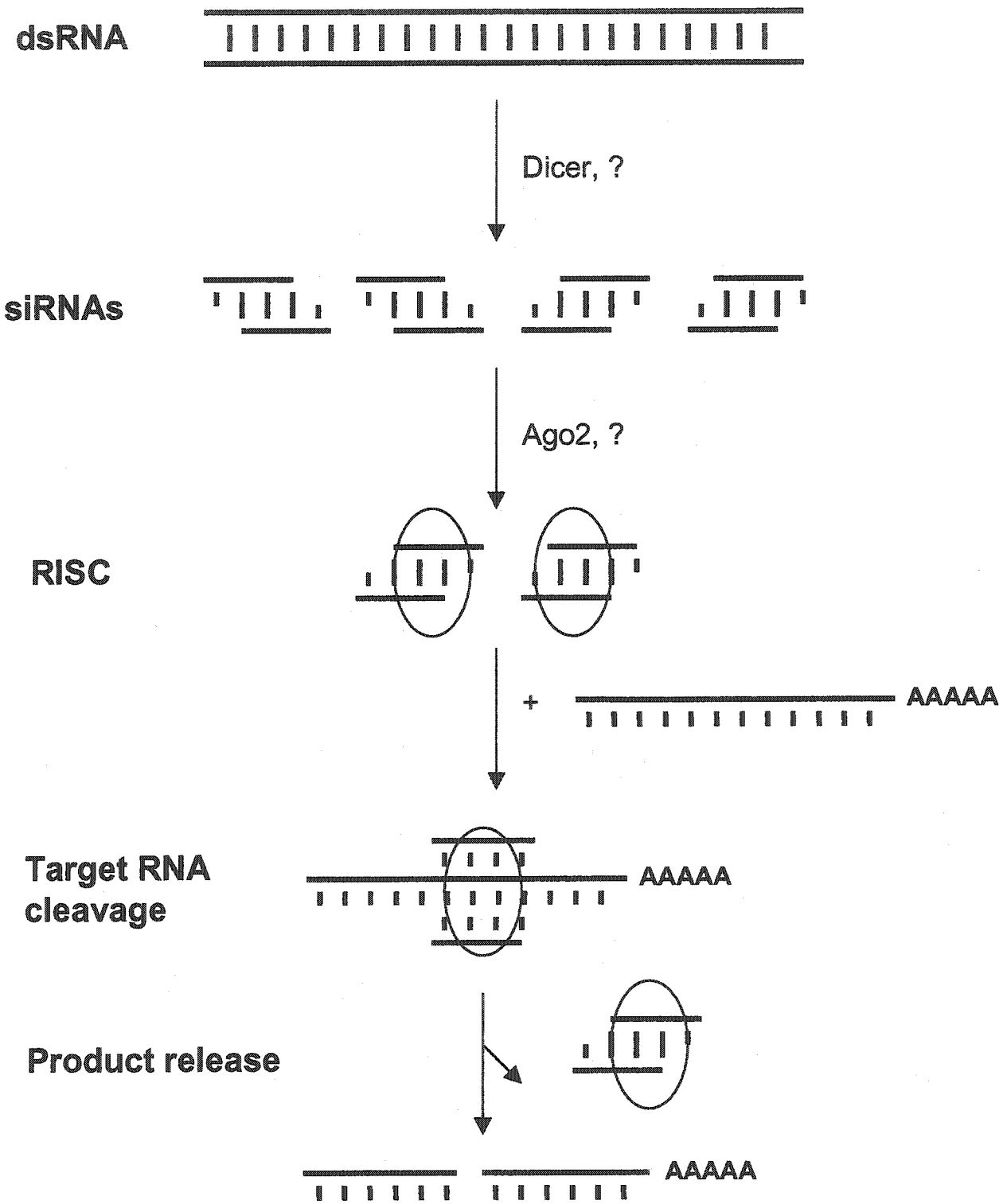

dsRNA
Dicer, ?
siRNAs
Ago2, ?
RISC
+
AAAAA
Target RNA cleavage
AAAAA
Product release
AAAAA

Figure 2.3 Model for RNA interference

DsRNA is processed to 21- to 23-nt siRNA duplexes by Dicer RNase III and like other unidentified factors. The siRNA duplexes are incorporated into RISC that targets the degradation of homologous mRNAs. RISC formation requires Argonaute2 (Ago2) and other yet uncharacterized proteins.

addition RNAi is a rapid method of performing genetic analysis with knockdown occurring in as little as 24 hours post siRNA duplex transfection (Harborth et al. 2001).

Antisense oligonucleotides, small molecule inhibitors, and the overexpression of dominant negatives are all commonly used techniques to assess the protein function. However these techniques have a number of disadvantages when comparing them to RNAi. Problems with toxicity, poor stability, and concerns about specificity are all drawbacks to antisense oligonucleotides (Bennett 2002). Chemical inhibitors also pose a problem of specificity, especially with kinase inhibitors (Lorimer et al. 2002). When overexpressing dominant-negative forms of proteins, there is a possibility that they can block upstream functions. For example, dominant-negative protein kinases may act as pseudosubstrates that interfere with upstream kinases. This phenomenon leads to non-specificity as the affected substrate cannot interact with other distinct proteins. Therefore, one is no longer speficially examining the function of the protein of interest. Such may be the case with dominant negative PKB/Akt that binds to PDK1, preventing it from activating kinases other than PKB/Akt (Vanhaesebroeck and Alessi 2000). It must be said that these techniques have been very useful in studying mammalian gene function. RNAi however, seems to circumvent the drawbacks to the commonly used genetic analysis methodologies and heighten the levels of specificity. Due to the many advantages of RNA interference we have begun to use this technique for our studies.

2.1.8 Hypothesis

The role of atypical PKC iota in glioblastoma multiforme has not been extensively

examined. Given atypical PKC iota's role in glioma cell cycle and protective role in other cancers and the suggested role of its close relative atypical PKCξ in glioblastoma, we hypothesize that atypical PKC iota may contribute to glioblastoma pathogenicity. The PI3-K pathway is constitutively activated in glioblastoma due to a number of genetic aberrations (Benjamin et al. 2003). Given the fact that the atypical PKCs are activated in a PI3-K/PDK-1 dependent manner, it is likely that they play a role in gliomagenesis. The fact that previous work in our lab has shown atypical PKC iota to be the only atypical PKC isoform present in the human glioblastoma cell line, U87MG, suggested that this cell line would be an effective model to study the physiological role of atypical PKC iota in glioblastoma. Using RNA interference, a highly specific post-translational gene silencing mechanism (Elbashir et al. 2002), we hope to examine the physiological role of atypical PKC iota in glioblastoma multiforme using U87MG as a model cell line. We hypothesize that inhibition of atypical PKC iota in glioblastoma cell lines will sensitize these cells to chemotherapeutic agents and alter their aberrant proliferative and invasive properties.

2.1.9 Specific objectives

(a) Determine the role of atypical PKC iota in glioblastoma using RNA interference to specifically knock-down atypical PKC iota protein expression levels in U87MG, U87MGEGFRvIII and SF-295 human glioblastoma cell lines to examine the resultant effects on glioblastoma proliferation signaling pathways involved in atypical PKC iota chemosensitization and invasion.

2.1.10 Significance

The development of new molecular based therapeutics is essential in overcoming the innate chemoresistance and pathogenicity of glioblastoma. In focusing research in this area, patients will benefit from more effective, highly specific and less toxic treatment regimens likely increasing mean survival times. Our goal is to examine atypical PKC iota as a target for glioblastoma therapy. This will be achieved by using RNAi to specifically knock-down atypical PKC iota protein expression levels. The methodology of RNA interference is based on small interfering double stranded RNA, of approximately 21 nucleotides in length, which silences the expression of a gene of interest that is highly homologous to either of the RNA strands in the duplex (Elbashir, Harborth et al. 2002). This technique has the advantages of being highly specific and remarkably potent only requiring a few dsRNA molecules per cell for effective interference. Other techniques such as antisense oligonucleotides, kinase inhibitors, and dominant negatives do pose some difficulties when used such as toxicity, non-specificity, and low intracellular uptake. When dealing with kinases, especially the PKC family of 13 closely related isoforms, conventional methods like kinase inhibitors pose a significant problem of non-specificity. RNAi is a robust knockdown technology making studies on mammalian gene function more specific, informative, and with major implications for therapeutic applications. In the case of the PKC family, this technique will allow for the direct examination of the specific function of atypical PKC iota in glioblastoma proliferation, invasion, and chemoresistance.

2.2 Materials and Methods

2.2.1 Cell lines

The glioblastoma cell lines U87MG, SF-295 and U87MGΔEGFR were used. The human glioblastoma cell lines U87MG and SF-295 were obtained from ATCC (Manassas, VA, USA) U87MGΔEGFR was made by stably transducing U87MG cells with a retroviral vector containing the cDNA for the mutant epidermal growth factor receptor (Nishikawa et al. 1994). U87MGΔEGFR cell line was obtained from Dr. W. Cavenee, Ludwig Institute for Cancer Research, La Jolla, CA. Each cell line was cultured in Dulbecco's modified Eagle's medium. The medium was supplemented with 7.5% donor bovine serum, 2.5% fetal calf serum, 100 units/ml penicillin, 100μg/ml streptomycin, 2 mM glutamine and cultured at 37°C and 5% CO_2. U87MG cells are mutated at the INK4a locus (Lee et al. 2000) and do not express p16/INK4A, p14/Arf, nor PTEN (Pore, Liu et al. 2003).

2.2.2 RNA interference

A short interfering double-stranded RNA (siRNA) against atypical protein kinase C iota was chemically synthesized (Dharmacon research Inc., CO, USA). The sequence for the RNA duplex corresponds to the region +480 to +500 (AAG UGC AUC AAC UGC AAA CUC) with respect to the translational start codon of the atypical Protein Kinase C iota mRNA. Scramble II non-specific siRNA (Dharmacon Research, Inc., CO, USA) was used as a negative control for siRNA activity. The scramble target sequence 5'- GCG CGC TTT GTA GGA TTC G -3' is not present in mammalian cells as determined by BLAST search at

NCBI: http://www.ncbi.nlm.nih.gov/BLAST/ (Altschul et al. 1990). U87MG and U87MGΔEGFR were seeded in 6 well dishes at 1 X 10^5 cells and maintained in DMEM supplemented with 7.5% donor bovine serum and 2.5% fetal calf serum and 1% 200 mM glutamine without antibiotics. siRNAs were transfected into 30% confluent U87MG and U87MGEGFRvIII cells using Oligofectamine (InVitrogen, CA, USA) and cells were harvested 48h and 72h post-transfection for analysis. Briefly, 10 µl 0.02 µM siRNA duplex was added to 175 µl OptiMEMI. In a separate tube, 4 µl Oligofectamine was added to 11 µl of OptiMEMI, mixed gently and incubated for 10 minutes at room temperature. The diluted Oligofectamine was added to the diluted siRNA duplex, mixed gently and incubated at room temperature for 20 minutes. The cells were then washed with warm OptiMEM after which 800 µl of OptiMEMI was added to each well. The combined Oligofectamine and siRNA duplex solution (200 µl) was then added to the cells and mixed gently for 30 seconds by lightly rocking of the plate. The cells were incubated for 4 hours at 37°C in a 5% CO_2 humidified chamber after which the media was replaced with fresh DMEM supplemented with 10% fetal and donor bovine serum and 1% 200 mM glutamine without antibiotics

2.2.3 Western blot analysis

Cells in monolayer culture were washed twice in ice-cold PBS and then lysed with 50 µl hot 2X Western Blot Lysis Buffer (5 mM Tris, pH 6.8, 4% sodium dodecyl sulphate, 2% glycerol, 0.2 M dithiothreitol). The cells were then scraped into microcentrifuge tubes, boiled for 10 minutes, cooled for 10 minutes, vortexed at high speed for 30 seconds,

sonicated, and boiled for 5 minutes. The Bio-Rad Protein Assay solution (Bio-Rad Laboratories, Hercules, CA) was used to determine the protein concentration of each sample. Cell lysates were prepared by adding 1X Laemmli buffer with beta-mercaptoethanol to the lysates and subjected to Western blot analysis. Briefly, 20 µg to 40 µg of protein was subjected to electrophoresis through a 10%-12% sodium dodecyl sulfate polyacrylamide gel and transferred to nitrocellulose membrane. Staining membranes for total protein with a solution of 1% amido black in 50% methanol and 10% acetic acid assessed protein loading and transfer. Membranes were washed with H_20, blocked with 5% skim milk powder and TBST (10mM TrisHCL, pH 7.6, 150 mM Nacl, 0.05% Tween 20) for 1 hour. The membranes were then incubated for 1 hour with specific primary antibody. Primary antibodies used were 1:200 nPKC. (C-20)-G goat polyclonal (also recognizes the same sequence in atypical PKCι) (Santa Cruz Biotechnology, CA, USA), 1:500 phospho-specific p44/p42 Map K (T202/T204) E10 mAb (Cell Signaling Technology, MA, USA), 1:4000 anti-pan ERK mAb (Pharmagen, CA, USA), 1:1000 phospho-specific Akt (ser 473) (Cell Signaling Technology, MA, USA), 1:200 Akt-1 (C-20) goat polyclonal IgG (Santa Cruz Biotechnology, CA, USA), and 1:200 p27 Ab-1 mAb (Medicorp, QC, CAN). Membranes were then washed for 6 minutes with 1X TBST four times then incubated with the appropriate secondary antibody at 1:5000 and washed as before once again. Blots were developed with SuperSignal West Pico Chemiluminescent Substrate detection reagent (Pierce Biotechnology, IL, USA).

2.2.4 Chemicals and reagents

Etoposide (Calbiochem, CA, USA), a topoisomerase II inhibitor, was dissolved in dimethyl sulfoxide and stored at –20 °C as 100 mM stock solution. The final concentration of dimethyl sulfoxide in growth media did not affect cell growth or any other parameters measured. Cisplatin (Ottawa Regional Cancer Centre Pharmacy, ON, CAN), a DNA damaging agent that introduces inter- and intrastrand crosslinks in DNA, was purchased as a 1mg/ml saline working solution and stored at room temperature protected from light. The concentration of etoposide and cisplatin used in each experiment was 50 μM. Both etoposide and cisplatin treatments were initiated 48 hours after RNA interference and were continued for 24 hours.

2.2.5 Cell cycle analysis

U87MG or U87MGΔEGFR were plated in 6 well culture plates at 1×10^5 cells. At approximately 30% confluency, the cells were transiently transfected with a control scramble non-specific RNA duplex, a specific atypical PKCι RNA interfering duplex, or with no RNA duplex (only Oligofectamine). 48 and 72 hours following RNA interference, both adherent and non-adherent cells were collected, washed twice with PBS containing 0.4% serum, and centrifuged at 1500 rpm for 5 minutes between washings. The cells were then fixed in 1 ml of 70% (v/v) ethanol/PBS at 4°C for up to two weeks. The nuclei of cells were then stained in 500 μl of a solution containing 180 μg/ml propidium iodide in 1X PBS and 400 μg/ml RNase A 10X propidium iodide stock diluted down to a 1X

working solution, and incubated at 4°C for 30 minutes. Ten thousand cells were analyzed on a FACScan BD LSR flow cytometer (Becton Dickinson, San Jose, CA) under each experimental condition. Data acquisition was done using Cell Quest software (Becton Dickinson, Sanjose, CA). DNA histograms were obtained and analyzed by Mod Fit LT software (Verity Software House Inc., Sopsham, ME). Histograms plotted on the log scale were used to analyze the percentage of cell in the sub-G population. Mod Fit LT software was used to analyse the G1-M phases of the cell cycle.

2.2.6 Scratch-wound assay

U87MG, U87MGEGFRvIII, and SF-295 cells were plated on gelatin-coated coverslips at 1 X 10^5 cells/well in six well plates. Approximately 24 hours later, upon reaching approximately 30% confluency, the cells were transfected with either a control non-specific scrambled siRNA duplex or a siRNA duplex specific for atypical PKC iota as described above. 48 hours post transfection, cultures were injured with a single scratch from a pipette tip. The scratch ran straight across the coverslip. 24 hours after the initial injury, cells were fixed in 4% paraformaldehyde for 15 minutes at room temperature, washed in PBS, dehydrated by sequential submersion for 30 sec once in 80% ethanol, twice in 95% ethanol, once in 100% ethanol, and three times in Xylene. They were then mounted for phase contrast microscopy analysis. Untransfected cells exposed only to Oligofectamine were also used for this assay.

2.2.7 Immunocytochemistry

U87MG grown in coverslips were transfected with either a control non-specific scrambled siRNA duplex or a siRNA duplex specific for atypical PKC iota using Oligofectamine, as previously described. 48 hours post transfection, the cells were prepared for immunocytochemistry to determine the cellular localization of the cdk inhibitor, $p27^{Kip1}$. Briefly, cells were washed three times in cold phosphate buffer saline (PBS), fixed in 4% paraformaldehyde for 1 hour at room temperature, washed three times for 10 minute in PBS and then permeabilized with 0.2% Triton X-100 (in PBS) for 10 minutes at room temperature. After three washes for 10 minutes in PBS, cells were blocked with 5% normal goat serum for 30 minutes at room temperature followed by a 1-hour incubation at room temperature with the relevant primary antibody. As a control, a mouse IgG1a mouse monoclonal antibody was used at a concentration of 4 µg/ml (Neomarkers 200 µg/ml stock). A $p27^{Kip1}$ Ab-1 mouse monoclonal clone 72.56 was also used at a concentration of 4 µg/ml (Neomarkers 200 µg/ml stock). After washing the cells three times for 5 minutes in PBS, the cells were stained for 1 hour at room temperature with a secondary goat anti-mouse antibody (Alexafluor 594-Molecular Probes 2mg/ml stock) at a concentration of 4 µg/ml. The cells were then washed for 5 minutes in PBS and counterstained with 4', 6-diamidino-2-phenylindole (DAPI) (1 µl in 50 ml PBS) for 5 minutes at room temperature. Cells were then washed three times for 10 minutes in PBS, rinsed in ddH_20 and coverslips were mounted on slides using Mount KPL Mounting Medium (Kirkegaard & Perry Laboratories, Maryland, USA). Immunofluorescence was performed using a Zeiss Axioskop2 fluorescence

microscope and images were captured with a CCD camera. Images were deconvolved using Axiovision 3.0 software (Carl Zeiss, Inc., NY, USA).

2.2.8 Immunoprecipitation

U87MG were transfected with either a control non-specific scrambled II siRNA duplex or a siRNA duplex specific for atypical PKC iota using oligofectamine, as previously described. 48 post transfection, the cells were prepared for immunoprecipitations of $p27^{KIP1}$. Briefly, cells from an entire 6 well tissue culture dish were scraped into 1 ml cold lysis buffer (20 mM Tris-HCl pH 7.4, 150 mM Nacl, 1% T X 100). After being passed through a 27 gauge needle, samples were centrifuged at 14,000 rpm for 10 minutes at 4 °C. 300 µl of the scrambled and PKC iota RNAi U87MG supernatants were incubated with either 2 µg/µl negative control IgG and p27 Kip1 IgG for 2-3 hours at 4 °C. 200 µl of Gamma-bind Sepharose (400 µl stock washed 4X in 1 ml lysis buffer, resuspended in 1 ml total) were added to each sample for 2 hours. Then immunocomplexes were washed five times with 750 µl lysis buffer and finally resuspended in 50µl 2X Laemmli, boiled for 5 minutes and stored at -20 °C.

2.3 Results

2.3.1 RNA interference of atypical PKC iota in glioblastoma cell lines

We were interested in examining the physiological role of atypical Protein Kinase C iota (aPKCι) in glioblastoma with the intention of possibly exploiting aPKCι as a novel

therapeutic target. Previous RT-PCR work done in our lab has shown atypical PKC iota to be the only isoform from the atypical PKC subfamily present in U87MG, a glioblastoma cell line. Therefore we felt confident in our results to pursue U87MG as a useful model to study the role that atypical PKC iota may play in glioblastoma proliferation and apoptosis, an area that has not been greatly examined.

To begin to study the physiological role of aPKCι in glioblastoma we chose to use RNA interference to efficiently and specifically knock-down the expression of aPKCι in three glioblastoma cell lines: U87MG, U87MGEGFRvIII, and SF-295. RNA interference is a post-transcriptional gene silencing mechanism that uses dsRNA to trigger degradation of homologous mRNA sequences (Elbashir et al. 2002). For our studies, we chose a sequence from the aPKCι cDNA open reading frame from position +480 to +500 with respect to the translational start codon with approximately a 50% G/C content following the sequence criteria 5'-AA(N19)UU. Our selected sequence falls into the criteria to obtain the most efficient and specific RNAi (Elbashir et al. 2002). The selected region should ideally be at least 50 to 100 nt downstream of the start codon in order to avoid 5' or 3' untranslated regions (UTRs) or regions close to the start codon that may be richer in regulatory protein binding sites. It is thought that UTR-binding proteins and/or translational initiation complexes could interfere with binding of RISC to the target RNA. The selected mRNA sequence should preferably be 5'-AA(N19)UU with approximately a 50% G/C content. Sequences rich in G tend to form G-quartet structures that could interfere with binding to the target RNA, and thus they should be avoided. Our sequence selection was AAG UGC AUC AAC UGC AAA GUC UU. A short interfering double-stranded RNA (siRNA) was

chemically synthesized as 5'-(N19)TT by Dharmacon Research Inc.who also supplied us with a non-specific scrambled II dsRNA duplex control **(Figure 2.4 A and B)**.

The efficiency of RNAi in U87MG glioblastoma cell lines was previously assessed in our lab using a cy3-labelled RNA duplex (Lavictoire et al. 2003). Immunofluorescence was used to analyze the RNAi transfection efficiency using Oligofectamine. Our work has shown that 95% of U87MG cells were successfully transfected with the cy3-labelled RNA duplex. Thus for my work we used Oligofectamine to transfect the glioblastoma cell lines with either: 1) no siRNA, 2) the control non-specific scrambled siRNA or 3) the siRNA specifically targetting aPKCι. The transfected cells were then harvested 48 and 72 hours post transfection for immunoblot analysis using an antibody against the carboxy terminus of atypical PKC zeta/iota. Immunoblot analysis shows that RNA interference of aPKCι in U87MG, U87MGEGFRvIII, and SF-295 resulted in a significant decrease in atypical PKC iota protein expression 48 and 72 hours post-transfection **(Figure 2.5 A).** As controls we probed the cell lysates exposed to Oligofectamine alone or with the non-specific scrambled II siRNA. In both cases, the levels of atypical PKC iota protein expression remained unchanged. The extent of atypical PKC iota depletion due to siRNA transfection was quantitatively assessed by comparing protein expression levels between 2 μg, 4 μg, 6 μg, and 10 μg of cell lysate from U87MG cells 72 hours (Day 3) post tranfection with the control scramble II RNA duplex to 20 μg U87MG cell lysate 72 hours (Day 3) post transfection with the atypical PKC

Figure 2.4

A

Atypical Protein Kinase C iota mRNA

agcggttttg ggcccgggcg gctgtagagg cggcggcgcc tacgggcagt gggaggagcc gcgcggttcc ggctgctccg gcgaggcgac ccttgggtcg gcgctgcggg cgaggtgggc aggtaggtgg gcggacggcc gcggttctcc ggcaagcgca ggcggcggag tcccccacgg cgcccgaagc gccccccgc accccggcc tccagcgttg aggcggggga gtgaggagat gccgacccag agggacagca gcaccatgtc ccacacggtc gcaggcggcg gcagcgggga ccattcccac caggtccggg tgaaagccta ctaccgcggg gatatcatga taacacattt tgaaccttcc atctcctttg agggcctttg caatgaggtt cgagacatgt gttcttttga caacgaacag ctcttcacca tgaaatggat agatgaggaa ggagacccgt gtacagtatc atctcagttg gagttagaag aagcctttag actttatgag ctaaacaagg attctgaact cttgattcat gtgttccctt gtgtaccaga acgtcctggg atgccttgtc caggagaaga taaatccatc taccgtagag gtgcacgccg ctggagaaag ctttattgtg ccaatggcca cactttccaa gccaagcgtt tcaacaggcg tgctcactgt gccatctgca cagaccgaat atggggactt ggacgccaag gatataagtg catcaactgc aaactcttgg ttcataagaa gtgccataaa ctcgtcacaa ttgaatgtgg gcggcattct ttgccacagg aaccagtgat gcccatggat cagtcatcca tgcattctga ccatgcacag acagtaattc catataatcc ttcaagtcat gagagtttgg atcaagttgg tgaagaaaaa gaggcaatga acaccaggga aagtggcaaa gcttcatcca gtctaggtct tcaggatttt gatttgctcc gggtaatagg aagaggaagt tatgccaaag tactgttggt tcgattaaaa aaaacagatc gtatttatgc aatgaaagtt gtgaaaaaag agcttgttaa tgatgatgag gatattgatt gggtacagac agagaagcat gtgtttgagc aggcatccaa tcatcctttc cttgttgggc tgcattcttg ctttcagaca gaaagcagat tgttctttgt tatagagtat gtaaatggag gagacctaat gtttcatatg cagcgacaaa gaaaacttcc tgaagaacat gccagatttt actctgcaga aatcagtcta gcattaaatt atcttcatga gcgagggata atttatagag atttgaaact ggacaatgta ttactggact ctgaaggcca cattaaactc actgactacg gcatgtgtaa ggaaggatta cggccaggag atacaaccag cactttctgt ggtactccta attacattgc tcctgaaatt ttaagaggag aagattatgg tttcagtgtt gactggtggg ctcttggagt gctcatgttt gagatgatgg caggaaggtc tccatttgat attgttggga gctccgataa ccctgaccag aacacagagg attatctctt ccaagttatt ttggaaaaac aaattcgcat accacgttct atgtctgtaa aagctgcaag tgttctgaag agttttctta ataaggaccc taaggaacga ttgggttgtc ttcctcaaac aggatttgct gatattcagg gacacccgtt cttccgaaat gttgattggg atatgatgga gcaaaaacag gtggtacctc cctttaaacc aaatatttct ggggaatttg gtttggacaa ctttgattct cagtttacta atgaacgtgt ccagctcact ccagatgacg atgacattgt gaggaagatt gatcagtctg aatttgaagg ttttgagtat atcaatcctc ttttgatgtc tgcagaagaa tgtgtctgat cctcattttt caaccatgta ttctactcat gttgccattt aatgcatgga taaacttgct gcaagcctgg atacaattaa ccattttata tttgccacct acaaaaaaac acccaatatc ttctcttgta gactatatga atcaattatt acatctgttt tactatgaaa aaaaaattaa tactactagc ttccagacaa tcatgtcaaa atttagttga actggttttt cagtttttaa aaggcctaca gatgagtaat gaagttatct tttttgttta aaaaaaaaaa aaaaa

AA-N19 mRNA target 5'→3

AAG UGC AUC AAC UGC AAA CUC

siRNA duplex

GUG CAU CAA CUG CAA ACU C dTdT

dTdT CAC GUA GUU GAC GUU UGA G

B

Scramble II non-specific siRNA duplex

AA-N19 mRNA target 5'→3

AAG CGCGCUUUGUAGGAUUCG

siRNA duplex

GCG CGC UUU GUA GGA UUC G dTdT

dTdT CGC GCG AAA CAU CCU AAG C

Figure 2.4 Design of small interfering RNA duplexes for RNA interference

A. The duplex targeting atypical PKC iota was designed from region +480 to +500 (orange) with respect to the translational start codon (green). The translational stop codon is also indicated (red). **B.** Scramble II non-specific siRNA (Dharmacon Research, Inc.) used as a negative control

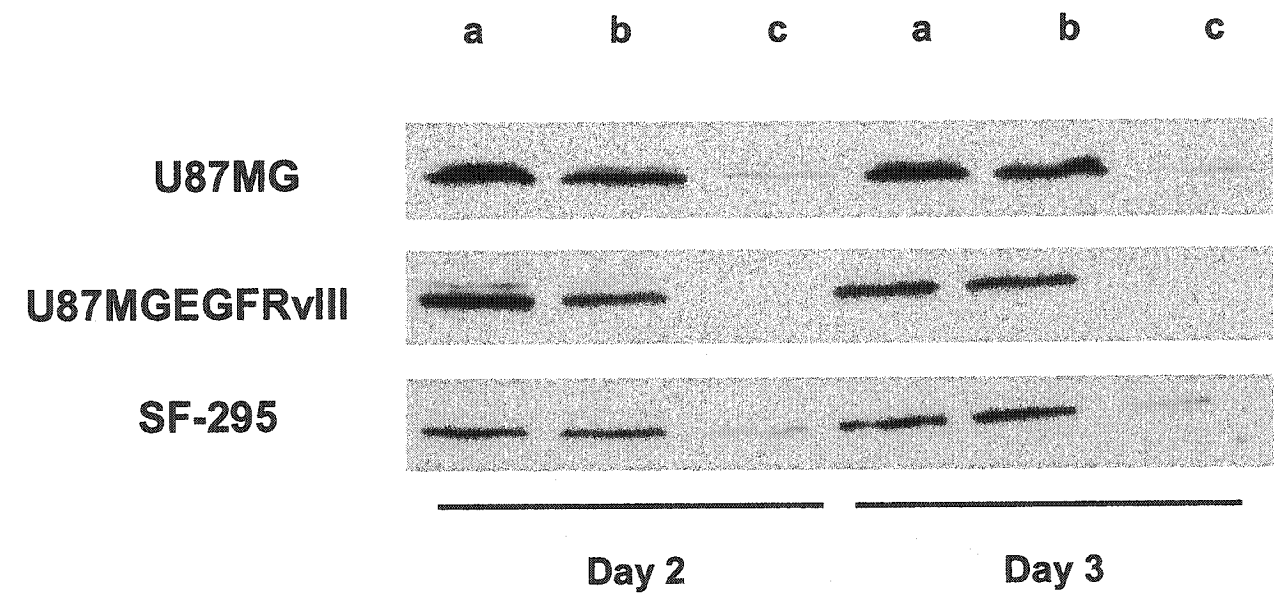
iota targetting duplex. Our results indicate that the atypical PKC iota expression level in U87MG cells transfected with the atypical PKC iota duplex was comparable to the levels of atypical PKC iota expressed in that of 2 μg to 4 μg of control U87MG cell lysates. Therefore there is an 80% to 90% depletion in the levels of atypical PKC iota expression due to RNAi **(Firgure 2.5 B)**.

2.3.2 RNA interference of atypical PKC iota causes a G0/G1 cell cycle arrest in U87MG but not in U87MGΔEGFR and SF-295 glioblastoma cells

U87MG, U87MGEGFRvIII and SF-295 glioblastoma cells were transfected with either: 1) no siRNA, 2) the control non-specific scrambled II siRNA or 3) the siRNA specifically targetting aPKCι. 48 and 72 hours post-transfection the cells were harvested for flow cytometry analysis. We used propidium iodide, a fluorescent nuclear dye, to stain whole cells and then used flow cytometry to analyze DNA content in order to assess the distribution of cells in each phase of the cell cycle. Only in the case of U87MG 48 hours post transfection was there a significant increase in the percentage of cells in G0/G1 and a concomitant decrease in G2/M and S phase under conditions of atypical PKC iota RNA interference. These results are indicative of growth inhibition due to the specific depletion of atypical PKC iota. The trend was also seen in U87MGEGFRvIII however much attenuated and not statistically significant. In U87MG, atypical PKC iota RNA interference caused an increase in G0/G1 from 53% to 72%, a decrease in G2/M from 4% to 2.4% and a

Figure 2.5

A


a
b
c
a
b
c
U87MG
U87MGEGFRvIII
SF-295
Day 2
Day 3

B

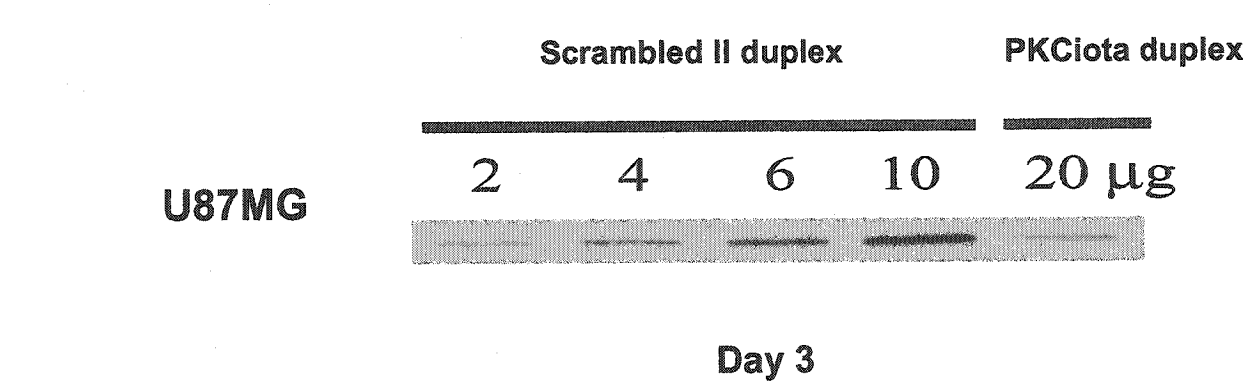

Scrambled II duplex
PKCiota duplex
2
4
6
10
20 µg
U87MG
Day 3

Figure 2.5 Atypical PKC iota siRNA efficiently reduces atypical PKC iota expression in U87MG, U87MGEGFRvIII, and SF-295 glioblastoma cell lines

A. Chemically synthesized siRNA duplexes were used. Cells were transfected with siRNA duplexes and harvested 2 and 3 days post transfection and 25 μg proteins of each cell lysates were separated on 12% acrylamide gels and immunoblotted with an antibody recognizing the C-terminal of PKC iota. **Lane a**, no siRNA transfected cells (cells exposed to Oligofectamine alone); **lane b**, negative control scramble II siRNA transfected cells; and **lane c**, atypical PKC iota siRNA duplex transfected cells. **B**. U87MG cells were transfected with the control scramble II siRNA duplex and the atypical PKC iota siRNA duplex. The cells were harvested 3 days post tranfection. 2 μg, 4 μg, 6 μg, and 10 μg protein of the control cell lysate and 20 μg protein from the atypical PKC iota RNAi cell lysate samples were separated on a 12% acrylamide gel and immunoblotted for atypical PKC iota.

decrease in the S phase from 43% to 25%. However, in U87MGEGFRvIII atypical PKC iota RNA interference caused an increase in G1/G0 from 46% to 52%, a decrease in G2/M from 12% to 9.5% and a decrease in the S phase from 42% to 39% (**Table 2.1**).

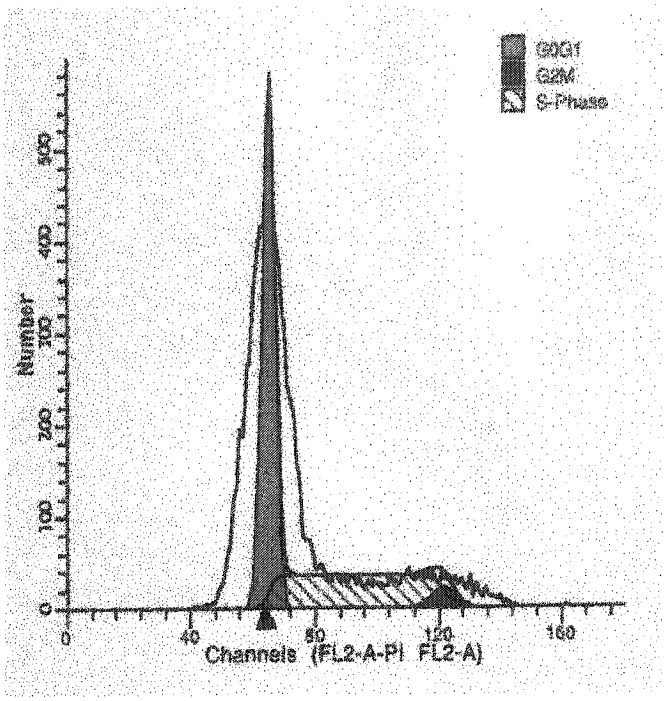
These observations were not as pronounced by day 3 post RNAi transfection. By day 3, the cells slow down in proliferation and are not dividing as fast likely due to high cell density, which may explain this observation. Due to this fact, for the remainder of our analysis we focus on day 2 samples. In the case of SF-295 glioblastoma cells, duplicate experiments showed that atypical PKC iota RNA interference did not cause any significant modulations in the cell cycle. Perhaps this is due to this cell line carrying a different set of mutations than U87MG and U87MGEGFRvIII that allows it to circumvent the apparent role that atypical PKC iota plays in U87MG; however the genetic background of SF-295 is unknown (**Table 2.1**).

Western blot analysis shows that atypical PKC RNAi causes a decrease in the levels of atypical PKC iota expression in U87MG, U87MGΔEGFR, and SF-295 glioblastoma cells. In order to assess whether atypical PKC iota depletion was affecting basal levels of apoptosis in the glioblastoma cell lines examined, we assessed the subG population of glioblastoma cell lines, U87MG and U87MGΔEGFR by flow cytometric analysis Day 2 post atypical PKC iota RNAi transfection (**Table 2.2**). The decrease in expression of atypical PKC iota by RNA interference did not increase the percentage of cells in subG. Therefore these results indicate that atypical PKC iota depletion on its own is not inducing apoptosis in the glioblastoma cell lines examined.

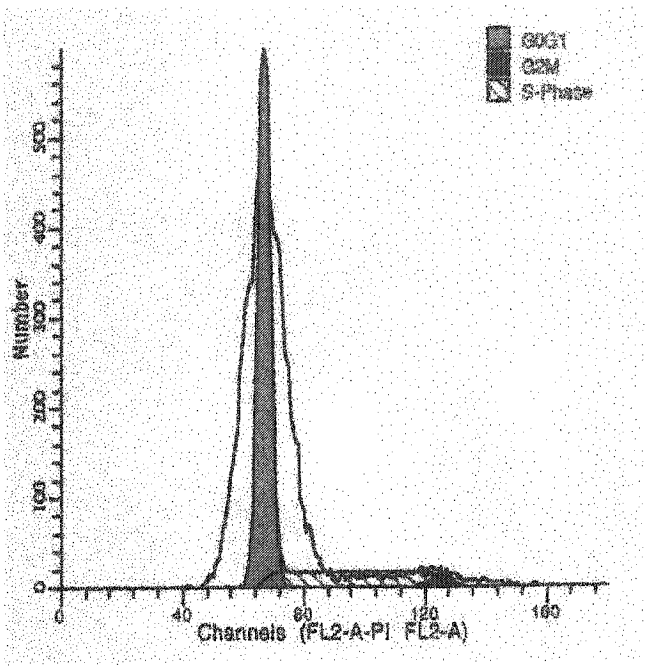
Table 2.1

U87MG

Scrambled II duplex



PKC iota duplex



	%G0G1	%G2M	%S
Scrambled II duplex	52.97 ± 4.39	4.03 ± 0.55	43.03 ± 4.34
PKC iota duplex	72.42 ± 3.70	2.36 ± 0.47	25.22 ± 3.26

U87MGEGFRVIII

	%G0G1	%G2M	%S
Scrambled II duplex	46.00 ± 5.84	11.9 ± 2.32	42.06 ± 6.17
PKC iota duplex	51.96 ± 9.75	9.48 ± 1.44	38.57 ± 8.89

SF-295

	%G0G1	%G2M	%S
Scrambled II duplex	26.31 ± 1.41	8.12 ± .014	65.58 ± 1.28
PKC iota duplex	23.51 ± 0.22	5.68 ± 0.85	70.81 ± 1.08

Table 2.1 Cell cycle distribution of U87MG, U87MGΔEGFR, and SF-295 glioblastoma cell lines subject to atypical PKC iota RNA interference

U87MG, U87MGΔEGFR, and SF-295 cells were harvested for flow cytometry using propidium iodide analysis 2 days post atypical PKC iota siRNA RNAi. Mod Fit LT software was used to analyse the G1-M phases of the cell cycle (FACS profile only shown for U87MG cells).

Table 2.2

% sub G

	U87MG	U87MGEGFRvIII
Scramble II duplex	7.71 ± 3.17	12.08 ± 4.46
PKC iota duplex	9.13 ± 3.65	15.31 ± 5.62

Table 2.2 % Apoptosis of U87MG and U87MGΔEGFR glioblastoma cell lines subject to atypical PKC iota RNA interference

U87MG and U87MGΔEGFR cells were harvested for flow cytometry using propidium iodide analysis 2 days post atypical PKC iota RNAi.

2.3.3 RNA interference of atypical PKC iota causes an increase in $p27^{Kip1}$ in U87MG cells

Due to our cell cycle results we further assessed the role of atypical PKC iota in the glioblastoma cell cycle by examining the modulation of $p27^{Kip1}$, a cdk inhibitor, under conditions of atypical PKC iota RNA interference in U87MG and U87MGEGFRvIII. Once again we transfected U87MG and U87MGΔEGFR with either: 1) the control non-specific scrambled siRNA or 2) the siRNA specifically targetting atypical PKC iota. 48 and 72 hours post transfection we harvested our samples for immunoblot analysis. Using an anti-$p27^{Kip1}$ antibody we assessed the levels of $p27^{Kip1}$ in both cell lines. Our results demonstrated that both U87MG and U87MGΔEGFR had higher levels of p27 expression under conditions of atypical PKC iota RNAi, both 48 and 72 hours post-transfection. The basal and induced levels of $p27^{Kip1}$ in U87MGΔEGFR cells were never as high as the basal and induced levels of $p27^{Kip1}$ in U87MG cells. This observation is in agreement with previous groups that showed EGFRvIII to increase cell proliferation in part by the down-regulation of p27 through the constitutive activation of the PI3-K/Akt pathway (Narita, Nagane et al. 2002). Our results show that a decrease in expression of atypical PKC iota by RNAi causes an upregulation in the expression of $p27^{Kip1}$. $p27^{Kip1}$ inhibits cell cycle progression through G1 to S. Thus the increase in the levels of $p27^{Kip1}$ due to atypical PKC iota RNAi may partly explain the significant increase of U87MG cellular population in G0/G1 under such conditions. These results further validate the role of atypical PKC iota in U87MG glioblastoma cell proliferation **(Figure 2.6)**.

Figure 2.6

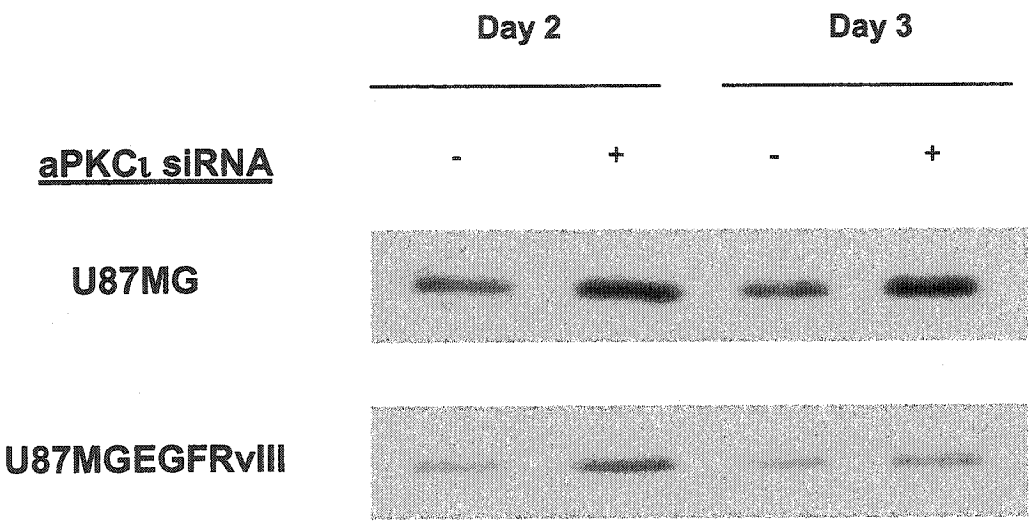


Figure 2.6 p27 expression levels in U87MG and U87MGEGFRvIII under atypical PKC iota RNAi

Chemically synthesized siRNA duplexes were used. Cells were transfected with siRNA duplexes, scramble II and atypical PKC iota, and harvested 2 and 3 days post transfection. 25 μg proteins of each cell lysate were used. Samples were separated on 12% acrylamide gels and immunoblotted for p27 expression levels.

2.3.4 $p27^{Kip1}$ localization under conditions of atypical PKC iota RNA interference in U87MG cells

$p27^{Kip1}$ is an important regulator of progression through G1. $p27^{Kip1}$ is an active cdk inhibitor when it is localized in the nucleus. It is highly expressed in G0, where it inhibits cyclin E-cdk 2 (Hengst et al. 1994). In mid-G1, $p27^{Kip1}$ plays a role in the assembly and nuclear import of cyclin D-cdk complexes (LaBaer et al. 1997). Transcriptional controls, ubiquitin-dependent proteolysis, and phosphorylation regulate $p27^{Kip1}$ levels and localization. $p27^{Kip1}$ is also transcriptionally regulated by AFX, a Forkhead Transcription factor that regulates longevity in *C. elegans*. AFX regulates cellular proliferation in part by integrating PI3-K signaling and Ras/Ral signaling to transcriptionally activate and increase protein levels of $p27^{Kip1}$ (Medema et al. 2000). The levels of $p27^{Kip1}$ decrease through G1 to S progression (Pagano et al. 1995). It has been suggested that p27 nuclear export is required for its degradation, however, the relationship between p27 localization and its degradation remains unclear (Connor et al. 2003).

After 48 hours PKC iota RNAi U87MG exhibited a decrease in proliferation as noted by an increase in the percentage of cells in G0/G1and an increase in the expression of the cdk inhibitor $p27^{Kip1}$. However, the increase in the expression of $p27^{Kip1}$ persisted even up to 72 hours post PKC iota RNAi. We became interested in examining the relationship between atypical PKC iota and $p27^{Kip1}$ by immunocytochemistry. We hypothesized that the decrease in proliferation in U87MG 48 hours post atypical PKC iota RNAi is partly due to the nuclear localization of $p27^{Kip1}$. Our results show that the U87MG cells transfected with the scramble II duplex, exhibited primarily cytoplasmic $p27^{Kip1}$ staining, while atypical PKC iota depletion

by RNAi does in fact cause an increase in nuclear $p27^{Kip1}$ translocation in U87MG cells as seen by an increase in nuclear staining of $p27^{Kip1}$. To prove that $p27^{Kip1}$ was actually undergoing nuclear translocation under atypical PKC iota RNAi, we counterstained our cells with the nuclear stain DAPI. Our results clearly show that the increase in $p27^{Kip1}$ signal under atypical PKC iota RNAi is occurring in the nuclei **(Figure 2.7)**. As a control, we stained our U87MG cells transfected with both the scramble II control duplex and the atypical PKC iota duplex with control IgG. Our control isotypes matched IgG samples only exhibited a faint background staining thus validating our results.

2.3.5 RNA interference of atypical PKC iota: effects on PKB/Akt and ERK

As discussed earlier, the PI3-K/PKB/Akt pathway is constitutively activated in multiple forms of cancer, glioblastoma being one of them, leading to aberrant survival and proliferation(Paez and Sellers 2003). As previously mentioned, it has been also been shown that $p27^{Kip1}$ transcription is repressed by PKB/Akt phosphorylation of the forkhead transcription factor, AFX (Medema et al. 2000; Liang et al. 2002). Mao *et al.* have demonstrated that atypical PKCζ and, to a lesser extent, atypical PKC ι/λ negatively regulate PKB/Akt by physical and functional interaction in breast cancer cells. Such an interaction causes an inhibition of activation-dependent phosphorylation of Akt at Ser 473 and Thr 308 (Mao et al. 2000). Using a phospho-specific PKB/Akt antibody, we examined if atypical PKC iota RNAi would affect basal expression and phosphorylation

Figure 2.7

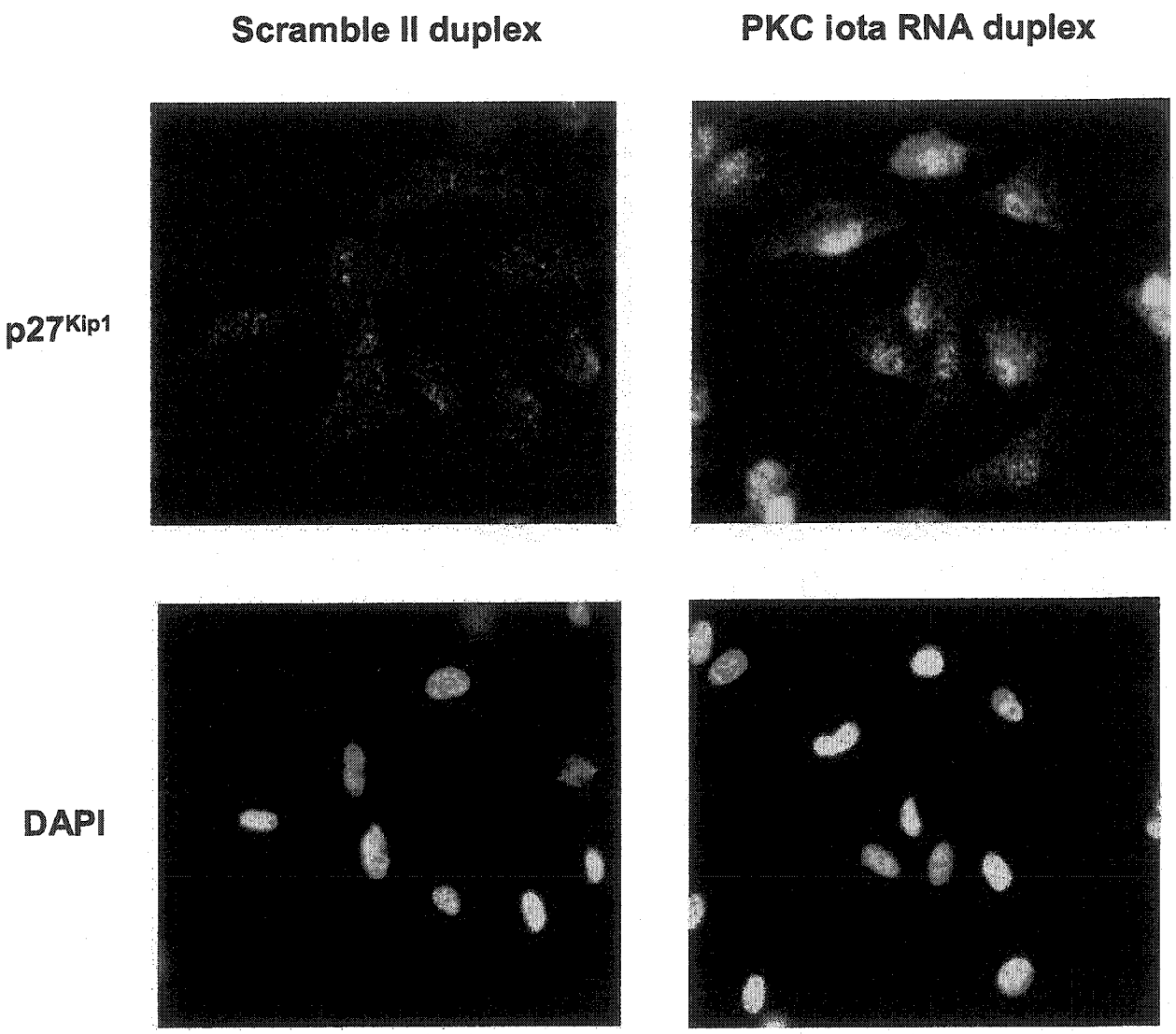


Figure 2.7 U87MG glioblastoma cell $p27^{Kip1}$ nuclear localization under conditions of atypical PKC iota RNAi

U87MG glioblastoma cells were transfected with a control scramble II non-specific siRNA duplex or with the atypical PKC iota siRNA duplex. 48 hours post transfection, cells were fixed and stained with an anti-p27 antibody (top panels) and counterstained with DAPI to show nuclear staining. (bottom panels). The top panels are images of U87MG cells that have been deconvoled as described under "Materials and Methods".

levels of PKB/Akt. Briefly, we transfected U87MG and U87MGΔEGFR with either: 1) the control non-specific scrambled siRNA or 2) the siRNA specifically targetting atypical PKC iota. 48 and 72 hours post transfection we harvested our samples for immunoblot analysis. Our results showed that atypical PKC iota RNA interference did not modulate the basal levels or levels of phosphorylated PKB/Akt in both U87MG and U87MGEGFRvIII (**Figure 2.8**). Thus PKCι does not appear to regulate signaling through the PI3-K/PKB/Akt signaling cascade **(Figure 2.2D)**. However, examination of PKB/Akt activity and subsequent phosphorylation levels of downstream PKB/Akt targets would also need to be analyzed.

The Erk pathway is an important proliferative pathway that is also constitutively activated in multiple forms of cancer, including glioblastoma multiforme (Seger and Krebs 1995; Lorimer and Lavictoire 2001; Barbero et al. 2003). In view of the decrease in proliferation we noted in U87MG subjected to atypical PKC iota RNAi, we decided to examine Erk signaling properties under these conditions. Under 10% serum conditions, atypical PKC iota RNA interference caused a transient decrease in the phosphorylation levels of ERK1/ERK2 (p44/p42 Map Kinase) in U87MG 48 hours post-transfection. By 72 hours post transfection, the levels of phospho-ERK were equivalent in both the control U87MG cells and the atypical PKC iota RNA interfered cells. Interestingly this observation was not seen in the U87MGΔEGFR cells **(Figure 2.8)**. Therefore while in the case of U87MG atypical PKC iota appears to act upstream of ERK1/ERK2, as seen by the decrease in the phosphorylation levels, the presence of EGFRvIII seems to obliterate this phenomenon. It is known that EGFRvIII constitutively activates the ERK signaling pathway, one mechanism by which this

Figure 2.8

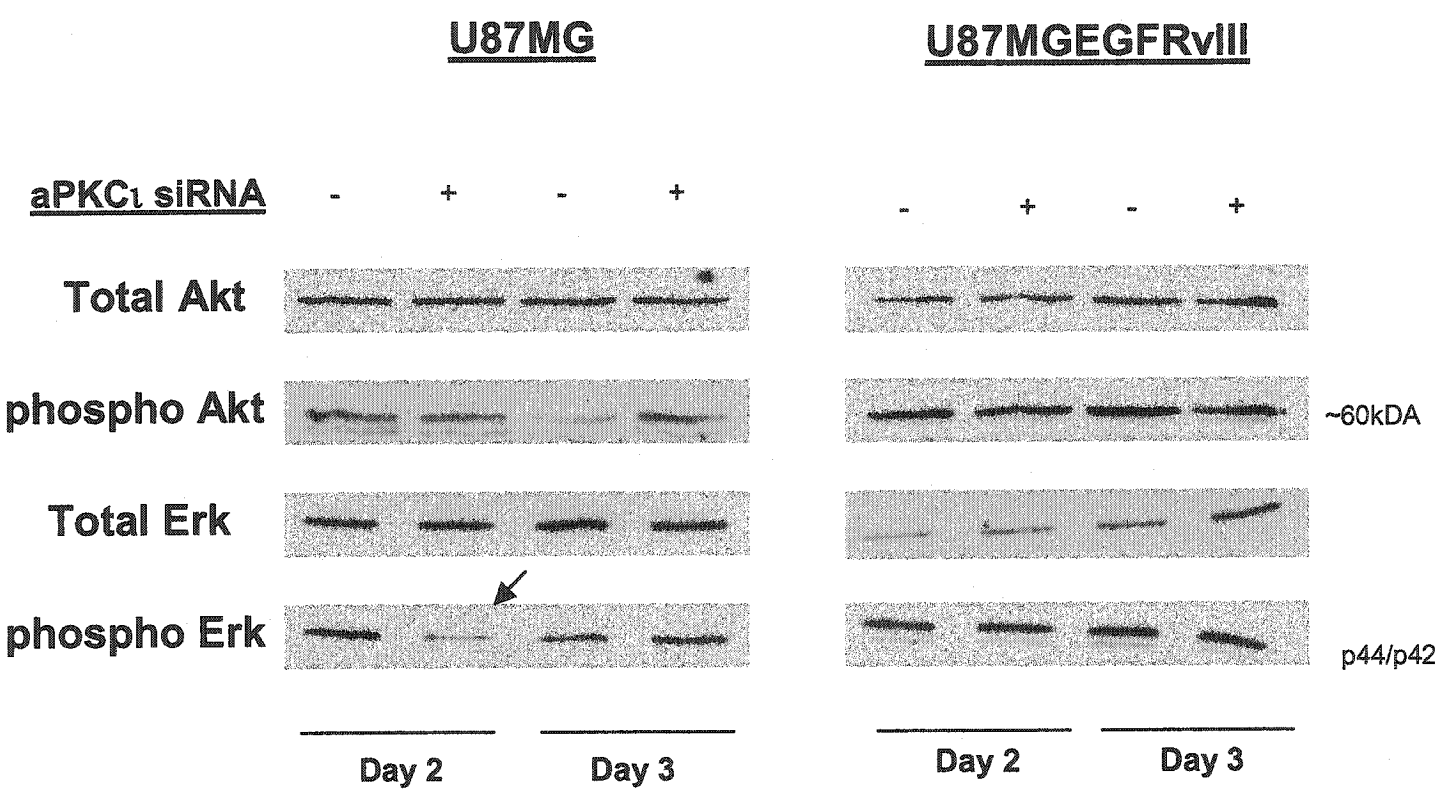

U87MG
U87MGEGFRvIII
aPKCι siRNA
- + - +
- + - +
Total Akt
phospho Akt
Total Erk
phospho Erk
~60kDA
p44/p42
Day 2
Day 3
Day 2
Day 3

Figure 2.8 Immunoblot analysis of U87MG and U87MGEGFRvIII signaling properties under atypical PKC iota RNAi

Chemically synthesized siRNA duplexes were used. Cells were transfected with siRNA duplexes, scramble II and atypical PKC iota, and harvested 2 and 3 days post transfection. 15μg, 25μg and 40 μg proteins of each cell lysates was used for total and phospho Erk, total Akt and phospho Akt, respectitively. Samples were separated on 12% acrylamide gels and immunoblotted with antibodies specified.

ongocenic factor allows for increased proliferation and survival (Lorimer and Lavictoire 2001). Therefore EGFRvIII may be so strongly activating ERK that it is able to compensate for the decrease in atypical PKC and its affect on the phosphorylation levels of ERK1/ERK2. In addition, RNA interference is at best 90-95% effective in knocking-down protein expression therefore EGFRvIII may still be so strongly signaling through the remaining atypical PKC iota that the levels of phosphorylated Erk was not affected. Since our SF-295 cell cycle analysis under conditions of atypical PKC iota RNA interference did not demonstrate atypical PKC iota as an important molecule for SF-295 proliferation, we decided to not include this cell line in our signaling studies.

2.3.6 Atypical PKC iota depletion sensitizes U87MG glioblastoma cells to cisplatin but not to etoposide

In order to assess if RNA interference of atypical PKC iota will chemosensitize glioblastoma, we have treated U87MG, U87MGEGFRvIII, and SF-295 cells that have had RNA interference of atypical PKC iota for a period of 48 hours with etoposide and cisplatin, two clinically relevant glioblastoma chemotherapeutic agents to which glioblastoma are frequently refractory (Jendrossek et al. 2001). Etoposide is a topoisomerase II inhibitor that inhibits DNA synthesis causing arrest in the G1 and mainly the late S and G2 phases of the cell cycle. Etoposide induces single and double stranded breaks in DNA causing DNA lesions. The lesions are due to the interference of etoposide with the scission-reunion reaction of topoisomerase II (Della Torre et al. 2003). Cisplatin is a platinum-containing,

broad activity antineoplastic agent that inhibits DNA replication by causing the formation of intra and interstand crosslinks in DNA (Della Torre et al. 2003). 50 μM of etoposide and 50 μM cisplatin were separately used to treat U87MG, U87MGEGFRvIII and SF-295 glioblastoma cells that were transfected with either: 1) no siRNA 2) the control non-specific scrambled siRNA or 3) the siRNA specifically targetting aPKCι. Drug treatments were performed on 48 hour post transfected cells for a period of 24 hours. Chemosensitization was assessed by the comparison of the percentage of apoptotic cells under each condition as determined by flow cytometry analysis using propidium iodide as a fluorescent nuclear dye to quantitatively analyze DNA content. For all three cell lines there was no increase in the percentage of apoptotic cells with etoposide when comparing the untransfected or the control non-specific II dsRNA transfection to the transfection with the dsRNA duplex specifically targeting atypical PKC iota. For all three cell lines, cisplatin by itself did increase the percentage of apoptotic cells. However, PKC iota depletion did chemosensitize U87MG to cisplatin induced apoptosis. U87MGΔEGFR and SF-295 cell lines did not exhibit the noted increase in apoptosis under PKCiota RNAi to cisplatin. Therefore, the presence of EGFRvIII in U87MGΔEGFR is likely imparting a protective effect to the drug perhaps by its differential signaling capacities or its ability to signal through the 5-10% remaining atypical PKC iota in the cells. In terms of SF-295, this glioblastoma cell line likely carries a different set of yet uncharacterized mutations than U87MG, which may be allowing it to be resistant to the effects of cisplatin **(Table 2.3)**.

Table 2.3

% Apoptosis

U87MG

	50 μM Etoposide	50 μM Cisplatin
Non transfected	21.6 ± 1.25	6.405 ± 0.05
Scramble II duplex	24.44 ± 0.96	6.30 ± 0.59
PKC iota duplex	25.49 ± 1.16	18.19 ± 0.02

U87MGEGFRvIII

	50 μM Etoposide	50 μM Cisplatin
Non transfected	10.99 ± 0.44	9.51 ± 3.26
Scramble II duplex	11.75 ± .84	7.77 ± 0.84
PKC iota duplex	10.60 ± 0.60	6.14 ± 0.79

SF-295

	50 μM Etoposide	50 μM Cisplatin
Non transfected	17.87 ± 1.98	17.15 ± 1.27
Scramble II duplex	20.13 ± 0.05	18.69 ± 2.00
PKC iota duplex	22.15 ± 2.67	20.13 ± 1.7

Table 2.3 Chemosensitization experiments of U87MG, U87MGΔEGFR, and SF-295 subject to atypical PKC iota RNA interference

Cells were transfected with either the scramble II siRNA or atypical PKC iota siRNA duplexes. 2 days post transfection, the cells were treated to either 50μM etoposode or cisplatin for 24 hours. Cells were then harvested for analysis by flow cytometry using propidium iodide. These results are representative of 3 experiments done in duplicate. The baseline % apoptosis values varied per experiment however, the trends were reproducible.

2.3.7 Scratch-wound/invasion assay

Recent evidence has highlighted the role of atypical PKC in complexes with Par 6 and Par 3 in the establishment and maintenance of cell polarity and directed cell migration (Etienne-Manneville and Hall 2003). The association between atypical PKC and Par 6 is direct and occurs at the amino-terminal domains of each protein. In epithelial cells, Par6, Par 3 and atypical PKC co-localize at cell-cell contacts (Ohno 2001). In addition during astrocyte migration, Par6 and atypical PKC are both localized to the leading edge of migrating cells (Etienne-Manneville and Hall 2001). The function of the Par6-Par3-aPKC complex has been shown to be essential for the polarization of *C.elegans* embryogenesis (Watts, Etemad-Moghadam et al. 1996), the asymmetric division of neuroblasts in Drosophila, oocyte differentiation (Huynh et al. 2001), and has been conserved in *Xenopus*, where it localizes to the animal pole of oocytes (Nakaya et al. 2000). Cell-cell adhesion is an important factor determining the invasiveness of cancer cells. In primary ovarian carcinomas, there is increased expression of E-cadherin, a calcium dependent transmembrane protein key to cell-cell adhesion of epithelial cells (Maines-Bandiera and Auersperg 1997). Interestingly, it has been shown that Par3 expression levels are upregulated as a consequence of E-cadherin mediated adhesion, possibly implicating atypical PKC in the process. Over-expression of Par3 was shown to suppress contact-mediated inhibition of cell migration in epithelial cells (Mishima et al. 2002). Given these important roles in cell migration and polarity it is possible that these processes are aberrant in cancerous cells allowing for increased invasive and migratory capacities. We therefore became interested in studying the

role of atypical PKC iota in glioblastoma invasion through inhibition studies using atypical PKC siRNA duplexes.

The capacity of the U87MG to migrate under conditions of PKC RNAi was assessed by the scratch wound assay. At the start of the experiment an initial straight-line injury is inflicted to the cells. The ability to fill in the wound area is an indication of the cells migratory capacity. The migratory capacities of U87MG cells transfected with the scrambled II siRNA duplex were compared to those transfected with the atypical PKC iota siRNA duplex 48 hours post transfection. No major differences in the capability to migrate into the wound were observed under either condition, with almost full recovery at the time of fixation **(Figure 2.9)**. Therefore our results suggest that the depletion of atypical PKC iota by RNAi in U87MG cells does not affect their ability to migrate. However, a more quantitative assay such as the Matrigel invasion assay (Hendrix et al. 1987) might show small differences that are not detected with the more qualitative scratch-wound assay.

2.4 Discussion

The specific role of atypical PKC iota in glioblastoma multiforme has not been extensively examined. The present work was intended to examine the role of atypical PKC iota in glioblastoma multiforme as a first step to determine if it could serve as a target for glioblastoma multiforme therapy. As proven by RT-PCR in our lab, U87MG glioblastoma cells only express atypical PKC iota; we therefore used this cell line as our model system to examine the role of atypical PKC iota.

Figure 2.9

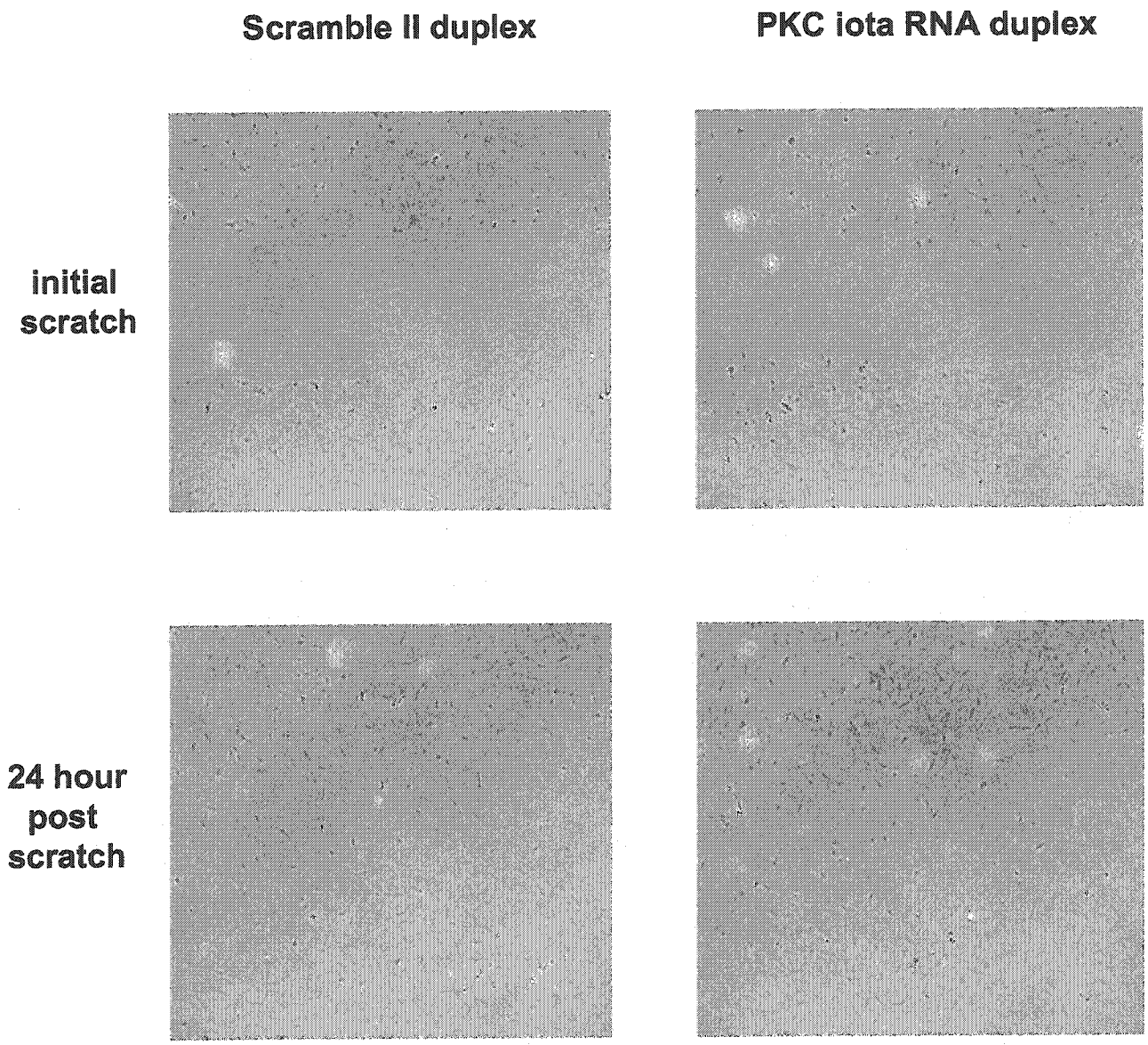

Scramble II duplex
PKC iota RNA duplex
initial scratch
24 hour post scratch

Figure 2.9 Scratch-wound assay of U87MG subject to atypical PKC iota RNAi

U87MG cells were transfected with either the scramble II siRNA or atypical PKC iota siRNA duplexes. 48 hours post transfection, the cell were subjected to a straight-line scratch from a pipet tip. Cells were then fixed in 4% paraformaldehyde 24 hours after the initial scratch.

Using RNA interference (RNAi), we have analyzed the role of atypical PKC iota in U87MG, U87MGΔEGFR, and SF-295 glioblastoma cell lines. The specificity of our atypical PKC iota targetting duplex is supported by the results of a BLAST search of the 21 nt antisense strand not fully matching with any other PKC mRNA, in particular that of atypical PKC zeta. Thus the decrease in expression due to RNAi in all our glioblastoma cells lines is in fact that of only atypical PKC iota. In addition, upon examination of the basal level of expression of other kinases in the glioblastoma cells lines, namely PKB/Akt and ERK, their respective levels were not affected due to the atypical PKC iota RNAi. Our atypical PKC iota targeting duplex is therefore highly specific and efficient in depleting the levels of atypical PKC iota. By this highly specific method, we were successful in decreasing the expression of atypical PKC iota in U87MG, SF-295, and U87MGΔEGFR cell lines by approximately 80-90% for at least 72 hours post transfection.

Earlier studies using dominant negative mutants, selective pseudosubstrate inhibitors, or antisense oligonucleotides of atypical PKC suggest a possible role for these enzymes in controlling cell proliferation either by the positive regulation of ERK (Sajan et al. 1999) and NF-κB (Wooten 1999), negative regulation of PKB/Akt (Wen et al. 2003) or by association with cyclin dependent kinase 7 (Acevedo-Duncan et al. 2002). We therefore examined the effects of atypical PKC iota depletion on the proliferation of U87MG, U87MGΔEGFR, and SF-295 glioblastoma cells. Under conditions of atypical PKC iota RNAi, we saw a significant accumulation of the U87MG cellular population in G0/G1. These results are indicative of growth inhibition due to the specific inhibition of atypical PKC iota in U87MG

glioblastoma cells. Our noted effects of atypical PKC iota on proliferation are therefore in agreement with the earlier atypical PKC inhibition studies.

Atypical PKC iota RNAi did not cause any significant growth inhibition in the U87MGΔEGFR and SF-295 glioblastoma cell lines. In terms of U87MGΔEGFR cells, our data suggest that the presence of EGFRvIII allows for cell cycle progression despite the depletion of atypical PKC iota. EGFRvIII is an oncogene known to participate in aberrant sustained proliferative signaling (Lorimer and Lavictoire 2001; Lorimer 2002). Therefore EGFRvIII in U87MGΔEGFR glioblastoma cells likely prevents a decrease in proliferation under conditions of atypical PKC iota RNAi by circumventing the depleted levels of atypical PKC iota via its aberrant signaling capacities or its ability to signal through the remaining depleted levels of atypical PKC iota.

The cell cycle is highly regulated by the activity of cyclin-dependent kinases (cdks). $p27^{Kip1}$ is a negative regulator of nuclear cdk2. Cdk2 is essential for the G1 to S cell cycle transition. Therefore, by inhibiting cdk2 in the nucleus, $p27^{Kip1}$ blocks the cell cycle at G1 (Slingerland and Pagano 2000). $p27^{Kip1}$ has been shown to be a tumor suppressor gene as $p27^{Kip1}$-/- mice spontaneously develop pituitary tumors (Nakayama et al. 1996) . We have shown that the reduction of atypical PKC iota by RNA interference led to an increase in the total level of $p27^{Kip1}$ and an increase in the level of nuclear $p27^{Kip1}$ in U87MG glioblastoma cells coinciding with a decrease in proliferation. Our data suggests that the increase in $p27^{Kip1}$ due to atypical PKC iota RNAi contributes to U87MG growth inhibition by increasing the cellular population of U87MG in G0/G1. Atypical PKCs have already been shown to modulate the cell cycle via the cyclin cdk inhibitor $p21^{CIP1}$ (Scott et al. 2002).

Atypical protein kinase C fractionated from mammalian cells phosphorylates $p21^{Cip1}$ at the Ser146 site. Expression of atypical PKC zeta or activation of the endogenous kinase by 3-phosphoinositide dependent protein kinase-1 (PDK1) decreased the half-life of p21. Conversely, dnPKCzeta or dnPDK1 increased p21 protein half-life, and a PDK1-dependent increase in the rate of p21 degradation was mediated by aPKC. Thus, aPKC acts as a modulator of the cell cycle by providing a signal for the $p21^{Cip1}$ degradation. Our results support a possible novel mechanism by which atypical PKC iota can modulate the cell cycle and promote U87MG proliferation by negatively mediating $p27^{Kip1}$ activity by altering its expression and cellular localization. Additional atypical PKC iota overexpression and knock-out studies will need to be performed to elucidate the role of atypical PKC iota modulation of the cell cycle via $p27^{Kip1}$. Such studies will allow for a more detailed look at the expression level, half-life and nuclear localization of $p27^{Kip1}$ due to atypical PKC iota.

Atypical PKC iota RNAi in U87MGΔEGFR cells also caused an increase in $p27^{Kip1}$ expression, however basal levels and upregulated levels were significantly lower than U87MG parental cells. Narita *et al.* have shown that the constitutive activation of the PI3-K/Akt pathway by EGFRvIII inhibits the expression level of $p27^{Kip1}$ thereby contributing to enhanced proliferation of human glioblastoma cells lines (Narita et al. 2002). Therefore our observations of the low basal and atypical PKC iota RNAi stimulated levels of $p27^{Kip1}$ in U87MGΔEGFR cells may be due to EGFRvIII oncogenic mediated downregulation of $p27^{Kip1}$ through the PI3-K/Akt pathway. In such a way EGFRvIII allows for uncontrolled proliferation and thus U87MGΔEGFR under atypical PKC iota RNAi may be able to prevent

a significant increase in G0/G1.

Two post-transcriptional mechanisms regulating $p27^{Kip1}$ activity have been described. Firstly, the activation of specific signaling pathways can induce ubiquitin proteasomal degradation of $p27^{Kip1}$ (Shirane et al. 1999). Secondly, the overexpression of cdks (Sandhu and Slingerland 2000) or the activation of cellular signaling pathways can lead to the cytoplasmic sequestration of $p27^{Kip1}$ preventing nuclear cdk2 inhibition and growth arrest (Shin et al. 2002). Transcriptional regulation of $p27^{Kip1}$ by the forkhead transcription factor has also been shown (Medema et al. 2000). Studies have reported that the subcellular localization of $p27^{Kip1}$ mediates its function in cancer-cell proliferation (Shin et al. 2002). In terms of our work, the increase in $p27^{Kip1}$ expression under conditions of atypical PKC iota RNAi may be due to an increase in protein stabilization or a change in translational or transcription regulation.

Cytoplasmic redistribution of $p27^{Kip1}$ has been reported in Barrett's-associated adenocarcinoma, colorectal tumors, and breast cancers(Liang et al. 2002) and has been linked to decreased patient survival (Zeng et al. 2000). Whether $p27^{Kip1}$ cytoplasmic redistribution is a marker for glioblastoma multiforme patient survival remains to be to elucidated. Cytoplasmic sequestration of $p27^{Kip1}$ has been shown to be controlled in part by phosphorylation of threonine 157 (Liang et al. 2002; Shin et al. 2002; Viglietto et al. 2002). Such data suggested that PKB/Akt was responsible for this phosphorylation in the cytoplasmic localization sequence of $p27^{Kip1}$ (Liang et al. 2002; Shin et al. 2002). One possible explanation for our results is that atypical PKC iota could directly phosphorylates $p27^{Kip1}$ at threonine 157, either instead or in addition to PKB/Akt, thereby directly causing

cytoplasmic redistribution of $p27^{Kip1}$ in U87MG. A recent study of the cdk inhibitor $p21^{WAF1/CIP1}$, highlights common phosphorylation sites for both PKB/Akt and atypical PKC iota (Scott et al. 2002). Atypical PKC iota was shown to phosphorylate $p21^{WAF1/CIP1}$ at the underlined serine in GRKRRQTSM. PKB/Akt phosphorylates $p21^{WAF1/CIP1}$ at the adjacent threonine residue. In $p27^{Kip1}$, the proposed PKB/Akt phosphorylation site is IRKRRPATD. The latter matches considerably to the atypical PKC iota site in $p21^{WAF1/CIP1}$ when comparing the basic residues and their spacing from the phosphoylated residue. However, our preliminary immunoprecipitation data suggests that atypical PKC iota does not affect phosphorylation of $p27^{Kip1}$ at threonine 157 in U87MG cells.

Another possibility is that atypical PKC iota may modulate $p27^{Kip1}$ cellular localization by phosphorylation at a site other than threonine 157. To begin to elucidate the phosphorylation of $p27^{Kip1}$, we are currently performing immunoprecipitations of $p27^{Kip1}$ in U87MG and U87MGΔEGFR cells to perform immunoblot analysis using a phospho-(Ser/Thr) Akt substrate antibody. This antibody recognizes phosphorylated Akt substrates with a Ser/Thr in a conserved motif characterized by Arg at positions –5 and –3.

It is also possible that atypical PKC iota may indirectly modulate $p27^{Kip1}$ subcellular localization. Atypical protein kinase C isozymes lambda and zeta have been implicated in Ras mediated reorganization of the actin cytoskeleton and cyclin D1-induction (Hellbert et al. 2000). Perhaps, atypical PKC iota RNAi may decrease the levels of cyclin D, thereby allowing for an increase in the unbound form of $p27^{Kip1}$ and its nuclear translocation.

Despite the many possibilities of $p27^{Kip1}$ regulation in glioblastoma multiforme, our results and data from other labs suggest that both atypical PKC iota and PKB/Akt are able to

regulate $p27^{Kip1}$ expression and localization. However as previously mentioned, whether the phosphorylation by these two serine/threonine kinases occurs on the same site remains to be examined. The hypothesis that best fits our data is the following: U87MG cells carry a PTEN mutation allowing for low levels of PI3-K activity. Under these conditions, atypical PKC iota would be mainly responsible for $p27^{Kip1}$ phosphorylation resulting in its depletion and decrease in proliferation; U87MGEGFRvIII cells carry both a mutation in PTEN and EGFRvIII allowing for high levels of PI3-K activity (Lorimer and Lavictoire 2001). Thus the high levels of activated PKB/Akt allow for $p27^{Kip1}$ phosphorylation by PKB/Akt. Thus may explain why we saw no effect on proliferation when atypical PKC iota was depleted by RNAi in U87MGEGFRvIII cells; the effect of atypical PKC iota depletion in conjunction with an increase in $p27^{Kip1}$ was circumvented by the high level of PI3-K activity.

Our results suggesting atypical PKC iota affects proliferation by the negative modulation of $p27^{Kip1}$ expression and its cytoplasmic redistribution is likely not the only method by which atypical PKC iota can affect proliferation. There are likely several other methods by which atypical PKC iota can affect proliferation. Atypical PKC has been shown to phosphorylate the cdk inhibitor $p21^{WAF1/CIP1}$ and lead to its degradation in a PDK1-dependent fashion. Research has also shown atypical PKCs to positively regulate Erk, a kinase that has been implicated in cell survival (Berra et al. 1993), an observation that is in agreement with our transient decrease in Erk phosphorylation under atypical PKC iota RNAi in U87MG.

Inhibition of atypical PKCζ by stable transfection of a kinase dead dominant negative PKCζ in human leukemic and colon cancer cells sensitized these cells to both etoposide and

cisplatin induced apoptosis, respectively (Filomenko et al. 2002) . We were able to sensitize U87MG with depleted atypical PKC iota to cisplatin. Cisplatin, a platinum containing antineoplastic alkylating agent, inhibits DNA synthesis by cross-linking strands of DNA and carries out its activity in late G1 and early S phases of the cell cycle. Entering the cell by diffusion, cisplatin's chloride is replaced with water forming the active species which reacts with DNA to form intra and interstrand crosslinks thereby inhibiting DNA replication (Lawrence et al. 2003). Given the fact that atypical PKC iota RNAi caused U87MG cellular population accumulation in G0/G1, these cells were likely more susceptible to cisplatin's cytotoxic effects. We were therefore able to sensitize these cells. Our results therefore indicate that atypical PKC iota plays a role in U87MG glioblastoma cell survival.

Atypical PKC iota RNAi in U87MGΔEGFR and SF-295 glioblastoma cells had no effect on their response to cisplatin. In the case of U87MGΔEGFR, EGFRvIII is a known chemoresistant factor to cisplatin in part due to its constitutive activation of the Akt/PI3-K and Erk pathways promoting uncontrolled proliferation and survival (Nagane et al. 2001). EGFRvIII could therefore possibly circumvent the depleted levels of atypical PKC iota or even signal through the atypical PKC iota remaining after RNAi. Our results indicate that the distinct set of mutations carried by these two cells lines allows them to maintain resistance to cisplatin under conditions of depleted atypical PKC iota, unlike the U87MG glioblastoma cell line.

We were unable to sensitize U87MG, U87MGΔEGFR and SF-295 with atypical PKC iota RNAi to etoposide. Etoposide is a topoisomerase II inhibitor that inhibits cell division by producing protein-associated DNA double stranded breaks. Etoposide acts in late S and

early G2 phases of the cell cycle (Arimondo and Helene 2001). We have shown that atypical PKC iota RNAi causes a decrease in proliferation of U87MG glioblastoma cells due to the inhibition of $p27^{Kip1}$ activity. The cells subsequently accumulate in G0/G1. Therefore it is logical that these cells would not be sensitized to etoposide, a G2/S acting drug. In actuality, U87MG cells under atypical PKC iota RNAi would have a decrease proportion of cells in G2/S thereby even further lessening the effects of etoposide on these cells. U87MG, U87MGΔEGFR, and SF-295 glioblastoma cell lines may also utilize other uncharacterized pathways to maintain survival to etoposide under our experimental conditions.

Due to atypical PKC iota's apparent role in U87MG glioblastoma proliferation, we examined two signaling pathways involved in survival and proliferation namely, PI3-K/PKB/Akt and ERK. Atypical PKCs have been shown to negatively regulate PKB/Akt (Mao et al. 2000). Atypical PKC ζ and, to a lesser extent, atypical PKC ι/λ negatively regulate PKB/Akt by physically and functionally interaction in breast cancer cells. Such an interaction causes an inhibition of activation dependent phosphorylation of Akt at Ser 473 and Thr 308 (Mao et al. 2000). Using a phospho-specific PKB/Akt antibody, we found that atypical PKC iota RNA interference did not modulate phosphorylation levels of PKB/Akt on Ser 473 in both U87MG and U87MGΔEGFR. Our data suggests that atypical PKC iota does not alter PKB/Akt at the level of phosphorylation. Thus the activation of PKC iota through the PI3-K pathway likely does not serve to limit signaling through the PI3-K/PKB/Akt signaling cascade. Our results possibly implicate atypical PKCζ as the more effective negative regulator of PKB/Akt as suggested by Mao *et al.* (Mao et al. 2000). One possibility is that the cross regulation between PKB/Akt and PKC signaling is isoform specific. If in

fact, atypical PKC ζ is the main regulator of PKB/Akt, U87MG cells that only express atypical PKC iota, under atypical PKC iota RNAi should not have decreased levels of phosphorylated PKB/Akt. However, examination of PKB/Akt activity and subsequent phosphorylation levels of downstream PKB/Akt targets would need to be analyzed in U87MG. In addition, analysis of phosphorylation levels of PKB/Akt in cells solely expressing atypical PKC iota ζ and atypical PKC ι, under RNA interference would also help clarify our results.

Atypical PKC iota RNA interference did cause a transient decrease in the phosphorylation levels of ERK1/ERK2 (p44/p42 Map Kinase) in U87MG but not in U87MGEGFRvIII. These results suggest atypical PKC iota to be a positive regulator of Erk signaling, a finding that is in agreement with recent literature (Berra et al. 1993). It is known that EGFRvIII constitutively activates the ERK signaling pathway, one mechanism by which this oncogenic factor allows for increased proliferation and survival (Lorimer and Lavictoire 2001). Therefore in the case of the U87MGΔEGFR cells examined, EGFRvIII may be so strongly activating ERK that it is able to compensate for the decrease in atypical PKC and its affect on the phosphorylation levels of ERK1/ERK2. In addition, RNA interference is not 100% effective in knocking-down protein expression (Elbashir et al. 2002) therefore EGFRvIII may still be so strongly signaling through the remaining atypical PKC iota that the levels of phosphorylated ERK was not affected. Therefore while in the case of U87MG atypical PKC iota modulates phosphorylation levels of ERK1/ERK2, the presence of EGFRvIII in U87MGΔEGFR seems to obliterate this phenomenon. Despite the fact that ERK does play a role in proliferation, we do not believe that this transient decrease in

ERK1/ERK2 phosphorylation is responsible for the growth inhibition of U87MG under atypical PKC iota RNAi. The more compelling hypothesis is that the atypical PKC iota directly phosphorylates $p27^{Kip1}$ and causes $p27^{Kip1}$ cytoplasmic sequestration at low levels of PI3-K activity.

Atypical PKC iota RNAi did not decrease the migratory capacity of U87MG cells. These results were not expected given the role atypical PKCs play in cellular polarization, migration, and invasion (Etienne-Manneville and Hall 2001; Suzuki et al. 2001; Yamanaka et al. 2001; Etienne-Manneville and Hall 2003). The remaining 10-20% of atypical PKC iota remaining after RNAi may be adequate to maintain a functional formation of the Par3/Par6/atypical PKC iota complexes with increased half-lives thereby still maintaining its role in migration. Also, these results may indicate that atypical PKC iota does not form part of this complex. All literature points to atypical PKC zeta forming such functional complexes to play a role in migration (Etienne-Manneville and Hall 2001; Etienne-Manneville and Hall 2003). Our results therefore may illustrate the functional differences of each specific PKC isoform.

Overall, our work has shown a role for atypical PKC iota in proliferation and chemosensitization to cisplatin in U87MG glioblastoma cells. Unfortunately the depletion of atypical PKC iota levels has no effect on proliferation and chemosensitization in U87MGΔEGFR and SF-295. Despite the fact that our observations do not seem to be a universal phenomenon in glioblastoma multiforme, it may be occurring in certain glioblastoma subsets. Therefore, a combination of atypical PKC iota depletion by RNAi, agents targetting specific aberrations, radiation and chemotherapy may prove to be a more

effective approach to inhibit proliferation and chemosensitize specific subsets of glioblastoma multiforme.

Further studies are required to determine whether targeting atypical PKC iota is an effective means of sensitizing glioblastoma multiforme to chemotherapy. A wider panel of glioblastoma cell lines would provide a more general examination of the physiological role of atypical PKC iota in glioblastoma. A more detailed examination of the signaling pathways affected by atypical PKC iota RNAi would provide more insight into the mechanism by which this kinase can possibly affect proliferation possibly by regulation of pro- and anti-apoptotic molecules, like NFκB and XIAP (Garcia-Cao et al. 2003), and cdk inhibitors, like $p27^{Kip1}$ and $p21^{Cip1}$ (Scott et al. 2002). Overexpression and knock-out studies would provide further valuable information on the role of atypical PKC iota in glioblastoma proliferation. In terms of chemosensitization studies, the design of pharmacologically useful atypical PKC iota inhibitors to test in animal models is an area that would provide much insight into the role of atypical PKC iota in glioblastoma multiforme.

In summary, we have shown atypical PKC iota to play a role in U87MG proliferation, apoptosis, and chemoresistance. Additionally, we have likely identified a novel mechanism by which atypical PKC iota can regulate U87MG cell proliferation. Our results suggest that atypical PKC iota can regulate both the levels and subcellular localization of $p27^{Kip1}$ possibly by direct phosphorylation of $p27^{Kip1}$. Given that atypical PKC iota RNAi caused a decrease in U87MG proliferation and chemosensitized these cells to cisplatin-induced apoptosis, our results therefore support atypical PKC iota as a possible novel therapeutic target in glioblastoma multiforme.

REFERENCES

Acevedo-Duncan, M., R. Patel, et al. (2002). "Human glioma PKC-iota and PKC-betaII phosphorylate cyclin-dependent kinase activating kinase during the cell cycle." Cell Prolif **35**(1): 23-36.

Adey, N. B., L. Huang, et al. (2000). "Threonine phosphorylation of the MMAC1/PTEN PDZ binding domain both inhibits and stimulates PDZ binding." Cancer Res **60**(1): 35-7.

Aguzzi, A., S. Brandner, et al. (1995). "Transgenic and gene disruption techniques in the study of neurocarcinogenesis." Glia **15**(3): 348-64.

Altschul, S. F., W. Gish, et al. (1990). "Basic local alignment search tool." J Mol Biol **215**(3): 403-10.

Arimondo, P. B. and C. Helene (2001). "Design of new anti-cancer agents based on topoisomerase poisons targeted to specific DNA sequences." Curr Med Chem Anti-Canc Agents **1**(3): 219-35.

Bajenaru, M. L., Y. Zhu, et al. (2002). "Astrocyte-specific inactivation of the neurofibromatosis 1 gene (NF1) is insufficient for astrocytoma formation." Mol Cell Biol **22**(14): 5100-13.

Balendran, A., A. Casamayor, et al. (1999). "PDK1 acquires PDK2 activity in the presence of a synthetic peptide derived from the carboxyl terminus of PRK2." Curr Biol **9**(8): 393-404.

Baltuch, G. H., N. P. Dooley, et al. (1995). "Protein kinase C isoform alpha overexpression in C6 glioma cells and its role in cell proliferation." J Neurooncol **24**(3): 241-50.

Baltuch, G. H., N. P. Dooley, et al. (1995). "Protein kinase C and growth regulation of malignant gliomas." Can J Neurol Sci **22**(4): 264-71.

Baltuch, G. H. and V. W. Yong (1996). "Signal transduction for proliferation of glioma cells in vitro occurs predominantly through a protein kinase C-mediated pathway." Brain Res **710**(1-2): 143-9.

Barbero, S., R. Bonavia, et al. (2003). "Stromal cell-derived factor 1alpha stimulates human glioblastoma cell growth through the activation of both extracellular signal-regulated kinases 1/2 and Akt." Cancer Res **63**(8): 1969-74.

Barradas, M., A. Monjas, et al. (1999). "The downregulation of the pro-apoptotic protein Par-4 is critical for Ras-induced survival and tumor progression." Embo J **18**(22): 6362-9.

Bass, B. L. (2001). "RNA interference. The short answer." Nature **411**(6836): 428-9.
Behin, A., K. Hoang-Xuan, et al. (2003). "Primary brain tumours in adults." Lancet **361**(9354): 323-31.

Bello, L., V. Lucini, et al. (2001). "Simultaneous inhibition of glioma angiogenesis, cell proliferation, and invasion by a naturally occurring fragment of human metalloproteinase-2." Cancer Res **61**(24): 8730-6.

Benjamin, R., J. Capparella, et al. (2003). "Classification of glioblastoma multiforme in adults by molecular genetics." Cancer J **9**(2): 82-90.

Bennett, C. F. (2002). "Efficiency of antisense oligonucleotide drug discovery." Antisense Nucleic Acid Drug Dev **12**(3): 215-24.

Berra, E., M. T. Diaz-Meco, et al. (1993). "Protein kinase C zeta isoform is critical for mitogenic signal transduction." Cell **74**(3): 555-63.

Besson, A. and V. W. Yong (2000). "Involvement of p21(Waf1/Cip1) in protein kinase C alpha-induced cell cycle progression." Mol Cell Biol **20**(13): 4580-90.

Biondi, R. M., A. Kieloch, et al. (2001). "The PIF-binding pocket in PDK1 is essential for activation of S6K and SGK, but not PKB." Embo J **20**(16): 4380-90.

Botturi, M. and L. Fariselli (1998). "Clinical results of unconventional fractionation radiotherapy in central nervous system tumors." Tumori **84**(2): 176-87.

Bredel, M. and I. F. Pollack (1997). "The role of protein kinase C (PKC) in the evolution and proliferation of malignant gliomas, and the application of PKC inhibition as a novel approach to anti-glioma therapy." Acta Neurochir (Wien) **139**(11): 1000-13.

Brown, P. D. (2000). "Ongoing trials with matrix metalloproteinase inhibitors." Expert Opin Investig Drugs **9**(9): 2167-77.

Cacace, A. M., M. Ueffing, et al. (1998). "Overexpression of PKCepsilon in R6 fibroblasts causes increased production of active TGFbeta." J Cell Physiol **175**(3): 314-22.

Caplen, N. J. (2003). "RNAi as a gene therapy approach." Expert Opin Biol Ther **3**(4): 575-86.

Carey, I., C. L. Williams, et al. (1999). "Overexpression of protein kinase C-alpha in MCF-7 breast cancer cells results in differential regulation and expression of alphavbeta3 and alphavbeta5." Int J Oncol **15**(1): 127-36.

Chen, W. S., P. Z. Xu, et al. (2001). "Growth retardation and increased apoptosis in mice with homozygous disruption of the Akt1 gene." Genes Dev **15**(17): 2203-8.

Choe, G., S. Horvath, et al. (2003). "Analysis of the phosphatidylinositol 3'-kinase signaling pathway in glioblastoma patients in vivo." Cancer Res **63**(11): 2742-6.

Connor, M. K., R. Kotchetkov, et al. (2003). "CRM1/Ran-Mediated Nuclear Export of p27(Kip1) Involves a Nuclear Export Signal and Links p27 Export and Proteolysis." Mol Biol Cell **14**(1): 201-13.

Couldwell, W. T., J. P. Antel, et al. (1992). "Protein kinase C activity correlates with the growth rate of malignant gliomas: Part II. Effects of glioma mitogens and modulators of protein kinase C." Neurosurgery **31**(4): 717-24; discussion 724.

Couldwell, W. T., D. R. Hinton, et al. (1994). "Protein kinase C inhibitors induce apoptosis in human malignant glioma cell lines." FEBS Lett **345**(1): 43-6.

Couldwell, W. T., J. H. Uhm, et al. (1991). "Enhanced protein kinase C activity correlates with the growth rate of malignant gliomas in vitro." Neurosurgery **29**(6): 880-6; discussion 886-7.

da Rocha, A. B., D. R. Mans, et al. (2000). "Protein kinase C-mediated in vitro invasion of human glioma cells through extracellular-signal-regulated kinase and ornithine decarboxylase." Pathobiology **68**(3): 113-23.

da Rocha, A. B., D. R. Mans, et al. (2002). "Targeting protein kinase C: new therapeutic opportunities against high-grade malignant gliomas?" Oncologist **7**(1): 17-33.

Dai, C. and E. C. Holland (2003). "Astrocyte differentiation states and glioma formation." Cancer J **9**(2): 72-81.

Della Torre, S., G. Procopio, et al. (2003). "Current treatments of neuroendocrine tumors role of biotherapy and chemotherapy." Tumori **89**(2): 111-6.

Di Cristofano, A., B. Pesce, et al. (1998). "Pten is essential for embryonic development and tumour suppression." Nat Genet **19**(4): 348-55.

Di Rocco, F., R. S. Carroll, et al. (1998). "Platelet-derived growth factor and its receptor expression in human oligodendrogliomas." Neurosurgery **42**(2): 341-6.

Diaz-Meco, M. T., M. M. Municio, et al. (1996). "The product of par-4, a gene induced during apoptosis, interacts selectively with the atypical isoforms of protein kinase C." Cell **86**(5): 777-86.

Diaz-Meco, M. T., M. M. Municio, et al. (1996). "Lambda-interacting protein, a novel protein that specifically interacts with the zinc finger domain of the atypical protein kinase C isotype lambda/iota and stimulates its kinase activity in vitro and in vivo." Mol Cell Biol **16**(1): 105-14.

Dominguez, I., L. Sanz, et al. (1993). "Inhibition of protein kinase C zeta subspecies blocks the activation of an NF-kappa B-like activity in Xenopus laevis oocytes." Mol Cell Biol **13**(2): 1290-5.

Donehower, L. A., M. Harvey, et al. (1992). "Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours." Nature **356**(6366): 215-21.

Donson, A. M., A. Banerjee, et al. (2000). "Protein kinase C zeta isoform is critical for proliferation in human glioblastoma cell lines." J Neurooncol **47**(2): 109-15.

Dooley, N. P., G. H. Baltuch, et al. (1998). "Apoptosis is induced in glioma cells by antisense oligonucleotides to protein kinase C alpha and is enhanced by cycloheximide." Neuroreport **9**(8): 1727-33.

Downward, J. (1998). "Ras signalling and apoptosis." Curr Opin Genet Dev **8**(1): 49-54.

Dutil, E. M., A. Toker, et al. (1998). "Regulation of conventional protein kinase C isozymes by phosphoinositide-dependent kinase 1 (PDK-1)." Curr Biol **8**(25): 1366-75.

Elbashir, S. M., J. Harborth, et al. (2001). "Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells." Nature **411**(6836): 494-8.

Elbashir, S. M., J. Harborth, et al. (2002). "Analysis of gene function in somatic mammalian cells using small interfering RNAs." Methods **26**(2): 199-213.

Elbashir, S. M., W. Lendeckel, et al. (2001). "RNA interference is mediated by 21- and 22-nucleotide RNAs." Genes Dev **15**(2): 188-200.

Elbashir, S. M., J. Martinez, et al. (2001). "Functional anatomy of siRNAs for mediating efficient RNAi in Drosophila melanogaster embryo lysate." Embo J **20**(23): 6877-88.

English, J. M., G. Pearson, et al. (1998). "Identification of substrates and regulators of the mitogen-activated protein kinase ERK5 using chimeric protein kinases." J Biol Chem **273**(7): 3854-60.

Esteve, P. O., O. Robledo, et al. (2002). "Induced expression of MMP-9 in C6 glioma cells is inhibited by PDGF via a PI 3-kinase-dependent pathway." Biochem Biophys Res Commun **296**(4): 864-9.

Etienne-Manneville, S. and A. Hall (2001). "Integrin-mediated activation of Cdc42 controls cell polarity in migrating astrocytes through PKCzeta." Cell **106**(4): 489-98.

Etienne-Manneville, S. and A. Hall (2003). "Cell polarity: Par6, aPKC and cytoskeletal crosstalk." Curr Opin Cell Biol **15**(1): 67-72.

Filomenko, R., F. Poirson-Bichat, et al. (2002). "Atypical protein kinase C zeta as a target for chemosensitization of tumor cells." Cancer Res **62**(6): 1815-21.

Fire, A., S. Xu, et al. (1998). "Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans." Nature **391**(6669): 806-11.

Fisher, G. H., S. Orsulic, et al. (1999). "Development of a flexible and specific gene delivery system for production of murine tumor models." Oncogene **18**(38): 5253-60.

Franke, T. F., D. R. Kaplan, et al. (1997). "Direct regulation of the Akt proto-oncogene product by phosphatidylinositol-3,4-bisphosphate." Science **275**(5300): 665-8.

Fruman, D. A., R. E. Meyers, et al. (1998). "Phosphoinositide kinases." Annu Rev Biochem **67**: 481-507.

Fueyo, J., C. Gomez-Manzano, et al. (1998). "Suppression of human glioma growth by adenovirus-mediated Rb gene transfer." Neurology **50**(5): 1307-15.

Garcia-Cao, I., M. J. Lafuente, et al. (2003). "Genetic inactivation of Par4 results in hyperactivation of NF-kappaB and impairment of JNK and p38." EMBO Rep **4**(3): 307-12.

Gasparetto, E. L., D. Warszawiak, et al. (2003). "Glioblastoma multiforme of the pineal region: case report." Arq Neuropsiquiatr **61**(2B): 468-72.

Guha, A., K. Dashner, et al. (1995). "Expression of PDGF and PDGF receptors in human astrocytoma operation specimens supports the existence of an autocrine loop." Int J Cancer **60**(2): 168-73.

Guo, Q., W. Fu, et al. (1998). "Par-4 is a mediator of neuronal degeneration associated with the pathogenesis of Alzheimer disease." Nat Med **4**(8): 957-62.

Hammond, S. M., S. Boettcher, et al. (2001). "Argonaute2, a link between genetic and biochemical analyses of RNAi." Science **293**(5532): 1146-50.

Harborth, J., S. M. Elbashir, et al. (2001). "Identification of essential genes in cultured mammalian cells using small interfering RNAs." J Cell Sci **114**(Pt 24): 4557-65.

Hayashi, Y., K. Ueki, et al. (1997). "Association of EGFR gene amplification and CDKN2 (p16/MTS1) gene deletion in glioblastoma multiforme." Brain Pathol 7(3): 871-5.

Hellbert, K., S. Kampfer, et al. (2000). "Implication of atypical protein kinase C isozymes lambda and zeta in Ras mediated reorganization of the actin cytoskeleton and cyclin D1-induction." Adv Enzyme Regul **40**: 49-62.

Hendrix, M. J., E. A. Seftor, et al. (1987). "A simple quantitative assay for studying the invasive potential of high and low human metastatic variants." Cancer Lett **38**(1-2): 137-47.

Hengst, L., V. Dulic, et al. (1994). "A cell cycle-regulated inhibitor of cyclin-dependent kinases." Proc Natl Acad Sci U S A **91**(12): 5291-5.

Holland, E. C. (2000). "A mouse model for glioma: biology, pathology, and therapeutic opportunities." Toxicol Pathol **28**(1): 171-7.

Holland, E. C. (2001). "Gliomagenesis: genetic alterations and mouse models." Nat Rev Genet **2**(2): 120-9.

Holland, E. C., W. P. Hively, et al. (1998). "A constitutively active epidermal growth factor receptor cooperates with disruption of G1 cell-cycle arrest pathways to induce glioma-like lesions in mice." Genes Dev **12**(23): 3675-85.

Hunter, T., T. Hunt, et al. (1975). "The characteristics of inhibition of protein synthesis by double-stranded ribonucleic acid in reticulocyte lysates." J Biol Chem **250**(2): 409-17.
Hurley, J. H., A. C. Newton, et al. (1997). "Taxonomy and function of C1 protein kinase C homology domains." Protein Sci **6**(2): 477-80.

Hussaini, I. M., L. R. Karns, et al. (2000). "Phorbol 12-myristate 13-acetate induces protein kinase ceta-specific proliferative response in astrocytic tumor cells." J Biol Chem **275**(29): 22348-54.

Huynh, J. R., M. Petronczki, et al. (2001). "Bazooka and PAR-6 are required with PAR-1 for the maintenance of oocyte fate in Drosophila." Curr Biol **11**(11): 901-6.

Ikeda, J., M. Tada, et al. (2001). "Restoration of endogenous wild-type p53 activity in a glioblastoma cell line with intrinsic temperature-sensitive p53 induces growth arrest but not apoptosis." Int J Cancer **94**(1): 35-43.

Ikemoto, H., E. Tani, et al. (1995). "Apoptosis of human glioma cells in response to calphostin C, a specific protein kinase C inhibitor." J Neurosurg **83**(6): 1008-16.

Ivanchuk, S. M., S. Mondal, et al. (2001). "The INK4A/ARF locus: role in cell cycle control and apoptosis and implications for glioma growth." J Neurooncol **51**(3): 219-29.

Izumi, Y., T. Hirose, et al. (1998). "An atypical PKC directly associates and colocalizes at the epithelial tight junction with ASIP, a mammalian homologue of Caenorhabditis elegans polarity protein PAR-3." J Cell Biol **143**(1): 95-106.

Jen, J., J. W. Harper, et al. (1994). "Deletion of p16 and p15 genes in brain tumors." Cancer Res **54**(24): 6353-8.

Jendrossek, V., W. Kugler, et al. (2001). "Erucylphosphocholine-induced apoptosis in chemoresistant glioblastoma cell lines: involvement of caspase activation and mitochondrial alterations." Anticancer Res **21**(5): 3389-96.

Karin, M. (1999). "The beginning of the end: IkappaB kinase (IKK) and NF-kappaB activation." J Biol Chem **274**(39): 27339-42.

Kleihues, P., D. N. Louis, et al. (2002). "The WHO classification of tumors of the nervous system." J Neuropathol Exp Neurol **61**(3): 215-25; discussion 226-9.

Kleihues, P. and H. Ohgaki (1999). "Primary and secondary glioblastomas: from concept to clinical diagnosis." Neuro-oncol 1(1): 44-51.

Kleihues, P. and L. H. Sobin (2000). "World Health Organization classification of tumors." Cancer **88**(12): 2887.

Kolesnick, R. N. and M. Kronke (1998). "Regulation of ceramide production and apoptosis." Annu Rev Physiol **60**: 643-65.

Konishi, H., S. Kuroda, et al. (1995). "Molecular cloning and characterization of a new member of the RAC protein kinase family: association of the pleckstrin homology domain of three types of RAC protein kinase with protein kinase C subspecies and beta gamma subunits of G proteins." Biochem Biophys Res Commun **216**(2): 526-34.

Kraus, J. A., J. Felsberg, et al. (2002). "Molecular genetic analysis of the TP53, PTEN, CDKN2A, EGFR, CDK4 and MDM2 tumour-associated genes in supratentorial primitive neuroectodermal tumours and glioblastomas of childhood." Neuropathol Appl Neurobiol **28**(4): 325-33.

LaBaer, J., M. D. Garrett, et al. (1997). "New functional activities for the p21 family of CDK inhibitors." Genes Dev **11**(7): 847-62.

Lakka, S. S., C. S. Gondi, et al. (2003). "Synergistic down-regulation of urokinase plasminogen activator receptor and matrix metalloproteinase-9 in SNB19 glioblastoma cells efficiently inhibits glioma cell invasion, angiogenesis, and tumor growth." Cancer Res **63**(10): 2454-61.

Lallena, M. J., M. T. Diaz-Meco, et al. (1999). "Activation of IkappaB kinase beta by protein kinase C isoforms." Mol Cell Biol **19**(3): 2180-8.

Lang, F. F., D. C. Miller, et al. (1994). "Pathways leading to glioblastoma multiforme: a molecular analysis of genetic alterations in 65 astrocytic tumors." J Neurosurg **81**(3): 427-36.

Lavictoire, S. J., D. A. Parolin, et al. (2003). "Interaction of Hsp90 with the nascent form of the mutant epidermal growth factor receptor EGFRvIII." J Biol Chem **278**(7): 5292-9.

Lawrence, T. S., A. W. Blackstock, et al. (2003). "The mechanism of action of radiosensitization of conventional chemotherapeutic agents." Semin Radiat Oncol **13**(1): 13-21.

Lee, S. H., M. S. Kim, et al. (2000). "Growth inhibitory effect on glioma cells of adenovirus-mediated p16/INK4a gene transfer in vitro and in vivo." Int J Mol Med **6**(5): 559-63.

Leslie, N. R., R. M. Biondi, et al. (2001). "Phosphoinositide-regulated kinases and phosphoinositide phosphatases." Chem Rev **101**(8): 2365-80.

Liang, J., J. Zubovitz, et al. (2002). "PKB/Akt phosphorylates p27, impairs nuclear import of p27 and opposes p27-mediated G1 arrest." Nat Med **8**(10): 1153-60.

Lorimer, I. A. (2002). "Mutant epidermal growth factor receptors as targets for cancer therapy." Curr Cancer Drug Targets **2**(2): 91-102.

Lorimer, I. A. and S. J. Lavictoire (2001). "Activation of extracellular-regulated kinases by normal and mutant EGF receptors." Biochim Biophys Acta **1538**(1): 1-9.

Lorimer, I. A., D. A. Parolin, et al. (2002). "Induction of apoptosis in glioblastoma cells by an atypical protein kinase C pseudosubstrate peptide." Anticancer Res **22**(2A): 623-31.

Luo, X., X. Gong, et al. (2003). "Suppression of EGFRvIII-mediated proliferation and tumorigenesis of breast cancer cells by ribozyme." Int J Cancer **104**(6): 716-21.

Macleod, K. F. and T. Jacks (1999). "Insights into cancer from transgenic mouse models." J Pathol **187**(1): 43-60.

Maehama, T. and J. E. Dixon (1998). "The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate." J Biol Chem **273**(22): 13375-8.

Maines-Bandiera, S. L. and N. Auersperg (1997). "Increased E-cadherin expression in ovarian surface epithelium: an early step in metaplasia and dysplasia?" Int J Gynecol Pathol **16**(3): 250-5.

Mandil, R., E. Ashkenazi, et al. (2001). "Protein kinase Calpha and protein kinase Cdelta play opposite roles in the proliferation and apoptosis of glioma cells." Cancer Res **61**(11): 4612-9.

Mao, M., X. Fang, et al. (2000). "Inhibition of growth-factor-induced phosphorylation and activation of protein kinase B/Akt by atypical protein kinase C in breast cancer cells." Biochem J **352 Pt 2**: 475-82.

Markert, J. (2003). "Glioblastoma multiforme: introduction." Cancer J **9**(2): 71.

Matsumoto, T., E. Tani, et al. (1995). "Effects of protein kinase C modulators on multidrug resistance in human glioma cells." Neurosurgery **36**(3): 565-71; discussion 572.

Medema, R. H., G. J. Kops, et al. (2000). "AFX-like Forkhead transcription factors mediate cell-cycle regulation by Ras and PKB through p27kip1." Nature **404**(6779): 782-7.

Mellor, H. and P. J. Parker (1998). "The extended protein kinase C superfamily." Biochem J **332 (Pt 2)**: 281-92.

Mishima, A., A. Suzuki, et al. (2002). "Over-expression of PAR-3 suppresses contact-mediated inhibition of cell migration in MDCK cells." Genes Cells **7**(6): 581-96.

Moscat, J. and M. T. Diaz-Meco (2000). "The atypical protein kinase Cs. Functional specificity mediated by specific protein adapters." EMBO Rep **1**(5): 399-403.

Moscat, J., L. Sanz, et al. (2001). "Regulation and role of the atypical PKC isoforms in cell survival during tumor transformation." Adv Enzyme Regul **41**: 99-120.

Moscatello, D. K., G. Ramirez, et al. (1997). "A naturally occurring mutant human epidermal growth factor receptor as a target for peptide vaccine immunotherapy of tumors." Cancer Res **57**(8): 1419-24.

Murray, N. R. and A. P. Fields (1997). "Atypical protein kinase C iota protects human leukemia cells against drug-induced apoptosis." J Biol Chem **272**(44): 27521-4.

Myers, M. P., J. P. Stolarov, et al. (1997). "P-TEN, the tumor suppressor from human chromosome 10q23, is a dual-specificity phosphatase." Proc Natl Acad Sci U S A **94**(17): 9052-7.

Nagane, M., A. Asai, et al. (1999). "Expression pattern of chemoresistance-related genes in human malignant brain tumors: a working knowledge for proper selection of anticancer drugs." Jpn J Clin Oncol **29**(11): 527-34.

Nagane, M., A. Levitzki, et al. (1998). "Drug resistance of human glioblastoma cells conferred by a tumor-specific mutant epidermal growth factor receptor through modulation of Bcl-XL and caspase-3-like proteases." Proc Natl Acad Sci U S A **95**(10): 5724-9.

Nagane, M., H. Lin, et al. (2001). "Aberrant receptor signaling in human malignant gliomas: mechanisms and therapeutic implications." Cancer Lett **162 Suppl**: S17-S21.

Nagashima, G., R. Suzuki, et al. (1999). "Graphic analysis of microscopic tumor cell infiltration, proliferative potential, and vascular endothelial growth factor expression in an autopsy brain with glioblastoma." Surg Neurol **51**(3): 292-9.

Nakanishi, H. and J. H. Exton (1992). "Purification and characterization of the zeta isoform of protein kinase C from bovine kidney." J Biol Chem **267**(23): 16347-54.

Nakaya, M., A. Fukui, et al. (2000). "Meiotic maturation induces animal-vegetal asymmetric distribution of aPKC and ASIP/PAR-3 in Xenopus oocytes." Development **127**(23): 5021-31.

Nakayama, K., N. Ishida, et al. (1996). "Mice lacking p27(Kip1) display increased body size, multiple organ hyperplasia, retinal dysplasia, and pituitary tumors." Cell **85**(5): 707-20.

Narita, Y., M. Nagane, et al. (2002). "Mutant epidermal growth factor receptor signaling down-regulates p27 through activation of the phosphatidylinositol 3-kinase/Akt pathway in glioblastomas." Cancer Res **62**(22): 6764-9.

Newcomb, E. W., M. Alonso, et al. (2000). "Incidence of p14ARF gene deletion in high-grade adult and pediatric astrocytomas." Hum Pathol **31**(1): 115-9.

Ng, H. K. and P. Y. Lam (1998). "The molecular genetics of central nervous system tumors." Pathology **30**(2): 196-202.

Ng, T., D. Shima, et al. (1999). "PKCalpha regulates beta1 integrin-dependent cell motility through association and control of integrin traffic." Embo J **18**(14): 3909-23.

Nishikawa, R., F. B. Furnari, et al. (1995). "Loss of P16INK4 expression is frequent in high grade gliomas." Cancer Res **55**(9): 1941-5.

Nishikawa, R., X. D. Ji, et al. (1994). "A mutant epidermal growth factor receptor common in human glioma confers enhanced tumorigenicity." Proc Natl Acad Sci U S A **91**(16): 7727-31.

Nishizuka, Y. (1988). "The molecular heterogeneity of protein kinase C and its implications for cellular regulation." Nature **334**(6184): 661-5.

Nishizuka, Y. (1989). "The Albert Lasker Medical Awards. The family of protein kinase C for signal transduction." Jama **262**(13): 1826-33.

O'Driscoll, K. R., K. K. Teng, et al. (1995). "Selective translocation of protein kinase C-delta in PC12 cells during nerve growth factor-induced neuritogenesis." Mol Biol Cell **6**(4): 449-58.

Ohno, S. (2001). "Intercellular junctions and cellular polarity: the PAR-aPKC complex, a conserved core cassette playing fundamental roles in cell polarity." Curr Opin Cell Biol **13**(5): 641-8.

Okada, Y., E. E. Hurwitz, et al. (2003). "Selection pressures of TP53 mutation and microenvironmental location influence epidermal growth factor receptor gene amplification in human glioblastomas." Cancer Res **63**(2): 413-6.

Okamoto, I., L. C. Kenyon, et al. (2003). "Expression of constitutively activated EGFRvIII in non-small cell lung cancer." Cancer Sci **94**(1): 50-6.

Okano, J., I. Gaslightwala, et al. (2000). "Akt/protein kinase B isoforms are differentially regulated by epidermal growth factor stimulation." J Biol Chem **275**(40): 30934-42.

Olapade-Olaopa, E. O., D. K. Moscatello, et al. (2000). "Evidence for the differential expression of a variant EGF receptor protein in human prostate cancer." Br J Cancer **82**(1): 186-94.

Ono, Y., T. Tamiya, et al. (1996). "Malignant astrocytomas with homozygous CDKN2/p16 gene deletions have higher Ki-67 proliferation indices." J Neuropathol Exp Neurol **55**(10): 1026-31.

Paez, J. and W. R. Sellers (2003). "PI3K/PTEN/AKT pathway. A critical mediator of oncogenic signaling." Cancer Treat Res **115**: 145-67.

Pagano, M., S. W. Tam, et al. (1995). "Role of the ubiquitin-proteasome pathway in regulating abundance of the cyclin-dependent kinase inhibitor p27." Science **269**(5224): 682-5.

Peng, X. D., P. Z. Xu, et al. (2003). "Dwarfism, impaired skin development, skeletal muscle atrophy, delayed bone development, and impeded adipogenesis in mice lacking Akt1 and Akt2." Genes Dev **17**(11): 1352-65.

Perletti, G. P., M. Folini, et al. (1996). "Overexpression of protein kinase C epsilon is oncogenic in rat colonic epithelial cells." Oncogene **12**(4): 847-54.

Persad, S., S. Attwell, et al. (2001). "Regulation of protein kinase B/Akt-serine 473 phosphorylation by integrin-linked kinase: critical roles for kinase activity and amino acids arginine 211 and serine 343." J Biol Chem **276**(29): 27462-9.

Philip, P. A. and J. A. Zonder (1999). "Pharmacology and clinical experience with bryostatin 1: a novel anticancer drug." Expert Opin Investig Drugs **8**(12): 2189-2199.

Ponting, C. P. and P. J. Parker (1996). "Extending the C2 domain family: C2s in PKCs delta, epsilon, eta, theta, phospholipases, GAPs, and perforin." Protein Sci **5**(1): 162-6.

Pore, N., S. Liu, et al. (2003). "PTEN mutation and epidermal growth factor receptor activation regulate vascular endothelial growth factor (VEGF) mRNA expression in human glioblastoma cells by transactivating the proximal VEGF promoter." Cancer Res **63**(1): 236-41.

Qiu, R. G., A. Abo, et al. (2000). "A human homolog of the C. elegans polarity determinant Par-6 links Rac and Cdc42 to PKCzeta signaling and cell transformation." Curr Biol **10**(12): 697-707.

Reilly, K. M., D. A. Loisel, et al. (2000). "Nf1;Trp53 mutant mice develop glioblastoma with evidence of strain-specific effects." Nat Genet **26**(1): 109-13.

Sajan, M. P., M. L. Standaert, et al. (1999). "Protein kinase C-zeta and phosphoinositide-dependent protein kinase-1 are required for insulin-induced activation of ERK in rat adipocytes." J Biol Chem **274**(43): 30495-500.

Sanchez, P., G. De Carcer, et al. (1998). "Localization of atypical protein kinase C isoforms into lysosome-targeted endosomes through interaction with p62." Mol Cell Biol **18**(5): 3069-80.

Sandhu, C. and J. Slingerland (2000). "Deregulation of the cell cycle in cancer." Cancer Detect Prev **24**(2): 107-18.

Schmidt, E. E., K. Ichimura, et al. (1994). "CDKN2 (p16/MTS1) gene deletion or CDK4 amplification occurs in the majority of glioblastomas." Cancer Res **54**(24): 6321-4.

Scott, M. T., A. Ingram, et al. (2002). "PDK1-dependent activation of atypical PKC leads to degradation of the p21 tumour modifier protein." Embo J **21**(24): 6771-80.

Seger, R. and E. G. Krebs (1995). "The MAPK signaling cascade." Faseb J **9**(9): 726-35.
Sells, S. F., D. P. Wood, Jr., et al. (1994). "Commonality of the gene programs induced by effectors of apoptosis in androgen-dependent and -independent prostate cells." Cell Growth Differ **5**(4): 457-66.

Sharif, T. R. and M. Sharif (1999). "Overexpression of protein kinase C epsilon in astroglial brain tumor derived cell lines and primary tumor samples." Int J Oncol **15**(2): 237-43.
Shin, I., F. M. Yakes, et al. (2002). "PKB/Akt mediates cell-cycle progression by phosphorylation of p27(Kip1) at threonine 157 and modulation of its cellular localization." Nat Med **8**(10): 1145-52.

Shirane, M., Y. Harumiya, et al. (1999). "Down-regulation of p27(Kip1) by two mechanisms, ubiquitin-mediated degradation and proteolytic processing." J Biol Chem **274**(20): 13886-93.

Shuey, D. J., D. E. McCallus, et al. (2002). "RNAi: gene-silencing in therapeutic intervention." Drug Discov Today **7**(20): 1040-6.

Slingerland, J. and M. Pagano (2000). "Regulation of the cdk inhibitor p27 and its deregulation in cancer." J Cell Physiol **183**(1): 10-7.

Stambolic, V., T. W. Mak, et al. (1999). "Modulation of cellular apoptotic potential: contributions to oncogenesis." Oncogene **18**(45): 6094-103.

Stambolic, V., A. Suzuki, et al. (1998). "Negative regulation of PKB/Akt-dependent cell survival by the tumor suppressor PTEN." Cell **95**(1): 29-39.

Strommer, K., M. F. Hamou, et al. (1990). "Cellular and tumoural heterogeneity of EGFR gene amplification in human malignant gliomas." Acta Neurochir (Wien) **107**(3-4): 82-7.

Suzuki, A., T. Yamanaka, et al. (2001). "Atypical protein kinase C is involved in the evolutionarily conserved par protein complex and plays a critical role in establishing epithelia-specific junctional structures." J Cell Biol **152**(6): 1183-96.

Swannie, H. C. and S. B. Kaye (2002). "Protein kinase C inhibitors." Curr Oncol Rep **4**(1): 37-46.

Tang, C. K., X. Q. Gong, et al. (2000). "Epidermal growth factor receptor vIII enhances tumorigenicity in human breast cancer." Cancer Res **60**(11): 3081-7.

Tohma, Y., C. Gratas, et al. (1998). "PTEN (MMAC1) mutations are frequent in primary glioblastomas (de novo) but not in secondary glioblastomas." J Neuropathol Exp Neurol **57**(7): 684-9.

Toker, A. and A. C. Newton (2000). "Cellular signaling: pivoting around PDK-1." Cell **103**(2): 185-8.

Ueffing, M., J. Lovric, et al. (1997). "Protein kinase C-epsilon associates with the Raf-1 kinase and induces the production of growth factors that stimulate Raf-1 activity." Oncogene **15**(24): 2921-7.

Uhrbom, L., C. Dai, et al. (2002). "Ink4a-Arf loss cooperates with KRas activation in astrocytes and neural progenitors to generate glioblastomas of various morphologies depending on activated Akt." Cancer Res **62**(19): 5551-8.

Vanhaesebroeck, B. and D. R. Alessi (2000). "The PI3K-PDK1 connection: more than just a road to PKB." Biochem J **346 Pt 3**: 561-76.

Viglietto, G., M. L. Motti, et al. (2002). "Understanding p27(kip1) deregulation in cancer: down-regulation or mislocalization." Cell Cycle **1**(6): 394-400.

Villano, J. L., A. M. Mauer, et al. (2003). "A case study documenting the anticancer activity of ZD1839 (Iressa) in the brain." Ann Oncol **14**(4): 656-8.

Vives, K. P. and J. M. Piepmeier (1999). "Complications and expected outcome of glioma surgery." J Neurooncol **42**(3): 289-302.

Watts, J. L., B. Etemad-Moghadam, et al. (1996). "par-6, a gene involved in the establishment of asymmetry in early C. elegans embryos, mediates the asymmetric localization of PAR-3." Development **122**(10): 3133-40.

Wen, H. C., W. C. Huang, et al. (2003). "Negative regulation of phosphatidylinositol 3-kinase and Akt signalling pathway by PKC." Cell Signal **15**(1): 37-45.

Wong, A. J., J. M. Ruppert, et al. (1992). "Structural alterations of the epidermal growth factor receptor gene in human gliomas." Proc Natl Acad Sci U S A **89**(7): 2965-9.

Wooten, E. C., D. Fults, et al. (1999). "A study of loss of heterozygosity at 70 loci in anaplastic astrocytoma and glioblastoma multiforme with implications for tumor evolution." Neuro-oncol **1**(3): 169-76.

Wooten, M. W. (1999). "Function for NF-kB in neuronal survival: regulation by atypical protein kinase C." J Neurosci Res **58**(5): 607-11.

Xiao, H., D. A. Goldthwait, et al. (1994). "The identification of four protein kinase C isoforms in human glioblastoma cell lines: PKC alpha, gamma, epsilon, and zeta." J Neurosurg **81**(5): 734-40.

Yamanaka, T., Y. Horikoshi, et al. (2001). "PAR-6 regulates aPKC activity in a novel way and mediates cell-cell contact-induced formation of the epithelial junctional complex." Genes Cells **6**(8): 721-31.

Yao, R. and G. M. Cooper (1995). "Requirement for phosphatidylinositol-3 kinase in the prevention of apoptosis by nerve growth factor." Science **267**(5206): 2003-6.

Yazaki, T., S. Ahmad, et al. (1996). "Treatment of glioblastoma U-87 by systemic administration of an antisense protein kinase C-alpha phosphorothioate oligodeoxynucleotide." Mol Pharmacol **50**(2): 236-42.

Yong, V. W., N. P. Dooley, et al. (1994). "Protein kinase C in cultured adult human oligodendrocytes: a potential role for isoform alpha as a mediator of process outgrowth." J Neurosci Res **39**(1): 83-96.

Zachary, I. (2003). "Vascular endothelial growth factor and anti-angiogenic peptides as therapeutic and investigational molecules." Idrugs **6**(3): 224-31.

Zamore, P. D., T. Tuschl, et al. (2000). "RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals." Cell **101**(1): 25-33.

Zellner, A., M. R. Fetell, et al. (1998). "Disparity in expression of protein kinase C alpha in human glioma versus glioma-derived primary cell lines: therapeutic implications." Clin Cancer Res 4(7): 1797-802.

Zeng, Y., K. Hirano, et al. (2000). "Minimal requirements for the nuclear localization of p27(Kip1), a cyclin-dependent kinase inhibitor." Biochem Biophys Res Commun 274(1): 37-42.

Zheleva, D. I., D. P. Lane, et al. (2003). "The p53-Mdm2 pathway: targets for the development of new anticancer therapeutics." Mini Rev Med Chem 3(3): 257-70.