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The role of atypical protein kinase c iota in glioblastoma multiforme

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The role of atypical protein kinase c iota in glioblastoma multiforme

by

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Graduate Program
in
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Abstract

Glioblastoma multiforme is the most aggressive form of primary brain tumour occurring in adults. Despite aggressive multimodal therapy which includes surgery, radiation and chemotherapy it remains largely incurable and patients have a mean survival time of 12-14 months. Therefore the need for improved therapeutic strategies is imperative. Identifying molecular mechanisms involved in the development and progression of glioblastoma will not only improve our overall understanding of the disease but also identify potential targets that may be exploited therapeutically. The phosphoinositide 3-kinase (PI3K) pathway is often constitutively active in glioblastoma tumours due to overexpression and mutation of the epidermal growth factor receptor, and deletion/loss of function of the tumour suppressor PTEN. The atypical protein kinase Cs (PKCι (iota) and PKCζ (zeta)) have been shown to be activated downstream of PI3K and contribute to the malignant phenotype of several types of human cancers, including lung and colon cancer. However they have not been studied in glioblastoma. Our analysis identified that glioblastoma cell lines express PKCι and have no detectable PKCζ. PKCι is activated in glioblastoma cells and its protein expression is increased compared to normal human astrocytes. To study the role of PKCι in glioblastoma cells RNA interference was used to specifically reduce its expression. Depletion of PKCι in glioblastoma cells decreased their proliferation, motility and invasiveness and partially enhanced their sensitivity to cisplatin-induced cell death. To identify potential mechanisms through which PKCι promotes these malignant characteristics gene expression microarray analysis was performed on U87MG glioblastoma cells depleted of PKCι. This analysis identified that PKCι affects many classes of genes involved in a variety of cellular processes. Within the set of genes that were negatively regulated by PKCι, glia maturation factor beta (GMFβ) and ras homology family member B (RhoB) were investigated further. GMFβ has been shown to enhance p38MAPK activation and signaling. The p38MAPK pathway has been previously identified as a key mediator of cisplatin cytotoxicity. Our results demonstrate that PKCι suppresses cisplatin-induced p38MAPK activation causing an enhancement in resistance through the repression of GMFβ. Overexpression of GMFβ in glioblastoma cells causes an enhancement of p38MAPK activation and cell death in response to cisplatin treatment. PKCι was also found to repress the expression of RhoB, which unlike its other Rho family members functions in the repression of tumourgenicity. Examination of RhoB repression by PKCι showed that this significantly increases the motility and invasiveness of glioblastoma cells as overexpression of RhoB decreased these properties. Additionally, our results have identified a mutually antagonistic relationship between PKCι activity and RhoB expression that may be a sensitive switch between a motile and non-motile phenotype. Lastly, live cell imaging of glioblastoma cells stably depleted of PKCι revealed that it is involved in the dynamics of leading edge formation and plays a role in mitosis. This work has demonstrated that inhibiting PKCι may be a useful therapeutic strategy for glioblastoma, either alone or in combination with other treatment modalities, such as chemotherapy or radiation, to improve the poor patient outcomes associated with this disease.

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List of Abbreviations

AID	Atypical PKC interacting domain
AJ	Adherens junction
APC	Adenomatous polyposis coli
ARF	$p14^{Arf}$
ASIP	Atypical PKC specific interacting protein
ATG	Aurothioglucose
ATM	Aurothiomalate
Ca^{2+}	Calcium
CDK4	Cyclin-dependent kinase 4
CML	Chronic myelogenous leukemia
CNS	Central nervous system
DAG	Diacylglycerol
DNA	Deoxyribonucleic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EGFRvIII	Epidermal growth factor receptor variant 3
ERK	Extracellular regulated kinase
FGF	Fibroblast growth factor
Gab1	Grb2-associated binder 1
GBD	GTP binding domain
GBM	Glioblastoma multiforme
GDP	Guanine diphosphate
GEF	Guanine exchange factor
Grb2	Growth factor receptor bound protein 2
GSK3β	Glycogen synthase kinase 3 beta
GTP	Guanine triphosphate
IκB	Inhibitory kappa B
INK4a	$p16^{INK4a}$
JAM	Junctional adhesion molecule
K	Lysine
Lgl	Lethal giant larvae
MAPK	Mitogen activated protein kinase
MDCK	Madin-Darby canine kidney
MEK	MAPK/ERK kinase
M/HDM2	Murine/human double minute 2
MTOC	Microtubule organizing center
NRG	Neuregulins
NSCLC	Non-small cell lung cancer
ORC	Octicosa peptide repeat
PAK1	p21-activated kinase 1
Par-3	Partitioning defective mutant 3
Par-4	Prostate apoptosis responsive 4
Par-6	Partitioning defective mutant 6
PB1	Phox and bem 1
PC	Phox and cdc
PDGF	Platelet-derived growth factor
PDGFR	Platelet-derived growth factor receptor
PDK1	Phosphoinositide-dependent kinase 1
PDZ	PSD95/Discs-large/Zona occludens 1
PH	Pleckstrin homology
PI3K	Phosphoinositide 3-kinase
PIP_2	Phosphatidylinositol 4, 5 bisphosphate
PIP_3	Phosphatidylinositol 3, 4, 5 trisphosphate
PKB	Protein kinase B
PKC	Protein kinase C
PLCγ	Phospholipase C gamma
PTB	Phosphotyrosine binding
PS	Pseudosubstrate
PTEN	Phosphatase and tensin homology on chromosome 10
R	Arginine
RB	Retinoblastoma protein
RTK	Receptor tyrosine kinase
S	Serine
SH2	Src homology 2
SHIP1	SH2-containing phosphatase 1
SHIP2	SH2-containing phosphatase 2
SOS	Son of sevenless
STAT	Signal transducer and activator of transcription
T	Threonine

TGFβ	Transforming growth factor beta
TJ	Tight junctions
TNFα	Tumour necrosis factor alpha
ZIP	Zeta interacting protein

1. Introduction

1.1 Brain tumours

The brain is one of the most complex organs in the human body, whose main function is to regulate and integrate the body's responses to external and internal stimuli. The brain, together with the spinal cord, forms the core of the central nervous system (CNS) (1). The mammalian CNS is composed of two main cell types: neurons and glial cells (1-3). The main function of neurons is to transmit information throughout the body. Glial cells are the supporting cell types that are found in the central nervous system. They are subdivided into four main populations: astrocytes, microglia, ependymal and oligodendrocytes (1). Each of these cell types has distinct morphologies and functions that are critical for normal CNS functioning.

Brain tumours are one of the most devastating and difficult to treat cancers affecting both men and women. This is related to their location, and also to the lack of understanding of the biological mechanisms that drive the development and progression of these tumours. It is estimated that 1700 deaths will occur from brain tumours in Canada this year (Statistics Canada 2008). The underlying cause of brain tumours is currently unknown.

Gliomas are the most common primary brain tumours occuring in adults, representing approximately 80% of all human CNS malignancies (4). Gliomas are heterogeneous tumours that are subdivided into three main categories based on their cellular characteristics: astrocytomas, oligodendrogliomas, or mixed oligoastrocytomas (4-6). Astrocytomas are the most common of all the brain tumour types (7). The World Health Organization classification system of CNS tumours identifies four grades of astrocytomas

based on their increasing aggressiveness (Grade I-IV) (8). Grade I (pilocytic astrocytomas) tumours are relatively benign with a low incidence of brain infiltration or distant spread and are curable by surgical resection. Diffuse astrocytomas (Grade II) are slow growing highly differentiated tumours that have increased infiltration into the surrounding normal brain (7;9). Most Grade II gliomas progress to more aggressive malignancy (Grades III and IV) associated with the accumulation of genetic alterations. Anaplastic astrocytomas (Grade III) arise from less aggressive astrocytomas and show increased cellularity and mitotic activity (4). The most aggressive and most common form of astrocytoma is glioblastoma multiforme (GBM), which has a Grade IV classification (8). The histological characteristics of GBM include nuclear atypia, increased mitosis and proliferation, angiogenesis, and central necrosis (4;6;9). Grades II to IV tumours are incurable and their grades are associated with increasingly poor prognosis and survival time: Grade II tumours are associated with a 5-15 year survival, grade III often less than 3 years, and grade IV have a median survival of 1 year making them one of the most lethal of all cancer types (4;5).

1.2 Origin of brain tumours

The cell of origin for malignant gliomas is currently unknown. The common, although unproven, theory is that astrocytomas originate from astrocytes, oligodendrocytes give rise to oligodendrogliomas, and mixed oligoastrocytomas have a mixed population consisting of both these cell types based on morphological and genetic characteristics (5;6). However, the lack of evidence of cells undergoing division within the adult brain suggests an alternative mechanism exists for tumour development (5;6).

The discovery that the adult brain contains a small population of neural progenitor cells (neural stem cells) has given a new dimension to the origin of brain tumours. These progenitor cells have been isolated from normal brain, as well as from both diffuse astrocytoma and GBM tumour tissue (10). These cells have the ability to self-renew, form neurospheres, differentiate, and form tumours in mice (10-12). In transgenic mice, targeted expression of oncogenic Ras and Akt in neural progenitor cells, but not in mature astrocytes, resulted in tumours that histologically resembled GBM (13). While these results suggest that a neural progenitor cell may give rise to brain tumours, there is evidence that supports the original theory. Primary astrocytes isolated from transgenic mice lacking the INK4a/Arf gene, a commonly mutated gene in GBM, have an increased proliferative capacity. When treated with epidermal growth factor, these cells can dedifferentiate and form neurospheres *in vitro* (14). Additionally, overexpression of constitutively active mutant epidermal growth factor receptor (EGFR) in both neural progenitor cells and astrocytes lacking INK4a/Arf equally induced tumour formation in mice (14). Although this occurs in a specific genetic context, it does show that astrocytes can serve as glioma precursor cells and identifies a unique dedifferentiation property that astrocytes possess. These studies have identified a plasticity of astrocytes that was previous unknown and uncovered a potential obstacle that may have to be overcome in the development of effective therapies for this devastating disease.

1.3 Glioblastoma multiforme

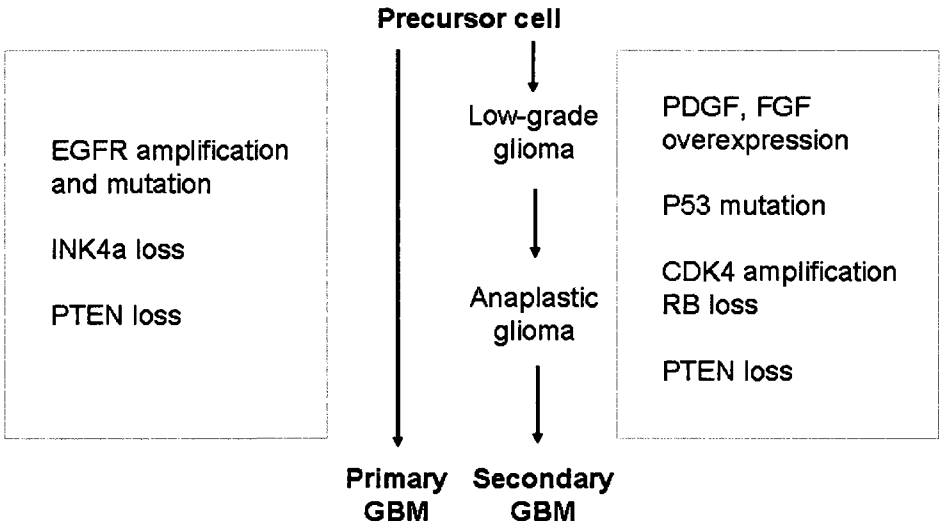
1.3.1 Molecular etiology of glioblastoma multiforme

Glioblastoma multiforme is the most aggressive (Grade IV) and most frequently

occurring brain tumour in adults, accounting for at least 80% of all malignant gliomas (4). The exact cause of GBM is not known, yet numerous genetic abnormalities have been identified that contribute to their development and progression. Glioblastomas are subdivided into two types, primary and secondary GBM, based on the development and genetic makeup of the malignancy (4;15;16). Primary GBM commonly occurs in older patients (60-70 years of age) arising *de novo* without evidence of a low grade precursor lesion (4). In contrast, secondary GBM progresses slowly from a preexisting lower grade lesions and occurs in younger patients (50-60 years of age) (4). The time to progression has a broad range from months to decades. Primary and secondary GBM cannot be distinguished morphologically, but they do contain distinct genetic profiles (Figure 1).

Primary GBMs are the most commonly occurring of these malignancies, and although they progress rapidly, their progression is not a result of a single transformation, but rather the acquisition of multiple genetic abnormalities. These are characterized into two types: mutations affecting receptor tyrosine kinase pathways and mutations altering cell cycle regulators. Alterations to both of these processes cooperate and seem to be required for tumourgenesis. Mutations in the EGFR, phosphatase and tensin homology (PTEN), platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF) play roles in GBM tumourgenesis (4;6;11;15-17). Primary GBM are characterized genetically by gene amplification (in 40-50% of cases) and overexpression (in 60% of cases) of the EGFR which only rarely occurs in secondary GBM (15;16). The EGFR is also often mutated in GBM, producing a ligand independent constitutively active receptor designated EGFR variant III (EGFRvIII, 9;15;16;18-20). Mutational loss of expression of PTEN is also

Figure 1: Schematic of primary and secondary GBM progression. Summary of key genetic abnormalities in the development of primary and secondary GBM (Adapted from Holland et al. 2001 (6)).


Precursor cell
EGFR amplification and mutation
INK4a loss
PTEN loss
Low-grade glioma
Anaplastic glioma
PDGF, FGF overexpression
P53 mutation
CDK4 amplification
RB loss
PTEN loss
Primary GBM
Secondary GBM

common in GBM and is found almost exclusive in primary GBM (11;15;16;21;22). PTEN is an important negative regulator of EGFR and other receptor tyrosine kinase signaling. Mutations of both EGFR and PTEN lead to aberrant mitogenic signaling downstream which can promote tumour progression. In secondary GBM, overexpression of the platelet-derived growth factor receptor (PDGFR) is a common genetic alteration (16). This is often seen in low-grade astrocytomas, and is thought to be one of the first genetic alterations that occurs (23). Similar to the EGFR, the PDGFR can activate similar downstream signaling pathways, leading to increased cell proliferation, survival and tumour progression.

Persistent growth factor signaling can be modulated by key regulators of the cell cycle that can induce arrest (in G1) and/or apoptosis. Therefore, the transformation process must also involve genetic alterations to cell cycle regulators. Commonly mutated cell cycle genes in GBM are $p16^{INK4a}$ (INK4a), retinoblastoma proteins (RB), cyclin-dependent kinase 4 (CDK4), cyclin D1, p53, $p14^{ARF}$ (ARF) and murine/human double minute 2 (M/HDM2, 6;11;15;16;24). Each of these mutations has specific contributions to the loss of cell cycle control. Mutations in INK4a and RB are common in both primary and secondary GBM (16). The *INK4a* gene encodes both INK4a and ARF, which control RB and p53, respectively (6). Inactivating mutations of INK4a and RB, or activating mutations of CDK4 and cyclin D1 facilitate uncontrolled cell cycle progression in response to mitogenic signals. Mutation of the *p53* gene occurs in approximately 60% of low grade diffuse astrocytomas, and is a characteristic feature which distinguishes primary from secondary GBMs (15;16). *p53* gene mutations do occur in primary GBMs, but at a low frequency (<30%, 16). Mutations in the *p53* gene are detected early in

secondary GBMs suggesting it is a critical event in the initiation and progression to a high-grade malignancy (6). Additionally, specific correlations exist in GBM between the mitogenic alterations and cell cycle alterations. EGFR and p53 mutations rarely coincide with each other in GBM (15;16). In contrast, EGFR and INK4a mutations share a positive correlation (15;16). This suggests that there is cooperation between the genetic alterations that occur in GBM that contribute to the progression to a more infiltrative/invasive phenotype.

1.3.2 Treatment of glioblastoma

Despite ongoing research into identifying new therapeutic options and diagnostic techniques for GBM, there have been no significant increases in survival times over the past 30 years and this disease remains incurable. The standard treatment for GBM is multimodal, consisting of surgical resection and radiotherapy (7;25-27). Recently, adjuvant chemotherapy has been added to the treatment regimen. Treatment of GBM with the DNA alkylating agent temozolomide, in combination with radiotherapy following surgical resection, increased median survival time and 2-year survival rates, 2.5 months and 16.1% respectively compared to a surgery and radiotherapy combination (28). However, these response rates are quite modest considering the aggressiveness of these treatments and, ultimately, the tumour recurs. This is in part due to the challenges of delivering drugs into the central nervous system, but is mainly due to the enhanced resistance of these cells (29). The basis of this intrinsic resistance in not fully understood. Therefore, there is a need to develop new treatments for this disease that either bypass or decrease the resistance of these cells to chemotherapy. The new therapeutic strategies for

the treatment of GBM have focused on identifying and modulating molecular targets that are involved in the progression and resistance of these tumours.

1.3.3 Glioblastoma cell invasion

The highly invasive nature of GBM cells has limited the success of surgical resection, radiation and chemotherapy in the treatment of this devastating disease. This contributes to the poor prognosis associated with GBM and renders it presently an incurable disease. GBM tumours progress with the formation of a core tumour mass that is surrounded by very diffusely infiltrating tumour cells into the normal brain tissue, making it almost impossible to discern tumour margins (30). As a result recurrence of GBM is almost inevitable and often occurs within 2 cm of the surgical resection margin. These cells also have the potential to travel long distances and lesions are often found several centimeters away from the primary tumour or in the contralateral hemisphere (31). GBM has a distinct pattern of invasion, invading as single cells within the brain. Only in very rare circumstances do they metastasize outside of the brain. In contrast, systemic malignant tumours that metastasize to the brain have sharply defined borders against the surrounding normal brain tissue and these can often be completely surgically resected (11;32). Additionally, these tumours invade only short distances and often in groups of cells rather than single cells. The invasion of GBM cells involves migration along pre-existing paths within the brain, often along white matter tracts, through the subpial glial space and along peripheral blood vessel walls, rather than through blood vessel and tissue (11;32). GBM cells have an inability to penetrate through vessel walls. It is unclear whether these paths represent the path of least resistance within the brain or if they are

rich in substrates that mediate adhesion and migration of GBM cells. In addition, it is thought that these invading cells may be more resistant to radiation and chemotherapy either due to location or slowed proliferation (31). Therefore, understanding the mechanisms involved in the migration of GBM cells could identify novel therapeutic targets and strategies enabling successful treatment of this incurable disease.

There are numerous proteins both intracellular and extracellular that may play a role in GBM invasiveness, including extracellular (ECM) matrix components, cell surface adhesion proteins, proteases, cytoskeletal proteins and growth factor receptor signaling (30-35). Targeting deregulated growth factor pathways is of particular interest because their effects converge on both cell migration and cell survival. Aberrant EGFR signaling is a common characteristic in GBM due to genetic alterations. EGFR signaling has been shown to positively influence the migration and invasion of GBM cells (30). This leads to activation and expression of downstream proteins that are involved in promoting an invasive phenotype (5;33-35). Inhibition of downstream effectors of the EGFR pathway have been shown to decrease GBM cell migration and increased sensitivity to apoptosis (36;37). Therefore, targeting the EGFR pathway represents a promising therapeutic strategy for the treatment of GBM by affecting both cell invasion and survival.

1.4 Epidermal growth factor receptor

The most frequently altered receptor tyrosine kinase (RTK) in GBM is the EGFR. The EGFR family belongs to the RTK superfamily and consists of four members: EGFR (also known as ErbB1/HER-1), ErbB2/Neu/HER-2, ErbB3/HER-3 and ErbB4/HER-4. These receptors are essential for development, as knockout mice of each of the EGFRs

display embryonic/perinatal lethality (19). The ErbBs are transmembrane tyrosine kinase receptors and are activated by the binding of extracellular ligands (growth factors) belonging to the EGF-family. These growth factors can be produced by the same cell that expresses the EGFR or by surrounding cells, acting in an autocrine or paracrine fashion. There are at least 16 different ligands that can activate the EGFRs, and they have different specificities for each of the four members. EGF, transforming growth factor alpha (TGFα) and amphiregulin bind specifically to EGFR. Betacellulin, heparin-binding growth factor and epiregulin can activate EGFR and ErbB4. The neuregulins (NRG) have four subtypes: NRG-1 and -2 can activate ErbB3 and all four NRGs can bind and activate ErbB4 (38;39). ErbB2 on the other hand has no known ligand, yet it is a functional RTK. EGFR receptor activation involves binding of a ligand to its extracellular binding domain. Upon ligand binding a conformational change in the inactive structure of the EGFR exposes a dimerization loop that facilitates homo- or hetero-dimerization (40;41). ErbB2 is an exception to this, as its dimerization loop is constitutively exposed due to its extracellular structure and studies have shown that ErbB2 is the preferred binding partner for other ligand bound receptors (38;39). Dimerization induces tyrosine *trans*-autophosphorylation of the intracellular kinase domain. These phosphotyrosines serve as essential recruitment sites for proteins containing Src homology 2 (SH2) and phosphotyrosine binding (PTB) domains that initiate and mediate activation of intracellular signaling pathways (38;39). EGFR receptor activation leads to the activation of a number of downstream signaling pathways including PI3K, Ras, STAT and PLCγ, which are involved controlling cellular proliferation, survival, apoptosis and migration (39;42).

The EGFR has been shown to promote the development of a number of tumour types (19;42). While *EGFR* gene amplification and protein overexpression is common in GBM, the EGFR is also mutated in a significant proportion of these tumours. The most common of such mutations is a deletion of exons 2-7 (amino acids 6-273, 18). This truncated EGFR, called EGFR variant III (EGFRvIII), has a truncated extracellular ligand binding domain that render it ligand-independent and constitutively active (15;16;43;44). *In vitro* experiments have shown that activation of EGFR, whether it is via mutation or overexpression, confers enhanced tumourgenicity in glioblastoma cells (45;46). Additionally, overexpression of EGFRvIII specifically in astrocytes or neuronal precursor cells in mice causes glioma-like lesions *in vivo*. However, this only occurs in mice lacking INK4a/ARF supporting the idea that cooperation of genetic events is required for GBM tumour development and progression (14;47). Interestingly the mutant receptor preferentially activates the PI3K pathway and can enhance the resistance of GBM cells to radiation and chemotherapy (48;49). However, EGFRvIII has also been shown to confer resistance to EGFR-targeted therapy (50). This has highlighted the importance of examining downstream pathways of the EGFR to identify potential therapeutic targets.

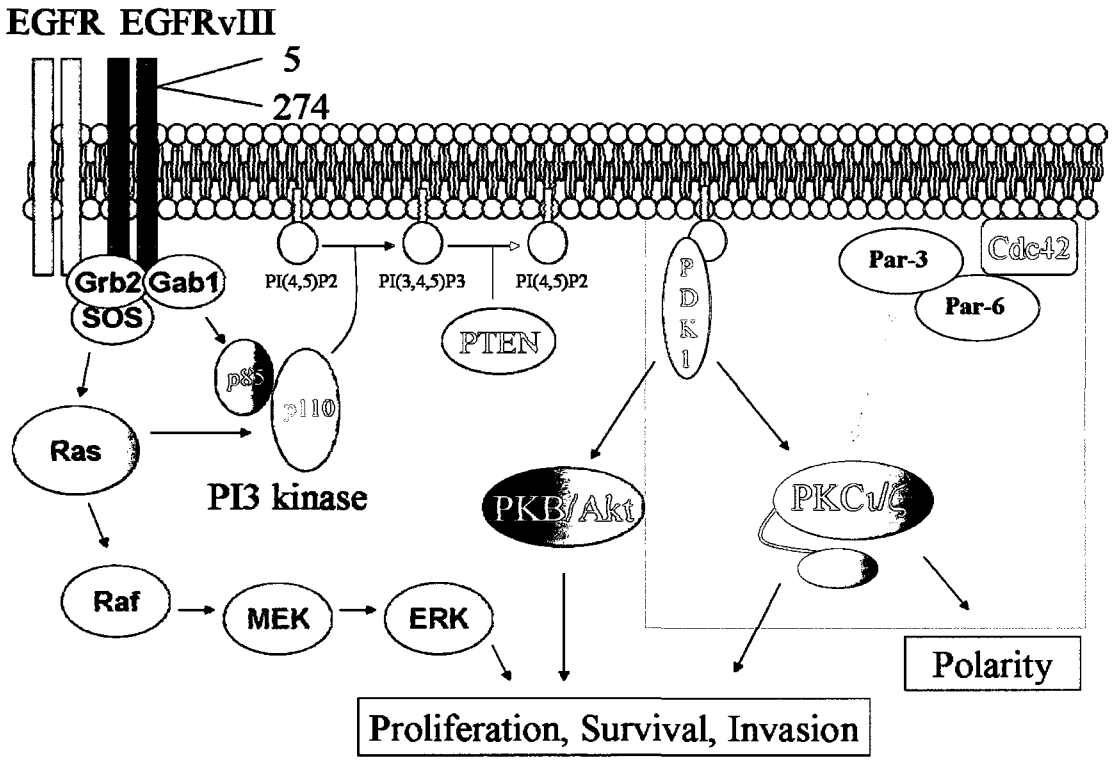
1.4.1 EGFR signaling

The EGFR can interact with and/or phosphorylate a number of intracellular signaling pathways simultaneously. Although these pathways are usually described individually, evidence has shown that they are often functionally linked and form complex networks (39). The PI3K and Ras/MAPK are key downstream pathways activated by EGFR and are responsible for generating signals promoting cellular proliferation, growth, survival,

anti-apoptosis and migration (Figure 2). Aberrant EGFR activity is involved in the development of human cancers and often associated with a more aggressive disease and poorer clinical outcome (42).

Phosphoinositide 3-kinase (PI3K) is a heterodimeric protein containing a regulatory (p85) and catalytic (p110) subunit. The p110 subunit has a high catalytic activity; however, as a monomer it is unstable (51). The p85 regulatory subunit binds the p110 catalytic subunit maintaining it in a low activity state. PI3K is recruited to and activated by the EGFR through indirect association with the EGFR via adaptor proteins (51;52). This involves the association of PI3K with the intracellular adaptor proteins, growth factor receptor bound protein 2 (Grb2) and Grb2-associated binder 1 (Gab1). Grb2 interacts with a phosphotyrosine on the receptor and with Gab1. The p85 regulatory subunit of PI3K in turn interacts with Gab1 via an SH2 interaction and this induces an allosteric activation of the p110 catalytic subunit by release of the inhibitory effect of the p85 subunit. Alternatively, PI3K can also be activated by Ras GTPase (discussed below). These mechanisms localize activated PI3K to its lipid membrane substrate phosphatidylinositol-4,5-bisphosphate (PIP_2). The activated PI3K converts PIP_2 to phosphatidylinositol-3,4,5-trisphosphate (PIP_3) by the phosphorylation of the inositol ring at position 3 (53). This triphosphorylation serves as a binding site for proteins containing a pleckstrin homology (PH) domain (52). The main effector kinase that initiates signaling from PIP_3 is phosphoinositide-dependent kinase 1 (PDK1). PDK1 is localized to the plasma membrane via PH domain interaction with PIP_3. The precise activation mechanism of PDK1 is unknown, however it is thought to retain a constitutive activity and its localization is the determining factor of substrate activation (54). PDK1 is

Figure 2: Schematic of key PI3K signal transduction pathways.


EGFR EGFRvIII
5
274
Grb2
Gab1
SOS
PI(4,5)P2
PI(3,4,5)P3
PI(4,5)P2
PTEN
p85
p110
Ras
PI3 kinase
PDK1
Par-3
Cdc42
Par-6
PKB/Akt
PKCι/ζ
Raf
MEK
ERK
Polarity
Proliferation, Survival, Invasion

responsible for the phosphorylation and activation of a number of downstream serine/threonine kinases, the most studied being protein kinase B (PKB/Akt). However, it can also phosphorylate and activate other protein kinases, including the atypical protein kinase Cs (PKC), p70 and p90S6 kinase (55;56). Activation of PDK1 substrates can enhance cell proliferation, survival, migration, protein synthesis and glucose uptake (53;54;56;57).

The activity of PI3K is highly regulated by the lipid phosphatases, PTEN, SH2-containing phosphatase 1 (SHIP1), and SHIP2 which can dephosphorylate the inositol ring, converting PIP_3 to PIP_2, to inactivate signaling (53;57). SHIP1 and 2 dephosphorylate PIP_3 at position 5, generating phosphatidylinositol-3,4-bisphosphate which only partially reduces PI3K signaling. PTEN dephosphorylates PIP_3 at position 3, and this causes a significant down regulation of PI3K signaling (53). PTEN was originally identified as a tumour suppressor gene as mutations have been observed in a variety of tumour types (57;58). The expression of PTEN is lost in approximately 50% of GBM tumours (15;16). Therefore, this along further contributes to amplifying PI3K signaling in GBM.

The EGFR can also activate the membrane bound small guanine triphosphate (GTP) binding protein, Ras. Ras is activated through an interaction with the adaptor protein Grb2. Following EGFR activation, Grb2 either interacts directly or indirectly through Shc with phosphotyrosine on EGFR. Grb2 is constitutively complexed with the guanine nucleotide exchange factor (GEF), son of sevenless (SOS). This localizes the exchange factor to membrane bound Ras and facilitates the exchange of GDP for GTP resulting in Ras activation (39). Activated Ras in turn activates the serine/threonine kinase Raf-1

which initiates the mitogen activated protein kinase (MAPK) signaling cascade (52). These can phosphorylate and activate downstream kinases MAPK/ERK kinases 1/2 (MEK1/2), which is the dual kinase that phosphorylates ERK1/2. Activated ERK 1/2 can phosphorylate and activate a number of cytoplasmic downstream targets, including p90 ribosomal S6 kinases and inhibitory κB (IκB), and it can also translocate to the nucleus where it can phosphorylate and activate nuclear transcription factors (Elk-1, Ets-1, c-Jun, c-Myc) (52;59). Activated Ras can facilitate the activation of PI3K through an interaction with the p110 catalytic subunit which induces a conformational change required for activation (59b). This represents an alternative mechanism of EGFR-mediate PI3K activation and a potential site of further enhancement of PI3K signaling. The downstream PI3K effector, PKCι, has also been shown to be activated downstream of Ras (potentially by Ras- activated PI3K) and is involved in downstream activation of MEK1/2 and ERK1/2 (60;61). Ras-induced transformation was shown to require PKCι activity (62). Additionally, ERK1/2 can in turn promote the expression of PKCι generating a positive feedback loop (63). These results demonstrate the complexity of these signaling pathways and the interactions that exist between downstream effectors to amplify the signaling cascade. In GBM, EGFR-induced Ras activation is a common feature, where its level of activation is similar to that observed in oncogenic Ras transformed fibroblasts (64). While oncogenic Ras mutations are a common occurrence in many human tumours, they are rarely detected in GBM and its activation is completely dependent on RTK activity, EGFR or PDGFR (17;64). Although targeting Ras has been shown to enhance sensitivity of GBM cells to chemotherapy *in vitro*, the agents used are more effective against oncogenic Ras and the concentrations required for effective

inhibition *in vivo* would be extremely toxic (64). Therefore, it may be a more useful strategy to target upstream or downstream of Ras in the treatment of GBM.

1.5 Targeted therapy for the treatment of glioblastoma

There are several characteristics that are thought to contribute to the resistance of GBM to systemic therapy, including aberrant growth factor signaling, altered cell cycle regulation, deregulated apoptosis, increased expression of drug efflux transporters, and the presence of a neural stem cells (27;29;64). The overexpression and mutational activation of the EGFR and its role in cell growth, survival and therapy-resistance has made it an attractive therapeutic target for the treatment of GBM (65-68). Additionally, a key negative regulator of EGFR signaling, PTEN, is mutationally lost in GBM causing an even greater enhancement of signaling through this pathway (26;65;67-70). Specifically targeting the EGFR in lung cancer has shown a significant therapeutic benefit in patients (68). Targeting the EGFR using small molecule inhibitors or monoclonal antibodies showed effectiveness *in vitro*, decreasing the proliferation and radiation resistance of GBM cells lines (69;71;72). However, in the clinical setting these inhibitors have had limited success in the treatment of GBM, showing only partial responses in combination with radiation and chemotherapy (26;69;70). These inhibitors also failed to consistently inhibit the phosphorylation and activation of the EGFR as well as the downstream effector, PKB/Akt (17). This limited response to EGFR inhibition may be caused by ineffective drug concentration reaching the tumour, lack of sensitivity of the receptor to inhibition, or pathway crosstalk which amplifies downstream signaling. Similarly, targeting one of the main direct effectors of the EGFR that initiates downstream

signaling, PI3K has also been shown to be effective in GBM cells *in vitro* (66). However, these compounds have been proven to be difficult to work with *in vivo* due to high toxicity (73;74). As a result there have been no PI3K inhibitors that have progressed through clinical trials (75). Therefore, therapies aimed at targeting further downstream of the EGFR and PI3K may alleviate the potential side effects associated with targeting the upstream effector and may increase specificity. Additionally, identifying downstream effectors that cause resistance of these tumours will allow for the development of multi-target strategies which may ultimately be needed to effectively treat GBM.

1.6 Atypical protein kinase C

1.6.1 Protein kinase C

The protein kinase Cs (PKC) are a conserved family of serine/threonine (S/T) kinases that are key mediators of intracellular signaling downstream of RTKs. The mammalian PKC family has at least 13 different members in total, which are divided into three main subclasses: classical/conventional, novel, and atypical PKCs (55). PKCs are single polypeptides divided into two major domains, an N-terminal regulatory and C-terminal kinase domain (55). The composition of the regulatory domain identifies each class of PKC and also specifies cofactors involved in their activation. Classical/conventional PKCs (α, βI, βII, γ) are sensitive to calcium (Ca2+), phospholipid, diacylglycerol (DAG) and phorbol esters which promote their activation. Novel PKCs (PKCδ, ε, η/L, θ, μ) are DAG or phorbol ester sensitive, but do not require Ca2+. Atypical PKCs (ι/λ, ζ) are insensitive to DAG, phorbol esters and Ca2+ both *in vitro* and *in vivo*, but rely on other lipid cofactors for activation, including PIP_3, ceramide and phosphatidic acid (76-78).

The regulatory region of classical PKCs contains 2 conserved domains (C1 and C2) (79). The C1 region contains two cysteine rich motifs that bind to DAG and phorbol esters. The C2 domain contains Ca2+ and phospholipid binding domains, and together with the C1 domain they function to regulate the activity of these enzymes. The regulatory domain of the novel PKCs is structurally similar to the classical PKCs, however they contain a unique C2 domain which does not bind Ca2+, rendering them Ca2+ insensitive. The regulatory domain of the atypical PKCs differs significantly from the classical and novel PKCs. The C1 domain contains only a single cysteine rich motif and they lack a C2 domain (55). Additionally, they contain an N-terminal Phox and Bem 1 (PB1) domain which is important for mediating protein-protein interactions (79). All PKCs contain a pseudosubtrate (PS) domain that immediately preceeds the C1 domain. This PS domain is a short stretch of amino acids that resembles a substrate recognition sequence and has a high affinity for the substrate binding pocket causing inhibition of the enzyme. It contains an alanine residue in the respective position of the serine (S) or threonine (T) of the substrate. Binding to specific lipid cofactors and proteins at the membrane produces the energy required to release the pseudosubstrate, allowing kinase phosphorylation and substrate interaction.

The C-terminal catalytic domain of the PKC enzymes, the C3 and C4 domains, contain the ATP and substrate binding sites respectively. They contain the conserved S and T residues that are phosphorylated for activation. Activation of PKC enzymes requires phosphorylation of conserved residues in their activation loop, which is dependent on the upstream kinase PDK1 (80). Stable enzyme activation may also require an autophosphorylation of a second S or T residue in the turn or hydrophobic motifs

(55;81). The PKCs enzymes can then phosphorylate several downstream substrates, including Raf-1, inhibitor κB (IκB), and glycogen synthase kinase 3β (GSK3β, 82;83). The PKC family of enzymes has been shown to affect cell survival and apoptosis, depending on the isoform. The activities of PKCδ, θ and μ have been associated with apoptosis (84-86), while overactivation of other family members is involved in tumourgenesis and has been associated with increased cell survival, motility and invasion (80). This has been shown in several tumour types including colon, prostate and brain tumours. Despite their role in tumourgenesis, only the atypical PKC enzyme, PKCι, has been identified as a human oncogene (87;88).

1.6.2 Atypical PKCs structure

The atypical PKC subfamily of kinases is composed of two members, PKCι (PKCλ in mouse) and PKCζ. These proteins are highly related, with a 72% overall amino acid identity and up to 84% in the catalytic domain (89). However, within the C1 domain there is a lower sequence homology (47%) between PKCι and PKCζ, which may allow for isotype specific protein interactions (90). Atypical PKCs contain an N-terminal PB1 domain which has several motifs: an octicosa peptide repeat (OCR), phox and Cdc (PC), and atypical PKC interacting domain (AID), that are collectively called the OPCA motif (79;91;92). These form an interacting surface that link the atypical PKCs to other PB1 domain containing proteins and influence their localization and activation. The best characterized PB1 domain interaction involving atypical PKC is with Partitioning defective mutant-6 (Par-6), which plays an important role in regulating cellular polarity (79;91;92). The PB1 domain also mediates interactions with ZIP/p62 and MEK5, linking

atypical PKCs to tumour necrosis factor α (TNFα) and MAPK mitogenic signaling, respectively (79;92).

The catalytic domain of the atypical PKCs also contains some unique features compared to classical and novel PKCs. Within the ATP binding domain (C3), both classical and novel PKCs contain a glycine rich loop (GXGXXG) which is highly conserved in many protein kinases (81). This is thought to provide the flexibility required to anchor an ATP molecule in the binding site. However, the atypical PKCs contain a conserved alanine residue in the third glycine (GXGXXA) position of this loop which reduces flexibility and forces a more open conformation. This has been shown to be essential for activation loop phosphorylation (79;81). Additionally, mutation of a conserved lysine (K) residue in the ATP binding domain to arginine (R) causes inactivation of most protein kinases. In contrast, mutation of this K in PKCι (K274R) and in PKCζ (K281R) does not affect kinase activity (81;93). However, mutation of this same residue to tryptophan abolishes the kinase activity of both PKCι and PKCζ (79;93;94). Addition of this bulkier amino acid may sterically hinder the open conformation required for phosphorylation. The hydrophobic motif of the atypical PKCs is a short amino acid sequence (FEGFEY) that mediates interaction with PDK1, allowing for activation loop T phosphorylation (77;95). One unique feature of the hydrophobic motif in atypical PKCs is the presence of a conserved glutamate residue, which in classical and novel PKCs is either a S or T (79;81). This residue is thought to act as a phosphorylation mimic and may prime atypical PKC activation (81). Finally, the activation loop and the turn motifs of PKCι and PKCζ contain conserved T residues that are phosphorylated for activation. These are T403 and 410 for PKCι and PKCζ

respectively in the activation loop and T555 and 560 for these kinases in the turn motif (77;95). Phosphorylation of both of these sites is thought to mediate stable activation of the enzymes (81). These differences in the catalytic domain of the atypical PKCs suggest that these enzymes have a unique mechanism of ATP binding which may be useful for the development of specific inhibitors.

1.6.3 Activation of Atypical PKCs

The mechanism of activation for atypical PKCs is not completely understood but has been shown to require both phosphorylation and interaction with key cofactors. The best characterized mode of activation for atypical PKCs is through the PI3K pathway. The atypical PKCs are directed to the plasma membrane, localizing to activated PI3K. At the membrane it is thought that the PS domain is displaced from the substrate binding pocket of the atypical PKC through an interaction with PIP_3 (96). This is not a PH domain interaction as seen with PKB/Akt; the characteristics of the PIP_3-PS domain interaction are not known. This interaction alters the conformation of the atypical PKC and allows for interaction with PDK1. PDK1 directly phosphorylates T403 and 410 on PKCι and PKCζ respectively (77;95;97). This in turn leads phosphorylation of T555 of PKCι or of T560 of PKCζ, by either autophosphorylation or by other protein kinases resulting in full activation of the enzyme (77). Although it is unknown how phosphorylation occurs at these sites, it is thought that they are critical for stabilizing enzyme activity (79;81;98). Inhibition of atypical PKC activity can be achieved by the expression of a kinase inactive PDK1 or the use of a PI3K specific inhibitors (77;97;99).

Unlike PKB/Akt, the activation of the atypical PKCs has also been shown to be influenced both positively and negatively through interactions with multiple cellular proteins which include, Ras, ZIP/p62, Par-6, Par-3 and Par-4.

i. Ras GTPase

Ras activation has been shown to enhance activity of both PKCι and PKCζ *in vitro* and *in vivo* (61;62;100). Ras has been shown to bind directly to the regulatory domain of PKCζ, and this interaction is thought to be involved in the localization of atypical PKCs to active PI3K (100). Additionally, Ras itself can also directly activate PI3K which amplifies activation of downstream effectors (39). Ras can regulate cellular morphology through alteration of the actin cytoskeleton, a process that has been shown to involve the activity of both atypical PKC enzymes (101;102). Overexpression of Ras causes disassembly of actin stress fibers (102). This loss of stress fiber formation can be reversed by inhibition of PI3K signaling using chemical inhibitors, wortmannin and LY294002, or by expression of dominant negative PKCλ or PKCζ.

ii. p62/zeta interacting protein

Zeta interacting protein (ZIP)/p62 is thought to function as a scaffolding protein linking the atypical PKCs to other proteins and signaling pathways. It was first identified by a screen for PKCζ interacting proteins (103). Structurally, ZIP contains a PB1 domain and a zinc-finger domain which are important for protein-protein interactions (92;103). ZIP has been shown to bind both PKCι and PKCζ through a PB1-PB1 domain interaction and regulates their activation (104). Through its zinc-finger domain, ZIP links the

atypical PKCs to activated tumour necrosis factor α receptor (TNFR) and activated interleukin-1 receptor (IL-1R) causing NFκB activation (92;95;104).

iii. Partitioning defective mutant-6

Partitioning defective mutant-6 (Par-6) is a multidomain containing protein that links the atypical PKCs to cell polarity and form a conserved polarity complex (92;105). Par-6 was first identified in a genetic screen for proteins involved in partitioning in *C. elegans* (106). Par-6 contains a conserved PSD95/Discs-large/ZO1 (PDZ) domain which mediates interaction with its family member, Par-3 (105). Par-6 also contains a PB1 domain that facilitates its interaction with either PKCι or PKCζ regulating their localization and activation (107). Par-6 dependent activation of atypical PKC has been shown to be dependent on the presence of the small GTPase Cdc42. Par-6 binds and inhibits atypical PKC activation. This is thought to be the result of a steric inhibition caused by a GTP binding domain (GBD) on Par-6 (92;105;108). Subsequent interaction of the atypical PKC/Par-6 complex with Cdc42-GTP induces a conformational change in Par-6 and relieves the inhibitory effect of the GBD domain, resulting in increased atypical PKC activity (92;105). This mechanism of atypical PKC activation appears to be PI3K dependent, because it requires PDK1 phosphorylation and because Cdc42 is also activated downstream of PI3K (99;109). Therefore, Par-6 acts as an adaptor that couples Cdc42 and atypical PKC signaling.

iv. Partitioning defective mutant-3

Partitioning defective mutant-3 (Par-3), also known as atypical PKC-specific interacting protein (ASIP), can interact with both PKCι and PKCζ (95). Par-3, like Par-6, is involved in the regulation of cell polarity and was found to colocalize with Par-6 at the anterior pole of the *C. elegans* embryo (105;110). Par-3 contains three conserved N-terminal PDZ domains, the first of which interacts with Par-6 while the others can link to other PDZ containing proteins (110). Par-3 can interact with the atypical PKCs indirectly though association with Par-6, or directly by interaction with the catalytic domain (111;112). This interaction was shown to inhibit the activity of the atypical PKCs (111). Par-3 is thought to play an important scaffolding role, where it regulates the proper localization of the atypical PKC/Par-6 complex in the establishment of cellular polarity (95;105). Additionally, the inhibitory function of the Par-3 on atypical PKC kinase activity may serve to spatially regulate its activity (105).

v. Prostate apoptosis responsive-4

Prostate apoptosis response-4 (Par-4) was initially identified as a gene that was induced during apoptosis in prostate cancer cells (95;113). It is an endogenously expressed protein that is regulated by a number of cellular processes, including transformation and apoptosis. Although very little is known about the function of Par-4 it has been shown to negatively regulate atypical PKC activity. Par-4 interacts with the C1 cysteine-rich motif of both PKCι and PKCζ, and inhibits their kinase activity (114). Overexpression of Par-4 has been shown to not only inhibit atypical PKC activation, but also to induce apoptosis (114). Additionally, it is thought that Par-4 may act as a tumour

suppressor by inhibiting the action of the atypical PKCs (76). Par-4 is downregulated in a number of cancers and restoration of expression enhances sensitivity to apoptotic stimuli, including chemotherapies (113). This introduces a further layer of complexity to the regulation of the atypical PKCs.

1.6.4 Cellular functions of atypical PKCs

Since their discovery more than 15 years ago, many studies have described overlapping functions for the atypical PKCs. However, more recently and with the advancement of specific reagents and techniques these enzymes have been shown to have unique roles in cells and are not functionally redundant. The most striking evidence that the atypical PKC isoforms have highly specific roles has come from the development of knockout mice (115). PKCζ knockout mice are viable and their development is essentially normal (116). In contrast, knocking out PKCλ in mice results in embryonic lethality (117). The exact reason for this embryonic lethality is not fully understood, and may be caused by loss of PKCλ specific regulation of cellular signaling and/or polarity. Examination of mouse embryonic fibroblasts from these mice shows that PKCζ-/- cells have impaired NFκB activation, whereas PKCλ-/- cells show normal activity (116;117). Additionally, PKCλ and PKCζ show differential expression within tissues during development, suggesting specific roles within distinct tissues (115). In the developing mouse embryo, PKCλ is expressed at higher levels compared to PKCζ at all stages. The pattern of expression is also different between these isozymes, PKCλ shows a more ubiquitous expression although at varying levels (115). PKCζ, on the other hand, displays a more regional pattern of expression localized to distinct areas within these

tissues (115). Particularly in the brain and lung, there is evidence of preferential expression of PKCλ, where it is either exclusively expressed or expressed at the same or higher levels than PKCζ (115). There are numerous studies that group these enzymes together and this may have misattributed particular functions. Therefore, it is very important to carefully distinguish which atypical PKC isoform is being studied in a particular context because they clearly have distinct functions with cells.

1.6.5 Atypical PKC signaling

The atypical PKCs have been shown to influence cellular proliferation and survival through the regulation and phosphorylation of several intracellular effectors. The atypical PKCs have been linked to cell proliferation through the activation of MEK1/2 and subsequently ERK1/2 through two mechanisms (60;61). Firstly, aPKC (most likely ι) activates Raf-1 which directly phosphorylates MEK1/2 (61) and secondly, through an association of PKCι with Rac1 leading to the activation of PAK1 (p21-activated kinase 1) which can phosphorylate both MEK1/2 or ERK1/2 (60;62). Both of these mechanisms show a requirement for Ras activation upstream of the PKCι. ERK can in turn phosphorylate and activate a variety of intracellular proteins that regulate survival. Further, it can translocate to the nucleus where it can regulate the activity of transcription factors (52;59). Interestingly, the promoter of PKCι contains an Elk-1 transcription factor binding site and Elk-1 is a well characterized substrate that is activated by ERK (63). Therefore, ERK activation of Elk-1 enhances the transcriptional activity and expression of PKCι, forming a positive feedback loop (63). PKCζ has also been shown to regulate ERK activation, however the mechanism of regulation is not fully understood

(62). The atypical PKCs also have been shown to activate an alternate MAPK pathway, MEK/ERK5. Both atypical PKCs associate with MEK5 and facilitate its activation (118). The catalytic activity of the atypical PKCs is not required to activate MEK5, suggesting this interaction may relieve an autoinhibition or that it localizes MEK5 to the kinases responsible for its activation. This interaction links the atypical PKCs to the mitogenic signaling of ERK5 (119;120). Additionally, there may be cell type specific roles for the atypical PKCs in proliferation as T-cells derived from PKCλ-/- mice show no impairment in proliferation, suggesting a cell type specific function of PKCλ in proliferation (117).

The atypical PKCs have also been shown to associate with the TNFR and IL-2R through adaptor proteins to mediate NFκB activation. Both PKCι and PKCζ have been shown to activate NFκB (116;121;122). This is thought to occur through direct phosphorylation of the inhibitory kappa B kinase β (IKKβ), which in turn phosphorylates inhibitory kappa B (IκB) relieving NFκB inhibition allowing it to dimerize and translocate to the nucleus (123;124). In the nucleus NFκB induces transcription of a number of genes that enhance cell survival (122;125). However, there also appears to be a cell type dependence for PKCι or PKCζ-induced NFκB activity (60;116;117;121;122). Specifically, mouse embryonic fibroblasts (MEF) isolated from PKCλ-/- mice retain normal NFκB activation, whereas MEFs isolated from PKCζ-/- mice exhibit an impairment of NFκB activity when treated with TNFα (116;117). Atypical PKC activity can also antagonize apoptotic signaling. Ultraviolet radiation causes PKCζ inactivation, which in turn leads to an inhibition of ERK activation, and a simultaneous increase in p38MAPK activation, which signals cell death in many cell types (126;127).

Additionally, Par-4, which negatively regulates atypical PKC activity, can induce apoptosis (128;129). This appears to be exclusively through its ability to inhibit atypical PKC activity (130). Recently, it has been shown that atypical PKCs can repress the activity of key apoptotic effectors. PKCι phosphorylates and inhibits the activity of the proapoptotic protein Bad on multiple S residues. Bad is also a substrate for PKB/Akt kinase, however, it only phosphorylates a single S, suggesting that PKCι may be a more potent inhibitor of Bad activity (131). Similarly, PKCζ was shown to phosphorylate and inhibit Bax (132). The phosphorylation of Bad and Bax interferes with their ability to bind and inhibit the antiapoptotic activity of Bcl2/Bcl-XL and mitochondrial membrane permeability (131;132). Bad and Bax phosphorylation appear to be specific to PKCι and PKCζ respectively, supporting the functional diversity between these isotypes.

The atypical PKCs have also been shown to be involved in regulating cell cycle progression by affecting the expression of several cell cycle proteins, including cyclin dependent kinase (CDK) inhibitors and cyclins (133;134). The CDK inhibitors, $p21^{Waf1/Cip1}$ and $p27^{Kip1}$, when upregulated or overexpressed reduce proliferation and induce accumulation of cells in G1 (135;136). Both of these proteins have been shown to be negatively regulated by the atypical PKCs, as well as PKB/Akt. Atypical PKC phosphorylates a S residue immediately adjacent to the T residue that is phosphorylated by PKB/Akt on $p21^{Waf1/Cip1}$ (133). These modifications individually negatively regulate the expression of p21 $^{Waf1/Cip1}$ by signaling proteosomal degradation (133). Additionally, siRNA depletion of PKCι in GBM cell lines negatively regulated the expression and nuclear accumulation of $p27^{Kip1}$ (*unpublished observation*). Similarly, PKB/Akt also negatively regulates the expression of $p27^{Kip1}$. This occurs through direct

phosphorylation of $p27^{Kip1}$, and prevents its nuclear accumulation and promotes its degradation (137-139). PKCι may also promote $p27^{Kip1}$ protein degradation, however this has not yet been shown. PKCι may also promote cell cycle progression through regulation of cyclin expression. In ovarian cancer tumours elevated PKCι expression is associated with increased cyclin E expression and in GBM cells depletion of PKCι results in a reduction in cyclin D1 expression (*unpublished observation*) (134). Similarly, overexpression of a constitutively active PKB/Akt increased the stability of cyclin D1 (139). This seems to occur through an inhibitory phosphorylation of GSK3β, which functions to phosphorylate cyclin D1 causing cyclin D1 to be exported from the nucleus and degraded in the cytoplasm (139). PKCι has also been shown to phosphorylate GSK3β at the same site as PKB/Akt, therefore it may also regulate cyclin D1 by a similar mechanism (99).

Overall, these data demonstrate the importance of PKCι and PKCζ in regulating key effector proteins involved in signaling cellular proliferation, apoptosis and regulating the cell cycle. They highlight the distinct functions that these highly homologous proteins possess and the existence of cell type specific functions. They also show that the atypical PKCs and PKB/Akt, while having distinct roles in cell signaling, share some overlapping functions and that inhibition upstream of these kinases is insufficient to distinguish between their functions. It is also possible that in different cell types and tissues there may be a preference to rely on one of these enzymes over the others. Therefore, results obtained from the inhibition of shared upstream activators of these kinases should be interpreted cautiously to limit the possibility of misattributing functions.

1.6.6 Atypical PKC and polarity

Polarity is an important aspect of cell function in all eukaryotic cells, allowing for controlled cell division and differentiation, development of tissues, and cellular migration. The atypical PKCs are an essential component of the machinery involved in cell polarity which appears to be evolutionarily conserved. In the establishment of cell polarity, the atypical PKCs cooperate with a family of proteins called the partitioning defective or Par proteins. These were first identified in a genetic screen for proteins affecting the asymmetric division of the one-celled *C. elegans* embryo (106). There are six Par proteins (Par-1 to 6) and each has a unique function (140). The atypical PKCs form a conserved polarity complex with Par-6 and Par-3 that has been observed in *C. elegans*, *Drosophila melanogaster* and mammalian cells (107). Atypical PKC and Par-6 make up the core of this complex and Par-3 associates with the plasma membrane and functions as a scaffolding protein responsible for their recruitment to a particular membrane region. The kinase activity of atypical PKC is also essential for formation of an active polarity complex (141). The functioning of the atypical PKC/Par-6 complex in polarity requires association with the small GTPase, Cdc42, which interacts directly with Par-6 (142;143). Cdc42 responds to both extracellular and intracellular stimuli to regulate both actin and microtubule organization, and therefore links the atypical PKC/Par-6 complex to cytoskeletal dynamics (142). Together the polarity complex and Cdc42 have been shown to regulate several cell polarity processes, such as asymmetric cell division, apical-basal polarity and cell migration.

Asymmetric cell division involves not only the formation of two unequally divided cells with respect to size, but also the unequal distribution of intracellular molecules

(105). This is important for the maintenance and generation of a self-renewing cell and a cell with a predetermined fate. In both *C. elegans* and *Drosophila* oocytes, the atypical PKC (PKC3 in *C. elegans*, atypical PKC in *Drosophila*)/Par-6/Par-3 (Bazooka in *Drosophila*) complex interacts with Cdc42 to establish anterior-posterior regions (140;144). Active Cdc42 recruits the polarity complex to the membrane of the anterior pole (108;145). Binding of Cdc42 to Par-6 induces a conformational change in Par-6 leading to atypical PKC activation (108;142). Loss of Cdc42 in embryos disrupts the asymmetric localization of the atypical PKC/Par-6/Par-3 complex (145). Subsequently, posterior pole components, including other Par family members (Par-1 and Par-2), become properly localized (144;146). This orientation is thought to regulate the pulling forces on the microtubules required for proper asymmetric cell division (110).

A key substrate of the atypical PKC that has recently been shown to regulate *Drosophila* oocyte polarity is Lethal giant larvae, Lgl (147). Lgl is a membrane associated protein that was first identified in *Drosophila* as a tumour suppressor (148). It is phosphorylated and inactivated in the anterior region by atypical PKC and this is thought to spatially regulate Lgl activity. Therefore, active Lgl is restricted to the posterior pole where it regulates the accumulation of proteins required to maintain the integrity of anterior-posterior polarity (ie. Par-1, 147). The precise mechanism by which Lgl promotes this asymmetric distribution is unclear. Loss of any of these proteins results in *C. elegans* or *Drosophila* causes polarity defects. Additionally, this also occurs in *Drosophila* neural progenitor cells (neuroblasts, 149). Cdc42 and the polarity complex polarize to the apical cortex and direct the accumulation of differentiating factors to the basal cortex (149;150). Lgl activity is restricted to the basal cortex where it is required

for the accumulation of cell-fate determinants such as Numb and Pros (151). This results in unequal cell division that generates a large self-renewing neuroblast and a small basal ganglion cell that differentiates into either a neuron or glial cell. Loss of function or localization of Cdc42, Par-6, Par-3, atypical PKC or Lgl has been shown to cause abnormal neuroblast proliferation and the formation of tumours (reviewed in (150)). Interestingly, it has recently been shown that atypical PKC also plays a role in the regulation of *Drosophila* neuroblast self-renewal, where it can also promote symmetric cell division (152). This function may have potential implications in the maintenance of a progenitor cell population in tumours, including GBM.

The establishment of apical-basolateral polarity is a necessary process in the formation of a functional epithelial cell barrier. This is highly dependent on laterally localized cell-cell contacts called Adherens junctions (AJ) and Tight junctions (TJ, 153). The primary role of AJ is the maintenance of cell-cell association, while TJ form a selective barrier that segregates specific membrane constituents to the apical or basolateral surface of the epithelial cell (154;155). The exact mechanism of TJ formation is unclear, however there is accumulating evidence that polarity complex plays a central role in their formation (107;140;155). Tight junctions contain specific transmembrane proteins called occludins, claudins and junctional adhesion molecules (JAM1-3), which interact with the adjacent cell extracellularly and with the actin binding proteins, zona occludins (ZO1-3) intracellularly. JAM1 is recruited to primitive cell-cell contacts during the formation of the junction (155). In mammalian MDCK (Madin-Darby canine kidney) epithelial cells, Par-3 has been shown to bind directly to JAM1 via PDZ domain interaction (140). This allows for the recruitment of atypical PKC/Par-6 to the junction (107;155). Expression of

kinase inactive mutants of PKCλ or PKCζ in mouse epithelial cells or MDCK cells inhibit the formation of TJ and is associated with Par-3 misslocalization (154;156;157). Additionally, *in vivo*, the loss of PKCλ specifically in the brain of mice causes a disruption in neuroepitheial architecture and a loss of cell-cell adhesions (158). Lgl also plays a role in regulating apical-basolateral polarity of epithelial cells. Lgl competes with Par-3 for binding to PKCλ/Par-6 thus sequestering it away from Par-3, inhibiting TJ formation (153). Lgl has also been shown to inhibit the interaction of Par-6 with Cdc42, which may affect the activation of PKCλ (159). Further, the activation of PKCλ interferes with Lgl association allowing for assembly of the active polarity complex. Phosphorylation of Lgl by atypical PKC in the apical cortex restricts its activity to the basolateral domain (140). This antagonistic interaction between atypical PKC and Lgl drives the maintenance of epithelial cell polarity. The maintenance of cell-cell adhesions is essential to proper epithelial development and function. Loss of this organization is implicated in the epithelial to mesenchymal transition associated with the invasive phenotype that many tumour cells exhibit.

Cell migration is a highly coordinated process that involves changes in cell morphology including development of membrane protrusions, reorganization of intracellular organelles, formation of adhesion complexes and cytoskeletal contraction to generate forward movement. Although the atypical PKCs play a critical role in the establishment of cell polarity, their exact roles in cell migration are not well understood. However, PKCζ has been implicated in the migration of several cell types including, astrocytes, intestinal epithelia, mammary epithelia, endothelial cells and fibroblasts (160-166). The role of PKCι in cell migration has not been directly studied.

Cell movement involves the reorganization of the nucleus, microtubule organizing center (MTOC) and Golgi apparatus (Golgi). When a cell is stimulated to migrate, the nucleus shifts from the center of the cell to the rear lagging edge, with respect to the direction of migration. The MTOC and the Golgi both reorganize to the leading edge of the nucleus (77;167). Microtubules emanate outward from the MTOC to the cell's leading edge. This is thought to facilitate the delivery of key mediators of cell migration to the leading edge surface. In primary rat astrocytes, Cdc42 activity is required for the polarization of the MTOC and Golgi following stimulation of migration (160). Active Cdc42 was shown to recruit PKCζ and Par-6 to the leading edge of the cell. Further, both PKCζ and Par-6 are essential for the reorganization of the MTOC and Golgi in both astocytes and fibroblasts stimulated to move (160;163). PKCζ activity is also required, as kinase inactive PKCζ significantly inhibited their polarization (160). Interestingly, in the astrocytes, PKCλ appeared to have no effect on the polarization of the MTOC.

A critical downstream target of the activated PKCζ/Par-6 complex is GSK3β. GSK3β was shown to be phosphorylated preferentially at the leading edge of migrating astrocytes colocalizing with Cdc42, PKCζ and Par-6 (168). This phosphorylation negatively regulates the activity of GSK3β, suggesting that its local inactivation is important for cell migration. GSK3β has also been shown to be inhibited through protein-protein interaction which may be more important than inhibition by phosphorylation (162). Regardless of the mechanism, inhibition of GSK3β impairs MTOC and Golgi reorganization (162;168). GSK3β is a key component of a number of signaling pathways where its activation has been shown to regulate gene transcription (168). With respect to cell migration, it has been shown to regulate the activity of an important microtubule

associated protein, adenomatous polyposis coli (APC). APC interacts with microtubule plus ends and regulates their localization (169;170). Additionally, APC localizes to the leading edge of the cell where active Cdc42 and PKCζ/Par-6 reside in the migrating cell (168). Therefore, this links Cdc42 and PKCζ/Par-6 to the regulation of microtubule dynamics in a migrating cell.

The small GTPases, Cdc42 and Rac become localized to the leading edge of a migrating cell where they coordinate the formation of filopodia and lamellipodia, respectively (171). These membrane protrusions extend from the leading edge of the cells and initialize cellular movement. Lamellipodia are short, highly branched actin filaments located at the leading edge of the cell. In contrast, filopodia are long, unbranched actin filaments that are arranged in tight parallel bundles (172). Filopodia sample the extracellular environment that lies ahead of the leading edge in search of a favoured path. It has previously been shown that Cdc42 can regulate the activity of Rac1 through the activation of atypical PKCζ (173). PKCζ activates a specific guanine nucleotide exchange factor, Tiam1/2 which in turn activates Rac1 (174). This is directly associated with the regulation of lamellipodia formation. The atypical PKCs and Cdc42 can also regulate actin cytoskeletal dynamics. A loss of actin stress fibre formation is associated with increased cell migration (101;102;175). Expression of a constitutively active Cdc42 induces a loss in actin stress fibre formation in fibroblasts (101). The co-expression of a kinase inactive PKCλ or PKCζ with active Cdc42 abrogates this effect. Similarly, expression of constitutively active PKCλ or PKCζ results in a loss of stress fibres in these fibroblasts (102). PKCλ$^{-/-}$ MEFs also exhibit a pronounced increase in actin stress fibres (117). However, this was not observed in fibroblasts isolated from

PKCζ$^{-/-}$ mice (116). Recently, it has also been shown that PKCλ may be involved in the regulation of integrin-mediated adhesions at the leading edge of migrating cells (176). Expression of a kinase inactive PKCλ decreased the turnover of adhesions at the leading edge and reduced cell migration. These results demonstrate that PKCι and PKCζ may have distinct functions in cell migration and suggest different cell types may rely on one enzyme over the other to promote in this process.

The literature on cell polarity largely assumes that PKCι and PKCζ have redundant functions. Clearly *in vitro*, there are distinct functions and cell type specificities for these enzymes. However, *in vivo* only PKCλ has been shown to potentially affect cell polarity (117;158). The PKCλ$^{-/-}$ mice exhibit embryonic disorganization which may be due to defects in establishing embryo polarity, although this has not been proven. Additionally, the distinct expression profiles of PKCι and PKCζ support the idea of cell or tissue-specific dependence on either PKCι or PKCζ. Cellular polarity is a multifaceted process that encompasses localization of intracellular components as well as the orientation of the entire cell. The atypical PKCs play an important role in controlling many aspects of cell polarity.

1.6.7 Atypical PKCs and cancer

The atypical PKCs play significant roles in cell proliferation, survival and polarity in normal cells, therefore it is not surprising that they play a role in cancer. Activities of one or both of the atypical PKCs have been implicated in several cancer types, including NSCLC, ovarian cancer, leukemia, colon cancer and glioblastoma (62;87;177-180). The specific functions of PKCι and PKCζ have been shown to be distinct among these

cancers. The expression of a kinase inactive mutant of PKCζ in both leukemia (U937) and colon cancer (HT-29) cell lines has been shown to accelerate cell death in response to chemotherapeutic agents (etoposide and cisplatin) both *in vitro* and *in vivo* (177). This effect was attributed to the attenuation of NFκB activation (117). However, this pro-survival effect of PKCζ in these leukemia cells seems stimulus specific, as phorbol ester (TPA) treatment of U937 cells overexpressing PKCζ had enhanced sensitivity to this treatment (181). The overexpression of PKCζ has also been shown to have an anti-tumourigenic effect in other tumour cell lines. In a breast cancer cell line (MDA-MB-468), the overexpression of PKCζ inhibited epidermal growth factor (EGF)-stimulated cell growth signaling (182). Additionally, the overexpression of PKCζ in human colon cancer cells (Caco-2) inhibited both cell growth and anchorage-independent colony formation in soft agar, and promoted apoptosis (183). Downregulation of PKCζ has also been observed in human colon tumours, thus the loss of PKCζ favours tumour development in this model. This evidence suggests PKCζ plays distinct roles within and between tumour types.

In contrast, PKCι has been strictly associated with pro-tumourgenic activities. In a distinct leukemia cell line, K562, overexpression of PKCι causes resistance to drug-induced (taxol and okadaic acid) apoptosis (121;178;184). PKCζ protein expression was undetectable in this cell line and its overexpression had no protective effects. The resistance observed in these cells was shown to be the result of Bcr-Abl-induced activation of PKCι, as leukemia cells that do not express Bcr-Abl have an attenuated PKCι activation in response to drug treatment (184). Bcr-Abl signaling also positively

regulates PKCι expression through the activation of ERK1/2, forming a positive feedback which may further promote PKCι-induced drug resistance (63). Surprisingly, this protective effect of PKCι was also linked to NFκB activation (121). This suggests that in some tissues PKCι is capable of activating the NFκB pathway while others rely on PKCζ. PKCι has also been shown to contribute to cell growth and tumourgenicity in human non-small cell lung cancer (NSCLC), were it has also been shown to be overexpressed (60). Expression of a kinase-deficient PKCι in A549 NSCLC cells significantly inhibited colony formation in soft agar and growth of xenografts in nude mice. This was associated with an inhibition in ERK activity and no alterations to NFκB activation, consistent with PKCλ-\- MEFs (60;117). In addition to its role in promoting tumourgenicity and chemoresistance, PKCι can induce migration and invasion. Depletion of PKCι in NSCLC cells using RNA interference significantly inhibited wound closure and invasion through Transwell chambers *in vitro*. This occurs through a novel mechanism in which PKCι regulates the activity and secretion of proteases (calpains) in response to nicotine treatment (185). Similarly, PKCι promotes the expression of the matrix metalloproteinase-10 in NSCLC cells, which contributes to both anchorage-independent colony formation and invasion *in vitro* (186). Recently, Stallings-Mann *et al.* have identified a novel small molecule inhibitor of PKCι (187). This compound, aurothiomalate, has previous been used for the treatment of rheumatoid arthritis. It was shown to interfere with the association of PKCι with Rac1 thereby inhibiting PKCι-mediated Rac1 activation, and blocking tumourgenic growth both *in vitro* and *in vivo*

(187). This suggests that PKCι may represent an effective therapeutic target for the treatment of NSCLC.

PKCι has also been shown to play a role in the transformation of cells. In a chemically-induced colon cancer mouse model, elevated expression and activity of PKCι was observed following treatment with the inducing agent (62). When these mice were crossed with mice expressing a constitutively active PKCι, it increased their sensitivity to tumour induction. As a result, they developed colon tumours with a higher incidence compared to control mice. Similarly, PKCι activity was also shown to be required for Ras-induced transformation of rat intestinal epithelial cells *in vitro* and the development of colonic precursor lesions in a Ras-induced colon cancer mouse model (62;164). This evidence supports a potential pro-tumourigenic role for PKCι in human cancers.

The role of PKCι in human cancer has been emphasized by the recent observation that it may be a critical oncogene in the development of both lung and ovarian cancer (87;134;179). PKCι is the first and only of the PKC family member to be classified as an oncogene. The PKCι gene lies on chromosome 3q26 (87;134;179). Mutations in this region occur with a frequency of approximately 20% in NSCLC and 44% in ovarian cancers (88;179). This region has also been shown to be mutated in other cancers, including head and neck and cervical cancers (88;188). In ovarian cancer, both an increased PKCι gene copy number, as well as increased protein expression and activation correlated with poor prognosis (134). Expression levels inversely correlated with patient survival (87). This increased PKCι expression and activation resulted in defects in apical-basal polarity due to mislocalization in the ovarian surface epithelium. The expression of Cyclin E was also found to be increased in these ovarian tumours, which

may increase the proliferative capacity of these cells (134). Additionally, PKCι enhances the Ras-induced tumourgenicity of primary murine ovarian surface epithelium *in vitro* (179). In NSCLC cells expression of a kinase-defective PKCι inhibited colony growth in soft agar of A549, H1299 and ChaGo cells. PKCι mRNA and protein expression is elevated in tumours compared to normal lung tissue (87). Additionally, frequent amplification of the PKCι gene, along with elevated protein expression, was observed in primary lung tumour samples compared to normal lung tissue controls (87). These results are consistent with a causal role for PKCι in both lung and ovarian cancer. In contrast, PKCι protein amplification has also been observed in colon cancer and CML without any detectable gene amplification (62;63). Recently, it was shown that PKCι protein expression may be elevated in primary brain tumours compared to normal brain tissue (180). However, it is not known whether this increase in protein expression is the result of gene amplification (180). Overall, this evidence suggests that PKCι may contribute to the onset and progression of these tumours.

1.7 Rationale and hypothesis

Glioblastoma multiforme is the most common and most aggressive form of primary brain tumours occurring in adults. Despite the ongoing research to understand the complexity of this devastating disease it remains largely incurable. Aggressive therapeutic strategies combining surgery, radiation, and chemotherapy have only modestly improved median survival times. Glioblastoma cells exhibit an intrinsic resistance and are highly invasive, which contributes to the ineffectiveness of the current therapies. Ultimately, tumour recurrence results in patient death. The underlying mechanisms of this resistance and invasiveness are poorly understood. Due to the limited and ineffective treatments for glioblastoma it is especially important to identify new therapeutic targets and strategies for this devastating disease.

Two common genetic mutations that have been identified in GBM are mutation of PTEN and the amplification and mutation of the EGFR (16). Both these mutations cause aberrant activation of the PI3K pathway. Direct inhibition of the EGFR has been shown to be less effective than originally thought in many patients (70). This is most likely due to the fact that the PI3K pathway can be activated by alternate mechanisms. Further, direct inhibition of PI3K has been shown to be highly toxic (74). Therefore, targeting downstream effectors of PI3K might generate a more specific and effective response. PKCι is activated downstream of PI3K, and is expressed predominantly in the lung and brain (89). It has been shown to be a critical oncogene in the tumourigenesis of both lung and ovarian cancer, and may be overexpressed in primary glioblastoma tumours (87;134;179;180). Therefore, PKCι represents a promising downstream target of the PI3K pathway that has not been studied in glioblastoma.

Hypothesis

PKCι plays a critical role in glioblastoma cell survival, motility and invasion; therefore, depletion/inhibition of PKCι in glioblastoma cells will enhance their sensitivity to chemotherapy and inhibit the invasiveness of glioblastoma cells.

Aim of thesis

The primary objective of this thesis is to identify and characterize the role of PKCι in glioblastoma cells by:

1. Examining the contribution of PKCι to the survival and chemoresistance of glioblastoma cells
2. Identify the potential mechanism of PKCι-induced chemoresistance
3. Investigating the role of PKCι in glioblastoma cell motility and invasion
4. Identify the mechanism of PKCι-induced motility and invasion
5. Assessing the consequence of stable PKCι repression in glioblastoma cells and its effects on cell growth and motility

2. PROTECTION OF GLIOBLASTOMA CELLS FROM CISPLATIN CYTOTOXICITY VIA PROTEIN KINASE Cι - MEDIATED ATTENUATION OF p38 MAP KINASE SIGNALING *

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Running Title: PKCι-mediated resistance to cisplatin cytotoxicity

Keywords: glioblastoma; protein kinase C ι; glia maturation factor β; p38 MAP kinase; cisplatin

Contribution of Authors

The content of this manuscript was written by R. M. Baldwin with the help of Dr. I. A. J. Lorimer. All of the experiments presented in this manuscript are the work of R. M. Baldwin with the exception of Figure 3E and Figure 5. Figure 3E was performed by M. Garratt-Lalonde (Masters student of I. A. J. Lorimer). The microarray analysis performed to generate Figure 5 is the work of P. M. Kryzanowski (Ph. D. student of M. A. Andrade). M. Garratt-Lalonde and D. A. E. Parolin initially characterized siPKCιA and performed the PCR analysis of PKCι and PKCζ expression in U87MG cells.

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Abstract

Glioblastoma multiforme is an aggressive form of brain cancer that responds poorly to chemotherapy and is generally incurable. The basis for the poor response of this cancer to chemotherapy is not well understood. The atypical protein kinases C (PKCι and PKCζ) have previously been implicated in leukaemia cell chemoresistance. To assess the role of atypical protein kinase C in glioblastoma cell chemoresistance, RNA interference was used to deplete human glioblastoma cells of PKCι. Transfection of cells with either of two different RNA duplexes specific for PKCι caused a partial sensitisation to cell death induced by the chemotherapy agent cisplatin. To screen for possible mechanisms for PKCι-mediated chemoresistance, microarray analysis of gene expression was performed on RNA from glioblastoma cells that were either untreated or depleted of PKCι. This identified sets of genes that were regulated either positively or negatively by PKCι. Within the set of genes that were negatively regulated by PKCι, the function of the gene coding for GMFβ, an enhancer of p38 MAP kinase signaling, was investigated further, as the p38 MAP kinase pathway has been previously identified as a key mediator of cisplatin cytotoxicity. The expression of both GMFβ mRNA and protein increased upon PKCι depletion, and this was accompanied by an increase in cisplatin-activated p38 MAP kinase signaling. Transient overexpression of GMFβ increased cisplatin-activated p38 MAP kinase signaling and also sensitised cells to cisplatin cytotoxicity. The increase in cisplatin cytotoxicity seen with PKCι depletion was blocked by the p38 MAP kinase inhibitor SKF86002. These data show that PKCι can confer partial resistance to cisplatin in glioblastoma cells by suppressing GMFβ-mediated enhancement of p38 MAP kinase signaling.

Introduction

Glioblastoma multiforme is the most common type of brain tumor found in adults. It is usually incurable, and patients have a median survival time after diagnosis of one year. Both surgery and radiation increase survival times but are rarely curative, because of local or distant recurrence within the central nervous system. As well, chemotherapy now has an accepted role in the treatment of glioblastoma, with the demonstration that the DNA alkylating agent temozolomide enhances survival (Stupp *et al.*, 2005). However, the survival benefit with this drug is quite modest, and glioblastoma is resistant to most other standard cancer chemotherapeutics. The basis for this resistance is not fully understood.

In other types of cancers, several studies have linked members of the atypical protein kinase C family to chemoresistance (Murray and Fields, 1997; Filomenko *et al.*, 2002). Primarily this has been studied in leukemia cells, although there is also some evidence that the atypical protein kinases C have a role in colon cancer chemoresistance. A possible role for the atypical protein kinases C in glioblastoma chemoresistance has not been examined. In humans there are two closely-related members of the atypical protein kinase C family, designated PKCι and PKCζ. Although much of the early literature does not distinguish between these two enzymes, studies in knockout mice now show that they have distinct functions (Soloff *et al.*, 2004), and perhaps also distinct activation mechanisms. The atypical protein kinases C are both distinguished from other members of the PKC family by their lack of dependence on diacylglycerol for activation; instead they appear to be activated by phosphorylation and by association with different protein complexes. For PKCι (the focus of this paper), activation appears to involve both

phosphorylation by phosphoinositide-dependent kinase 1 (PDK1) and an association with an activated member of the Cdc42 family of GTPases (Kanzaki *et al.*, 2004). PDK1 and Cdc42 are both activated downstream of multiple growth factor receptor tyrosine kinases. For PDK1 this occurs via activation of PI 3-kinase (Vanhaesebroeck and Alessi, 2000). The epidermal growth factor receptor is frequently activated in glioblastoma by amplification and mutation (Lorimer, 2002), which leads to constitutive activation of the PI 3-kinase pathway (Moscatello *et al.*, 1998). Mutations in PTEN, a negative regulator of the PI 3-kinase pathway, are also common in glioblastoma (Sansal and Sellers, 2004). PKCι could therefore potentially mediate some of the downstream consequences of these oncogenic mutations. Multiple other mechanisms of atypical protein kinase C activation have also been reported (Moscat and Diaz-Meco, 2000), and there is evidence for kinase-independent signaling functions for the protein as well (Diaz-Meco and Moscat, 2001). It is generally not clear if these mechanisms are used by both of the atypical protein kinases C, or are PKCζ-specific.

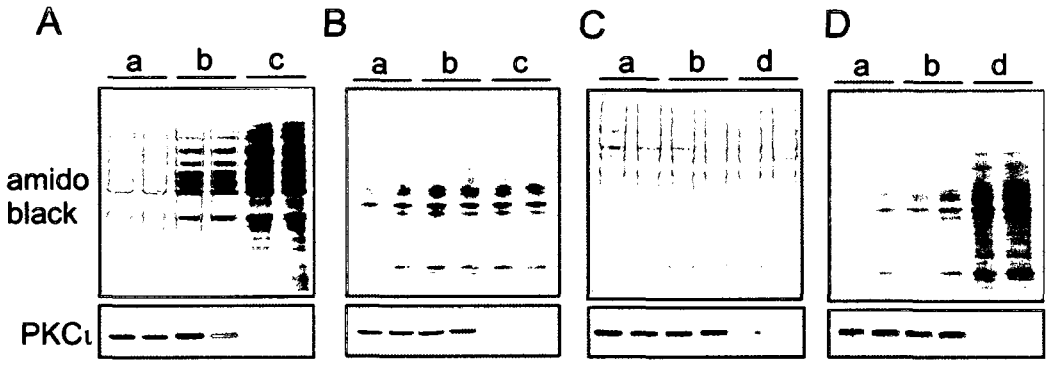
The protein kinase C family has at least thirteen different family members in total and previously it has been difficult to determine precise roles for its specific members in human cell lines. This paper describes the use of RNA interference to address this problem and assess the role of PKCι in glioblastoma cell chemoresistance. PKCι was found to confer partial resistance to cisplatin, a widely-used chemotherapy agent. Microarray analysis of gene expression identified a novel linkage between PKCι and the p38 MAP kinase pathway that contributes to glioblastoma cell chemoresistance.

Results

PKCι depletion by RNA interference: Expression of aPKCs in U87MG cells was initially characterized by RT-PCR. Using mRNA prepared from U87MG glioblastoma cells as template, RT-PCR with the primer pair IOTA1 and IOTA2 (see Materials and Methods) gave a product of the expected size (data not shown). Sequencing of the PCR product confirmed that PKCι cDNA had been amplified. RT-PCR using U87MG mRNA and two different primer pairs for PKCζ did not give any PCR products, although both primer pairs gave products of the expected size when a plasmid containing the cDNA for PKCζ was used as template. U87MG cells therefore express PKCι mRNA, but no detectable PKCζ mRNA.

To assess the role of PKCι in human glioblastoma cells, RNA interference was used to reduce PKCι levels in U87MG cells. Two RNA duplexes, designated siPKCιA (sense strand sequence GUGCAUCAACUGCAAACUC) and siPKCιB (sense strand sequence UGAGGUUCGAGACAUGUGU), were synthesized. Each strand contained a 3' overhang of two 2-deoxythymidines. BLAST searches (Altschul *et al.*, 1997) with the sense sequence of each of these duplexes showed that there were no exact matches to either sequence in the GenBank, EMBL, DDBJ and PDB databases, other than PKCι itself. U87MG glioblastoma cells were transfected with a random control RNA duplex or the siPKCιA RNA duplex, and levels of PKCι mRNA were assessed by quantitative PCR. This showed that the siPKCιA duplex reduced PKCι mRNA levels by 75 ± 4 % two days after transfection (data not shown). Western blot analysis showed that PKCι protein levels were also reduced two days after transfection in both U87MG cells (Figure 1A) and a second human glioblastóma cell line, A172 (Figure 1B). A similar level of

Figure 1. Depletion of PKCι in glioblastoma cells using RNA interference. A - D; Glioblastoma cells were mock transfected (a), transfected with control RNA (b), transfected with siPKCιA (c) or siPKCιB (d) RNA duplexes. Total cell lysates were collected 48 h post transfection and analyzed by Western blotting for expression of PKCι. Amido black staining of membranes was used to assess total loading and transfer of proteins. A and B show PKCι depletion with siPKCιA in U87MG and A172 cells respectively. C and D show PKCι depletion with siPKCιB in U87MG and A172 cells respectively. E; Quantitation of PKCι depletion. Different amounts of total cell lysate from U87MG cells transfected with a random RNA duplex (control), siPKCιA or siPKCιB were analyzed by Western blotting for levels of PKCι and ERK (as a loading control). F and G; U87MG cells (F) and A172 cells (G) treated as above were analyzed by Western blotting with an antibody that recognizes PKCα, PKCβI and PKCβII, with an antibody that recognizes PKCδ, and with an antibody to ERK (used as a loading control).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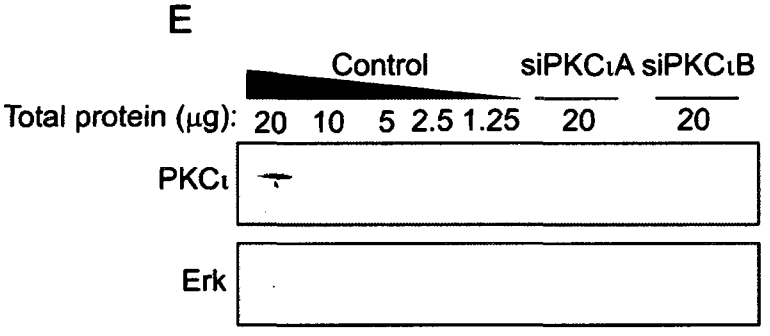
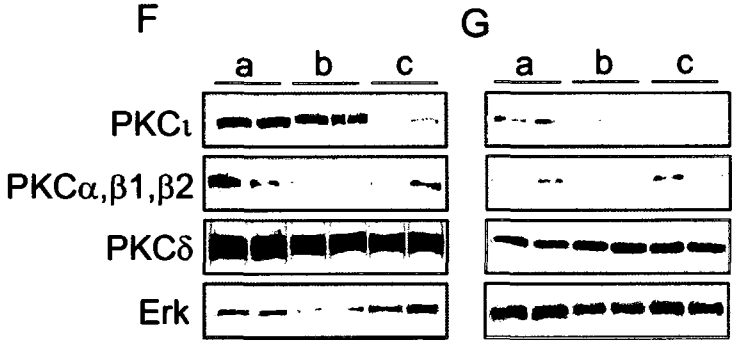
amido black
PKCι


E
Control
siPKCιA
siPKCιB
Total protein (μg):
20
10
5
2.5
1.25
20
20
PKCι
Erk


F
G
a
b
c
a
b
c
PKCι
PKCα,β1,β2
PKCδ
Erk

depletion was maintained at three days after transfection (the longest time point examined, data not shown). Western blot analyses of different amounts of cell extract showed that PKCι levels were reduced by 75 - 90 % in different experiments (Figure 1E). The effects on PKCι mRNA and protein levels were seen using concentrations of RNA duplex below those reported to cause non-specific effects in other systems (Semizarov *et al.*, 2003). The second RNA duplex, siPKCιB, also reduced the levels of PKCι in both U87MG and A172 cells (Figures 1C and 1D). However, on average this duplex did not deplete PKCι protein levels as effectively as the siPKCιA duplex (Figure 1E). Protein levels of other PKC family members examined (PKCα, PKCβI, PKCβII and PKCδ) were unaffected by RNA interference targeted to PKCι (Figure 1F and G). Microarray analysis data also showed that mRNA levels for other PKC members were unaffected (see below).

PKCι depletion sensitizes glioblastoma cells to cisplatin: To determine whether PKCι conferred resistance to cell death induced by chemotherapy agents, U87MG cells were transfected with random or siPKCιA RNA duplexes and then treated with the chemotherapy agent cisplatin. Depletion of PKCι resulted in sensitization of U87MG glioblastoma cells to cisplatin, as assessed by MTT assays (Figure 2A). The IC_{50} for cisplatin on U87MG cells was 60 μg/ml for mock and control RNA duplex-transfected cells, and 30 μg/ml for siPKCιA transfected cells. Cisplatin potency has been reported to be very different depending on the cell culture media used (Hanigan *et al.*, 2005); these values were all determined using the cell culture conditions described in Materials and Methods. Further experiments were done using a cisplatin concentration of 20 μg/ml, as the difference in cytotoxicity between control and PKCι-depleted cells was pronounced at

this concentration. Figure 2B-G shows the morphology of cells treated with cisplatin with or without PKCι depletion. Cells that were depleted of PKCι and treated with cisplatin were more rounded up and exhibited more blebbing than cells treated with cisplatin alone, or depleted of PKCι only. Determination of live cell counts showed that PKCι depletion with either siPKCιA or siPKCιB significantly enhanced the cytotoxicity of cisplatin, in both U87MG cells (Figure 2H and 2J) and A172 cells (Figure 2I and 2K). Flow cytometry was used to directly assess effects of PKCι depletion on apoptosis. PKCι depletion on its own had no effects on levels of apoptosis in U87MG cells (data not shown). PKCι depletion did significantly increase the amount of apoptosis induced by 20 μg/ml cisplatin compared to that seen in mock or control RNA duplex-transduced cells. This was seen in both A172 cells (Figures 3A-C and 3D) and U87MG cells (Figure 3E). These results show that PKCι has a role in protecting U87MG glioblastoma cells from apoptosis induced by cisplatin.

Effects of PKCι on activation of the NFκB pathway: The atypical protein kinase C-mediated chemoresistance seen in leukemia cells was shown to be due to its role in the activation of the NFκB pathway (Lu *et al.*, 2001). To determine if the same mechanism functioned in glioblastoma cells, the effects of PKCι depletion on NFκB activation was assayed. Levels of the NFκB inhibitor IκBα, which is rapidly degraded upon activation of this pathway, were used as a readout. Treatment of U87MG cells with TNFα caused a rapid degradation of IκBα (Figure 4A). This was seen within 10 min after exposure; levels of IκBα started to recover after 60 min, probably because of the enhanced IκBα

Figure 2. Effects of PKCι depletion on cisplatin cytotoxicity. A. U87MG cells were seeded in 96 well tissue culture plates. The following day, U87MG cells were mock transfected (♦), transfected with control RNA (■), or transfected with siPKCιA (▲). 48 h later, cells were treated for 24 h with 0-80 μg/ml cisplatin. Cell viability was assessed using MTT assays as described in Materials and Methods. Values shown are the means determined from three separate experiments performed in triplicate +/- SEM. (* $p < 0.05$). B-G. U87MG cells were examined by phase contrast microscopy 48 h after mock (B, E), control RNA duplex (C, F) and siPKCιA (D, G) transfection in the absence (B-D) or presence (E-G) of 24 h cisplatin (20μg/mL) treatment. H-K. Cell counts were determined for mock transfected cells (black bars), control RNA duplex - transfected cells (dark gray bars), siRNAιA transfected cells (white bars) and siRNAιB transfected cells (light gray bars). Transfections were done as in A. 48 h later, cells were treated for 24 h with 0-80 μg/ml cisplatin. H and J show results for U87MG cells, while I and K show results for A172 cells. Experimental means are values calculated from three experiments performed in triplicate +/- SEM (* $p < 0.01$).

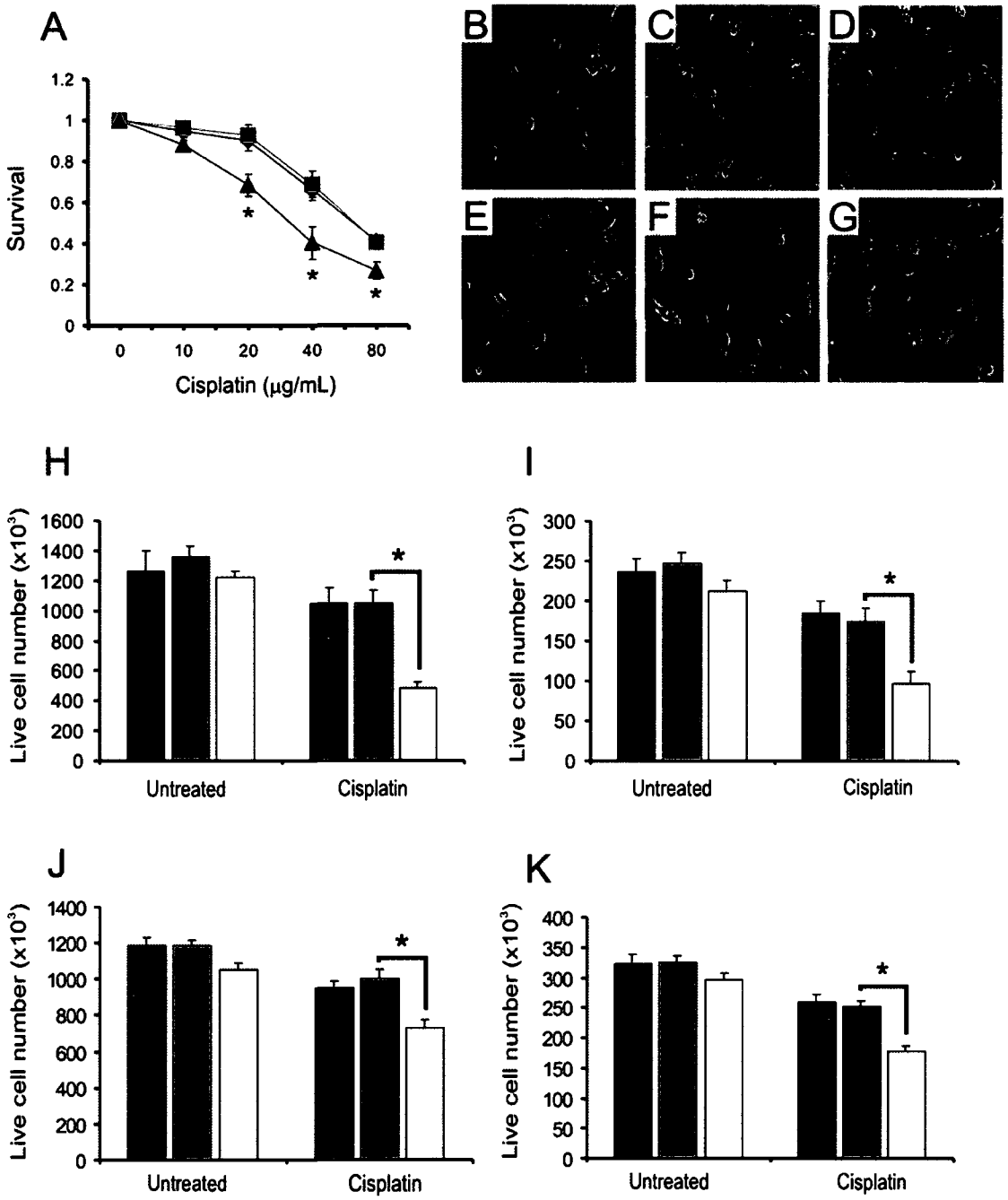
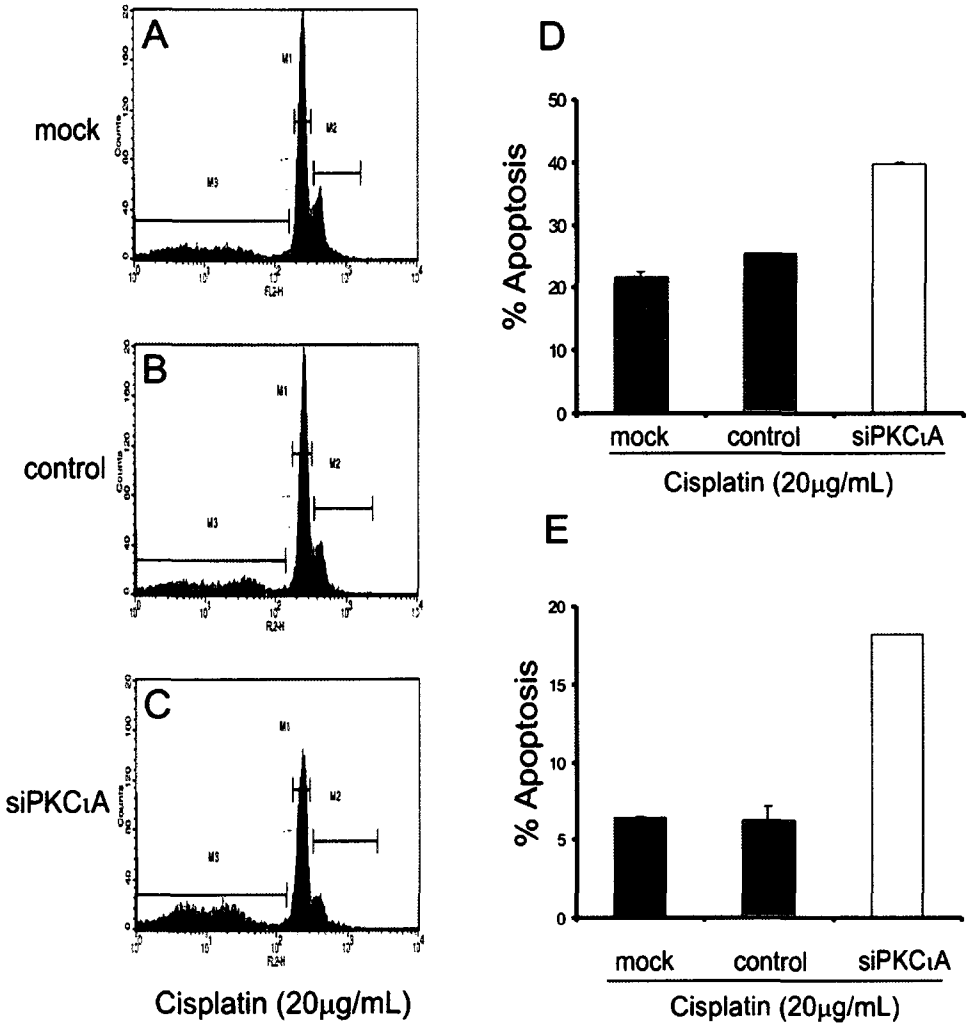

A
Survival
1.2
1
0.8
0.6
0.4
0.2
0
0
10
20
40
80
Cisplatin (μg/mL)
B
C
D
E
F
G
H
Live cell number (x10³)
1600
1400
1200
1000
800
600
400
200
0
Untreated
Cisplatin
I
Live cell number (x10³)
300
250
200
150
100
50
0
Untreated
Cisplatin
J
Live cell number (x10³)
1400
1200
1000
800
600
400
200
0
Untreated
Cisplatin
K
Live cell number (x10³)
400
350
300
250
200
150
100
50
0
Untreated
Cisplatin

Figure 3. Flow cytometric analysis of cisplatin - treated glioblastoma cells with and without PKCι depletion. A172 glioblastoma cells were mock transfected (A), control RNA duplex transfected (B), or transfected with siPKCιA (C). 48 h later, cells were treated with 20μg/ml cisplatin for 24 h. Representative flow cytometric analyses of A172 cells following cisplatin treatment are shown in A, B and C. D shows the percentage of subG1 (apoptotic) nuclei for A172 cells, determined from flow cytometry data as described in Materials and Methods. E shows the same for U87MG cells. The graphs show representative results from one of three independent experiments for each cell line.

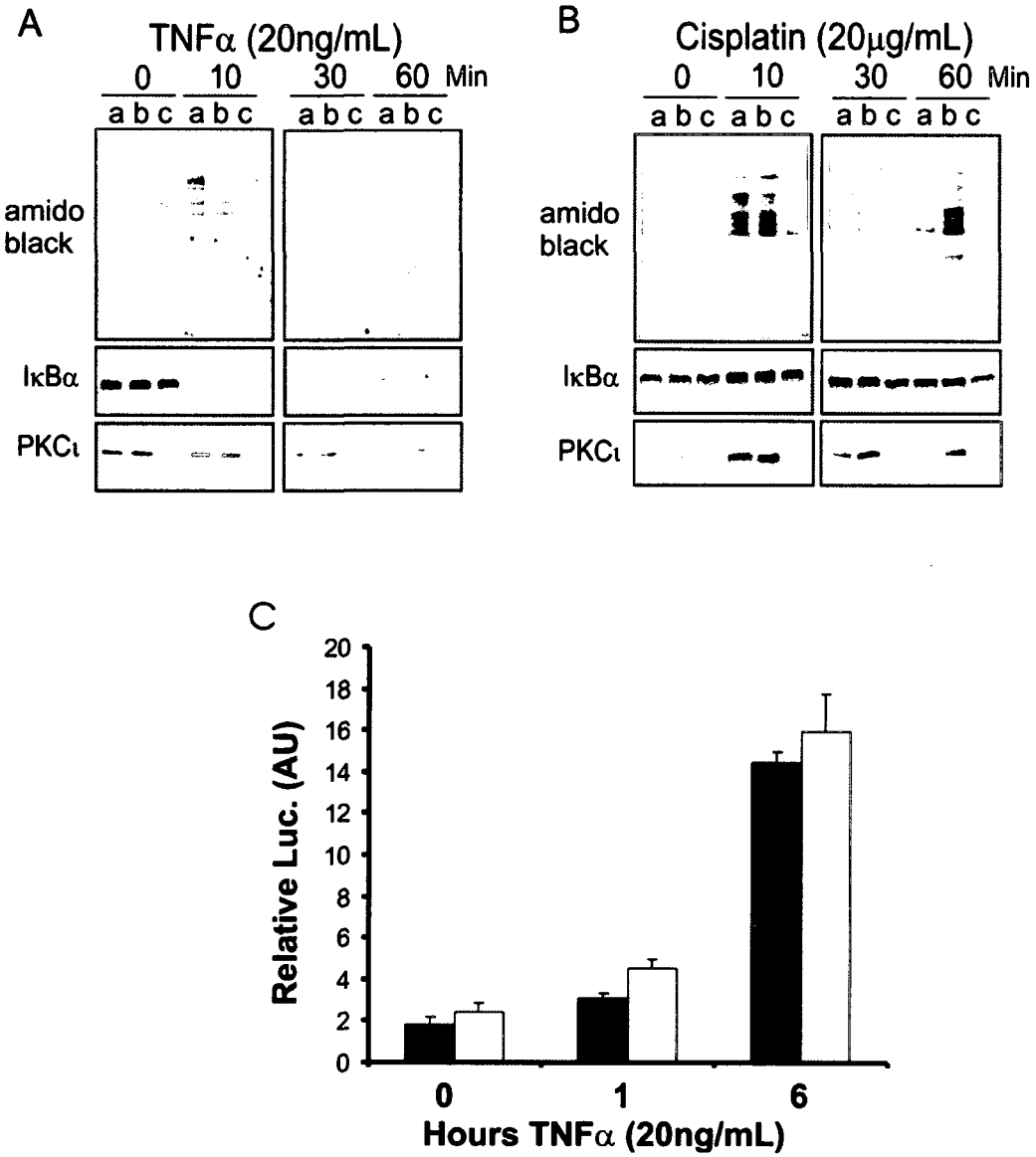

A
mock
B
control
C
siPKCιA
Cisplatin (20μg/mL)
D
% Apoptosis
mock
control
siPKCιA
Cisplatin (20μg/mL)
E
% Apoptosis
mock
control
siPKCιA
Cisplatin (20μg/mL)

transcription that is known to occur with prolonged stimulation of this pathway (Le Bail *et al.*, 1993). Depletion of PKCι had no effects on the kinetics of IκBα degradation and recovery. Cisplatin did not induce the degradation of IκBα in U87MG cells (Figure 4B), although activation of NFκB by chemotherapy has been reported in other cell types (Mayo and Baldwin, 2000).

As a second method to assess NFκB activity, control or PKCι-depleted cells were transfected with an NFκB reporter plasmid. PKCι depletion had no significant effect on basal or TNFα-stimulated NFκB activity (Figure 4C). Taken together, these data show that NFκB pathway activation was not grossly affected by PKCι depletion, and that enhanced activation of the NFκB pathway is not a major mechanism for PKCι-mediated chemoresistance in glioblastoma cells.

Microarray analysis of PKCι - mediated resistance to cisplatin: To screen for other possible mechanisms by which PKCι could confer resistance to cisplatin, microarray analysis of gene expression was performed, as described in Materials and Methods. Four conditions were analyzed: (i) U87MG cells transfected with a control RNA duplex; (ii) U87MG cells transfected with a control RNA duplex and treated for 6 h with cisplatin; iii) U87MG cells transfected with a siPKCιA RNA duplex; (iv) U87MG cells transfected with a siPKCιA RNA duplex and treated for 6 h with cisplatin. This experiment was performed in triplicate on the same day. RNA was isolated, converted to cDNA, *in vitro* transcribed and hybridized to the Human Genome U133 set of microarray chips as described in Materials in Methods. The Affymetrix Human Genome U133 Set is designed to analyze the expression of greater than 33,000 human genes (www.affymetrix.com). All

Figure 4. NFκB activation following PKCι depletion. A; U87MG cells were mock (a), control (b) or siPKCιA (c) transfected for 48 h and then treated with 20 ng/ml TNFα. Total cell lysates were collected following 0, 10, 30, 60 min of treatment. IκB and PKCι levels were determined by Western blot analyses. Amido black staining of membranes (top panels) was used to assess total loading and transfer of proteins. B; U87MG cells were transfected as in A and then treated with 20 μg/ml cisplatin. IκB and PKCι levels were determined as in A. C; U87MG cells were transfected with control (black bars) or siPKCιA (white bars) RNA duplexes. 24 h later they were transfected with an NFκB reporter plasmid. Cells were treated with TNFα as indicated, and assayed for NFκB activity as described in Materials and Methods.


A
TNFα (20ng/mL)
0 10 30 60 Min
a b c a b c a b c a b c
amido black
IκBα
PKCι
B
Cisplatin (20μg/mL)
0 10 30 60 Min
a b c a b c a b c a b c
amido black
IκBα
PKCι
C
Relative Luc. (AU)
20
18
16
14
12
10
8
6
4
2
0
0
1
6
Hours TNFα (20ng/mL)

comparisons described below are between means derived from the three independent experiments. As one initial test of the quality of the data, the changes in levels of PKCι mRNA determined by this method were checked. The levels of PKCι decreased 8.3 fold in siPKCιA transfected cells compared to the control RNA duplex transfected cells (an 88% decrease, Figure 5B). PKCι mRNA also decreased 8.1 fold in siPKCιA transfected cells treated with cisplatin compared to the control RNA duplex transfected cells (data not shown). This is in agreement with data from qRT-PCR analysis and Western blot analysis described above. Both changes were deemed significant by significance analysis of microarray (SAM; Tusher *et al.*, 2001) at a False Discovery Rate of 0.05. In terms of specificity of the siPKCιA RNA duplex, hybridization values for probe sets corresponding to other PKC family members did not change significantly (Figure 5A). One exception to this was probe set 215195_at, one of the three probe sets for PKCα. Since 215195_at hybridizes at a much lower level than another probe set for this gene, 213093_at, which is unchanged, 215195_at hybridization probably does not accurately represent PKCα mRNA levels. This is supported by the unchanged protein levels of PKCα (Figure 1F and G).

The microarray strategy was designed to identify sets of genes that were: (i) regulated by basal PKCι function; (ii) regulated by cisplatin; (iii) regulated by cisplatin in a PKCι-dependent fashion. This study focuses on the set of genes regulated by basal PKCι function. SAM was used to identify genes that were differentially expressed in PKCι - depleted samples as compared with controls (Figure 5B). Out of 4897 probe sets that were identified as significantly changed on HG-U133A (FDR = 0.05), overall decreases greater than two-fold were detected in 354 probe sets; 19 of these exhibited decreases of

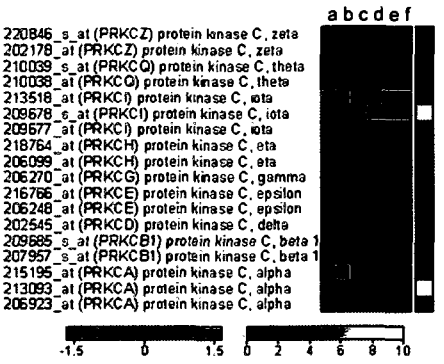
more than five-fold, while the maximum decrease in expression observed was 12 fold. Significant increases greater than two-fold were detected with 1389 probe sets; 143 of these exhibited increases of more than five-fold, while the maximum increase in expression was 34 fold. These data suggest that PKCι influences cell behaviour more by repression, rather than activation, of gene transcription. For both activation and repression, no obvious single class of genes with related functions was affected.

PKCι represses Glia maturation factor β (GMFβ) expression: The stress-activated kinase p38 MAP kinase has been shown to mediate cisplatin cytotoxicity in various cancer cell lines (Losa *et al.*, 2003). Microarray analysis showed that PKCι depletion increased the levels of GMFβ, a protein which has previously shown to be an enhancer of p38 MAP kinase signaling (Figure 5B, Lim and Zaheer, 1996). This suggested that PKCι could protect cells from apoptosis by preventing GMFβ-mediated enhancement of p38 MAP kinase signaling. To determine whether changes in GMFβ mRNA levels led to changes in protein expression, Western blot analysis of GMFβ was performed on cells treated with cisplatin in the absence or presence of PKCι depletion. This showed that PKCι depletion also caused an increase in GMFβ protein levels (Figure 6A and B); this was seen with the two different RNAi duplexes against PKCι.

PKCι attenuates p38 MAP kinase signaling: Total cell extracts from control or siPKCιA transfected cells treated with cisplatin were also probed for levels of p38 MAP kinase, ERK, and their activated, phosphorylated forms. Cisplatin caused an increase in both

Figure 5. Microarray analysis of gene expression in U87MG cells following PKCι depletion. Hybridization values were obtained for three replicates transfected with control RNA duplexes (a-c) and three replicates transfected with siPKCιA RNA duplexes (d-f). A. Heat map showing hybridization values of probe sets corresponding to PKC isoform transcripts. Probe set identifiers are shown on the left. The heat map using the green/red scale shows changes in hybridization between control and PKCι-depleted cells (median centered). The greyscale heat map on the right shows the average expression level across all replicates. B. Table showing the 50 probe sets whose hybridization values changed most significantly between the two types of samples according to SAM (False Discovery Rate = 3.2%). Hybridization values (Control and siPKCιA) are expressed on a Log2 scale. Fold change ratios were calculated between the average hybridization values of probe sets in the control replicates and in the siPKCιA depleted replicates; values above one point to genes that are up-regulated upon depletion of siPKCιA, and values below one point to genes that are down-regulated upon depletion of siPKCιA. As expected, one of the significantly down-regulated hybridization values is for a probe set corresponding to PKCι (with a fold change of 0.12). A probe set for GMFβ also shows a fold change in hybridization value of 13.42 indicating strong up-regulation upon PKCι depletion. The original .CEL files for the six samples can be downloaded from http://www.ogic.ca/projects/Baldwin05/.

A



B

Probe ID	Symbol	Gene Name	Control RNA (Log2)	siPKCA (Log2)	Fold Change
216705_s_at	ADA	adenosine deaminase	3.27	8.35	33.81
205792_at	WISP2	WNT1 inducible signaling pathway protein 2	3.34	8.26	30.40
211976_at	LOC401255	Similar to oocyte specific homeobox 6 (mouse)	2.08	6.55	22.12
201764_at	MGC5576	hypothetical protein MGC5576	1.59	6.04	21.73
202048_s_at	CBX6	chromobox homolog 6	1.85	6.07	18.68
213158_at	Hypothetical	Homo sapiens, clone IMAGE:4214654, mRNA	3.58	7.74	17.79
205943_at	TDO2	tryptophan 2,3-dioxygenase	2.89	7.01	17.35
201427_s_at	SEPP1	selenoprotein P, plasma, 1	4.81	8.81	16.00
208877_at	PAK2	p21 (CDKN1A)-activated kinase 2	3.90	7.87	15.75
203834_s_at	TGOLN2	trans-golgi network protein 2	3.04	6.95	15.03
208737_at	ATP6V1G1	ATPase, H+ transporting, lysosomal 13kDa, V1 subunit G isoform 1	4.46	8.35	14.78
211332_x_at	HFE	hemochromatosis	2.88	6.73	14.45
201601_x_at	IFITM1	interferon induced transmembrane protein 1	2.93	6.73	13.98
202543_s_at	GMFB	glia maturation factor, beta	3.74	7.49	13.42
204639_at	ADA	adenosine deaminase	5.60	9.33	13.27
213306_at	MPDZ	multiple PDZ domain protein	3.05	6.68	12.31
203124_s_at	SLC11A2	solute carrier family 11, member 2	3.81	7.37	11.80
206087_x_at	HFE	hemochromatosis	2.77	6.33	11.76
202479_s_at	TRIB2	tribbles homolog 2 (Drosophila)	3.31	6.86	11.73
201308_s_at	40787	septin 11	3.61	7.13	11.51
213156_at	Hypothetical	Homo sapiens, clone IMAGE:4214654, mRNA	3.23	6.72	11.33
212444_at	Hypothetical	CDNA clone IMAGE:6025865, partial cds	3.22	6.65	10.86
204501_at	NOV	nephroblastoma overexpressed gene	4.24	7.57	10.03
218519_at	SLC35A5	solute carrier family 35, member A5	3.41	6.71	9.86
212299_at	NEK9	NIMA (never in mitosis gene a)- related kinase 9	2.34	5.62	9.70
209884_s_at	SLC4A7	solute carrier family 4, sodium bicarbonate cotransporter, member 7	4.00	7.24	9.43
203588_s_at	TFDP2	transcription factor Dp-2 (E2F dimerization partner 2)	2.80	5.95	8.87
208770_s_at	EIF4EBP2	eukaryotic translation initiation factor 4E binding protein 2	5.39	8.54	8.83
218881_s_at	FOSL2	FOS-like antigen 2	4.13	7.27	8.82
213358_at	KIAA0802	KIAA0802	3.19	6.32	8.74
212040_at	TGOLN2	trans-golgi network protein 2	4.30	7.40	8.63
212158_at	SDC2	syndecan 2	3.79	6.89	8.58
205410_s_at	ATP2B4	ATPase, Ca++ transporting, plasma membrane 4	3.42	6.50	8.49
205387_s_at	CGB	chorionic gonadotropin, beta polypeptide	4.31	7.40	8.49
212043_at	TGOLN2	trans-golgi network protein 2	7.55	10.63	8.46
205568_at	AQP9	aquaporin 9	4.77	7.85	8.45
203123_s_at	SLC11A2	solute carrier family 11, member 2	3.93	6.99	8.37
214808_at	Hypothetical	MRNA; cDNA DKFZp762N156 (from clone DKFZp762N156)	2.97	6.03	8.31
209109_s_at	TM4SF6	transmembrane 4 superfamily member 6	4.17	7.22	8.28
212099_at	RHOB	ras homolog gene family, member B	3.54	6.58	8.26
206825_at	OXTR	oxytocin receptor	4.88	7.89	8.22
207824_s_at	MAZ	MYC-associated zinc finger protein	4.29	7.32	8.17
219321_at	MPP5	membrane protein, palmitoylated 5 (MAGUK p55 subfamily member 5)	3.68	6.69	8.07
206874_s_at	AL138761	Similar to Ste20-related kinase	3.00	6.01	8.07
212535_at	MEF2A	MADS box transcription enhancer factor 2, polypeptide A	3.24	6.18	7.67
201018_at	EIF1AX	eukaryotic translation initiation factor 1A, X-linked	2.68	5.61	7.60
213518_at	PRKCI	protein kinase C, iota	5.87	2.81	0.12
201277_s_at	HNRPAB	heterogeneous nuclear ribonucleoprotein A/B	7.46	4.35	0.12
216976_s_at	RYK	RYK receptor-like tyrosine kinase	5.80	2.58	0.11
202020_s_at	LANCL1	LanC lantibiotic synthetase component C-like 1	7.20	3.65	0.09

phosphorylated p38 MAP kinase and ERK, as described previously in other cell types ((Figure 6C and 6D, Losa *et al.*, 2003; Choi *et al.*, 2004). In cells depleted of PKCι, basal levels of phosphorylated p38 MAP kinase were similar to control duplex-transfected cells; however, cisplatin consistently induced higher levels of p38 MAP kinase phosphorylation relative to cells with normal levels of PKCι. Levels of activated ERK were, in contrast, decreased.

GMFβ enhances p38 MAP kinase signaling and cisplatin cytotoxicity: A retroviral vector was constructed that expressed GMFβ under control of the CMV promoter as described in Materials and Methods. Transient transfection of this construct into 293T cells showed that it was able to drive expression of GMFβ. However retroviral transduction of U87MG cells, followed by puromycin selection, did not give any resistant cells while empty control vector did, suggesting that *sustained* high overexpression of GMFβ is toxic to U87MG cells. This has been reported previously for other cell types, and is consistent with a pro-apoptotic function for GMFβ (Kaimori *et al.*, 2003). To circumvent this problem, U87MG cells were transiently transfected with the GMFβ-expressing vector. This induced some cell death on its own, but enough cells survived to allow the analysis of effects of GMFβ expression on p38 MAP kinase activity and cisplatin cytotoxicity. The GMFβ transgene protein product was larger in size than the endogenous GMFβ, due to the introduction of an inframe upstream initiation site and an additional 14 amino terminal codons during subcloning (Figure 7A). Cells expressing the GMFβ transgene showed an increased level of p38 MAP kinase phosphorylation in response to cisplatin. Overall levels of p38 MAP kinase were either unaffected or reduced in different

Figure 6. Effects of PKCι depletion on GMFβ protein levels and p38 MAP kinase activation. A. U87MG cells were transfected with control or siPKCιA RNA duplexes. 48 h later, cells were treated with vehicle or 20 μg/ml cisplatin for 6 h. Total cells lysates were collected 48 h post transfection and analyzed for GMFβ and PKCι expression by Western blotting. B; As in A, only U87MG cells were transfected with control or siPKCιB RNA duplexes. GMFβ levels were the same in mock transfected and control RNAi transfected cells (not shown). C. As in A, except that total cell lysates were analyzed by Western blotting for levels of phosphorylated p38 MAP kinase, total p38 MAP kinase, phosphorylated Erk1/2 and total Erk 1/2. D; As in C, except that U87MG cells were transfected with control or siPKCιB RNA duplexes.

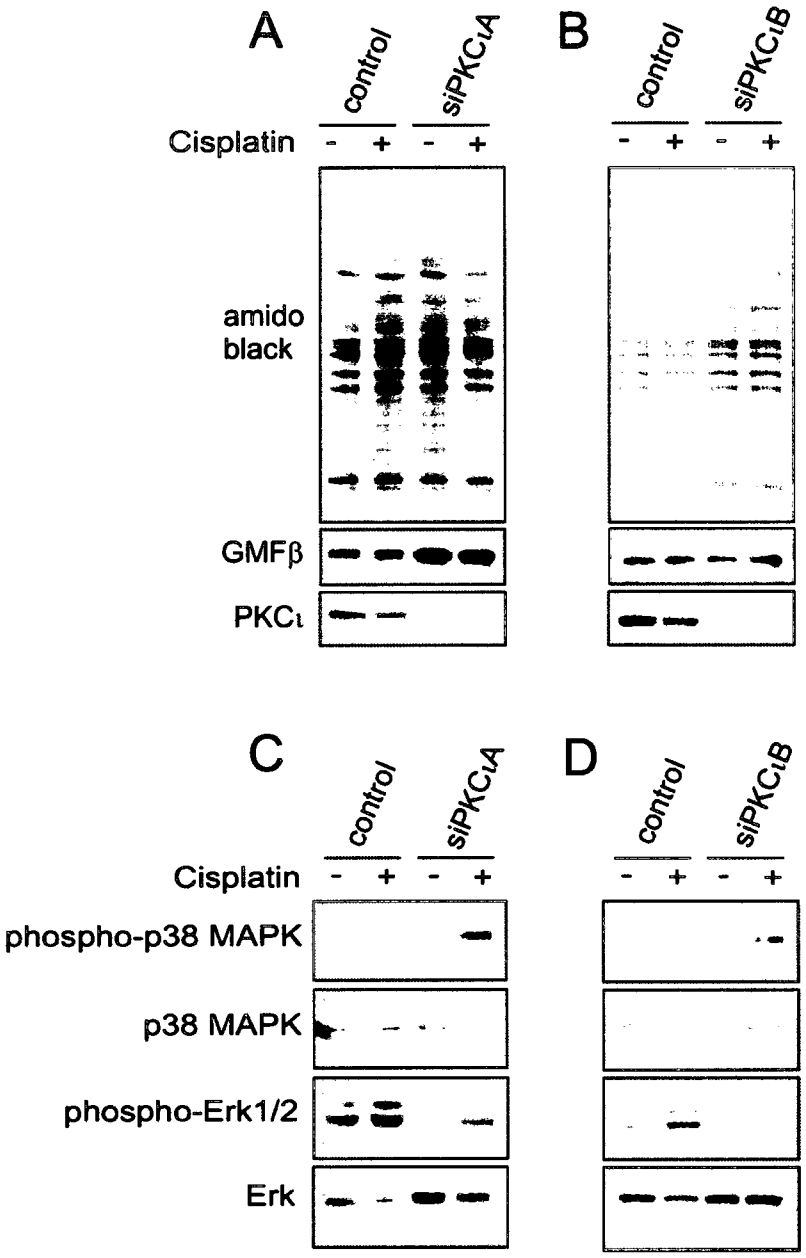
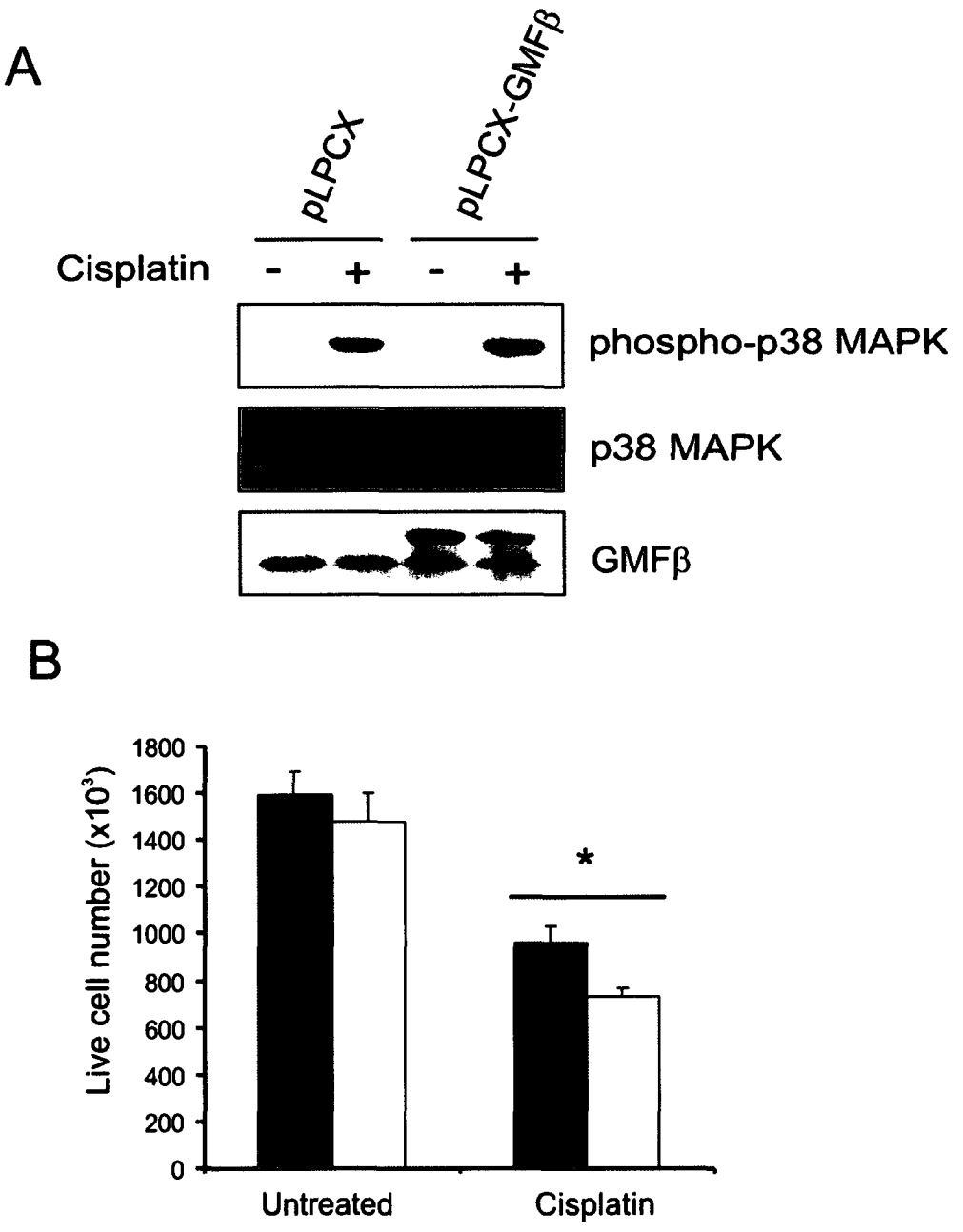

A
control
siPKCιA
Cisplatin
- + - +
amido black
GMFβ
PKCι
B
control
siPKCιB
- + - +
C
control
siPKCιA
Cisplatin
- + - +
phospho-p38 MAPK
p38 MAPK
phospho-Erk1/2
Erk
D
control
siPKCιB
- + - +

Figure 7. Effect of increased GMFβ expression on p38 MAP kinase activation and cisplatin cytotoxicity. A. U87MG cells were transfected with empty vector (black bars) or vector expressing GMFβ (white bars). 48 h later, cells were treated with 20 μg/ml cisplatin for 6 h. Total cell lysates were collected and analyzed by Western blotting for expression of phosphorylated p38 MAP kinase, total p38 MAP kinase and GMFβ. B. U87MG cells were transfected and treated with 20 μg/ml cisplatin as in A. Numbers of live cells were then determined as described in Materials and Methods. The data shown are the results of three independent experiments (* $p < 0.05$).

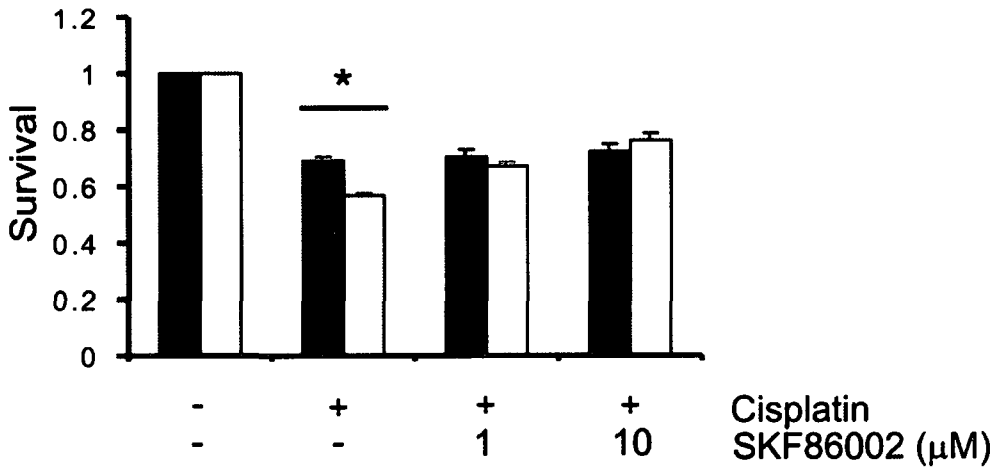

A
pLPCX
pLPCX-GMFβ
Cisplatin
-
+
-
+
phospho-p38 MAPK
p38 MAPK
GMFβ
B
Live cell number (x10³)
1800
1600
1400
1200
1000
800
600
400
200
0
*
Untreated
Cisplatin

experiments. Expression of the GMFβ transgene also consistently caused a partial sensitization of cells to cisplatin cytotoxicity, as assessed by cell counts (Figure 7B). The extent of the sensitization was less than seen with PKCι depletion; this may be due to differences in the level of GMFβ expression in the GMFβ transient transfections, as compared to the levels of GMFβ in cells depleted of PKCι using the siPKCιA.

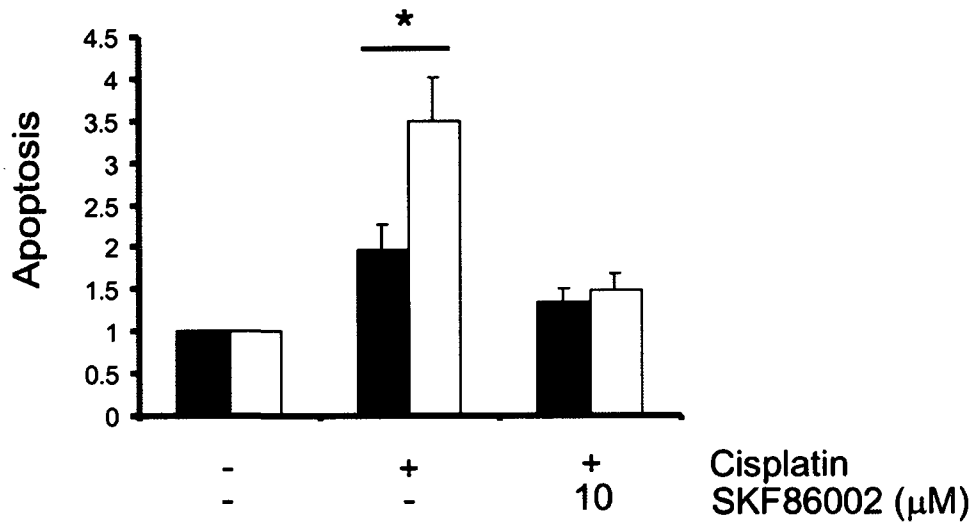
p38 MAP kinase mediates the enhanced cisplatin cytotoxicity seen with PKCι depletion: To determine if p38 MAP kinase had a role in the enhanced cisplatin-induced cell death seen with PKCι depletion, U87MG cells were treated with the p38 MAP kinase inhibitor SKF86002, which is active against all four p38 MAP kinase family members (Losa *et al.* 2003). Pretreatment with this drug effectively blocked the increase in cisplatin-induced cell death seen in PKCι-depleted cells, as assessed by both MTT assays (Figure 8A) and flow cytometry (Figure 8B).

Figure 8. Effects of p38 MAP kinase inhibition on cisplatin sensitivity. U87MG cells were transfected with control RNA or siPKCιA. 48 h later, cells were pretreated for 1hr with SKF86002 and then treated for 24 h with 20 μg/ml cisplatin. For A and B, dark bars are values for control RNA duplex-transfected U87MG cells; white bars are values for siPKCιA-transfected cells. A. Assessment of live cell numbers by MTT assays after treatment with 0, 1, and 10 μM SKF68002. Values shown are means calculated from three experiments performed in triplicate +/- SEM. B. Flow cytometry assessment of cells treated with SKF86002 and cisplatin. The graph shows the results from three independent experiments. Percentages of subG1 (apoptotic) cells were normalized to the percentage seen in untreated, control RNA - transfected cells. (* $p < 0.05$).

A


Survival
1.2
1
0.8
0.6
0.4
0.2
0
*
- + + +
- - 1 10
Cisplatin
SKF86002 (μM)

B


Apoptosis
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
*
- + +
- - 10
Cisplatin
SKF86002 (μM)

Discussion

The data presented here show that PKCι confers partial resistance to cisplatin. This was observed in two different human glioblastoma cell lines. While other studies have shown a role for PKCι in chemoresistance, the chemoresistance seen in glioblastoma cells appeared to be mechanistically different, as NFκB pathway activation was not affected by PKCι depletion. This conclusion was based on the fact that PKCι depletion did not obviously affect TNFα-induced degradation of IκBα, and also did not affect basal or TNFα-induced NFκB activity. This result is in agreement with results reported recently for PKCι knockout mice (Soloff *et al.*, 2004). There is clear evidence from PKCζ knockout mice that this enzyme is involved in NFκB activation (Leitges *et al.*, 2001); it therefore appears likely that only the PKCζ enzyme, and not PKCι, has a role in NFκB activation. If this is the case, the fact that the glioblastoma cells used here do not express PKCζ would provide a simple explanation for a lack of any NFκB activation in response to cisplatin and the lack of any apparent role for NFκB in cisplatin chemoresistance.

The experiments described here define a novel mechanism by which PKCι can protect cells from cisplatin. This is based on the finding from microarray analysis that PKCι negatively regulates the expression of GMFβ mRNA. Although originally thought to be an extracellular peptide, GMFβ is now known to be an intracellular protein that is found primarily in brain, but also in other tissues including lung, spleen, colon, thymus and kidney (Kaimori *et al.*, 2003; Inagaki *et al.*, 2004). GMFβ has an important role in normal brain, as knockout mice that do not express GMFβ, although viable, show defects

in motor performance and learning (Lim *et al.*, 2004). This beneficial effect comes at a price, as GMFβ sensitizes both astrocytes and proximal tubular kidney cells to oxidative injury (Kaimori *et al.*, 2003; Zaheer *et al.*, 2004). At least part of this sensitization is due to GMFβ's ability to enhance p38 MAP kinase activity (Kaimori *et al.*, 2003; Zaheer *et al.*, 2004), which appears to involve a physical association between GMFβ and this kinase in cells (Lim and Zaheer, 1996).

The regulation of GMFβ mRNA by PKCι also led to changes in GMFβ protein expression. This was seen with two different RNA duplexes specific for PKCι, providing strong evidence that PKCι is responsible for this effect. This repression of GMFβ gene expression by PKCι may occur either by a direct mechanism, or indirectly as a consequence of the regulation of other genes. Because GMFβ has been shown to enhance p38 MAP kinase signaling (Lim and Zaheer, 1996), it was predicted that the increase in GMFβ levels upon PKCι depletion would lead to an enhanced p38 MAP kinase activation in response to cisplatin. This was the case, as cisplatin activated p38 MAP kinase to a greater extent in cells depleted of PKCι. This result is consistent with previous observations on the effect of atypical PKC inhibition by Par-4, an endogenous inhibitor of PKCζ and PKCι (Berra *et al.*, 1997; Garcia-Cao *et al.*, 2003); inhibition of PKCι by this method also enhanced p38 MAP kinase signaling. Overexpression of GMFβ in U87MG cells reproduced the effects of PKCι depletion on both p38 MAP kinase activation and cisplatin cytotoxicity. PKCι-mediated repression of GMFβ expression is therefore sufficient to account for these effects. Pharmacological blockade of p38 MAP kinase signaling blocked the increase in cell death seen in PKCι-depleted cells, showing

that attenuation of p38 MAP kinase signaling by PKCι is necessary and sufficient for its effects on cisplatin cytotoxicity. Other groups have reported that the MEK inhibitor PD98059, which blocks ERK activation, sensitizes glioblastoma cells to cisplatin (Zhan and O'Rourke, 2004). PKCι depletion decreased ERK activation by cisplatin, suggesting that this might be a second mechanism by which PKCι could affect cisplatin cytotoxicity. However this did not appear to be the case, as the effects of PKCι depletion were completely reversed by p38 MAP kinase inhibition. It is possible that the magnitude of the effects of PKCι depletion on ERK activation is not sufficient for this to be a major factor. p38 MAP kinase inhibition did not completely inhibit cisplatin cytotoxicity, suggesting that other mechanisms also contribute to the toxicity of this drug in glioblastoma. Overexpression of PKCι in U87MG cells did not further repress GMFβ levels (data not shown). Consistent with the mechanism we have shown here, PKCι overexpression also did not increase resistance to cisplatin further. A likely explanation for this is that PKCι downstream signaling is already saturated, so that further increases in PKCι signaling do not make any difference.

The repression of GMFβ expression, leading to attenuation of pro-apoptotic p38 MAP kinase signaling, is one mechanism by which PKCι can repress apoptosis. This presumably can function in concert with, or independently of, other anti-apoptotic pathways mediated by PKCι. These include the recently described function of PKCι in phosphorylating and inactivating the pro-apoptotic protein Bad (Jin *et al.*, 2005). The suppression of GMFβ expression by PKCι also potentially provides a mechanism by which glioblastoma cells can protect themselves from oxidative damage, based on the established role of GMFβ in oxidative stress. While oxidative damage is thought to be

mutagenic, and therefore tumor-promoting, in the early stages of cancer, in later stages of tumor growth oxidative damage limits the growth of tumor cells (Benhar *et al.*, 2002). While more studies would be required to validate this, PKCι-mediated suppression of GMFβ may be a mechanism by which the PI 3-kinase pathway prevents oxidative damage-induced senescence in glioblastoma cells.

The microarray analysis performed here also identified a large number of other genes that are potentially regulated by PKCι. A survey of the data shows that these genes do not fall into any one particular class - instead the data suggest that PKCι is very pleiotropic in its cellular function. PKCι appears to affect the expression of mRNAs for a wide array of other signaling molecules, including members of the CCN family (CCN3/NOV, CCN5/WISP2)(Brigstock, 2003) and the Wnt coreceptor RYK (Lu *et al.*, 2004). This suggests that, aside from the linkage between PKCι and p38 MAP kinase described here, there is cross-talk between PKCι and multiple other signaling pathways in glioblastoma cells.

Materials and Methods

Chemicals and antibodies: Custom RNA interference duplexes were synthesized by Dharmacon RNA Technologies Inc. (Lafayette, CO, USA). TNFα, propidium iodide and MTT were from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). SKF86002 was from Calbiochem (San Diego, CA, USA). Goat polyclonal antibody nPKCζ (C-20) used to detect PKCι was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Mouse monoclonal antibody to GMFβ was from R & D Systems Inc. (Minneapolis, MN, USA). Phospho-p38 MAP kinase and phospho-Erk monoclonal antibodies and p38 MAP kinase and IκB rabbit polyclonal antibodies were from Cell Signaling Technology (Beverly, MA, USA). Pan Erk monoclonal antibody was from Transduction Laboratories (Lexington, KY, USA). Cisplatin was obtained from the Ottawa Hospital Regional Cancer Centre pharmacy.

Cell lines: The human glioblastoma cell line U87MG was obtained from Dr. W. Cavenee (Ludwig Institute for Cancer Research, La Jolla, CA). A172 cells were from the American Type Culture Collection. Both these cell lines are wild type for p53, mutant for PTEN and mutated at the INK4A/ARF locus (Ishii *et al.*, 1999); this set of mutations is common in *de novo* glioblastoma (Holland, 2001). Cells were cultured at 37°C and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine and 10% (v/v) of a 2:1 mixture of donor bovine serum and fetal bovine serum.

RT-PCR: PolyA+ mRNA was isolated from cells using the Micro-FastTrack 2.0 mRNA Isolation Kit (Invitrogen, Carlsbad, CA). First strand cDNA synthesis was performed with the cDNA Cycle Kit (Invitrogen, Carlsbad, CA). The following primer pairs were used for PCR: for PKCζ the primer pair PKCZ5 CCCAAGATGGAAGGGAGCGGCGGC and PKCZA CCGACTAGTCGTCTGGA AGCAGGAGTG also the primer pair PKCZB ACGACTAGTCGGTTGTTCCTG GTCATTGAG and PKCZ3 GCCTCACACCGACTCCTCGGTGGACAG; for PKC ι the primer pair IOTA1 ACCATGTCCCACACGGTCGCAGGC and IOTA2 ATCCTGAAGACCTAGACTGGATGAAGC. For quantitative RT-PCR, total RNA was isolated using RNEasy Mini Kits (Qiagen Inc., Mississauga, Ontario, Canada) and cDNA was synthesized using First Strand cDNA Synthesis Kits (MBI Fermentas, Burlington, Ontario, Canada) using random hexamer primers. Quantitative PCR was performed in a LightCycler (Roche, Basel, Switzerland) using FastStart DNA Master SYBR Green I kits (Roche, Basel, Switzerland). $MgCl_2$ concentrations and PCR temperatures and cycle times were optimized for each primer pair used. Data were analyzed using LightCycler analysis software (Roche, Basel, Switzerland). For quantitative PCR of PKCι the primer pair IOTAL (CGGCATGTGTAAGGAAGGAT) and IOTAR (TCGGAGCTCCC AACAATATC) was used; for β 2- microglobulin, used as a reference gene, the primer pair B2MFOR (TGCTGTCTCCATGTTTGATGTATCT) and B2MREV (TCTCTGCT CCCCACCTCTAAGT) was used.

RNA transfections: RNA duplexes were obtained from Dharmacon Research Inc. (Lafayette, CO). Two control duplexes were used: the first had the sense RNA sequence

GCGCGCUUUGUAGGAUUCGdTdT (Scramble II duplex, Dharmacon Research, Inc.) and the second had the sense RNA sequence AUUCUAUCACUAGCGUGACUU (Non-specific control duplex X (42 % GC content), also from Dharmacon Research, Inc.). RNA duplex concentrations were determined by measuring absorbance at 260 nm and calculating concentrations using extinction coefficients provided by the manufacturer. Glioblastoma cells were plated in six well dishes at a density of 100 000 cells/plate the day before transfection, in DMEM supplemented with 10 % serum and no antibiotics. On the day of transfection, 10 µl of RNA duplex solution was added to 175 µl OptiMEM I (Invitrogen Canada, Inc., Burlington, ON). In a separate tube, 4 µl of Oligofectamine (Invitrogen Canada, Inc., Burlington, ON) was added to 11 µl of OptiMEM I. This was mixed gently and incubated 10 min at room temperature. The diluted Oligofectamine was then added to the RNA duplex in OptiMEM I and incubated for another 20 min at room temperature after gentle mixing. Media was removed from the cells, and the cells were washed once with 3 ml of warm OptiMEM I. 800 µl of OptiMEM I was added per well, and the Oligofectamine/RNA duplex mix was then added (200 µl per well) and mixed by rocking the plate from side-side for 30 sec. Final concentrations of RNA in the transfections were 5.3 nM of siPKCιA and 20 nM for siPKCιB. Control RNA concentrations were matched to the specific siRNA duplex used. Cells were incubated 4 h at 37°C, after which 2 ml of DMEM supplemented with 10 % serum was added. For transfections in 10 cm plates and 96 well plates, cells were plated at densities of $1x10^6$ and $1x10^4$ cells/well, respectively, and reagent volumes were adjusted accordingly.

Western blot analysis: Western blotting was performed as described previously (Lorimer and Lavictoire, 2000). After electrophoretic transfer from the gel, membranes were stained with amido black to confirm that equal sample loading and transfer was achieved.

MTT assays. Cells in 96 well tissue culture plates were transfected with RNA duplexes as above. 48 h after transfection, the media was replaced with 100 μL of media containing increasing concentrations of cisplatin (0-80 μg/ml). 24 h later, cell survival was assessed using the MTT (3-(4,5-cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay. Cells were exposed to 25 μl of MTT (5mg/ml in sterile PBS, Sigma-Aldrich, Canada) and incubated for 2-3 hours at 37°C and 5% CO_2. The formazan product was formed and dissolved by adding 100 μl of lysis buffer (20% SDS (BioShop Canada Inc., Burlington, ON)) and DMF (VWR International, Mississauga, ON, Canada). Absorbance was measured at 570 nm in a Dynex MRX microplate reader. Background values were determined by carrying out the assay in wells containing media without cells.

Cell counts. Live cell number was determined using a Beckmann Coulter counter (Beckman Coulter Canada, Inc., Mississauga, ON, Canada).

Flow cytometry: After transfection with different RNA duplexes, adherent and non-adherent cells were harvested and fixed with 70% (v/v) ethanol in PBS. Cell nuclei were stained with propidium iodide and DNA content was analyzed by flow cytometry using a BD LSR flow cytometer (Becton Dickinson, San Jose, CA). Data acquisition was done

using Cell Quest software (Becton Dickinson, San Jose, CA) and the data were analyzed using Mod Fit LT software (Verity Software House, Inc., Sopsham, ME).

Reporter plasmid assay for NFκB activity. Cells in 6 well tissue culture plates were transfected with RNA duplexes as above. 24 h after RNA transfection, cells were co-transfected with 3 μg of the reporter plasmid pNF-κB-Luc (Stratagene) and 3 ng pRL-CMV (Promega), which contains the CMV promoter upstream of the Renilla luciferase gene, to serve as an internal control for transfection efficiency. Transfections were performed using GeneJuice Transfection Reagent (Novagen, Madison, WI) as recommended by the manufacturer. 24 h following DNA transfection, the culture media was replaced with 1 mL of media containing 20 ng/mL of TNFα. The cells were then harvested at various times, 0, 1 and 6 h after treatment. Firefly and Renilla luciferase assays were performed using the Dual-Luciferase Reporter Assay System (Promega, Madison WI). Samples were analyzed on a EG&G Berthold Lumat LB9507 luminometer (Berthold Technologies, Bad Wildbad, Germany).

Microarray analysis: RNA was isolated from cells on 10 cm plates using Qiagen RNEasy kits (Qiagen Inc, Mississauga, ON, Canada) according to the manufacturer's protocol for total RNA isolation from animal cells. RNA was converted to cDNA, *in vitro* transcribed and hybridized to the Human Genome U133 Set of microarray chips (Affymetrix, Santa Clara, CA) at the Ontario Genomics Innovation Centre, Ottawa, Canada.

Microarray data analysis: Expression values were computed from .CEL files by applying GCRMA (v1.1.3) under R (v2.0.0) [http://www.r-project.org/] within sets of replicate samples. Statistical testing was performed with the Significance Analysis of Microarrays (SAM) (Tusher *et al.*, 2001) function in SIGGENES (v1.2.11). The GCMRA and SIGGENES packages are part of the Bioconductor project (Gentleman *et al.*, 2004). Expression data will be deposited in the Gene Expression Omnibus database (GEO) (http://www.ncbi.nlm.nih.gov/geo/).

Construction of GMFβ expression vector: Full length cDNA for human GMFβ was obtained from the American Type Culture Collection (MGC-12462). The cDNA was isolated after digestion with Eco RI and Not I, and ligated into the retroviral vector pLPCX (Clontech, Palo Alto, CA) that had been digested with the same restriction enzymes. Retrovirus containing GMFβ cDNA was made as described previously (Lorimer and Lavictoire, 2000). For transient overexpression of GMFβ in U87MG cells, transfections were performed using GeneJuice Transfection Reagent (Novagen, Madison, WI) .

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3. REGULATION OF GLIOBLASTOMA CELL INVASION BY PKCι AND RHOB

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***Running Title*: PKCι in glioblastoma cell invasion**

Keywords: atypical PKC; RhoB; PI 3-kinase; PTEN; glioblastoma

Contribution of Authors

The content of this manuscript was written by R. M. Baldwin with the help of Dr. I. A. J. Lorimer. All of the experiments presented in this manuscript are the work of R. M. Baldwin with the exception of Figure 1E and Figure 4E. Immunofluorescence to generate Figure 1E and 4E was performed by D. A. E. Parolin.

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Abstract

Glioblastoma multiforme is the most aggressive form of primary brain tumor and remains largely incurable, in large part due to its highly invasive nature. The phosphoinositide (PI) 3-kinase pathway is often constitutively active in these tumours due to activating mutations in the epidermal growth factor receptor, or deletion/loss of function of the tumour suppressor PTEN. PKCι, a member of the atypical protein kinase C family, is activated by the PI 3-kinase pathway and is an important downstream mediator. Here we have assessed the role of PKCι in glioblastoma cell invasion. Depletion of PKCι with RNA interference caused an increase in actin stress fibers and a decrease in cell motility and invasion. Gene expression microarray analysis of U87MG cells showed that PKCι repressed expression of mRNA for RhoB, which has previously been shown to have a role in actin stress fiber formation. Western blot analysis showed that both PKCι depletion and pharmacological inhibition of PKCι caused an increase in the protein levels of RhoB, as did inhibition of PI 3-kinase. Expression of RhoB from a constitutive promoter caused changes in actin stress fibers and cell invasion that were similar to those seen with PKCι depletion. These data show that PKCι, activated as a consequence of aberrant upstream PI 3-kinase signaling, mediates glioblastoma cell motility and invasion, and that repression of RhoB is key downstream event in PKCι signaling leading to enhanced cell motility. In addition, constitutive expression of RhoB repressed PKCι activity, as assessed by its phosphorylation status on Thr555. PKCι and RhoB are therefore mutually antagonistic, potentially creating a sensitive switch between invasive and non-invasive phenotypes.

Introduction

Glioblastoma multiforme is the most common form of brain tumor occurring in adults. This devastating disease is usually incurable and patients have a mean survival time of approximately one year after diagnosis. The highly invasive nature of glioblastoma makes surgical resection non-curative, and it has also been proposed that invading cells may be more resistant to radiation and chemotherapy (Giese et al. 2003). Glioblastoma cells invade as single cells; these travel along white matter tracts and also along blood vessel walls and through the subpial glial space, with some cells traveling long distances. They do not generally invade through blood vessel walls or bone and glioblastomas only very rarely metastasize outside the brain. This is distinct from the invasive behaviour of cancer that has metastasized to the brain from other sites (Bellail et al. 2004). These metastases are generally more delineated from surrounding tissue, invade short distances (often as groups of cells), and invade through blood vessel walls and bone.

Two common genetic changes that have been identified in glioblastoma are mutation of PTEN and the amplification and mutation of the gene for the epidermal growth factor receptor (EGFR; (Ohgaki and Kleihues 2007)). Both of these mutations have been linked to increased motility and invasion that is a consequence of their aberrant activation of the phosphoinositide 3-kinase (PI3K) pathway (Tamura et al. 1999; Cai et al. 2005). The PI3K pathway activates multiple downstream kinases including PKCι, a member of the atypical protein kinase C family (Akimoto et al. 1996). PKCι activation involves phosphorylation by PDK1 and binding to a Cdc42 family protein (Kanzaki et al. 2004). The potential importance of PKCι as a downstream mediator in the PI 3-kinase pathway has been emphasized by the recent finding that it can itself function as an oncogene in

both lung and ovarian cancer (Regala et al. 2005b; Eder et al. 2005). Previous studies have shown that the atypical PKCs have a role in cell motility (Xu and Deng 2006); however it is not known whether they have a role specifically in glioblastoma cell motility, and the downstream events involved in atypical PKC-mediated motility have not been determined.

To assess the role of PKCι in glioblastoma, we previously performed a microarray analysis of gene expression in human glioblastoma cells depleted of PKCι by RNA interference (Baldwin et al. 2006). This identified a number of candidate genes that are potentially regulated by PKCι. One of these was the small GTPase RhoB, a member of the Rho GTPase family of proteins that regulates a variety of cellular processes in including actin organization, proliferation and differentiation (Wheeler and Ridley 2004). RhoB has been shown to exhibit tumor repressive activity (Huang and Prendergast 2006). In human brain tumors, RhoB protein expression decreases with increasing tumor grade (Forget et al. 2002) suggesting that RhoB repression may have a role in increasing the aggressiveness of this cancer.

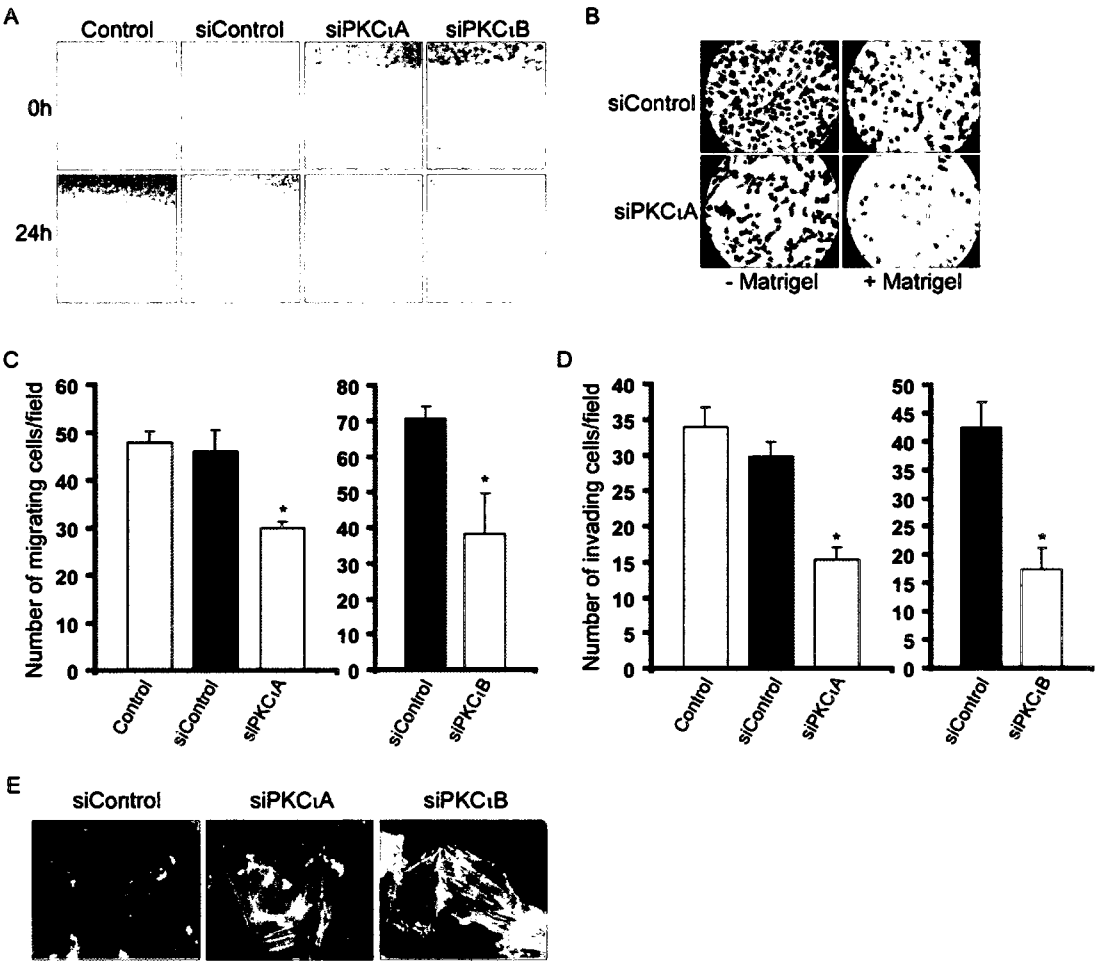
In this paper we describe a role for PKCι in glioblastoma cell motility and invasion. We show that PKCι negatively regulates the expression of RhoB, and that restoration of RhoB expression inhibits glioblastoma cell motility. We also show that RhoB is able to inhibit PKCι activity; the two proteins therefore are mutually antagonistic and may function as a switch for cell motility.

Results

PKCι depletion inhibits glioblastoma motility and invasion: The ability of glioblastoma cells to invade into normal surrounding tissue is influenced by both their motility and their ability to penetrate through tissue barriers such as extracellular matrix. To assess the role of PKCι in glioblastoma cell motility, we used "scratch wound" assays together with RNA interference to reduce PKCι protein levels. We have previously shown that RNA interference effectively reduces PKCι mRNA and protein levels 75-90% without affecting mRNA levels of other PKC family members (Baldwin et al. 2006). These assays showed that U87MG cells depleted of PKCι with two different RNA duplexes did not fill in a scratch as rapidly as cells treated with a control RNA duplex, suggesting decreased cell motility (Figure 1A and S1A). However a caveat with these assays is that PKCι depletion slows the growth of glioblastoma cells; this gives rise to significant differences in cell densities over the four day period in which these assays are done, and some of the differences seen may be a consequence of this, rather than reflecting true effects on motility.

To overcome this problem, we also assessed glioblastoma cell motility using Transwell chambers. In these assays, control cells and cells depleted of PKCι are replated into Transwell chambers *at the same density* and 24 h later the numbers of cells that have crossed the chamber membrane are counted. The effects of PKCι depletion on cell number were not significant at 24 h. Depletion of PKCι in U87MG cells using siPKCιA or siPKCιB resulted in 37% and 46% decreases, respectively, in the number of cells that crossed the chamber membrane, indicating a true decrease in motility (Figure 1B and C).

Figure 1. PKCι enhances glioblastoma cell motility and invasion. U87MG cells were mock-transfected (control), transfected with a control RNA duplex, or transfected with two different duplexes targeting PKCι. Cell motility was analyzed in scratch-wound assays (A) and using Transwell chambers without Matrigel (B and C). Invasion was analyzed using Transwell chambers with Matrigel (B and D). B shows representative pictures of U87MG cells that have passed through the Transwell chamber -/+ Matrigel. C and D show the migrating cell counts in the absence or presence of Matrigel respectively. Data are from one of two independent experiments that gave similar results. F. U87MG cells stained for actin filaments after PKCι depletion.


A
Control
siControl
siPKCιA
siPKCιB
0h
24h
B
siControl
siPKCιA
- Matrigel
+ Matrigel
C
Number of migrating cells/field
Control
siControl
siPKCιA
siControl
siPKCιB
D
Number of invading cells/field
Control
siControl
siPKCιA
siControl
siPKCιB
E
siControl
siPKCιA
siPKCιB

Similar decreases were also observed in a second glioblastoma cell line, A172 (42% and 45% decreases with siPKCιA and siPKCιB, respectively; Figure S1B and C).

To assess the effects of PKCι on the overall invasive ability of glioblastoma cells (*i.e.* their motility and their ability to penetrate through tissue barriers), the effects of PKCι depletion on the migration of glioblastoma cells through Transwell chamber membranes coated with Matrigel was also assayed. Depletion of PKCι also caused a significant decrease in the ability of glioblastoma cells to pass through Matrigel-coated membranes (for U87MG cells, 55 % and 59 % decreases with siPKCιA and siPKCιB (Figure 1B and D); for A172 cells, 56 % and 51 % (Figure S1B and D)). Comparing the data for motility alone (*i.e.* no Matrigel) and overall invasion (*i.e.* with Matrigel), the reductions in the numbers of cells crossing the Transwell chamber membranes are higher in the presence of Matrigel, but the overall differences between values seen with and without Matrigel are not large. This shows that PKCι primarily increases invasion by promoting cell motility, but may also increase the ability of glioblastoma cells to break down surrounding extracellular matrix as well. Consistent with a role for PKCι in cell motility, PKCι also regulated actin stress fiber formation in glioblastoma cells (Figure 1E).

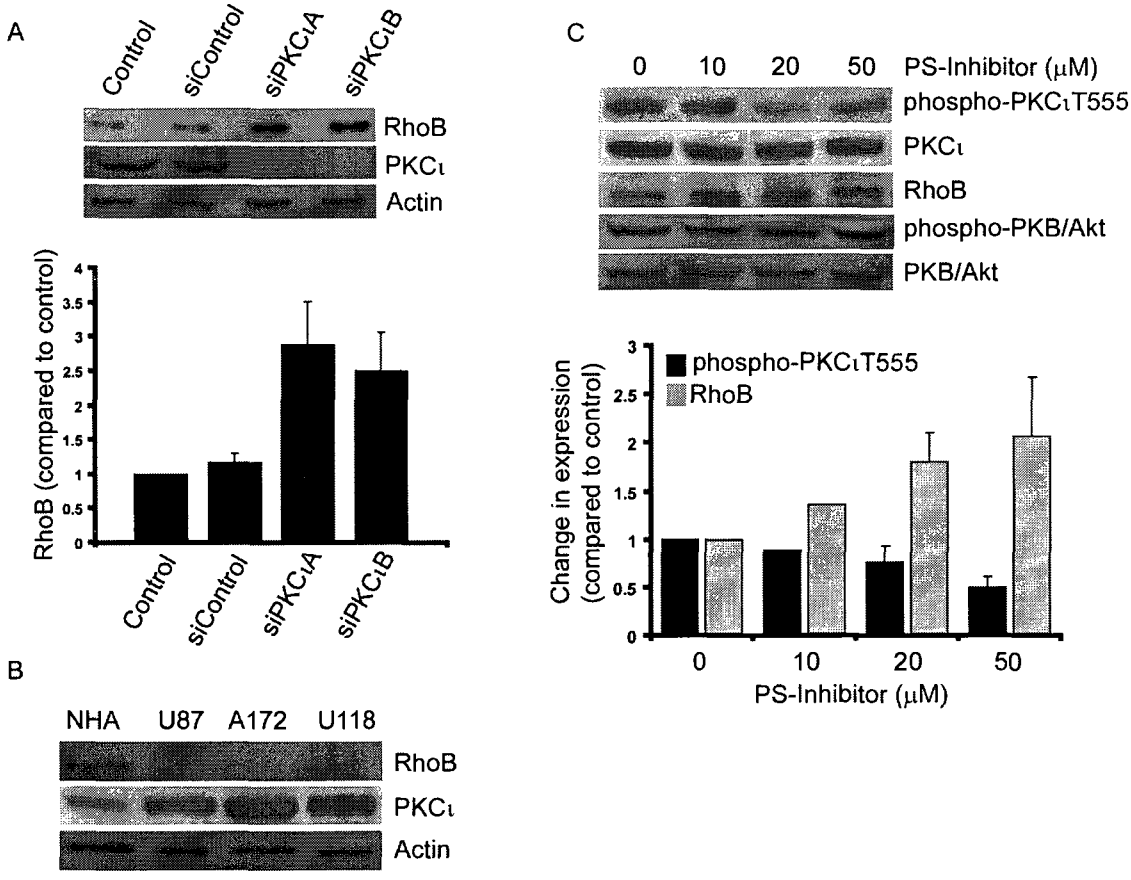
RhoB expression is repressed in glioblastoma: Microarray expression analysis was previously performed on U87MG cells that were depleted of PKCι by RNA interference (Baldwin et al. 2006). Supplementary Table 1 shows microarray analysis data for RhoB mRNA expression. Depletion of PKCι alone increased the levels of RhoB mRNA expression by 8-fold. PKCι had no effect on RhoA or RhoC mRNA levels (data not shown). Quantitative RT-PCR showed that PKCι depletion increased RhoB mRNA

levels 4.4 ± 0.8 fold (mean of three separate determinations ± standard deviation). This confirms the regulation of RhoB mRNA levels by PKCι, but indicates that the magnitude of the change was overestimated in the microarray analysis.

Depletion of PKCι also caused a two-four fold increase in RhoB protein expression in U87MG (Figure 2A) and A172 cells (Figure S2A). Expression of RhoB was also assessed in normal human astrocytes (NHA) and glioblastoma cell lines (U87MG, A172, U118). RhoB protein expression was repressed in each of the glioblastoma cell lines compared to the NHA (Figure 2B). In contrast, levels of PKCι were higher in each of the three glioblastoma cell lines compared to NHA.

Inhibition of PKCι activity enhances RhoB expression: The effects of pharmacological inhibition of PKCι on RhoB expression were assessed using a myristoylated atypical PKC pseudosubstrate peptide (Kanzaki et al. 2004). For these experiments, PKCι activity was monitored with an antibody that recognizes Thr555-phosphorylated PKCι. This phosphorylation site lies in the turn motif that is conserved across the AGC family of kinases to which PKCι belongs; phosphorylation at this site stabilizes an active conformation (Messerschmidt et al. 2005) and is likely due to autophosphorylation that follows phosphorylation by PDK1 on the activation loop (Newton 2003). Treatment of glioblastoma cells with inhibitor caused a dose-dependent decrease in PKCι phosphorylation and a corresponding increase in RhoB levels (Figure 2C and S2B). To examine the role of PI3K in activating PKCι and the repression of RhoB in human glioblastoma cells, LY294002 was used to pharmacologically inhibit PI3K. This also caused a decrease in PKCι phosphorylation and an increase in RhoB levels (Figure 3A).

Figure 2. PKCι represses RhoB. A. Total cell lysates of U87MG cells were analyzed for levels of RhoB, PKCι and actin (as a loading control) after depletion of PKCι. The top panel shows a representative Western blot and the bottom panel shows quantitation of changes in RhoB levels by densitometry (mean ± range of two independent experiments). B. Western blot analysis of RhoB and PKCι levels in normal human astrocytes (NHA) and three human glioblastoma cell lines. C. U87MG in serum containing media were treated with increasing concentrations of atypical PKCζ-pseudosubstrate peptide inhibitor for 2 h. Total cells lysates were analysed by Western blotting for the indicated proteins. Quantitation by densitometry shows the mean ± range of two independent experiments.


A
Control
siControl
siPKCιA
siPKCιB
RhoB
PKCι
Actin
RhoB (compared to control)
4
3.5
3
2.5
2
1.5
1
0.5
0
Control
siControl
siPKCιA
siPKCιB
B
NHA
U87
A172
U118
RhoB
PKCι
Actin
C
0
10
20
50
PS-Inhibitor (μM)
phospho-PKCιT555
PKCι
RhoB
phospho-PKB/Akt
PKB/Akt
Change in expression (compared to control)
phospho-PKCιT555
RhoB
3
2.5
2
1.5
1
0.5
0
0
10
20
50
PS-Inhibitor (μM)

In addition, PTEN was reintroduced into U87MG cells (both U87MG and A172 do not express PTEN due to mutational inactivation (Ishii et al. 1999)) using retroviral transduction. Restoration of PTEN decreased levels of PKCι phosphorylation; this was also accompanied by an increase in RhoB expression levels (Figure 3B).

To determine whether the effects of PKCι on RhoB involved p38MAP kinase, U87MG cells depleted of PKCι were treated with the p38MAP kinase inhibitor SKF86002, which is active against all four p38MAP kinase members (Losa et al. 2003). SKF6002 had no effect on RhoB levels in U87MG cells, and did not effect the increase in RhoB that is seen with PKCι depletion (Figure 3C and D). Inhibition of ERK also did not effect RhoB levels (data not shown).

RhoB inhibits glioblastoma cell motility and invasiveness: To examine the effect of RhoB on glioblastoma cell motility we made U87MG and A172 cells that constitutively express high levels of RhoB (Figure 5A and B, top panels). These cells showed significant reductions in motility in both scratch wound assays (Figure 4A and S3A) and Transwell assays (35% decrease with U87MG cells (Figure 4B and C); 44 % with A172 cells (Figure S3B and C)). Glioblastoma cells constitutively expressing high levels of RhoB also had significantly reduced invasion through Matrigel (71% decrease in U87MG cells (Figure 4B and D); 54 % decrease in A172 cells (Figure S3B and D)). U87MG and A172 cells constitutively expressing high levels of RhoB also showed a reduction in growth rate compared to empty vector containing control cells (Figure SE and F). The Transwell motility and invasion assays described above are not affected by these differences in proliferation rates, as no significant difference in proliferation is observed at 24 h.

Figure 3. Effects of PI 3-kinase and p38 MAP kinase inhibition on RhoB expression.
A. Western blot analysis of U87MG cells for phospho-PKCιT555, total PKCι and RhoB after treatment for 2 h with increasing concentrations of LY294002. B. Western blot analysis of U87MG cells stably expressing PTEN C. Western blot analysis of U87MG cells after PKCι depletion and treatment with SKF86002 (10 μM) for 1 h. D. Western blot analysis of U87MG cells for phospho-HSP27, total HSP27 and PKCι following a 1 h pretreatment with SKF86002 (10 μM) and 1 h anisomycin (10 μM) treatment, showing that SKF86002 effectively blocks p38 MAP kinase activation in U87MG cells. All inhibitor treatments were carried out on cells in serum containing media.

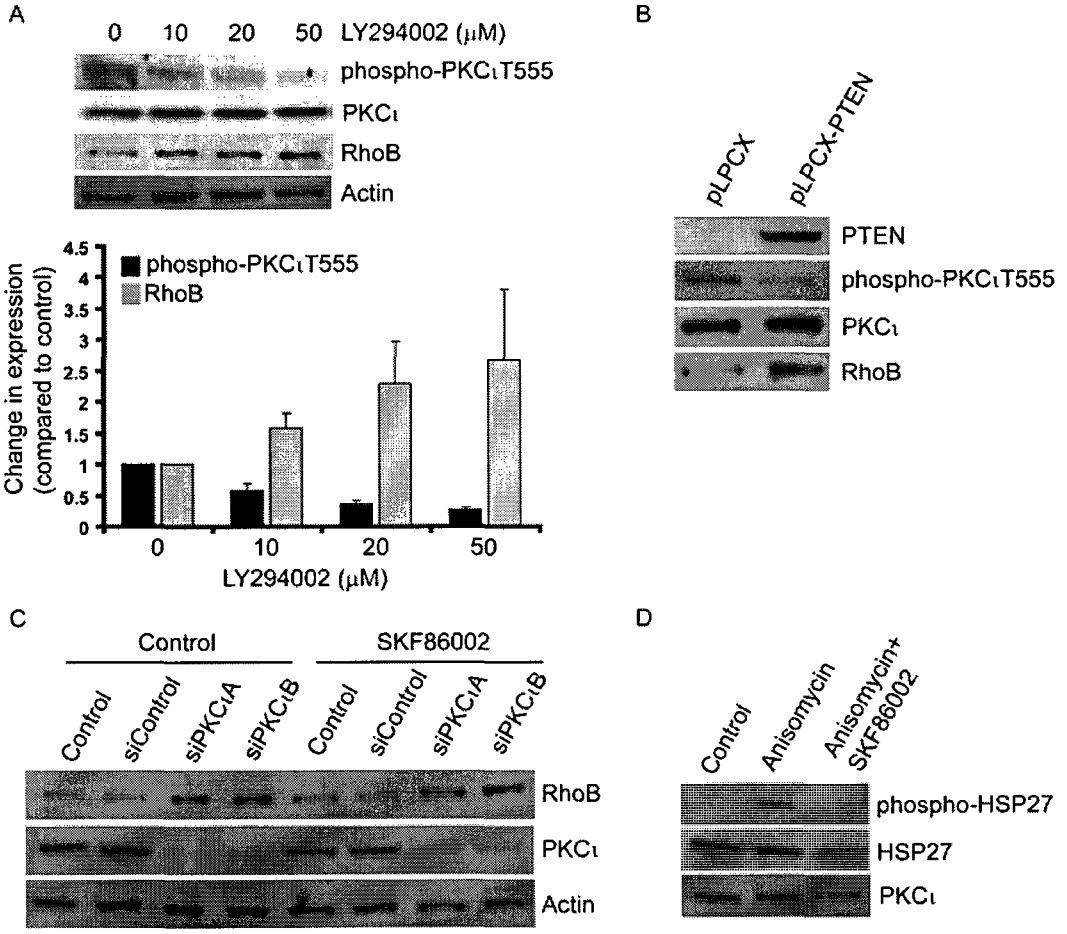
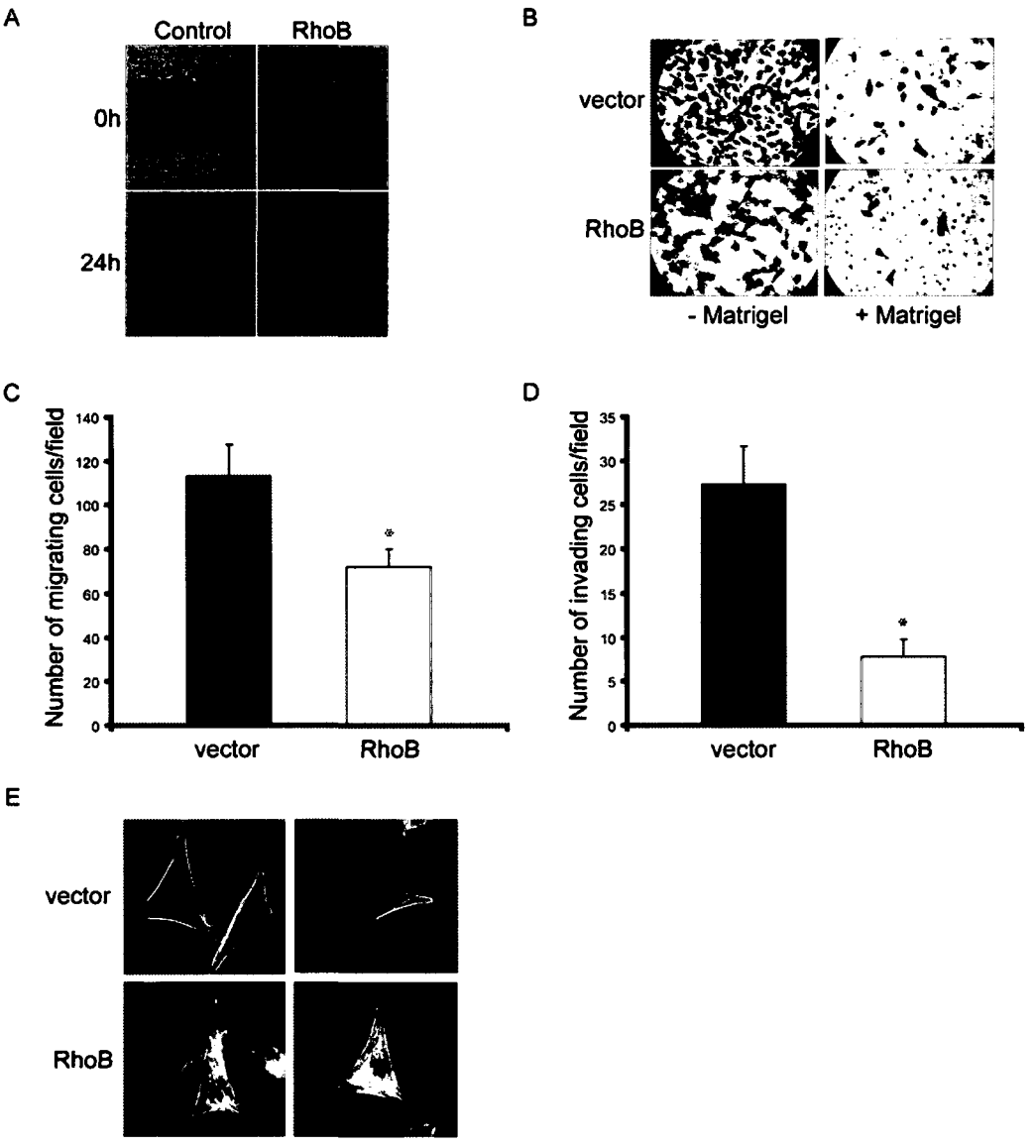

A
0 10 20 50 LY294002 (μM)
phospho-PKCιT555
PKCι
RhoB
Actin
phospho-PKCιT555
RhoB
Change in expression (compared to control)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
0 10 20 50
LY294002 (μM)
B
pLPCX
pLPCX-PTEN
PTEN
phospho-PKCιT555
PKCι
RhoB
C
Control
SKF86002
Control
siControl
siPKCιA
siPKCιB
Control
siControl
siPKCιA
siPKCιB
RhoB
PKCι
Actin
D
Control
Anisomycin
Anisomycin+ SKF86002
phospho-HSP27
HSP27
PKCι

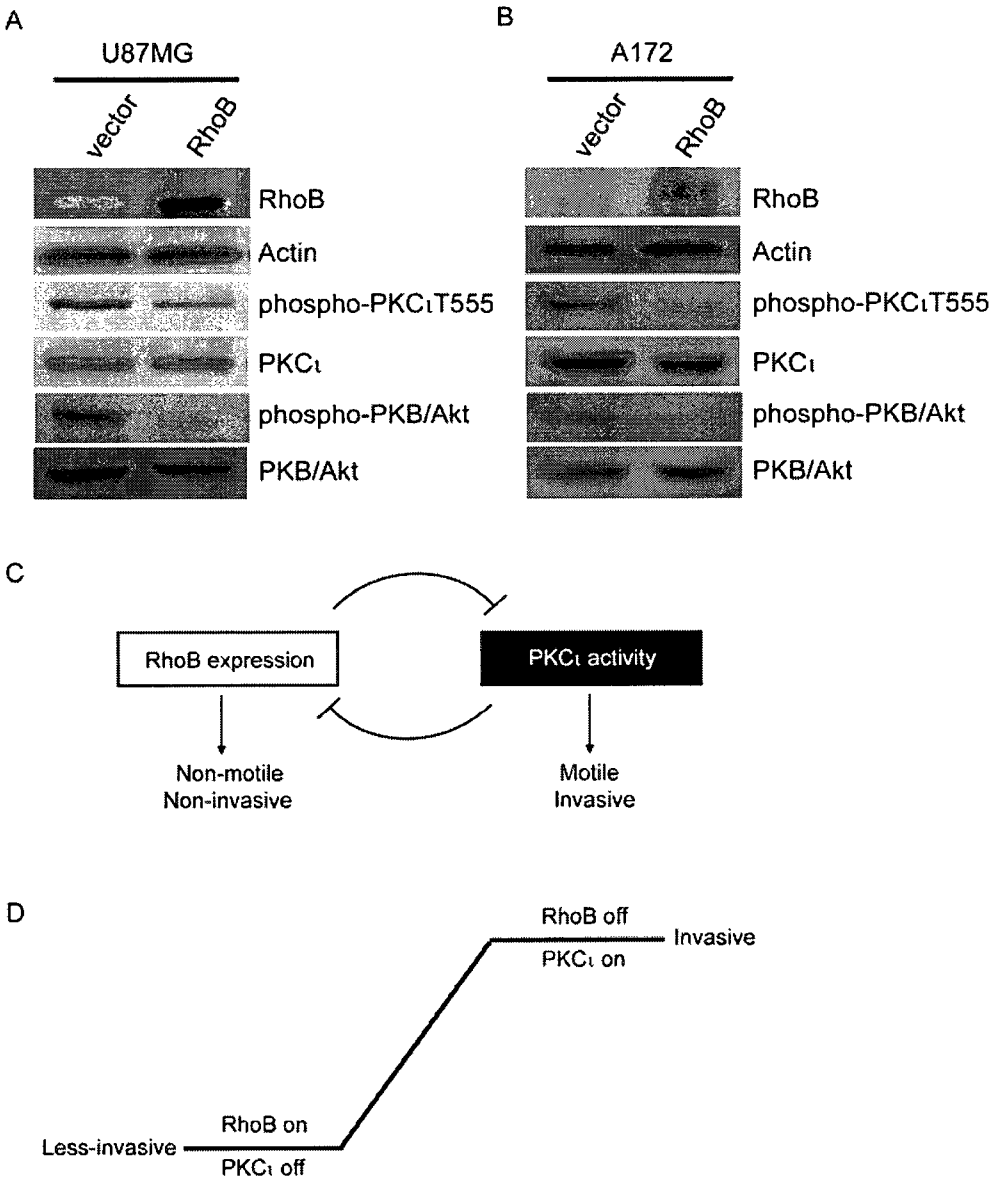
Figure 4. RhoB inhibits glioblastoma cell motility and invasion. U87MG cells were transduced with empty vector or vector containing cDNA for RhoB. Cell motility was analysed using scratch-wound assays (A) and Transwell chambers without Matrigel (B and C). Invasion was analyzed using Transwell chambers with Matrigel (B and D). B shows representative pictures of U87MG cells that have passed through the Transwell chamber -/+ Matrigel. C and D show the migrating cell counts in the absence or presence of Matrigel, respectively. Data are from one of two independent experiments that gave similar results. E. Actin filament staining in U87MG cells transduced with empty vector and vector containing cDNA for RhoB.


A
Control
RhoB
0h
24h
B
vector
RhoB
- Matrigel
+ Matrigel
C
Number of migrating cells/field
140
120
100
80
60
40
20
0
vector
RhoB
D
Number of invading cells/field
35
30
25
20
15
10
5
0
vector
RhoB
E
vector
RhoB

Consistent with the previously described ability of Rho proteins to induce actin stress fiber formation (Wheeler and Ridley 2004), overexpression of RhoB in glioblastoma cells also caused an increase in actin stress fibers when compared to control cells (Figure 4E).

RhoB expression inhibits activation of PKCι : Previously it has been reported that RhoB has the ability to negatively regulate PI3K pathway signaling in some cell types (Liu and Prendergast 2000). To evaluate the effect of RhoB expression on PKCι activity, glioblastoma cells stably expressing RhoB from a constitutive promoter were examined for phosphorylation of PKCι at Thr555. RhoB overexpression in glioblastoma cell lines resulted in a reduced phosphorylation of PKCι at Thr555 (Figure 5A and B), showing that RhoB can regulate the activity of this enzyme. Consistent with previous reports we also observed a decrease in PKB/Akt phosphorylation (Figure 5A and B), indicating that RhoB regulation of these activities occurs upstream of these enzymes.

Figure 5. RhoB inhibition of PKCι activity. Total cell lysates from RhoB-transduced U87MG (A) and A172 (B) cells were assessed for levels of the indicated proteins using Western blot analysis. Actin serves as a loading control for these experiments. C. Model illustrating mutual antagonism between PKCι activity and RhoB expression in glioblastoma cells. D. The kinetic consequence of a mutually antagonistic network is that analog signals are converted to a binary output.


A
U87MG
vector
RhoB
RhoB
Actin
phospho-PKCιT555
PKCι
phospho-PKB/Akt
PKB/Akt
B
A172
vector
RhoB
RhoB
Actin
phospho-PKCιT555
PKCι
phospho-PKB/Akt
PKB/Akt
C
RhoB expression
PKCι activity
Non-motile
Non-invasive
Motile
Invasive
D
RhoB off
PKCι on
Invasive
Less-invasive
RhoB on
PKCι off

Discussion

Glioblastoma multiforme is almost invariably a fatal disease, primarily because of its invasive nature. Here we show that PKCι has a role in promoting glioblastoma cell invasion. There is precedent for this from previous studies showing that atypical protein kinase C is involved in the polarized migration of cultured astrocytes (Etienne-Manneville et al. 2005) and that PKCι is involved in the nicotine-activated migration and invasion of H1299 human non-small cell lung cancer cells (Xu and Deng 2006). The actin cytoskeleton is a dynamic element within cells that undergoes changes that are essential to cell motility (Lambrechts et al. 2004). PKCι depletion resulted in the formation of long actin stress fibers; similar changes in stress fibers were described by Soloff *et al.* in PKCι knockout mouse embryonic fibroblasts (Soloff et al. 2004) and other studies have also implicated the atypical protein kinase C family in stress fiber loss (Coghlan et al. 2000; Uberall et al. 1999).

Our previous microarray analysis of PKCι-mediated gene expression identified RhoB as a gene that was negatively-regulated by PKCι. RhoB expression is known to be induced by a number of cellular stresses (Fritz et al. 1995). The stress-inducible fraction of RhoB expression is in some cases dependent on p38 MAP kinase (Gerhard et al. 2005). RhoB levels are also controlled by a second mechanism that involves the PI 3-kinase pathway (Jiang et al. 2004). We show here that PKCι is a downstream mediator in this pathway in glioblastoma cells, with both pharmacological inhibition of PKCι and depletion of PKCι using RNA interference pointing to a role for PKCι in repressing RhoB expression. Inhibition of PI 3-kinase, either pharmacologically or with transduced PTEN, also caused an increase in RhoB levels that was similar in magnitude to that seen

with PKCι inhibition. These data indicate that aberrant activation of the PI 3-kinase pathway in glioblastoma represses RhoB expression primarily via activation of PKCι. Previously it has been reported that in NIH3T3 mouse fibroblasts and human pancreatic cancer cells, PKB/Akt represses RhoB expression downstream of PI 3-kinase activation (Jiang et al. 2004). Inhibition of PKCι with myristoylated pseudosubstrate peptide increased RhoB levels without any effect on PKB/Akt phosphorylation (Figure 2C), showing that the role of PKCι in RhoB regulation is independent of PKB/Akt in glioblastoma cells. Recent literature suggests that PKCι and PKB/Akt share some similar downstream functions - for example both enzymes can phosphorylate the anti-apoptotic protein Bad (Jin et al. 2005). It is conceivable that different tissues may rely more on one enzyme than the other for RhoB regulation. PKCι has been shown to be expressed at higher levels in brain tissues compared to other tissues (Selbie et al. 1993). Therefore, in brain tissue and potentially brain tumours, cells may preferentially signal through PKCι as the dominant downstream PI3K effector. In addition, the tissue specific expression of adaptor or anchoring proteins may play a role in the dependence on one enzyme over another in particular cell types. It is also possible that the dominant negative PKB/Akt used in the previous study functions in part as a pseudosubstrate inhibitor of PDK1, which could potentially result in the inhibition of multiple kinases downstream from PDK1 including PKCι.

We found that RhoB levels were higher in normal human astrocytes levels compared to those seen in three different glioblastoma cell lines. This is consistent with the previous observation that levels of RhoB are repressed in tissue samples from high grade glioblastomas compared to those of a lower grade and normal brain (Forget et al. 2002).

PKCι protein levels were higher in the glioblastoma lines. Increased levels of PKCι protein have been reported in lung and ovarian cancer due to increased copy number of the PKCι gene (Regala et al. 2005a; Zhang et al. 2006); additionally increased levels of PKCι protein have been observed in leukemia cell lines, where there is an increase in transcription of the PKCι gene (Gustafson et al. 2004). We do not currently know if one of these mechanisms is also responsible for the increased PKCι levels in glioblastoma.

To bypass the repression of RhoB by PKCι, we transduced glioblastoma cells with a cDNA that expressed RhoB from a constitutive promoter. As with PKCι depletion, these cells showed reduced motility and invasion along with increased numbers of actin stress fibers. Therefore it appears that the effects of PKCι on both motility and actin dynamics are a consequence of its ability to repress RhoB expression.

It has been reported that RhoB is able to suppress activation of PKB/Akt in human breast and pancreatic cancer cells, but not fibroblasts (Liu and Prendergast 2000). As this suggests a role for RhoB in regulating PI 3-kinase pathway signaling, we determined whether RhoB constitutive expression also suppressed the activity of PKCι. This was the case, as RhoB repressed phosphorylation of PKCι on Thr555 in two different human glioblastoma cell lines. The exact mechanism of how RhoB accomplishes this remains unclear, but as we also see repression of PKB/Akt phosphorylation it is likely to occur at a point upstream of these kinases. RhoB has been shown to recruit PDK1 to endosomes (Flynn et al. 2000); one possibility is that PDK1 in endosomes is unable to activate PKCι and PKB/Akt effectively. Secondly, the regulation could be at the receptor level as it has been suggested that RhoB may control endosomal targeting and trafficking of the EGFR (Gampel et al. 1999). Our data show that PKCι and RhoB are mutually antagonistic, with

PKCι repressing RhoB mRNA levels and RhoB repressing PKCι activation (Figure 5C and D). The kinetic consequence of mutually antagonistic networks is that analog signals are converted into a binary output. This has been demonstrated both theoretically and experimentally with the construction of an artificial "toggle" switch in bacteria containing two repressible promoters arranged in a mutually inhibitory network (Gardner et al. 2000). The mutual antagonism between PKCι and RhoB may therefore provide the basis for a "switch", such that a cell is either motile or non-motile without an intermediate state. Our data show only a partial reduction in cell motility with PKCι depletion or constitutive RhoB expression; analysis of individual cells using live cell imaging would determine whether this represents a change in the proportion of motile to non-motile cells (consistent with a "switch" model) or a decrease in the rate of movement of individual cells.

In summary our data define a role for PKCι in glioblastoma cell motility and invasion. Mechanistically we show that PKCι is activated in glioblastoma as a consequence of aberrant upstream PI 3-kinase signaling and that repression of RhoB is key downstream event in PKCι signaling leading to enhanced cell motility. The repression of RhoB expression by PKCι also provides a mechanism for: (1) the previously reported downregulation of RhoB in human glioblastoma; and (2) the PKCι-mediated loss of actin stress fibers that has been observed in a number of different cell types. These results and our previous study (Baldwin et al. 2006) suggest that PKCι should be evaluated further as a potential drug target for glioblastoma therapy.

Materials and Methods

Chemicals and antibodies: Custom RNA interference duplexes were from Dharmacon RNA Technologies Inc. (Lafayette, CO, USA). RhoB rabbit polyclonal and actin monoclonal antibodies were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Mouse monoclonal anti-PKCι and phospho-PKCι Thr555 rabbit monoclonal were from BD Biosciences (Mississauga, ON, CAN). Phospho-Akt/PKB, Akt/PKB and PDK1 rabbit polyclonal antibodies were from Cell Signaling Technology (Beverly, MA, USA). Pan Erk monoclonal antibody was from Transduction Laboratories. LY294002, SKF86002 and the atypical PKC inhibitor peptide were from Sigma (Oakville, ON, CAN), Calbiochem (San Diago, CA, USA) and Biosource (Camarillo, CA, USA), respectively.

Cell lines: Normal human astrocytes were purchased from Cambrex Bioscience (Walkersville, MD, USA) and were maintained in astrocyte growth media (Lonza Group Ltd, Basel, Switzerland) supplemented as recommended by the manufacturer. The human glioblastoma cell line U87MG was obtained from Dr. W. Cavenee (Ludwig Institute for Cancer Research, La Jolla, CA, USA). A172 and U118 cells were from the American Type Culture Collection. Cells were cultured as described previously (Lavictoire et al. 2003).

RNA transfections: Depletion of PKCι using RNA interference was done as described previously (Baldwin et al. 2006). The control RNA duplex had the sense RNA sequence

UAGCGACUAAACACAUCAA (siControl, Dharmacon Research, Inc. (Lafayette, CO, USA)).

Western blot analysis: Western blotting was performed as described previously (Baldwin et al. 2006). After electrophoretic transfer from the gel, blots were stained with amido black to confirm that equal sample loading and transfer was achieved.

Construction of PTEN and RhoB expression vector: Full length cDNA for human PTEN was obtained from the American Type Culture Collection. PTEN cDNA was cloned into the retroviral vector pLPCX (Clontech, Palo Alto, CA, USA). Retroviral MSCVpac and MSCVpac-RhoB vectors were a generous gift from Dr. George Prendergast (Lankenau Institute for Medical Research, Wynnewood, PA, USA). Retrovirus containing PTEN or RhoB cDNA was made as described previously (Lavictoire et al. 2003). Cells were gown in media containing puromycin (1 μg/mL) to select for transductants.

Cell counts: Live cell number was determined using a Vi-Cell XR cell viability analyzer (Beckman Coulter Canada Inc., Mississauga, ON, CAN).

Cell motility and invasion assays: Scratch wound assays were done as described (Sang et al. 2007). For Transwell chamber assays, BD Biocoat Matrigel invasion chambers (BD Biosciences) were used. Details of the procedures are given in the legend to Figure S1.

Statistical analysis: All results were expressed as the mean ± S.D. Statistical analysis was performed using the two-tailed Student's t test, and $p < 0.05$ was considered statistically significant.

Figure S1. PKCι enhances glioblastoma cell motility and invasion in U87MG and A172 cells. A. Quantitative analysis of scratch-wound assays done on U87MG cells after PKCι depletion. B. A172 cells were assessed for motility and invasion after PKCι depletion using Transwell chambers with or without a layer of Matrigel. The top panel shows stained cells that have passed through the membrane of the Transwell chamber and the bottom panel shows migrating cell counts without Matrigel (C) and with Matrigel (D). Data are from one of two independent experiments that gave similar results.

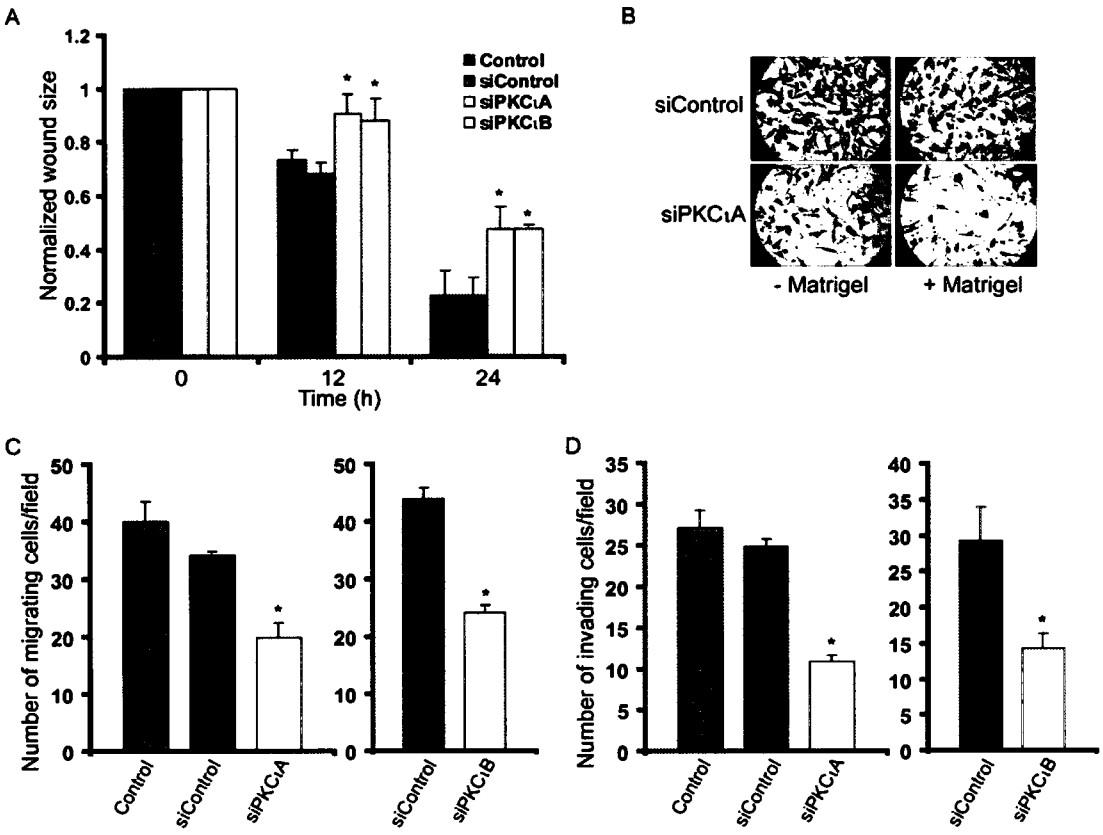

A
Normalized wound size
1.2
1
0.8
0.6
0.4
0.2
0
Control
siControl
siPKCιA
siPKCιB
0
12
24
Time (h)
B
siControl
siPKCιA
- Matrigel
+ Matrigel
C
Number of migrating cells/field
50
40
30
20
10
0
Control
siControl
siPKCιA
siControl
siPKCιB
D
Number of invading cells/field
35
30
25
20
15
10
5
0
40
35
30
25
20
15
10
5
0
Control
siControl
siPKCιA
siControl
siPKCιB

Figure S2. Effect of PKCι on RhoB protein expression in A172 cells. A. Total cell lysates of A172 cells were analyzed for levels of RhoB, PKCι and actin (as a loading control) after depletion of PKCι. The top panel shows a representative Western blot and the bottom panel shows quantitation of changes in RhoB levels by densitometry (mean ± range of two independent experiments). B. A172 in serum containing media were treated with increasing concentrations of atypical PKCζ-pseudosubstrate peptide inhibitor for 2 h. Total cells lysates were analysed by Western blotting for the indicated proteins. Quantitation by densitometry shows the mean ± range of two independent experiments.

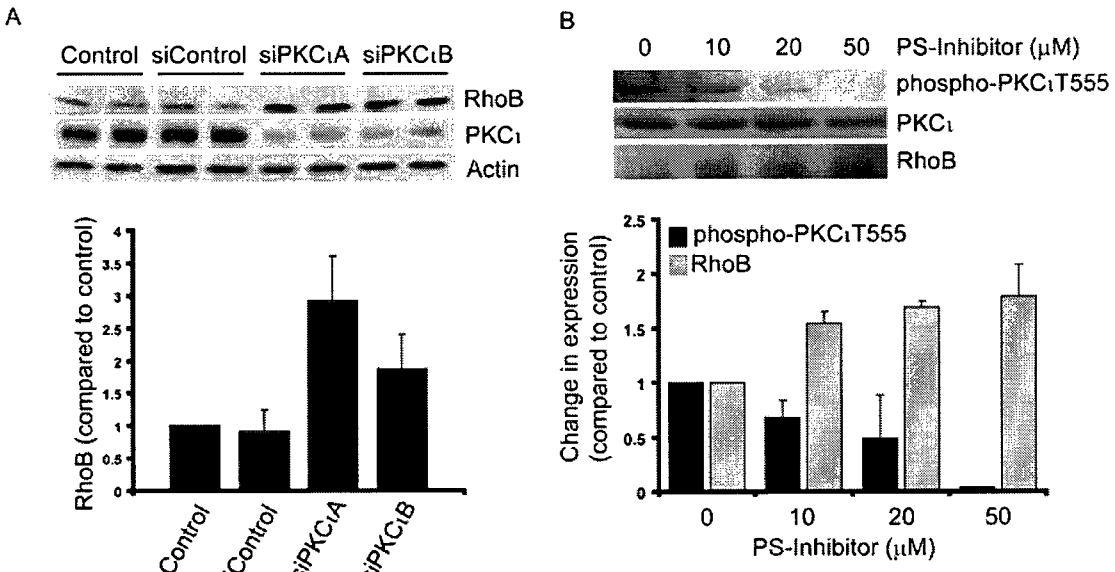
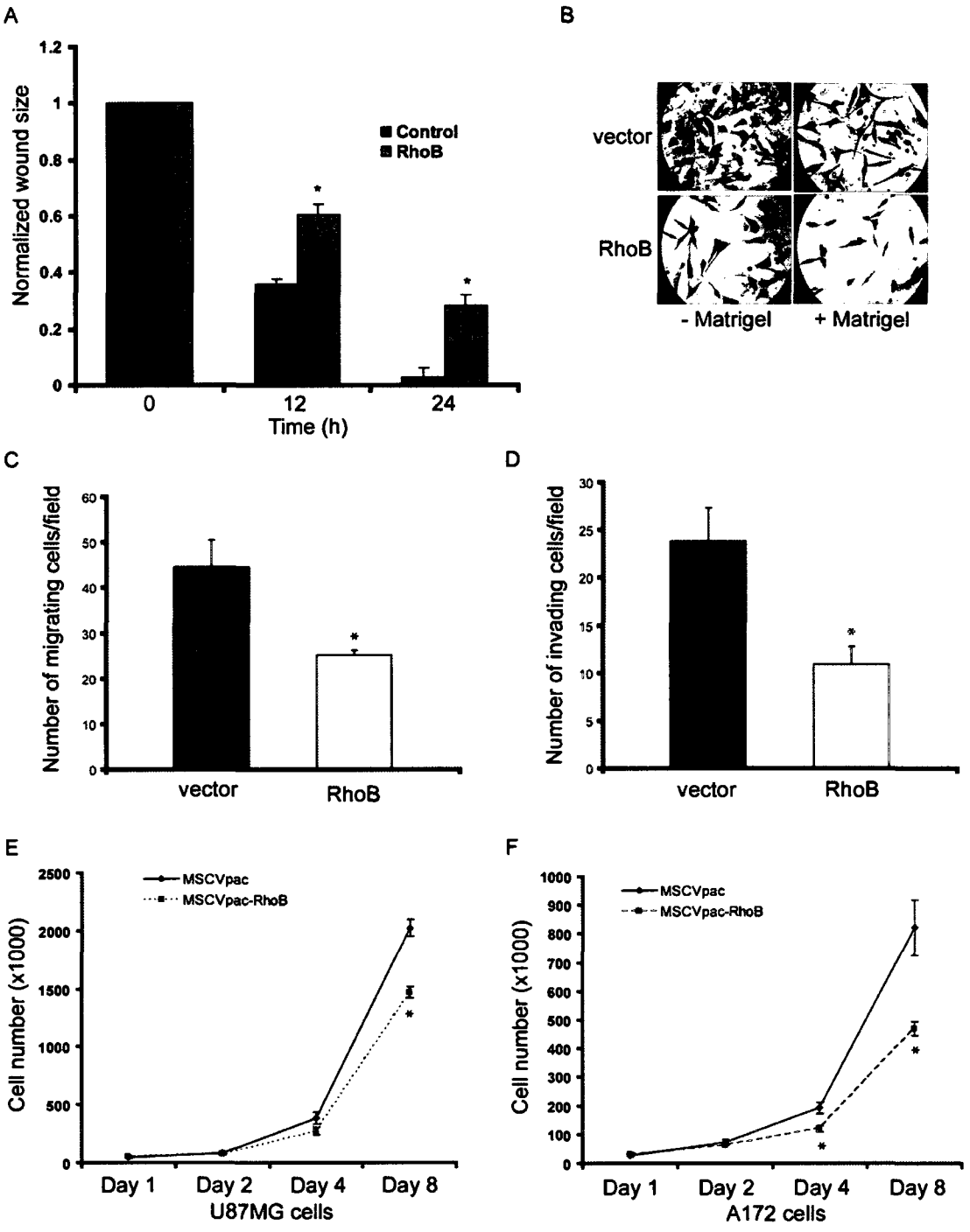

A
Control
siControl
siPKCιA
siPKCιB
RhoB
PKCι
Actin
RhoB (compared to control)
Control
siControl
siPKCιA
siPKCιB
B
0
10
20
50
PS-Inhibitor (μM)
phospho-PKCιT555
PKCι
RhoB
Change in expression (compared to control)
phospho-PKCιT555
RhoB
PS-Inhibitor (μM)

Figure S3. RhoB inhibits glioblastoma cell motility and invasion in A172 cells. A. Quantitative analysis of scratch-wound assays done on U87MG cells transduced with RhoB. B. Transwell chamber assay of A172 cells transduced with a control vector or vector containing RhoB cDNA C. Analysis of A172 cell motility and invasion using Transwell chambers without (C) or with (D) a layer of Matrigel. Data are from one of two independent experiments that gave similar results. Growth of U87MG (E) and A172 (F) cells transduced with a control vector or vector containing RhoB cDNA.


A
Normalized wound size
1.2
1
0.8
0.6
0.4
0.2
0
Control
RhoB
*
*
0
12
24
Time (h)
B
vector
RhoB
- Matrigel
+ Matrigel
C
Number of migrating cells/field
60
50
40
30
20
10
0
*
vector
RhoB
D
Number of invading cells/field
30
25
20
15
10
5
0
*
vector
RhoB
E
Cell number (x1000)
2500
2000
1500
1000
500
0
MSCVpac
MSCVpac-RhoB
*
Day 1
Day 2
Day 4
Day 8
U87MG cells
F
Cell number (x1000)
1000
900
800
700
600
500
400
300
200
100
0
MSCVpac
MSCVpac-RhoB
*
Day 1
Day 2
Day 4
Day 8
A172 cells

Supplementary Table 1

	Gene name	*Control RNA: log2*	*Control RNA + Cisplatin: log2*	*PKCι RNAi: log2*	*PKCι RNAi + Cisplatin: log2*	*Genbank accession number(s)*
PRKCI	Protein kinase c, iota	5.87	6.76	2.81	2.86	NM002740
RHOB	Ras homology gene family, B	3.54 (-)	8.67 (35)	6.58 (8)	9.52 (63)	NM004124

Table S1. Microarray analysis of PKCι effects on RhoB mRNA expression. Numbers is parentheses indicate fold changes compared to control RNA transfected cells. Changes in RhoB mRNA levels were also assessed using quantitative RT-PCR. Total RNA was isolated using RNEasy Mini Kit (Qiagen Inc.). Reverse transcription was carried out with 2 μg of RNA with random hexamer primers using the First Strand cDNA Synthesis Kit (MBI Fementas). qPCR was performed with a LightCycler (Roche) using FastStart DNA Master SYBR Green I Kit (Roche). Data were analysed using the LightCycler analysis software. For qPCR of RhoB the primer pair RhoBF (5'-GGTCCCCTGAGCATGCTTTTCTGA) and RhoBR (5'-GCCACACTCCCGCGCCAATCTC)was used; for β 2-microglobulin, used for reference, the primer pair B2MFor (5'- TGCTGTCTCCATGTTTGATGTATCT) and B2MRev (5'-CTCTGCTCCCCACCTCTAAGT) was used.

Acknowledgments

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4. STABLE DEPLETION OF PKCι IMPAIRS GLIOBLASTOMA CELL MOTILITY AND CELL DIVISION

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***Running Title*: PKCι inhibition causes impairment of cell motility and division**

Keywords: atypical PKC; RhoB; PI3-kinase; PTEN; glioblastoma

Contribution of Authors

The content of this chapter was written by R. M. Baldwin with the help of Dr. I. A. J. Lorimer. All of the experiments presented in this manuscript are the work of R. M. Baldwin with the exception of Figure 5 and Supplementary Figure 2. Immunofluorescence to generate Figure 5 and Supplementary Figure 2 was performed by D. A. E. Parolin.

Abstract

The invasiveness of glioblastoma cells is an unfavorable characteristic for the prognosis and treatment of this devastating disease. Enhanced activation of the PI3K pathway is observed in glioblastoma due to mutations in PTEN and EGFR, and promotes its invasive properties. PKCι is a key downstream effector of PI3K that potentially links the activity of this pathway to glioblastoma cell motility and invasion. PKCι is part of a conserved polarity complex with Par-6 which can regulate several events that are critical to the cell invasion process. Here we have assessed the role of stable PKCι depletion on glioblastoma cell invasion. Stable suppression of PKCι in glioblastoma cells was accomplished using an expression plasmid containing a PKCι-targeted short hairpin. This caused a significant decrease in the proliferation of U87MG and A172 glioblastoma cells. Stable suppression of PKCι also caused an increase in actin stress fibre formation and a decrease in cell motility and invasion as assessed using Transwell chamber assays. Cell motility and invasion is a very dynamic process; live cell imaging was used to examine the effects of PKCι depletion on the coordination of cell movement. Control U87MG cells migrate in the direction of a coordinated leading edge lamellipodia. In contrast, U87MG cells stably depleted of PKCι have a reduced ability to migrate and have a more rounded morphology. They also show a loss in the ability to coordinate the formation of a functional leading edge lamellipodia and instead they generate projections from all sides of the cell. This suggests that there is a loss in the organization required for cell motility to proceed. Additionally, we observed a potential impairment of cell division with the loss of PKCι in U87MG cells. Morphologically these cells appear to enter mitosis, however they remain rounded for an extended period of time. This

contributes to the reduced proliferation observed. Therefore, PKCι may play a role in the regulation of cell division. Live cell imaging has allowed for a real-time evaluation of the consequences of PKCι depletion in glioblastoma cells. This assessment has demonstrate that PKCι plays a functional role in the coordination of glioblastoma cell movement, and gives more insight into the benefit of therapeutically targeting it in the treatment of glioblastoma.

Introduction

Glioblastoma is currently an incurable primary brain tumour with a very poor prognosis. Despite the use of aggressive therapeutic approaches combining surgery, radiation and chemotherapy, the median survival time for patients is only 12-14 months (Stupp et al. 2005). Ultimately, recurrence of the tumour is lethal to patients. The highly invasive nature of glioblastoma cells allows them to evade these types of treatments. Invasive tumour cells can blur tumour margins making complete surgical resection impossible (Nakada et al. 2007). Additionally, it is thought that invading cells may be more resistant to radiation and chemotherapy (Giese et al. 2003). It is therefore an important therapeutic strategy to inhibit glioblastoma cell invasion in order to maintain a more localized lesion that may be more effectively treated by these therapies.

Cell migration is a dynamic process that is highly regulated in normal cells and loss of this control drives the motile phenotype of cancer cells. The phosphoinoside 3-kinase (PI3K) pathway is often constitutively active in glioblastoma as a result of mutations in PTEN, as well as mutation and amplification of the epidermal growth factor receptor (EGFR; Ohgaki and Kleihues 2007). These genetic alterations have been shown to promote motility and invasion of glioblastoma cells through aberrant activation of the PI3K pathway (Tamura et al. 1999; Cai et al. 2005; Gunther et al. 2003; Merlo and Bettler 2004). Inhibition of this pathway can significantly reduce cell motility (Merlo and Bettler 2004). The PI3K pathway can activate multiple downstream effectors that can regulate cell migration and invasion, including protein kinase C iota (PKCι). The activation of PKCι involves direct phosphorylation by PDK1 and association with Cdc42, a small GTPase that is extensively involved in cell migration (Kanzaki et al. 2004;

Etienne-Manneville 2004; Raftopoulou and Hall 2004). It has recently been shown that PKCι may be overexpressed in primary glioblastoma tumours compared to normal brain samples, suggesting it may contribute to the aggressiveness of this disease (Patel et al. 2008).

The atypical PKCs have been shown to play a role in the establishment of multiple forms of cell polarity, such as asymmetric cell division, apical-basal polarity and cell migration in C. elegans, Drosophila and mammalian systems (Etienne-Manneville and Hall 2001; Henrique and Schweisguth 2003; Etienne-Manneville and Hall 2003). They form a conserved polarity complex with the partitioning protein, Par-6 (Henrique and Schweisguth 2003; Moscat et al. 2006). Par-6 is responsible for facilitating the interaction of the atypical PKCs with Cdc42. This interaction links PKCι activity to cytoskeletal dynamics which is directly involved in the regulation of cell motility. The contribution of PKCι to cancer cell invasiveness has only recently been identified (Xu and Deng 2006; Frederick et al. 2008). PKCι has been shown to promote the invasiveness of lung cancer cells (Xu and Deng 2006). We have also demonstrated that transient inhibition of PKCι using RNA interference (RNAi) can reduce the invasiveness of glioblastoma cells through the regulation of the small Rho GTPase, RhoB (Xu and Deng 2006; Baldwin et al. 2008). These studies have given insight into the role of PKCι in cellular invasion, however they have relied on a static analysis of invasion, which is a very dynamic process.

In this study, we have investigated the effects of stable PKCι depletion on glioblastoma motility and invasion and have shown that it promotes these processes, consistent with our previous model. To gain futher insight into the role PKCι plays in the

regulation of glioblastoma cell motility we have used live cell imaging. These studies showed that PKCι plays a critical in the coordination of cell motility in U87MG cells. Additionally, live cell imaging also revealed that the loss of PKCι causes an impairment in the ability of these cells to complete mitosis, suggesting it may also be involved in the coordination of glioblastoma cell division.

Results

Downregulation of PKCι expression by shRNA: To determine the anti-tumour effect of stably depleting PKCι in glioblastoma, two unrelated PKCι targeting siRNA expression plasmids (pshPKCιA and pshPKCιB) were prepared and infected into human glioblastoma cell lines using retrovirus (Figure 1A). This generated stable populations of glioblastoma cells after two weeks of selection in puromycin. In Western blotting analysis of U87MG cells expressing the pshPKCιA plasmid, PKCι protein expression was stably repressed approximately 60%. However, a second PKCι targeting plasmid, pshPKCιB, was inefficient in repressing PKCι protein expression, reducing it by only 25% (Figure 1B). In a second glioblastoma cell line, A172, expression of the pshPKCιA plasmid effectively repressed PKCι protein expression, reducing it by approximately 67% (Figure 1C).

Inhibition of cell motility and invasion in U87MG cells stably depleted of PKCι: The effects of stable depletion of PKCι on glioblastoma cell motility and invasion were initially assessed using Transwell chambers. To examine cell motility, control and PKCι depleted U87MG cells were seeded at the same density in Transwell chambers, 24 h later the number of cells that crossed through the chamber were counted. Stable depletion of PKCι resulted in a 64% decrease in the number of cells that crossed through the chamber (Figure 2A and B). To assess the effects on invasion, control and PKCι depleted U87MG cells were seeded at equal densities into Transwell chambers that were coated with a Matrigel layer. Stable depletion of PKCι also caused a significant reduction (61%) in the number of cells that were able to pass through the Matrigel-coated chambers (Figure 2A

Figure 1. Western blot analysis of PKCι expression in glioblastoma cells stably expressing PKCι-targeted shRNA. Control GFP and PKCι targeting sequences were cloned into the pSUPER.retro.puro backbone (A). Western analysis of PKCι expression in U87MG (B) and A172 (C) glioblastoma cells following selection of stably expressing cell populations. Densitometry for the westerns normalizing to U87MG (B) or A172 (C) lanes are shown in the lower panels. Actin or total ERK were used as loading controls.

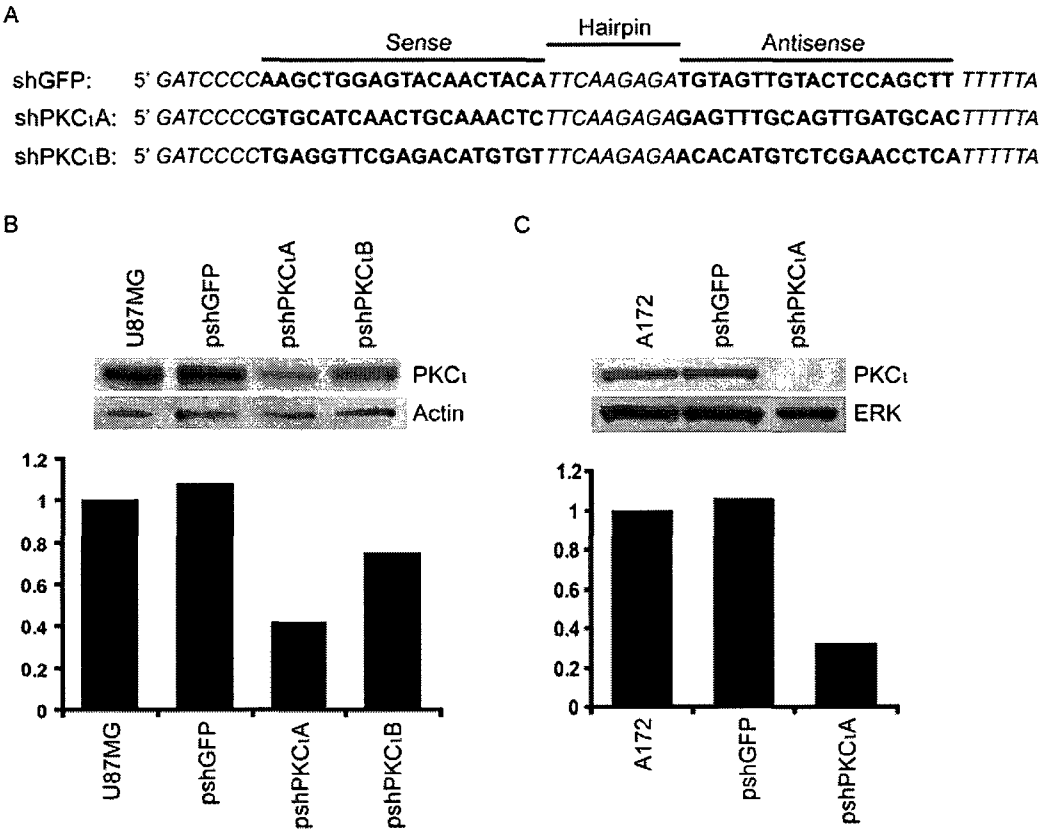
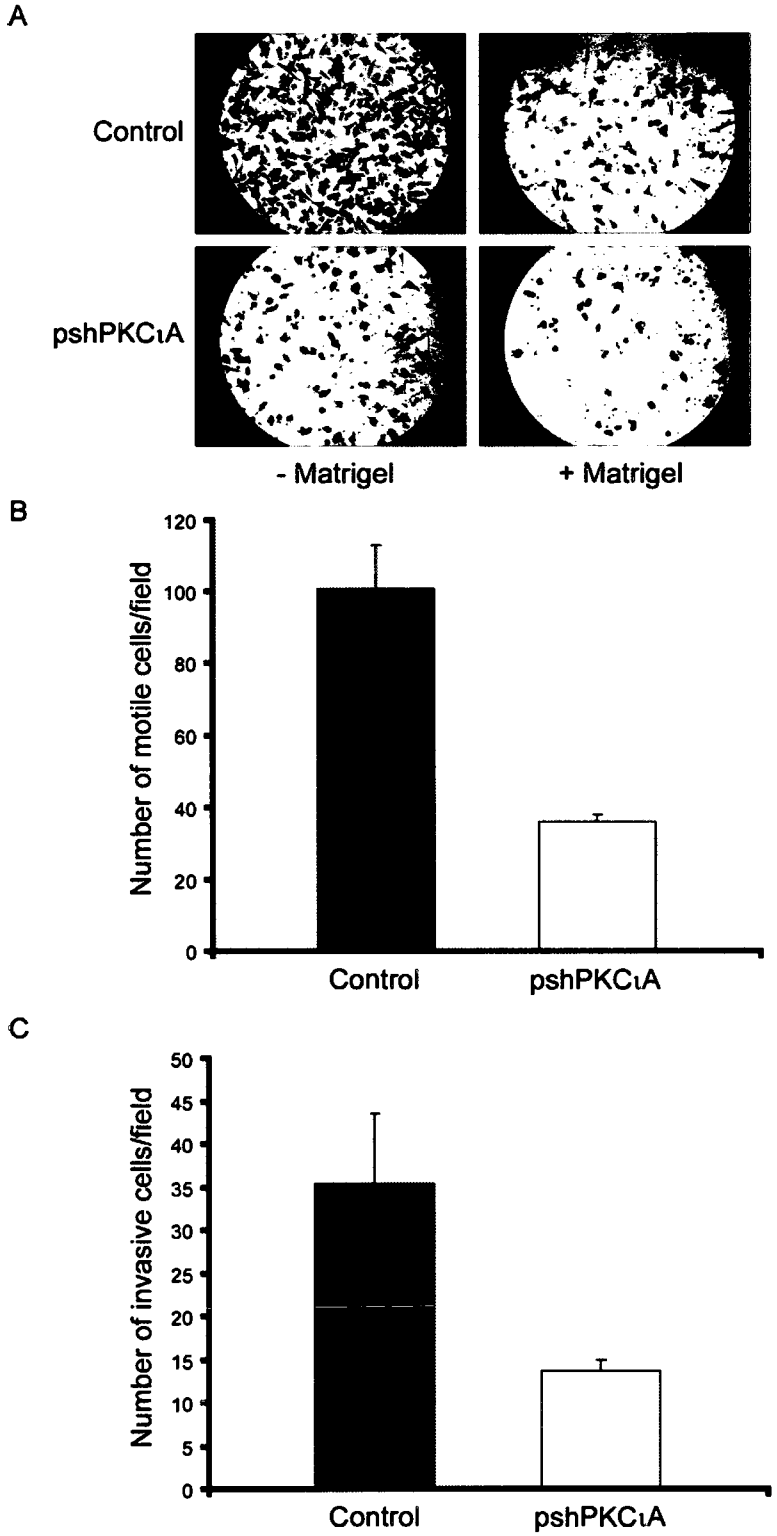

A
Sense
Hairpin
Antisense
shGFP: 5' GATCCCCAAGCTGGAGTACAACTACATTCAAGAGATGTAGTTGTACTCCAGCTT TTTTTA
shPKCιA: 5' GATCCCCGTGCATCAACTGCAAACTCTTCAAGAGAGAGTTTGCAGTTGATGCACTTTTTA
shPKCιB: 5' GATCCCCTGAGGTTCGAGACATGTGTTTCAAGAGAACACATGTCTCGAACCTCATTTTTA
B
U87MG
pshGFP
pshPKCιA
pshPKCιB
PKCι
Actin
1.2
1
0.8
0.6
0.4
0.2
0
U87MG
pshGFP
pshPKCιA
pshPKCιB
C
A172
pshGFP
pshPKCιA
PKCι
ERK
1.2
1
0.8
0.6
0.4
0.2
0
A172
pshGFP
pshPKCιA

Figure 2. Stable repression of PKCι inhibits glioblastoma cell motility and invasion. Control U87MG cells and U87MG cells stably depleted of PKCι were seeded into Transwell chambers to assess cell motility and invasion. Cell motility was analyzed using Transwell chambers without Matrigel (A and B). Invasion was analyzed using Transwell chambers with Matrigel (A and C). A shows representative pictures of U87MG cells that have passed through the Transwell chamber -/+ Matrigel. B and C show the migrating cell counts in the absence or presence of Matrigel respectively. Data are from one of two independent experiments that gave similar results.


A
Control
pshPKCιA
- Matrigel
+ Matrigel
B
Number of motile cells/field
120
100
80
60
40
20
0
Control
pshPKCιA
C
Number of invasive cells/field
50
45
40
35
30
25
20
15
10
5
0
Control
pshPKCιA

and C). The fact that the differences in the number of cells that crossed through the chamber in the presence or absence of Matrigel are similar suggests that PKCι effects the invasion of glioblastoma cells by promoting cell motility. Although these experiments give an indication of the influence PKCι has on cell motility, this is only a static observation of this active process.

To gain more insight into the effects PKCι has on glioblastoma cell motility, live cell imaging was used. Control and PKCι depleted U87MG cells were plated into a live cell imaging plate at a density of 10^3 cells to allow space for migration. Phase contrast images of the cells were taken at 5 min intervals for 20 h and compiled to generate a time-lapse movie. Initial assessment of these two cell lines showed that PKCι depleted cells had a significantly altered morphology compared to controls. A significant proportion of these cells have a more rounded morphology with multiple filopodia-like processes projecting out from the cells. Control U87MG cells showed two distinct morphologies of cell movement; an elongated morphology with a single leading process, and more commonly a fibroblast-like morphology with a broad, ruffling lamellipodia and a long trailing tail (Figure 3A, Suzuki and Iwaki 2005). Examination of live cell movies of these two cell lines revealed that PKCι depleted cells have a significant impairment in cell motility. Control cells display coordination to their movement, with initial extension and formation of a leading or lamellipodia, followed by translocation of the nucleus toward the lagging edge and finally retraction of the trailing process. In contrast, cells stably depleted of PKCι seem to lack this coordination. A significant proportion of these cells seem to have an impaired ability to generate a substantial leading lamellipodia. Instead, there are multiple short filopodial extensions emanating from all sides of the cell.

To quantify the migration rate (migration distance per minute) between control and PKCι depleted cells, two independent time-lapse movies of each cell line were analyzed using the Zeiss LSM image browser software. The migration of 20 cells of each cell line were followed by tracking the nucleus and an arbitrary distance unit (AU) was assigned per unit time. This analysis showed that cells stably depleted of PKCι show a 48% reduction in migration distance per minute (2.6 units/min) compared to the control cells (5.0 units/min, Figure 3B and C).

Altered cell morphology and actin cytoskeleton in U87MG stably depleted of PKCι: U87MG cells stably depleted of PKCι show alterations in cell morphology when compared to control cells (Figure 3A). These cells are more rounded and exhibit numerous membrane attachment points when compared to their control counterparts as assessed by phase contrast microscopy. To investigate the basis for this difference in morphology, fluorescence microscopy was used to examine components of the cytoskeleton; actin fibres, microtubules and non-muscle myosin type-II a (NMMIIa). No significant differences in staining patterns of NNMIIa and microtubules were observed between controls and PKCι depleted cells (Figure 4). In contrast, staining of F-actin with phalloidin revealed a higher abundance of stress fibers in U87MG cells stably depleted of PKCι compared to control cells (Figure 4). This is consistent with what we have previously shown with transient depletion of PKCι using RNA interference and what is observed in the PKCλ knockout mouse embryonic fibroblasts (Soloff et al. 2004; Baldwin et al. 2008).

Figure 3. Stable depletion of PKCι alters cell morphology and inhibits cell motility as assessed by live cell imaging. A. Phase contrast image analysis of Control versus PKCι depleted U87MG cells. Control cells show two migratory morphologies: broad leading edge (→) and single leading processes (>). Migration distance per minute (AU/min) was quantitated and using Zeiss LSM image browser. B. Represents the mean (+/- SD) from two independent live cell imaging trials of Control and PKCι depleted U87MG cells (20 cells in total were analyzed for each condition). C. Representative figure of a migrating control cell and a non-motile PKCι depleted cell examined for 300 min.

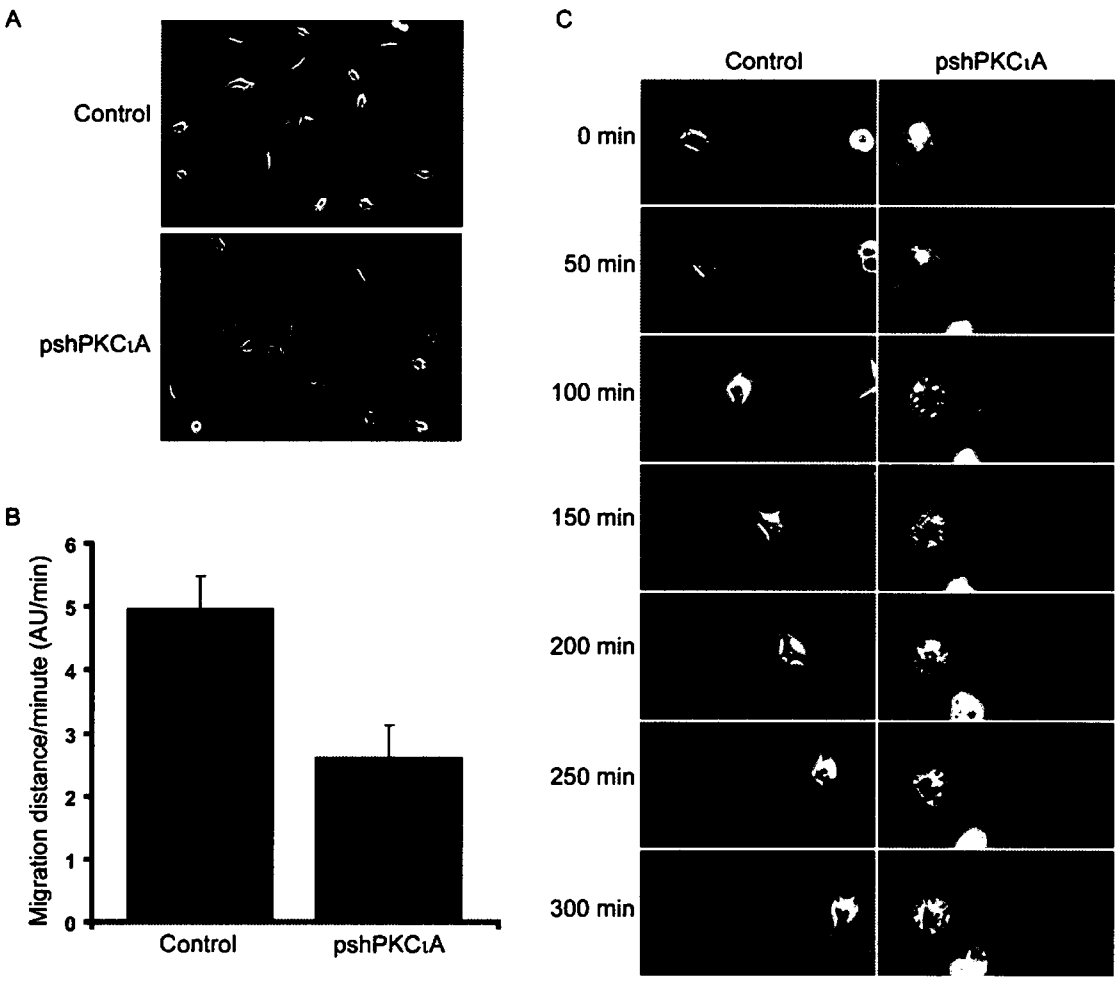
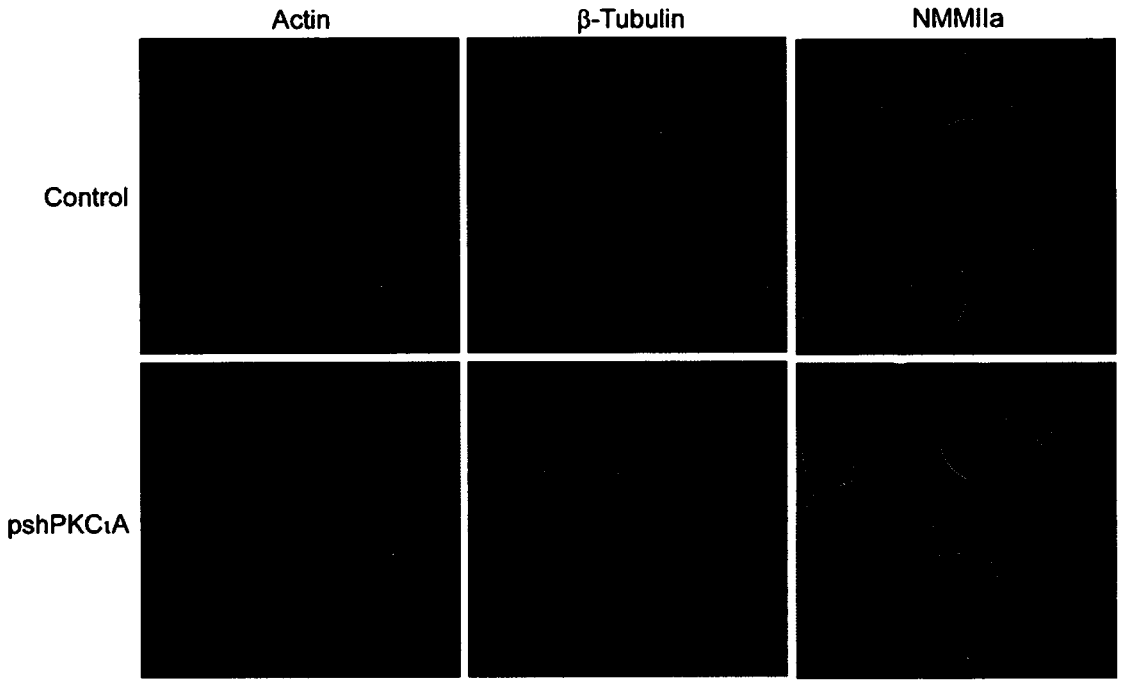

A
Control
pshPKCιA
B
Migration distance/minute (AU/min)
0
1
2
3
4
5
6
Control
pshPKCιA
C
Control
pshPKCιA
0 min
50 min
100 min
150 min
200 min
250 min
300 min

Figure 4. Effects of stable PKCι depletion on the cytoskeletal organization. U87MG cells expressing a shRNA targeting PKCι were examined by fluorescence for changes in three cytoskeletal components, actin, microtubules (MT) and non-muscle myosin type IIa (NMMIIa). Cells expressing a shorthairpin targeting GFP were used as controls. Actin was assessed using conjucated TRITC-phalloidin. Microtubules and NMMIIa were examined using immunofluorescence as described in the "Materials and Methods". Nuclei were stain using DAPI. Cells were fixed and visualized by confocal microscopy.

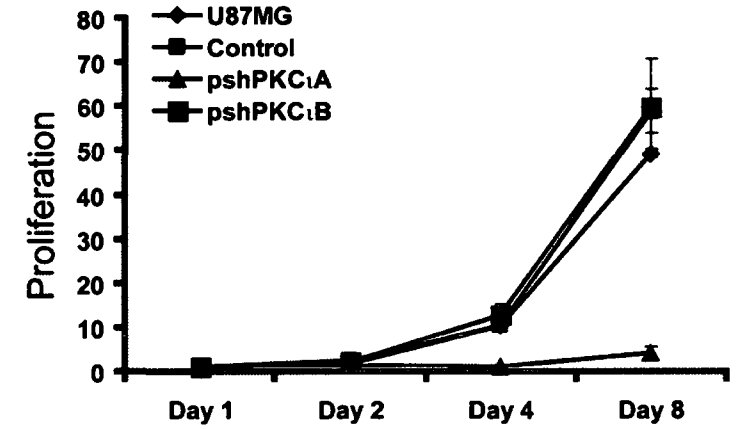

Actin
β-Tubulin
NMMIIa
Control
pshPKCιA

Stable depletion of PKCι in glioblastoma impairs cell growth: The effects of stable PKCι depletion on glioblastoma cell viability and proliferation were evaluated by trypan blue exclusion. Viable cell number was counted 1, 2, 4 and 8 days following plating control and PKCι depleted cells at equal densities. Stable depletion of PKCι in both U87MG and A172 cells significantly reduced growth rate compared to control cells (Figure 5A and B). This is consistent with results observed by targeting PKCι with transient siRNA depletion (Supplementary Figure 1A and B; Patel et al. 2008). The minimal depletion of PKCι in U87MG cells expressing the pshPKCιB plasmid showed no effect on cell proliferation possibly because it inefficiently suppressed PKCι expression (Figure 1B and 5A).

Impaired cell division in U87MG cells depleted of PKCι: Interestingly, the time-lapse movie analysis showed that the stable depletion of PKCι in U87MG cells impairs cell division. Control U87MG cells undergo a rapid cell rounding and divide into two separated cells in approximately 250 min (Figure 6A). PKCι depleted cells, while undergoing the characteristic rounding, have an inability to divide and remain rounded for an extended period of time (>865 min; Figure 6A). Cell numbers were compared at the beginning and end point of the time-lapse movie. In control U87MG cells a range of 11-13 mitotic events were counted in 20 h (Figure 6A). U87MG cells stably depleted of PKCι have a significantly reduced number of events, ranging from 0-1 in a 20 h time period (Figure 6B). This may be the cause of the reduced proliferation observed.

Figure 5. Stable depletion of PKCι inhibits glioblastoma cell proliferation. Control and PKCι depleted U87MG (A) and A172 (B) cells were plated at equal densities and live cell numbers using trypan blue exclusion were determined at Day 1, 2, 4 and 8 as an assessment of proliferation.

A


U87MG
Control
pshPKCιA
pshPKCιB
Proliferation
80
70
60
50
40
30
20
10
0
Day 1
Day 2
Day 4
Day 8

B

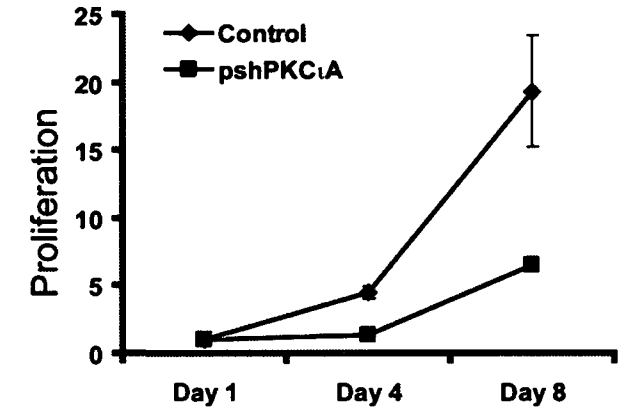
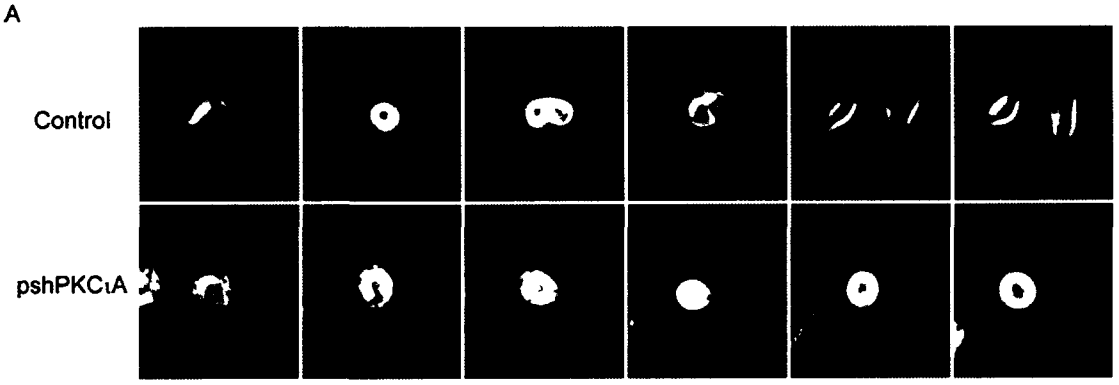
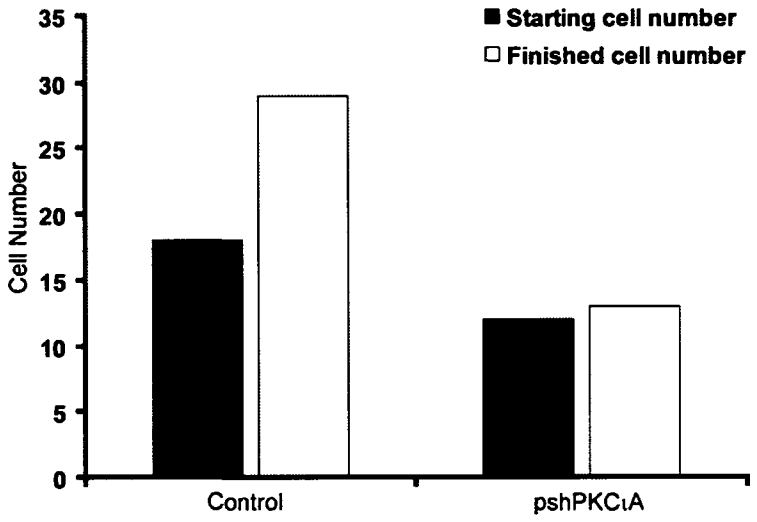

Control
pshPKCιA
Proliferation
25
20
15
10
5
0
Day 1
Day 4
Day 8

Figure 6. Effects of stable PKCι depletion on U87MG cell division. A. Representative images of a control and PKCι depleted U87MG cell undergoing cell division. B. Quantitation of the number of mitotic events observed during live cell image analysis. Data are from one of two independent live cell experiments.

A


Control
pshPKCιA

B


Starting cell number
Finished cell number
Cell Number
35
30
25
20
15
10
5
0
Control
pshPKCιA

Discussion

The invasive property of glioblastoma cells is a major factor contributing to the lethality of this disease. Unlike other tumours which generally invade short distances as groups of cells through barriers such as blood vessels and bone, glioblastoma cells often migrate as single cells down pre-existing paths such as myelinated fiber tracts (Tysnes and Mahesparan 2001). Recurrence often occurs immediately adjacent to the surgically resected boundaries due to the diffuse nature of these tumours; however, these tumour cells also have the ability to travel long distances within the brain along these tracts (Giese et al. 2003). The mechanisms underlying their ability to migrate are only beginning to be uncovered.

Here we have stably repressed PKCι expression in glioblastoma and studied its effects on cell motility and invasion. Consistent with our previous model using transient repression, stable repression of PKCι caused a significant inhibition of cell invasion as assessed using Transwell chambers. This analysis showed that PKCι plays a significant role in promoting glioblastoma invasion, and it mainly affects cell motility. This does not rule out the possibility that PKCι can increase the ability of glioblastoma cells to breakdown extracellular matrix, as it can regulate the expression, activity and secretion of proteases that serve this function in other tumour types (Xu and Deng 2006; Frederick et al. 2008). These results offer only a static assessment of cell motility, which is a highly organized dynamic process. Therefore, to gain more insight into the effect of PKCι loss on glioblastoma cell motility live cell imaging was used.

Live cell imaging is a powerful technique that allows for the study of cells migration in real-time and gives valuable insight into the morphological processes that take place in

order for cells to move. Cell migration involves a coordinated polarization, which reorganizes cell shape and intracellular components that drive cell movement (Jaffe and Hall 2005). A non-motile cell has a characteristic rounded morphology and the nucleus is centrally located within the cell. In contrast, a motile cell has a more elongated shape with broad leading edge lamellipodia and multiple filopodia extending from the leading edge. The trailing edge narrows with a long trailing tail. Control U87MG cells constitutively expressing a shRNA targeting GFP showed a significantly higher proportion of cells that have the morphological characteristics of a motile cell. However, cells expressing the shRNA targeting PKCι had a more non-motile morphology. When observed in real-time using live cell imaging control U87MG cells moved rapidly in the field of view. These cells undergo changes in morphology including a shift in the nucleus to the lagging edge, development of a leading edge filopodia and lamellipodia, and migration in the direction of lamellipodia. The movement observed was random and associated with rapid changes in direction. In contrast, stable PKCι depletion significantly reduced the proportion of cells that are able to develop a motile phenotype and migrate effectively. As a result they traveled shorter distances and had a reduced migration distance traveled per minute. These cells also show a significant impairment in the generation of leading edge lamellipodia. Instead they appear to generate miscoordinated filopodia extending and retracting from all sides of the cell. These images have shown that PKCι plays a central role in the coordination of glioblastoma cell motility and its loss is a detriment to this process.

There are several intracellular functions that have been described for the atypical PKCs that are related to its role in cell motility. The atypical PKCs are part of a

conserved polarity complex that has been shown to play a critical role in the establishment of the intracellular polarity required for cell migration (Etienne-Manneville and Hall 2001; Henrique and Schweisguth 2003; Nakayama et al. 2008). Proper localization and activation of this complex is an essential event for its function in cell motility. In astrocytes, upon stimulation to migrate by wounding, PKCζ is localized to the leading edge of the cell where it interacts with Cdc42 (Etienne-Manneville and Hall 2001; Nakayama et al. 2008). This is mediated by the interaction of the atypical PKC scaffolding partner, Par-6, directly with Cdc42. This interaction facilitates the activation of atypical PKC (Kanzaki et al. 2004). PI3K also localizes to the leading edge of migrating cells, where it can promote the activation of both Cdc42 and atypical PKC (Merlot and Firtel 2003; Weiner 2002).

The Rho GTPases, Cdc42 and Rac1 are highly active at the leading edge of a migrating cell and drive actin polymerization to form filopodia and lamellipodia, respectively (Hall 2005). These leading edge processes are essential for cell migration. It has previously been shown that Cdc42 can regulate the activity of Rac1 through the activation of atypical PKC (Nakayama et al. 2008). Atypical PKC can activates a specific guanine nucleotide exchange factor, Tiam1/2 which in turn activates Rac1. Therefore, the loss of PKCι expression may negatively impact the activation of Rac1, which could disrupt lamellipodia formation. This may play a role in the phenotype observed in the PKCι depleted U87MG cells, where they have an impaired ability to for a leading edge lamellipodia. Additionally, it has recently been shown that atypical PKC can regulate the turnover of integrin-mediated adhesions at the leading edge by influencing their endocytosis (Nishimura and Kaibuchi 2007). The breakdown of

adhesions at the leading edge is required for the forward progression of the cell. These results demonstrate the importance the atypical PKC activity at the leading edge of a migrating cell.

The activation of atypical PKC plays an essential in the polarization of intracellular organelles in preparation for cell movement. In a non-motile cell, the microtubule organizing center (MTOC) and Golgi apparatus (Golgi) are located adjacent to the nucleus. When the cell is stimulated to migrate, the MTOC and Golgi relocalize between the leading edge lamellipodia and the nucleus. This process of relocalization has been shown to be dependent on atypical PKC activity as well as its association with Cdc42 in both astrocytes and 3T3 cells (Etienne-Manneville and Hall 2001; Nakayama et al. 2008; Gomes et al. 2005; Lee et al. 2005). This is thought to facilitate the transport and localization of key mediators of cell migration to the leading edge. Cell migration is also highly dependent on the reorganization of the cellular cytoskeleton. The interaction of the atypical PKCs with Cdc42 links them to cytoskeletal dynamics. We have observed that the stable depletion of PKCι in U87MG cells leads to a greater abundance of actin stress fibres compared to control cells. This has also been observed for PKC λ (mouse ι) knockout mouse embryonic fibroblasts and in glioblastoma cells transiently depleted of PKCι (Soloff et al. 2004; Baldwin et al. 2008). The atypical PKCs have been shown to be involved in the regulation actin stress fiber stability. PKCλ and PKCζ when activated, interact with active Cdc42 (GTP bound) to promote stress fiber loss (Coghlan et al. 2000; Uberall et al. 1999). Consequently, the loss of stress fiber formation has been shown to increase the ability of cells to migrate (Sahai et al. 2001). Examination of other cytoskeletal components, the MTs and NMMIIa, showed no change in U87MG cells

stably depleted of PKCι, also consistent with the mouse knockout fibroblasts (Soloff et al. 2004). However, PKCι may play a role in MT regulation in glioblastoma cells as we have observed an alteration in MT organization with transient depletion of PKCι using RNAi (Supplement Figure 2). This difference may be a result of insufficient depletion in the stable cells compared to transients where we observe 60% and 80% reduction in protein respectively (Baldwin et al. 2006). Suggesting that there may be threshold amount of PKCι required for different process within U87MG cells or these cells adapt a PKCι-independent mechanism of regulating MT organization.

Additionally, live cell imaging revealed that the observed effect of PKCι loss on glioblastoma cell proliferation may be the result of impaired mitosis. A significant reduction in the number of mitotic events over a 20 h imaging period was observed in U87MG cell stably depleted of PKCι. These cells show a characteristic loss of adhesion and rounding, which is indicative of entry into mitosis (Pugacheva et al. 2006). However, a significant proportion of these cells do not divide, instead they retain a rounded morphology for an extended period of time. There is evidence that atypical PKCs may influence mitosis, with the finding that PKCζ localizes to the mitotic spindle in Chinese hamster ovary and epithelial cells (Lehrich and Forrest, Jr. 1994). As well, it has been suggested that during development, the atypical PKC/Par-6 complex may function to help specify a cellular division plane (Mishima et al. 2001). However, the role of PKCι in cell division is not known and requires further study to understand its function in this process.

Live cell imaging can therefore provide valuable information into the cellular dynamics of cell movement. Specifically, it can be used as a technique to examine the

importance of a particular protein to the dynamics of cell motility at the whole cell level. Further, it can use to evaluate the effects of inhibition of potential therapeutic targets that promote the motility and invasion of cancer cells. The use of live cell imaging here has provided direct evidence that PKCι is a critical mediator of the mechanics of glioblastoma cell motility, a key component of glioblastoma cell invasion. However, these observations were examined using a single short hairpin targeting PKCι and need to be confirmed using an additional short hairpin that effectively depletes PKCι or an alternate method to target and inhibit PKCι. The inhibition of PKCι may represent a promising therapeutic strategy for the treatment of glioblastoma.

Methods and Materials

Chemicals and antibodies: Custom RNA interference duplexes were synthesized by Dharmacon RNA Technologies Inc. (Lafayette, CO, USA). Phospho-PKCι T555 (rabbit polyclonal), total PKCι (mouse monoclonal) and total ERK (mouse monoclonal) antibodies were from BD Biosciences (Mississauga, ON, Canada). Anti-mouse Actin, mouse Beta-tubulin and rabbit NMMIIa antibodies were from Sigma-Aldrich Inc.

Cell lines: Human glioblastoma cell line U87MG was obtained from Dr. W. Cavenee (Ludwig Institute for Cancer Research, La Jolla, CA). A172 cells were from the American Type Culture Collection. Cells were cultured at 37°C and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine and 10% (v/v) of a 2:1 mixture of donor bovine serum and fetal bovine serum.

Construction of PKCι short hairpin expression vector: Short hairpin DNA target sequences were designed and ordered from Integrated DNA Technolgies (Coralville, IA, USA). Sense and antisense strands were annealed and subcloned into the pSUPER.retro.puro backbone according to the manufacturers specifications (OligoEngine, Seattle, WA, USA). Retroviruses containing pshGFP, pshPKCιA or pshPKCιB were made as described previously (Lorimer and Lavictoire 2000). Cells were grown in media containing puromycin (1 μg/mL) to select for transductants.

Western blot analysis: Western blotting was performed as described previously (Baldwin et al. 2006). After electrophoretic transfer from the gel, blots were stained with amido black to confirm that equal sample loading and transfer was achieved.

Cell counts: Live cell number was determined using a Vi-Cell XR cell viability analyzer (Beckman Coulter Canada Inc., Mississauga, ON, Canada).

Fluorescence microscopy: Cells were plated in 6-well plates containing coverslips in complete DMEM. Cells were washed with cold PBS and fixed in a 4% paraformaldehyde in PBS solution. Cells were incubated with beta-tubulin (mouse monoclonal, 1:500; Sigma clone2.1), rabbit NMMIIa (1:200; Sigma Inc.) for 1 h. Then washed for 3 x 10 min with PBS. Cells were then incubated in the dark with secondary antibody AF594 (goat anti-mouse; 2ug/mL) or AF488 (chicken anti-rabbit; 2ug/mL) respectively for 1 h. Conjucated-phalloidin was used to visualize actin fibres. Fluorescent labeling was observed using a Zeiss Observer.Z1 microscope (63X/1.40 oil DIC M27 objective) connected to a Zeiss LSM 510 Meta confocal unit. Alexa Fluor 555 was excited using the He-Ne 543 laser set at 50% power and channeled through an HFT 488/548 main dichroic filter, an NFT 545 secondary dichroic filter and a BP 560-615 IR. Images were captured using Zeiss' ZEN (version 4.5) software for the confocal microscope. AF 488 was excited with the Argon laser set at 20% power and channeled through an HFT 488/548 main dichroic filter and a BP 505-530 filter. DAPI was excited using the He-Ne 405 laser set at 20% power channeled through an HFT 405/488 main

dichroic filter and a BP 420-480 filter. Image stacks were collected with the software Pinhole set at 1 Airy unit and a slice interval of 0.41 um.

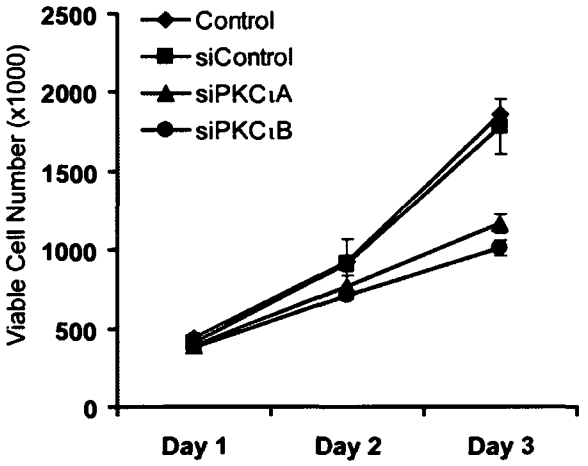
Cell motility and invasion assays: Chambers (BD Biocoat Matrigel invasion chambers, BD Biosciences, Mississauga, ON, Canada) were rehydrated and equilibrated for 2 h with 500 μL of serum free DMEM medium. After 2 h, the medium in the inserts was aspirated and inserts were placed into the wells containing complete DMEM (10% FBS:DBS). Chambers that were not coated with Matrigel (control inserts) were used to measure motility. Each chamber contains a membrane with 8 μm pores. U87MG stably expressing PKCι short hairpin were counted and resuspended in serum free DMEM medium at 1×10^5 cells/mL. Five hundred microlitres of cell suspension (50 000 cells) were added to each chamber. The chambers were incubated for 22 h at 37°C in a 5% CO_2 atmosphere. The media was then removed and the upper surface of the membrane was scrubbed ten times with a cotton swab. Cells on the lower surface of the scrubbed membranes were fixed in 10% methanol and stained with Diff-Kwik (Dade-Behring) according to the manufacturer's instructions. Three random fields were counted from three chambers under the light microscope at 40X magnification.

Live cell imaging: Control and PKCι depleted U87MG cells were plated into a live cell imaging plate in 2 mL of complete DMEM at a density of 10^3 cells to allow space for migration. Live cell imaging was done using an inverted microscope (Ziess Axiovert 200M) equipped with phase-contrast microscopy using a 10X objective. Images were acquired with a CCD camera (AxioCam HRm) driven by Zeiss Axiovision 4.5 software.

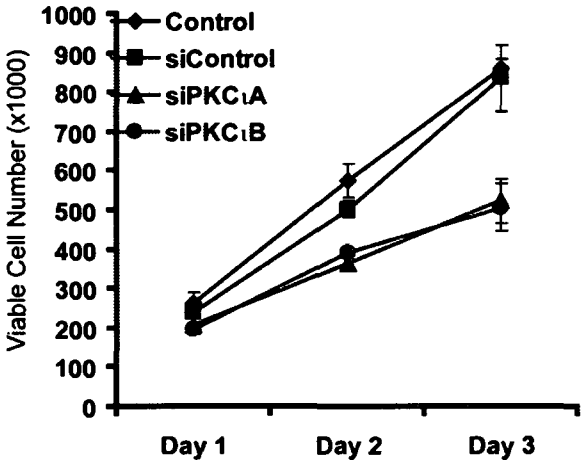
Phase contrast images of the cells were taken at 5 min intervals for 20 h and compiled to generate a time-lapse video. To quantify the migration distance per minute, 20 cells in total from two independent time-lapse video (10 cells from each video) of each cell line were analyzed using the Zeiss LSM image browser software. Cell nuclei were tracked to determine migration distance and divided by the travel time.

Supplementary figure 1. Effect of transient PKCι depletion on cell proliferation. U87MG (A) and A172 (B) cells were untransfected (Control), control RNA transfected (siControl) or transfected with one of two unrelated siRNAs targeting PKCι (siPKCιA or siPKCιB) and count at Day 1, 2 and 3 to assess proliferation.

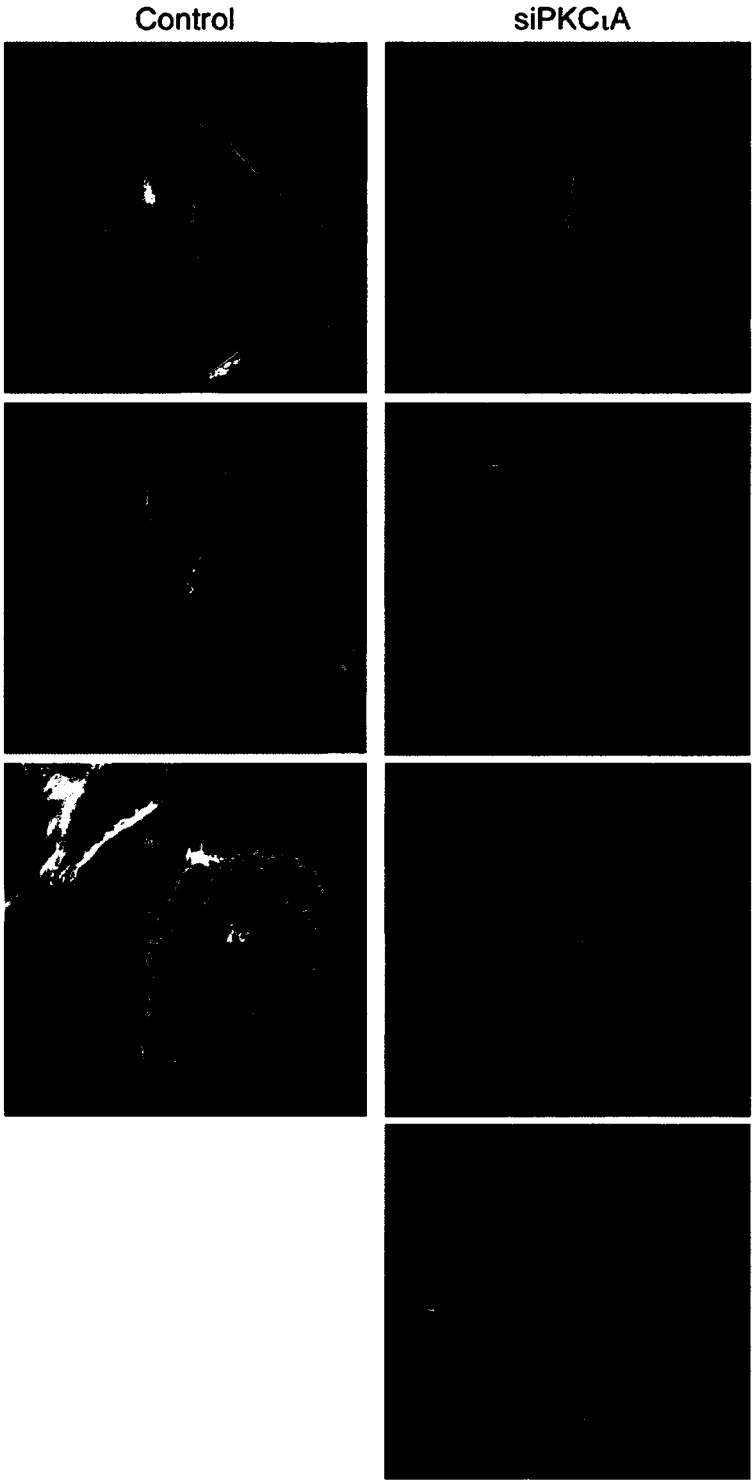
A


Control
siControl
siPKCιA
siPKCιB
Viable Cell Number (x1000)
2500
2000
1500
1000
500
0
Day 1
Day 2
Day 3

B


Control
siControl
siPKCιA
siPKCιB
Viable Cell Number (x1000)
1000
900
800
700
600
500
400
300
200
100
0
Day 1
Day 2
Day 3

Supplementary figure 2. Effect of transient PKCι depletion on MT organization in U87MG cells. U87MG were control RNA transfected (siControl) or transfected with an siRNA targeting PKCι (siPKCιA) for 48 h in six well plates containing glass coverslips. MT organization was assessed using immunofluorescence using beta-tubulin (mouse monoclonal antibody) by confocal microscopy. Nuclei were visualized by DAPI staining.


Control
siPKCιA

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5. Discussion

Hanahan and Weinberg (203) describe six essential alterations that are acquired by cancer cells and together impact malignant growth: 1) Self-sufficiency in growth signals; 2) Insensitivity to antigrowth signals; 3) Evasion of apoptosis; 4) Limitless replicative potential; 5) Sustained angiogenesis, and 6) Tissue invasion and metastasis. Discovering key mediators of these characteristics within cancer cells will help in identifying novel therapeutic strategies. The aim of this thesis was to assess the role of PKCι in GBM cells with a view of determining its potential as a therapeutic target. Two common genetic alterations that occur in GBM are mutation of PTEN and the amplification and mutation of the gene for the EGFR (16). Both of these mutations have been linked to increased proliferation, chemoresistance, and invasion of cancer cells as a result of aberrant activation of the PI3K pathway, in which PKCι is activated downstream. The recent identification of PKCι as a critical oncogene in the development of NSCLC and ovarian cancer has demonstrated its impact in tumour development (87;134;179). We hypothesized that in GBM, PKCι is a key downstream mediator of the PI3K pathway and has a significant impact on promoting the proliferation/growth, chemoresistance and invasiveness of GBM cells. The first step in determining the role of PKCι in GBM cells was to examine its expression and activation.

5.1 PKCι is activated in GBM

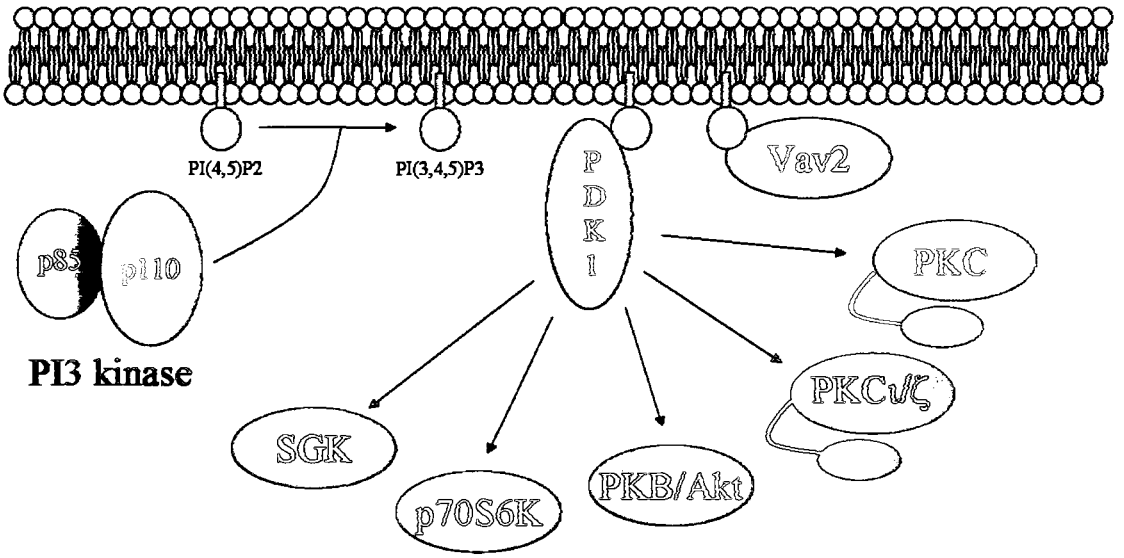
Assessment of atypical PKC mRNA expression in U87MG cells showed that only PKCι is expressed, with no detectable PKCζ. PKCι protein expression is also elevated in GBM cells compared to normal human astrocytes. This is consistent with the recent

observation that PKCι may also be overexpressed in brain tumour tissue (180). The reason for this overexpression is not known for GBM; however, recent analysis of ovarian and NSCLC tumours has revealed that the region of the genome that PKCι lies on (3q26) is frequently amplified in both these cancers (87;88;134;179). Therefore, subsequent analysis of GBM tumours may identify gene amplifications of PKCι. Alternatively, the increased expression of PKCι in GBM cells may be the result of increased transcriptional activity. In leukemia, expression of Bcr-Abl enhances the activation of ERK, which in turn enhances the activation of the Elk-1 transcription factor and directly promotes PKCι expression (63). In GBM cells, activation of EGFR by stimulation or mutation (EGFRvIII expression) causes a constitutive activation of ERK through PI3K (204). Thus, enhanced ERK activity in GBM cells may contribute to the elevated PKCι expression observed. GBM cells exhibit amplified PI3K signaling due to genetic alterations of key regulators of this pathway. Pharmacological inhibition using a small molecule inhibitor of PI3K, LY294002, effectively inhibited the phosphorylation of PKCι at threonine (T) 555 in GBM cells. Additionally, the use of an atypical PKC-specific pseudosubstrate peptide also effectively inhibited PKCι phosphorylation at this site. This phosphorylation site has been shown to be critical in stabilizing the active conformation of the catalytic subunit (81). It is postulated that when unphosphorylated, the turn motif spatially interferes with the active site of the enzyme and phosphorylation pushes a segment of this domain away from the active site allowing for full activation and substrate interaction (81). Our results show that PKCι is activated in GBM cells and may contribute to their malignant phenotype.

While PKCι is a downstream target of the PI3K pathway, it should also be acknowledged that PI3K can initiate the activation of multiple downstream effectors that can influence a number of cellular processes including cell survival, protein synthesis, cell cycle, membrane trafficking, and actin cytoskeletal organization. However, much of the literature focuses on a single downstream effector of this pathway, PKB/Akt, as the main mediator of the downstream signaling. This has potentially caused significant misinterpretation and misattributed functions to this single downstream effector.

The PI3K enzymes are responsible for the production of 3′ phosphorylated phosphatidylinositol (PI) lipids (73). There are three classes of PI3K enzymes and each generates a different form of phosphorylated PI lipid. Class I PI3K is responsible for the generation of the well characterized PIP_3, while class II and III both generate $PI(3)P_1$. Class II PI3K may also be responsible for the production $PI(3,4)P_2$ from $PI(4)P_1$. 3′-phosphorylated PI bind an assortment of proteins containing PH, PX and FYVE domains that are recruited to the membrane and are involved in mediating downstream signaling from PI3K (205). PIP_3 is responsible for the recruitment of PDK1, which is a primary effector of Class I PI3K signaling and is a starting point of downstream signal amplification. PDK1 not only phosphorylates and activates PKB/Akt, but has also been shown to play a central role in activating many AGC (cAMP-dependent, cGMP-dependent and PKC) kinase family members including p70S6 kinase (206), p90RSK (207), SGK (208) and the atypical PKCs (Figure 1, 99;133). Activation of these substrates causes distinct effects on a number of intracellular processes including cell survival, proliferation, cell cycle progression, and protein synthesis (56). Adding further diversity to the downstream effectors of PI3K is the fact that GEFs and GAPs of

Figure 1: *Diversity of PI3K signaling.* The PI3K pathway affects numerous biological processes within cells. This occurs through its ability to promote the generation of the lipid second messenger PIP_3. This activates several downstream effectors that influence cell growth and survival, cell migration, transcription and metabolism (99, 133, 206, 207, 208).


PI(4,5)P2
PI(3,4,5)P3
p85
p110
PI3 kinase
PDK1
Vav2
PKC
PKCζ
SGK
p70S6K
PKB/Akt

the Ras and Rho families of GTP-binding proteins contain PH domains and are PI3K dependent (209). In particular, the Vav GEF family member Vav2, which is Rac specific, has been shown to be activated in response to EGF stimulation. This activity is PI3K-dependent as pharmacological inhibition of PI3K activity inhibited Vav2 exchange activity and effected actin cytoskeletal dynamics (210). Additionally, proteins containing PX and FYVE domains interact primarily with $PI(3)P_1$. Several of these proteins have been implicated in the regulation of membrane trafficking events which can also impact the activation of intracellular signaling pathways (211).

While many of the downstream effectors of the PI3K pathway have distinct functions, there is also evidence for overlapping roles within cells. This is particularly true for PKCι and PKB/Akt. For example, both enzymes can phosphorylate GSK3β and the anti-apoptotic protein Bad (99;131). We have shown that PKCι can regulate RhoB expression in GBM cells (**Chapter 3**). RhoB expression is also affected by PKB/Akt activity in other cell types (212). It is conceivable that different tissues may rely more on one enzyme than the other in the regulation of these downstream effectors. PKCι has been shown to be expressed at higher levels in brain and lung compared to other tissues (89). Therefore, in brain tissue and potentially brain tumours, cells may preferentially signal through PKCι as the dominant downstream PI3K effector. In addition, the tissue specific expression of adaptor or anchoring proteins may play a role in a cell's dependence on one enzyme over another in a particular cell type. Interaction of kinases with these regulatory proteins can alter not only their activity, but also subcellular localization and downstream substrate specificity (213;214). This may also explain how the regulation of Bad or RhoB can be influenced by either PKCι or PKB/Akt in different tissues.

This shows the complexity and diversity of the PI3K signalling pathway and, therefore, it is necessary to keep this in mind when examining the intracellular consequences of its inhibition. Understanding the specific contribution of each of these downstream effectors to the growth and survival of tumour cells will aid in identifying novel and effective therapeutic strategies. Here we specifically examined the role of PKCι to the malignant phenotype of GBM cells.

5.2 Contribution of PKCι to the malignant phenotype of GBM cells

5.2.1 Cell proliferation

The increased expression and activation of PKCι in GBM cells suggests that it may have a significant impact on cell proliferation, as its downstream signaling has been linked to this process (60-62). PKCι has been shown to be involved in the activation of ERK, which affects several physiological functions including proliferation and cell survival (62;215). Our results demonstrate that PKCι is a key contributor to the proliferation of GBM cells, as its depletion both transiently and stably caused a significant reduction in the proliferation of these cells *in vitro*. Consistently, we also observed an inhibition of ERK activation with PKCι depletion, which may contribute to this reduced proliferation. Live cell imaging has also revealed a potential role for PKCι in the progression of cells through mitosis. U87MG cells stably depleted of PKCι show a reduced number of cell divisions (**Chapter 4**). Morphologically it appears that cells enter mitosis, by rapidly rounding however, once rounded they fail to successfully divide into two cells. This has identified a novel function of PKCι but requires further analysis to validate and identify its exact role in mitosis. Additionally, preliminary observations

suggest the PKCι effects the expression of key cell cycle regulators. Depletion of PKCι causes increased expression of the cyclin dependent kinase inhibitor, $p27^{Kip1}$ and a decrease in the expression of cyclin D1 (*unpublished observations*). Both of these proteins are key regulators of cell cycle progression. $p27^{Kip1}$ binds and inhibits cyclin A/E/CDK2 complexes thereby inhibiting entry of cells into the G1 phase of the cell cycle (216). Overexpression of $p27^{Kip1}$ in GBM cell lines has been shown to decrease their proliferation both *in vitro* and tumour growth *in vivo* (217). In contrast, cyclin D1 binding to its CDK partners, CDK4 and 6, is involved in promoting the progression of the cell cycle from G1 to S phase (218). Cyclin D1 has been identified as an oncogene in several cancer types and has been shown to be overexpressed in malignant brain tumours (219). It has also been shown to contribute to increased proliferation of GBM cells (220). These results demonstrate that PKCι is a key upstream mediator of GBM cell proliferation and potentially plays a role at multiple levels in regulating this process.

5.2.2 Evasion of apoptosis

The atypical PKCs have been shown to contribute to the protection of cells from apoptosis-inducing agents in several types of cancers including leukemia and prostate (121;177;178;184). Our results demonstrate that depletion of PKCι using RNA interference in GBM cells causes a partial enhancement of cisplatin cytotoxicity in both U87MG and A172 GBM cell lines. In leukemia, PKCι is specifically involved in the resistance to apoptosis through the activation of NFκB (121); however, this is not the mechanism in GBM cells. To identify potential mechanisms by which PKCι could confer resistance to cisplatin, microarray analysis of gene expression was performed on

U87MG cells following PKCι depletion. This analysis showed that PKCι is pleotropic in its cellular functions, affecting the expression of a large number of genes both positively and negatively. It also revealed that PKCι represses more genes than it enhances in U87MG cells. Further analysis would be required to understand the mechanisms by which PKCι regulates these genes.

The repression of GMFβ identified a novel mechanism by which PKCι enhances the resistance of GBM cells to cisplatin-mediated cell death (**Chapter 2**). The interest in this gene stemmed from its role in regulating the stress-activated protein kinase, p38MAPK, which when activated promotes death in many cancer cell types (221). This was consistent with a previous study showing that Par-4 knockout MEFs have repressed p38MAPK activity compared to their wildtype counterpart (129). Additionally, p38 MAPK activation has been shown to be a specific result of cisplatin-based therapy, and repression of p38 MAPK activity confers resistance to this treatment (222). Exactly how GMFβ serves this function is poorly understood; however, it has been shown to interact directly with p38MAPK, which may positively influence its activity (223). The regulation of p38MAPK activity is also influenced by specific phosphatases that are downstream targets of intracellular signaling pathways. ERK can regulate, through phosphorylation, the activity of a p38MAPK-specific phosphatase, MAPK phosphatase 7 (224;225). PKCι depletion in glioblastoma cells also resulted in an inhibition of basal and cisplatin-induced ERK activation. Therefore, this may also affect p38MAPK activity and contribute to the reduced proliferation and enhanced cell death observed. The repression of p38MAPK activation has been observed in several cancer cell types, including ovarian, lung and breast cancers and further GBM with our analysis (226-228).

This has been shown to play a role in the progression of cancer and enhances cell survival under stress conditions such as cytotoxic insult, contributing to their inherent resistance (227;229;230). Oncogenic activation in cancer results in a shift in the cell survival-cell death balance in favour of cell survival and therefore drives tumour progression. This has been demonstrated with several oncogenes, such as EGFR, Ras and BCR-ABL which are all implicated in tumour development. The specific inhibition of the activity of these oncogenes in cell culture systems cause a significant downregulation of growth signals and a subsequent increase in p38MAPK activation resulting in cell death (231). Therefore, PKCι represents one vulnerability point that may be exploited to shift the cell survival-cell death balance toward cell death. However, the cisplatin sensitivity observed in response to cisplatin was only partial, suggesting that other signaling pathways participate in the resistance of these cells. Thus, it may be necessary to target multiple cell survival effectors to ensure therapeutic effectiveness due to the signaling overlap associated with these pathways.

5.2.3 Motility and invasion

The depletion of PKCι using RNA interference significantly decreased the motility and invasiveness of GBM cells. This also caused an enhacement of actin stress fibre formation within U87MG cells, consistent with what is observed in PKCλ knockout MEFs (117). PKCλ and Cdc42 have previously been shown to interact and regulate actin dynamics (101). Cdc42 bound to GTP (activated) interacts with activated PKCλ and functions to properly localize it, and this contributes to actin stress fibre disassembly (101). Our results demonstrate the PKCι suppresses the expression of RhoB, which

contributes to increased motility and invasion of GBM cells (**Chapter 3**). RhoB, unlike its family members RhoA and C, seems to suppress cancer progression. RhoB has been shown to be a negative regulator and tumour formation in several cancers, including lung, ovarian and pancreatic cancer (212;232-236). Interestingly in gliomas, RhoB expression is not only repressed but its repression inversely correlates with tumour grade (237). The repression of RhoB in ovarian cancer cells was shown to be functionally permissive for tumour cell growth *in vivo*, and adenoviral delivery of RhoB to established intraperitoneal tumours significiantly repressed tumour cell growth and cured approximately 50% of mice (236). These data suggest that the ability of a therapeutic agent to increase the expression of RhoB may offer therapeutic benefit to patients harbouring RhoB repressed tumours. RhoB expression has been shown to be negatively regulated by enhanced signaling through the PI3K pathway (212;238). Our data show that RhoB and PKCι have a mutually antagonistic relationship in GBM cells, where PKCι negatively regulates RhoB expression and RhoB in turn negatively impacts PKCι activity. Constitutive expression of RhoB inhibited the activation of both PKCι and PKB/Akt. This suggests that RhoB regulation of these enzymes occurs upstream. Additionally, RhoB overexpression has been previously shown to induce apoptosis and inhibit transformed cell growth both *in vitro* and *in vivo* (235). The exact mechanism RhoB plays in suppressing tumourgenesis in not clear, however it has been linked to the trafficking of key oncogenic mediators, specifically those that reside in the PI3K pathway. RhoB has been shown to regulate the trafficking of activated EGFR from endosomes to pre-lysosomes and this may lead to decreased cell surface receptor expression and activity (232;239). The PI3K effector PDK1 is also recruited to endosomes by RhoB

sequestering it away from key upstream activating signals (240). This could lead to decreased signaling downstream resulting in the observed decrease in PKCι and PKB/Akt activation. Therapeutic agents that enhance RhoB expression could have a substantial impact in tumours that exhibit elevated PI3K signaling, such as GBM. The expression of RhoB has also been shown to be critical in mediating cell death in transformed cells as RhoB knockout MEFs show an increased resistance to DNA damaging agents (241). Therefore, repression of RhoB in cancer cells may impact multiple levels of the tumourigenic capacity of cells.

The repression of RhoB expression is one mechanism by which PKCι influences cell motility and invasion of GBM cells. The use of live cell imaging has revealed that PKCι has a considerable influence on the dynamics of their movement (**Chapter 4**). While PKCζ has been shown to play a critical role in the polarization of the MTOC, nucleus and golgi in astrocytes, PKCι was not shown to contribute to this organization (160). Examination of the role of PKCι in glioblastoma cell movement identified that it functions in the coordination of membrane organization, and its loss significantly interferes with the ability of U87MG cells to migrate. Stable depletion of PKCι in U87MG cells caused impairment in the development of a leading edge lamellipodia. The proper development of this process is essential for the cell movement. Lamellipodia formation has been shown to be dependent on the activity of the RhoGTPase, Rac1 (171). The atypical PKCs have been implicated in regulating Rac1 activity, which is one mechanism by which PKCι may be regulating this process (173). These observations are the first evidence suggesting that PKCι is involved in this process.

Our findings show that PKCι contributes to the malignant phenotype of GBM cells. PKCι plays a role in at least three of the characteristic hallmarks of cancer, affecting cell growth, sensitivity to cell death, and invasion. These findings therefore justify further assessment of PKCι as a potential therapeutic target for GBM.

5.3 *In vivo* testing of PKCι inhibition

The studies described here have demonstrated that PKCι may have potential therapeutic value for the treatment of GBM; however, these experiments have been performed in an *in vitro* setting. An extension of this work will be to evaluate the inhibition of PKCι in an *in vivo* model. The loss of PKCι in mice is embryonic lethal; however, the effects of its loss in adult tissues has not been examined (117). Therefore, it would be useful to examine the effects of inhibition of PKCι in adult tissues of mice to identify if there are adverse effects associated with its loss. Our results show that specific depletion of PKCι can be achieved in cells by transient or stable RNA interference. An *in vivo* RNAi approach may be useful in examining the loss of PKCι expression in adult tissues. An inducible shRNA strategy has been successfully used in the development of a reversible *in vivo* model of diabetes (242). Mice were engineered to ubiquitously express a doxycycline-inducible shRNA that targets the insulin receptor. Treatment of mice with doxycycline induced expression of the shRNA and effectively depleted insulin receptor expression in the tissues examined in a dose dependent manner. This caused a concomitant increase in blood glucose levels and was completely reversible by the elimination of doxycycline treatment. A system similar to this would be useful in indentifying potential toxicities associated with systemic PKCι inhibition in adult mice.

Further, it allows for the controlled expression and the effects of both short and long term inhibition of PKCι *in vivo* could be evaluated.

Several *in vivo* model systems have been used to study GBM, including subcutaneous and orthotopic xenografts of established human GBM cell lines in mice which provide a simple and effective method for evaluating tumour growth and allow for the assessment of the therapeutic effectiveness of a target. It is not known whether targeting PKCι would cause a similar effect in an *in vivo* model. Both subcutaneous and intracranial injections of GBM cells have been used to evaluate potential therapeutics and targets (201;243;244). Interestingly, a recent paper examined the consequences of matrix metalloproteinase-2 (MMP-2) inhibition on intracranial xenograft tumour growth using viral delivery of an siRNA to downregulate the expression MMP-2 in these tumours (243). The expression of MMP-2 was successfully decreased in these tumours using this method. We have effectively targeted PKCι using an siRNA approach in our studies, therefore we could exploit this method to effectively inhibit PKCι in intracranial xenografts which would provide valuable insight into its role in tumour cell growth *in vivo*. Further, several genetically engineered mouse models have been created that produce GBM tumours that resemble those found in human patients. For example, a combination of EGFRvIII overexpression and INK4a/Arf knockout specifically in astrocytes or neural progenitor cells induces glioma-like lesions in mice (47). Similarly, activated Ras expressed specifically in astrocytes or neural progenitor cells of mice lacking INK4a/Arf resulted in the formation of GBM tumours (245). These models have shown the requirement for at least two cooperative genetic modifications in the development of GBM, which is thought to be required for human GBM tumour

development as well. These models also display similar genetic characteristics to those arising in human GBM, which may allow for a more accurate pre-clinical assessment of potential therapeutic targets. The use of genetically engineered mice has been successful for the evaluation of the alkylating agent temozolomide for the treatment of PDGF-induced GBM (246). These models may subsequently be utilized in additional evaluation of targeting PKCι *in vivo*.

5.4 PKCι targeted drug development

The identification of PKCι as a critical oncogene in the development of lung and ovarian cancer has driven the identification of strategies to target this enzyme. Alan Field's group recently identified two related compounds, aurothioglucose (ATG) and aurothiomalate (ATM), as functional inhibitors of PKCι (187). Using a high throughput screen of compounds, the ability to block the PB1-PB1 domain interaction between PKCι and Par-6 was used as a readout. Functionally, interference with this association inhibits PKCι-induced Rac1 activation, which has been shown to be important in both lung and colon tumourgenesis (60;62;164). Treatment of A549 cells with ATG inhibited Rac1 activation and cell growth both *in vitro* and in subcutaneous lung tumour cell xenografts. Additionally, these compounds are already in use for the treatment of rheumatoid arthritis, therefore their toxicity profiles in humans are known. ATM is currently in a Phase I clinical trial for use in the treatment NSCLC.

Recently, a group of small molecule inhibitors specific for PKCι have been generated (Dillon, C. *et al. unpublished observation*). Within this group there are both ATP-competitive and allosteric compounds. These compounds exhibit potencies of inhibiting

purified PKCι enzyme that range from 16-83 nM. Assessment of their effectiveness in cells has shown that these inhibitors reduced cell viability and motility of A549 lung cancer cells at concentrations ranging from 3-24 μM. However, the effects on the inhibition of PKCι within these cells is not clear. We have shown that PKCι is overexpressed and active in GBM cells through the examination of phosphorylated T555 (**Chapter 3**). Therefore, these small molecule inhibitors of PKCι may also have therapeutic benefit in GBM cells and detection of phosphorylated T555 may give some indication of their effectiveness of inhibiting PKCι. We are characterizing the effectiveness of these inhibitors in GBM cells, in collaboration with Dr. Jon Roffey (Cancer Research Technologies, UK).

In addition to developing selective inhibitors of PKCι, it is also necessary to identify specific biological markers that can be used to evaluate their effectiveness. These are not only useful for the pre-clinical assessment of novel therapeutics but also can be translated into the clinical evaluation of successful candidate therapies (247). Studies examining PKCι in cancer cells have identified several potential biological markers that could be used as an indication of PKCι inhibition, which include Rac1 activation, Bad phosphorylation, and calpain protease activation (131;185;187). Our studies in GBM cells have identified PKCι T555 phosphorylation, GMFβ expression, and RhoB expression as potential and additional biomarkers that may be used in the assessment of PKCι activity. Additionally, our microarray analysis may contain several more targets that may be useful biomarkers for the evaluation of PKCι targeting agents, although these need to be characterized further to ensure their validity and reproducibility as markers.

5.5 Conclusions

The data presented here have improved our understanding of the role of PKCι in GBM cells. They show that PKCι is involved in GBM cell growth, resistance to cisplatin, motility, and invasion. This work has demonstrated that inhibiting PKCι may be a useful therapeutic strategy in GBM, either alone of in combination with other strategies, such as chemotherapy or radiation, to improve the poor patient outcomes associated with this disease.

6. References

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7. Appendix A: Activation of p38MAPK contributes to expanded polyglutamine-induced cytotoxicity

Activation of p38MAPK Contributes to Expanded Polyglutamine-Induced Cytotoxicity

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Abstract

Background: The signaling pathways that may modulate the pathogenesis of diseases induced by expanded polyglutamine proteins are not well understood.

Methodologies/Principal Findings: Herein we demonstrate that expanded polyglutamine protein cytotoxicity is mediated primarily through activation of p38MAPK and that the atypical PKC iota (PKCι) enzyme antagonizes polyglutamine-induced cell death through induction of the ERK signaling pathway. We show that pharmacological blockade of p38MAPK rescues cells from polyglutamine-induced cell death whereas inhibition of ERK recapitulates the sensitivity observed in cells depleted of PKCι by RNA interference. We provide evidence that two unrelated proteins with expanded polyglutamine repeats induce p38MAPK in cultured cells, and demonstrate induction of p38MAPK in an *in vivo* model of neurodegeneration (spinocerebellar ataxia 1, or SCA-1).

Conclusions/Significance: Taken together, our data implicate activated p38MAPK in disease progression and suggest that its inhibition may represent a rational strategy for therapeutic intervention in the polyglutamine disorders.

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Introduction

The polyglutamine diseases encompass at least 9 different disorders including Huntington's disease (HD) and five spinocerebellar ataxias (SCA-1, SCA-2, SCA-3, SCA-6 and SCA-7 (reviewed in [1]). These are dominantly inherited diseases typically detected in the third or fourth decade of life. No effective therapeutic interventions are currently available, and the polyglutamine diseases are generally fatal. Polyglutamine disorders arise from expansion of a CAG repeat within the coding region of genes such that the length of the encoded polyglutamine stretch exceeds a critical threshold. At the ultrastructural level, disease progression features heat shock protein (HSP)-containing nuclear ubiquitinated inclusions [2] that have accumulated an assortment of cellular host components in association with the polyglutamine-containing protein [3]. There is evidence from experiments performed in cultured mammalian cells and animal models of disease that polyglutamine expanded proteins adversely affect basic biological processes (reviewed in [4]). Their expression has been associated with impaired proteolysis [5], loss of transcriptional control mechanisms [6] and with altered regulation of cell death/survival pathways (reviewed in [7]).

The mitogen-activated protein kinases (MAPK) are involved in the integration and processing of multiple extracellular signals and their induction triggers diverse biological responses (reviewed in [8,9]). While the activation of the extracellular regulated kinase 1/2 (hereafter referred to as ERK) by mitogenic and proliferative stimuli is coupled to cell survival [10], stress inducible kinases JNK and p38MAPK respond to environmental stress and their sustained activation transduces signals leading to cell death (reviewed in [11]). Protein kinase C (PKCs) family members have been positioned upstream of ERK and are potent modulators of its activation (reviewed in [12]). With the current exception of the stress-inducible kinase JNK whose excessive activation has been well documented in neurodegenerative diseases [13] and reviewed in [14], the mechanistic relationship between the stress inducible host signaling pathways and expanded polyglutamine-induced toxicity remain controversial. It has been shown, for example, that the mutant huntingtin (Htt) protein causes aberrant activation of epidermal growth factor receptor (EGFR) signaling [15], a finding which has been contradicted by more recent reports in which EGFR signaling was disrupted by expression of the expanded polyglutamine protein [16,17]. In a *Drosophila* model of polyglutamine toxicity, the mutant Htt protein has been shown to disrupt EGFR signaling through interference with the ERK cascade [18] while in a cell culture model it has been shown to activate the pro-survival pathway mediated through ERK [19]. All these anomalies are consistent with gain of function effects of expanded polyglutamine proteins. There is ample evidence from experimental systems that a simple polyglutamine tract can be toxic

without the context of its natural surrounding protein sequence [20,21] but possible loss of function effects in polyglutamine proteins must also be considered. The normal huntingtin protein, for example, has been shown to increase transcription of brain-derived neurotrophic factor (BDNF), which is required for survival of striatal neurons [22,23]. Loss of this activity in the mutant protein may therefore contribute to neuronal loss in diseased individuals. Insulin-like growth factor I also has neuroprotective activity in the context of polyglutamine-induced cytotoxicity [24,25], and like BDNF activates the survival pathway mediated through the phosphoinositide 3-kinase (PI3-K) [26–28]. Kinases activated downstream in this pathway include PKB/Akt and the atypical protein kinase C iota (PKCι) [29,30,31–34]. The toxicities of huntingtin and ataxin-1 gene products are modulated by their phosphorylation states [35,36], but while the role of PKB/Akt activity has been studied in this context nothing is known of the role of PKCι.

As a starting point the current study sought to address the role of MAPK signaling pathways in polyglutamine disorders including Huntington's disease and SCA-1. Our findings suggest that expanded polyglutamine proteins mediate adverse effects through activation of p38MAPK signaling and that this cytotoxicity is antagonized by PKCι, which enhances protective signaling through the ERK pathway. We show that pharmacological inhibition of p38MAPK rescues cells from polyglutamine-induced cell death whereas inhibition of ERK signaling or depletion of PKCι by RNA interference enhances cytotoxicity.

Methods

Reagents and antibodies

Custom RNA interference duplexes were synthesized by Dharmacon RNA Technologies Inc. (Lafayette, CO, USA). A control duplex having the following sense RNA sequence AUUCUAUCACUAGCGUGACUU (non-specific control duplex) was purchased from Dharmacon Research, Inc and used as a control. RNA duplex concentrations were determined by measuring absorbance at 260 nm and calculating concentrations using extinction coefficients provided by the manufacturer. Propidium iodide and MTT reagents were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). P38MAPK inhibitors, SKF86002 and SB202190 were purchased from Calbiochem (San Diego, CA, USA) and Biosource (Camarillo, CA, USA) respectively. The MEK inhibitor U0126 was from Promega (Madison, WI, USA). The goat polyclonal antibodies nPKCæ (used to detect PKCι) and ataxin-1 were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). The mouse monoclonal phospho-p38MAPK and phospho-ERK 1/2 antibodies and the rabbit polyclonal p38MAPK antibody were from Cell Signaling Technology (Beverly, MA, USA). Pan ERK monoclonal antibody was from Transduction Laboratories (Lexington, KY, USA). GFP, Htt-25 and Htt-103 were detected with a mouse monoclonal AFP antibody purchased from Quantum Biotechnologies Inc.(Montréal, Québec, Canada). Phospho-ATF2 and total ATF2 levels were detected with rabbit polyclonal antibodies purchased from Cell Signaling Technology (Beverly, MA, USA). The mouse monoclonal actin antibody was purchased from Sigma-Aldrich Canada (Oakville, ON).

Expression constructs and transgenic mice

The pEGFP-N1 expression construct which served as a control in transient transfection experiments was purchased from Clontech (Palo Alto, California, USA). The Htt-25 and Htt-103 expression constructs (gifts from Dr. Ron Kopito) contain a synthetic insert encoding exon 1 of human Huntingtin containing a polyglutamine tract of either 25Q or 103Q fused to the yellow fluorescent reporter protein (YFP). The plasmids encoding the full length human ataxin-1 proteins with a polyglutamine tract of 30Q or 83Q were a gift from Dr. Huda Zoghbi. The origin of the B05 transgenic line carrying a mutant Ataxin-1 allele with 82 CAG repeats and the A02 line with a CAG repeat of 30 codons was described in a paper from the laboratory of Dr. Harry Orr [37], from whom these lines were obtained.

Cell culture and transfections

The human U87MG cell line (a gift from Dr. W. Cavenee, Ludwig Institute for Cancer Research, La Jolla, CA) was maintained at 37°C and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine and 10% (v/v) of a 2:1 mixture of donor bovine serum and fetal bovine serum. For RNA interference experiments, cells were transfected using Oligofectamine (Invitrogen Canada, Inc., Burlington, ON) as per the supplier's protocol. Final concentrations of RNA in the transfections were 5.3 nM for siPKCιA and 20 nM for siPKCιB. Control RNA concentrations were matched to the specific siRNA duplex used in the experiment. For transient transfections, cells were plated in either 96- or 6 well dishes 24 hours prior to transfections. Subsequently, they were transfected using GeneJuice Transfection Reagent (Novagen, Madison, WI, USA) as per the supplier's protocol. 0.5 μg of plasmid DNA was used in each well of a 96 well dish. A total amount of 3 μg of plasmid DNA was used in each well of a 6 well dish. For p38MAPK inhibition experiments using SKF86002 and SB202190, cells in 96 well plates were transfected with RNA duplexes. 24 hours post-transfection, cells were pre-treated for 2 h with 20 μM of the respective inhibitor. ERK inhibition experiments were performed in a similar manner using the MEK inhibitor U0126 at a final concentration of 20 μM. Following this incubation period cells were transiently transfected with various expression constructs.

Survival assays

Survival assays were performed by MTT, trypan blue exclusion and flow cytometry. For MTT assays, cells in 96 well microtitre plates were transfected with RNA duplexes as described above. 6 h post-transfection, they were transiently transfected with the GFP control vector, Htt-25, Htt-103, Atx-30 or Atx-83 as indicated. 24 h post-transfection of the plasmid DNA cell survival was assessed using the MTT (3-(4,5-cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay as described previously [38]. Background values were determined by carrying out the assay in wells containing media without cells. Toxicity was measured by trypan blue exclusion in pooled fractions consisting of attached and detached cells. For flow cytometry experiments, adherent and non-adherent cells were harvested and fixed with 70% (v/v) ethanol in PBS. Cell nuclei were stained with propidium iodide. DNA content was analyzed by flow cytometry using a BD LSR flow cytometer (Becton Dickinson, San Jose, CA). Data was acquired using Cell Quest software (Becton Dickinson, San Jose, CA) and were analyzed using Mod Fit LT software (Verity Software House, Inc., Sopsham, ME).

Western blot analysis

U87MG cells were harvested in protein lysis buffer consisting of 100 mM Tris pH 6.8, 20 mM DTT, 4% SDS, 5% glycerol. Protein concentrations were determined using the Bradford assay reagents (Bio-Rad, Hercules, CA, USA). Reduced proteins were separated through 4–12% bis-tris polyacrylamide gels using an

Xcell II min cell system (Invitrogen, San Diego, CA, USA). Proteins were transferred onto PVDF nylon membranes (Amersham Pharmacia Biotech, Buckinghamshire, UK) and stained with amido black prior to probing with the appropriate primary antibody. Proteins were detected using the HRP method and SuperSignal West Pico Chemiluminescent Substrate reagents (Pierce, Rockford, IL, USA). Proteins were visualized using the GeneGnome (Syngene, Frederick, MD,USA). Sequential probing of membranes was performed after stripping with the use of Western Blot Stripping Buffer (Pierce, Rockford, IL, USA) for 30 min at room temperature. Mouse cerebella were harvested by homogenization in protein lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 1% NP-40 and 20% glycerol) containing the following protease and phosphatase inhibitors: 200 μg/ml phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 2 μg/ml aprotinin, 200 μM NaF, 200 μM NaPPi and 10 mM NEM. Soluble protein was quantified as described above. Proteins were resolved on a 10% SDS-polyacrylamide gel and electroblotted onto a Hybond C nitrocellulose membrane (Amersham Biosciences Corp, Baie d'Urfé, QC). The membranes were stained with Ponceau S prior to immunoblotting with the appropriate primary antibody. Proteins were visualized as described above.

Immunohistochemistry

Cerebella from age-matched nontransgenic, A02 and B05 mice were excised and fixed in 10% phosphate-buffered formalin overnight at room temperature. Tissues were paraffin-embedded and sectioned sagitally using a microtome at a thickness of 5 μm. Deparaffinized sections were heated in a solution of 10 mM sodium citrate (pH 6.0) in 700W microwave for 10 minutes. Endogenous peroxidase activity was blocked by incubating in methanol containing 3% hydrogen peroxide for 20 minutes. Sections were washed with PBS (pH 7.4) and incubated for 30 minutes with 1.5% normal goat serum (Santa Cruz Biotechnologies Inc., SC, CA, USA) to block nonspecific binding. Sections were then incubated overnight at 4°C with the phospho-p38MAPK antibody (Cell Signaling Technology, Beverly, MA, USA). The reaction product was visualized with the ABC system (DAKO Diagnostics Canada Inc.). The use of animals in these experiments followed the guidelines of the Animal Care Committee of the University of Ottawa and was approved under protocol number ME-212.

Statistical analysis

Unless otherwise indicated, all values are represented as the average of three independent experiments performed in triplicate, with error bars indicating standard error of the mean. Statistical significance was determined by a two tailed Student's t-test. Values were considered significant when $P<0.05$.

Results

PKCι modulates the sensitivity of cells to polyglutamine-induced cellular death

We used a previously described siRNA strategy [38] to investigate the role of PKCι depletion in polyglutamine-induced cytotoxicity. This method specifically depletes PKCι RNA and protein with no effect on other PKC enzymes [38]. We used U87MG cells which have been shown to have an elevated basal ERK activity as a result of increased signaling through the EGFR pathway [39]. We reasoned that if ERK was protective such a cell model would be less sensitive to expanded polyglutamine induced toxicity. The use of cells from a glial as opposed to neuronal lineage is unlikely to be of consequence in that similar results were obtained in glioblastoma and neuroblastoma cell lines (as described below). To assess whether the depletion of PKCi would affect cell survival in the presence of an expanded polyglutamine protein, U87MG cells were transfected with a control or one of two siPKCι RNAs (siPKCιA and siPKCιB). Cells were then transiently transfected with either a GFP control plasmid or constructs encoding exon 1 of the Huntingtin protein containing a normal polyglutamine tract of 25Qs (hereafter referred to as Htt-25) or with a pathogenic tract of 103Qs (hereafter referred to as Htt-103) fused to the yellow fluorescent protein (YFP) reporter. Similar expression constructs encoding exon 1 of the Htt protein with an expanded polyglutamine tract have been previously used in cell culture models of polyglutamine toxicity [6,40] and in the generation of the well characterized R6/2 transgenic mouse line [41]; R6/2 mice develop a progressive neurological phenotype with motor symptoms resembling those in HD [42]. By phase contrast microscopy, a pronounced effect was observed in PKCι depleted cells expressing Htt-103 wherein a significant increase in the number of shrunken, rounded and detached cells was noted (Figure 1A). Analogous to other cell culture systems used in the study of polyglutamine biology (3T3, PC12, SHY-5Y cells, etc), U87MG cells expressing Htt-103 were found to accumulate visible nuclear inclusions as early as 24 hours post-transfection (Figure 1B). No such inclusions were observed in cells expressing GFP alone or Htt-25 (Figure 1B). Depletion of PKCι was assessed by Western blot analysis with an antibody recognizing PKCι in extracts from U87MG cells transfected with either the control or siPKCιA and siPKCιB; a reduction in the protein levels was observed at 24 and 48 hours post-transfection (Figure 1C). The transfection efficiency of the Htt proteins in U87MG cells was estimated at ~80% as assessed by fluorescence microscopy (Figure 1B). Similar levels of expression of GFP, Htt-25 and Htt-103 were confirmed by Western blot analysis of extracts from transfected cells with an antibody specific for the fluorescent protein reporter (Figure 1D). Quantification of survival with the use of a metabolic assay (MTT) revealed that the depletion of PKCι sensitized cells to the expression of Htt-103 such that survival was reduced by approximately 25% when compared to cells transfected with the control RNA (Figure 2B). The survival of U87MG cells transfected with an Htt-25 expression plasmid was no different then that of cells expressing GFP alone (Figure 2A). When compared to GFP transfectants, the depletion of PKCι mildly sensitized cells to the expression of Htt-25 but to a lesser extent than did expression of Htt-103 (Figure 2A and B). The data obtained by MTT analysis were consistent with survival as measured by the trypan-blue exclusion method (Figure 2C) and by flow cytometric analysis (Figure 2D) of Htt-103 transfected cells, both of which revealed an increase in cell death in PKCι depleted cells when compared to control RNA transfectants. These data suggested that the depletion of PKCι was sensitizing cells to the expression of expanded polyglutamine proteins. As assessed by MTT, the exogenous expression of flag-tagged PKCι was found to modestly increase the resistance of cells to the toxic effects associated with expression of Htt-103 (Figure 2E). The overexpression of PKCι in these stable transfectants was confirmed by Western blot analysis with both the PKCι and flag tag antibodies respectively (Figure 2F).

Impaired ERK activation sensitizes cells to polyglutamine-expanded proteins

It has been previously reported that PKCι is positioned upstream of the mitogen-regulated kinase ERK [43] and it was therefore conceivable that PKCι depletion would affect ERK activation. To test this hypothesis, we examined the basal levels of

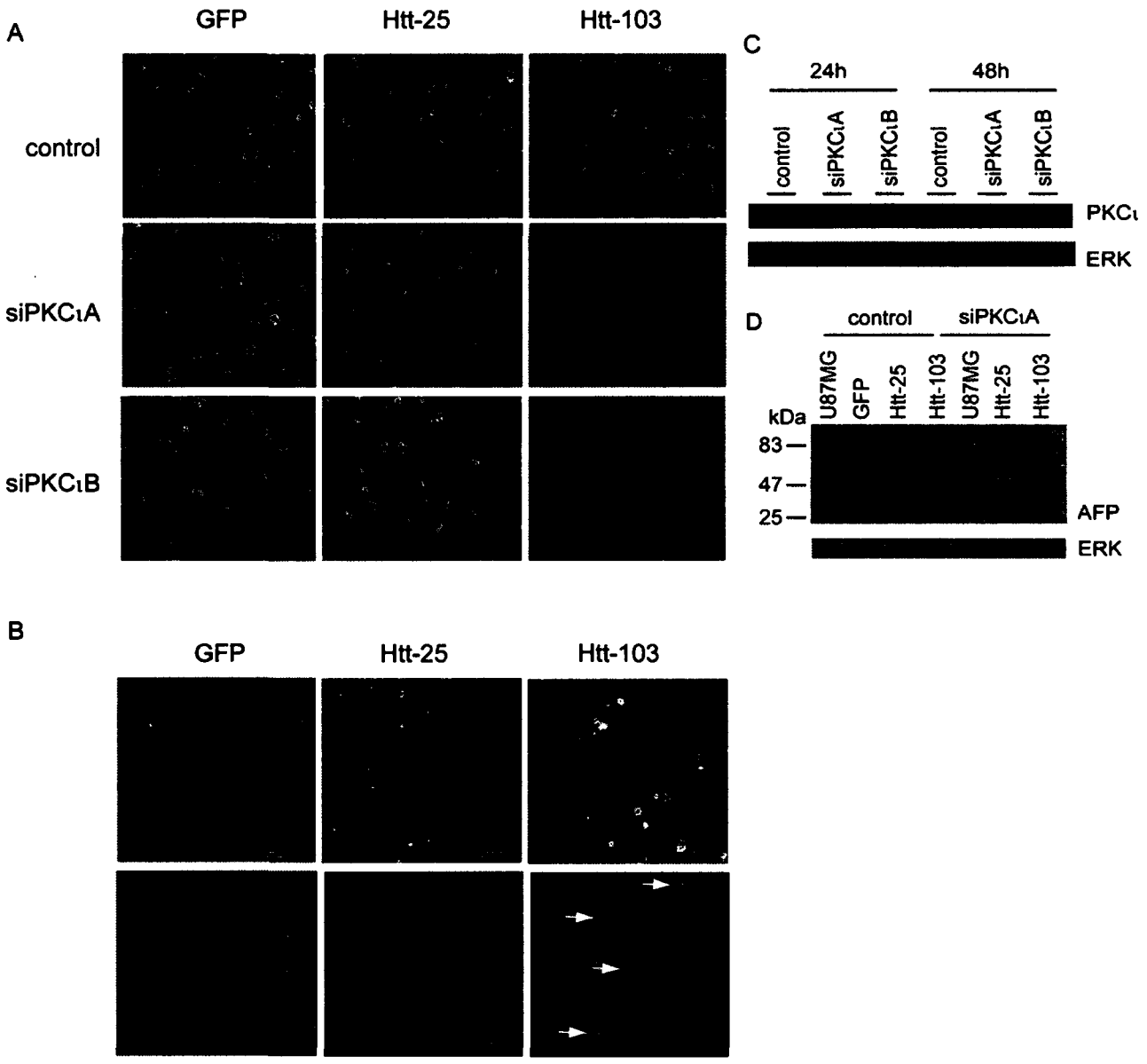

Figure 1. Morphological alterations in PKCι depleted cells expressing Htt-103. A) U87MG cells transfected with the control or with siPKCιA and siPKCιB were transiently transfected with plasmids encoding GFP, Htt-25 or Htt-103 for 24 hours. Cell morphology was assessed by phase contrast microscopy. An increase in the number of shrunken, rounded and detached cells was observed in PKCι depleted cells expressing Htt-103 when compared to control RNA transfected cells or cells expressing GFP or Htt-25. Magnification was 40×. B) U87MG cells expressing GFP, Htt-25 or Htt-103 for 24 hours were visualized under fluorescence (bottom panel) to assess transfection efficiency. Upper panels represent the same field of view visualized under white light. Arrowheads demonstrate nuclear inclusions in Htt-103 expressing cells. Scale bars represent 100 μm. C) Western blot analysis with a PKCι specific antibody of cell extracts from U87MG cells transfected in duplicate with either the control or siPKCιA and siPKCιB showing the reduction in the protein levels of PKCι at 24 and 48 hours post-transfection. The membrane was re-probed with an antibody directed against Pan-ERK which served as a loading control. D) Western blot analysis of cell extracts from cells transfected with either control or siPKCιA expressing GFP, Htt-25 or Htt-103 with an antibody raised against AFP. No significant difference in the protein levels of GFP, Htt-25 and Htt-103 were observed in extracts from control and PKCι transfected cells. The membrane was re-probed with an antibody directed against Pan-ERK which served as a loading control.
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activated ERK in PKCι depleted cells. Cell extracts from control or siPKCιA RNA transfected cells were analyzed by Western blot analysis with an antibody recognizing phospho-ERK. The analysis revealed a reduction in ERK phosphorylation in PKCι depleted cells when compared to the levels in U87MG cells transfected with the control RNA (Figure 3A). These data suggested that U87MG cells have elevated basal levels of activated ERK most probably due to the constitutively active EGFR pathway and that PKCι depletion affects ERK induction. To investigate specifically whether the loss of ERK signaling due to PKCι depletion was the basis for the increased sensitivity observed in PKCι depleted cells to expanded polyglutamine protein expression, we made use

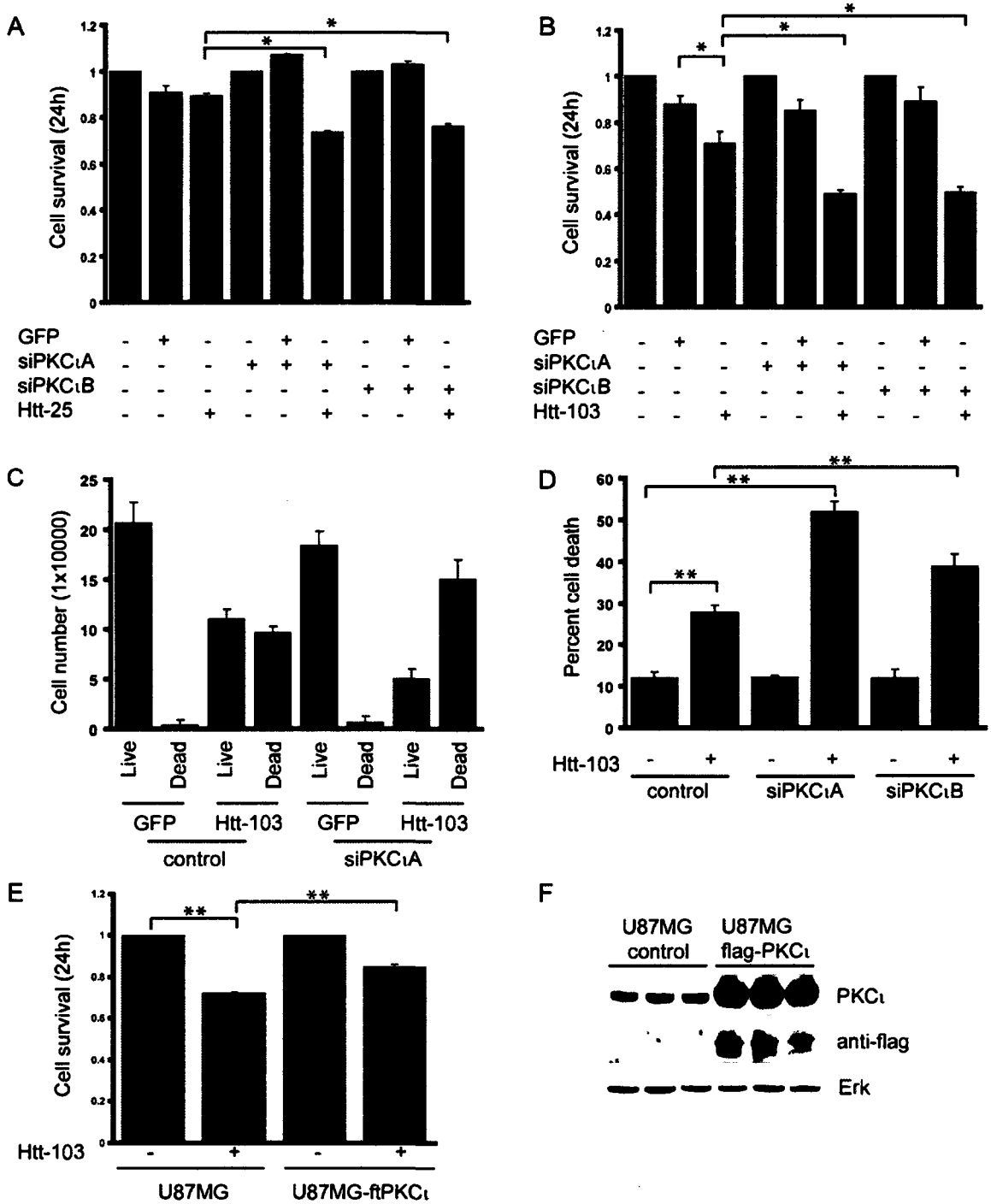

Figure 2. Depletion of PKCι sensitizes cells to polyglutamine induced toxicity. A) and B) U87MG cells transfected for 24 hours with either control or siPKCιA and PKCιB were plated in 96 well dishes. Subsequently, cells were transfected with expression constructs encoding GFP, Htt-25 (A) and Htt-103 (B) and cell survival was measured by MTT assay. A) The survival of mock RNA transfected cells expressing Htt-25 was comparable to cells expressing GFP alone. A slight decrease in cell survival was observed in PKCι depleted cells expressing Htt-25 when compared to GFP transfectants (* $p<0.05$). B) A marked decrease in cell survival was observed in control RNA transfected cells expressing Htt-103 that was further pronounced in PKCι depleted cells (* $p<0.05$). C) Survival as measured by trypan blue exclusion of U87MG cells transfected with control or siPKCιA in the presence or absence of Htt-103. In accordance with the MTT assay, an increase in the population of dead cells was observed in control transfected U87MG cells expressing Htt-103 which was further increased in PKCι depleted cells. D) Flow cytometric analysis of cells transfected with either the control or siPKCι expressing Htt-103 showing an increase in the number of dead cells in PKCι depleted cells when compared to control transfected cells. Data represents the average of three independent experiments, with error bars indicating standard deviation (** $p<0.01$). E) U87MG cells stably expressing flag epitope tagged PKCι (ftPKCι) were transiently transfected with the Htt-103 expression construct for 24 hours. Survival as assessed by MTT

analysis revealed a modest increase in survival in PKCι transfectants when compared to the parental U87MG cells (** $p<0.01$). F) Western blot analysis of triplicate cell extracts from untransfected U87MG and cells stably expressing flag-tagged PKCι with antibodies raised against PKCι and the flag epitope tag respectively. The flag tag specific antibody detected ectopically expressed PKCι in transfected cells, which was absent in the untransfected control cell extracts. The PKCι antibody detected endogenous and exogenous PKCι in lysates from U87MG cells and cells stably expressing PKCι. Pan-ERK served as the loading control. MTT and trypan blue data are represented as the average of three independent experiments performed in triplicate, with errors bars indicating standard error of the mean.
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of U0126, a specific inhibitor of MEK (positioned directly upstream of ERK). U87MG cells were either untreated or treated with U0126 prior to transfection with the GFP control vector, Htt-25 or Htt-103 plasmids. Twenty-four hours post-transfection, cell survival was assessed by the MTT assay. The data presented in figure 3B revealed that blockade of ERK recapitulated the findings in PKCι depleted cells: the survival of cells expressing Htt-103 was significantly compromised (Figure 3B). The survival of Htt-25 expressing cells treated with the inhibitor was comparable to that of GFP transfectants (Figure 3B). The efficient blockade of ERK activation in U0126 treated cells was confirmed by Western blot analysis of cell extracts from cells transfected with Htt-103 with the phospho-ERK specific antibody (Figure 3C). The data in Figure 3C also revealed that the expression of the expanded polyglutamine protein has no effect on ERK induction when compared to GFP transfectants. Taken together, they suggest that the status of ERK is strictly dependent on PKCι and not the expression of the expanded polyglutamine protein.

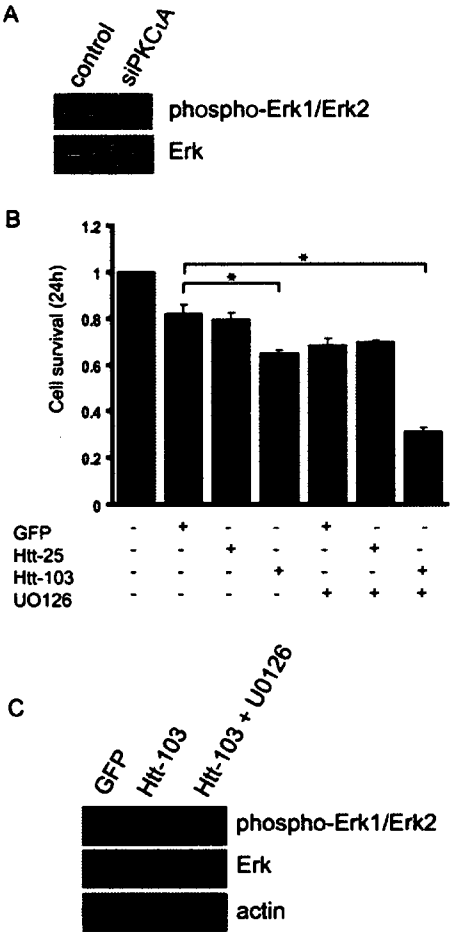

Figure 3. PKCι-mediated ERK activation protects cells from expanded polyglutamine-induced cytotoxicity. A) Western blot analysis of cell extracts from control and siPKCιA transfectants with the phospho-ERK specific antibody. The basal levels of ERK phosphoprotein were significantly reduced in PKCι depleted cells when compared to control RNA transfectants. Total ERK levels were assessed with the pan ERK antibody which also served as a loading control. B) U87MG cells were pre-treated with the MEK inhibitor U0126 for 2 hours prior to transfection with GFP, Htt-25 and Htt-103. Cell survival was assessed by MTT 24 hours post-transfection. Blockade of ERK in Htt-103 expressing cells resulted in a significant reduction in cell survival when compared to untreated Htt-103 expressing cells (* $p<0.05$). Data are represented as the average of three independent experiments performed in triplicate, with error bars indicating standard error of the mean. C) Western blot analysis of extracts from untreated and U0126 treated U87MG cells expressing Htt-103 with the phospho-ERK antibody confirming the blockade of ERK phosphorylation in U0126 treated cells. Pan-ERK was used to detect total ERK levels and actin served as a loading control.
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Expression of Htt-103 is associated with induction of p38MAPK and its pharmacological blockade rescues cells from polyglutamine-induced toxicity

Given that the blockade of ERK signaling preferentially sensitized Htt-103 expressing cells when compared to Htt-25 transfectants, we reasoned that the expanded polyglutamine may be affecting stress-inducible pro-apoptotic pathways. The activation of the p38MAPK pathway in response to environmental and genotoxic stress is well characterized [44–46] and its induction in response to amyloid beta treatment has been well documented [47,48]. Expanded polyglutamine proteins have recently been shown to induce death in cell culture models (reviewed in [49]) but the role of p38MAPK has not been investigated. To investigate the role of this kinase, we analyzed cell extracts from control and PKCι depleted cells expressing GFP, Htt-25 and Htt-103 by Western blot analysis with a phospho-p38MAPK antibody. The analysis revealed that the expression of Htt-103 resulted in a similar increase in p38MAPK phosphorylation in both the control and siPKCιA transfected cells. These data suggested that the status of PKCι has no effect on expanded polyglutamine induced p38MAPK activation (Figure 4A) and that the increased sensitivity observed in PKCι depleted cells was a reflection of a diminished activation of ERK. The levels of phospho-p38MAPK remained unchanged in GFP expressing cells and were minimally affected in Htt-25 transfectants (Figure 4A). This suggested that the activation of p38MAPK may be the basis for the increased cell death observed in Htt-103 expressing cells and that interfering with its phosphorylation may rescue cells from polyglutamine-induced toxicity. Inhibition of p38MAPK with the use of SKF86002, a specific p38MAPK inhibitor, resulted in a significant rescue of Htt-103 expressing U87MG control and siPKCιA transfected cells such that their survival was comparable to Htt-25 and GFP transfectants treated with the inhibitor (Figure 4B). Similar results were obtained by blockade of p38MAPK with the use of SB202190, a different p38MAPK inhibitor; its inhibition resulted in a statistically significant increase in cell survival of PKCι depleted cells expressing Htt-103 (Figure 4C). The efficient blockade of p38MAPK activation in SKF86002 treated cells was confirmed by Western analysis of cell extracts from Htt-103 expressing cells with a phospho-ATF2 antibody, a downstream

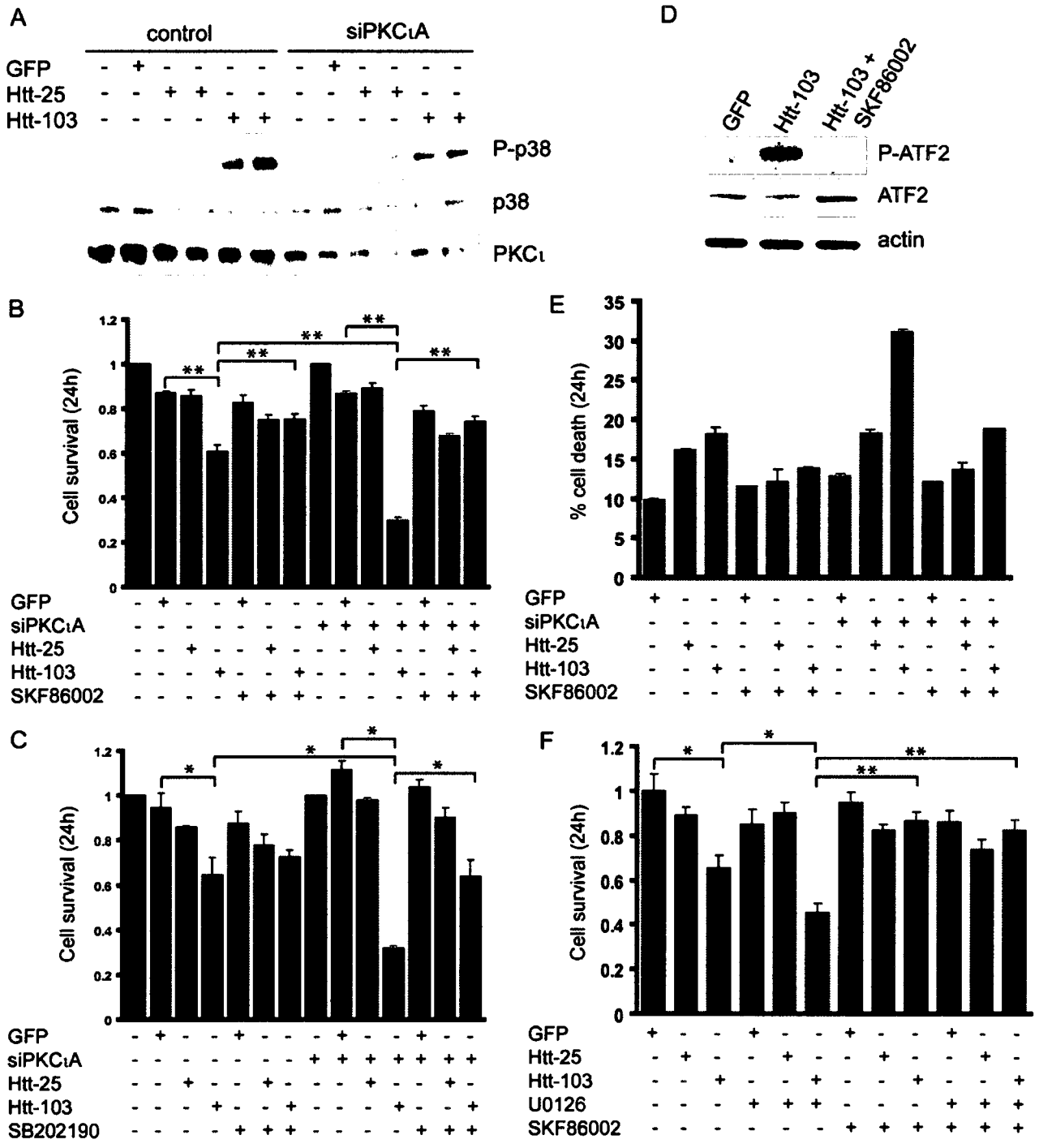

Figure 4. Expanded polyglutamine proteins induce p38MAPK. A) Western blot analysis of extracts from control and siPKCιA transfected cells expressing GFP, Htt-25 or Htt-103 with the phospho-38MAPK antibody showing the phosphorylation of p38MAPK in extracts from control and siPKCιA transfected cells expressing Htt-103. Phospho-p38MAPK levels were slightly increased in extracts from cells transfected with Htt-25 when compared to GFP transfectants. The levels of total p38MAPK remained unchanged in all extracts as assessed by Western blot analysis with the p38MAPK antibody. Efficient depletion of PKCι was confirmed by re-probing the membrane with the PKCι specific antibody. B) and C) Control or siPKCιA transfected cells were either left untreated or were pre-treated with p38MAPK inhibitors, SKF86002 (B) and SB202190 (C) for 2 hours prior to transfection with GFP, Htt-25 or Htt-103. 24 hours post-transfection, cell survival was assessed by MTT which revealed an increase in survival of Htt-103 expressing cells by treatment with SKF86002 in both control and siPKCιA transfected cells (** $p<0.01$). The survival of Htt-103 expressing cells treated with SB202190 was less pronounced when compared to SKF86002 treated cells but was still statistically increased in PKCι depleted cells when compared to the untreated counterparts (* $p<0.05$). D) Western blot analysis of cell extracts from cells expressing Htt-103 that were either untreated or treated with SKF86002 with the phospho-ATF2 antibody. The analysis revealed an abrogation of ATF2 phosphorylation in Htt-103 expressing cells treated with SKF86002. Re-probing the membrane with an ATF-2 antibody revealed no significant difference in the total levels of ATF2 protein. Actin

served as a loading control. E) Flow cytometric analysis of control and siPKCιA transfected cells expressing Htt-103 that were either left untreated or treated with SKF86002. The survival of SKF86002 treated, Htt-103 expressing cells was significantly improved when compared to the untreated counterparts and was comparable to the survival of GFP and Htt-25 transfectants in both the mock and PKCι depleted cells. Data is represented as the average of three independent experiments performed in duplicate with error bars indicating standard error of the mean. F) U87MG cells transfected with GFP, Htt-25 and Htt-103 were treated with SKF86002 in combination with U0126. Survival was assessed by MTT assay 24 hours post-transfection. As described above, treatment of Htt-103 expressing cells with U0126 resulted in reduced viability (* $p<0.05$) whereas treatment with SKF86002 alone or in combination with U0126 rescued cells from polyglutamine toxicity to a level that was similar to p38MAPK inhibition alone (** $p<0.01$). Data are represented as the average of three independent experiments performed in triplicate, with error bars indicating standard error of the mean.

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target of p38MAPK (Figure 4D). Flow cytometric analysis of Htt-103 expressing cells treated with SKF86002 revealed that inhibiting p38MAPK increased the survival of Htt-103 transfectants in both control and PKCι depleted cells (Figure 4E). To further dissect the relative importance of ERK and p38MAPK in polyglutamine-induced death, we treated GFP, Htt-25 and Htt-103 expressing U87MG cells with SKF86002 in combination with U0126. By MTT analysis, we found that pharmacological inhibition of p38MAPK alone or in combination with ERK inhibition resulted in a similar and significant rescue of cells from death associated with expression of Htt-103 (Figure 4F). These data suggest that the induction of p38MAPK contributes to polyglutamine-induced cytotoxicity and that whether in the presence or absence of activated ERK, its inhibition is sufficient to block cell death.

Full-length expanded human Ataxin-1 protein induces cell toxicity in a p38MAPK dependent manner

To investigate whether the depletion of PKCι and p38MAPK pathways represent a general mechanism of expanded polyglutamine toxicity, we transfected control or PKCι depleted cells with an expression construct encoding the full length ataxin-1 gene product with an expanded polyglutamine tract of 83Q (hereafter referred to as Atx-83). The length of the polyglutamine repeat in normal, unaffected humans is from 6 to 40 residues and mice expressing full length ataxin-1 with 30Qs (Atx-30) show no phenotype effects [37]; the Atx-30 expression was therefore a suitable control for the expanded (83Q) protein in our experiments. We were unable to detect expression of an ataxin-1 protein with only 2 glutamine residues and speculate that this variant may be unstable (data not shown). Western blot analysis of cell extracts from Atx-30 and Atx-83 transfected cells with the phospho-p38MAPK antibody revealed an increase in p38MAPK activation in Atx-83 expressing cells when compared to Atx-30 and parental U87MG cells (Figure 5A). Additionally, the ectopic expression of Atx-30 and Atx-83 resulted in an increase in total levels of p38MAPK as assessed by Western analysis of the same membrane with the antibody raised against total p38MAPK (Figure 5A). An increase in p38MAPK activation in response to ectopic expression of Atx-83 was also observed in NIH-3T3 fibroblasts and HT4 neuroblastoma cells suggesting that its induction represents a cell type independent mechanism of polyglutamine cytotoxicity (Figure 5B). By MTT assay, we found that the survival of control RNA transfected U87MG cells expressing Atx-83 was reduced when compared to cells expressing the non-expanded Atx-30 counterpart 24 hours post-transfection (Figure 5C). The sensitivity of cells expressing Atx-83 was significantly increased in PKCι depleted cells; survival was reduced by approximately 20% when compared to control RNA-transfected cells expressing an empty vector control (Figure 5C). Pharmacological inhibition of p38MAPK with the use of SKF86002 in Atx-83 expressing cells recapitulated the findings in Htt-103 transfectants; a statistically significant increase in cell survival was observed in control RNA transfectants and was more pronounced in PKCι depleted cells (Figure 5C). To confirm that the rescue observed in SKF86002 treated cells was attributable to blockade of p38MAPK signaling, we transiently co-transfected U87MG cells with the Atx-30 or Atx-83 plasmids in conjunction with constructs encoding either flag tagged wild-type p38 alpha (wt p38) or its dominant-negative kinase dead counterpart (KD p38). These expression constructs have previously been used to examine the contribution of p38MAPK signaling in cultured cells [50,51]. By MTT analysis we found that expression of the kinase dead p38MAPK increased survival of Htt-103 expressing cells in a similar manner to blockade with SKF86002 suggesting that the decrease in survival is due to activation of p38MAPK. The expression of wt p38 had no significant impact on survival of Atx-83 expressing cells (Figure 5D). The expression levels of Atx-30 and Atx-83 were similar as assessed by Western blot analysis with an ataxin-1 specific antibody (Figure 5E).

Expanded polyglutamine protein induced p38MAPK in the cerebella of SCA-1 transgenic mice

The *in vivo* induction of p38MAPK was examined in the previously characterized B05 mouse model of spinocerebellar ataxin-1 (SCA-1). In this model a human ataxin-1 cDNA with an expanded CAG tract encoding 82 glutamines is specifically expressed in Purkinje neurons (reviewed in [52]). The A02 transgenic strain expressing a similar construct with a non-pathological expansion of 30 glutamines served as a control. Western blot analysis of cerebellar extracts from aged-matched 3 month old mice with the phospho-p38MAPK antibody revealed phosphorylation of p38MAPK in extracts from nine B05 mice (five of which are shown in Figures 6A and B). In agreement with the findings in cultured cells, the phosphorylation of p38MAPK in lysates from A02 mice was lower than that detected in B05 extracts but slightly increased when compared to lysates from nontransgenic controls (Figure 6A). Contrary to what was observed in lysates from U87MG cells transfected with Atx-30 and Atx-83, re-probing the membrane with the antibody raised against total p38 revealed that total p38MAPK levels remained unchanged in A02 and B05 lysates when compared to nontransgenic control lysates (Figure 6A). We speculate that the induction of total p38MAPK levels may simply represent a response of cultured cells to the expression of Atx-30 and Atx-83. In B05 mice we observed a significant induction in p38MAPK phosphorylation at 3 months of age, while mice at 1 and 2 months of age show little or no detectable p38MAPK phosphorylation (Figure 6C). This activation correlates well with the onset of behavioral and anatomical anomalies in the mouse model of SCA-1. We examined the localization of phosphorylated p38MAPK by immunohistochemistry in cerebella of 3 month old nontransgenic and B05 mice. We found that phosphorylated p38MAPK was primarily localized to the cytoplasm and nucleus of Purkinje neurons (Figure 6D), showing that the increase in the levels of activated p38MAPK (as detected by Western analysis) could be attributed to expanded polyglutamine expression in those cells.

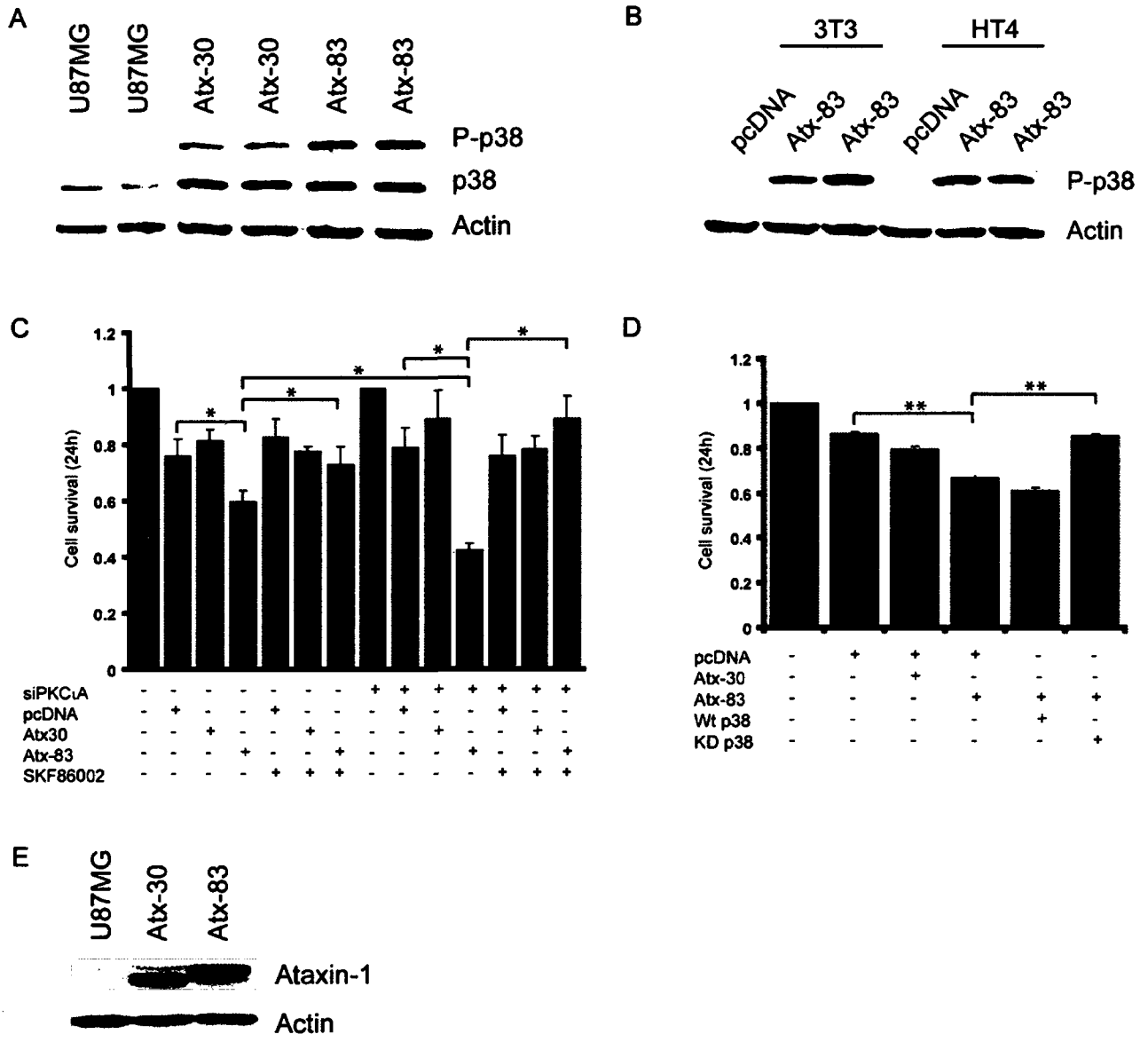

Figure 5. Expanded ataxin-1 toxicity is mediated through induction of p38MAPK. A) Western blot analysis of duplicate cell extracts from U87MG cells expressing Atx-30 and Atx-83 with the phospho-p38MAPK antibody. An increase in phosphorylated p38MAPK was observed in lysates from Atx-83 expressing cells when compared to lysates from mock transfected or cells expressing Atx-30. Total levels of p38MAPK were increased in lysates from cells transfected with either Atx-30 and Atx-83 when compared to mock transfected cells as assessed by re-probing of the membrane with the p38MAPK antibody. Actin served as a loading control. B) HT4 and NIH-3T3 cells were transiently transfected in duplicate with the Atx-83 expression construct. Cell extracts were analyzed by western blot analysis with the phospho-p38MAPK antibody. p38MAPK activation was observed in cell extracts from both NIH-3T3 and HT4 cells expressing Atx-83. The induction of p38MAPK was not observed in lysates from cells expressing an empty vector control. C) Untreated or SKF86002 treated control or siPKCιA transfected cells were transfected with Atx-30 or Atx-83 for 24 hours and cell survival was assessed by MTT. The analysis revealed a decrease in survival of Atx-83 expressing cells in control RNA transfected cells that was significantly more pronounced by PKCι depletion. Blockade of p38MAPK increased survival of Atx-83 expressing cells in both control and siPKCι transfectants such that it was comparable to the survival of Atx-30 expressing cells. Data represent the average of three independent experiments performed in triplicate, with error bars indicating standard error of the mean (* $p<0.05$). D) U87MG cells were co-transfected with Htt-103 and either empty vector alone or expression constructs encoding wild-type (wt p38) or dominant-negative (KD) p38MAPK alpha. 24 hours post-transfection, cells were analyzed by MTT which revealed a statistically significant increase in survival in cells co-expressing Htt-103 and dominant-negative p38MAPK alpha (** $p<0.01$). The survival of cells expressing empty vector control alone or co-expressing Htt-25 with empty vector was not significantly different. E) Western blot analysis of extracts from Atx-30 and Atx-83 transfected cells with an ataxin-1 specific antibody revealing a similar level of expression. Actin served as a loading control.
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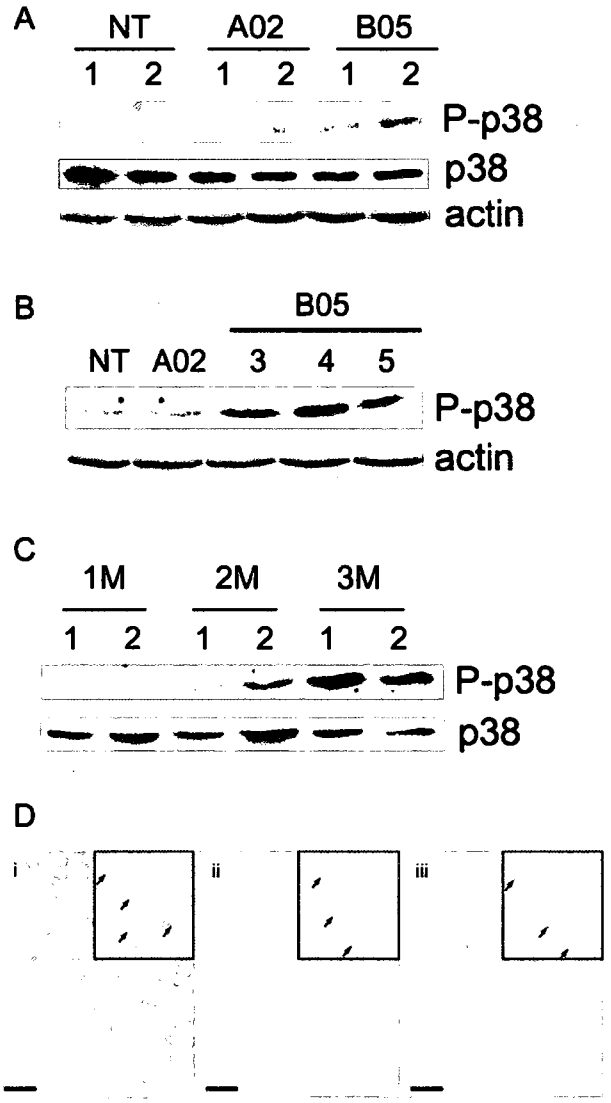

Figure 6. In vivo induction of p38MAPK in SCA-1 mice. A) and B) Western blot analysis of cerebellar extracts from 3 month-old non-transgenic, A02 and B05 mice with the phospho-p38MAPK antibody. An increase in the protein levels of phospho-p38 was detected in the extracts from B05 mice when compared to A02 and nontransgenic control extracts. Total p38MAPK levels were similar as assessed by re-probing the membrane with the p38MAPK antibody or actin. C) Western blot analysis of cerebellar extracts from B05 mice at 1,2 and 3 months of age with the phospho-p38MAPK antibody. The analysis revealed a detectable induction in p38MAPK activation in lysates from mice at 3 month of age. Total levels of p38MAPK were assayed by re-probing the membrane with the p38MAPK antibody which also served as a loading control. D) Immunohistochemistry of mouse cerebella with the phospho-p38MAPK antibody. Panel i) cerebellum of a B05 animal and panel ii) cerebellum of a nontransgenic animal. Immunoreactivity was observed in the cytoplasm and nucleus of Purkinje neurons in B05 and nontransgenic mice. Panel iii) section from a B05 animal stained with the secondary antibody alone demonstrating the absence of immunoreactivity by omission of the primary antibody. Scale bars represent 25 μm.
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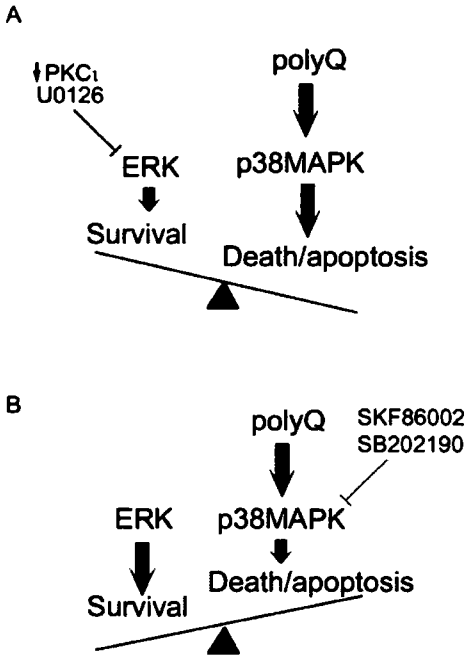

Figure 7. Model of polyglutamine induced toxicity. Activation p38MAPK signaling is counteracted by PKCι-mediated ERK activation in expanded polyglutamine expressing cells. The pharmacological blockade of ERK with U0126 or by PKCι depletion sensitizes cells to polyglutamine-induced death through a mechanism of exaggerated induction of p38MAPK (A). In contrast, inhibition of p38MAPK phosphorylation by SKF86002 or SB202190 rescues cells from polyglutamine toxicity (B). The blockade of both signaling pathways in cells expressing expanded polyglutamine proteins recapitulates blockade of p38MAPK (B) indicating a causative association between p38MAPK induction and polyglutamine induced death.
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Discussion

Clear evidence for the essential role of protein kinase C family members in neuronal homeostasis has been provided by neurodegeneration attributable to a loss of function mutation in the *PKCγ* gene in spinocerebellar ataxia type 14 (SCA-14, [53]). No such genetic disorder has been mapped to the *PKCι* gene, but evidence from overexpression studies indicates that PKCι can be protective against a variety of cytotoxic insults including UV damage and chemotherapy [38,54] and neurotoxic insults including beta amyloid [55]. Conversely, inhibition of PKCι and the closely related PKCζ by the prostate apoptosis-response 4 (PAR-4) protein has been recently shown to increase proteolytic processing of amyloid precursor protein [56,57] and to exacerbate Aβ accumulation and toxicity in mouse models of Alzheimer's disease [58,59] suggesting a role for PKCι in modulating survival.

Using specific MAP kinase inhibitors we have established that p38MAPK is activated in expanded polyglutamine expressing cells and that PKCι-mediated ERK activation can antagonize polyglutamine-induced cell death in a cell culture model. Our data are in accordance with a recent report demonstrating the protective effects of ERK activation in expanded polyglutamine expressing cells [19]. Based on our findings, we propose a mechanism (schematically depicted in Figure 7) wherein p38MAPK induction contributes significantly to the toxicity observed in expanded polyglutamine expressing cells while ERK activation serves to counteract its effects. The fate of cells

expressing polyglutamine proteins would therefore seem to be determined, in part, by comparing the activation state of the two signaling cascades. In this model the ERK cascade would generate a pro-survival signal in response to PKCι-mediated input. The p38MAPK cascade would generate a pro-death output specifically in response to the expanded polyglutamine protein. If the p38MAPK signal outweighed the ERK signal (as is the case by expression of expanded polyglutamine proteins or by blockade of ERK and/or PKCι signaling) the cell would respond by activating its cell death program. In the presence of expanded polyglutamine proteins the simultaneous blockade of both ERK and p38MAPK signaling pathways was found to be functionally equivalent to blockade of p38MAPK alone suggesting that the inhibition of p38MAPK was sufficient to block cell death regardless of the presence or absence of activated ERK (Figure 4F). To promote the survival of neurons in neurodegenerative disorders it may therefore suffice to block p38MAPK signaling (there may be no added therapeutic benefit in promoting the ERK-mediated survival signal, despite previously published evidence that ERK activation promotes survival of polyQ-expressing PC12 cells [19]). Consistent with our supposition that p38MAPK blockade should be the therapeutic objective is recent evidence demonstrating that the promotion of ERK-mediated signaling may ultimately compromise neuronal viability ([60–62] and reviewed in [63,64]).

A recent report has implicated activated stress inducible JNK in a cell culture model of HD [19] and its pharmacological blockade resulted in a statistically significant but partial inhibition of cell death [19]. Our data do not allow us to formally exclude a role for JNK, and it is conceivable that the concerted action of both these pathways mediate adverse effects on polyglutamine expressing cells. Whether or not this is the case the almost complete rescue of cell death by inhibition of p38MAPK under our experimental design suggests a significant contribution of this kinase in mediating toxicity.

The model presented in Figure 7 is based on data from polyglutamine tracts in two quite different contexts (an expanded polyglutamine tract appended to exon 1 of the huntingtin protein and the pathogenic form of full length ataxin-1), suggesting that it may have applicability to expanded polyglutamine proteins in general. The activation of p38MAPK was detected in cultured mammalian cells of different origins (glioblastoma, fibroblasts and cells of neural lineage) and more importantly in cerebellar Purkinje neurons of transgenic mice expressing the neuropathogenic ataxin-1 cDNA at the age of onset of pathology (Figure 6 and [65]). In conjunction with recent reports demonstrating p38MAPK induction in cellular [66] and animal models of Alzheimer's disease [67,68] and amyotrophic lateral sclerosis [69–71], our data suggest that blockade of p38MAPK may have broad utility in delaying the progress of neurodegenerative diseases, even those that do not involve expanded polyglutamine proteins. Consistent with this supposition is the finding that inhibition of p38MAPK is beneficial in mouse models of disease [72,73] and in the suppression of human inflammatory conditions [74–76]. Here we demonstrate that pharmacological blockade of p38MAPK may potentially be an efficacious intervention for the polyglutamine disorders. Such an intervention may not only attenuate regional inflammation (reviewed in [77]) and decrease the phosphorylation of HSP27 (a downstream target of p38/MAPKAP 2/3 whose phosphorylation status has been shown to modulate the cytotoxity of polyglutamine expressing cells, [78]), but may delay or preclude the otherwise inexorable neuronal loss that is associated with these diseases.

Other therapeutic modalities may become apparent as the events downstream of p38 MAPK activation by polyglutamine proteins become known. At this point it is not at all clear how many and which of the several pathogenic mechanisms might be affected by p38MAPK signaling. One plausible scenario is that p38MAPK activation leads to transcriptional dysregulation through negative effects on pivotal transcriptional regulators. For example, the levels of the p300/CBP histone acetyltransferase enzymes are known to be affected by expanded polyglutamine proteins [6,79–81], and their loss correlates with reduced expression of a set of target genes whose importance to neuronal homeostasis is well established [79,82]. It has recently been shown that p300 is degraded by the proteasome in response to p38MAPK activation [83] and that partial inhibition of proteolysis may delay the loss of p300/CBP in the SCA-1 model [84]. Consistent with this model, inhibitors of histone deacetylases (HDACs) have been shown to have beneficial effects in counteracting polyglutamine protein toxicity [85–88] and recently reviewed in [89]. The histone acetyltransferases would therefore seem promising as downstream targets of p38MAPK, and we are currently seeking a deeper understanding of this relationship.

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Author Contributions

Conceived and designed the experiments: MT RB. Performed the experiments: MT RB MT. Analyzed the data: DG MT RB MT IL. Contributed reagents/materials/analysis tools: DG IL. Wrote the paper: DG MT RB MT IL.

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Curriculum Vitae

R. Mitchell Baldwin

Curriculum Vitae

Education

Sept. 2003-present

Doctor of Philosophy, supervisor Dr. Ian Lorimer
Department of Biochemistry, University of Ottawa
Ottawa, ON

- R. Mitchell Baldwin. The role of atypical protein kinase C iota in glioblastoma multifome. Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biochemistry, University of Ottawa, 2008.

1998-2003

Honours Bachelor of Science, Biology and Pharmacology Co-op Program
McMaster University
Hamilton, ON

- Lab experience includes organ bath, Schild analysis, and patch clamping techniques
- Acquired knowledge in detergent solublization, polyacrylamide gel electrophoresis and immuno-precipitation techniques

1993-1998

Ontario Secondary School Diploma
St. Joseph Scollard Hall Catholic Secondary School
North Bay, ON

- Recognized for highest achievement in chemistry in Ontario Academic Credit year
- Developed a natural method of measuring air quality that received a placing of third in the North Bay Regional Science Fair

Work Experience

Sept.-Dec. 2002 — Undergraduate thesis student, supervisor Dr. Roger Moorehead
Department of Biomedical Sciences, University of Guelph,
Guelph, ON

- Accepted to complete undergraduate senior thesis component of the Biology and Pharmacology program
- Pursued senior thesis investigating the role of insulin-like growth factor two in human pulmonary adenocarcinoma
- Defended February 2003

2002
(Jan-Aug)
Co-op — Lab Researcher
Department of Biomedical Sciences, University of Guelph
Guelph, ON

- Performed an eight month work term from Jan-Aug 2002
- Supervised and taught techniques to summer and project students as well as other members of the department
- Experimental development, including literature searching and protocol adaptations
- Cell culture, including MTT, soft agar colony forming assays
- Protein and RNA extraction, southern and western blotting
- Two-dimensional gel electrophoresis
- PCR and Microarray
- Transfections
- Tissue embedding and sectioning
- Contributed to the organization of the laboratory and some operating grant applications

2001
(May-Aug)
Co-op — Research Technician I
Division of Experimental Therapeutics, Ontario Cancer
Institute/Princess Margaret Hospital, University Health Network
Toronto, ON

- Involved in organizing and developing experiments of two projects working both independently and in cooperation with collegues
- Innovatively developed a novel ex-vivo analysis of apoptosis using fluorescence and confocal microscopy
- Cell culturing, including clonogenic survival assays
- DNA extraction
- Mice handling and dissection
- Immunocytochemistry
- Developed skills in data analysis and organization
- Presented poster to the Ontario Cancer Institute department of Experimental Therapeutics

AWARDS

Canadian Cancer Society-Ontario Division Travel Award, 2007
National Cancer Institute of Canada Terry Fox Foundation Studentship, 2006-2009
Ontario Graduate Scholarship, 2006 *(Declined)*
Ottawa Health Research Institute graduate student poster day 2nd prize, 2005
Ottawa Health Research Institute graduate student poster day 3rd prize, 2004
Ontario Graduate Scholarship in Science and Technology (OGSST), 2004-2005
Stem Cell Network Graduate Studentship, 2003-2004
McMaster University Deans Honour List, 2003
McMaster University Science Incentive Bursary, 1998

ACADEMIC ACCOMPLISHMENTS

Manuscripts

R. Mitchell Baldwin, Doris A. E. Parolin, and Ian A. J. Lorimer. (2008). Stable depletion of PKCiota impairs glioblastoma cell motility and cell division. *(in preparation).*

Maria Tsirigotis$^{\xi}$, **R. Mitchell Baldwin**$^{\xi}$, Matthew Y. Tang, Ian A.J. Lorimer and Douglas A. Gray. (2008). Activation of p38MAPK contributes to expanded polyglutamine-induced cytotoxicity. ($^{\xi}$authors contributed equally to manuscript). *PLoS One*, **3** (5), 1-13.

R. Mitchell Baldwin, Doris A. E. Parolin, and Ian A. J. Lorimer. (2008). Regulation of glioblastoma cell invasion by PKCiota and RhoB. *Oncogene,* **27**, 3587-3595.

R. Mitchell Baldwin, Michelle Garratt-Lalonde, Doris A. E. Parolin, Paul M. Krzyzanowski, Miguel A. Andrade and Ian A. J. Lorimer. (2006). Protection of glioblastoma cells from cisplatin cytotoxicity via protein kinase c iota – mediated attenuation of p38 MAP kinase signaling. *Oncogene,* **25**, 2909-2919.

Nicolle M. Linnerth, **Mitch Baldwin**, Craig Campbell, Melissa Brown, Heather McGowan and Roger A. Moorehead. (2005). IGF-II induces CREB phosphorylation and cell survival in human lung cancer cells. *Oncogene*, **24**, 7310-7319.

Roger A. Moorehead, Otto H. Sanchez, **R. Mitchell Baldwin**, and Rama Khokha. (2003). Transgenic overexpression of IGF-II induces spontaneous lung tumours: a model for human lung adenocarcinoma. *Oncogene*, **22**, 853-857.

Abstracts and Presentations

R. Mitchell Baldwin, Doris A. E. Parolin, and Ian A. J. Lorimer. Regulation of glioblastoma cell invasion by PKCiota and RhoB. Cold Spring Harbour Laboratory PTEN/PI3K Pathway Signaling 2008. *(poster presentation).*

R. Mitchell Baldwin, Doris A. E. Parolin, and Ian A. J. Lorimer. Regulation of glioblastoma cell invasion by PKCiota and RhoB. *Making Connections*: A Canadian Cancer Research Conference Celebrating the National Cancer Institute of Canada's 60th Anniversary 2007. *(poster presentation).*

R. Mitchell Baldwin, Doris A. E. Parolin, and Ian A. J. Lorimer. Examining the role of PKCι in the regulation of RhoB expression and glioblastoma cell survival. Keystone Symposia: Molecular Targets for Cancer 2007. *(poster presentation).*

R. Mitchell Baldwin and Ian A.J. Lorimer. Examining the role of PKCι in the regulation of RhoB expression and glioblastoma cell survival. University of Ottawa, Department of Biochemistry, Microbiology and Immunology Graduate Research Symposia 2007. *(platform presentation).*

R. Mitchell Baldwin. The role PKCι in glioblastoma chemoresistance: PKCι repression of p38 MAPK. 6th Annual Ottawa Health Research Institute Research Day 2006. *(Invited speaker).*

R. Mitchell Baldwin and Ian A.J. Lorimer. The role of atypical protein kinase c iota in glioblastoma cell chemoresistance. University of Ottawa, Department of Biochemistry, Microbiology and Immunology Graduate Poster Day 2006. *(poster presentation).*

R. Mitchell Baldwin and Ian A.J. Lorimer. The role of atypical protein kinase c iota in glioblastoma cell chemoresistance. 96th Annual American Association for Cancer Research 2005. *(poster presentation).*

R. Mitchell Baldwin and Ian A.J. Lorimer. The role of atypical protein kinase C in the chemoresistance of glioblastoma cells to cisplatin. 5th Annual Ottawa Health Research Institute Research Day 2005. *(poster presentation).*

R. Mitchell Baldwin and Ian A.J. Lorimer. The role of atypical protein kinase c iota in the chemoresistance of glioblastoma cells. University of Ottawa, Department of Biochemistry, Microbiology and Immunology Departmental seminar 2005. *(seminar presentation).*

R. Mitchell Baldwin and Ian A.J. Lorimer. The role of atypical protein kinase c iota in glioblastoma cell chemoresistance. 4th Annual Ottawa Health Research Institute Research Day 2004. *(poster presentation).*

R. Mitchell Baldwin and Ian A.J. Lorimer. The role of atypical protein kinase c iota in glioblastoma cell chemoresistance. University of Ottawa, Department of Biochemistry, Microbiology and Immunology Graduate Poster Day 2004. *(poster presentation).*

R. Mitchell Baldwin and Roger A. Moorehead. IGF-II induced cell survival in human lung ademocarcinoma. 94th Annual American Association for Cancer Research 2003. *(poster presentation).*