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PKC-ι promotes glioblastoma cell survival by phosphorylating and inhibiting BAD through a phosphatidylinositol 3-kinase pathway

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ABSTRACT

The focus of this research was to investigate the role of protein kinase C-iota (PKC-ι) in regulation of Bad, a pro-apoptotic BH3-only molecule of the Bcl-2 family in glioblastoma. Robust expression of PKC-ι is a hallmark of human glioma and benign and malignant meningiomas. The results were obtained from the two human glial tumor derived cell lines, T98G and U87MG. In these cells, PKC-ι co-localized and directly associated with Bad, as shown by immunofluorescence, immunoprecipitation, and Western blotting. Furthermore, *in-vitro* kinase activity assay showed that PKC-ι directly phosphorylated Bad at phospho specific residues, Ser-112, Ser-136 and Ser-155 which in turn induced inactivation of Bad and disruption of Bad/Bcl-XL dimer. Knockdown of PKC-ι by siRNA exhibited a corresponding reduction in Bad phosphorylation suggesting that PKC-ι may be a Bad kinase. PKC-ι knockdown also induced apoptosis in both the cell lines. Since, PKC-ι is an essential downstream mediator of the PI (3)-kinase, we hypothesize that glioma cell survival is mediated via a PI (3)-kinase/PDK1/PKC-ι/Bad pathway. Treatment with PI (3)-kinase inhibitors Wortmannin and LY294002, as well as PDK1 siRNA, inhibited PKC-ι activity and subsequent phosphorylation of Bad suggesting that PKC-ι regulates the activity of Bad in a PI (3)-kinase dependent manner. Thus, our data suggest that glioma cell survival occurs through a novel PI (3)-kinase/PDK1/PKC-ι/BAD mediated pathway.

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1. Introduction

Glioblastoma multiforme is the most lethal form of all primary brain tumors with median survival time being less than a year. Rapid development, aggressive proliferation and susceptibility to invasion and metastasis cause poor prognosis in glioblastoma. Progress in glioblastoma therapy has remained stagnant despite rigorous therapeutic methods such as surgery, radiation and chemotherapy [1,2]. Thus, there is a pressing need to identify novel molecular targets that might help improve survival of glioblastoma patients.

Rapid glioma growth and survival have been attributed to inherently high levels of protein kinase Cs (PKC) [3]. The PKC family is comprised of twelve distinct isozymes classified on their requirement of specific activators and co-factors [4–6]. PI (3)-kinase is frequently augmented in glioblastoma due to activating mutations or amplifications in epidermal growth factor receptor (36% of the cases) or loss of function/deletion of PTEN (25% of the cases) [7,8]. Over activation of PI (3)-kinase is involved in glioblastoma cell proliferation and invasion [9,10]. The atypical PKC family member, PKC-ι is a targeted mediator in the PI (3)-kinase signal transduction repertoire [11]. PKC-ι was of special interest as it has been shown to be involved in tumor proliferation, angiogenesis, cell invasion and cell survival as well as induction of chemoresistance in several types of cancers [12–17]. PI (3)-kinase induces activation of phosphoinositide-dependent kinase (PDK) 1 which in turn phosphorylates and activates atypical PKCs [18]. Importance of PKC-ι as a signaling mediator is attributed to the fact that it can function as an oncogene [19,20].

Bcl-2 (B cell lymphoma-2) family members are vital regulators of the mitochondrial cell death machinery and are comprised of 15 members [21]. Bcl-2 and Bcl-XL promote cell survival whereas Bax, Bak and several BH3 only proteins promote apoptosis [22,23]. Among the BH3 only protein, Bad lacks the hydrophobic C-terminal domain which prevents its direct mitochondrial interaction [24,25]. Bad activity is dependent on three prime residues—Ser112, Ser136 and Ser155 [26]. Upon external death signals, these residues remain dephosphorylated which enables Bad to associate with Bcl-2/Bcl-XL [27]. Survival kinases phosphorylate these residues deactivating and disrupting Bad/Bcl-XL dimerization [28]. Dissociated Bad further binds to 14-3-3 scaffold protein and is subsequently degraded [29]. Such imbalance among the Bcl-2 family of proteins with more dependency on anti-apoptotic members is often observed to occur in cancer cells [30,31].

We have previously reported that PKC-ι is highly expressed in transformed phenotypes of human glioma and benign and malignant meningioma [32]; however, little is understood about its role in glioma cell survival. PKC-ι has been shown to inhibit the pro-apoptotic function

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of Bad in non-small cell lung cancer (NSCLC) cells when activated by nitrosoamine 4-(methylnitrosamino)-1-(3-pyridyl)-1butanone (NNK) [33]. In this study we provide evidence that PKC-ι inhibited the pro-apoptotic function of Bad to promote glioma cell survival. PI (3)-kinase inhibition, PDK1 inhibition and knockdown of PKC-ι activity upon siRNA treatment exhibited corresponding reduction in Bad phosphorylation preventing glioma cell survival. Thus, our data suggest the presence of a novel PI (3)-kinase/PDK1/PKC-ι/Bad signaling cascade. Targeting this pathway may be a possible glioblastoma targeted therapy.

2. Materials and methods

2.1. Materials

Recombinant active PKC-ι, PKC-ζ and primary antibodies for Bad and PKC-ζ were purchased from Millipore (Temecula, CA). PKC-ι primary antibody was purchased from BD Biosciences (San Jose, CA). Bad, phospho-Bad Ser-112, phospho-Bad Ser-136, phospho-Bad Ser-155, β-actin, and histone H1 primary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Bcl-XL, 14-3-3, PDK1, Poly adipose ribose polymerase (PARP), cysteine aspartic acid proteases (Caspase)-3, cytochrome C and survivin primary antibodies were purchased from Cell Signaling Technology (Danvers, MA). Horseradish peroxidase (HRP) conjugated bovine anti-goat IgG and HRP goat anti-rabbit IgG, as well as secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). HRP conjugated goat anti-mouse Goat × Mouse IgG secondary antibody was purchased from Accurate (Westbury, NY). Normal rabbit IgG was purchased from Upstate (Upstate, NY). Wortmannin, LY294002 and anti-rabbit IgG (whole molecule)-conjugated with agarose beads (1:1 v/v) were purchased from Sigma-Aldrich (St. Louis, MO). PKC-ι silencing RNA (siRNA), PKC-ζ siRNA and PDK1 siRNA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). All the chemicals were purchased from Sigma-Aldrich, Fisher Scientific (Norcross, GA), Pierce (Rockford, MO), Bio-Rad (Richmond, CA), Invitrogen (Carlsbad, CA) unless otherwise stated.

2.2. Cell culture

T98G (CRL-1690) and U87MG (HTB-14) cells were obtained from the American Tissue Culture Collection (Rockville, MD, USA). The T98G cell line was isolated as a spontaneous variant of the parental T98G cells which were derived from a glioblastoma multiforme tumor of a 61-year-old Caucasian male. The cells have a hyperpentaploid chromosome count and display immortality but are not tumorigenic in nude mice. The doubling time for T98G cells is approximately 18 h. The trait that distinguishes T98G cells from fully transformed cells is that they behave similarly to normal cells and can become arrested and stationary in G_1 phase [34]. The U87MG is classified as a grade IV astrocytoma and was isolated from highly malignant glioblastoma of a 44-year-old Caucasian female. The cells have a hypodiploid chromosome count and display epithelial-like morphology and are tumorigenic in nude mice. The doubling time of U87MG cells is approximately 32 h. Both cell lines were cultured as a monolayer in 75 cm^2 flasks containing Eagles minimum essential medium (MEM), 10% fetal bovine serum (FBS) and antibiotics (penicillin 10 U/ml and streptomycin 10 μg/ml) at 37 °C in a humidified atmosphere containing 5% CO_2.

2.3. Cell-cycle time course and cell fractionation

T98G and U87MG cells were cultured as monolayers in 150 mm tissue culture plates until 50–60% confluent, followed by serum starvation for 48 h. Subsequently, starvation media was removed and complete media was added to each plate to allow cells to complete the cell cycle (24 h for T98G and 36 h for U87MG). T98G cells were harvested every 2 h and U87MG cells were harvested every 3 h by placing the flask on ice and washing twice with ice cold 1×. Cells were subsequently scraped, re-suspended in 500 μl of homogenization buffer (50 mM HEPES, pH 7.5), 150 mM sodium chloride, 0.5% Triton X-100, 1 mM EDTA (ethylenediaminetetraaecetic acid) and 2 mM EGTA [glycol-bis(2-aminoethylether)-N, N, N′, N′-tetraacetic acid], 1 mM orthovanadate, 0.5 M sodium fluoride, 0.2 M PMSF (phenylmethylsulphonyl fluoride), 1 mM DTT (dithiothreitol), and 0.15 U/ml aprotinin [35]. Cell suspensions were subsequently sonicated and centrifuged at 16,000*g*. Protein content was measured according to Bradford [35].

2.4. Immunoprecipitation and Western blot analysis

Total protein (1 mg) was immunoprecipitated (IP) using anti-Bad or anti-PKC-ι primary antibody and subsequently subjected to Western blot analysis. Both protocols were followed according to Win H. et al. [36].

2.5. PKC activity assay

PKC activity assay was performed by suspending recombinant active PKC-ι, recombinant active PKC-ζ (0.5 μg each) individually in 200 μl of PKC kinase buffer [37] to test the ability of each PKC to phosphorylate recombinant as well as endogenously immunoprecipitated Bad. The PKC kinase buffer consisted of 20 mM Tris–HCl (pH 7.5), 6 mM magnesium acetate, phosphotidylserine (5 μg) and adenosine triphosphate (ATP) (0.96 μg). The reaction was terminated after incubation for 30 min at 30 °C by addition of sample loading buffer by placing the samples on ice. Proteins were subsequently fractionated by SDS-PAGE and analyzed using Western blot analysis.

2.6. Immunofluorescence

Approximately 1×10^6 cells were plated and grown in 2-well culture slides (Collagen type I coated). Twenty-four hours post-plating, cells were washed with cold 1× DPBS and fixed with 1:1 concentration of methanol and acetone for 5 min at −20 °C. Cells were incubated with 8% normal goat serum blocking buffer for 45 min followed by incubation with anti-mouse antibody against PKC-ι for 90 min and subsequently incubated with anti-mouse secondary antibody for 30 min followed by staining with fluorescein isothiocyanate (FITC) dye diluted in its blocking buffer (Vector Laboratories, Burlingame, CA) for 30 min. Cells were washed 3 times with 1× DPBS followed by blocking with 8% normal horse serum for 45 min. Subsequently, cells were incubated with primary anti-rabbit antibody against Bad for 90 min and then incubated with anti-rabbit secondary antibody for 30 min followed by staining with Texas red diluted in its blocking buffer. Cells were washed

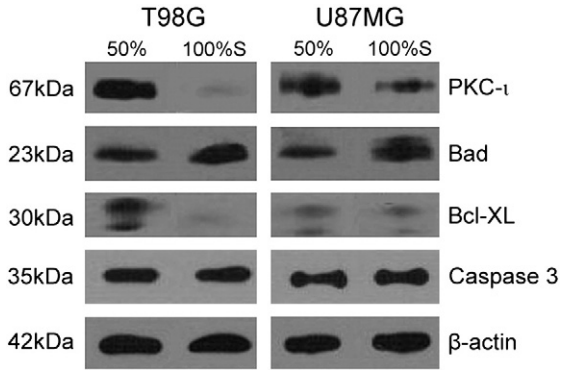

Fig. 1. Expression profile of PKC-ι, Bad and Bcl-XL in T98G and U87MG cells. PKC-ι, Bad, Bcl-XL and Caspase-3 expression levels in actively proliferating and contact inhibited plus serum starved T98G and U87MG cells were determined by Western blot analysis. β-actin indicates equal loading of the total lysate. Data is representative of N=3 independent experiments.

3 times with 1× DPBS and cell nuclei were visualized with mounting medium containing DAPI (4′, 6-diamidino-2-phenylindole) staining blue. Cells were observed with a Nikon Eclipse TE2000-U microscope. Pictures were captured in NIS-Elements F Version 2.10. To illustrate subcellular regions of protein co-localization, individual red and green-stained images derived from the same field were merged in Image-Pro Express Version 5.1.

2.7. Inhibition of gene expression

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

663: 5′-CAAGCCAAGCGUUUCAACA-3′
5′-UGUUGAAACGCUUGGCUUG-3′

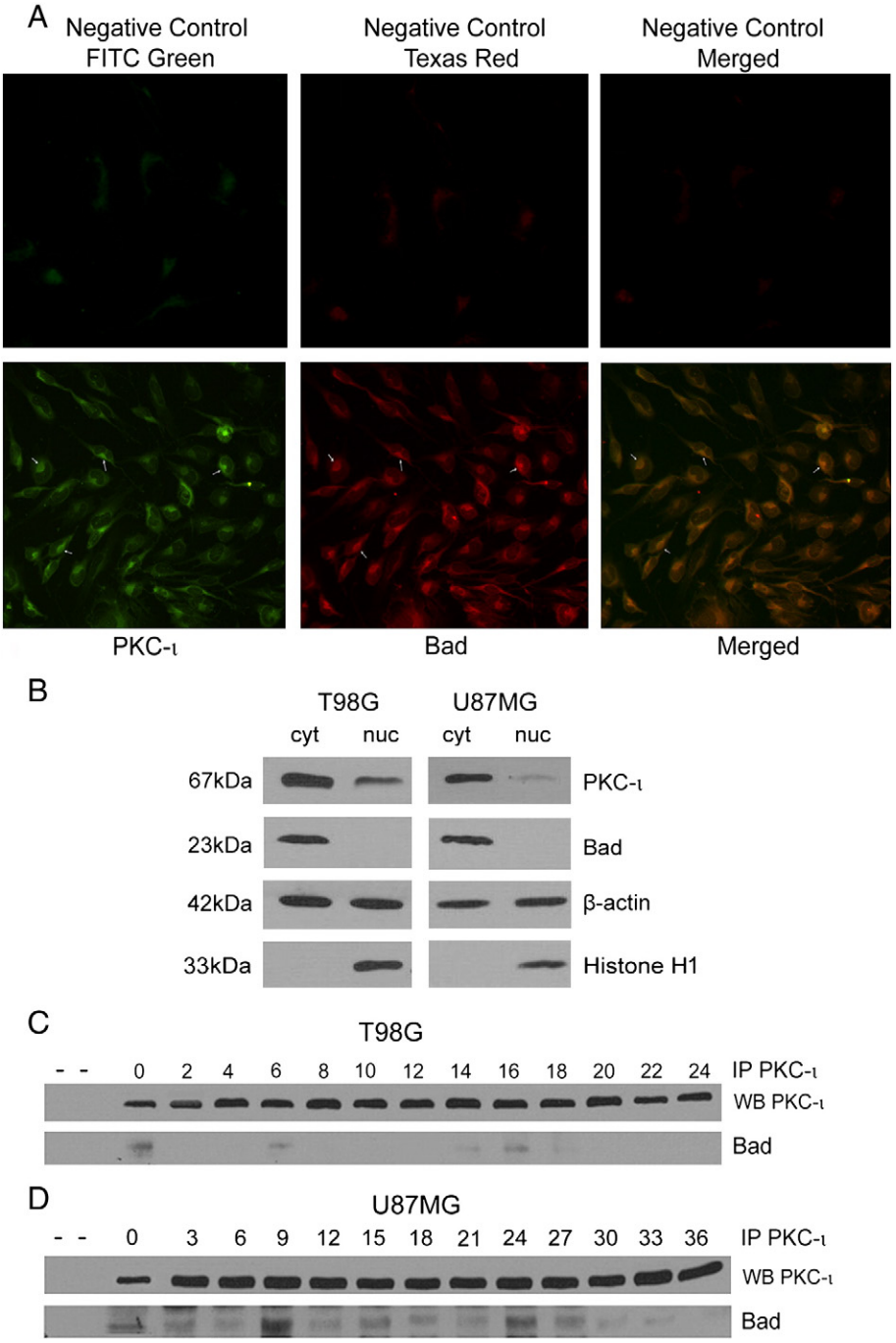

Fig. 2. PKC-ι co-localizes and directly associates with Bad. (A, top panel) Displays IF controls incubated without primary antibody. (A, bottom panel) Represents the fluorescence from FITC green used to detect PKC-ι and the fluorescence from Texas Red to detect Bad. As reported by Jin et al. [33], the merged image of green and red fluorescence (yellow color) depicts co-localization of PKC-ι and Bad in the cytosol. IF data represented are that of N=3 independent experiments. (B) Both T98G and U87MG cells were fractionated into cytoplasmic and nuclear fraction as explained in the "Materials and methods" section and Western blot analysis was performed to determine the expression of the proteins. PKC-ι is expressed in the nuclear and cytoplasmic nuclear fraction of both cells, whereas Bad is expressed only in the cytoplasmic fraction. β-actin was used as loading control. Histone H1 was used as the purity control for the nuclear fraction. (C–D) Whole cell extracts (1 mg) from each time point (every 2 h for T98G and 3 h for U87MG) were IP with PKC-ι antibody. Proteins were separated by SDS-PAGE and were immunoblotted for PKC-ι and Bad. The first negative control (−) contains whole cell extract plus rabbit IgG whole molecule (50 μl of 1:1 v/v) and the second negative control (−) contains whole cell extract plus rabbit IgG whole molecule (50 μl) and normal rabbit IgG serum (5 μg). (C) PKC-ι transiently associates with Bad in T98G cells in its 24 h cell cycle. (D) PKC-ι in U87MG cells associates with Bad at most time points compared to T98G though its expression varies throughout the cell cycle (36 h). Data represent $N=3$ independent experiments.

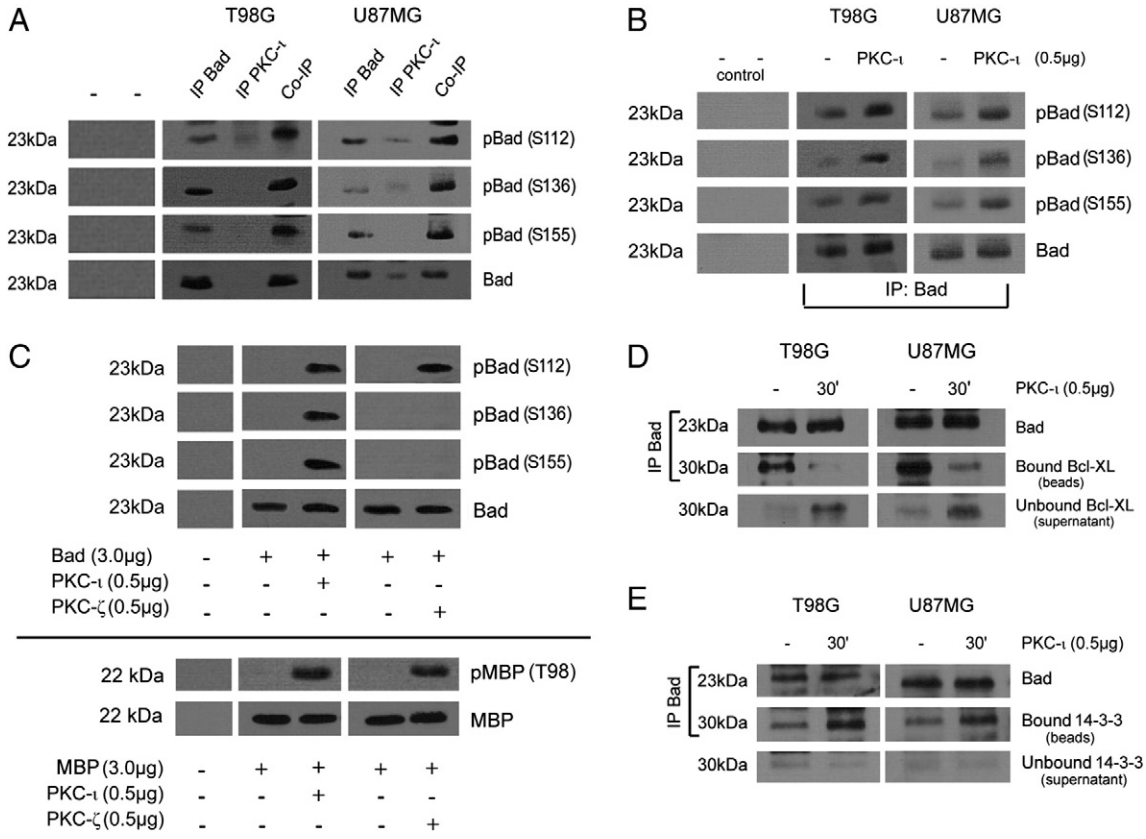

Fig. 3. PKC-ι induces direct phosphorylation of Bad. The first negative control (−) contains whole cell extract plus rabbit IgG whole molecule (50 μl of 1:1 v/v) and the second negative control (−) contains whole cell extract plus rabbit IgG whole molecule (50 μl) and normal rabbit IgG serum (5 μg). (A) IP Bad, IP PKC-ι and co-IP from T98G and U87MG cells were subjected to kinase activity assay. Phosphorylation of Bad at S112, S136, S155 and Pan Bad were quantified using Western blot analysis. (B) IP Bad from both T98G and U87MG cells was incubated with active PKC-ι (0.5 μg) in an *in-vitro* kinase activity assay as explained in "Materials and methods." Western blot analysis was performed to determine phosphorylation of Bad at S112, S136 and S155 and Pan Bad. (C, upper panel) Recombinant Bad (3.0 μg) was incubated with purified, active PKC-ι and PKC-ζ (0.5 μg) for 30 min in an *in-vitro* kinase assay. Western blot analysis was performed to determine pBad at S112, S136, S155 and Pan Bad. (C, lower panel) Recombinant MBP (3.0ug) was incubated with active PKC-ι and PKC-ζ (0.5 μg) for 30 min in an *in-vitro* kinase assay followed by Western blot analysis to determine pMBP at T98. (D) Bad/Bcl-XL complex was IP from T98G and U87MG cells and subsequently incubated with active PKC-ι (0.5 μg) for 30 min in an *in-vitro* kinase activity assay. The samples were centrifuged at 16,000*g* for 15 min. Western blot analysis was performed on the resulting supernatant and the beads to determine the amount of bound Bcl-XL, unbound Bcl-XL and Pan Bad. (E) Bad/14-3-3 complex was IP from both the cell lines and subsequently incubated with active PKC-ι (0.5 μg) for 30 min in an *in-vitro* kinase activity assay. The samples were centrifuged at 16,000*g* for 15 min. Western blot analysis was performed on the resulting supernatant and the beads to determine the amount of bound 14-3-3, Pan Bad. Western blots are representative of three independent experiments.

729: 5′-GGAACGAUUGGGUUGUCAU-3′
5′-AUGACAACCCAAUCGUUCC-3′
2137: 5′-CCCAAUAUCUUCUCUUGUA-3′
5′-UACAAGAGAAGAUAUUGGG-3′

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

The experiments performed with siRNA are as follows:

Approximately, 4×10^3 T98G cells were grown in 96 well tissue culture plates. Twenty-four hour post-plating, cells were exposed to a increasing concentrations of either PKC-ι or control siRNA (25 nM–125 nM) for 48 h. Cell proliferation was assessed using MTS assay.

Approximately, 1×10^6 cells were grown in 100 mm tissue culture plates. Twenty-four hour post-plating, cells were treated with either PKC-ι or control siRNA (100 nM). Cells were harvested every 24 h and cell viability was quantified using trypan blue exclusion assay. Western blot analysis with total protein (30 μg) was performed to determine the expression of PKC-ι and PKC-ζ. Total protein (30–100 μg) was also separated by Western blot analysis to investigate the expression of several apoptotic markers such as anti-whole PARP, anti-PARP cleavage, anti-Caspase 3, anti-cytochrome C and anti-survivin antibodies.

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

2.8. Trypan blue exclusion assay

The protocol for trypan blue exclusion assay was carried out as explained under Win. H. et al. [36].

2.9. (3-(4, 5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (MTS) assay

Approximately, 4×10^3 T98G cells were grown in 96 well tissue culture plates. Twenty-four hours post-plating, cells were treated with either PKC-ι or control siRNA (25 nM–125 nM) for 24–48 h. At the specific time points, cells were incubated for 3 h with MTS reagent according to manufacturer's instructions (Promega). Cell viability was

quantified by measuring the absorbance at 490 nm using a microtiter plate reader.

2.10. Subcellular fractionation

The protocol for subcellular fractionation (nuclear and cytoplasmic extraction) was performed according to Win H. et al. [38]. Nuclear and cytoplasmic extracts were subjected to Western blot analysis to determine PKC-ι, Bad, β-actin and histone H1 expression.

2.11. Densitometry

Intensity of each band was measured using the Quantity One, 1-D analysis software (Bio-Rad Laboratories). In order to achieve the correct intensity of each band, the background intensity was subtracted from the intensity of each band. Mean absorbance of three independent studies were compared by means of Student's *t*-test.

3. Results

3.1. PKC-ι is over expressed in actively proliferating glioma cells

In 50% confluent T98G cells (actively proliferating cells), there was an increase in the expression of PKC-ι (90-fold, $P=0.001$) and Bcl-XL (66-fold, $P=0.006$) when compared to 100% confluent plus serum starved cells (contact inhibited). Similarly, in U87MG cells, an increase in the levels of PKC-ι (50-fold, $P=0.008$) and Bcl-XL (20-fold, $P=0.01$) was observed in actively proliferating cells compared to serum starved cells. In contrast, the expression of Bad was enhanced in serum starved cells versus actively proliferating cells (44-fold in T98G, $P=0.004$ and 52-fold in U87MG, $P=0.002$). Caspase-3 expression was equal in 50% confluent and 100% serum starved cells (Fig. 1). Caspase-3 expression levels were analyzed to ensure that the decrease in the expression of PKC-ι was not an outcome of cells undergoing apoptosis. β-actin was used as the loading control.

3.2. PKC-ι co-localizes and directly associates with Bad

Cellular distribution of Bad and Bcl-XL in T98G cells was determined by double immunofluorescence (IF). Negative controls for FITC green, Texas red and their merged image exhibited minimal background immunofluorescence (IF) (Fig. 2A, top panel). PKC-ι had some nuclear staining but was stronger in the cytoplasmic region (FITC green) whereas Bad stained only in cytoplasmic fraction of the cells (Texas red) (Fig. 2A, bottom panel). Merging of the two individual frames showed that PKC-ι predominantly co-localized with Bad in the cytoplasmic region of the cells as displayed by yellow coloration (Fig. 2A, bottom panel). Small crescent shaped structures were observed near the perinuclear region of the cells, as indicated by the arrows. The identity of this structure requires further investigation. Subcellular fractionation of both T98G and U87MG cells showed that PKC-ι was present in both cytoplasmic as well as nuclear fractions ($P<0.01$ for T98G and U87MG), whereas, Bad was present only in the cytoplasmic fraction of the cells ($P=0.002$). β-actin was used as loading control. Histone H1 was used as a purity control for nuclear fraction (Fig. 2B).

To further demonstrate that PKC-ι directly associates with Bad, PKC-ι was immunoprecipitated. In T98G cells, PKC-ι was constitutively expressed at all time points and directly associated with Bad. The association, however, was transient as observed at T0, T6, T14, T16 hours (Fig. 2C). Although PKC-ι associated with Bad in U87MG cells at most time points (T0–T36 h), distinct variation in the Bad expression was observed (Fig. 2D). The reason for such expression requires further investigation.

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

Since, our data showed that PKC-ι directly associates with Bad in both T98G and U87MG cells (Fig. 2C, D), we examined whether PKC-ι is a probable kinase to Bad in glioblastoma. Significant increase in the levels of pBad at S112, S136 and S155 in co-IP (PKC-ι and Bad) samples compared to individual IP samples (Fig. 3A) suggest that Bad might be a direct downstream substrate to PKC-ι in T98G and U87MG cells. Further, when purified active PKC-ι was incubated with IP Bad in an *in-vitro* kinase assay, active PKC-ι directly phosphorylated Bad at all three serine residues (Fig. 3B, $P<0.05$ for all the residues). As reported by Jin et al. [33], our data also showed that active PKC-ι phosphorylated recombinant Bad at all three residues ($P<0.01$ for all residues) (Fig. 3C, upper panel). In contrast, when, another atypical PKC, active PKC-ζ (70% homologous to PKC-ι) was incubated with recombinant Bad in an *in-vitro* kinase assay, PKC-ζ phosphorylated Bad at only S112 residue ($P=0.02$) (Fig. 3C, upper panel) suggesting that it could also regulate Bad but would require other kinases in order to completely abrogate the pro-apoptotic function of Bad. To confirm that the activity of PKC-ζ was not compromised, PKC-ι and PKC-ζ were subjected to an *in-vitro* kinase activity assay using a common substrate, myelin basic protein (MBP). Results showed that both PKC-ι and PKC-ζ induced equivalent phosphorylation of MBP at T98, demonstrating equal activity ($P<0.01$) (Fig. 3C, bottom panel).

Furthermore, when Bad-associated Bcl-XL (bound Bcl-XL) was IP and incubated with purified active PKC-ι (0.5 μg) in an *in-vitro* kinase assay, we found decrease in the amounts of bound Bcl-XL and increase in the levels of unbound Bcl-XL (Fig. 3D) in both T98G ($P=0.013$) and U87MG cells ($P=0.002$). Additionally, when, Bad-associated 14-3-3 (bound 14-3-3) was IP and incubated with purified active PKC-ι (0.5 μg) in an *in-vitro* kinase assay, we found higher amounts of 14-3-3 in the samples incubated with PKC-ι, suggesting that increased phosphorylation of Bad (Fig. 3B) as well as Bad/Bcl-XL dissociation (Fig. 3D) led to

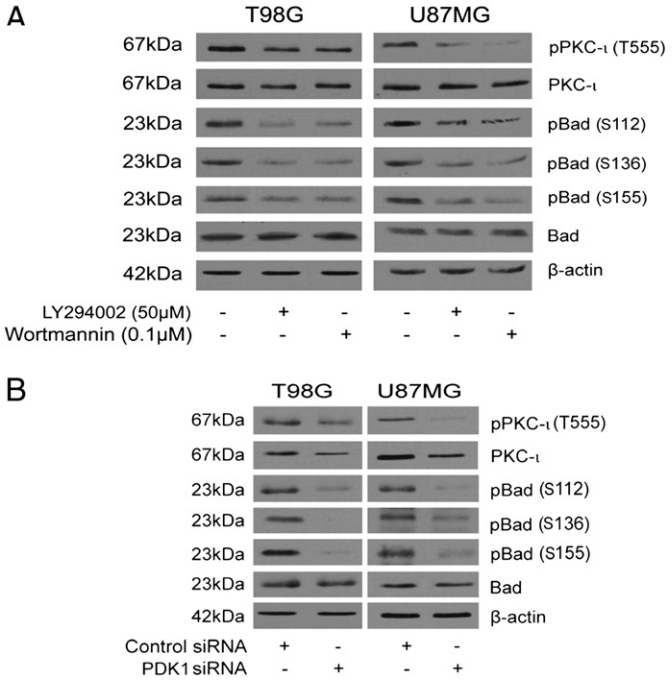

Fig. 4. Effect of PI (3)-kinase inhibition and PDK1 knockdown in T98G and U87MG cells. (A) Western blot analysis of T98G and U87MG cells for phospho-PKC-ι (T555), Pan PKC-ι, phospho-Bad (S112), (S136), (S155) and Pan Bad after individual treatment for 2 h with LY294002 (50 μM) and Wortmannin (0.1 μM). (B) Both the cells were treated with PDK1 siRNA (100 nM for 24 h) followed by Western blot analysis to detect phospho-PKC-ι (T555), phospho-Bad (S112), (S136), (S155) and Pan PKC-ι and Bad. Data are representative of three independent experiments.

increased Bad/14-3-3 dimerization (Fig. 3E) in both T98G ($P=0.03$) and U87MG cells ($P=0.023$). These findings indicated that PKC-ι is a potential upstream kinase to Bad.

3.4. Inhibition of PKC-ι activity leads to corresponding reduction in Bad phosphorylation

Previous studies showed that pharmacological inhibition of PI (3)-kinase blocked PKC-ι activity, suggesting that PI (3)-kinase is an upstream regulator of PKC-ι in glioblastoma [9]. Similarly, our results indicated that treatment of both T98G and U87MG cells with LY294002 (50 μM) and Wortmannin (0.1 μM) for 2 h blocked PKC-ι activity in the form of diminished phosphorylation at T555 residue (53% with LY294002 and 68% with Wortmannin in T98G cells), (69% with LY294002 and 83% with Wortmannin in U87MG cells). Marked reduction in the amount of Bad phosphorylation at S112, S136 and S155 was also observed (Fig. 4A).

PDK1 is a downstream mediator of PI (3)-kinase and previous studies have shown that PDK1 interacts and regulates the activity of atypical PKCs [18]. Thus, we investigated the effect of PDK1 knockdown on PKC-ι (70% homologous to PKC-ζ) activity in glioma cells. Our data showed that inhibition of PDK1 by siRNA (100 nM) inhibited the phosphorylation of PKC-ι at T555. Subsequent reduction in the levels of Bad phosphorylation at S112, S136 and S155 was also observed (Fig. 4B). Additionally, it has also been shown that in embryonic stem cells, where PDK1is knocked out, expression of atypical PKC isoforms (PKC-ζ, PRK1 and PRK2) was significantly reduced [39]. Thus, we investigated the expression levels of PKC-ι in glioma cells that were treated with PDK1 siRNA. Results showed marked reduction in expression of PKC-ι in PDK1 knockdown cells suggesting that PDK1 could also regulate PKC-ι expression; however further investigation may be required to explore this possibility.

3.5. PKC-ι siRNA potently depletes cell proliferation, cell viability and promotes apoptosis

Treatment of T98G cells with PKC-ι siRNA (25 nM–125 nM) over a 48 h time course was performed using MTS assay. The data showed that PKC-ι siRNA significantly inhibited cell proliferation of T98G cells with 71.4% at 24 h using a concentration of 100 nM ($P<0.001$) (Fig. 5A). This data confirmed the effective dose treatment to be used for further experiments. Trypan blue exclusion assay also showed that PKC-ι siRNA (100 nM) significantly inhibited cell viability of T98G (74% at 24 h and 63% at 48 h) and U87MG (85% at 24 h and 72% at 48 h) cells (Fig. 5B). Western blot analysis illustrated that PKC-ι siRNA treated cells showed diminished expression of PKC-ι in T98G (77% at 24 h and 61% at 48 h) and U87MG (80% at 24 h and 69% at 48 h) cells compared to control siRNA treated cells (Fig. 5C). The silencing effect of PKC-ι was highly specific as no cross reactivity was observed when the membrane was immunoblotted for PKC-ζ (70% homologous to PKC-ι) (Fig. 5C). In addition, PKC-ι siRNA (100 nM) treated cells displayed distinct morphological changes such as loss of cell membrane symmetry and cell shrinkage as well as detachment of

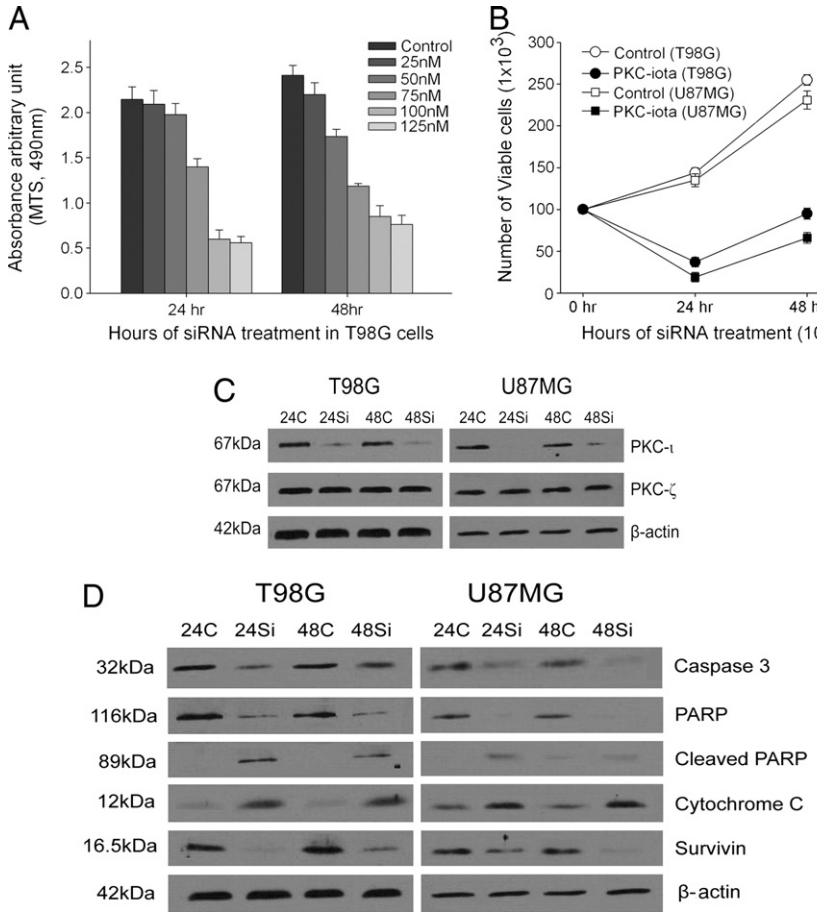

Fig. 5. PKC-ι is required for glioma cell proliferation and survival. (A) T98G cells were treated with increasing concentration of either control siRNA or PKC-ι siRNA (25 nM–125 nM) for 24 and 48 h. At each time point, cell proliferation was detected using MTS assay. Data represent $N=3$ independent studies. (B) Both T98G and U87MG cells were treated either with control siRNA or PKC-ι siRNA (100 nM) for 24–48 h. At the indicated time points, cell viability was quantified using trypan blue exclusion assay. Data represent three independent studies. (C) Western blot analysis was performed to determine the expression of PKC-ι and PKC-ζ. β-Actin displays equal loading of protein. Western blots representing three independent studies. (D) Control siRNA or PKC-ι siRNA (100 nM for 24 h) treated T98G and U87MG cells were harvested and total protein (100 μg) was analyzed by Western blot to detect PARP, PARP cleavage, Caspase-3, cytochrome C and survivin. β-Actin displays equal protein loading. Data represent $N=3$ independent experiments.

cells was observed (data not shown) indicating that cells might be undergoing apoptosis. Several apoptotic markers such as Caspase-3 activation, PARP cleavage, release of cytochrome C as well as decrease in expression of survivin were also observed (Fig. 5D).

3.6. PKC-ι knockdown diminished Bad phosphorylation, augmented Bad/Bcl-XL association and inhibited Bad/14-3-3 dimerization

When T98G and U87MG cells were treated with PKC-ι siRNA (100 nM for 24 h), Bad phosphorylation at S112, S136 and S155 was significantly inhibited in both the cell lines (Fig. 6A). Furthermore, incubation with purified, active PKC-ι (0.5 μg) restored the amount of Bad phosphorylation in PKC-ι siRNA treated cells (Fig. 6A). Also, Bad-associated Bcl-XL (bound Bcl-Xl) increased ($P=0.02$) and Bad-associated 14-3-3 decreased ($P=0.004$) in PKC-ι siRNA treated cells (Fig. 6B). In addition, when T98G and U87MG cells were treated with PKC-ζ siRNA, Bad phosphorylation at S112 ($P<0.05$) was inhibited while phosphorylation at S136 and S155 remained constitutive (Fig. 6C). These results indicated that PKC-ζ does not have the ability to completely inhibit Bad function.

4. Discussion

Glioblastoma multiforme is the most fatal of all brain tumors and new targeted therapies are critically needed. Our data supports previous findings that have acknowledged the role of recognize PKC-ι as a potential molecular target because it is involved in glioma cell proliferation, invasion and metastasis [9,32]. In the current study, we observed that PKC-ι also mediates cell survival in glioblastoma. In order to elucidate the signaling mechanism by which PKC-ι mediates its effect in glioblastoma, we investigated the role of the Bcl-2 family proteins, Bad and Bcl-XL [40].

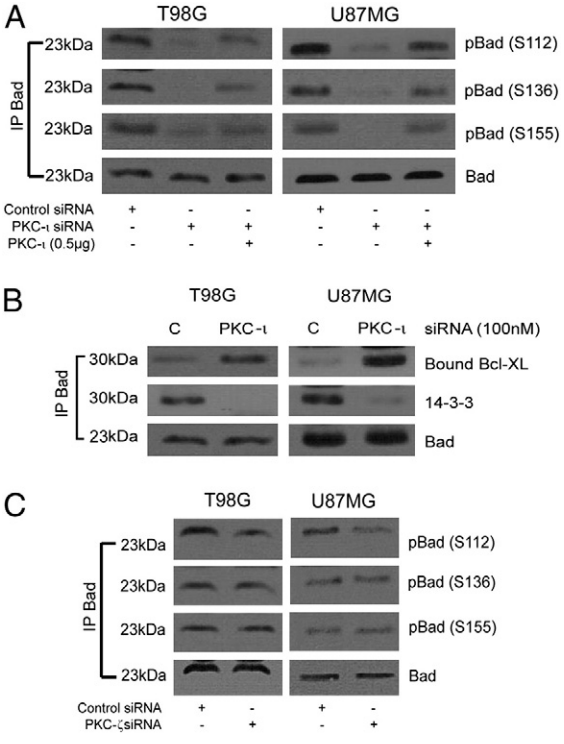

Fig. 6. Knockdown of PKC-ι exhibited corresponding reduction in Bad phosphorylation and led to increase in Bad/Bcl-XL interaction. (A) Both T98G and U87MG cells were treated with either control siRNA or PKC-ι siRNA (100 nM) for 24 h. Subsequently, Bad was IP and subjected to kinase activity assay followed by Western blot to detect pBad at S112, S136 and S155 and Pan Bad. Another set of IP Bad was incubated with purified, active PKC-ι in an *in-vitro* kinase assay to detect its effect on the levels of pBad in PKC-ι siRNA treated samples. (B) Bad was IP from both T98G and U87MG cells treated with either control or PKC-ι siRNA. Bad-associated Bcl-XL (bound Bcl-XL), Bad-associated 14-3-3 (bound 14-3-3) and Pan Bad were analyzed using Bcl-XL, Pan 14-3-3 and Bad antibodies respectively. (C) Both T98G and U87MG cells were treated with either control siRNA or PKC-ζ siRNA (100 nM) for 24 h. Subsequently, Bad was IP and subjected to kinase activity assay followed by Western blot to detect pBad at S112, S136 and S155 and Pan Bad. Data are representative of three independent experiments.

Our present data showed an inverse relation between the expression levels of PKC-ι and Bad in glioma cells, indicating a potential correlation between these two proteins. In actively proliferating cells, PKC-ι is highly expressed, complementing our previous findings [32], whereas the levels of Bad were found to be diminished. However, under serum starved conditions, where the cells were quiescent [32], the expression of PKC-ι was significantly decreased while that of Bad was higher. Recent findings of Fernando et al. have shown that overexpression of Bad in G1phase may not necessarily promote apoptosis, but it could inhibit cell proliferation [41]. Additionally, Bad has been shown to arrest G1-S progression of MCF7 breast cancer cells and this function depends on the phosphorylation state of Bad. Thus, we hypothesized that, in glioma cells, PKC-ι might be regulating the activity of Bad by phosphorylation. In order to test our hypothesis, we analyzed the association between PKC-ι and Bad in these cells. Since, we observed transient association between PKC-ι and Bad, we speculated that there could be a cell cycle dependent involvement of the two proteins. As predicted, there was transient but direct association of PKC-ι and Bad at different time points in the cell cycle. Further investigation may be required to comprehend the mechanism which induces such variable Bad expression in glioma cells. Since, the function of Bad is dictated by its three phosphorylation sites [27] and as PKC-ι directly associated with Bad, we hypothesize that PKC-ι might be an upstream kinase to Bad in glioblastoma. Our results showed that endogenous PKC-ι not only co-localized and associated with Bad but also directly phosphorylated Bad at S112, S136 and S155. Additionally, purified, active PKC-ι induced *in-vitro* phosphorylation of recombinant Bad, demonstrating that it is a potential Bad kinase. This inactivation of Bad promoted its dissociation from Bcl-XL and inhibited its ability to quench the survival function of Bcl-XL. Furthermore, phosphorylated Bad dimerized with 14-3-3, a known proteosomal scaffold protein.

PKC-ι knockdown reduced Bad phosphorylation, increased Bad/Bcl-XL interaction and decreased Bad/14-3-3 dimerization. Furthermore, active PKC-ι re-stimulated the phosphorylation of Bad in these cells confirming that PKC-ι is a potent regulator of Bad function. Such a cell survival mechanism has been previously shown in NSCLC; however, the activation of PKC-ι in these cells was NNK dependent [33]. In contrast, our results demonstrated that endogenous PKC-ι is highly activated in glioblastoma and it promotes survival of cells independent of any external stimulation.

PI (3)-kinase has been shown to induce activation of PDK1 which subsequently phosphorylates and activates PKC-ι [9,11,18]. PI (3)-kinase and PDK1 inhibition not only blocked PKC-ι activity but also inhibited Bad phosphorylation. This suggests that glioma cell survival occurs through a PI (3)-kinase signaling pathway. Intriguingly, PDK1 knockdown also significantly inhibited the endogenous expression of PKC-ι in our cells. Previous studies have shown that in embryonic stem cells, where PDK1 was knocked out, there was a marked reduction in the expression of atypical PKC isoforms (PKC-ζ, PRK1 and PRK2) [39]; however, regulation of PKC-ι expression by PDK1 has never been shown before. The mechanism by which PDK1 regulates PKC-ι expression will require further investigation.

Our data also showed that knockdown of PKC-ι by PKC-ι siRNA led to corresponding decrease in cell proliferation and initiation of apoptosis in cells grown in tissue culture which is consistent with the finding of Baldwin et al. [10]. These mechanisms contrast with those of other cancers where such an effect was observed only in anchorage independent cells signifying a tissue specific role of PKC-ι [12,42].

Collectively, our data suggest that PKC-ι promotes glioblastoma cell survival by regulating the pro-apoptotic function of Bad through

PI (3)-kinase in absence of any external stimulation. Thus, we show that PKC-ι expression may be used as a prognostic marker for the identification of glioblastoma patients that may benefit from anti-PKC-ι therapy for personalized medicine.

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References

[1] C. Parlato, M. Barbarisi, M. Moraci, A. Moraci, Surgery, radiotherapy and temozolomide in treating high-grade gliomas, Front. Biosci. 11 (2006) 1280–1283.
[2] R. Stupp, W.P. Mason, M.J. van den Bent, M. Weller, B. Fisher, M.J. Taphoorn, K. Belanger, A.A. Brandes, C. Marosi, U. Bogdahn, et al., Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma, N. Engl. J. Med. 352 (2005) 987–996.
[3] W. Couldwell, J.P. Antel, M.J.L. Apuzzo, V.W. Yong, Inhibition of growth of established human glioma cell lines by modulators of the protein kinase C system, J. Neurosurgery. 73 (1990) 594–600.
[4] M. Inoue, A. Kishimoto, Y. Takai, Y. Nishizuka, Studies on cyclic nucleotide-independent protein kinase and its proenzyme in mammalian tissues. II. Proenzyme and its activation by calcium-dependent protease from rat brain, J. Biol. Chem. 252 (1977) 7610–7616.
[5] A. Kishimoto, Y. Takai, T. Mori, U. Kikkawa, Y. Nishizuka, Activation of calcium and phospholipid-dependent protein kinase by diacylglycerol, its possible relation to phosphotidylinositol turnover, J. Biol. Chem. 255 (1980) 2273–2276.
[6] M. Castagna, Y. Takai, K. Kaibuchi, K. Sano, U. Kikkawa, Y. Nishizuka, Direct activation of calcium-activated, phospholipid-dependent protein kinase by tumor-promoting phorbol esters, J. Biol. Chem. 257 (1982) 7847–7851.
[7] Ohgaki Hiroko, Genetic pathways to glioblastomas, Neuropathology 25 (2005) 1–7.
[8] S. Kesari, N. Ramakrishna, C. Sauvageot, C.D. Stiles, P.Y. Wen, Targeted molecular therapy of malignant gliomas, Curr. Neurol. Neurosci. Rep. 5 (2005) 186–197.
[9] R.M. Baldwin, D.A.E. Parolin, I.A.J. Lorimer, Regulation of glioblastoma cell invasion by PKCι and RhoB, Oncogene 27 (2008) 1–9.
[10] R.M. Baldwin, G.M. Barrett, D.A.E. Parolin, J.K. Gillies, J.A. Paget, S.J. Lavictoire, D.A. Gray, I.A.J. Lorimer, Coordination of glioblastoma cell motility by PKCι, Mol. Cancer 9 (2010) 233–245.
[11] K. Akimoto, R. Takahashi, S. Moriya, N. Nishioka, J. Takayanagil, K. Kimura, Y. Fukuil, S. Osada, K. Mizuno, S. Hirai, A. Kazlauskas, S. Ohno, EGF or PDGF receptors activate atypical PKCλ through phosphatidylinositol 3-kinase, EMBO J. 15 (1996) 788–798.
[12] N.R. Murray, A.P. Fields, Atypical protein kinase C iota protects human leukemia cells against drug induced apoptosis, J. Biol. Chem. 272 (1997) 27521–27524.
[13] R.P. Regala, C. Weems, L. Jamieson, J.A. Copland, E.A. Thomson, A.P. Fields, Atypical protein kinase Cι plays a role in human lung cancer cell growth and tumorigenicity, J. Biol. Chem. 180 (2005) 31109–31115.
[14] R.M. Baldwin, M. Garette-Lalonde, D.A.E. Parolin, P.M. Krzyzanowski, M.A. Andrade, I.A.J. Lorimer, Protection of glioblastoma cells from cisplatin cytotoxicity via protein kinase Cι-mediated attenuation of p38 MAP kinase signaling, Oncogene 25 (2006) 2909–2919.
[15] R.P. Regala, R.K. Davis, A. Kunz, A. Khoor, M. Leitges, A.P. Fields, Atypical protein kinase C iota is required for bronchioalveolar stem cell expansion and lung tumorigenesis, Cancer Res. 69 (2009) 7603–7611.
[16] E.M. Rodriguez, E.E. Dunham, G.S. Martin, Atypical protein kinase C activity is required for extracellular matrix degradation and invasion by Src-transformed cells, J. Cell. Physiol. 22 (2009) 171–182.
[17] R. Takagawa, K. Akimoto, Y. Ichikawa, H. Akiyama, Y. Kojima, H. Ishiguro, Y. Inayama, I. Aoki, C. Kunisaki, I. Endo, Y. Nagashima, S. Ohno, High expression of atypical protein kinase C lambda/iota in gastric cancer as a prognostic factor for recurrence, Ann. Surg. Onco. 17 (2010) 81–88.
[18] A. Balendran, R.M. Biondi, P.C.F. Cheung, A. Casamayor, M. Deak, D.R. Alessi, A 3-phosphoinositide-dependent protein kinase-1 (PDK1) docking site is required for the phosphorylation of protein kinase Cζ (PKCζ) and PKC-related kinase 2 by PDK1, J. Biol. Chem. 275 (2000) 20806–20813.
[19] R.P. Regala, C. Weems, L. Jamieson, A. Khoor, E.S. Edell, C.M. Lohse, A.P. Fields, Atypical protein kinase Cι is an oncogene in human non-small cell lung cancer, Cancer Res. 65 (2005) 8905–8911.
[20] L. Zhang, J. Huang, N. Yang, S. Liang, A. Barchetti, A. Giannakakis, M.G. Cadungog, A. O'Brien-Jenkins, M. Massobrio, K.F. Roby, et al., Integrative genomic analysis of protein kinase C (PKC) family identifies PKCiota as a biomarker and potential oncogene in ovarian carcinoma, Cancer Res. 66 (2006) 4627–4635.
[21] Z. Chen-Levy, J. Nourse, M.L. Cleary, The bcl-2 candidate protooncogene product is a 24-kilodalton integral-membrane protein highly expressed in lymphoid cell lines and lymphomas carrying the t(14;18) translocation, Mol. Cell. Biol. 9 (1989) 701–710.
[22] M. Wei, W. Zong, E. Cheng, T. Lindsten, V. Panoutsakopoulou, A. Ross, K. Roth, G. MacGregor, C. Thompson, S. Korsmeyer, Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death, Science 292 (2001) 727–730.
[23] M. Suzuki, R.J. Youle, N. Tjandra, Structure of Bax coregulation of dimer formation and intracellular localization, Cell 103 (2000) 645–654.
[24] A. Kelekar, C.B. Thompson, Bcl-2-family proteins: the role of the BH3 domain in apoptosis, Trends Cell Biol. 8 (1998) 324–330.
[25] A.M. Petros, E.T. Olejniczak, S.W. Fesik, Structural biology of the Bcl-2 family of proteins, Biochimi. Biophys. Acta. 1644 (2004) 83–94.
[26] Y. Tan, M.R. Demeter, H. Ruan, M.J. Comb, BAD Ser 155 phosphorylation regulates BAD/Bcl-XL Interaction and cell survival, J. Biol. Chem. 275 (2005) 25865–25869.
[27] D.C. Huang, A. Strasser, BH3-only proteins—essential initiators of apoptotic cell death, Cell 103 (2000) 839–842.
[28] I. Hirai, H.G. Wang, Survival-factor-induced phosphorylation of Bad results in its dissociation from Bcl-xL but not Bcl-2, Biochem. J. 359 (2001) 345–352.
[29] S.R. Datta, A. Kaisov, L. Hu, A. Petros, S.W. Fesik, M.B. Yaffe, M.E. Greenberg, 14-3-3 Proteins and survival kinases cooperate to inactivate BAD by BH3 domain phosphorylation, Mol. Cell 6 (2000) 41–51.
[30] P. Juin, O. Genesteb, E. Raimbaudb, J.A. Hickman, Shooting at survivors: Bcl-2 family members as drug targets for cancer, Biochim. Biophys. Acta. 1644 (2004) 251–260.
[31] H. Strik, M. Deininger, J. Streffer, E. Grote, J. Wickboldt, J. Dichgans, M. Weller, R. Meyermann, BCL-2 family protein expression in initial and recurrent glioblastomas: modulation by radiochemotherapy, J. Neurol. Neurosurg. Psychiatry 67 (1999) 763–768.
[32] R. Patel, H. Win, S. Desai, K. Patel, J.A. Matthews, M. Acevedo-Duncan, Involvement of PKC-ι in glioma proliferation, Cell Prolif. 41 (2008) 122–135.
[33] Z. Jin, M. Xin, X. Deng, Survival function of protein kinase Cι as a novel nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone-activated Bad kinase, J. Biol. Chem. 280 (2005) 16045–16052.
[34] J. Ponten, E.H. MacIntyre, Long term culture of normal and neoplastic glia, Acta Pathol. Microbiol. Scand. 74 (1968) 465–486.
[35] M.M. Bradford, A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the property of protein-dye binding, Anal. Biochem. 72 (1976) 248–254.
[36] H.Y. Win, M. Acevedo-Duncan, Role of protein kinase C-iota in transformed non-malignant RWPE-1 cells and androgen-independent prostate carcinoma DU-145 cells, Cell Prolif. 42 (2009) 182–194.
[37] J.S. Davis, M.R. Clark, Activation of protein kinase in the bovine corpus luteum and Ca^{2+}, Biochem. J. 214 (1983) 569–574.
[38] H.Y. Win, M. Acevedo-Duncan, Atypical protein kinase C phosphorylates IKKαβ in transformed non-malignant and malignant prostate cell survival, Cancer Lett. 270 (2008) 302–311.
[39] B.J. Collins, M. Deak, V. Murray-Tait, K.G. Storey, D.R. Alessi, In vivo role of the phosphate groove of PDK1 defined by knockin mutation, J. Cell Sci. 118 (2005) 5023–5034.
[40] N.N. Danial, BAD: undertaker by night, candyman by day, Oncogene 27 (2009) S53–S70.
[41] R. Fernando, J.S. Foster, A. Bible, A. Ström, R.G. Pestell, M. Rao, A. Saxton, S. Joon Baek, K. Yamaguchi, R. Donnell, M. Cekanova, J. Wimalasena, Breast cancer cell proliferation is inhibited by BAD: regulation of cyclin D1, J. Biol. Chem. 282 (2007) 28864–28873.
[42] M.L. Scotti, W.R. Bamlet, T.C. Smyrk, A.P. Fields, N.R. Murray, Protein kinase Cι is required for pancreatic cancer cell transformed growth and tumorigenesis, Cancer Res. 70 (2010) 2064–2074.