Regulation of Cdk7 activity through a phosphatidylinositol (3)-kinase/PKC-\(\i\)-mediated signaling cascade in glioblastoma

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The objective of this research was to study the potential function of protein kinase C (PKC)-\(\i\) in cell cycle progression and proliferation in glioblastoma. PKC-\(\i\) is highly overexpressed in human glioma and benign and malignant meningioma; however, little is understood about its role in regulating cell proliferation of glioblastoma. Several upstream molecular aberrations and/or loss of PTEN have been implicated to constitutively activate the phosphatidylinositol (PI) (3)-kinase pathway. PKC-\(\i\) is a targeted mediator in the PI (3)-kinase signal transduction repertoire. Results showed that PKC-\(\i\) was highly activated and overexpressed in glioma cells. PKC-\(\i\) directly associated and phosphorylated Cdk7 at T170 in a cell cycle-dependent manner, phosphorylating its downstream target, cdk2 at T160. Cdk2 has a major role in inducing G1–S phase progression of cells. Purified PKC-\(\i\) phosphorylated both endogenous and exogenous Cdk7, PKC-\(\i\) downregulation reduced Cdk7 and cdk2 phosphorylation following PI (3)-kinase inhibition, phosphatidylinositol-dependent kinase 1 knockdown as well as PKC-\(\i\) silencing (by siRNA treatment). It also diminished cdk2 activity. PKC-\(\i\) knockdown inhibited overall proliferation rates and induced apoptosis in glioma cells. These findings suggest that glioma cells may be proliferating through a novel PI (3)-kinase/PKC-\(\i\)/Cdk7/cdk2-mediated pathway.

Introduction

Glioblastoma multiforme is the most lethal form of primary brain tumor having a median survival time of 1 year. Its high propensity of invading the surrounding healthy brain prevents good prognosis in glioblastoma patients. Aggressive proliferation, rapid infiltration and late diagnosis are hallmarks of glioblastoma that have rendered glioblastoma incurable. Despite advances in various therapeutic methods such as surgery, radiation and chemotherapy, the etiology of glioblastoma has remained constant (1,2). Therefore, a better understanding of glioblastoma cells, proliferative and survival mechanisms and methods to achieve its inhibition are appropriate strategies to improve glioblastoma treatment.

Inherent overexpression of protein kinase Cs (PKC) (3,4) is a characteristic of many cancers. Several studies have shown that PKC hyperactivity is concordant with the malignant growth rates in gliomas. The PKC family of Ser/Thr kinases comprises 12 distinct isoforms classified in three classes based on their requirement of specific activators and cofactors (5–7). The atypical PKCs-\(\i\) and \(\zeta\) are stimulated by protein–protein interaction and by phospholipids that are involved in mechanistic pathways that control cellular responses such as growth, proliferation, survival and apoptosis (8).

Frequent mutations/deletion in the PTEF gene (15–40% of the cases) as well as overexpression (40% of the cases) or amplifications (60–70% of the cases) in epidermal growth factor receptor occur in glioblastoma (9,10). These constitutively augment phosphatidylinositol (PI) (3)-kinase activity stimulating uncontrolled growth and survival in glioblastoma (11,12). PI (3)-kinase induces activation of phosphatidylinositol-dependent kinase (PDK) 1 which in turn phosphorylates and activates atypical PKCs (13). PKC-\(\i\) is a targeted mediator in the PI (3)-kinase signal transduction repertoire (14). PKC-\(\i\) is the most common genomic amplicon as identified by comparative genomic hybridization and has been recognized as an oncogene (15–17). It stimulates cell survival and prevents apoptosis in non-small-cell lung cancer, prostate cancer and gastric carcinoma (18–20). PKC-\(\i\) hyperactivation is also responsible for chemoresistance in chronic myelogenous leukemia cells and glioblastoma (21,22).

Cdk7, a member of cyclin-dependent protein kinase family, is a master cell cycle regulator (23). It is part of a trimeric CAK (cyclin-dependent kinase activating kinase) complex (comprising of Cdk7, cyclin H and MAT1) that phosphorylate and activate downstream cdks (24). Cdk7 is the only CAK identified that regulates eukaryotic cell cycle (25). Among all the cdks activated by Cdk7, cdk2 co-ordinates the transition from Gap 1 (G1) phase to the DNA synthesis (S) phase of the cells (26) and regulates multiple cell cycle events such as DNA replication, centrosome duplication and transcription. Although, cdk2 is activated during cell cycle progression, it is the phosphorylation of its substrates that regulates the ordered cellular changes for cell division (27).

PKC-\(\i\) is highly expressed in transformed phenotypes of human glioma and benign and malignant meningioma (28,29); and a cross talk occurs between PKC-\(\i\) and Cdk7 in glioma and prostate cancer cells. Nevertheless, the role of PKC-\(\i\) in cell cycle regulation is not yet completely defined. In the current study, we provide evidence that PKC-\(\i\) directly associated and phosphorylated Cdk7 in a cell cycle-dependent manner. We also demonstrate that PI (3)-kinase and PDK1 inhibition suppressed PKC-\(\i\) activity and exhibited corresponding reduction in Cdk7 and cdk2 phosphorylation. Direct PKC-\(\i\) knockdown by silencing RNA (siRNA) not only inhibited Cdk7, cdk2 phosphorylation but also induced apoptosis and abrogated glioma cell proliferation. Thus, our data suggest that glioma cells may be proliferating through a novel PI (3)-kinase/PKC-\(\i\)/Cdk7 signaling cascade that can be targeted for glioblastoma therapy.

Material and methods

Materials

Recombinant active PKC-\(\i\) and primary antibody for PKC-\(\zeta\) were purchased from Millipore (Temecula, CA). PKC-\(\i\) primary antibody was purchased from BD Biosciences (San Jose, CA). Cdk7, cdk2 and \(\beta\)-actin primary antibodies, PKC-\(\i\) siRNA and PDK1 siRNA were purchased from Santa Cruz Biotechnolog (Santa Cruz, CA). pcdk7 primary antibody was purchased from Abcam (Cambridge, MA). pcdk2, caspase 9 and cleaved caspase 9 primary antibodies were purchased from Cell Signaling Technology (Danvers, MA). Secondary antibodies were purchased from Santa Cruz Biotechnology. Wortmannin, LY294002 and anti-rabbit IgG (whole molecule)-conjugated with agarose beads (1:1 vol/vol) were purchased from Sigma–Aldrich (St Louis, MO). Apodetc Annexin V-FITC kit and Vybrant Carboxyfluorescein diacetate succinimidyl ester (CFDA-SE) Cell kit were bought from Invitrogen (Grand Island, NY). Nuclear and cytoplasmic kit was purchased from Pierce (Rockford, MO).

Cell culture

T98G (CRL-1690) and U87MG (HTB-14) cells, obtained from the American Tissue Culture Collection (Rockville, MD), were developed by Ponten et al. (30). Both cell lines were cultured as a monolayer in 75 cm\(^2\) flasks containing Eagles minimum essential medium, 10% fetal bovine serum and antibiotics (penicillin 10 U/ml and streptomycin 10 \(\mu\)g/ml) at 37°C in a humidified atmosphere containing 5% CO\(_2\).

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

Cell fractionation, immunoprecipitation and western blot analysis
Cells were harvested by placing the flask on ice and washing twice with ice-cold 1× PBS. Cells were subsequently scraped, resuspended in 500 μl of homogenization buffer (50 mM HEPES, pH 7.5), 150 mM sodium chloride, 0.5% Triton X-100, 1 mM ethylenediaminetetraacetic acid and 2 mM ethyl-enediacyloxy-2-(aminoethyl)ethylenediamine (N, N’, N’-tetraacetic acid, 1 mM orthophosphate, 0.5 M sodium fluoride, 0.2 M phenylmethylsulphonyl fluoride, 1 mM dithiothreitol and 0.15 U/ml aprotinin. Cell suspensions were sonicated and centrifuged at 16 000 g for 16 h. Total protein content was measured according to Bradford (31) and whole cell lysates were prepared and subsequently separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and analyzed using western blot analysis. Total protein (1 mg) was immunoprecipitated (IP) using anti-Cdk7 or anti-PKC-1 or both primary antibodies and subsequently separated by SDS–PAGE and analyzed using western blot analysis. Blots were blocked with 5% non-fat dry milk for 2 h followed by incubation with Cdk7, cdk2, pCdk7, pcdk2, PKC-1 and β-actin primary antibodies and subsequently with horseradish peroxidase-conjugated secondary antibodies. Protein expressions were detected by enhanced chemiluminescence.

PKC activity assay
PKC activity assay was performed by suspending recombinant active PKC-1 (0.5 μg each) in 200 μl of PKC kinase buffer (32) to test the ability of each PKC to phosphorylate endogenously IP Cdk7. The PKC kinase buffer consisted of 20 mM Tris–HCl (pH 7.5), 6 mM magnesium acetate, phosphatidylserine (0.5 μg/ml) and adenosine triphosphate (0.06 μg/ml). The reaction was terminated after incubation for 30 min at 30°C by addition of sample loading buffer and placing the samples on ice. Proteins were subsequently fractionated by SDS–PAGE and analyzed using western blot analysis to detect Cdk7, phospho-Cdk7 (T170) and PKC-1.

Inhibition of PKC-1 gene expression by siRNA
PKC-1 siRNA was a pool of three combined RNA sequences for targeting PKC-1 (28) and control siRNA contained a scrambled sequence, which did not lead to specific degradation of any known cellular messenger RNA and whose sequence is proprietary of Santa Cruz Biotechnology. The experiments performed with siRNA are as follows.

Approximately, 1×10^6 cells were grown in 100 mm tissue culture plates. Twenty-four hours post-plating, cells were transfected with either PKC-1 or control siRNA (100 nM) using siRNA transfection reagent (formulation is proprietary of Santa Cruz Biotechnology). Cells were harvested after 24 h treatment and western blot analysis with total protein (30 μg) was performed to determine the expression of PKC-1, pPKC-1 (T555), Cdk7 and pCdk7 (T170), cdk2 and pckd2 (T160).

Subcellular fractionation
Cells were harvested in a cell cycle-dependent manner and subsequently separated into nuclear and cytoplasmic extracts. The protocol for subcellular fractionation was performed according to manufacturers instructions. Extracts were subjected to western blot analysis to determine PKC-1, Cdk7, cdk2 and histone H2 expression.

Annexin VPI staining apoptosis detection
The cells were stained according to the manufacturer’s instructions with Annexin V-fluorescein isothiocyanate (FITC) at room temperature for 10 min. Following a wash with binding buffer, the cells were stained with PI and immediately analyzed via Canto II (BD Biosciences) and FACsDIVA software. Ultraviolet (UV) irradiated T98G cells, the apoptosis-positive controls, were generated by UV light exposure via a transilluminator (UVP) for 20 or 25 min and 20 000 events were collected for each sample for analysis.

CFDA-SE staining dilation assay
One day prior to siRNA transfection experiments, T98G cells were resuspended in 1 μM of CFDA-SE solution (Molecular Probes/Invitrogen, Carlsbad, CA) and incubated for 15 min at 37°C. Following an incubation in complete medium for 30 min, the CFDA-SE-labeled cells were seeded into six-well plates at 2×10^5 cells per well. T98G cells were harvested at 48 h post-siRNA or mock siRNA transfections and stained with 7-Aminoactinomycin D (7-AAD) (BD Biosciences, San Jose, CA) for examination by two color flow cytometry of cell division and exclusion of dead cells with CFDA-SE and 7-AAD, respectively. The live gate was established by excluding the 7-AAD-stained cells. Ten thousand events were collected for each sample and analysis was performed via Canto II and FACsDIVA software.

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

Densitometry
Intensity of each band was measured using the Quantity One, 1-D analysis software (Bio-Rad Laboratories). Experiments were performed a total of three times in triplicate. Mean absorbance of the three independent studies was compared by means of Student’s t-test.

Results
PKC-1, Cdk7 and cdk2 are overexpressed in rapidly proliferating glioma cells
The expression of PKC-1, Cdk7 and cdk2 were higher in 50% confluent T98G cells (rapidly proliferating cells) than in 100% confluent plus serum-starved cells (contact inhibited) (Figure 1A). A similar but less robust decrease in the levels of PKC-1, Cdk7 and cdk2 in 100% confluent cells occurred in U87MG cells (Figure 1A). Constitutive expression of Caspase-3 showed that the decrease in the expression of PKC-1 was not due to cells undergoing apoptosis. β-actin was used as the loading control. The analyses of the cytoplasmic and nuclear expression of these proteins revealed a higher expression profile in actively proliferating glioma cells (Figure 1B). In T98G cells, expression of PKC-1 and cdk2 were significantly depleted in 100% confluent plus serum-starved cells (contact inhibited) versus proliferating cells (Figure 1B, T98G). Whereas, Cdk7 expression was reduced by only 2-fold in serum-starved T98G cells. The overall difference in the levels of PKC-1 and cdk2 was not especially robust in the subcellular fractions of U87MG cells (actively proliferating versus serum starved) in comparison with T98G cells (Figure 1B, U87MG). Thus, the results suggest that PKC-1, Cdk7 and cdk2 may be involved in glioma cell proliferation.
PKC-ι, Cdk7 and cdk2 regulate glioma cell cycle

Since, our data in Figure 1 suggested a potential cross talk between PKC-ι, Cdk7 and cdk2 in proliferation of glioma cells and Cdk7 and cdk2 are known cell cycle regulators, it was important to understand whether there was a correlation between these proteins in regulating glioma cell cycle. We analyzed the DNA content of both T98G and U87MG cells at time points over 36 h. Initially, the cells were populated (87% in T98G and 65% in U87MG) in the Gap1 (G1) phase. There was progression into S phase from 18 until 24 h, followed by increase in Gap2 (G2 phase) by 30 h, suggesting that the cells were proliferating. Overall, these relationships were less evident in U87MG compared with T98G cells because of the innate asynchronous nature of U87MG cells. A robust non-specific band was observed below the phosphorylated form of Cdk7 (Figure 2C and G). Determining the identity of this band requires further investigation.

PKC-ι is an upstream Cdk7 kinase

We recently demonstrated that PKC-ι is an in vitro kinase to Cdk7 (33). Here, we investigated whether PKC-ι/Cdk7-mediated signaling may also be occurring in glioma cells. We measured a significant increase in pCdk7 at T170 in co-IP (PKC-ι and Cdk7) samples compared with individual IP samples (Figure 3A). In contrast, pcdk2 at T160 was observed only in IP Cdk7 samples and not in IP PKC-ι samples suggesting that PKC-ι may not be a cdk2 kinase. Moreover, a 3-fold increase in pcdk2 at T160 was observed in co-IP samples (PKC-ι and Cdk7), suggesting that the presence of PKC-ι probably increased Cdk7 activity, which may have promoted a more robust downstream phosphorylation and activation of cdk2. In addition, when purified, active PKC-ι was incubated with IP Cdk7 in an in vitro kinase assay, direct phosphorylation of Cdk7 at T170 was observed (Figure 3B, P < 0.05). These results suggest that PKC-ι is an upstream Cdk7 kinase and that glioma cells may be progressing through PKC-ι/Cdk7/cdk2-mediated signaling.

PKC-ι knockdown led to reduction in expression and activity of Cdk7 and cdk2

To further prove the involvement of a PKC-ι/Cdk7/cdk2 proliferation pathway in glioma cells, both T98G and U87MG cells were treated with PKC-ι siRNA (100 nM for 24 h). Western blot analysis illustrated that PKC-ι siRNA-transfected cells showed diminished expression of PKC-ι in T98G (81%, P < 0.05) and U87MG (77%, P < 0.05) cells compared with control siRNA-transfected cells (Figure 4A). The Cdk7 phosphorylation at T170 and cdk2 phosphorylation at T160 was significantly inhibited in both cell lines when transfected with PKC-ι siRNA (Figure 4A, P < 0.005). In addition, a significant reduction in the expression of total Cdk7 and cdk2 was also seen. This result supports our earlier data (Figures 1 and 2) (34) that showed that the expression of total Cdk7 and cdk2 is crucial for glioma cell proliferation. Furthermore, the reduction in PKC-ι induced Caspase 9 cleavage (Figure 4B, P < 0.05) suggesting that the cells are undergoing apoptosis via the intrinsic mitochondrial pathway. PKC-ι knockdown also decreased the phosphorylation of the retinoblastoma (Rb), a tumor suppressor gene (P < 0.05) and increased the amount of p27kip1, a known Cdk inhibitor (P < 0.05). These results suggest that PKC-ι is involved in regulating Rb and p27kip1, which play essential roles in cell cycle machinery.

Inhibition of PKC-ι activity preceded a reduction in Cdk7/cdk2 phosphorylation

Treatment with LY294002 (50 μM) or Wortmannin (0.1 μM) for 2 h blocked PKC-ι activity by diminishing its phosphorylation at the T555 residue in both T98G cells and U87MG cells (Figure 5A, P < 0.05). Marked reduction in the amount of Cdk7 phosphorylation at T170 and cdk2 phosphorylation at T160 was also observed (Figure 5A, P < 0.05), suggesting the PKC-ι regulates the expression of these proteins as previously observed (Figures 2–4). β-actin was used as a loading control. Inhibition of PDK1 by siRNA (100 nM) diminished the endogenous amount of PKC-ι, which subsequently inhibited the activity of PKC-ι by 2.5-fold (pPKC-ι at T555) (Figure 5B, P < 0.05). Correspondingly, a reduction in the levels of Cdk7 phosphorylation at T170 and cdk2 phosphorylation at T160 was observed (Figure 5B, P < 0.05). A modest reduction in total Cdk7 (20% in T98G and 28% in U87MG) and cdk2 (27% in T98G and 30% in U87MG) levels were also observed, further supporting our data that PKC-ι might be regulating the expression of Cdk7 and cdk2. These results indicate that the PI (3)-kinase/PDK1 regulate the activity and expression of PKC-ι, Cdk7 and cdk2 in these cells.

PKC-ι knockdown potently inhibited cell proliferation, cell progression and promoted apoptosis

The distribution pattern of cell cycle phases following PKC-ι silencing in T98G cells demonstrated significant changes in cell cycle phases. A significant (2-fold) increase in G2 phase was seen following 48 h post-siRNA transfection compared with control cells. In
addition, a significant increase in cell death was observed suggesting that the cells might be going from G2/M arrest directly into apoptosis after 48 h PKC-i siRNA treatment (Figure 6A). PKC-i downregulation along with the subsequent inhibition of cell proliferation and increase in cell death led us to examine the specific assessment of cell death rates after siRNA transfection in T98G cells. The average death rate obtained with the Trypan blue method demonstrated similar cell death rates to those of the Annexin V-FITC/PI assay for each treatment group under investigation (Figure 6B and C). PKC-i siRNA treatment and both UV exposures generated significantly higher percentages of overall cell death compared with the no treatment group and the control siRNA group (Figure 6B). Nevertheless, the 25 min UV exposure produced significantly more overall cell death than any of the treatment groups (Figure 6B). Further analysis of Annexin V-FITC/PI data provided an additional perspective on the change in the cell death distribution patterns following the downregulation of PKC-i in T98G cells. The T98G cells were so resilient that UV irradiation for <20 min did not induce much cell death (data not shown). PKC-i siRNA exposure or 25 min exposure to UV irradiation induced a 34% overall apoptotic rate which was significantly more than that observed in untreated cells or the control siRNA-treated cells (Table I). Notably, it was found that 20 min was the threshold for the T98G cell’s capacity to maintain membrane integrity during continuous exposure to UV irradiation. Both UV exposure times induced significantly more late apoptosis than PKC-i siRNA-treated T98G cells whereas PKC-i silencing produced the most significant level of early apoptosis in comparison with all treatments (Table I). Furthermore, both exposure times to UV irradiation led to significantly more necrotic T98G cells than any other treatments (Table I).

It was important to gain further understanding of the extent to which PKC-i modulates the mitotic phase of the cell cycle (as seen in Figure 6A). Therefore, the proliferation rate was measured by the
CFDA-SE dilution assay (i.e. a shift demonstrating a decrease in fluorescence intensity). By 48 h, the proliferation rates for the no treatment group and control siRNA group (also fixed CFDA-SE-positive control) were significantly greater than that of PKC-i-silenced T98G cells (Figure 6D and E). By simultaneously measuring the actively proliferating cells and non-viable cells, it was confirmed that many of the cells were going from G2/M arrest directly to apoptosis after PKC-i inhibition (Figure 6D and F).

**Discussion**

PKC-i, an atypical isoform of PKC family, is a key regulator of cell survival, invasion and chemoresistance in glioblastoma (11,22,35). Previous data from our lab had demonstrated the role of PKC-i in glioma cell proliferation and involvement in the malignant phenotype of glioblastoma. However, the mechanistic signaling remained unclear (28).

PKC isotype profiling in previous studies demonstrated that PKC-i was highly expressed in actively proliferating neuroblastoma, glioblastoma and prostate cells. However, under serum-starved conditions, the cells were quiescent, PKC-i was significantly decreased (33–35). Our current studies also displayed similar overexpression of PKC-i in proliferating glioma cells (T98G and U87MG) and reduced levels in serum-starved cells. Cdk7 and cdk2 levels were also reduced in the serum-starved conditions. In addition, the analyses of the cytoplasmic and nuclear expression of these proteins (Figure 1B) revealed that even though Cdk7 was constitutively present in both cell fractions, there was reduced cdk2 expression (in both T98G and U87MG cells) in the absence of PKC-i, indicating that PKC-i may be essential for regulating the expression of cdk2 (in the presence of cdk7) thereby triggering the cell proliferation. Thus, the data suggest the importance of cross talk between these proteins for glioma cell growth and proliferation. This putative mechanism of regulation of cdk2 expression in glioma cells has never been reported before, however, further experimentation may be required to confirm this data.

Cdk7 regulates the eukaryotic cell cycle and is an *in vitro* upstream kinase to cdk2 (26,27). Phosphorylation and activation of cdk2 controls the transition of cells from G1–S phase. Our results showed PKC-i, Cdk7 and cdk2 levels were increased continuously as cells progressed to S and subsequently G2 phase. This suggests a potential correlation between these proteins in a cell cycle-dependent manner. Thus, we hypothesized that PKC-i might be regulating the expression and activity of Cdk7. To test this hypothesis, we analyzed the association between PKC-i and Cdk7 in these cells. The majority of PKC-i associated with and phosphorylated Cdk7 at T18–T30 h suggesting that

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Fig. 2. Continued
PKC-ι regulates cell cycle progression of glioma cells. There was also a cell cycle-dependent increase and translocation of PKC-ι, Cdk7, and Cdk2 into the nucleus further supporting this hypothesis. Although, an overall correlation was observed in both cell lines, U87MG did not show a similar pattern to T98G because U87MG cells are highly asynchronous in nature.

Recent findings by Pillai et al. (33) have shown that PKC-ι is an in vitro upstream kinase to Cdk7. In the current studies, we found that PKC-ι not only associated with Cdk7 but also directly phosphorylated Cdk7 at T170 endogenously as well as exogenously suggesting that PKC-ι may also be an upstream Cdk7 kinase in glioblastoma. PKC-ι knockdown by siRNA treatment has been reported to inhibit glioma cell survival by suppressing the proapoptotic function of Bad (34). Moreover, in this study, we observed that PKC-ι knockdown reduced Cdk7 and Cdk2 phosphorylation suggesting that PKC-ι is also required for glioma cell survival but may also be crucial for glioma cell proliferation. Such a mechanism has been shown previously in prostate cancer; however, PKC-ι did not directly associate with Cdk7 in DU-145 cells and only transiently associated with Cdk7 in RWPE-1 cells implying that PKC-ι may not be a direct upstream kinase and that other kinases may be required to completely inhibit proliferation in these cells (35). PKC-ι knockdown also regulated the expression of the known cdk2 substrates, Rb (36,37) and p27kip1 (38), implying that PKC-ι inhibition may lead to cell cycle arrest. PKC-ι depletion also triggered Caspase-9 cleavage suggesting induction of the intrinsic pathway mediated apoptosis in glioma cells, complementing our recently published data (34). Furthermore, PKC-ι knockdown also affected the total expression of Cdk7 and cdk2. According to our current results, PKC-ι appears to regulate the activity of

Fig. 3. PKC-ι induces direct phosphorylation of Cdk7. (A and B) Western blot analysis of T98G and U87MG cells for pPKC-ι (T555), pCdk7 (T170), pcdk2 (T160), Pan Cdk7, Pan cdk2 and PKC-ι following individual treatments for 2 h with LY294002 (50 μM) and Wortmannin (0.1 μM).

B) PDK1 knockdown (100 nM for 24 h) cells were analyzed by western blotting to detect phospho-PKC-ι (T555), total PKC-ι, phospho-Cdk7 (T170) phospho-cdk2 (T160), Pan Cdk7, Pan cdk2. Data are representative of three independent experiments.

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Fig. 4. PKC-ι silencing diminished Cdk7 and cdk2 phosphorylation. (A) Both T98G and U87MG cells were treated with either control siRNA or PKC-ι siRNA (100 nM) for 24 h. Subsequently, Cdk7 was IP and subjected to kinase activity assay followed by western blot to detect pCdk7 at T170, cdk2 at T160, total Cdk7 and total cdk2. (B) Whole cell lysate were from PKC-ι-silenced cells were analyzed by western blotting to detect Caspase 9, cleaved Caspase 9, pRb and p27kip1.

Fig. 5. PKC-ι knockdown preceded a reduction in Cdk7 and cdk2 (A) Western blot analysis of T98G and U87MG cells for pPKC-ι (T555), pCdk7 (T170), pcdk2 (T160), Pan Cdk7, Pan cdk2 and PKC-ι following individual treatments for 2 h with LY294002 (50 μM) and Wortmannin (0.1 μM).

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Recent findings by Pillai et al. (33) have shown that PKC-ι is an in vitro upstream kinase to Cdk7. In the current studies, we found that PKC-ι not only associated with Cdk7 but also directly phosphorylated Cdk7 at T170 endogenously as well as exogenously suggesting that PKC-ι may also be an upstream Cdk7 kinase in glioblastoma. PKC-ι knockdown by siRNA treatment has been reported to inhibit glioma cell survival by suppressing the proapoptotic function of Bad (34). Moreover, in this study, we observed that PKC-ι knockdown reduced Cdk7 and cdk2 phosphorylation suggesting that PKC-ι is not only required for glioma cell survival but may also be crucial for glioma cell proliferation. Such a mechanism has been shown previously in prostate cancer; however, PKC-ι did not directly associate with Cdk7 in DU-145 cells and only transiently associated with Cdk7 in RWPE-1 cells implying that PKC-ι may not be a direct upstream kinase to Cdk7 and that other kinases may be required to completely inhibit proliferation in these cells (35). PKC-ι knockdown also regulated the expression of the known cdk2 substrates, Rb (36,37) and p27kip1 (38), implying that PKC-ι inhibition may lead to cell cycle arrest. PKC-ι depletion also triggered Caspase-9 cleavage suggesting induction of the intrinsic pathway mediated apoptosis in glioma cells, complementing our recently published data (34). Furthermore, PKC-ι knockdown also affected the total expression of Cdk7 and cdk2. According to our current results, PKC-ι appears to regulate the activity
Fig. 6. PKC-i depletion reduced cell proliferation and induced apoptosis in T98G cells. (A) The distribution pattern of cell cycle phases in PKC-i knockdown cells is compared with control cells over 48 h treatment period. (B) Cell death induced by PKC-i inhibition was detected using the Trypan blue exclusion method (left panel) and the Annexin V-FITC/PI assay (right panel). UV treatment was used as positive control for apoptosis detection. (C) The distribution pattern on cell death by PKC-i knockdown was compared with control siRNA-treated and -untreated cells. UV treatment was used as positive control for apoptosis detection. Representative dot plots of the Annexin V/PI analysis for overall apoptotic rate, late apoptotic rate, early-apoptotic rate and necrotic rate. Experiments were repeated four times with a total N = 8 for all treatment groups. UV treatment groups had N = 3–5. The significance is explained in Table I for this data. (D) Proliferation inhibition after PKC-i silencing was assessed by the CFDA-SE dilution assay with live gate analysis. The proliferation rate of PKC-i-silenced cells were compared with control siRNA-treated and -untreated cells. Fixed CFDA-SE stained cells were used as positive control. (E) PKC-i-silenced cells and control cells were analyzed by 7-AAD staining to determine the average percentage of dead cells. (F) Average percentage of proliferating cells was measured by CFDA-SE dilution (examined from the Live Gate) from PKC-i knockdown and control cells. The experiment was repeated twice with a total N = 4. *P < 0.05, Student’s t-test for specified treatment groups, **P < 0.05, Student’s t-test for all treatment groups.
and expression of Cdk7 and cdk2 in a cell cycle-dependent manner. In addition, PKC-\(\text{\textit{i}}\) silencing led to increase in cell cycle arrest and induction of apoptosis suggesting that the activity and expression of these cell cycle proteins are dependent on the activity and expression of PKC-\(\text{\textit{i}}\). To date, this is the first report that suggests that PKC-\(\text{\textit{i}}\) may be regulating Cdk7 and cdk2 expression but the exact mechanism has not been explored yet. It is possible that different signaling mechanisms may be involved; however, further investigation is required to confirm such possibilities.

PKC-\(\text{\textit{i}}\)-silenced glioma cells as well as UV-exposed glioma cells generated different distribution patterns of cell death. To our knowledge, these findings are the first evidence that PKC-\(\text{\textit{i}}\) inhibition generates early apoptosis and late apoptosis. However, a conventional method of apoptosis induction, UV irradiation, produced mostly late apoptosis. The CFDA-SE/7-AAD analysis for the first time revealed the activity of the T98G cells that resisted cell death following PKC-\(\text{\textit{i}}\) silencing. These resistant T98G cells were viable but experienced hampered cell division due to G2/M arrest. A similar distribution pattern was also observed in the cell cycle analysis data wherein, a 2-fold increase in G2/M cell population suggested that the cells might be going from G2/M arrest directly to apoptosis following RNA silencing. Such effects were observed in cells grown in tissue culture (also demonstrated by Baldwin et al. (11), contradicting the observations in other cancer models where such an effect was seen only in anchorage independent cells signifying a tissue specific role of PKC-\(\text{\textit{i}}\) (21,39).

**Table I. Distribution pattern of cell death after the silencing of PKC-\(\text{\textit{i}}\)**

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>Overall % apoptotic cells, average ± SD</th>
<th>% Late apoptotic (Annexin V + PI) cells, average ± SD</th>
<th>% Early apoptotic (Annexin V only) cells, average ± SD</th>
<th>% Necrotic (PI only) cells, average ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>No treatment</td>
<td>2.89 ± 0.70</td>
<td>2.26 ± 0.63</td>
<td>0.63 ± 0.21</td>
<td>1.21 ± 0.83</td>
</tr>
<tr>
<td>Control siRNA</td>
<td>7.43 ± 1.06*</td>
<td>5.01 ± 0.95*</td>
<td>2.41 ± 0.39*</td>
<td>2.89 ± 0.77*</td>
</tr>
<tr>
<td>PKC-(\text{\textit{i}}) siRNA</td>
<td>33.69 ± 12.00**</td>
<td>16.19 ± 8.59**</td>
<td>17.50 ± 9.93**</td>
<td>10.46 ± 4.26**</td>
</tr>
<tr>
<td>UV treatment (20 min)</td>
<td>33.82 ± 12.08**</td>
<td>30.54 ± 14.05***</td>
<td>3.28 ± 2.13*</td>
<td>18.84 ± 4.17***</td>
</tr>
<tr>
<td>UV treatment (25 min)</td>
<td>75.77 ± 3.02***</td>
<td>72.30 ± 3.00***</td>
<td>3.47 ± 0.12**</td>
<td>17.90 ± 1.15***</td>
</tr>
</tbody>
</table>

\(P\), 0.05, Student’s t-test compared with untreated group; \(\ast \ast \ast \ P\), 0.05, Student’s t-test for all control groups; \(\ast \ast \ast \ast \ P\), 0.05, Student’s t-test for all treatment groups except the alternative UV treatment; \(\ast \ast \ast \ast \ast \ P\), 0.05, Student’s t-test for all treatment groups.
PI (3)-kinase, an upstream kinase of PDK1 phosphorylates and activates atypical PKCs (11,13,14). Pharmacological inhibition of PI (3)-kinase and PDK1 blocked PKC-1 activity and also inhibited Cdk7, Cdk2 phosphorylation. These results suggest that glioma cell proliferation may occur through a PI (3)-kinase-mediated signaling pathway. Intriguingly, PDK1 knockdown also inhibited the endogenous expression of PKC-1 and slightly that of Cdk7 and Cdk2 in our cells. Previous studies in embryonic cells showed that PDK1 knockdown markedly reduced the expression of atypical PKC isoforms (PKC-δ, PKR1 and PRK2) (13,40); however, regulation of PKC-1 by PDK1 was never explored. Thus, our findings are the first to postulate a role of PDK1 in the regulation of PKC-1. Further investigation is required to confirm this theory. Although, Cdk7 and Cdk2 expression was reduced upon PDK1 knockdown, their reduction was not as significant as that observed upon PKC-1 knockdown. This may be because there was lesser knockdown of PKC-1 upon PDK1 knockdown as compared with PKC-1 silencing itself. Since our results suggest that PKC-1 may be regulating the expression of Cdk7 and Cdk2, we postulate that the amount of PKC-1 unaffected upon PDK1 silencing may be promoting the expression of Cdk7 and Cdk2 in these cells. Thus, higher concentration of PDK1 siRNA may be required to completely knockdown the expression of Cdk7 and cdk2 in these cells. The knockdown of PDK1 siRNA may be required to completely knockdown the expression of Cdk7 and Cdk2. Furthermore, among the PKCs, only PKC-1 is found to be highly activated and overexpressed in glioblastoma cell lines (43). Other studies have shown that PKC-1 is overexpressed in several glioblastoma cell lines (43) and overexpression of dominant-negative PKC-ε inhibited proliferation of U373MG cells (44). Findings by Duncan et al. (29) showed that, although several PKCs were expressed in U373MG cells, only PKC-1 and PKC-βII phosphorylated and activated Cdk7 and Cdk2. Furthermore, among the PKCs, only PKC-1 was observed to be highly overexpressed in transformed benign, glioma and meningioma as well as glioma cells lines (28). To our knowledge, no other studies have shown the involvement of other PKC isoforms in mediating cell proliferation via Cdk7, suggesting that Cdk7 may be a unique substrate to PKC-1 in glioblastoma but whether it is a unique substrate to PKC-1 in other cancer models is yet to be determined. It would be interesting to determine whether other PKC isoforms could also phosphorylate Cdk7, however, the potential role of other PKCs in regulating Cdk7 cannot be included at this point.

In conclusion, these data suggest a role of PKC-1 in glioblastoma cell cycle progression and proliferation. We show a novel mechanism indicating that PKC-1 is highly activated and overexpressed in glioblastoma. PKC-1 induces uncontrolled glioma cell cycle progression and proliferation by modulating Cdk7/Cdk2 activity in a PI (3)-kinase-dependent manner. Furthermore, PKC-1 silencing studies demonstrated that glioma cells are highly resilient to conventional modalities suggesting that a combinatorial therapy may be required in the future to combat the apoptosis-resistant glioblastoma cells following PKC-1 downregulation. Collectively, these results suggest that PKC-1 is a potential predictive biomarker for personalized medicine and to identify glioblastoma patients that may benefit from anti-PKC-1 therapy.

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**References**


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