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TYROSINE 311 IS PHOSPHORYLATED BY c-Abl AND PROMOTES THE APOPTOTIC EFFECT OF PKC δ IN GLIOMA CELLS

Wei Lu^{1,*}, Susan Finnis^{1,*}, Cunli Xiang¹, Hae Kyung Lee¹, Yael Markowitz², Hana Okhrimenko², and Chaya Brodie^{1,2}

¹ William and Karen Davidson Laboratory of Cell Signaling and Tumorigenesis, Hermelin Brain Tumor Center, Department of Neurosurgery, Henry Ford Hospital, Detroit, MI

² The Mina & Everard Goodman Faculty of Life Sciences Faculty of Life-Sciences, Bar-Ilan University, Ramat-Gan Israel 52900

Abstract

In this study, we characterized the phosphorylation of tyrosine 311 and its role in the apoptotic function of PKC δ in glioma cells. We found that c-Abl phosphorylated PKC δ on tyrosine 311 in response to H₂O₂ and that this phosphorylation contributed to the apoptotic effect of H₂O₂. In contrast, Src, Lyn and Yes were not involved in the phosphorylation of tyrosine 311 by H₂O₂. A phosphomimetic PKC δ mutant, in which tyrosine 311 was mutated to glutamic acid (PKC δ Y311E), induced a large degree of cell apoptosis. Overexpression of the PKC δ Y311E mutant induced the phosphorylation of p38 and inhibition of p38 abolished the apoptotic effect of the PKC δ mutant. These results suggest an important role of tyrosine 311 in the apoptotic function of PKC δ and implicate c-Abl as the kinase that phosphorylates this tyrosine.

Keywords

PKC δ ; tyrosine phosphorylation; apoptosis; c-Abl; Src; H₂O₂; glioma

INTRODUCTION

Protein kinase C δ is a ubiquitously expressed novel PKC isoform which regulates various cellular functions. PKC δ plays a major role in the regulation of cell apoptosis in a cellular and stimulus-dependent manner [1]. Thus, PKC regulates cell apoptosis of various cell types in response to etoposide [2,3], cisplatin [4], UV radiation [5] and oxidative stress [6]. In contrast to its proapoptotic effect, PKC δ has been also reported to act as an anti-apoptotic kinase; in FGF-treated granulosa cells [7], in TRAIL-treated and Sindbis virus-infected glioma cells [8, 9] and in NO-treated macrophages [10].

One of the important factors that impacts the activity and functions of PKC δ is its phosphorylation on tyrosine residues. PKC δ has been reported to undergo tyrosine phosphorylation in response to a large variety of stimuli including PMA [11,12], PDGF [12, 13], substance P [14] and activation of the IgE receptor [15]. Tyrosine phosphorylation of

Address all correspondence to: Chaya Brodie, MD, Hermelin Brain Tumor Center, Dept of Neurosurgery, Henry Ford Hospital, Phone: 313-916-8619, Fax: 313-916-8755, Email: nscha@neuro.hfh.edu

*Equal contribution

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PKC δ is one of the earliest events that occur in response to various apoptotic stimuli and it has been shown to play a major role in the apoptotic function of PKC δ [1]. Indeed, tyrosines 311, 332 and 532 are phosphorylated in response to H₂O₂ [16,17] and tyrosines 311 and 332 are phosphorylated in response to ceramide [18]. In addition, phosphorylation of PKC δ on tyrosines 187 and 64 mediate the apoptotic effect of etoposide [3]. Although tyrosine phosphorylation is currently recognized as a critical event in the activation of PKC δ , the identification of tyrosine kinases that phosphorylate PKC δ and the role of specific tyrosine residues in the activation, localization and function of this isoform are just beginning to be understood.

In this study we characterized the phosphorylation of tyrosine 311 in glioma cells and examined its role in glioma cell apoptosis. We found that tyrosine 311 is phosphorylated by H₂O₂ in glioma cells and that c-Abl is involved in its phosphorylation. In addition, we found that a PKC δ Y311F mutant inhibited glioma cell apoptosis induced by H₂O₂, whereas a PKC δ Y311E mutant induced cell apoptosis. Finally, we found that the PKC δ Y311E mutant induced phosphorylation of p38 and that inhibition of this phosphorylation abolished the apoptotic effect of the PKC δ mutant.

MATERIALS AND METHODS

Materials

Polyclonal anti-PKC δ (C-20, C-17) anti-Src, anti-Lyn, anti-Yes and anti-c-Abl antibodies were obtained from Santa-Cruz (Santa-Cruz, CA). Anti-phospho-PKC δ Tyr311 antibody was obtained from Biosource, Invitrogen (Carlsbad, California). Anti-PARP, p38, phospho-p38, JNK, phospho-JNK, Erk1/2, phospho Erk1/2, XIAP, AKT and phospho-AKT antibodies were obtained from Cell Signaling (Beverly, MA). PP1, PP2 and SB203580 were obtained from EMD Biosciences (San Diego, CA) and STI571 was kindly provided by Novartis Switzerland.

siRNA transfection

Small interfering siRNA duplexes for Src, Yes, Lyn and c-Abl were obtained from Dharmacon (Lafayette, CO). A scrambled sequence was used as a negative control. Transfection of siRNAs was performed using OligoFectamine (Invitrogen, Carlsbad, California). Experiments were performed 72 h after transfection.

Site directed mutagenesis of PKC δ

PKC δ cloned into the pEGFP plasmid served as a template vector for the site-directed mutagenesis using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). Conversion of tyrosine residues at sites 311 into phenylalanine (Y311F) or to glutamic acid (Y311E) was performed using the following primers. Y311F: forward 5'-ca gag tct gtc gga ata TTC cag gga ttt gag aag aag-3'; reverse 5'-ctt ctt ctc aaa tcc ctg GAA tat tcc gac aga ctc tg-3'. Y311E: forward 5'-ca aca gag tct gtc gga ata GAA cag gga ttt gag aag aag cc-3'; reverse 5'-gg ctt ctt ctc aaa tcc ctg TTC tat tcc gac aga ctc tgt tg-3'. The mutations were confirmed by DNA sequencing. PKC δ and the PKC δ mutants were fused into the N-terminal enhanced GFP vector pEGFP-N1 (Clontech Laboratories, Palo Alto, CA) as previously described [3].

Cell apoptosis assays

Cell apoptosis was measured using propidium iodide staining and analysis by flow cytometry as previously described [3,8] and by ELISA using anti-histone antibodies.

For anti-histone ELISA (Cell Death Detection ELISA kit; Roche Applied Sciences, Indianapolis, IN), cellular extracts containing histone-associated DNA fragments were

incubated in 96-well plates coated with anti-histone antibodies. Plates were then incubated with anti-DNA antibodies conjugated to peroxidase for 2 h. Substrate solution was added and absorbance was measured at 405 nm.

Immunoblot analysis

Lysates were resolved by SDS-PAGE and were transferred to nitrocellulose membranes. The membranes were blocked with 5% dry milk and subsequently stained with the primary antibody. Specific reactive bands were detected using a goat anti-rabbit IgG conjugated to horseradish peroxidase (BioRad, Hercules, CA) and the immunoreactive bands were visualized by the ECL Western blotting detection kit (Amersham, Arlington Heights, IL).

Statistical analysis

The results are presented as the mean values $\pm$ S.E. Data were analyzed using ANOVA and a paired Student's t-test to determine the level of significance between the different groups.

RESULTS

The phosphorylation of PKC δ on tyrosine 311 in response to H₂O₂ is not mediated by Src, Yes or Lyn

Oxidative stress induces tyrosine phosphorylation of PKC δ in various cellular systems [16, 17]. Here, we examined the phosphorylation of PKC δ on tyrosine 311 in response to H₂O₂ in glioma cells. We found that H₂O₂ (1 mM) induced phosphorylation of PKC δ in the U87 cells already after 5 min and it persisted up to 30 min thereafter (Figure 1A). Similar results were obtained in the A172 glioma cells (data not shown).

To identify the tyrosine kinase that phosphorylates PKC δ in response to H₂O₂, we first employed the Src family inhibitor PP1 (10 μ M) and found that it significantly blocked the phosphorylation of tyrosine 311 by H₂O₂ (Figure 1B). Similar results were obtained with PP2 (data not shown). To further examine the role of Src in the phosphorylation of tyrosine 311 we silenced Src expression using siRNA duplexes. As presented in Figure 1C, transfection of U87 cells with Src siRNA decreased the expression of Src after 3 days; however, it did not significantly affect the phosphorylation of tyrosine 311 in response to H₂O₂. We then examined the role of two other Src-related kinases that are expressed in glioma cells, Lyn and Yes, in the phosphorylation of PKC δ on tyrosine 311. Silencing of Yes or Lyn with specific siRNA duplexes did not alter the phosphorylation of tyrosine 311 in response to H₂O₂ (data not shown).

c-Abl phosphorylates tyrosine 311 in H₂O₂-treated cells and this phosphorylation mediates the apoptotic effect of PKC δ

Since the Src inhibitors PP1 and PP2 have been recently reported to inhibit other kinases such as c-Kit, PDGFR and c-Abl [19,20], we examined the role of c-Abl in the phosphorylation of tyrosine 311. For these experiments we employed the c-Abl inhibitor STI571 [21] and siRNA duplexes directed against c-Abl siRNA. Treatment of the cells with STI571 significantly decreased the phosphorylation of tyrosine 311 in H₂O₂-treated cells (Figure 2A). Similarly, silencing of c-Abl reduced the phosphorylation of tyrosine 311 by H₂O₂, whereas a control scrambled siRNA did not exert significant effects (Figure 2B).

To examine the role of tyrosine 311 in the apoptotic effect of H₂O₂ we employed a PKC δ Y311F mutant. Overexpression of the PKC δ Y311F mutant partially reduced glioma cell death induced by H₂O₂ (Figure 2C). Thus, CV U87 cells exhibited 60% cell death whereas cells overexpressing the PKC δ Y311F mutant exhibited only 28% cell death. Similarly, the c-Abl inhibitor STI571 (5 μ M), partially protected glioma cells from H₂O₂-induced apoptosis (Figure 2D).

Mutation of tyrosine 311 to glutamic acid (PKC δ Y311E) induces cell apoptosis

To further examine the role of tyrosine 311 in glioma cell apoptosis, we mutated tyrosine 311 to glutamic acid (Y311E mutation), which is positively charged and is therefore phosphomimetic [22]. The expression of the PKC δ and the PKC δ mutants in the A172 cells is presented in Figure 3A. Using DNA fragmentation ELISA assay we found that PKC δ Y311E mutant induced a large degree of cell apoptosis in the transfected cells, whereas the CV, PKC δ WT or the PKC δ Y311F mutant did not induce significant cell death (Figure 3B). Similar results were obtained using propidium iodide staining and FACS analysis; about 55% of the PKC δ Y311E overexpressing cells were apoptotic (Figure 3C). The PKC δ Y311E mutant exerted cell apoptosis also in the U87 and HeLa cells (data not shown).

The PKC δ Y311E mutant induces apoptosis via phosphorylation of p38

To delineate the mechanisms by which the PKC δ Y311E induces cell apoptosis, we examined its effects on the expression and phosphorylation of various apoptosis-related proteins. As presented in Figure 4A, overexpression of the PKC δ Y311E mutant in the A172 cells increased the phosphorylation of p38, whereas it did not affect that of Erk1/2, JNK and AKT. In addition, the PKC δ Y311E mutant decreased the expression of XIAP.

To examine the role of the phosphorylation of p38 in the apoptotic effect of the PKC δ Y311E mutant, we employed the p38 inhibitor, SB203580. The A172 cells were transfected with the PKC δ Y311E mutant and following 6 hr of transfection, the cells were treated with SB203580 (20 μ M) for additional 12 hr. As presented in Figure 4B, treatment of the cells with SB203580, significantly decreased the apoptotic effect of the PKC δ Y311E mutant, as compared to untreated cells.

DISCUSSION

Tyrosine phosphorylation of PKC δ has been long recognized as an important factor in the regulation of PKC δ activity and function [12–14,23,24]. However, the identification of tyrosine kinases that mediate these phosphorylation events and the role of specific tyrosine residues of PKC δ in its apoptotic effects are just beginning to be understood.

In this study, we characterized the phosphorylation of tyrosine 311 (313 in the human PKC δ) and its role in the apoptotic function of PKC δ in glioma cells. We found that H₂O₂ induced phosphorylation of tyrosine 311 in the glioma cell lines U87 and A172, similar to the results described for other cell lines [11,16,17,25]. The phosphorylation of tyrosine 311 has been reported to be mediated by Src, mostly based on the use of different inhibitors of the Src family [11,18,25]. We also found that the Src inhibitor, PP1 inhibited the phosphorylation of tyrosine 311 by H₂O₂, however this phosphorylation was not inhibited by the silencing of Src, Yes or Lyn suggesting that a kinase other than Src-related kinases was involved in this phosphorylation. The different results that were obtained with the Src inhibitor and Src kinase silencing can be attributed to the ability of the various Src inhibitors to inhibit additional kinases including c-Abl [19,20]. Indeed, we found that silencing of c-Abl and its inhibition by STI571 significantly inhibited the phosphorylation of tyrosine 311 by H₂O₂, suggesting that in glioma cells c-Abl phosphorylates PKC δ on tyrosine 311 in response to oxidative stress.

We demonstrated that tyrosine 311 played a role in the apoptosis of glioma cells in response to H₂O₂. Mutation of tyrosine 311 to phenylalanine (PKC δ Y311F mutant) decreased the apoptotic effect of H₂O₂, and similar results were obtained with the c-Abl inhibitor STI571. A PKC δ Y311F mutant has been shown to protect dopaminergic neuronal cells from H₂O₂-induced cell death [25]. Our results that STI571 protected glioma cells from H₂O₂, similar to the PKC δ Y311F mutant, further support a functional role of the tyrosine phosphorylation of

PKC δ by c-Abl. The interaction of PKC δ and cAbl has been shown to play a role in the regulation of cell apoptosis in various cellular systems [26–28]. However, to the best of our knowledge, this is the first study that identifies tyrosine 311 as the tyrosine that is phosphorylated by c-Abl and is involved in PKC δ and c-Abl effects on H₂O₂-induced cell apoptosis.

The importance of tyrosine 311 in promoting glioma cell death was further demonstrated by using a phosphomimetic mutant of PKC δ , in which tyrosine 311 was mutated to glutamic acid. We found that the PKC δ Y311E mutant induced a large degree of cell apoptosis in glioma cells as compared to CV and PKC δ overexpressing cells.

The PKC δ Y311E mutant induced phosphorylation of p38 and decreased the expression of XIAP, whereas it did not affect the phosphorylation of AKT, Erk1/2 and JNK. The phosphorylation of p38 by the PKC δ Y311E mutant contributed to the apoptotic effect of this mutant since the p38 inhibitor, SB203580, abrogated the apoptotic effect induced by the PKC δ Y311E mutant. The mechanisms by which the PKC δ Y311E induces phosphorylation of p38 are currently not known, however PKC δ has been associated with the phosphorylation of p38 [29] and a recent study demonstrated that PKC δ was tyrosine phosphorylated by c-Abl in response to ionizing radiation which led to the activation of p38 [30]. The activation of p38 by the PKC δ Y311E mutant may be also associated with the decreased expression of XIAP as a recent report suggested that p38 inhibited Erk activity which is important for maintaining XIAP levels [31].

In summary, we identified c-Abl as the tyrosine kinase that phosphorylates PKC δ on tyrosine 311 in response to H₂O₂. This study also demonstrates the importance of tyrosine 311 in PKC δ apoptotic function in glioma cells; phosphorylation of PKC δ on tyrosine 311 by c-Abl mediated the apoptotic effect of H₂O₂ in glioma cells and a phosphomimetic mutant of PKC δ (PKC δ Y311E) induced cell apoptosis via a p38-dependent mechanism. Collectively, these results further emphasize the importance of the tyrosine phosphorylation of PKC δ as a key factor in the apoptotic function of PKC δ and in the regulation of downstream signaling pathways.

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Figure 1. Role of Src in the phosphorylation of tyrosine 311 in response to H₂O₂

U87 cells were treated with H₂O₂ (1 mM) and the phosphorylation of PKC δ on tyrosine 311 was determined using a tyrosine 311 phospho-specific antibody (A). The cells were pre-treated with PP1 (10 μ M) for 30 min and the phosphorylation of PKC δ on tyrosine 311 was determined in cells treated with H₂O₂ (1 mM) for 30 min (B). Cells were transfected with control scramble or Src siRNA duplexes. Following three days, the expression of Src and the phosphorylation of tyrosine 311 in response to H₂O₂ (1mM, 30 min) were determined (C). The results are from one representative out of four independent experiments.

Figure 2. Tyrosine 311 is phosphorylated by c-Abl and mediates the apoptotic effect of PKCδ in H₂O₂-treated cells

Cells were treated with H₂O₂ (1mM) for 30 min in the presence or absence of STI571 (5 μM) and the phosphorylation of PKCδ on tyrosine 311 was determined (A). Cells were transfected with c-Abl or control siRNA duplexes for 3 days and the level of c-Abl, PKCδ and actin and the phosphorylation of PKCδ on tyrosine 311 was determined (B). A172 cells transfected with CV, PKCδ and the PKCδ Y311F mutant were treated with H₂O₂ (1 mM) and cell apoptosis was determined (C). Cells were treated with STI571 (5 μM) for 30 min followed by treatment with H₂O₂ for an additional 24 h. Cell death was determined by propidium iodide and FACS analysis (D). The results represent the means ± SE of three independent assays (C,D) or are from one representative out of four independent experiments (A,B). *p<0.01; p<0.001

Figure 3. The PKCδ Y311 E mutant induces cell apoptosis

A172 cells were transfected with CV, PKCδ, PKCδ Y311F or PKCδ Y311E. The expression of PKCδ and the PKCδ mutants was examined by Western blot analysis (A). Following 24 h, the cells were analyzed for cell apoptosis using anti-histone ELISA (B) or by PI staining and FACS analysis (C). The results represent the mean ± SE of four separate experiments (B) or are from one representative out of four independent experiments (A,C). *p<0.0001

Figure 4. Role of p38 in glioma cell apoptosis induced by the PKC δ Y311E mutant

A172 cells were transfected with PKC δ , PKC δ Y311F and the PKC δ Y311E mutant. After 24 h, the expression and phosphorylation of various apoptosis-related proteins were analyzed using Western blot analysis (A). To examine the role of p38 in the apoptosis induced by the PKC δ Y311E mutant, cells were transfected with CV and the PKC δ Y311E mutant and after 6 hr were treated with SB203580 (20 μ M) for additional 12 hr. Cell apoptosis was determined using PI staining and FACS analysis (B). The results are from one representative experiment out of three similar experiments (A) or represent the mean $\pm$ SE of three separate experiments (B). * $p < 0.002$