Identification of a Novel Antiapoptotic Human Protein Kinase C δ Isoform, PKCδVIII in NT2 Cells†

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ABSTRACT: Protein kinase C (PKC) δ plays an important role in cellular proliferation and apoptosis where it is involved in the caspase-3 mediated apoptotic pathway. Cleavage of PKCδI by caspase-3 releases a catalytically active C-terminal fragment that is sufficient to induce apoptosis. In this paper, we identified a novel human PKCδ isozyme, PKCδVIII (Genbank accession number DQ516383) in human teratocarcinoma (NT2) cells that differentiate into hNT neurons upon retinoic acid (RA) treatment. Expression of PKCδVIII was confirmed by real-time RT-PCR analysis, and we observed that after an initial peak at 24 h following RA treatment, its expression gradually declined with prolonged RA treatment. PKCδVIII is generated via the utilization of an alternative 5′ splice site, and this results in an insertion of 31 amino acids in the caspase-3 recognition sequence DMQD. The function of PKCδVIII was examined by subcloning it into an expression vector and raising an antibody specific to PKCδVIII. Using in vivo and in vitro assays, we demonstrated that PKCδVIII is resistant to caspase-3 cleavage. Next, we sought to determine the role of PKCδVIII in apoptosis in NT2 cells. Overexpression of PKCδVIII and knockdown using PKCδVIII siRNA suggest an antiapoptotic function for the PKCδVIII isozyme. We demonstrate that antisense oligonucleotides (ASO) directed toward the 5′ splice site I promote the expression of the PKCδVIII isozyme. Our results indicated that ASO mediated PKCδVIII expression rescued NT2 cells from etoposide-induced apoptosis. We conclude that the novel human PKCδVIII splice variant functions as an antiapoptotic protein in NT2 cells.

Protein kinase C (PKC)[1] is a serine/threonine kinase that modulates diverse cellular functions such as proliferation, survival, and differentiation as well as apoptosis. PKCs also function as mediators of tumor growth, hormone secretion, and immune responses (*1*, *2*). The PKC family is comprised of different isozymes and their splice variants classified on the basis of their homology and sensitivity to their cofactors: conventional PKCs (cPKCs) include α, *β*, and *γ*, which are activated by calcium and diacyl glycerol (DAG); novel PKCs (nPKCs) include *δ*, *ϵ*, *η*, and *θ*, which are activated by DAG; and atypical PKCs (aPKCs) include *ζ*/*λ* and *ι*, which are activated by phorbol esters.

Since PKCs mediate signaling cascades in a variety of tissues and are central to regulating numerous cellular processes (*3*), it is no surprise that alternative splicing of PKC isozymes is becoming central in understanding how a cell modulates its response to external stimuli, often leading to cellular survival or death decisions. Alternative splicing is a process by which the genome generates diversity in its protein products. The first report of PKC isozymes known to undergo alternative splicing was in the PKC*β* gene, generating PKC*β*I and PKC*β*II mRNA. Alternative splicing of PKC*β* pre-mRNA and its regulation by insulin in skeletal muscle cells have been extensively studied. Insulin also regulates this event in adipocytes, hepatocytes, and fibroblasts (*4*−*7*). In recent years, additional PKC isozymes generated by alternative splicing have been described. PKC*θ* is alternatively spliced in mouse testis with PKC*θ*II being specifically expressed in the seminiferous tubules of mouse testis (*8*). PKCζII is described as a small molecule of PKCζ and is expressed in the mouse brain (*9*). PKC*δ*, so far, has the most described isozymes generated by alternative splicing. PKCδI is ubiquitously present in all species. PKCδII is present in mouse (*10*), while PKCδIII is specific for rat (*11*). PKCδIV, -V, -VI, and -VII were recently described in mouse testis (*12*), totaling six PKCδ isoforms in the mouse. The specific functions of these recently described isozymes remain to be elucidated.

The primary amino acid structure of PKCδ is composed of conserved domains (C1−C4) that serve as functional modules and are separated by variable domains (V1−V5) that contribute to its specificity and function (*13*). The hinge region of PKCδ is at the juxtaposition between the N-

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[1] Abbreviations: ASO, antisense oligonucleotides; DAG, diacylglycerol; PARP, poly (ADP-ribose) polymerase; PKC, protein kinase C; RA, all-*trans* retinoic acid; SR, serine−arginine-rich proteins.

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terminal regulatory domain and the C-terminal catalytic domain. PKCδ is activated by a diacylglycerol (DAG) mediated mechanism as well as by proteolytic cleavage at the hinge region (V3 domain). The catalytic domain of PKCδ is released through cleavage by caspase-3 and performs many of the functions attributed to PKCδ. For example, the C-terminal catalytic domain of PKCδ is sufficient to induce apoptosis (*14*).

Retinoic acid (RA), a vitamin A metabolite, is essential in controlling differentiation of the developing embryo (*15*). Moreover, RA regulates plasticity and nerve regeneration in the adult brain (*16*). Ntera-2 or NT2 cells, a human teratocarcinoma cell line, differentiate into hNT neurons (also called NT2-N neurons) when treated with all-*trans* RA. This represents a widely used model for the study of neurogenesis and the developing nervous system (*17, 18*). Exposure of NT2 precursors to RA not only activates the expression of genes necessary for neuronal differentiation but also signals NT2 cells to differentiate toward a neuronal phenotype. Apoptosis plays a critical role in sculpturing the neuronal network. Since PKCδ is implicated as a mediator of apoptosis, we studied the expression and function of PKCδ isoforms in NT2 cells upon treatment with RA. Here, we report a novel human PKCδ splice variant, PKCδVIII, whose expression is regulated by RA in NT2 cells. We further examine the function of PKCδVIII in human NT2 cells.

EXPERIMENTAL PROCEDURES

Cell Culture. NTERA-2 cl.D1 [NT2/D1] or NT2 cells, a human teratocarcinoma cell line, were purchased from ATCC (CRL-1973). NT2 cells differentiate into hNT neurons upon treatment with all-*trans* RA. NT2 cells begin showing the neuronal morphology after 4 weeks of RA treatment. NT2 cells were plated at a density of 10^6 and maintained in Dulbecco's Modified Eagle's Medium with 4 mM L-glutamine (modified by ATCC), supplemented with 10% fetal bovine serum and maintained at 37 °C in a humidified 5% CO_2, 95% air atmosphere in six-well plates. Media was changed every 3 days. The all-*trans* RA (10 μM) was added to NT2 cells as indicated in the experiments.

Cloning of PKCδVIII cDNA in the pcDNA 6.2/V5 Gateway Directional TOPO Vector. NT2 cells were treated with RA for 24 h, and the total RNA was extracted using RNA-Bee (Tel Test, Inc.) according to the manufacturer's instructions. RT-PCR was carried out using the high fidelity polymerase ExTaq from Takara, CA. The primers were forward primer CACCATGGCGCCGTTCCTG (which includes the ATG initiation codon) and reverse primer ATCTTCCAGGAG-GTGCTCGAATT. The PCR product, 2127 nucleotides, was extracted and purified from agarose gel and cloned into Invitrogen's pcDNA 6.2/V5 Gateway directional TOPO vector as per the manufacturer's instructions. Multiple clones were tested with restriction digestion and PCR analysis to confirm the orientation of the insert. Out of the positive clones, three were sent for sequencing to Moffitt Sequencing Core Lab, Tampa, FL. The expression vector is hereby referred to as PKCδVIII_GW.

Transient Transfections of Plasmid DNA. NT2 cells were plated at $\sim 10^6$ cells per 35 mm dish on the day prior to the transfections. The PKCδVIII_GW expression vector (2 μg/well of a six-well plate) was transfected in serum-free medium with Lipofectamine (Invitrogen, Life Technologies). Mock transfection was carried out simultaneously. The lipofectamine−DNA complex was incubated at room temperature for 20 min, diluted with the serum-free transfection medium, and added to the cells. Cells were placed at 37 °C for 4 h and then incubated in the growth medium at 37 °C for 48 h.

Transient Transfections of Antisense Oligonucleotides. NT2 cells were plated at $\sim 10^6$ cells per 35 mm dish the day prior to the transfections. Multiple 20-mer antisense oligonucleotides (ISIS Pharmaceuticals) that are fully modified 2′-0-(2-methoxy) ethyl (2′MOE) P═S were designed to span the 5′ splice sites I or −II of PKCδ exon 10 pre-mRNA. Scrambled antisense oligonucleotides (ASOs) were transfected as control. The 100 nM ASO was determined as the optimum concentration (data not shown) and was transfected in serum-free medium with Lipofectamine as described previously. The cells were then incubated in the growth medium at 37 °C for 48 h.

Transient Transfections of siRNA. NT2 cells were plated at $\sim 10^6$ cells per 35 mm dish the day prior to the transfections. Pre-designed and custom designed siRNAs for PKCδVIII were purchased from Ambion. The efficiency and conditions of transfections were validated by using silencer GAPDH siRNA from Ambion. The negative control siRNA was simultaneously transfected into NT2 cells. The Ambion siRNA ID 290535 worked most efficiently for PKCδVIII, and it was used for further studies. The 100 nM siRNAs were transfected in serum-free medium with Lipofectamine as described previously. The cells were then further incubated in the growth medium at 37 °C for 72 h.

RT-PCR Analysis. Total RNA was isolated from NT2 cells using RNA-Bee (Tel Test, Inc.) as per the manufacturer's instructions. A total of 2 μg of RNA was used to synthesize first strand cDNA using an Oligo(dT) primer and Omniscript kit (Qiagen). PCR was performed using 2 μL of RT reaction and Takara Taq polymerase. The primers were human PKCδ sense 5′ CACTATATTCCAGAAAGAACGC 3′ and antisense 5′ CCCTCCCAGATCTTGCC 3′ and huGAPDH 5′ CTTCATTGACCTCAACTACATG 3′ and antisense 5′ TGTCATGGATGACCTTGGCCAG 3′. Following 30 cycles of amplification in a Biometra T3000 thermocycler (human PKCδ: 94 °C, 30 s; 54 °C, 30 s; and 72 °C, 1 min and GAPDH: 94 °C, 30 s; 60 °C, 30 s; and 72 °C, 45 s), 5% of products was resolved on 6% PAGE gels and detected by silver staining. Briefly, gels were first soaked in 10% ethanol for 3 min, followed by soaking in 1% nitric acid for 3 min. The gel was then incubated in 0.1% $AgNO_3$ for 10 min. The developing solution (37% formaldehyde, 10 mg/mL sodium thiosulfate, 6% Na_2CO_3) was then added, and the gel was rocked until bands appeared. The reaction was stopped by the addition of 10% glacial acetic acid. The gel was then dried between cellophane sheets. This is an optimal visualization method for PCR products as it is efficient, sensitive, and allows quantification of mRNA (*19*−*25*). The PCR reaction was optimized for linear range amplification to allow for the quantification of products. Densitometric analyses of the bands were performed using the Un-Scan IT Software. Sequencing of the products (from three different PCRs in separate experiments) was carried out by the Moffitt Core

Sequencing Laboratory, Tampa, FL using forward and reverse primers separately.

Quantitative Real-Time RT-PCR. Total RNA was isolated from NT2 cells treated with RA for 0−4 days using RNA-Bee (Tel Test, Inc.) as per the manufacturer's instructions. Total RNA (50 ng) was reverse transcribed using an Omniscript kit (Qiagen) using equal amounts of Oligo $(dT)_{12-18}$ and random hexamers as primers. A 2 μL sample of cDNA was amplified by real-time quantitative PCR using Syber (SYBR) Green with an ABI PRISM 7900 sequence detection system (PE Applied Biosystems) to quantify the absolute levels of PKCδI and PKCδVIII mRNA in the samples. GAPDH was used as the endogenous control. Two sets of primer pairs for each transcript were designed to have an annealing temperature of ∼60 °C (*26*). These primers were initially tested using cDNA from NT2 cells as well as with the vector PKCδVIII_GW in a RT-PCR reaction using Taq polymerase (see method stated previously) to give distinct products corresponding to the respective transcripts. The primers selected for use in real-time RT-PCR are as follows: PKCδI sense primer: 5′GCCAACCTCTGCG-GCATCA 3′ and antisense primer: 5′ CGTAGGTCCCACT-GTTGTC|TTGCATG 3′ and PKCδVIII sense primer: 5′GC-CAACCTCTGCGGCATCA 3′ and antisense primer: 5′ CGTAGGTCCCACTGTTGTC|CTGTCTC 3′. These primers overlap the exon−exon boundary (indicated by the |) specific for each transcript. The primers for GAPDH were sense primer 5′ CTTCATTGACCTCAACTACAT 3′ and antisense primer 5′ TGTCATGGATGACCTTGGCCA 3′. Next, the optimal primer concentration was determined from a range of 50−900 nM. The final concentration of 300 nM was selected to ensure efficiency and specificity for its target based on the dissociation curve that showed a single, sharp peak, indicating that the primers amplify one specific target. For absolute quantification, a standard curve was generated for each gene in every assay. To do so, 100−0.4 ng of RNA was reverse transcribed as described previously. The resulting cDNA was used to obtain a standard curve correlating the amounts with the threshold cycle number (C_t values). A linear relationship ($r^2 > 0.96$) was obtained for each gene. Real-time PCR was then performed on samples and standards in triplicate. The plate setup also included a standard series, no template control, no RNA control, no reverse transcriptase control, and no amplification control. The dissociation curve was analyzed for each sample. Absolute quantification of mRNA expression levels for PKCδI and PKCδVIII was calculated by normalizing the values to GAPDH. The results were analyzed with a two-tailed Student's *t*-test using PRISM4 statistical analysis software (GraphPad). A level of $p < 0.05$ was considered statistically significant. Significance was determined after three or more experiments.

Subcellular Fractionation. NT2 cells were treated with RA for 0−48 h, then were washed twice with ice cold sterile 1X PBS, and centrifuged at 1000*g*. The cell pellet was suspended in cold (4 °C) buffer A (20 mM Tris pH 7.5, 0.25 M sucrose, 1.2 mM EGTA, 2.5 mM $MgCl_2$, 0.1 mM PMSF, Protease Inhibitor Cocktail), sonicated, and centrifuged in Beckman's TL100 ultra centrifuge at 105 000*g* for 30 min. Supernatant is the cytosolic fraction. The cell pellet was resuspended in cold buffer B (20 mM Tris pH 7.5, 0.25 M sucrose, 5 mM EGTA, 2 mM EDTA, 1% Triton X-100, 0.1 mM PMSF, Protease Inhibitor Cocktail), sonicated, and centrifuged in Beckman's TL100 ultra centrifuge as described previously. This supernatant is the membrane fraction. Fractions were subjected to Western blot analysis.

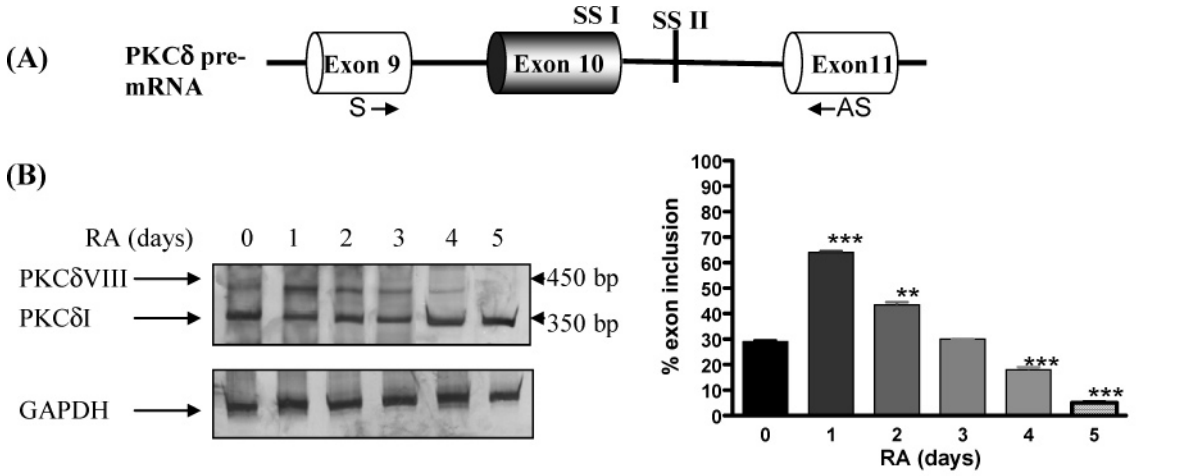
Western Blot Analysis. NT2 cell lysates (50 mg) and subcellular fractions were separated on 10% polyacrylamide gel electrophoresis-SDS (PAGE-SDS) as per Laemmli's protocol (*27*). Proteins were electrophoretically transferred to nitrocellulose membranes, blocked with Tris buffered saline/0.1% Tween 20 containing 5% nonfat dried milk, washed, and incubated with either a polyclonal antibody against anti-PARP (from Upstate), anti-XIAP (AnaSpec), anti-PKCδ C-terminal antibody (Biosource International, Inc.), or an antibody raised against the amino acids of the extended hinge region of human PKCδVIII. This region is absent in PKCδI. PKCδVIII-specific polyclonal antibody was raised in rabbits by Bio-Synthesis, Inc. to the synthetic peptide NH2- GEAGSIAPLRFLFPLRPKKGDC-COOH (amino acids 329−359, corresponding to the V3 hinge domain of PKCδVIII). Enzyme-linked immunosorbent assay (ELISA) was performed with the previous epitope region peptide. Then, 1:2 serial dilutions in BSA were carried out. The antibody serum had a titer up to 1:25 600. The antibody was characterized alongside unreactive preimmune antisera and was shown to recognize PKCδVIII in human NT2 cells. A peptide/antigen assay was also performed to confirm its specificity. Briefly, 2 μL aliquots of PKCδVIII antibody were taken in two separate tubes and diluted to 100 μL with sterile 1X PBS. A total of 10 μg of antigen was added to one tube. Both tubes were incubated at 37 °C for 1 h followed by incubation at 4 °C for 2 h. The tubes were centrifuged at 4 °C for 15 min, and the supernatant was used in Western blot analysis. Following incubation with anti-rabbit IgG-HRP, detection was performed using enhanced chemiluminesence (Pierce).

DNA Laddering. NT2 cells were lysed with TE lysis buffer, scraped, and harvested in treatment medium to ensure that all apoptotic cells detached from the plate were included in the analysis. An apoptotic DNA ladder detection kit (APT151) was bought from Chemicon International, and the protocol was performed according to the manufacturer's instructions. A total of 20 μL of the product was loaded onto a 1% agarose gel containing 0.5 μg/mL ethidium bromide. The gel was visualized by UV light and documented using Kodak Image Analysis software.

In Vitro Caspase-3 Assay. NT2 cells were rinsed with cold, sterile PBS and lysed using cleavage buffer (20 mM HEPES (pH 7.4), 1% NP40, 2 mM EDTA, 5 mM DTT, 1X protease inhibitor cocktail) as described by Halle et al. (*28*). A total of 30 μg of protein lysate was incubated with 20 U of caspase-3 (AnaSpec) at 30 °C for 0, 15, and 30 min. The caspase-3 inhibitor (AnaSpec) was added 30 min prior to the addition of caspase-3 as indicated in the lanes. Control samples were incubated under the same conditions in the absence of caspase-3. Samples were collected and immediately placed on ice, and PKCδVIII cleavage was determined by immunoblotting.

RESULTS

Novel Splice Variant PKCδVIII Is Expressed in Human NT2 Cells. Human teratocarcinoma cell line (NT2 cells) differentiates into hNT neurons following treatment with all-



(C)

cgcttcaacatcgacatgccgcaccgcttcaaggttcacaactacatgagccccaccttctgtgaccactgcggcagcctgctctggggactggtgaagc
agggattaaagtgtgaagactgcggcatgaatgtgcaccataaatgccgggagaaggtggccaacctctgcggcatcaaccagaagcttttggctgag
gccttgaaccaagtcacccagagagcctcccggagatcagactcagcctcctcagagcctgttgggatatatcagggtttcgagaagaagaccggagtt
gctggggaggacatgcaaggtgaagctgggtccattgccccattacggtttttattccccctgaggccaaagaaaggggactgtcctcccttccattgtca
agtgagacaggacaacagtgggacctacggcaagatctgggaggg

(D)

…HHKCREKVANLCGINQKLLAEALNQVTQRASRRSDSASSEPVGIYQGFEKKTGVAGE**DMQ**GEAG
SIAPLRFLFPLRPKKGDCPPFHCQVRH**D**NSGTYGKIWEGSSKCNINNFIFHKVLGKGSFGKVLLGELK
GRGE…

FIGURE 1: Identification of novel human PKCδVIII splice variant in NT2 cells. (A) Schematic shows the position of PCR primers on exons 9 and 11 of human PKCδ pre-mRNA. S: sense primer; AS: antisense primer; SSI: 5′ splice site I; and SSII: 5′ splice site II. (B) Human NT2 cells were treated with 10 μM all-*trans* RA for 0 (control), 1, 2, 3, 4, or 5 days. Total RNA was extracted and RT-PCR performed with primers as indicated. 5% of products was resolved on 6% PAGE gels and detected by silver staining as described in the Experimental Procedures. PKCδI was observed at 350 nucleotides, and a 443 nucleotide band was identified as PKCδVIII. The gel was quantified by densitometric scanning of the silver-stained gels from five experiments and represented on the graph. Percent exon inclusion was calculated as δVIII/(δVIII + δI) × 100. **: $p < 0.001$ and ***: $p < 0.0001$ (by two-tailed Student's *t*-test). (C) Sequencing data of the 443 bp PCR product shows inclusion of 93 bp (underlined) via usage of the downstream 5′ splice site II of exon 10 on PKCδ pre-mRNA in generating PKCδVIII mRNA. (D) Partial translated PKCδVIII protein sequence showing that the insertion of 31 amino acids (underlined) disrupts the human caspase-3 recognition sequence DMQD (bold).

trans RA (RA). This model represents an opportunity to study the early genes involved in neurogenesis. Apoptosis is an integrated mechanism in modeling of the neuronal network during neurogenesis. We studied the early events since molecular determinants of apoptosis are triggered immediately following RA treatment. The initial 24−48 h is crucial in determining cell fate as this coincides with the first detection of the neuronal phenotype in NT2 cells (*16*). Since protein kinase C (PKC) δ is implicated in the apoptotic process (via its C-terminal catalytic fragment), we studied expression of PKCδ isoforms in NT2 cells upon RA treatment for 5 days. RT-PCR analysis using human PKCδ primers (Figure 1A) corresponding to exon 9 (sense primer) and exon 11 (antisense primer) showed that PKCδI mRNA levels remained constant upon RA treatment. An additional product of 443 nucleotides was observed (Figure 1B). Purification of this 443 nucleotide product followed by sequencing indicated that this was a novel human PKCδ splice variant, hereby referred to as PKCδVIII (Genbank accession number DQ516383).

Sequencing and computational analysis of the PKCδVIII PCR product indicated that a downstream 5′ splice site of exon 10 was utilized to generate PKCδVIII mRNA, which resulted in the inclusion of 93 nucleotides of the intronic sequence between exons 10 and 11 of human PKCδ pre-mRNA (Figure 1C). This region corresponds to the V3 hinge domain of PKCδ. The partial translated protein sequence of PKCδVIII is shown in Figure 1D. It is observed that the 31 amino acids included in PKCδVIII disrupt the human caspase-3 recognition sequence, DMQD. The calculated molecular weight for PKCδVIII is 80.97 kDa.

PKCδ Splice Variant Specific Quantitative RT-PCR. To further validate that PKCδVIII is indeed a spliced variant of PKCδI, we performed quantitative, two-step real-time RT-PCR using Syber (SYBR) Green technology (*26*, *29*, *30*). The SYBR green method allows each transcript to be measured independently by using primer pairs that will amplify that transcript only. The primers are specific to the exon junctions of PKCδI mRNA and PKCδVIII mRNA as shown in Figure 2A. Each transcript is normalized to the endogenous control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), to obtain absolute quantification. Total RNA was extracted from NT2 cells treated with RA for 0−4 days, and real-time RT-PCR was performed. Our results indicated that PKCδVIII expression increased significantly following 24 h of RA and then gradually declined over time (Figure 2B). The expression of PKCδI remains almost constant over time (Figure 2C). These results further validate that PKCδVIII mRNA is generated via utilization of a downstream 5′ splice site of exon 10.

In simultaneous experiments, we performed Western blot analysis on NT2 cells treated with RA for 0−4 days to

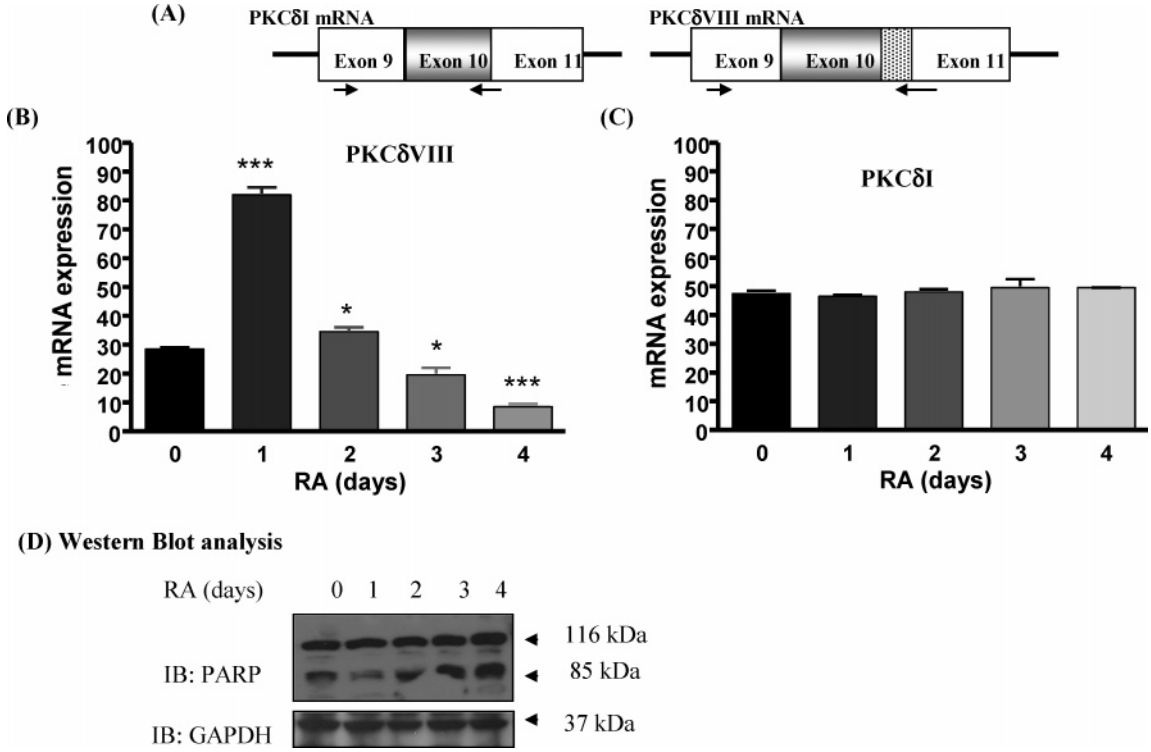


FIGURE 2: Real-time RT-PCR based quantitation of novel PKCδVIII transcript. A total of 50 ng of total RNA from NT2 cells treated with 10 μM RA for 0−4 days was analyzed using primers specific for each transcript. The arrows indicate the positions of the primers that span the exon−exon boundary for each transcript as shown in panel A. The black box following exon 10 in PKCδVIII mRNA represents the sequence included via utilization of the 5′ splice site II. Real-time RT-PCR analysis using SYBR green was performed in triplicate and repeated 3 times in separate experiments. The absolute mRNA expression of PKCδI and PKCδVIII transcripts are shown in panels B and C. PKCδVIII expression increases significantly following 24 h of RA treatment and then gradually declines over time. *: $p < 0.05$ and ***: $p < 0.0001$ (by two-tailed Student's *t*-test). (D) Western blot analysis was performed on whole cell lysates treated with RA for 0−4 days. The membranes were immunoblotted with anti-PARP antibody. The experiments were repeated 3 times with similar results.

observe ongoing apoptosis. In apoptotic cells, poly (ADP-ribose) polymerase (PARP) is cleaved by caspase-3 into an 85 kDa fragment that is detected in addition to the 116 kDa fragment using anti-PARP antibody in Western blot analysis. PARP is differentially processed in apoptosis and necrosis and can be used as a means of distinguishing the two forms of cell death (*31*). Hence, we used PARP cleavage as a means to monitor apoptosis in NT2 cells. We observed that PARP cleavage declined upon 1 day of RA treatment and then increased with prolonged RA treatment (Figure 2D).

The peak in PKCδVIII expression in NT2 cells coincided with the decrease observed in PARP cleavage, indicating decreased apoptosis. These data suggested that decreasing levels of PKCδVIII following 2 days of RA treatment allowed for increased apoptosis in NT2 cells.

PKCδVIII Is an Active PKC Isozyme. Protein kinase C isoforms are usually present in the cytosol. Upon stimulation, active PKCs translocate to the membrane. Translocation of PKCs from cytosol to membrane fraction is regarded as an indicator of activated PKC isoforms. To determine as to whether PKCδVIII functions as an activated protein kinase C, we performed subcellular fractionations of NT2 cells treated for 0−48 h with RA during which PKCδVII expression is increased. Cytosolic and membrane fractions were analyzed by Western blot analysis using a PKCδVIII-specific antibody. This antibody was raised against the 31 amino acids of PKCδVIII included in the mature protein due to alternative 5′ splice site selection on PKCδ pre-mRNA. This antibody is specific for PKCδVIII since it recognizes the intact, extended hinge region of PKCδVIII that is absent in PKCδI. Antibody specificity was confirmed by performing the peptide/antigen assay. It was observed that the peptide antigen could very effectively compete with the PKCδVIII antibody (Figure 3A). Further, the PKCδVIII levels increased in the membrane fraction and whole cell lysates in response to RA treatment (Figure 3B,C). This suggests that PKCδVIII translocated to the membrane fraction and thus functions as an active protein kinase C in accordance with other members of the novel PKC family.

PKCδVIII Is Resistant to Cleavage by Caspase-3 in Vivo. The V3 hinge domain of PKCδ is cleaved by caspase-3 to release the catalytically active C-terminal fragment that is sufficient to induce apoptosis (14). The inclusion of 93 nucleotides between exons 10 and 11 via usage of the 5′ splice site II in PKCδVIII resulted in the disruption of the human caspase-3 recognition sequence Asp-Met-Gln-Asp: DMQD (shown in Figure 1C). We sought to determine as to whether this disruption prevented proteolytic cleavage mediated by caspase-3. Since RA increased the expression of PKCδVIII, we treated NT2 cells with RA for 24 h. To determine if caspase-3 could cleave PKCδVIII, 20 U of caspase-3 was added, and the cells were returned to the incubator and maintained at 37 °C overnight. Whole cell lysates were assayed by Western blot analysis using an antibody raised against the extended hinge region of PKCδVIII. This antibody will detect the full-length PKCδVIII as well as any fragment that is generated if PKCδVIII is cleaved before or after the inserted amino acids. Full-length PKCδVIII was detected, but no cleavage products were observed (Figure 4A). To determine if the caspase-3 treat-

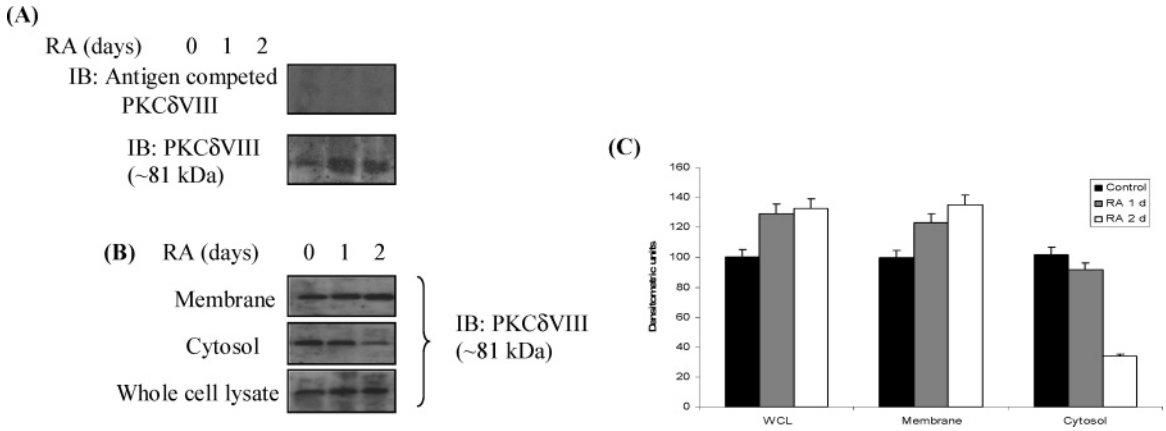


FIGURE 3: PKCδVIII translocates to the membrane fraction. (A) Peptide/antigen competition to determine PKCδVIII antibody specificity. PKCδVIII antibody (1:2000 dilution) was diluted in 100 μL of sterile PBS in two tubes. The PKCδVIII hinge region-specific antigen (10 μg) was added to one tube, mixed, and incubated for 37 °C for 1 h followed by 2 h at 4 °C. Western blot analysis was then performed using PKCδIII antibody with and without antigen competition. (B) NT2 cells were treated with 10 μM RA for 0−2 days, and subcellular fractions were obtained. Whole cell lysates were obtained from NT2 cells treated with 0 (control), 1, or 2 days of RA. Western blot analysis was performed using PKCδVIII-specific antibody. (C) Graph represents densitometric analysis of the results. Data are shown as the average of the mean ± SEM in three experiments. The experiments were performed 3 times with similar results.

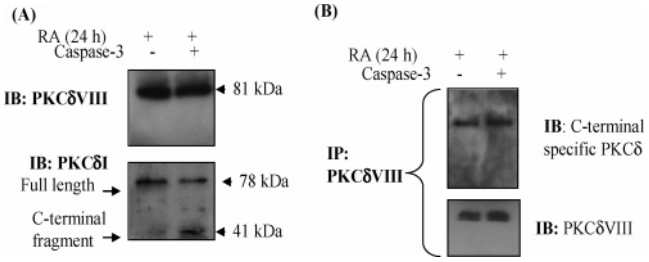


FIGURE 4: PKCδVIII is resistant to cleavage by caspase-3 in vivo. NT2 cells were treated with RA for 1 day (24 h). Caspase-3 was added, and cells were further incubated overnight at 37 °C. (A) Whole cell lysates were collected, and Western blot analysis was performed using PKCδVIII-specific antibody and PKCδI antibody. (B) In separate experiments, NT2 lysates were immunoprecipitated with PKCδVIII-specific antibody, followed by immunoblotting with C-terminal-specific PKCδI antibody and PKCδVIII-specific antibody. The experiments were repeated 4 times with similar results.

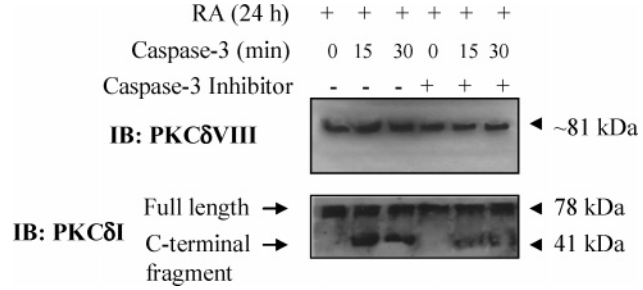


FIGURE 5: In vitro caspase-3 cleavage assay. NT2 cells were treated with 10 μM RA for 1 day, and an in vitro caspase-3 assay was carried out with the addition of caspase-3 for 0, 15, and 30 min. The caspase-3 inhibitor was added 30 min prior to the addition of caspase-3 as indicated in the figure. Western blot analysis was carried out using antibodies for anti-PKCδVIII and C-terminal anti-PKCδI. The experiment was repeated 5 times to ensure reproducibility.

ment cleaved PKCδI, we immunoblotted using a PKCδI antibody (Biosource International, Inc.) and observed full-length PKCδI (74 kDa) and a C-terminal 38 kDa fragment generated by caspase-3 cleavage. To further verify that PKCδVIII is not cleaved by caspase-3, we immunoprecipitated NT2 cell lysates treated with RA for 1−2 days using the PKCδVIII antibody. We then immunoblotted using the PKCδI antibody (that recognizes the C-terminal of PKCδ) to detect the presence of any cleaved fragments (Figure 4B). Our results indicated that PKCδVIII is resistant to cleavage by caspase-3.

In Vitro Caspase-3 Assay. A large number of caspase-3 substrates having the DXXD consensus sequence has been identified. Further, several proteins involved in apoptosis were cleaved by caspase-3. Since PKCδ plays an important role in apoptosis and it was shown that caspase-3 mediated release of the C-terminal catalytic fragment of PKCδ was sufficient to induce apoptosis (*32*), we sought to monitor the in vitro cleavage of PKCδ isoforms by human recombinant caspase-3 as described earlier (*28*, *33*). NT2 cells treated with RA for 24 h were washed with cold PBS and collected in a cleavage assay buffer. A total of 50 μg of proteins was incubated with 25 ng of recombinant caspase-3 for 0, 15, and 30 min at 30 °C. To further confirm that PKCδ cleavage was mediated by caspase-3, human caspase-3 inhibitor (Ac-DMQD-CHO) was added 30 min prior to the addition of caspase-3 to the samples in separate reactions. The samples were then assayed by Western blot analysis using a PKCδVIII-specific antibody as well as the PKCδ C-terminal antibody. PKCδVIII was not cleaved as detected by its specific antibody, while PKCδI was cleaved to a 38 kDa C-terminal fragment and detected in addition to the full-length PKCδI fragment (Figure 5). Hence, inclusion of the 31 amino acids disrupted the caspase-3 recognition motif.

*Role of PKCδ***V**III in Cellular Apoptosis. The release of the C-terminal catalytic fragment of PKCδ**I** by caspase-3 is sufficient to induce apoptosis (*32*). We have previously shown that NT2 cells have basal ongoing apoptosis and that overexpression of PKCδ**I** promotes apoptosis (*34*). Since our data indicated that increased PKCδVIII levels coincided with a decrease in apoptosis in NT2 cells and that PKCδVIII was resistant to caspase-3 cleavage, we investigated the function of PKCδVIII and its correlation to apoptosis in NT2 cells. PKCδVIII was cloned into Invitrogen's pcDNA 6.2/V5 Gateway directional TOPO vector as described in the Experimental Procedures, and this expression vector is referred to as PKCδVIII_GW. PKCδVIII was overexpressed by transient transfection of the PKCδVIII_GW expression

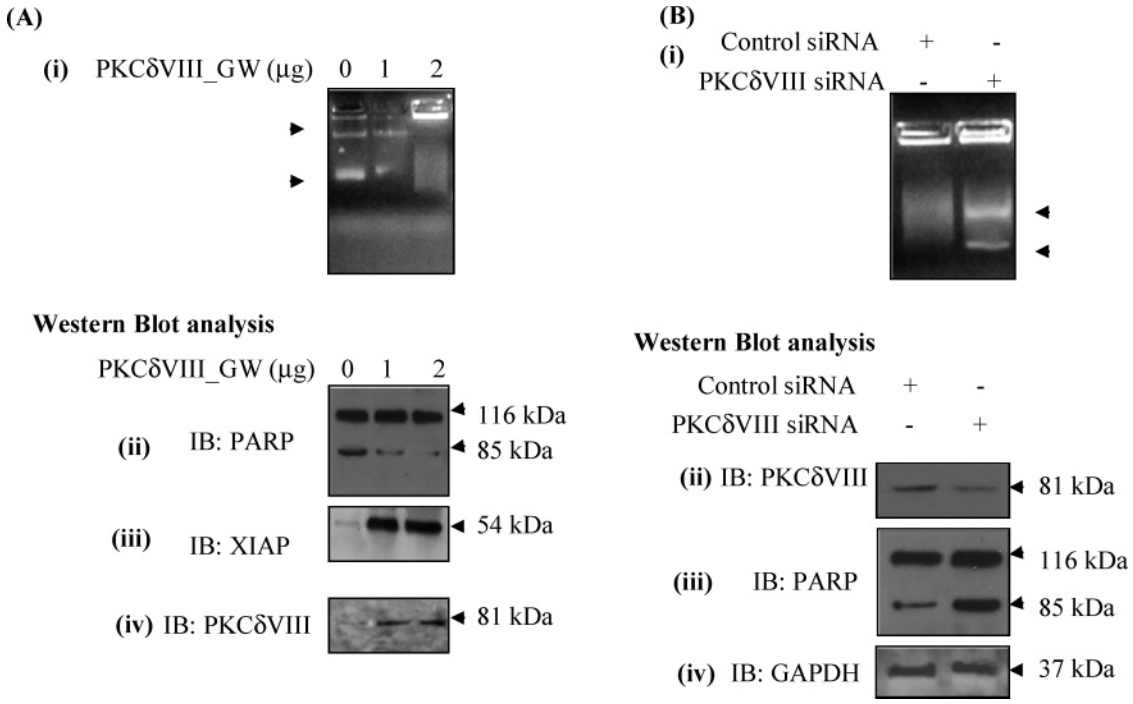


FIGURE 6: (A) PKCδVIII overexpression prevents apoptosis in NT2 cells. A total of 0 (mock transfection control), 1, or 2 μg of PKCδVIII_GW was transiently transfected into NT2 cells. (i) DNA fragmentation assay was carried out as described in the Experimental Procedures and resolved on 1% agarose gel stained with ethidium bromide. Arrowheads indicate high molecular weight fragments. Western blot analysis was performed on whole cell lysates transfected with 0, 1, or 2 μg of PKCδVIII_GW. The membranes were immunoblotted with (ii) anti-PARP antibody, (iii) anti-XIAP antibody, and (iv) anti-PKCδVIII antibody. The experiments were repeated 3 times with similar results. (B) Knockdown using PKCδVIII siRNA resulted in increased apoptosis in NT2 cells. PKCδVIII siRNA was transiently transfected in NT2 cells for 72 h along with its control siRNA. (i) DNA fragmentation was carried out in cells transfected with PKCδVIII siRNA or control siRNA as described in the Experimental Procedures and resolved on 1% agarose gel stained with ethidium bromide. Arrowheads indicate high molecular weight fragments. Western blot analysis was performed, and the membrane was immunoblotted with (ii) anti-PKCδVIII antibody, (iii) anti-PARP, or (iv) anti-GAPDH antibody. The experiments were repeated 3 times to ensure reproducibility of results.

vector in NT2 cells, and the extent of apoptosis was monitored by a DNA fragmentation assay, XIAP expression, and PARP cleavage. As established in previous studies (*35*), DNA from NT2 cells was cleaved into high molecular weight 50−300 kb fragments but was not cleaved further into smaller nucleotides (small DNA fragments), yet it showed typical nuclear morphological apoptotic changes. DNA fragmentation as a result of apoptosis in NT2 cells was observed as two bands of high molecular weight fragments (Figure 6A-i). Western blot analysis using the PARP antibody showed that PARP cleavage was decreased significantly in cells overexpressing PKCδVIII (Figure 6A-ii). Expression of the X-chromosome-linked inhibitor of apoptosis (XIAP) is also an indicator of ongoing apoptosis with decreased levels seen with increasing apoptosis (*36*). We show that XIAP expression is increased in cells overexpressing PKCδVIII (Figure 6A-iii). PKCδVIII levels were measured using the PKCδVIII-specific antibody (Figure 6A-iv). Thus, overexpression of PKCδVIII_GW decreased basal apoptosis in NT2 cells.

Next, we hypothesized that decreasing the amount of endogenous PKCδVIII should increase the extent of apoptosis. The use of siRNAs to specifically knockdown human PKCδ isoforms has been previously demonstrated (*37*). We transiently transfected siRNA specific to human PKCδVIII (Ambion siRNA ID 290535) along with its negative control into NT2 cells. Western blot analysis indicated a 70−75% decline in PKCδVIII levels. DNA fragmentation increased in NT2 cells transfected with PKCδVIII siRNA (Figure 6B-i). Moreover, we observed an increase in the cleavage of PARP in NT2 cells that were transfected with PKCδVIII siRNA. GAPDH levels remain unchanged (Figure 6B-ii−iv). These results suggested that an increase in basal apoptosis was observed in NT2 cells when the expression of PKCδVIII was reduced.

Antisense Oligonucleotides (ASO) Modulate Alternative Splicing of PKCδVIII. Since the alternatively spliced isoforms of human PKCδ, PKCδI, and PKCδVIII have distinct roles in apoptosis, we next determined as to whether modification of 5′ splice site selection by antisense oligonucleotides (fully modified 2′MOE, P═S; ISIS pharmaceuticals) could alter the expression of PKCδI or PKCδVIII mRNA. The 2′-0-(2-methoxy) ethyl (MOE) antisense oligonucleotide allows for the modulation of the message inside the cell, and it protects the phosphodiester backbone from nuclease degradation. The chemistry does not support RNase H mediated cleavage of hybridized mRNA. Multiple 20-mer antisense oligonucleotides (ASO) spanning the 5′ splice site I or 5′ splice site II of human PKCδ pre-mRNA exon 10 were tested for efficiency and dosage in NT2 cells. On the basis of these results (data not shown), ASO 71 and ASO 75 (generating PKCδVIII or PKCδI, respectively, schematic shown in Figure 7A) were selected to modulate 5′ splice site selection in PKCδ pre-mRNA. ASO 71 and ASO 75 along with its scrambled control were transiently transfected into NT2 cells. The total RNA was isolated, and RT-PCR analysis was performed. Our results demonstrated that ASO 71 masks 5′ splice site I, resulting in increased expression of PKCδVIII mRNA via the utilization of 5′ splice site II of PKCδ pre-mRNA exon 10. ASO 75 masked 5′ splice site II

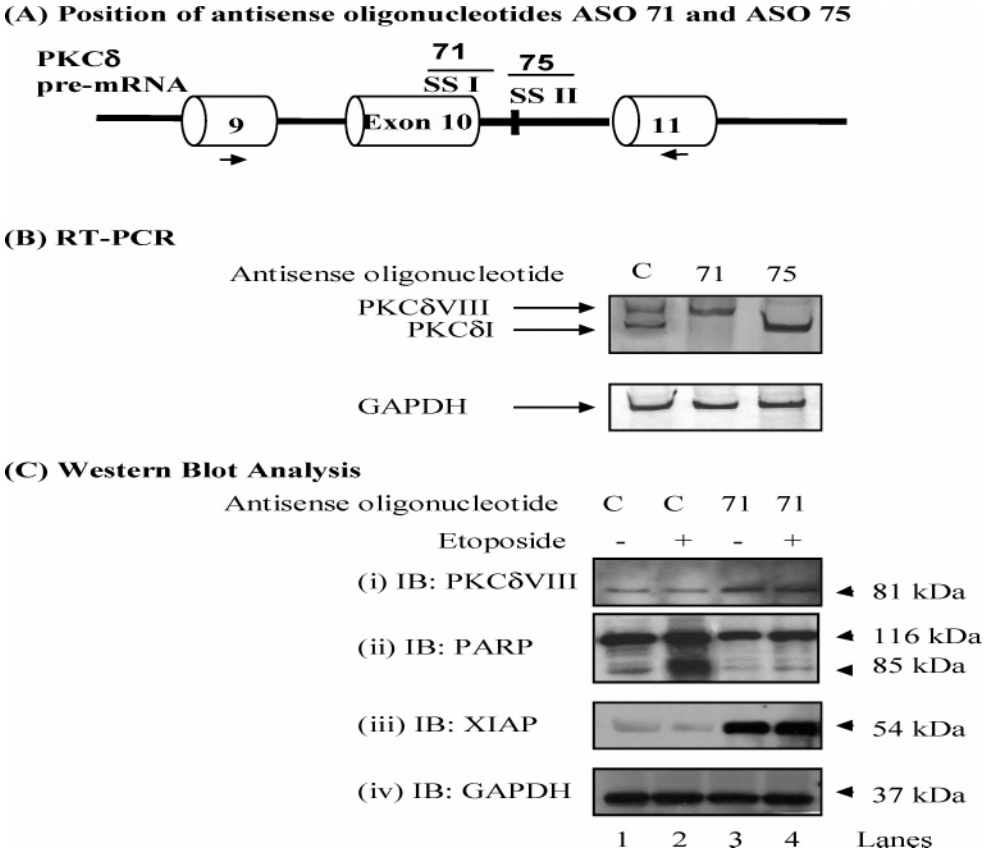


FIGURE 7: (A) Antisense oligonucleotides promote the expression of PKCδVIII in NT2 cells: schematic shows the position of 20-mer antisense oligonucleotides (ASO) on the PKCδ pre-mRNA. (B) ASO 71 and ASO 75 along with the scrambled control (−C) were transiently transfected for 48 h into NT2 cells. Total RNA was extracted, and RT-PCR was performed using PKCδ primers that detect PKCδI and −δVIII simultaneously as indicated in the Experimental Procedures. A total of 5% of products was resolved on 6% PAGE gels and detected by silver staining. ASO71 masks splice site I, resulting in an increased expression of PKCδVIII. ASO75 masks splice site II, resulting in expression of PKCδI. (C) ASO71 was transiently transfected along with its scrambled control in NT2 cells. Etoposide (25 μg/mL) was added to the cells post-transfection of ASO 71 for 12 h as indicated. Western blot analysis was performed on whole cell lysates and membrane immunoblotted using (i) anti-PKCδVIII antibody, (ii) anti-PARP, (iii) anti-XIAP antibody, or (iv) anti-GAPDH antibody (internal control). The experiments were repeated 4 times to ensure reproducibility of results. Lane numbers are indicated at the bottom.

and increased the expression of PKCδI mRNA as observed in Figure 7B. GAPDH was used as an internal control to confirm that these ASOs were specific for their target.

Etoposide, a topoisomerase II inhibitor, is a potent inducer of apoptosis. We wanted to determine as to whether increased expression of PKCδVIII could prevent the cells from undergoing apoptosis. In control NT2 cells, the addition of etoposide induced apoptosis as shown by increased PARP cleavage (Figure 7C-ii; lanes 1 and 2) and decreased expression of XIAP (Figure 7C-iii; lanes 1 and 2). Next, we transfected NT2 cells with ASO 71 and then treated them with etoposide for 12 h. Our results demonstrated that transfection of ASO 71 also increased the protein levels of PKCδVIII (Figure 7C-i). In NT2 cells transfected with ASO 71, we simultaneously observed decreased PARP cleavage (Figure 7C-ii; lanes 3 and 4) and increased expression of XIAP (Figure 7C-iii; lanes 3 and 4) as compared to the control NT2 cells treated with etoposide. The results suggest that increased expression of PKCδVIII mediated by ASO 71 protected the NT2 cells from etoposide-induced apoptosis.

DISCUSSION

In this study, we demonstrated that the novel human PKCδVIII isoform is generated via utilization of an alternative 5′ splice site downstream of exon 10 in human PKCδ pre-mRNA. Alternative splicing is a means of generating protein diversity. This process results in the production of distinct protein products that often differ in their functions. We have demonstrated here that the splice variants of human PKCδ, PKCδI, and PKCδVIII have opposing functions—PKCδI is pro-apoptotic, while PKCδVIII is antiapoptotic in NT2 cells.

A significant number of genes involved in apoptotic pathways is regulated by alternative splicing. This list includes, but is not limited to, the following proteins: Fas and FasL proteins of the extrinsic pathway (*38*), capase-activated DNAse (CAD) and inhibitor of CAD (ICAD) involved in DNA fragmentation (*39*), and caspases such as caspase-2, caspase-9, and caspase-10 that are integrated into the apoptotic pathway (*40*, *41*) as well as the Bcl2 family (*42*, *43*) of proteins. The differential expression of these alternatively spliced proteins can alter cellular apoptosis. A recent review covers the regulation of apoptosis by alternative pre-mRNA splicing and its implications (*44*). We have now placed human PKCδI and PKCδVIII in this list as isoforms participating in crucial decisions regarding cellular fate. There exists an optimal balance in the expression of these alternatively spliced isoforms. Our data suggest that PKCδVIII shields NT2 cells from onset of apoptosis. There exists an inverse relationship between apoptosis and expression of

PKCδVIII in NT2 cells. Interestingly, the expression of PKCδVIII is increased in T cell leukemia, myeloid leukemia, and breast cancer cell lines (unpublished observations, Patel laboratory). Tilting of the balance toward any PKCδ isoform could significantly contribute to the onset of diseases such as cancer and Alzheimer's disease, in which apoptosis plays a significant role.

PKCδ has been implicated in regulating cell apoptosis since the early 1990s. PKCδ has been shown to be activated by multiple apoptotic stimuli such as UV radiation and TNFα. Tyrosine phosphorylation can regulate the proteolytic activation of PKCδ in response to oxidative stress and promote apoptosis in dopaminergic neuronal cells (*45*). PKCδ is a target for caspase-3 mediated proteolysis and is also an activator of caspase-3 (*46*). Inhibition of PKCδ is shown to reduce the effects of apoptosis caused by external stimuli (*47*). On the other hand, PKCδ has been shown to have an antiapoptotic role in human neutrophils and granulosa cells (*48*−*50*). Pro-apoptotic and antiapoptotic effects of PKCδ have been nicely documented in a review explaining the regulation of cell apoptosis by PKCδ (*51*). This discrepancy of function in apoptosis attributed to PKCδ can be explained by the presence of distinct PKCδ isoforms. Most experiments involving PKCδ are conducted using the PKCδ antibody, which would detect multiple PKCδ isoforms or RT-PCR using primers that are not specific for splice variants. We have previously demonstrated that PKCδI is pro-apoptotic and is a mediator of apoptosis (*34*). Mouse-specific PKCδII isoform and human PKCδVIII isoform, described here, function as antiapoptotic proteins. Even though there is greater than 90% homology between mouse PKCδII and human PKCδVIII transcripts, the crucial V3 hinge domains are completely distinct. It is possible that PKCδVIII has additional functions as an intact protein in the cell. PKCδVIII could activate targets in the apoptotic pathway, which may also mediate apoptosis in cells. We are currently exploring these possibilities in our laboratory.

Utilization of the 5′ splice site II of exon 10 on human PKCδ pre-mRNA generates mature PKCδVIII mRNA. RA treatment increases the expression of PKCδVIII within 24 h. Prolonged exposure (3 days or more) of RA results in a significant reduction of PKCδVIII expression. We have identified putative splice factors that bind to exonic sequences of PKCδVIII pre-mRNA via computational analysis and consensus sequence determination (data not shown). Alternative splice site selection is governed by exonic and intronic sequences that serve as enhancers or suppressors (ESEs/ESSs or ISEs/ISSs). The interplay of these sequences with the splice factors that are a part of the spliceosome determine the outcome of pre-mRNA splicing. Splice factors such as serine−arginine-rich (SR) proteins that are complexed with the spliceosome machinery bind to these elements and regulate alternative splicing. Heteronuclear ribonucleoproteins (hnRNPs), polypyrimidine tract binding protein (PTB), and other RNA binding proteins also interact with these cis elements to regulate alternative splicing. RA may influence the binding of these splicing factors or alternatively influence the signaling pathways involved in the post-translational modification of these factors. Regulation of alternative splicing is another level of fine-tuning of the cell's genetic repertoire to adjust to its changing environment. The inclusion of 93 nucleotides in PKCδVIII pre-mRNA splicing translates to 31 amino acids in the hinge region of the mature protein. This falls into the class of modular exons that can be inserted or removed from the transcript without affecting the rest of the protein (*52*). Modular exons are more likely to be exact multiples of three nucleotides and have emerged as a significant decisive factor for functional alternative splicing events. There have been increasing reports of alternatively spliced isoforms involved in the functions of the brain, many of which are associated with neurological diseases (*53*).

Alternative splicing in neurons is now considered to be a central phenomenon in development, evolution, and survival of neurons (*54*). Interestingly, current literature suggests an emerging role of RA in alternative splicing events. In P19 embryonal carcinoma stem cells, during RA-induced differentiation, the co-activator CoAA rapidly switches to its dominant negative splice variant CoAM (*55*). In the same cells, the splicing pattern of the delta isoform of CaM kinase is also changed with RA-induced differentiation (*56*). It has been demonstrated that RA alters the expression of a dynamic set of regulatory genes at the early stages of differentiation (*57*). Our data led us to suggest that RA-induced expression of PKCδVIII in the early stages of differentiation may be necessary to activate a cascade of genes involved in the development and differentiation pathways in NT2 cells as they commit to the neuronal phenotype.

NT2 cells terminally differentiate into neurons upon treatment with RA. This differentiation is progressive, and its stages have been described earlier (*58*). The process of apoptosis was closely evaluated in NT2 cells treated with RA by Guillemain et al. (*59*). Their data using Annexin V/propidium iodide staining and the TUNEL method indicated that apoptosis declined slightly on day 1 of RA treatment as compared to control NT2 cells but increased significantly by day 3 of RA treatment. Our data concur with this. Expression of the antiapoptotic PKCδVIII isoform peaks at day 1 of RA treatment but declines thereafter, allowing PKCδI (the pro-apoptotic isoform) to mediate apoptosis in NT2 cells. Our demonstration of the use of antisense oligonucleotides to switch the expression of PKCδ isoforms in human NT2 cells opens the possibility of potential therapeutic application in diseases where apoptosis plays a prominent role.

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