Published in final edited form as:
Gene Expr. 2006 ; 13(2): 73–84.

PKCδ Alternatively Spliced Isoforms Modulate Cellular Apoptosis in Retinoic Acid-Induced Differentiation of Human NT2 Cells and Mouse Embryonic Stem Cells

NIKETA A. PATEL[*,†], **SHIJIE S. SONG**[*], and **DENISE R. COOPER**[*,†]

* *James A. Haley Veterans Hospital, Research Service, Tampa, FL 33612, USA*

† *Department of Molecular Medicine, College of Medicine, University of South Florida, Tampa, FL 33612, USA*

Abstract

NT2 cells are a human teratocarcinoma cell line that, upon treatment with retinoic acid (RA), begin differentiating into a neuronal phenotype. The transformation of undifferentiated NT2 cells into hNT neurons presents an opportunity to investigate the mechanisms involved in neurogenesis because a key component is cell apoptosis, which is essential for building neural networks. Protein kinase Cδ (PKCδ) plays an important role as a mediator of cellular apoptosis in response to various stimuli. PKCδ (δI) is proteolytically cleaved at its hinge region (V3) by caspase 3 and the catalytic fragment is sufficient to induce apoptosis in various cell types. Mouse PKCδII is rendered caspase resistant due to an insertion of 78 bp within the caspase recognition site in its V3 domain. No functional role has been attributed to these alternatively spliced variants of PKCδ. We sought to find a correlation between the onset of apoptosis, neurogenesis, and the expression of PKCδ isoforms. Our results indicate that RA regulates the expression of PKCδ alternative splicing variants in NT2 cells. Further, overexpression of PKCδI promotes apoptosis while PKCδII overexpression shields the cells from apoptosis. This is the first report to attribute physiological function to PKCδI and -δII isoforms. Next we demonstrated that mouse embryonic stem cells differentiate in vitro into dopaminergic neurons upon stimulation with RA and ciliary neurotrophic factor. These cells showed a simultaneous increase in tyrosine hydroxylase and PKCδII expression. We suggest that the molecular mechanisms regulating differentiation and apoptosis could be understood by alternative expression of PKCδ isoforms.

Keywords

Alternative splicing; Apoptosis; Mouse embryonic stem cells; NT2 cells; Neurons; PKCδ; Retinoic acid

INTRODUCTION

Apoptosis, or programmed cell death, is an integral part of the cell cycle. It reflects the ability to eliminate surplus cells and promote specific cells as required for development and differentiation at various steps of life. Apoptosis, as a mechanism in generating different cell populations during development, is significantly different from necrosis, in which the cause of cell death may be injury, infarction, infection, or inflammation. In apoptosis, a biochemical

Address correspondence to Niketa A. Patel, Ph.D., J. A. Haley Veterans Hospital (VAR 151), 13000 Bruce B. Downs Blvd., Tampa, FL 33612, USA. Tel: (813) 972-2000, ext. 7121; Fax: (813) 972-7623; E-mail: npatel@health.usf.edu.

cascade activates proteases that destroy molecules required for cell survival and others that mediate a program of cell death (14,18,19). Thus, regulation of apoptosis is a complex event involving activation of multiple signaling cascades culminating in cell death.

In the developing nervous system, elimination of neurons by apoptosis helps to restructure the neuronal network (29). The surviving neurons retain their ability to undergo apoptosis in adulthood as part of the aging process as well as in neurodegenerative diseases. Ntera-2 or NT2 cells, a human teratocarcinoma cell line, differentiate into hNT neurons (also called NT2-N neurons) when treated with all-*trans* retinoic acid (RA) and represents a potentially important source of cells to treat neurodegenerative diseases (25). RA not only activates the expression of genes necessary for neuronal differentiation, but also triggers apoptotic signals for early development of nervous system (1,16). This model mimics neuronal differentiation in vivo and represents early stages of progenitor cells. Because neurogenesis is a fine balance between differentiation, apoptosis, and cell survival, the transformation of undifferentiated NT2 cells into hNT neurons by RA presents an opportunity to investigate the molecular mechanisms involved in the early onset of apoptosis.

Protein kinase C (PKC), a serine/threonine kinase family, consists of 11 isoforms involved in the regulation of cellular differentiation, growth, and apoptosis. The primary amino acid structure of PKCs can be divided into conserved regions (C1–C4) separated by the variable regions (V1–V5). All PKCs have an N-terminal regulatory domain and a C-terminal catalytic domain separated by the V3 hinge region. The protein kinase C family is subdivided into three groups based upon their activation by calcium, phosphatidyl serine, diacyl glycerol, or phorbol esters: classical or conventional PKCs (α, βI, βII, and γ), novel PKCs (δ, ε, η, and θ), and atypical PKCs (ζ, λ/ι). PKCs are also activated by proteolytic cleavage at the V3 hinge region by calpain I, II or caspase 3 to generate a constitutively active catalytic domain of PKC (26, 28,30).

Protein kinase Cδ (PKCδ), a member of the novel PKC (nPKC) subfamily, plays an important role in the regulation of cell apoptosis. On one hand, PKCδ regulates the expression and function of apoptotic proteins such as p73β (37), DNA-dependent protein kinase (DNA-PK) (5), Rad9 (22), phospholipid scramblase 1 and 3 (12), and nuclear lamin B (9); on the other hand, PKCδ is itself a target for caspases. In response to apoptotic stimuli, PKCδ is proteolytically cleaved at its hinge region (V3) by caspase 3. The release of the catalytic fragment of PKCδ (or PKCδI) is sufficient to induce nuclear fragmentation and apoptosis (6). Furthermore, inhibition of the catalytic fragment of PKCδ abrogates caspase-induced apoptosis (38). PKCδ is also known to induce caspase expression, thus forming a positive feedback loop in cells marked for apoptosis. Although most studies indicate a proapoptotic role of PKCδ, a few studies implicate PKCδ in cell survival and antiapoptotic effects. Granulosa and PC12 cell apoptosis is prevented by basic fibroblast growth factor (bFGF) via PKCδ pathway (35). In human neutrophils, PKCδ plays a role in antiapoptotic effects of TNF-α (21). PKCδ antiapoptotic effects are also documented in glioma cells infected with a virulent strain of Sindbis virus (48). Thus, PKCδ has been shown to have dual effects—mediator of apoptosis and antiapoptosis effector—and therefore represents a switch that determines cell survival and fate.

Recently, an alternatively spliced variant of PKCδ has been reported in the mouse (PKCδII, GenBank Accession No. AB011812), which has an insertion of 78 bp (corresponding to 26 amino acids) in the caspase 3 recognition sequence of the V3 region of PKCδ (δI), thus rendering PKCδII caspase insensitive (39). This caspase-resistant isoform of PKCδ sheds light to the two opposite effects of PKCδ, inducing apoptosis or protecting cells from apoptosis. The caspase-resistant PKCδII isoform is not constitutively present in all cell types. The molecular

mechanisms regulating the differentiation and protection of cells from apoptosis could be understood by alternative expression of PKCδ isoforms.

In this study we examined the expression of PKCδ isoforms in human NT2 cells in the early stages of differentiation into neurons upon RA treatment and their correlation to onset of apoptosis. Overexpression of PKCδI and -δII alternatively spliced isoforms shed light on their physiological roles for the first time. We further extended our study to mouse embryonic stem (mES) cells because they possess the capacity of self-renewal and the ability to differentiate into practically any cell type. We show that mES cells can differentiate into dopaminergic neurons upon treatment with RA and ciliary neurotrophic factor (CNTF) and relate it to the expression of PKCδII isoform.

MATERIALS AND METHODS

Cell Culture

NTERA-2 cl.D1 (NT2/D1) or NT2 cells, a human teratocarcinoma cell line, was purchased from ATCC (CRL-1973). NT2 cells were plated at a density of 10^6 and maintained in Dulbecco's modified Eagle's medium (DMEM) with 4 mM L-glutamine (modified by ATCC), supplemented with 10% fetal bovine serum (FBS), and maintained at 37°C in a humidified 5% CO_2, 95% air atmosphere in six-well plates. The cells were replated and medium changed every 3 days.

Mouse embryonic stem cells, ES-D3, were derived from a strain of 129/Sv+C/+P mouse blastocyst, and purchased from ATCC (CRL-1934). ES-D3 cells were grown in DMEM with 4 mM L-glutamine (modified by ATCC), supplemented with 0.1 mM 2-mercaptoethanol, 10% FBS, and 10 ng/ml of leukemia inhibitory factor (LIF). The cells were harvested when they grew to confluence. To differentiate the cells in vitro, six-well plates were treated overnight with 0.1% polyethylenimine (PEI). After washing the plates with PBS, 20,000 cells/cm^2 in neurobasal medium supplemented with 4% FBS were added per well. Retinoic acid (RA; 5 μM), 10 ng/ml brain-derived neurotrophic factor (BDNF), 10 ng/ml epidermal growth factor (EGF), or 10 ng/ml ciliary neurotrophic factor (CNTF) was added. Medium was replaced every 3 days.

Transient Transfections of Plasmid DNA

NT2 cells were plated at ~10^6 cells per 35-mm dish the day prior to the transfections. The pTracer/CMV PKCδI or -δII expression vectors were transfected in serum-free medium with Lipofectamine™ (from Invitrogen, Life Technologies). The lipofectamine™–DNA complex was incubated at room temperature for 20 min, diluted with the serum-free transfection medium, and added to the cells. The cells were incubated at 37°C for 4 h and then incubated in the growth medium at 37°C for 18 h.

RT-PCR Analysis

Total RNA was isolated from cells using RNA-Bee™ (Tel Test, Inc.) as per manufacturer's instructions. RNA (2 μg) 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: mouse PKCδ sense 5′-CACCATCTTCCAGA AAGAACG-3′ and antisense 5′-CAACAACGGGA CCTATGGCAAG-3′; human PKCδ sense 5′-CACTATATTCCAGAAAGAACGC-3′ and antisense 5′-CCCTCCCAGATCTTGCC-3′; huGAPDH 5′-CTTCATTGACCTCAACTACATG-3′ and antisense 5′-TGTCATGGATGACCTTGGCCAG-3′; human nestin sense 5′-CCAGCTACTGGATC-3′ and antisense 5′-AGCCAGAAGGCTCAGCA-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; mouse

PKCδ: 94°C 30 s, 56°C 30 s, and 72°C 1 min; GAPDH: 94°C 30 s; 60°C 30 s, and 72°C 45 s; and nestin: 94°C 30 s; 55°C 30 s, and 72°C 1 min; for 30 cycles) 5% of products were resolved on 6% PAGE gels and detected by silver staining. The PCR reaction was optimized for linear range amplification to allow for quantification of products. Densitometric analyses of the bands were done using the Scan Analysis Software.

Western Blot Analysis

NT2 cell lysates (50 mg) were separated on 10% polyacrylamide gel electrophoresis-SDS (PAGE-SDS). Proteins were electrophoretically transferred to nitro-cellulose membranes, blocked with Tris-buffered saline/0.1% Tween 20 containing 5% nonfat dried milk, washed, and incubated with either a polyclonal antibody against PARP (from Upstate), PKCδ C-terminal antibody (Biosource International, Inc.), or an antibody raised against the hinge region of mouse PKC δII. PKCδII-specific polyclonal antibody was raised in rabbits by Bio-Synthesis, Inc. (Lewisville, TX) to the synthetic peptide NH2-GEAGSHISLKLSFP-COOH (977–1110, V3 hinge domain). The antibody was characterized alongside unreactive preimmune antisera and was shown to recognize PKCδII in mouse testis. 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. Apoptotic DNA ladder detection kit (APT151) was bought from Chemicon International and the protocol performed according to the manufacturer's instructions. The product (20 μl) was loaded onto a 1% agarose gel containing 0.5 μg/ml ethidium bromide. The gel was visualized by UV light and image documented using Kodak Image Analysis software.

Immunocytochemistry

For immunofluorescence, slides with NT2 cells were carefully washed with fresh cold 0.1 M PBS and treated for 1 h at room temperature in a mixture of 10% normal goat serum, 0.03% Triton X-100 in PBS. Cultures were incubated in primary antibodies overnight at 4°C with the primary antibody being omitted from the control slides. The following antibodies were used: nestin (BD Biosciences; 1:150, monoclonal), vimentin (Dako; 1:100, monoclonal), and TuJ1 (Covance; 1:1,500, polyclonal). For mES cells, rabbit anti-tyrosine hydroxylase (TH), AB151 polyclonal antibody, 1:3000, from Chemicon was used and counted in 20 fields per dish using Bioquant software.

After four PBS washes, primary antibodies were detected using appropriate secondary antibodies (Alexa Fluor 594 goat anti-rabbit, 1:2000; Alexa Fluor 546 goat anti-mouse 1:1000, Alexa Fluor 488 goat anti-mouse, 1:800; Molecular Probes, Eugene, OR). The slides were washed with cold PBS and coverslipped with 95% glycerol. To visualize cell nuclei, some cultures were coverslipped using Vectashield with DAPI counterstain (Vector Laboratories, Burlingame, CA). The sections were observed under epifluorescence and photographed on an Olympus BX60 microscope.

RESULTS

Expression of the Alternatively Spliced PKCδ Isoforms Is Cell Type Specific

Splicing of exon 9 and exon 10 (coding for the hinge region) in PKCδ pre-mRNA generates alternative products if additional downstream 5′ intronic sequence is included between the exons (Fig. 1A). The alternative expression of PKCδ isoforms has not been extensively reported. Thus, we examined four different cell lines representing both differentiated and

undifferentiated cells for PKCδ splice variants. Using primers in PCR that would detect the alternatively spliced products simultaneously, we observed that only PKCδI mRNA was expressed in the L6 rat skeletal muscle cells and A10 rat vascular smooth muscle cells. This was in agreement with earlier observations (39) that PKCδI is the predominant isoform in the rat while mouse tissues express PKCδI and -δII isoforms. The NIH-3T3-L1 cell line is an important fibroblast line established from disaggregated tissue of an albino Swiss mouse embryo. The cell line is preprogrammed for differentiation and is committed to adipocyte lineage (8). We next sought to compare these differentiated mouse cells with undifferentiated cells. Immortalized mouse embryonic fibroblasts (MEF) obtained from Dr. Morris Birnbaum (H.H.M.I., U. Penn) were derived from primary mouse fibroblasts established from E13.5 embryos (4). RT-PCR results showed the expression of only PKCδI isoform in 3T3-L1 cells while the MEF expressed both PKCδI and -δII isoforms (Fig. 1B). This indicates that the PKCδII isoform expression correlates to early embryonic stages of the cell. Once a cell is committed and is differentiated into its adult phenotype, PKCδII expression declines.

Expression of PKCδ Isoforms in NT2 Cells

NT2 cells, a human teratocarcinoma cell line, differentiate into hNT neurons (also called NT2-N neurons) when treated with all-*trans* retinoic acid (RA) and represent a potentially important source of cells to treat neurodegenerative diseases (25). The exposure of NT2 precursors to RA not only activates the expression of genes necessary for neuronal differentiation but also triggers apoptotic signals for early development of nervous system (1). Hence, NT2 cells serve as a model to study gene regulation in the central nervous system (CNS) because NT2 differentiation is comparable to normal neuronal differentiation in the brain. Morphologically, NT2 neurons, like CNS neuronal precursors, express a variety of neuronal phenotypes typical for young and mature neurons. Simultaneous studies were performed on NT2 cells treated with RA to observe its morphological changes and expression of PKCδ isoforms.

We first demonstrate morphologically that NT2 cells indeed differentiate into neurons upon treatment with 10 μM of RA. As shown in Figure 2A, NT2 cells are flat with irregular shaped bodies with large nuclei. Some cells are multinuclei while others are seen in various stages of mitosis. After 3 weeks of treatment with RA (Fig. 2B), the differentiated NT2 cells have smaller, bi- and multipolar cell bodies with extensive neuritic networks. Most NT2 precursor cells stained for early neural marker, vimentin (red) (Fig. 2C). Vimentin was localized in the cytoplasm with varying degrees of intensities while Dapi staining (blue) was used to visualize the nucleus. Virtually every cell coexpressed the early neural marker, vimentin, as well as TuJ1 (marker to detect healthy neural cells). However, the cells that stained stronger for TuJ1 showed no vimentin staining (Fig. 2C, arrow).

The signals for NT2 cells to differentiate into neurons are activated within 24–48 hours of RA treatment (1,13) and hence we looked for the expression of PKCδ isoforms in NT2 cells differentiating upon RA exposure for 1, 2, 3, and 7 days. RT-PCR analysis revealed alternatively spliced variants of PKCδ whose expression is regulated by RA (Fig. 2D). The 369 bp product corresponded to PKCδI and an additional splice variant was detected at 462 bp. The expression of the longer splice variant decreased upon RA (10 μM) exposure after 24–48 h and a 90% decline was observed after 7 days of RA treatment (see graph in Fig. 2G). This is the first report of PKCδ alternative splicing and its regulation upon differentiation by RA in human cells. Nestin, an early neural marker, was present in these NT2 cells treated with RA as seen by PCR (Fig. 2E). GAPDH levels served as an internal control (Fig. 2F).

Increased Apoptosis in NT2 Cells Upon RA Treatment

PKCδ splice variants have purported roles in apoptosis, which is an important aspect in differentiating neurons. We explored the relationship between the expression of PKCδ

isoforms, differentiation into neurons, and onset of apoptosis in human NT2 cells. Poly(ADP-ribose) polymerase (PARP) is differentially processed in apoptosis and necrosis and hence its activity can be used as a means of distinguishing the two forms of cell death (36). In apoptotic cells, 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. Hence we used PARP cleavage as a means to monitor apoptosis in NT2 cells. NT2 cells were treated with RA for 24–72 h and protein lysates were collected for Western blot analysis. A faint 85-kDa fragment is seen in control cells with no RA. Upon RA treatment for 0 (control)–3 days, an increase in the intensity of this shorter fragment was observed, implying an ongoing apoptotic process (Fig. 3). This observation, taken together with data from Figure 2, implies that in NT2 cells the proportion of cells undergoing apoptosis or differentiation is seen concurrent with increased expression of PKCδI mRNA.

Overexpression of PKCδII Prevents Apoptosis in NT2 Cells

Caspase-induced apoptosis is mediated by PKCδ in various cell types (15). PKCδI is cleaved by caspase 3 at its V3 domain (hinge region) to release the catalytic fragment that is shown to mediate its apoptotic effects (6). PKCδII isoform, which is speculated to have an opposing function to PKCδI, is rendered caspase resistant by insertion of 26 amino acids in the hinge region and consequently is not cleaved into catalytic and regulatory fragments by caspase 3. Hence, we sought to test if overexpression of the PKCδII isoform could render protection against apoptosis in NT2 cells. Mouse PKCδI and -δII were overexpressed in NT2 cells and the extent of apoptosis was monitored. PKCδI and -δII pTracer/CMV expression plasmids were kindly provided by Dr. Harutoshi Kizaki (Department of Biochemistry, Tokyo Dental College, Japan). NT2 cells were either transfected with pTracer/CMV PKCδI or -δII or mock transfected (control). Following 36 h of transfections, NT2 cells were treated with RA for 24–48 h and protein lysates were collected. Western blot analysis was performed using PKCδ C-terminal antibody (Fig. 4A), which would detect full-length and cleaved C-terminal catalytic fragment of PKCδ protein. Treatment with RA resulted in cleavage of PKCδI as observed as an additional 43-kDa band. Samples with overexpression of PKCδII did not exhibit this cleavage and release of the catalytic fragment. A faint cleavage fragment was seen in PKCδII-transfected cells, which reflect the endogenous levels of PKCδI isoform.

Onset of apoptosis was observed by analyzing PARP cleavage by Western blot analysis in NT2 cells that were either transfected with pTracer/CMV PKCδI or -δII or untransfected (control). Overexpression of PKCδII prevented cleavage of PARP by greater than 85% (Fig. 4B). Some apoptosis is seen due to the presence of endogenous PKCδI expression. Further, overexpression of PKCδII was verified using mouse PKCδII-specific antibody raised against the 26 amino acid insertion in the hinge region of PKCδII (Fig. 4C).

Simultaneously, onset of apoptosis was also detected by visualizing high molecular weight DNA fragmentation of NT2 cells. As established in previous studies (44), DNA from NT2 cells is cleaved into high molecular weight (HMV) 50–300 kb fragments but is not cleaved further into smaller nucleotides (small DNA fragments), yet show typical nuclear morphological apoptotic changes. Control NT2 cells and NT2 cells transfected with either pTracer/CMV PKCδI or -δII for 48 h were harvested in their treated medium to study the extent of apoptosis by detecting DNA fragmentation. Cells transfected with pTracer/CMV PKCδI showed an increase in HMV DNA fragmentation in comparison to untransfected cells or NT2 cells transfected with pTracer/CMV PKCδII (Fig. 4D). Etoposide, an inducer of apoptosis, was used as a control.

Expression of PKCδ Isoforms in Mouse Embryonic Stem Cells

Mouse embryonic stem (mES) cells are permanent cell lines established from the inner cell mass of the developing blastocysts and are widely used to characterize biological systems. Embryonic stem cells differ from adult stem cells in that they can proliferate without differentiating for long periods of time. Because our data indicated that PKCδII isoform expression is predominant at the embryonic stage (Fig. 1), we looked for its expression in mES cells. Further, the expression of PKCδ splice variants in embryonic cells is of interest because they represent a proliferating population without differentiating, which is distinct from NT2 cells. These mES cells have the ability to differentiate into any cell type and have been used by various researchers to elucidate the factors governing differentiation into specific cells. We used various differentiation and growth factors to stimulate the mES cells to differentiate into dopaminergic neurons in a manner comparable to NT2 cells differentiating into hNT neurons.

Tyrosine hydroxylase (TH) is the rate-limiting step in the synthesis of dopamine and hence we used TH expression levels to reflect the extent of mES differentiation. TH-positive cells also express dopamine transporter typical of nigral dopaminergic (DA) neurons (47). We simultaneously looked at the expression of PKCδ isoforms and TH-positive cells. The mES cells maintained in minimum media (N5) expressed PKCδI isoform predominantly with low PKCδII expression level (Fig. 5A). Ciliary neurotrophic factor (CNTF) is reported to enhance survival of neurons (11). RA has been shown to promote neuronal differentiation in models such as human NT2 cells. We sought to observe the effect of RA and CNTF in differentiation of mES cells into dopaminergic neurons and correlate it with the presence of PKCδ isoforms. CNTF treatment (which includes RA) increased the total percentage of TH-positive cells upto 30% (Fig. 5B, C). Interestingly, these cells expressed both the PKCδI and -δII isoforms, confirming that PKCδII expression was associated with neurogenesis. Addition of BDNF (and EGF) did not change the expression pattern with only PKCδI isoform being observed, and less than 1% of the cells were TH positive (see graph). This is the first report of CNTF treatment promoting mES cells to differentiate into dopaminergic neurons.

DISCUSSION

Alternative splicing is an important mechanism in generating protein diversity as emphasized by sequencing of the human genome, which demonstrated that 25,000–30,000 genes produce over 100,000 proteins. The physiological significance of a large number of alternatively spliced variants has not yet been studied. Alternative splicing of PKCδ producing PKCδI and -δII isoforms was described by Sakurai et al. (39) in mouse thymocytes while Saito et al. identified the rat PKCδIII mRNA, which results in a truncated protein without the catalytic domain (43). The alternative expression of PKCδ isoforms has not yet been studied in additional tissues and cell types. We pursued the functional roles of the mouse PKCδI and -δII isoforms and demonstrate for the first time that PKCδI is proapoptotic while PKCδII is antiapoptotic in NT2 cells. PKCδ has long been recognized as a mediator of apoptosis in various cell types (6). PKCδ is lethal to normal and neoplastic keratinocytes (24). Denning and colleagues (40) demonstrated that 1) overexpression of the catalytic fragment of PKCδ is sufficient to induce apoptosis in keratinocytes and 2) dominant negative PKCδ, in which the hinge region has been mutated, prevents apoptosis. Furthermore, a number of studies have also pointed to a role for PKCδ in cell survival and antiapoptotic responses in granulosa and PC12 cells (46). Recent studies show that a caspase-resistant mutant of PKCδ protects keratinocytes from UV-induced apoptosis (10). Hence, PKCδ is reported as both an antiapoptotic and a proapoptotic mediator. Studies in PKCδ$^{-/-}$ (23) mice show further evidence that PKCδ maintains a delicate balance between cell proliferation and apoptosis, which is also reviewed (23,42). Although the presence of PKCδII isoform had been established, its functional importance was left to speculation. We demonstrated that the expression of PKCδII isoform is developmentally regulated because its

expression is observed in embryonic fibroblasts but not in cells that are fully differentiated or committed to differentiation. PKCδI isoform is the predominant isoform in all adult tissues and it may be assumed that it reflects the isoform reported by researchers describing the proapoptotic effects of PKCδ.

A subtle balance of differentiation and apoptosis is crucial in determining cellular fate. As in the case of human NT2 cells, RA treatment promotes the differentiation into neuronal hNT cells, thus making it a model system that mirrors normal central nervous system development. Undifferentiated NT2 cells express two alternatively spliced products of human PKCδ. Treatment with RA results in the decrease of the long isoform while expression of the short isoform (PKCδI) remains constant. This reflects the differentiating state of these cells in which increasing apoptosis defines the population of cells committing to production of neurons. We studied the early events because molecular determinants of apoptosis are triggered immediately following RA treatment. The initial 24–48 h are crucial in determination of cell fate as this coincides with the first detection of the neuronal phenotype in NT2 cells (16). Retinoic acid has been shown to induce apoptosis in NT2 cells and its withdrawal enhances cell survival (45). This agrees with our data in which RA promotes the splicing of PKCδ to the short form (PKCδI), thereby promoting apoptosis.

In view of the fact that PKCδII, the caspase-insensitive mouse isoform, is expressed in undifferentiated or embryonal cells, we hypothesized that the biological function of PKCδII may be opposite to that of PKCδI (which is proapoptotic). To test this, we over-expressed mouse PKCδI and -δII in NT2 cells and showed that indeed the expression of PKCδII isoform is associated with decreased apoptosis. This is the first report to attribute physiological function to the two alternative spliced variants PKCδI and -δII. The opposing function of alternatively spliced products of PKCδ further underscores the importance of alternative splicing in generating protein diversity.

Embryonic stem cells that are pluripotent and hold the promise of differentiating into numerous cell types, depending on the stimulus, have been a subject of extensive research recently. We explored the ability of mouse embryonic stem cells differentiating into neurons and associated it to the expression of PKCδ isoforms. It was interesting to note that PKCδII was observed in cells treated with CNTF and RA, suggesting that alternative expression of PKCδII isoform may be crucial in directing neurogenesis. CNTF has been shown to regulate the expression of GFAP via STAT (41), although the mechanism by which CNTF and RA can induce the expression of PKCδII is still under investigation.

There is evidence of alternative splicing of other protein kinase C (PKC) isoforms. The PKCβ alternative splicing is regulated by insulin (7) in multiple cell types. Insulin regulates PKCβII-specific exon inclusion as well as activation of additional 5′ splice sites (32–34). Other alternatively spliced variants include isoforms of PKCθ (27) and PKCζ (31). PKCδ has two splice variants observed in mouse tissues and a third isoform in rat tissues. We have isolated and sequenced the long isoform of PKCδ observed in human NT2 cells, which is distinct from the previously described PKCδ isoforms (manuscript in preparation). Further work is presently being carried out by our laboratory to determine the molecular mechanisms involved in RA-regulated alternative splicing of PKCδ isoforms. A number of studies (13,17,20) have shown that RA treatment regulates gene expression of a variety of genes, but our work is among the first to demonstrate that RA regulates alternative splicing of PKCδ, thereby promoting the differentiation of human NT2 and mouse embryonic stem cells towards the neuronal phenotype. Regulation of alternative splicing by RA may be a cotranscriptional mechanism involving retinoic acid receptor (RARs or RXRs) binding to its response element on the promoter and recruitment of its cofactors. These coactivators bind to RNA polymerase II and sequester splicing factors such as SR proteins, thereby influencing the alternative splicing of

the gene (2,3). This hypothesis is currently under investigation in our laboratory. Elucidating the posttranscriptional mechanisms in the regulation of expression of the alternatively spliced PKCδ isoforms in the early phases of differentiation and neuronal fate could contribute to development of therapeutic agents for treatment of neurodegenerative diseases.

Acknowledgements

We thank Dr. Juan Sanchez-Ramos for his advice and discussions on this project. We also thank James E. Watson and André Apostolatos for technical support and Iwona Misiuta for early immunofluroscence work on NT2 cells. PKCδI and -δII pTracer/CMV expression plasmids were kindly provided by Dr. Harutoshi Kizaki, Department of Biochemistry, Tokyo Dental College, Japan. This work was supported by the Department of Veteran's Affairs Medical Research Service (N.A.P.) and (D.R.C.) and NIH DK 54393 (D.R.C.).

References

1. Andrews PW. Retinoic acid induces neuronal differentiation of a cloned human embryonal carcinoma cell line in vitro. Dev Biol 1984;103(2):285–293. [PubMed: 6144603]
2. Auboeuf D, Dowhan DH, Kang YK, Larkin K, Lee JW, Berget SM, O'Malley BW. Differential recruitment of nuclear receptor coactivators may determine alternative RNA splice site choice in target genes. Proc Natl Acad Sci USA 2004;101(8):2270–2274. [PubMed: 14982999]
3. Auboeuf D, Honig A, Berget SM, O'Malley BW. Coordinate regulation of transcription and splicing by steroid receptor coregulators. Science 2002;298(5592):416–419. [PubMed: 12376702]
4. Bae SS, Cho H, Mu J, Birnbaum MJ. Isoform-specific regulation of insulin-dependent glucose uptake by Akt/protein kinase B. J Biol Chem 2003;278(49):49530–49536. [PubMed: 14522993]
5. Bharti A, Kraeft SK, Gounder M, Pandey P, Jin S, Yuan ZM, Lees-Miller SP, Weichselbaum R, Weaver D, Chen LB, Kufe D, Kharbanda S. In-activation of DNA-dependent protein kinase by protein kinase Cdelta: implications for apoptosis. Mol Cell Biol 1998;18(11):6719–6728. [PubMed: 9774685]
6. Brodie C, Blumberg PM. Regulation of cell apoptosis by protein kinase c delta. Apoptosis 2003;8(1): 19–27. [PubMed: 12510148]
7. Chalfant CE, Mischak H, Watson JE, Winkler BC, Goodnight J, Farese RV, Cooper DR. Regulation of alternative splicing of protein kinase Cbeta by insulin. J Biol Chem 1995;270:13326–13332. [PubMed: 7768933]
8. Cooper DR, Watson JE, Patel N, Illingworth P, Duncan-Acevedo M, Goodnight J, Chalfant CE, Mischak H. Ectopic expression of protien kinase C beta II-delta and epsilon, but not betaI or zeta, provide for insulin stimulation of glucose uptake in NIH-3T3 cells. Arch Biochem Biophys 1999;372 (1):69–79. [PubMed: 10562418]
9. Cross T, Griffiths G, Deacon E, Sallis R, Gough M, Watters D, Lord JM. PKC-delta is an apoptotic lamin kinase. Oncogene 2000;19(19):2331–2337. [PubMed: 10822384]
10. D'Costa AM, Denning MF. A caspase-resistant mutant of PKC-delta protects keratinocytes from UV-induced apoptosis. Cell Death Differ 2005;12(3):224–232. [PubMed: 15618968]
11. Emsley JG, Hagg T. Endogenous and exogenous ciliary neurotrophic factor enhances forebrain neurogenesis in adult mice. Exp Neurol 2003;183(2):298–310. [PubMed: 14552871]
12. Frasch SC, Henson PM, Kailey JM, Richter DA, Janes MS, Fadok VA, Bratton DL. Regulation of phospholipid scramblase activity during apoptosis and cell activation by protein kinase Cdelta. J Biol Chem 2000;275(30):23065–23073. [PubMed: 10770950]
13. Freemantle SJ, Kerley JS, Olsen SL, Gross RH, Spinella MJ. Developmentally-related candidate retinoic acid target genes regulated early during neuronal differentiation of human embryonal carcinoma. Oncogene 2002;21(18):2880–2889. [PubMed: 11973648]
14. Friedlander RM. Apoptosis and caspases in neurode-generative diseases. N Engl J Med 2003;348 (14):1365–1375. [PubMed: 12672865]
15. Ghayur T, Hugunin M, Talanian RV, Ratnofsky S, Quinlan C, Emoto Y, Pandey P, Datta R, Huang Y, Kharbanda S, Allen H, Kamen R, Wong W, Kufe D. Proteolytic activation of protein kinase C delta by an ICE/CED 3-like protease induces characteristics of apoptosis. J Exp Med 1996;184(6): 2399–2404. [PubMed: 8976194]

16. Guillemain I, Fontes G, Privat A, Chaudieu I. Early programmed cell death in human NT2 cell cultures during differentiation induced by all-trans-retinoic acid. J Neurosci Res 2003;71:38–45. [PubMed: 12478612]

17. Harris MN, Ozpolat B, Abdi F, Gu S, Legler A, Mawuenyega KG, Tirado-Gomez M, Lopez-Berestein G, Chen X. Comparative proteomic analysis of all-trans-retinoic acid treatment reveals systematic posttranscriptional control mechanisms in acute promyelocytic leukemia. Blood 2004;104(5):1314–1323. [PubMed: 15142884]

18. Heidenreich KA. Molecular mechanisms of neuronal cell death. Ann NY Acad Sci 2003;991:237–250. [PubMed: 12846991]

19. Hengartner MO. The biochemistry of apoptosis. Nature 2000;407:770–776. [PubMed: 11048727]

20. Houldsworth J, Heath SC, Bosl GJ, Studer L, Chaganti RSK. Expression profiling of lineage differentiation in pluripotential human embryonal carcinoma cells. Cell Growth Differ 2002;13(6):257–264. [PubMed: 12114215]

21. Kilpatrick LE, Lee JY, Haines KM, Campbell DE, Sullivan KE, Korchak HM. A role for PKC-delta and PI 3-kinase in TNF-alpha-mediated antiapoptotic signaling in the human neutrophil. Am J Physiol Cell Physiol 2002;283(1):C48–57. [PubMed: 12055072]

22. Lee MW, Hirai I, Wang HG. Caspase-3-mediated cleavage of Rad9 during apoptosis. Oncogene 2003;22(41):6340–6346. [PubMed: 14508514]

23. Leitges M, Mayr M, Braun U, Mayr U, Li C, Pfister G, Ghaffari-Tabrizi N, Baier G, Hu Y, Xu Q. Exacerbated vein graft arteriosclerosis in protein kinase Cdelta-null mice. J Clin Invest 2001;108(10):1505–1512. [PubMed: 11714742]

24. Li L, Lorenzo PS, Bogi K, Blumberg PM, Yuspa SH. Protein kinase Cdelta targets mitochondria, alters mitochondrial membrane potential, and induces apoptosis in normal and neoplastic keratinocytes when overexpressed by an adenoviral vector. Mol Cell Biol 1999;19(12):8547–8558. [PubMed: 10567579]

25. Misiuta IE, Anderson L, McGrogan MP, Sanberg PR, Willing AE, Zigova T. The transcription factor Nurr1 in human NT2 cells and hNT neurons. Dev Brain Res 2003;145:107–115. [PubMed: 14519498]

26. Newton AC. Protein kinase C: Structure, function, and regulation. J Biol Chem 1995;270:28495–28498. [PubMed: 7499357]

27. Niino YS, Irie T, Takaishi M, Hosono T, Huh N, Tachikawa T, Kuroki T. PKCtheta II, a new isoform of protein kinase C specifically expressed in the seminiferous tubules of mouse testis. J Biol Chem 2001;276(39):36711–36717. [PubMed: 11470790]

28. Nishizuka Y. Studies and prespectives of protein kinase C. Science 1986;233:305–312. [PubMed: 3014651]

29. Oppenheim RW. Cell death during development of nervous system. Annu Rev Neurosci 1991;14:453–501. [PubMed: 2031577]

30. Parker PJ, Murray-Rust J. PKC at a glance. J Cell Sci 2004;117(Pt 2):131–132. [PubMed: 14676268]

31. Parkinson SJ, Le Good JA, Whelan RD, White-head P, Parker PJ. Identification of PKCzetaII: An endogenous inhibitor of cell polarity. EMBO J 2004;23(1):77–88. [PubMed: 14685273]

32. Patel NA, Apostolatos HS, Mebert K, Chalfant CE, Watson JE, Pillay TS, Sparks J, Cooper DR. Insulin regulates protein kinase CbetaII alternative splicing in multiple target tissues: Development of a hormonally responsive heterologous minigene. Mol Endocrinol 2004;18(4):899–911. [PubMed: 14752056]

33. Patel NA, Chalfant CE, Watson JE, Wyatt JR, Dean NM, Eichler DC, Cooper DC. Insulin regulates alternative splicing of protein kinase CbII through a phosphatidylinositol 3-kinase-dependent pathway involving the nuclear serine/arginine-rich splicing factor, SRp40, in skeletal muscle cells. J Biol Chem 2001;276:22648–22654. [PubMed: 11283022]

34. Patel NA, Yamamoto M, Illingworth P, Mancu D, Mebert K, Chappell DS, Watson JE, Cooper DR. Phosphoinositide 3-kinase mediates protein kinase C beta II mRNA destabilization in rat A10 smooth muscle cell cultures exposed to high glucose. Arch Biochem Biophys 2002;403(1):111–120. [PubMed: 12061808]

35. Peluso JJ, Pappalardo A, Fernandez G. Basic fibroblast growth factor maintains calcium homeostasis and granulosa cell viability by stimulating calcium efflux via a PKC delta-dependent pathway. Endocrinology 2001;142(10):4203–4211. [PubMed: 11564676]
36. Putt KS, Beilman GJ, Hergenrother PJ. Direct quantitation of poly(ADP-ribose) polymerase (PARP) activity as a means to distinguish necrotic and apoptotic death in cell and tissue samples. Chembiochem 2005;6:53–55. [PubMed: 15549722]
37. Ren J, Datta R, Shioya H, Li Y, Oki E, Biedermann V, Bharti A, Kufe D. p73beta is regulated by protein kinase Cdelta catalytic fragment generated in the apoptotic response to DNA damage. J Biol Chem 2002;277(37):33758–33765. [PubMed: 12097319]
38. Reyland ME, Anderson SM, Matassa AA, Barzen KA, Quissell DO. Protein kinase C delta is essential for etoposide-induced apoptosis in salivary gland acinar cells. J Biol Chem 1999;274(27):19115–19123. [PubMed: 10383415]
39. Sakurai Y, Onishi Y, Tanimoto Y, Kizaki H. Novel protein kinase C delta isoform insensitive to caspase-3. Biol Pharm Bull 2001;24(9):973–977. [PubMed: 11558579]
40. Sitailo L, Tibudan S, Denning MF. Bax activation and induction of apoptosis in human keratinocytes by protein kinase C delta catalytic domain. J Invest Dermatol 2004;123:434–443. [PubMed: 15304079]
41. Song MR, Ghosh A. FGF2-induced chromatin remodeling regulates CNTF-mediated gene expression and astrocyte differentiation. Nat Neurosci 2004;7(3):229–235. [PubMed: 14770186]
42. Steinberg SF. Distinctive activation mechanisms and functions for protein kinase Cdelta. Biochem J 2004;384(Pt 3):449–459. [PubMed: 15491280]
43. Ueyama T, Ren Y, Ohmori S, Sakai K, Tamaki N, Saito N. cDNA cloning of an alternative splicing variant of protein kinase C delta (PKC deltaIII), a new truncated form of PKCdelta, in rats. Biochem Biophys Res Commun 2000;269(2):557–563. [PubMed: 10708593]
44. Walker PR, Leblanc J, Carson C, Ribecco M, Skorska M. Neither caspase-3 nor DNA fragmentation factor is required for high molecular weight DNA degradation in apoptosis. Ann NY Acad Sci 1999;887:48–59. [PubMed: 10668463]
45. Wang S, Rosengren L, Hamberger A, Haglid K. Antisense inhibition of BCL-2 expression induces retinoic acid-mediated cell death during differentiation of human NT2N neurons. J Neurochem 2001;76(4):1089–1098. [PubMed: 11181829]
46. Wert MM, Palfrey HC. Divergence in the anti-apoptotic signalling pathways used by nerve growth factor and basic fibroblast growth factor (bFGF) in PC12 cells: Rescue by bFGF involves protein kinase C delta. Biochem J 2000;352(Pt 1):175–182. [PubMed: 11062070]
47. Zigova T, Barroso LF, Willing AE, Saporta S, McGrogan MP, Freeman TB, Sanberg PR. Dopaminergic phenotype of hNT cells in vitro. Brain Res Dev Brain Res 2000;122(1):87–90.
48. Zrachia A, Dobroslav M, Blass M, Kazimirsky G, Kronfeld I, Blumberg PM, Kobiler D, Lustig S, Brodie C. Infection of glioma cells with Sindbis virus induces selective activation and tyrosine phosphorylation of protein kinase C delta. Implications for Sindbis virus-induced apoptosis. J Biol Chem 2002;277(26):23693–23701. [PubMed: 11927579]

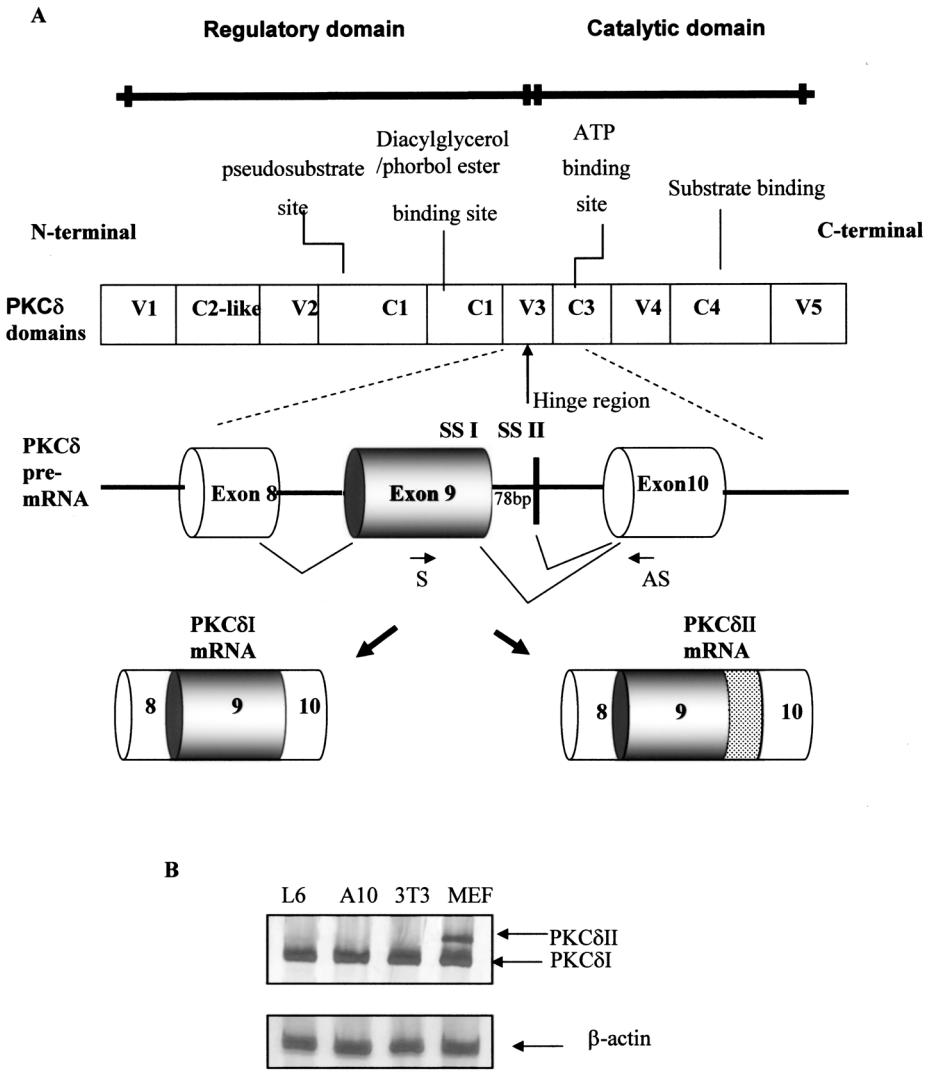
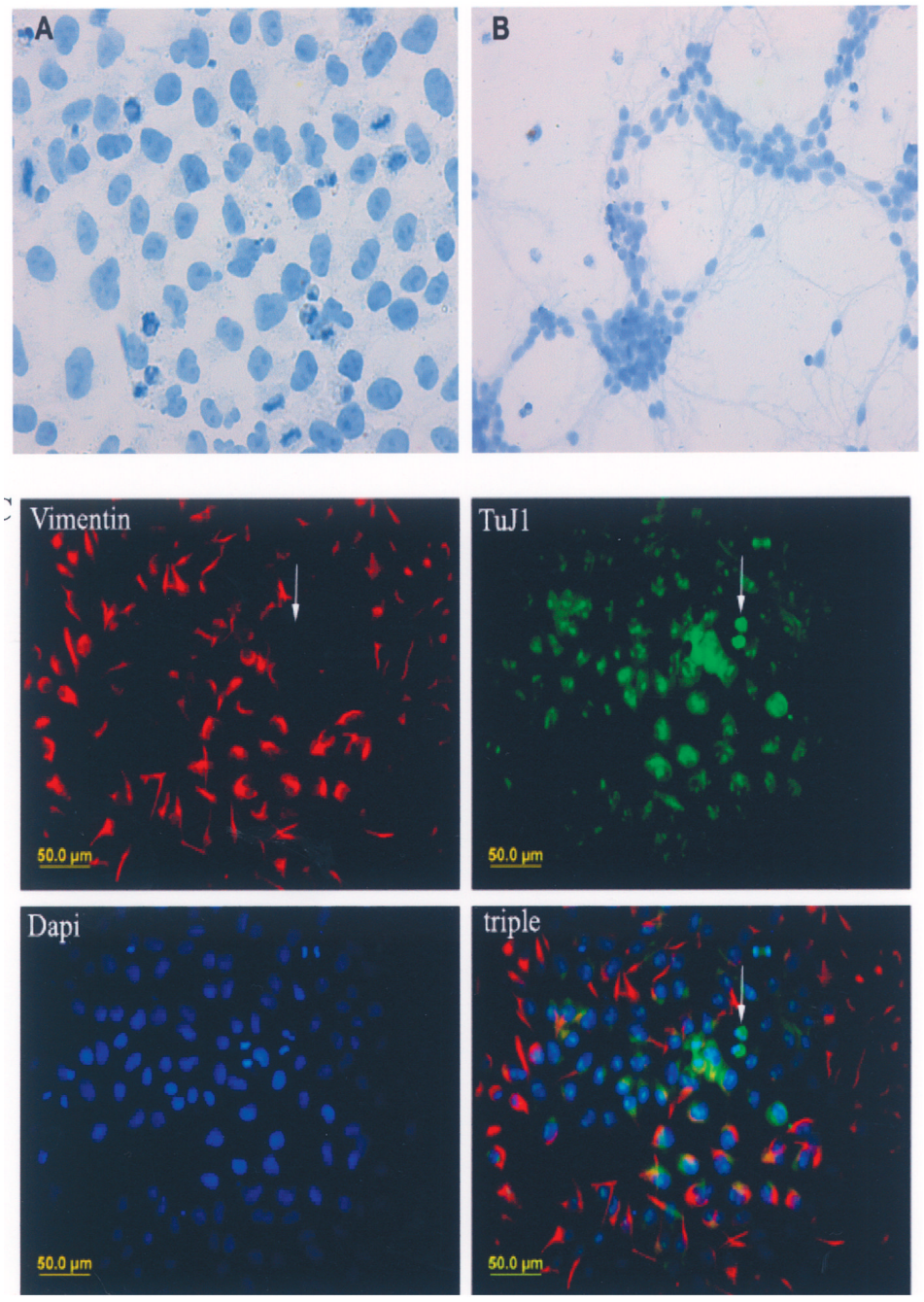


Figure 1.

(A) Schematic of alternative splicing of PKCδ isoforms. Schematic indicates the regulatory and catalytic domains of PKCδ separated by the V3 hinge region, where it is cleaved by caspase 3 to release the catalytic fragment. Alternative 5′ splice site selection in exon 9 of mouse PKCδ pre-mRNA results in the generation of the PKCδI and PKCδII mRNAs, which differ by 78 bp at their hinge region. SSI: splice site I; SSII: splice site II. This insertion renders PKCδII caspase resistant. PCR primer positions are indicated by arrows marked S (sense) and AS (antisense). (B) Total RNA was extracted from rat L6 skeletal muscle cells, rat A10 vascular smooth muscle cells, mouse NIH-3T3-L1-fibroblasts, and murine embryonic fibroblasts (MEF). RNA (2 μg) was used in RT-PCR analysis using PKCδ sense and antisense primers. Five percent of products was resolved on 6% PAGE gels and detected by silver staining. The products obtained were δI: 356 bp and δII: 434 bp. Primers for β-actin were used for normalization. The experiments were repeated 2–3 times for each cell type and similar results were obtained.



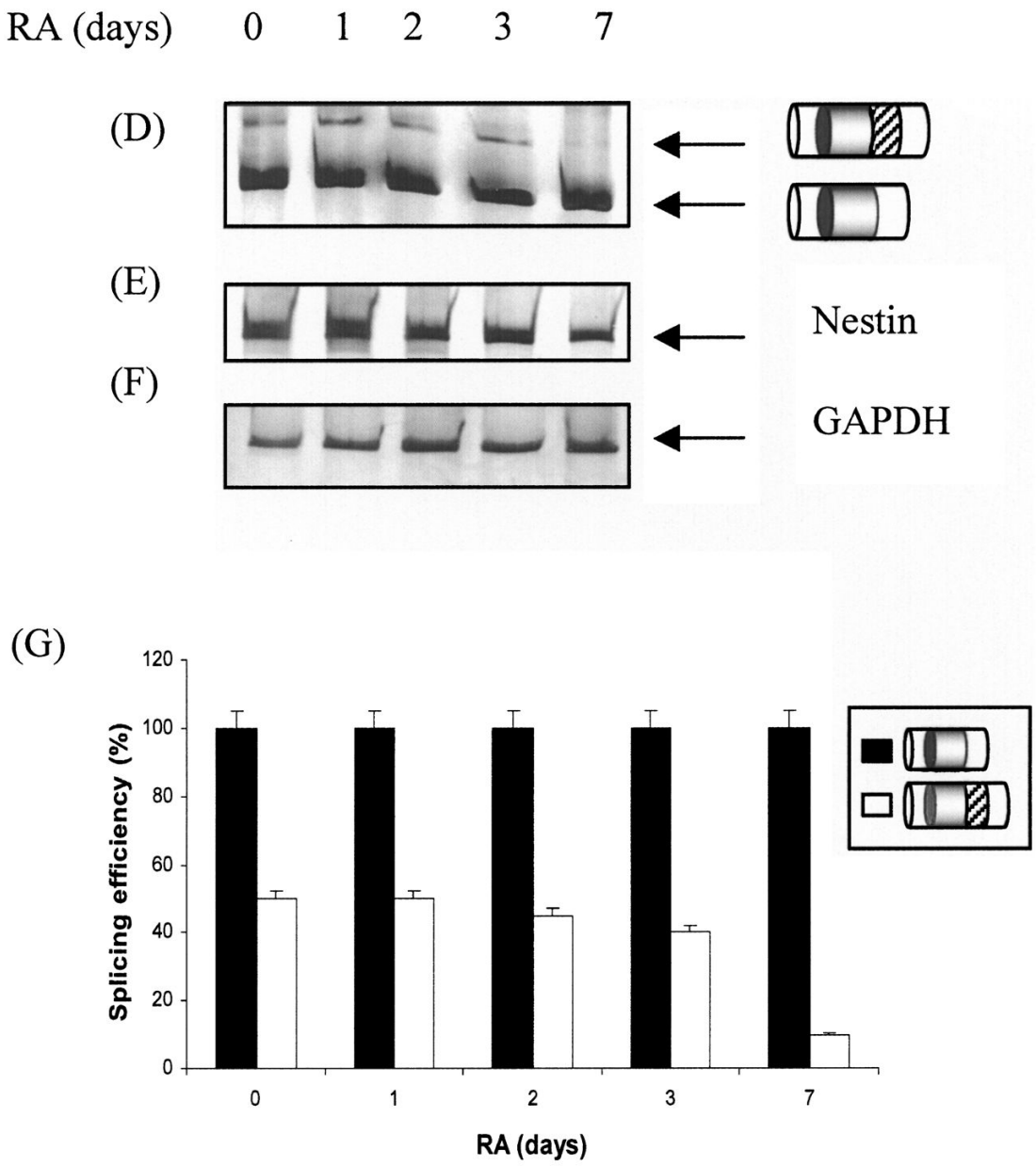


Figure 2.

(A–C) NT2 cells differentiate into neurons. To evaluate the morphology of NT2 cells (A), the slides were exposed to blue counterstain (R&D Systems, Minneapolis, MN) for 5 s, washed 10 times in tap water, and coverslipped using 90% glycerol. (B) NT2 cells stained following 3 weeks of RA exposure. (C) Immunoflorescence studies of NT2 cells following RA treatment for 4 days using vimentin (early neural marker), TuJI (healthy neural cell), and DAPI (nucleus). (D–G) NT2 cells express PKCδ isoforms. In separate experiments using the same passage of NT2 cells, total RNA was isolated from NT2 cells treated with RA for 0, 1, 2, 3, and 7 days and 2 μg of RNA was used in RT-PCR using human (D) PKCδ primers, (E) nestin primers, and (F) GAPDH primers. Five percent of products were resolved on 6% PAGE gels and detected by silver staining. The experiments were repeated five times to ensure reproducibility. (G) Splicing efficiency was quantified by densitometric scanning of the silver-stained gels

from three experiments and represented on the graph. Arbitrary value of 100% splicing efficiency is attached to 369 bp product (δI) in control (RA 0 day) sample.

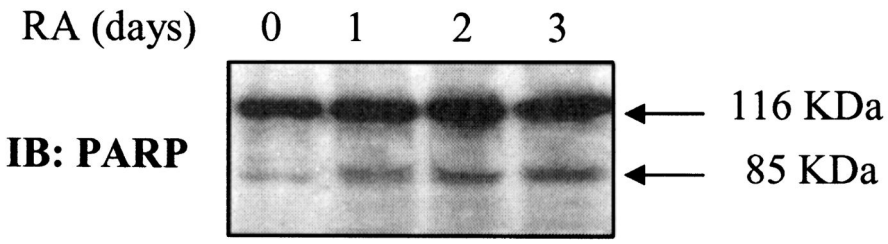


Figure 3.

NT2 cells undergo apoptosis upon RA treatment. Whole protein lysates were isolated from NT2 cells treated with RA for 0–3 days. Proteins were separated on SDS-PAGE and Western blot analysis was performed using PARP antibody. An additional cleaved fragment of PARP demonstrating ongoing apoptosis was detected with RA treatment. The experiment was repeated three times with similar results.

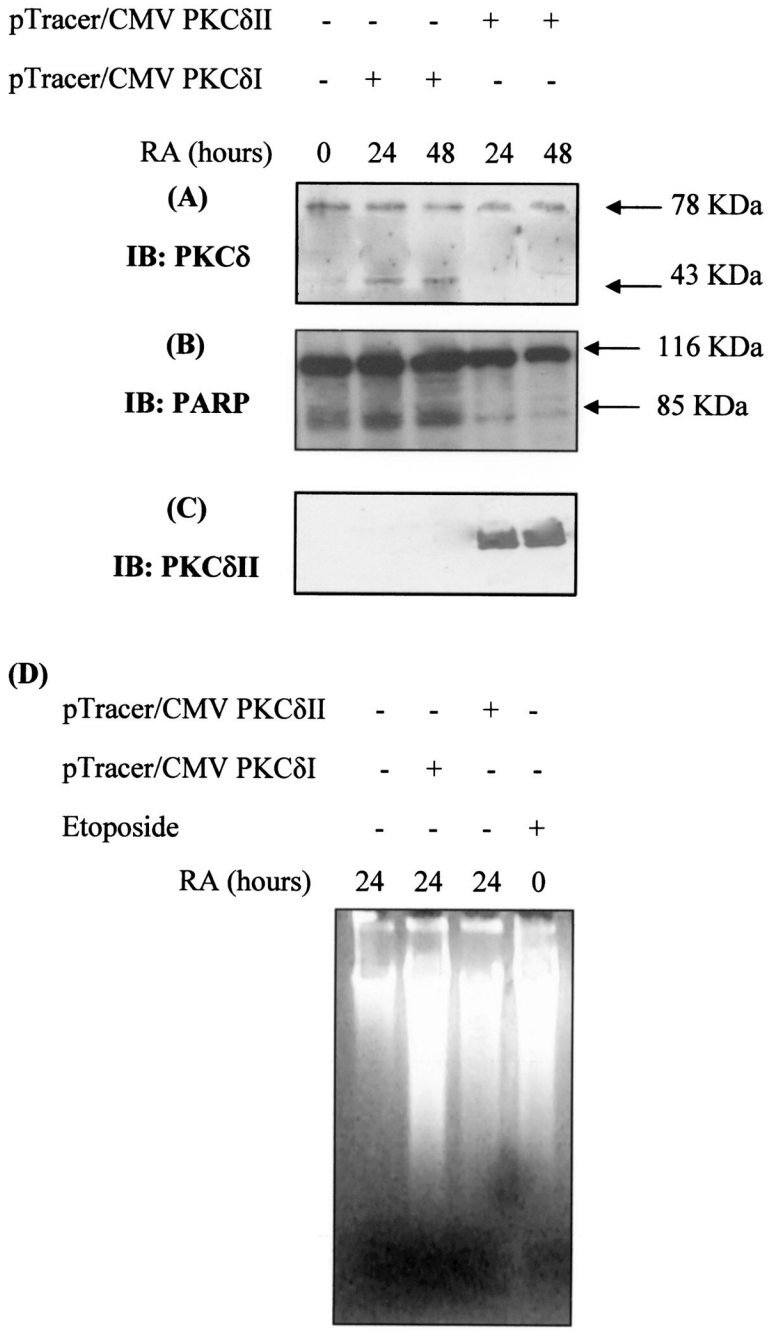


Figure 4.

Overexpression of PKCδII attenuates apoptosis in NT2 cells. Whole protein lysates were isolated from NT2 cells treated with RA for 0–48 h and transfected with either pTracer/CMV PKCδI or -δII. Western blot performed using (A) PKCδ C-terminal antibody, (B) PARP antibody, and (C) mouse PKCδII-specific antibody. (D) DNA was isolated for DNA fragmentation assay from NT2 cells treated with RA for 24 h and transfected with either pTracer/CMV PKCδI or -δII. Etoposide was used as a control for apoptosis. The experiment was repeated three times to ensure reproducibility.

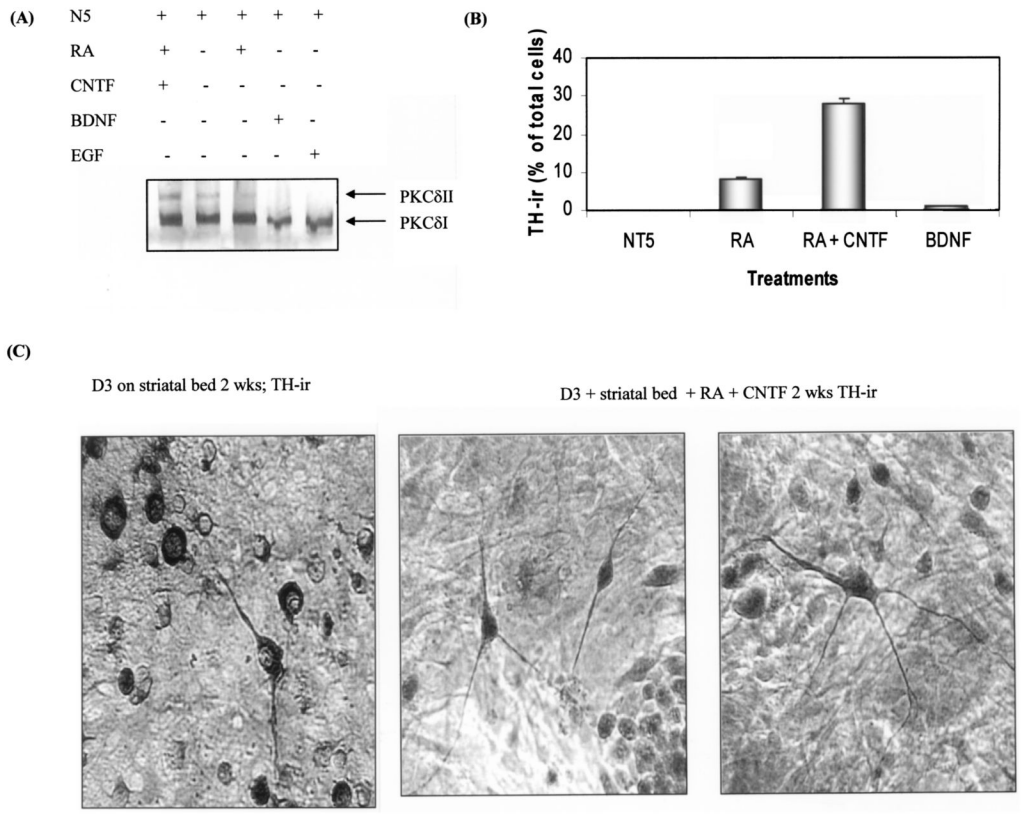


Figure 5.

Mouse embryonic stem cells differentiate into dopaminergic neurons with RA and CNTF treatment. Total RNA was isolated from mouse embryonic stem cells treated with RA, CNTF and RA, BDNF, or in neurobasal N5 medium. (A) RNA (2 μg) was used in RT-PCR using mouse PKCδ primers. Five percent of products were resolved on 6% PAGE gels and detected by silver staining. Simultaneously (B) TH-ir-positive cells were counted as a percent of total cells. (C) Right panel shows TH staining of mES cells on rat striatal bed for 2 weeks. The left panels show mES cells on rat striatal bed in a medium treated with RA and CNTF for 2 weeks in which the cells show neural outgrowth. Experiments were repeated twice with similar results.