

# Induction of p53-Dependent, Insulin-Like Growth Factor-Binding Protein-3-Mediated Apoptosis in Glioblastoma Multiforme Cells by a Protein Kinase C $\alpha$ Antisense Oligonucleotide

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Received June 12, 1998; accepted November 3, 1998

This paper is available online at <http://www.molpharm.org>

## ABSTRACT

Protein kinase C $\alpha$  (PKC $\alpha$ ) expression is related to tumor progression in glioblastoma multiforme (GBM), the most common malignant brain tumor in adults. To determine whether PKC $\alpha$  regulates an anti-apoptotic survival pathway in GBM, A172 GBM cells were treated with a PKC $\alpha$ -selective antisense oligonucleotide. PKC $\alpha$  antisense oligonucleotide treatment was accompanied by reduction in PKC $\alpha$  levels and the induction of wild-type p53 and insulin-like growth factor-binding protein-3 (IGFBP3) 24–72 h after treatment, a period that coincided with the appearance of apoptotic cell death as detected by DNA

fragmentation. There were no significant changes in the levels of Bcl-X<sub>L</sub>, Bax, and p21<sup>WAF1</sup>. Induction of p53 after PKC $\alpha$  down-regulation was not associated with increased mRNA expression, but increased IGFBP3 levels were accompanied by increased mRNA levels. Recombinant human IGFBP3 induced an apoptotic effect that was similar to the PKC $\alpha$  antisense oligonucleotide, and its effect was blocked by IGF-I. These results suggest that one mechanism by which PKC $\alpha$  produces its antiapoptotic activity in GBM cells is by suppressing the p53-mediated activation of IGFBP3.

Protein kinase C (PKC) is a multigene family consisting of the conventional ( $\alpha$ ,  $\beta_1$ ,  $\beta_2$ , and  $\gamma$ ), novel ( $\delta$ ,  $\epsilon$ ,  $\theta$ , and  $\eta$ ), and atypical ( $\lambda$ ,  $\zeta$ , and  $\iota$ ) classes of isoforms (Kikkawa et al., 1989) that participate in signal transduction pathways regulating growth and differentiation (Gescher, 1992; Blobe et al., 1994; Glazer, 1994). Elevated PKC activity is associated with the proliferation of glioblastoma multiforme (GBM) cells (Yong et al., 1988; Couldwell et al., 1991; Couldwell et al., 1992) and the anaplastic phenotype of primary GBM (Couldwell et al., 1992). High PKC $\alpha$  levels and lesser amounts of PKC $\beta_2$ ,  $\delta$ ,  $\epsilon$ , and  $\zeta$  were observed in several GBM cell lines including U-87, A172, U-251, U-138, U-373, and T98G (Misra-Press et al., 1992; Sharma et al., 1991; Ahmad et al., 1994; Mishima et al., 1994; Shen and Glazer, 1998), and a similar pattern has been observed in primary GBM (Todo et al., 1991; Mishima et al., 1994; Reifenberger et al., 1989). PKC activity correlated with the growth rate of several GBM cell lines and primary tumors (Couldwell et al., 1991, 1992), and has been proposed as a therapeutic target for the treatment of GBM

(Vertosick, 1992; Baltuch et al., 1993; Couldwell et al., 1993; Glazer, 1997). However, the participation of PKC in signal transduction pathways that regulate proliferation and apoptosis is likely to be a complex process because PKC is a multigene family consisting of 11 closely related isoforms. Therefore, it is important to develop methods to selectively inhibit PKC isoforms to evaluate their utility as therapeutic targets. One approach for determining the functional roles of PKC is the use of an isoform-specific antisense cDNA or antisense oligonucleotide (AON) (Van der Krol et al., 1988; Dean et al., 1996b). Inhibition of PKC $\alpha$  in U-87 cells with an antisense cDNA or treatment of mice with U-87 xenografts with a PKC $\alpha$  AON (ISIS3521) led to marked inhibition of cell and tumor growth without affecting the levels of other PKC isoforms (Ahmad et al., 1994; Yazaki et al., 1996; Glazer, 1997).

The role of PKC in maintaining cells in an antiapoptotic state has been deduced from several studies. Short-term exposure to phorbol ester activators of PKC-protected cells against apoptosis induced by radiation, glucocorticoids, and growth factor-deprivation (McConkey et al., 1989; Batistatou and Green, 1993; Motyka et al., 1993; Haimovitz-Friedman

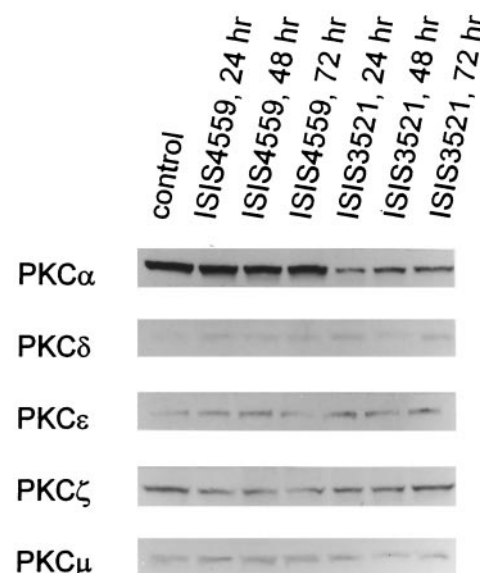
This work was supported by National Institutes of Health Grant NS34431 (to R.I.G.).

**ABBREVIATIONS:** AON, antisense oligonucleotide; GBM, glioblastoma multiforme; IGF-I; insulin-like growth factor-I; IGFBP3, IGF-binding protein-3; PKC, protein kinase C; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; ISEL, in situ end-labeling.

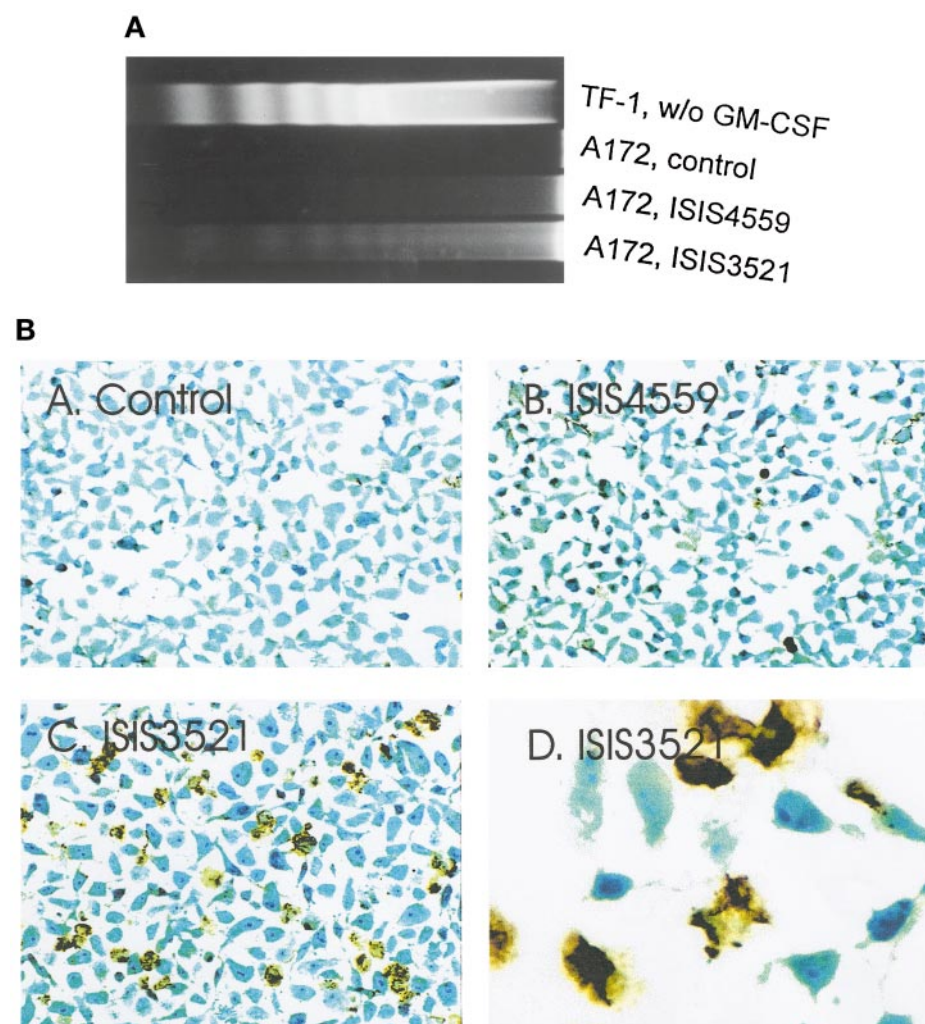
et al., 1994). Conversely, PKC inhibitors either alone or in combination with other chemotherapeutic drugs promoted apoptosis in neuroblastoma, GBM, and gastric cancer cell lines (Couldwell et al., 1994; Behrens et al., 1995; Ikemoto et al., 1995; Schwartz et al., 1995). Inhibition of PKC by the selective bisindolylmaleimide PKC inhibitor, Ro 31-8220, induced apoptosis in vitro in U-87 and A172 cells where PKC $\alpha$  was the predominant inhibitor-sensitive isoform (Shen and Glazer, 1998). Despite this evidence, the PKC isoform(s) and the downstream effectors that are involved in the resistance of GBM to apoptosis have not been defined. In this report, we show that antisense inhibition of PKC $\alpha$  induced accumulation of wild-type p53 and insulin-like growth factor-binding protein-3 (IGFBP3) that was associated with the onset of apoptosis, and that exogenous recombinant IGFBP3 produced a similar effect. These results define for the first time a specific apoptotic pathway that is negatively regulated by PKC $\alpha$ .

## Materials and Methods

**Chemicals and Reagents.** ISIS3521 and ISIS4559 were synthesized at Isis Pharmaceuticals (Carlsbad, CA). Antibodies and growth factors were obtained from the following suppliers: PKC $\alpha$ , PKC $\epsilon$ , and PKC $\zeta$  monoclonal antibodies from Transduction Laboratories (Lexington, KY); Bcl-X<sub>L</sub> and Bax polyclonal, and Bcl-2 and p21<sup>WAF1</sup>



**Fig. 1.** PKC $\alpha$  levels after treatment with a PKC $\alpha$  antisense oligonucleotide. A172 cells were treated for 24–72 h with 200 nM ISIS3521(antisense) or ISIS4559 (scrambled). Cell lysates (50  $\mu$ g) were separated in an 8% polyacrylamide gel by SDS-PAGE and transferred electrophoretically to nitrocellulose. Blots were developed using isoform-specific primary antibodies, and immunoreactive proteins were visualized by chemiluminescence.



**Fig. 2.** Inhibition of PKC $\alpha$  produces DNA fragmentation. A, A172 cells were treated for 48 h with vehicle (control), 200 nM ISIS3521 or ISIS4559, and soluble DNA was separated in a 1.5% agarose gel by electrophoresis. TF-1 denotes soluble DNA from apoptotic TF-1 leukemia cells (Borellini and Glazer, 1993). B, in situ DNA breakage in A172 cells determined after treatment with ISIS3521. Cells were treated for 48 h with either vehicle (control) (A), 200 nM ISIS4559 (B), or ISIS3521 (C and D). DNA breakage was determined by ISEL and is shown by yellow-brown stained cells against a background of cells counterstained with methylene green. Original magnification, 10 $\times$  (A–C); 40 $\times$  (D).

monoclonal antibodies from Santa Cruz Biotechnology, Inc. (San Diego, CA); p53 monoclonal antibody DO-1 from NeoMarkers (Fremont, CA); IGFBP3 polyclonal antibody, recombinant human IGFBP3 and IGF-I from Upstate Biotechnology. (Lake Placid, NY); horseradish peroxidase-conjugated goat anti-rabbit and goat anti-mouse IgG from Bio-Rad (Richmond, CA). Biotinylated anti-mouse and anti-rabbit IgG, Vectastain *elite* ABC Kit and Vector VIP, 3,3'-diaminobenzidine substrate kits were from Vector Labs, Inc. (Burlingame, CA). enhanced chemiluminescence detection reagents were purchased from Amersham Life Science (Arlington Heights, IL).

**Cell Culture.** A172 cells were obtained from the American Type Culture Collection (Manassas, VA). Cells were grown at 37°C under 5% CO<sub>2</sub> in Dulbecco's modified Eagle's medium (DMEM) medium (GIBCO/BRL, Gaithersburg, MD) supplemented with 10% fetal calf serum and 50 µg/ml gentamicin.

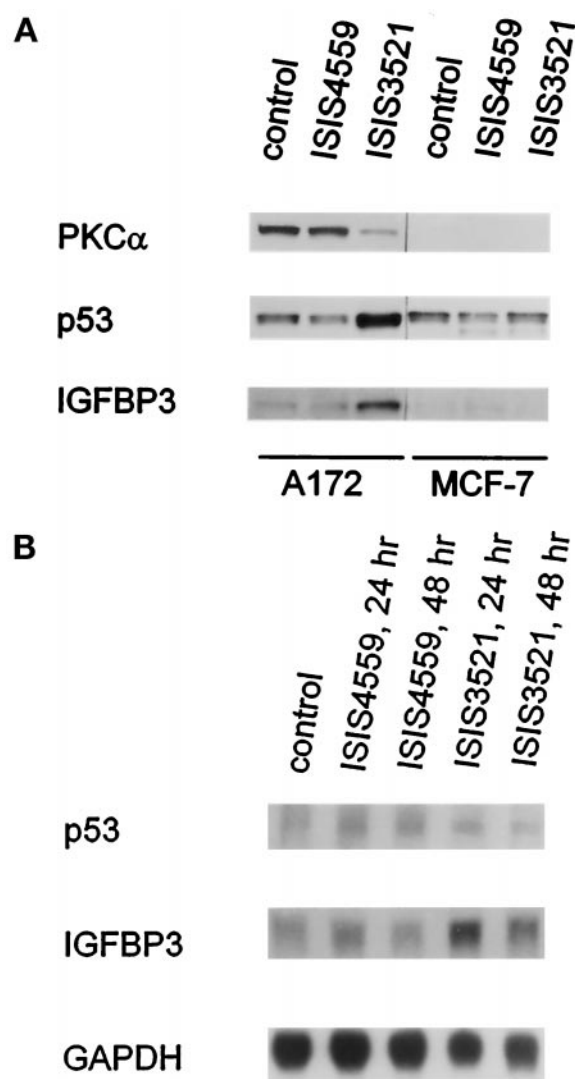
**Phosphorothioate AONs.** PKC $\alpha$  AON, ISIS3521, hybridizes to the 3'-untranslated sequence of human PKC $\alpha$  beginning at the TGA codon, and contains the sequence, 5'-GTTCTCGCTGGTGAGTTTCA-3'. ISIS4559 is a scrambled control oligo with the same base composition as ISIS3521 and contains the sequence, 5'-GGTTTTAC-

CATCGGTTCTGG-3'. ISIS4559 does not recognize any known human mRNA sequence based on a Blast search (Pedro's Biomolecular Research Tools; <http://www.public.iastate.edu>).

A172 cells were plated at 70% density 24 h before AON treatment. Cells were washed three times with serum-free DMEM and incubated for 4 h with 200 nM ISIS3521 or ISIS4559 in serum-free DMEM containing 5 µg/ml of Lipofectin (GIBCO/BRL). Cells were then incubated in DMEM medium containing 10% fetal bovine serum and 200 nM AON for 24–72 h. This concentration was based on determining the concentration that gave the greatest degree of selectivity between antisense and scrambled oligonucleotides with respect to reduction of PKC $\alpha$  levels.

**DNA Fragmentation Assay.** After treatment for 48 h with the AON, adherent and nonadherent cells were harvested, and soluble DNA was isolated from  $1 \times 10^6$  cells as described previously (Herrmann et al., 1994). DNA was separated by electrophoresis in a 1% agarose gel containing 0.5 µg/ml of ethidium bromide in TAE buffer [40 mM Tris-acetate (pH 8.0) and 2 mM EDTA] and visualized by UV fluorescence.

**Western Blot Analysis.** Cells were harvested, washed twice with phosphate-buffered saline (PBS), and suspended in a buffer contain-



**Fig. 3.** Induction of p53 and IGFBP3 by ISIS3521. A, cells were treated for 24 h with either vehicle (control), 200 nM ISIS4559, or ISIS3521. Cell lysates (50 µg) were prepared from A172 and MCF-7 cells, separated in a 10% polyacrylamide gel by SDS-PAGE, transferred to nitrocellulose and immunoreactive proteins visualized by chemiluminescence. B, A172 cells were treated for 24–48 h with either vehicle (control), 200 nM ISIS4559, or ISIS3521, and total RNA was prepared and analyzed by Northern blotting. Abundance of GAPDH mRNA served as a control for RNA loading. C, quantitation of the Northern blot in B by densitometry. p53 and IGFBP3 mRNA levels were normalized to GAPDH levels and expressed relative to control levels.



ing 50 mM Tris-HCl (pH 7.5), 2 mM EDTA, 1 mM EGTA, and 50  $\mu$ g/ml of phenylmethylsulfonyl fluoride. The cell suspension was homogenized by sonication, and protein concentrations were determined with the Coomassie Protein Assay Reagent (Pierce, Rockford, IL) using bovine serum albumin as a standard. Cell lysate (50  $\mu$ g) were separated by SDS-PAGE in either 8% or 12% polyacrylamide gels. Samples were transferred electrophoretically to nitrocellulose membranes, blocked with 5% fat-free dry milk in Tris-buffered saline with Tween 20 [50 mM Tris-HCl (pH 7.5), 0.15 M NaCl, 0.1% Tween-20] and incubated for 3 h with the appropriate diluted primary antibody in Tris-buffered saline with Tween 20. After incubation with a horseradish peroxidase-conjugated secondary antibody, immunoreactive proteins were visualized with the enhanced chemiluminescence detection system (Amersham). Membranes were re-probed with different primary antibodies after stripping the membrane in a buffer containing 62.5 mM Tris-Cl (pH 7.6), 2% SDS, and 100 mM  $\beta$ -mercaptoethanol at 50°C for 1 h.

**In Situ End-Labeling.** Cells were grown in a six-well plate and treated with AON as described above. Adherent and nonadherent cells were collected by centrifugation, washed twice with PBS, and fixed for 10 min in 10% formalin in PBS. Cells were washed twice with PBS and  $1-2 \times 10^5$  cells were resuspended in 200  $\mu$ l of PBS, spotted on a polylysine-coated glass slide, and allowed to air dry overnight. Cells were rehydrated in PBS, and DNA strand breakage was detected by in situ end-labeling (ISEL) (Berchem et al., 1995).

**Northern Blot Analysis.** Northern hybridization was carried out as described previously (Drici et al., 1996). The p53 probe was a 1.8-kb *Bam*HI fragment of the human p53 cDNA from plasmid pC53-SN3 (provided by Dr. Bert Vogelstein, Johns Hopkins University, Baltimore, MD). The IGFBP3 probe was a 1.3-kb *Eco*RI fragment of the IGFBP3 cDNA from plasmid pSP73/hIGFBP-3 (provided by Dr. David Powell, Baylor College of Medicine, Houston, TX). A 0.9-kb glyceraldehyde 3-phosphate dehydrogenase (GAPDH) probe was generated by reverse transcriptase-polymerase chain reaction with primers supplied by CLONTECH (Palo Alto, CA).

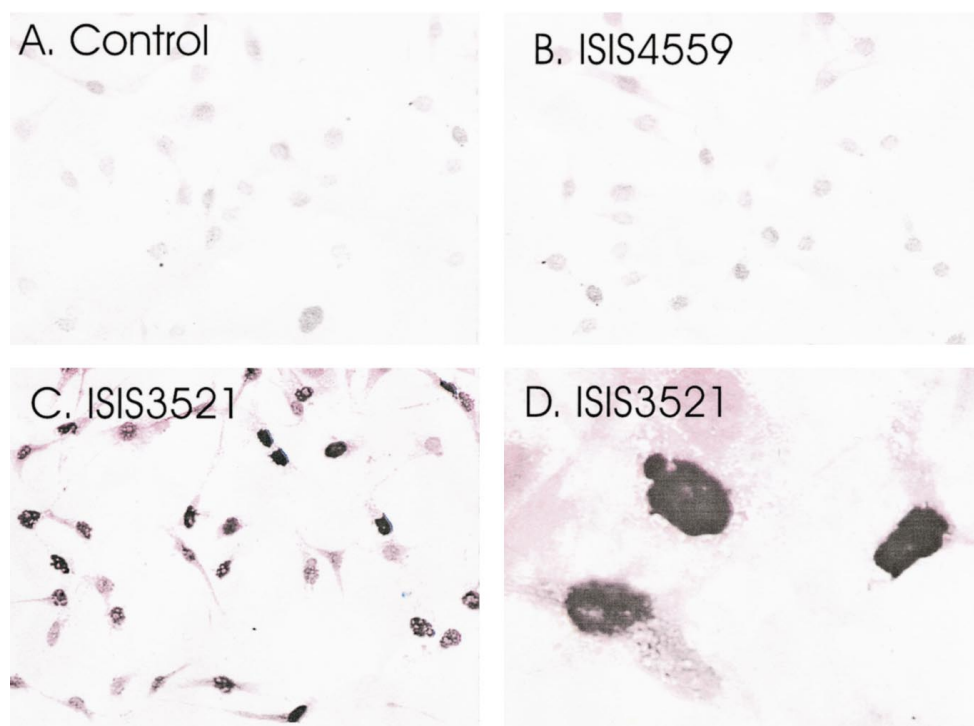
**Immunohistochemistry.** Cells were grown on chamber slides and treated with 200 nM ISIS3521 or ISIS4559 for varying time intervals. Cells were fixed for 10 min in 10% formalin in PBS containing 0.1% Triton X-100, washed three times with PBS, incubated

for 5 min in 0.5% Triton X-100 in PBS and washed three times with PBS. Fixed cells were incubated for 30 min at room temperature with either p53 monoclonal antibody DO-1 or an IGFBP3 polyclonal antibody and washed five times with PBS. Slides were incubated for 30 min with biotinylated anti-mouse or anti-rabbit IgG, washed five times with PBS and the antigen visualized with the immunoperoxidase-based system, Vectastain *elite* ABC kit (Vector Laboratories).

## Results

**PKC $\alpha$  Inhibition and Induction of Apoptosis.** The PKC $\alpha$  AON, ISIS3521, has pronounced antiproliferative and antitumor activity against U-87 cells and U-87 xenografts (Yazaki et al., 1996). Because A172 cells have a similar phenotype (Shen and Glazer, 1998), it was of interest to determine the effect of ISIS3521 on PKC isoform levels in this cell line (Fig. 1). Treatment of A172 cells with 200 nM ISIS3521 selectively reduced PKC $\alpha$  levels 24–72 h after treatment without appreciably affecting PKC  $\delta$ ,  $\epsilon$ ,  $\zeta$ , and  $\mu$  levels, whereas treatment with the scrambled control oligonucleotide, ISIS4559, did not affect PKC expression.

PKC inhibitors administered either alone or in combination with anticancer drugs promote apoptosis in GBM and other tumor cell lines (Couldwell et al., 1994; Behrens et al., 1995; Ikemoto et al., 1995; Schwartz et al., 1995; Shen and Glazer, 1998); however, the role of individual PKC isoforms in preventing apoptosis is not known. To address this question, A172 cells were treated with ISIS3521 and apoptosis was determined by measuring DNA fragmentation (Fig. 2A). A classic 180-bp nucleosomal DNA ladder appeared 48 h after treatment with ISIS3521, but not after treatment with ISIS4559. DNA fragmentation was also assessed by ISEL, which detects 3'-end cleavage of DNA in situ (Fig. 2B). After treatment for 48 h with ISIS3521, ~20% of the cells exhibited DNA breakage (Fig. 2, B–D), and cells treated with ISIS4559 (Fig. 2B) appeared similar to untreated cells (Fig. 2B, A). It



**Fig. 4.** Immunocytochemical detection of p53 in A172 cells. Cells were treated for 24 h with either vehicle (control) (A), 200 nM ISIS4559 (B), or ISIS3521 (C and D), fixed and stained for p53. Immunoreactive protein was visualized by staining as described in *Materials and Methods*. Nuclear p53 is indicated by dark purple staining. Original magnification, 10 $\times$  (A–C); 40 $\times$  (D).

should be noted that the apoptotic response was not related to oligonucleotide uptake because incubation with a fluorescein-tagged oligonucleotide produced nuclear fluorescence in virtually 100% of the cells within 4 h after treatment (results not shown).

**Induction of p53 and IGFBP3.** The possibility that PKC $\alpha$  suppressed expression of proapoptotic proteins such as p53 and IGFBP3 was next investigated in A172 cells by immunoblotting (Fig. 3A). As a control, MCF-7 cells, which contain very low levels of PKC $\alpha$  and contain wild-type p53 (Bartek et al., 1990; Yu et al., 1991), were treated similarly. p53 and IGFBP3 levels were markedly increased in A172 cells, but not in MCF-7 cells 24 h after treatment with ISIS3521 (Fig. 3A), and apoptosis was not observed in MCF-7 cells. The control-scrambled oligonucleotide did not produce a significant effect on PKC $\alpha$ , p53, and IGFBP3 levels in either cell line. To determine whether the increases in p53 and IGFBP3 levels were associated with comparable changes in mRNA levels, northern blotting was performed 24–48 h after treatment with ISIS3521 (Fig. 3B). p53 mRNA levels remained relatively unchanged throughout treatment, but IGFBP3 mRNA levels increased 4-fold 24 h after treatment (Fig. 3C); ISIS4559 did not produce a significant effect on either p53 or IGFBP3 mRNA levels (Fig. 3C). Immunohistochemical analysis of p53 distribution after AON treatment indicated that it accumulated mostly in the nucleus (Fig. 4, C and D). In contrast, IGFBP3 was associated exclusively with the cytosol and/or the plasma membrane (results not shown).

The levels of other p53-regulated proapoptotic proteins were also examined at varying times after ISIS3521 treatment (Fig. 5A). p53 levels were elevated >12-fold 24 h after ISIS3521 treatment, whereas IGFBP3 levels increased throughout treatment reaching a level that was 8-fold higher than control cells after 72 h. There were relatively minor changes in the level of Bax and Bcl-X<sub>L</sub> by ISIS3521, but p21<sup>WAF1</sup> levels increased by 3-fold 72 h after treatment (Fig. 5B). Treatment with the control-scrambled oligonucleotide produced only a transient effect on p53, IGFBP3, and p21 levels.

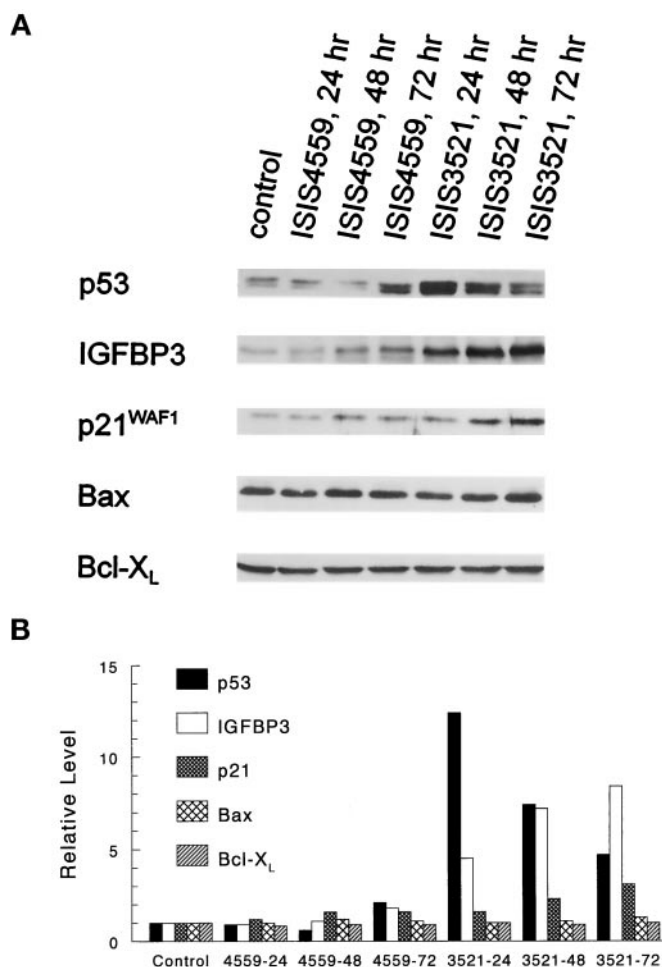
p53 undergoes rapid proteolysis and is often induced by DNA damage or genotoxic chemotherapeutic drugs through a post-translational mechanism (Canman and Kastan, 1997; Levine, 1997). Because p53 mRNA levels remained unchanged during p53 accumulation, it suggested that PKC $\alpha$  was controlling a post-translational process that resulted in an effect on p53 levels that was similar to that of proteasome and protease inhibitors (Maki et al., 1996; Lopes et al., 1997). Therefore, the effects of these inhibitors were compared with ISIS3521 alone and in combination (Fig. 6). Treatment with lactacystin or calpain inhibitor I increased p53 levels in a manner similar to ISIS3521, whereas calpain inhibitor II did not affect p53 levels. The combination of ISIS3521 and protease inhibitors did not exhibit additive effects suggesting that they acted on similar processes that regulate p53 turnover.

**Recombinant Human IGFBP3 Induces Apoptosis.** IGFBP3 is generally considered a negative regulator of cell growth that predisposes cells to apoptosis (Gill et al., 1997; Nickerson et al., 1997; Rajah et al., 1997). Because the induction of endogenous IGFBP3 was closely associated with apoptotic cell death in A172 cells, the effect of recombinant human IGFBP3 on apoptosis was examined (Fig. 7). After

incubation with IGFBP3 for 48 h, ~30% of the cells underwent apoptosis (Fig. 7B). Exogenous IGF-I blocked the apoptotic effect of IGFBP3 (Fig. 7C), suggesting that IGFBP3 was probably producing its apoptotic effect by sequestering IGF-I from the IGF-I receptor.

## Discussion

PKC $\alpha$  is a dominant tumor progression factor that is associated with GBM proliferation and invasiveness. The present study suggests that PKC $\alpha$  regulates survival by negatively regulating p53 and IGFBP3 levels and hence suppressing apoptosis. The selective inhibition of PKC $\alpha$ , the prevalent isoform in GBM cells (Misra-Press et al., 1992; Ahmad et al., 1994; Xiao et al., 1994; Yazaki et al., 1996), elicited apoptosis in A172 cells that was associated with the nuclear accumulation of wild-type p53 and induction of endogenous IGFBP3, a gene that has been shown previously to be induced by wild-type p53 (Buckbinder et al., 1995). These results suggest that the dependence of tumor cell proliferation on PKC $\alpha$ -dependent signaling pathways is a result of inhibition of a p53-dependent apoptotic pathway.



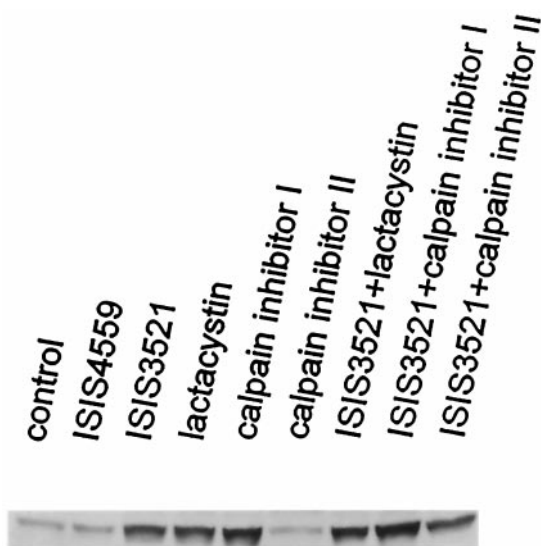
**Fig. 5.** Effect of ISIS3521 on p53, IGFBP3, p21<sup>WAF1</sup>, Bcl-X<sub>L</sub>, and Bax levels. A, A172 cells were treated as described in Fig. 1. Cell lysates (50  $\mu$ g) were separated in a 12% polyacrylamide gel by SDS-PAGE, transferred to nitrocellulose, and immunoreactive proteins visualized by chemiluminescence. B, quantitation of the Western blot in A by densitometry. p53, IGFBP3, p21, Bax, and Bcl-X<sub>L</sub> levels are expressed relative to control levels.



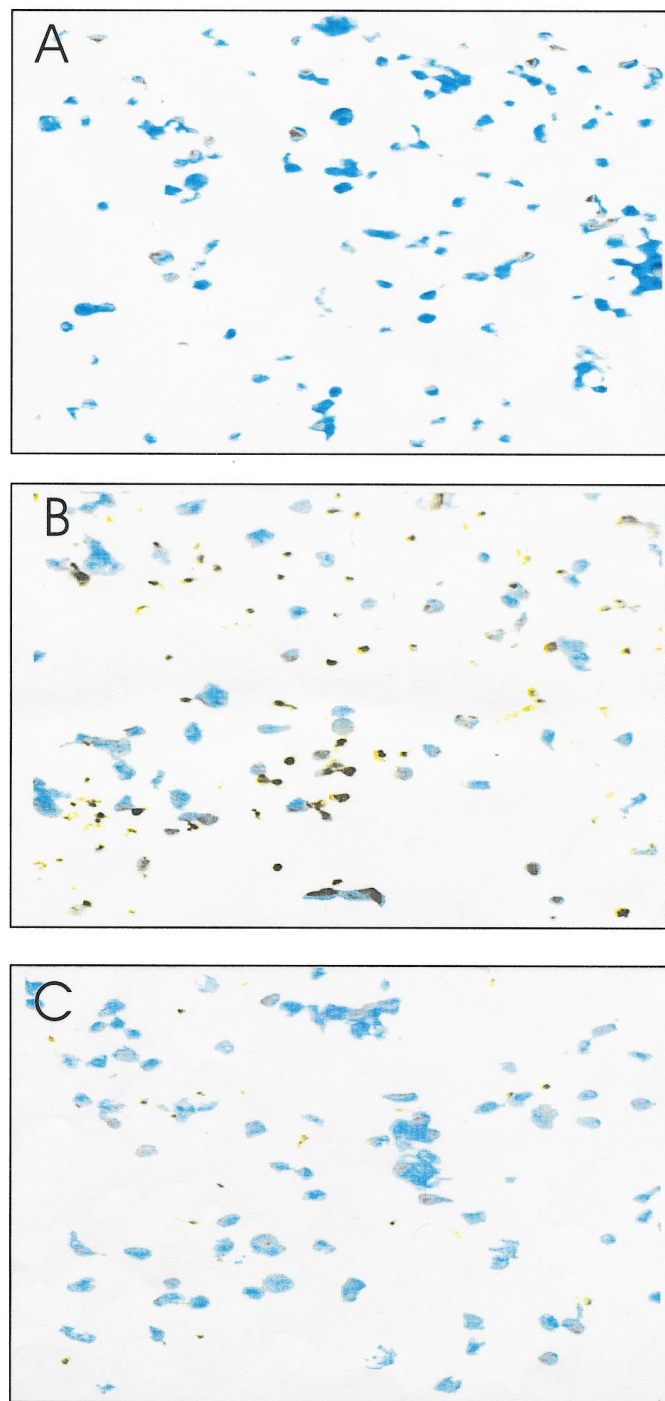
The role of PKC in maintaining GBM cells in an anti-apoptotic state to ensure a selective growth advantage has been implied by several studies. Short-term exposure to phorbol esters protects cells against apoptosis induced by radiation, glucocorticoids and growth factor deprivation (Batistatou and Green, 1993; Motyka et al., 1993; Haimovitz-Friedman et al., 1994). Conversely, PKC inhibitors either alone or in combination with anticancer drugs promote apoptosis in GBM and other tumor cell lines (Couldwell et al., 1994; Behrens et al., 1995; Ikemoto et al., 1995; Schwartz et al., 1995; Shen and Glazer, 1998). However, the PKC isoforms and the downstream effectors that are involved in the resistance of GBM to apoptosis have not been determined. The present data indicate that inhibition of PKC $\alpha$  with a PKC $\alpha$ -selective AON (Dean et al., 1994, 1996a,b) resulted in the nuclear accumulation of wild-type p53. This finding is reminiscent of the induction of wild-type p53 after heat shock (Matsumoto et al., 1994), and suggests that other cellular stresses besides DNA damage (Zhan et al., 1993) can induce p53. Because p53 is a central mediator of apoptosis and cell cycle arrest (Ko and Prives, 1996; Canman and Kastan, 1997; Levine, 1997), it is likely to be involved in the apoptotic effect resulting from PKC $\alpha$  inhibition.

The IGFBPs are a complex family of seven closely related proteins that act as modulators of IGF availability (Shimasaki et al., 1991; Oh, 1998). Among these proteins, only IGFBP3 has been identified as a p53-regulated gene whose induction is capable of inhibiting cell growth (Buckbinder et al., 1995) and directly inducing apoptosis in MCF-7 breast carcinoma cells (Nickerson et al., 1997). The present data show that IGFBP3 is a direct mediator of apoptosis downstream to PKC $\alpha$  that is reversible by addition of IGF-I, suggesting that IGFBP3 causes functional sequestration of this growth factor. However, this does not necessarily rule out IGF-independent pathways in the proapoptotic action of IGFBP3 as shown in IGF receptor-negative fibroblasts (Rajah et al., 1997) or its possible role as an accessory factor involved in apoptosis (Gill et al., 1997). Because IGF-I is a survival

factor that exerts its mitogenic effect in GBM through the IGF-I receptor, and inhibition of the IGFR elicits apoptosis in gliomas (Resnicoff et al., 1994; D'Ambrosio et al., 1996; Baserga et al., 1997), it appears that the negative regulatory effect of PKC $\alpha$  on this process provides a link between growth factor generated second messenger activators of PKC and cell survival.



**Fig. 6.** Effect of ISIS3521 and protease inhibitors on p53 levels. A172 cells were treated for 24 h with either vehicle (control), 200 nM ISIS4559, or ISIS3521, 20  $\mu$ M lactacystin, calpain inhibitor I or calpain inhibitor II alone or in combination. Lysates were analyzed by immunoblotting as described in Fig. 3.



**Fig. 7.** Apoptosis induced by recombinant IGFBP3. A172 cells were incubated for 48 h with medium (A), 20 nM recombinant human IGFBP3 (B), or 20 nM IGFBP3, and 120 nM recombinant human IGF-I (C). Apoptotic cells were detected by ISEL as described under *Materials and Methods*.

## Acknowledgments

We thank Dr. Bert Vogelstein (Johns Hopkins University, Baltimore, MD) for plasmid pC53-SN3, and Dr. David Powell (Baylor College of Medicine, Houston, TX) for providing recombinant IGFBP3 and plasmid pSP73/hIGFBP-3.

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