In Situ Effects of Interferon on Human Glioma Protein Kinase C-α and -β Ultrastructural Localization

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Abstract
Transmission electron microscopy was used to determine how immunogold labeling of PKC-α or -β is modulated by the antitumor drug IFN (HuIFN α-2b) in the cytoplasm, membrane structures, and nucleus of rapidly dividing and confluent human glioma U-373 cells. Results showed that except for nuclear localization, there were no specific cytoplasmic organelles that PKC-α or -β translocated to following HuIFN α-2b treatment. Electron micrographs of PKC-β in proliferating cells depicted 1.34-fold more PKC-β in the nucleus than in the cytoplasm and a 1-min HuIFN α-2b (500 units/ml) treatment transiently increased PKC-β immunoreactivity in the cytoplasm (1.95-fold) and nucleus (1.97-fold). In confluent cells, incubation with HuIFN α-2b for 2 min significantly decreased cytoplasmic PKC-β immunoreactivity by 37%, and no change was observed in nuclear PKC-β labeling. PKC-α labeling in proliferating cells showed similar immunoreactivity in both control cytoplasm and nucleus. Treatment of proliferating cells with HuIFN α-2b for 2 min decreased PKC-α in the cytoplasm (59%) and nucleus (44%). In confluent cells, cytoplasmic PKC-α labeling decreased 59% at 1 min, 61% at 2 min, and 76% at 10 min of HuIFN α-2b treatment. Nuclear PKC-α decreased by 65% at 1 min, 80% at 2 min, and 62% at 10 min after HuIFN α-2b treatment. Western blots of total PKC-α in proliferating and confluent cells and PKC-β in confluent cells showed similar results. However, Western blots of total PKC-α and -β in proliferating cells did not demonstrate any significant changes in either PKC-α or -β immunoreactivity following 1-min HuIFN α-2b treatment. These results suggest that treatment of proliferating U-373 cells with HuIFN α-2b for 1 min unfolds and exposes PKC-β antigenic sites (hinge region) and increases in situ PKC-β immunogold labeling.

Introduction
PKCβ is a family of 11 isozymes that are phospholipid-dependent serine/threonine kinases found in varying ratios in the cytosolic and membrane fractions of cells, depending on the type of tissue and its physiological state (1–3). Protein kinase C isozymes may regulate different cellular functions and proliferation by phosphorylating intracellular proteins in response to transmembrane signals from hormones, growth factors, neuro-transmitters, and pharmacological agents (4). Activation of PKC by various agonists (including IFNs) results in altered transcription of a considerable number of genes (5, 6). Protein kinase C is the major receptor for tumor-promoting phorbol esters, and PKC isozymes are abundant in brain tissue (7). However, the extent of PKC involvement in cellular malignancy is not clearly defined. Various studies indicate that increased tumorigenicity is associated with dysregulation of PKC activity, from changes in PKC concentration, or from both (8–12). Inherently high levels of PKC (10, 11) have been functionally linked with the rapid proliferation rates of high-grade gliomas.

Since there are 11 PKC isozymes, it is believed that PKC isozymes within a single cell type may be differentially regulated by extra- and intracellular stimuli and thereby influence growth, proliferation, differentiation, metabolism, and transformation via distinct mechanisms in different subcellular compartments (1, 2). However, direct ultrastructural evidence for the association of PKC isozymes with specific intracellular organelles, thereby suggesting specific functions for the different PKC isozymes, is limited. To date, the only PKC isoyme ultrastructural localization study reported is that of Chida et al. (13). They found that PKC-γ is localized on the rough endoplasmic reticulum of COS1 cells that transiently expressed PKC-γ. In addition, predominant expression of PKC-γ was found in differentiated or differentiating epithelial cells, suggesting a role for PKC-γ in epidermal differentiation.

Other studies have used cell fractionation techniques to determine the intracellular location of PKC isozymes, but these methods may artificially distort the true location of proteins, and immunofluorescent microscopy cannot distinguish between PKC translocation to the perinuclear, intranuclear, or microsomal membranes following agonist treatment (reviewed in Ref. 14). HuIFN α-2b was studied since it is antiproliferative towards glioma U-373 and activates PKC in these cells (15, 16). It is thought that the response of U-373 glioma cells to HuIFN α-2b is related to the redistribution (movement) of PKC isozymes within the cell. Here we demonstrate the use of immunogold labeling and TEM as a method to ultrastructurally study PKC activation and translocation within the cytoplasm, nucleus, membrane organelles, or cytoskeletal

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3 The abbreviations used are: PKC, protein kinase C; TEM, transmission electron microscopy; HuIFN α-2b, human IFN α-2b; RT-PCR, reverse transcription-PCR; DPBS, Dulbecco’s PBS.
structures following agonist treatment. The distribution of PKC-α and -β was evaluated in U-373 glioma cells at two different growth rates and saturation densities following HuIFN α-2b treatment.

It is well known that astrocytomas are extremely heterogeneous tumors and vary within standard tumor grades in their morphology, differentiation, and types and amounts of PKC isoforms (17, 18). The U-373 glioma cell line used here was originated by Ian Ponten from a Grade 3 (highly proliferative) astrocytoma (19). Protein kinase C-α and -β were analyzed because Western blots of U-373 total cell (cytosolic, membranous, and nuclear) PKC probed with polyclonal antibodies to PKC-α and -β demonstrated immunoreactivity for PKC-α and to a lesser degree for PKC-β (βI and βII) when compared to brain extracts. Todo et al. (18) also found moderately positive type II (PKC-β) immunoreactivity with mAbs in all their astrocytomas examined and weak immunoreactivity for type I (PKC-α) in four of seven astrocytomas (Grade 2) and two of two anaplastic astrocytomas (Grade 3). However, controversy exists surrounding the question as to whether PKC βI or βII are expressed in human astrocytomas. Other workers have not detected either PKC-βI or -βII by immunohistochemistry, Northern blots, or immunoblotting (20, 21). In this study, we used the technique of RT-PCR, which is more sensitive and specific to demonstrate expression of PKC-βI and -βII mRNA (22). To our knowledge, this is the first in situ immuno-ultrastructural study examining PKC-α and -β movement following agonist treatment.

Results

Demonstration of PKC-βI and -βII mRNA in U-373 Human Glioma Cells. Since glioma PKC-β expression was debatable, we demonstrated the presence of PKC-βI and -βII mRNA in U-373 glioma cells used here by RT-PCR (22, 23). Antisense PKC-βI and -βII oligonucleotide primers were synthesized to amplify the nucleotide sequences in the COOH-terminal variable (V5) regions of PKC-βI and -βII cDNA. A sense primer corresponding to the hinge or third variable region (V3) common to PKC-βI and -βII were used to amplify cDNA. These primers amplified the catalytic region of PKC-β including the alternative splice site for PKC-βI and -βII. Fig. 1 demonstrates that mRNA transcripts to PKC-βI and -βII are present in U-373 glioma cells.

Western Blots Demonstrate PKC-α, -βI, -βII, and -β in U-373 Human Glioma Cells. The presence of PKC-α, -βI, -βII, and β (βI and βII) in U-373 glioma cells was demonstrated with isozyme-specific antibodies (Fig. 2) by representative immunoblot. Direct comparison of the immunoreactive polypeptides demonstrated that U-373 total (cytosolic, membrane, and nuclear) immunoreactivity was PKC-α > PKC-β. For the ultrastructural studies and Western blots mentioned below, we used PKC-α and -β antipeptide antisera.

Monospecificity of PKC-α and -β Antipeptide Antisera. Confluent U-373 glioma cells were treated with HuIFN α-2b (500 units/ml) for 0, 1, 2, or 10 min, and total cell homogenates were used for Western blots. Peptides to PKC-α and -β were coincubated overnight with antipeptide antisera and used in Western blot blocking experiments. No immunoreactive staining was detected in total cell homogenates of U-373 cells treated with HuIFN α-2b for 0, 1, 2, or 10 min and probed with antipeptide antisera to PKC-α and -β (Fig. 3). These results demonstrate that antipeptide antisera are mono-specific for PKC-α and -β and that cross reactions with other U-373 glioma cellular proteins can be eliminated from consideration.

Immunofluorescence and Immunogold Electron Microscopy of U-373 Glioma Cells Treated with HuIFN α-2b. We initially used indirect immunofluorescent light microscopy to determine if changes in the intracellular localization of PKC-α or -β could be observed in U-373 glioma cells following HuIFN α-2b treatment. Rapidly dividing and confluent cultures of U-373 glioma cells were treated with HuIFN α-2b for 0, 1, 2, and 10 min and processed for immunofluorescence using fluorescein-conjugated second antibodies. Incubation of control U-373 glioma cells with nonimmune sera showed no detectable autofluorescence (Fig. 4A). Treatment of control U-373 glioma cells with antibodies to PKC-β demonstrated immunofluorescent labeling in the cytoplasm, nuclear, and perinuclear regions (Fig. 4B). Compared to controls, no dramatic changes in immunofluorescent PKC-α or -β patterns were observed for the entire series of HuIFN α-2b treated U-373 glioma cells (data not shown).

Immunogold electron microscopy was used to identify the intracellular sites to which PKC-α or -β associate following HuIFN α-2b treatment. As a positive control, glucocorticoid receptor translocation was used to optimize the technique of immunogold ultrastructural translocation. Glucocorticoids are used in glioma chemotherapy to inhibit tumor-induced edema and postoperative brain swelling. Glucocorticoid receptors are predominantly cytoplasmic in the absence of steroids. Upon binding of the glucocorticoids to the receptor, the hormone-receptor complexes
translocate to the nucleus, where they affect transcription and gene expression (Ref. 24; reviewed in Ref. 25). To test for ultrastructural translocation, U-373 glioma cells were incubated for various times with 0.025 µM dexamethasone (a synthetic glucocorticoid) and were processed for immunogold labeling using antibody to the glucocorticoid receptor. Controls for nonspecific labeling using nonimmune rabbit serum and control sections of U-373 glioma demonstrated minimal staining (Fig. 5A). As shown in Fig. 5B, control U-373 cells assayed with anti-glucocorticoid receptor showed sparse labeling restricted to the cytosol. To enhance the visibility of the gold particles, sections in Fig. 5, B and C, were not contrast stained with lead citrate. Examination of U-373 cells treated with dexamethasone for 2 min and labeled with the anti-glucocorticoid receptor showed the majority of the gold particles associated with the nucleolus (the site of nuclear transcription). These results suggest that agonist-induced ultrastructural translocation can be documented with immunogold labeling.

Subsequently, the ultrastructural effects of HuIFN α-2b on PKC-α and -β stimulation were evaluated in cross-sectional profiles of U-373 glioma cells treated with HuIFN α-2b for 0, 1, 2, and 10 min under the TEM. In rapidly dividing (75% confluent) U-373 glioma cells, the distribution of PKC-β immunogold particles in untreated cytoplasm and nucleus was significantly different (Fig. 6A). There was 1.34- and 1.27-fold more PKC-β labeling in the nucleus compared to the cytoplasm in proliferative and confluent cells, respectively (Fig. 6). However, treatment of rapidly dividing cells with HuIFN α-2b for 1 min abruptly and significantly increased PKC-β labeling in both the cytoplasm (1.95-fold) and nucleus (1.97-fold) compared to untreated cells (Fig. 6A). Following 2 min incubation of proliferating cells with HuIFN α-2b, cytoplasmic and nuclear PKC-β immunoreactivity transiently declined to near basal levels, and at 10 min after HuIFN α-2b treatment, cytoplasmic (44%) and nuclear (50%) PKC-β decreased to below basal levels (Fig. 6A). In contrast, no significant modulation
of nuclear PKC-\(\beta\) was observed in confluent cells at any of
the incubation time points examined (Fig. 6B). Cytoplasmic
PKC-\(\beta\) immunoreactivity in confluent cells did not change
at 1 min after HuIFN \(\alpha\)-2b. However, at 2 min after HuIFN
\(\alpha\)-2b treatment, there was a significant decrease (34\%) in
PKC-\(\beta\) labeling compared to controls. Cytoplasmic PKC-\(\beta\)
immunoreactivity approached basal levels at 10 min after
HuIFN \(\alpha\)-2b treatment.

The temporal effects of HuIFN \(\alpha\)-2b on PKC-\(\beta\) ultra-
structural localization in rapidly dividing cells showed
PKC-\(\beta\) labeling distributed between the cytoplasm and
nucleus under basal conditions (Fig. 7). Increased cyto-
plasmic and nuclear PKC-\(\beta\) labeling was observed at 1
min following HuIFN \(\alpha\)-2b treatment (Fig. 7, C and D). In
confluent cells, nuclear PKC-\(\beta\) labeling was approxi-
mately equal at all time points after HuIFN \(\alpha\)-2b treatment
(Fig. 8). In contrast, cytoplasmic PKC-\(\beta\) in confluent
cells decreased at 2 min following HuIFN \(\alpha\)-2b treatment.
Examination of subcellular organelle labeling in prolif-
erating and confluent cells (Figs. 7 and 8) depicted promi-
ient PKC-\(\beta\) staining in the nucleus (euchromatin, het-
erochromatin, and nucleolus). Immunogold particles
pecific to PKC-\(\beta\) were associated with plasma mem-
brane, mitochondria, lysosomes, lipid droplets, rough
and smooth endoplasmic reticulum, and Golgi
complexes.

Quantitative evaluation of PKC-\(\alpha\) immunogold labeling
in proliferating cells showed sparse immunoreactivity
equally distributed in both untreated cytoplasm and
nucleus (Fig. 9A). Incubation with HuIFN \(\alpha\)-2b for 1 min
yielded no significant change in either PKC-\(\alpha\) cytoplasmic
or nuclear distribution. Following 2 min of HuIFN \(\alpha\)-2b
treatment, cytoplasmic (59\%) and nuclear (44\%) PKC-\(\alpha\)
immunoreactivity decreased. By 10 min after HuIFN \(\alpha\)-2b

Fig. 4. Fluorescein indirect immunofluorescence micrographs of U-373
glioma cells. Control U-373 cells incubated with nonimmune rabbit
serum (A) and rabbit anti-PKC-\(\beta\) (B). See “Materials and Methods” for experimental
details. \(\times 450\).

Fig. 5. Immunogold transmission electron micrographs of U-373 human
glioma cells. Control U-373 cells incubated with nonimmune rabbit
serum (A) at 1:20 dilution and rabbit anti-glucocorticoid receptor (B) at 1:400
dilution. C, glioma U-373 treated with dexamethasone (0.025 \(\mu\)M) for 2 min
and assayed with rabbit anti-glucocorticoid receptor at 1:400 dilution. Col-
loidial gold protein A was diluted 1:50. A, \(\times 25287\); B, \(\times 25287\); C, \(\times 30742\).
C, cytoplasm; n, nucleus; Nu, nucleolus; gl, glial filaments; L, lipid droplets.
treatment, PKC-α labeling returned to near control levels in both the cytoplasm and nucleus. Quantitation of the temporal effects of HuIFN α-2b on PKC-α immunogold labeling in confluent cells demonstrated decreased labeling in both the cytoplasm and nucleus (Fig. 9B). Cytoplasmic PKC-α labeling decreased by 59% at 1 min, 61% at 2 min, and 76% at 10 min of HuIFN α-2b treatment. Nuclear PKC-α decreased by 65% at 1 min, 80% at 2 min, and 62% at 10 min after HuIFN α-2b treatment. Ultrastructural analysis of PKC-α organelle labeling pattern in proliferating cells (Fig. 10) illustrated enhanced staining in the nucleus and equal labeling between all other organelles at all HuIFN α-2b time points studied. Alternatively, analysis of PKC-α labeling in confluent cells (Fig. 11) showed random labeling in both the cytoplasm and nucleus and no specific PKC-α organelle association was found. Western Blots of Total PKC-α and PKC-β in Proliferating and Confluent U-373 Cells following HuIFN α-2b Treatments. To determine if modulation of PKC-α and -β immunoreactive gold particles in both proliferating and confluent U-373 cells following HuIFN α-2b treatment was due to either conformational changes in PKC-α or -β resulting in unfolding of the molecule and exposure of more antigenic sites (the “hinge” region) or to increased synthesis of PKC-α or -β. Western blots were performed, and the content of PKC-α and -β after HuIFN α-2b treatment was compared. If the changes in PKC-α or -β immunoreactive gold particles were due to conformational unfolding, then total (cytosolic, nuclear, and membranal) PKC content as judged by Western blots would remain the same. Alternatively, if the content of PKC-α or -β increased, Western blots would depict a change in PKC-α or -β immunoreactivity. Western blots of PKC-α and -β in both proliferating and confluent cells after HuIFN α-2b treatment are shown in Fig. 12. Immunoreactivity to PKC-β (Fig. 12, C and D) is greater than PKC-α (Fig. 12, A and B), possibly due to longer times of chemiluminescence exposure. Resulting densitometric scans of PKC-α in proliferating cells (Fig. 12A), confluent cells (Fig. 12B), and PKC-β in confluent cells (Fig. 12D) demonstrated decreases in immunoreactivity following HuIFN α-2b treatment. Since similar changes were noted in another experiment and no increases in PKC-α were observed at 1 min after HuIFN-α2b treatment, the differences between immunoblot data and in situ immunogold labeling data may reflect rapid turnover and degradation. The content of PKC-β immunoreactivity in proliferating cells remained constant at 1 min and decreased by 14% at 2 min and 31% at 10 min of HuIFN α-2b treatment. Again, no increase in PKC-β immunoreactivity was obtained at 1 min after HuIFN α-2b treatment. These results suggest that the increase in immunogold particles to PKC-β in proliferating U-373 is due to unfolding of antigenic sites (hinge region) and increases in in situ PKC-β immunogold labeling, whereas the decrease seen at 2 and 10 min after HuIFN α-2b suggests turnover and degradation.

Discussion
Previous efforts to determine the subcellular movement of PKC following activation have used immunofluorescent (26, 27) or subcellular fractionation techniques (28). A difficulty in using light microscopy and either immunofluorescent or immunohistochemical techniques for PKC localization after activation is that the precise subcellular distribution of PKC, i.e., nuclear or perinuclear, cytoplasmic or microsomal, microfilamentous or mitochondrial, is not known (Fig. 4B). Alternatively, subcellular fractionation of PKC before and after activation is also not optimal, since membrane isolation procedure involves cellular lysis, which may result in release and partial associa-
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Fig. 7. Immunogold transmission electron micrographs of PKC-β labeling in nonconfluent U-373 cells after HuIFN α-2b treatment. Cytoplasm (left) and nucleus (right) of cells treated with DPBS (control) or HuIFN α-2b (500 units/ml) for 0 min (A and B), 1 min (C and D), 2 min (E and F), and 10 min (G and H). Note increased gold particle density in both cytoplasm (C) and nucleus (D) of cells treated with HuIFN α-2b for 1 min. × 29,491. Antibody dilutions are as described in “Materials and Methods.”

Antibody dilutions are as described in “Materials and Methods.”

The present study has attempted to circumvent the aforementioned predicaments by using immunogold labeling.
and TEM to ultrastructurally define the movement and location of PKCs after activation. Previous studies have shown translocation of the glucocorticoid receptor to the nucleus following steroid binding (24, 25). We used the dexamethasone-glucocorticoid receptor model to demonstrate that ultrastructural translocation can be documented using immunogold labeling and TEM. These results depicted increased immunogold labeling of the
activity in rapidly growing proliferating cells and increased cytosolic PKC activity in confluent cells (29, 30). With the identification of numerous PKC isozymes, this area of research has evolved considerably. We have initially investigated the subcellular distribution of U-373 PKC-α and -β and are presently examining different classes and subspecies of PKCs to determine their organelle association and modulation by HuIFN-α.

The morphometric data presented herein illustrates that PKC-α and -β are present in the cytoplasm and nucleus of both proliferating and confluent cells, but their distribution is different. There was less total cytoplasmic and nuclear PKC-α in untreated proliferating U-373 cells than in confluent cells, but the ratio of cytoplasmic to nuclear PKC-α is similar in both untreated proliferating and confluent U-373 cells. In contrast, both proliferating and confluent cells have more nuclear PKC-β compared to cytoplasmic PKC-β. Exposure of proliferating cells to HuIFN α-2b increased immunoreactive cytoplasmic and nuclear PKC-β at 1 min and decreased cytoplasmic and nuclear PKC-α at 2 min. In confluent cultures, there was no significant change in nuclear PKC-β, whereas cytoplasmic PKC-β decreased at 2 min.

Western blots of total PKC-α in proliferating and confluent cells and PKC-β in confluent cells showed results that may reflect turnover and degradation. Western blots of PKC-β in proliferating cells demonstrated no detectable change in PKC-β immunoreactivity at 1 min of HuIFN α-2b treatment and a decline in PKC-β immunoreactivity at 2 and 10 min of HuIFN α-2b. These results suggest that the increase in PKC-β immunogold particles observed in electron micrographs of proliferating cells following 1 min of HuIFN α-2b treatment may be due to conformational unfolding of PKC-β and that the decline in PKC-α and -β at 2 and 10 min after HuIFN α-2b may be due to proteolytic turnover of PKC-β.

The conformational change in PKC-β may be due to exposure of its hinge region. This region is not exposed in unactivated PKC. Antibodies to PKC-β were raised to a peptide corresponding to the hinge-V3 region of PKC. This region is exposed upon membrane binding of the enzyme, and its exposure causes PKC to become more susceptible to proteolysis (31). The enhanced levels of *in situ* PKC-β immunogold labeling as detected with antibodies directed to the hinge region are consistent with PKC-β binding to membranes and its subsequent activation and turnover by proteolysis.

No translocation of either PKC-α or -β to specific subcellular organelles was observed in either proliferating or confluent cells following exposure to HuIFN α-2b. Unlike the translocation of PKC isoforms between the cytoplasm, nucleus, and other membranes, which is found following activation when conventional membrane separation techniques are used, PKC-α and -β in U-373 glioma cells appears to be activated without its actual movement. Therefore, PKC-α and -β must be in close proximity to intracellular membrane structures. The similarities in the lack of specific PKC-α and -β organelle translocation following HuIFN α-2b treatment of proliferating and confluent cells may reflect analogous functions of PKC isozymes within a family of PKC subspecies. However, we interpret the differences in nuclear distribution of PKC-α and -β to mean that PKC-α and -β may mediate similar but specialized functions within the cell. The differences in expression mode of PKC-α and -β following HuIFN α-2b treatment
may depend on the proliferative state of U-373 glioma cells, suggesting that activation of PKC isozymes in response to the antitumor effects of HuIFN α-2b is regulated by the cell cycle.

We are currently characterizing the ultrastructural effects of HuIFN α-2b on PKC-γ and the new and atypical PKCs as part of an intracellular description of the structural associations of PKC isozymes.

Fig. 10. Immunogold transmission electron micrographs of PKC-α labeling in nonconfluent U-373 cells after HuIFN α-2b treatment. Cytoplasm (left) and nucleus (right) of cells treated with DPBS (control) or HuIFN α-2b (500 units/ml) for 0 min (A and B), 1 min (C and D), 2 min (E and F), and 10 min (G and H). Note decreased gold particle density in both cytoplasm (C) and nucleus (D) of cells treated with HuIFN α-2b for 2 min. × 29,491. Antibody dilutions are as described in “Materials and Methods.”

Materials and Methods

Passage of U-373 Glioma Cells. Glioma U-373 cells were cultured according to Ponten and MacIntyre (19). Cells were seeded (1 × 10⁶) on 75 cm² flasks containing MEM, 10% FCS, 2 mM L-glutamine, and antibiotics (10 units/ml penicillin and 10 mg/ml streptomycin).
Isolation of RNA and RT-PCR. Total cellular RNA was obtained using a single-step method (23). Generation of single-stranded cDNA templates for RT-PCR was carried out on total RNA using SuperScript reverse transcriptase (Life Technologies, Inc.) according to product instruction for first-stranded synthesis. Controls for template contamination were as described. The cDNA was divided into two equal aliquots, and two primer sets were used to amplify PKC-βI and -βII as described (22): (a) sense primer corresponding to the V3 region of PKC-β (ATGAAACTGAC- CGATTTTACTTCTCTG) and antisense primer corresponding to the V5 region of PKC-βI (AAGAGTTTGT-CAGGGAGGACTCTCC); or (b) sense primer corresponding to the V3 region of PKC-β and antisense primer corresponding to the V5 region of PKC-βII (CGGAGGTC-TACAGATCTACCTAGCTCT). For all primer pairs, the cy-
clinging conditions were an initial 96°C melt for 5 min, followed by 58°C for 1 min, followed by 72°C for 3 min. Following 95°C for 1 min, this cycle was repeated 34 times, ending with 95°C, 1 min; 58°C, 1 min; and 72°C, 10 min. Specificity of the primers was verified with cDNA for PKC-β1 and -βII (from Dr. John Knopf, Genetics Institute, Inc., Cambridge, MA). The RT-PCR data demonstrate that PKC-β1 and -βII are present in U-373 human glioma cells.

The assays are not quantitative.

**Total Extraction of U-373 Glioma PKC.** For extraction of total U-373 glioma PKC, cells were washed with DPBS, and flasks were placed on ice. Monolayers were scraped at 4°C and sonicated in homogenization buffer (10 mM Tris/HCl (pH 7.5), 10 mM EGTA, 25 mM MgCl₂, 2 mM DTT, 0.25 mM sucrose, 2 mM phenylmethylsulfonyl fluoride, 0.4 mM leupeptin, and 0.15 unit/ml aprotinin) containing 1% Triton-X-100. Cell homogenates were centrifuged at 100,000 × g for 30 min to obtain total extractable PKC.

**Procedure for Immunoblotting.** Total U-373 extractable PKC (50–200 µg of protein) was subjected to SDS-PAGE (9% gel) according to the method of Laemmli (32). Proteins were electrophoretically transferred to nitrocellulose according to Towbin et al. (33). Equipment, reagents, and procedures were from Bio-Rad (Melville, NY). Immunoreactive bands were visualized with chemiluminescence (Amersham, Arlington Heights, IL).

**Antibodies and Specificity of Antipeptide Antisera to PKC-α and -β.** Peptides and antibodies to PKC-α, PKC-β (βI+βII), -βI, and -βII were produced, characterized, and kindly provided to us by Dr. Rayudu Gopalakrishna (University of Southern California, Los Angeles, CA). They were raised to peptides synthesized from oligopeptide sequences unique to PKC-α, -β, -βI, and -βII using previously published sequences reported to have good antigenicity (34, 35) at the USC Norris Comprehensive Cancer Center. Antibodies were raised to protein conjugated peptides in New Zealand White rabbits.

The PKC-α antibody was raised to amino acid residues 313–326 ([C]AGNKVISPSEDRRQ) that correspond to the hinge region of PKC-α. The PKC-α oligopeptide sequence was coupled to hemocyanin using m-maleimidobenzoyl N-hydroxysuccinimide (34). Antibodies to PKC-β were raised to amino acid residues C13-C39 (GPKTPEEKTAN-TISKD), which corresponds to the hinge or V3 region of PKC-β (35). This antibody does not distinguish between PKC-βI or -βII. Antibodies to PKC-βI and -βII were raised to the COOH-terminal peptide sequences of PKC-βI (SYTNPEFVINV) and βII (SFVSENFLKPEVKS), which couple to thyroglobulin using glutaraldehyde (35). The last 50 amino acids of PKC-βI are different from -βII due to alternative splicing of a common primary transcript.

Western blots to characterize the antipeptide antisera used alkaline phosphatase-conjugated anti-rabbit goat antibodies as the second antibody and gave positive staining at 1:1000 dilution compared to preimmune serum. Coincubation of antibodies with peptides to PKC-α and -β and not with unrelated peptides blocked positive staining.

Blocking experiments using Western blots were performed in the present study to demonstrate that antipeptide antisera were mono-specific for PKC-α and -β and that cross reactions with other U-373 glioma cellular proteins could be eliminated from consideration. Confluent U-373 glioma cells were treated with HuIFN α-2b (500 units/ml) for 0, 1, 2, or 10 min as stated below. Total cell homogenates containing either 100 µg of protein for assaying PKC-α or 200 µg of protein for probing PKC-β were loaded/lane on SDS-

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*R. Gopalakrishna, personal communication.*
PAGE for Western blots. Peptides to PKC-α and -β (300 μl containing 300 μg) were incubated overnight with 1 ml of milk and 50 μl of antibody. The antibody-peptide mixture was diluted with milk at 7:1000 and used for Western blots and enhanced chemiluminescence (Amersham Corp.).

In Vitro Treatment of U-373 Glioma Cells with HuIFN α-2b. In vitro studies were performed according to Acevedo-Duncan et al. (36). Subconfluent (75%) and confluent cells were washed three times with DPBS, 2 h prior to treatments with either DPBS (control) or HuIFN α-2b (500 units/ml) for 0, 1, 2, or 10 min. Treatments were terminated by fixing cells in 0.5 glutaraldehyde/2% paraformaldehyde.

Indirect Immunofluorescence Microscopy. Indirect immunofluorescence microscopy was performed by modification of the procedure of Chida et al. (13). Glioma U-373 were grown on Lab-Tek chamber slides (Nunc Inc., Naperville, IL). When the desired cell density was reached, cells were washed twice with 2 ml of PBS and treated for different time periods with HuIFN α-2b (2 ml of PBS) as described above. Treatment with HuIFN α-2b was terminated by fixation of cells in 3.7% formaldehyde for 15 min. After washing cells twice with PBS, slide were blotted and permeabilized with cold (−4°C) 100% ethanol for 10 min at −4°C. Cells were then air dried, and nonspecific sites were blocked with heat-inactivated 3% goat serum for 30 min. Cells were subsequently washed twice with PBS and incubated with either nonimmune serum, antipeptide PKC-α, or antipeptide PKC-β at a 1:20 dilution for 1 h. Cells were washed three times with PBS and incubated in the dark with fluorescein-conjugated goat anti-rabbit IgG (Hyclone, Logan, UT) for 1 h at 1:50 dilution. Cells were washed three times with PBS and twice with water to remove salts. Slides were mounted with Fluromount (Fisher, Pittsburgh, PA), and immunofluorescence was observed using a Lietz Labor Lux microscope with an incident fluorescence attachment (LEP, Inc.) and a filter cube appropriate for fluorescein. Kodak Tri-X Pan 400 film was used to photograph the fluorescence.

Procedure for Immunogold Labeling and TEM. Following specific treatments, cells were fixed in 0.5% glutaraldehyde/2% paraformaldehyde in DPBS for 10 min. Cells were washed three times in DPBS by centrifugation and fixed overnight in 2% paraformaldehyde in DPBS at 4°C. The next day, dehydration was accomplished by 5-min stepwise gradients up to 80% ethanol. Cells were infiltrated with a 2:1 mixture of LR white (Electron Microscopy Sciences, Fort Washington, PA) to 80% ethanol for 1 h at room temperature (37). Two 1-h infiltrations in 100% LR white followed, and a third infiltration in 100% LR white was left overnight at 4°C. The next day, cells were infiltrated in 100% LR white at room temperature and transferred to BEEM capsules for polymerization for 48 h in a light tight UVP box at room temperature.

Sectioning. Blocks were sectioned on a Jung Reichert Ultracut E ultramicrotome using a Diatome diamond knife. Approximately 80-nm (pale-gold) thin sections were collected in distilled water and transferred to nickle formvar-coated grids using a wire loop. After drying, the sections were labeled by immunogold technique described below.

Immunogold Technique. Sections were incubated (at room temperature) by floating them face down on droplets of solutions on paraffilm affixed to the bench top. The procedure of Haller et al. (38) was used. Sections were pre-treated with Tris-buffered saline (TBS) with 5% FCS for 20 min and washed with TBS-AGT (TBS containing 0.1% BSA, 0.2% coldwater fish gelatin (Sigma Chemical Company, St. Louis, MO) and 0.05% Tween 20) for 10 s each. Primary antibody (rabbit anti-PKC-α and -β) at 1:20 dilution in TBS-AGT was applied for 2 h and washed five times with TBS-AGT for 5 min each. Nonimmune rabbit serum at a 1:20 dilution was used as the control for the primary antibody. The second antibody (goat anti-rabbit IgG conjugated to 10-nm gold particles; Amersham) was at a 1:50 dilution and was incubated for 1 h in the dark. Sections were washed three times with TBS-AGT for 5 min each and then washed for 5 min in TBS, followed by distilled water, and allowed to dry on filter paper. Countering was performed with saturated uranyl acetate for 1 min and with 1 m lead citrate for 30 s to enhance the contrast of cellular ultrastructure. Controls were run concurrently using rabbit preimmune serum to test for antibody specificity. Sections were examined on a Phillips CM-10 electron microscope at 60KV to minimize resin instability. Micrographs were taken on Kodak electron microscope film (Estar thick base) and developed with Kodak D-19 developer. Prints were on Kodabrome RC paper developed with an Ektaomatic processor.

Morphometry. The morphogenetic effects of HuIFN α-2b on PKC-α and -β intracellular localization was assessed on serial sections cut from one block for each of the different time treatments. Two separate experiments were performed, and two separate sets of immunogold-labeled grids were photographed for analysis. Five cells from each set were photographed at ×21,000, and micrographs were printed at ×35,700. The number of immunogold particles present in three different cytoplasmic locations and three different nuclear locations/cell were quantified from micrographs of five different cells for each treatment. Particle densities were expressed as mean gold particles/micrometer square. Statistical determination of gold particle density was by Student’s t test using Minitab software (Minitab, Inc., State College, PA).

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