Protein Kinase C-β2 Inhibits Cycling and Decreases c-myc-induced Apoptosis in Small Cell Lung Cancer Cells

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Abstract
The overexpression of c-myc frequently accompanies the relapse of small cell lung cancer (SCLC) cells and contributes to the poor prognosis of this tumor. In this study, we confirm that transfected c-myc results in decreased homotypic cell aggregation and increased proliferative capacity of SCLC cells when nutrient conditions are adequate. We also find that c-myc contributes to apoptosis when cells are nutrient depleted, and slow cytometry suggests that this enhanced apoptosis is associated with a failure to halt cell cycling, consistent with the experience in other cell types. We previously found that protein kinase C-β (PKC-β) expression in NCI H209 (209) SCLC cells increases markedly with c-myc transfection (L. F. Barr et al., Cancer Res., 51: 5514–5519, 1991), and we hypothesized that PKC-β may mediate some of the effects of c-myc in these cells. We test this hypothesis by transfection of rat PKC-β1 and bovine PKC-β2 isoforms into 209 cells before and after transfection with c-myc. PKC-β1 transfection has no effect on these cells. However, PKC-β2 expression has distinct phenotypic consequences. In the parental cells, PKC-β2 expression results in increased homotypic cell aggregation and a prolonged doubling time. Furthermore, PKC-β2 expression increases the fraction of these cells in G0-G1. In the cells which express a transfected c-myc gene, PKC-β2 expression improves the survival of cells in low serum by decreasing myc-induced apoptosis. This effect was associated with, and may be mediated by, a selection for cells in the G0-G1 fraction. We postulate that transfection of c-myc into SCLC cells may select for those expressing the PKC-β2 gene because this signal transduction event protects against myc-induced apoptosis.

Introduction
The prognosis of SCLC is among the worst for solid tumors, and even treated patients have a median survival of less than 16 months (1). Ironically, even the majority of patients with extensive SCLC respond to the initial therapeutic regimens, but they ultimately relapse with a more refractory and aggressive tumor (1). A comparison of the biology of the post-treatment cells to the initially responsive tumor cells suggests that one contributor to this increasingly malignant behavior is high expression of the c-myc oncogene. Twenty percent of cell lines established from SCLC tumors from patients who have received therapy have c-myc gene amplification, whereas virtually none of the lines established prior to therapy overexpress this gene (2). Furthermore, the presence of c-myc amplification significantly worsens the patient’s prognosis (2).

The c-myc gene has been associated with cell proliferation (3), and, as with other cell types, transfection of c-myc into SCLC cells leads to a shortened doubling time and an increased cloning efficiency (4). Paradoxically, this gene can also induce apoptotic cell death when expressed in nutrient-deprived cells (5, 6). In tumors, cells are exposed to environments which may favor either response, and the outcome of c-myc overexpression in SCLC must represent a balance between modulation of cell proliferation and cell death by this gene. Elucidation of the components of pathways mediating these opposing effects and of factors which induce or repress these steps will be important to understanding the biology of the myc gene and the behavior of SCLC and other tumor types in which myc overexpression contributes to the malignant behavior.

In previous studies, we have shown that the expression of a transfected c-myc gene into SCLC cells is associated with a striking increase in the steady-state expression of the signal transduction factor, PKC-β (7). The PKC family of isoforms works downstream from a variety of environmental signals, and similar to the action of c-myc, activation or transfection of PKC can both positively and negatively affect cell growth (8, 9) and can collaborate with oncogenic ras to transform cells (10, 11). This led us to hypothesize that PKC-β and c-myc lie along the same pathway, and that PKC-β might mediate some of the effects of c-myc in SCLC cells. In the current study, we address this question by transfecting PKC-β1 and c-β2 isoforms into a “classic” SCLC cell line and after transfection of these cells with the c-myc gene. Our findings indicate that PKC-β2 may function,
in SCLC cells, to abrogate the apoptotic effects of c-myc in cells exposed to nutrient deprivation.

Results

c-myc Expression Increases Proliferation and Apoptosis of SCLC Cells. NCI H209 (209 cells) is a classic SCLC line characterized by cells growing in floating aggregates, having high neuroendocrine marker expression, and not expressing c-myc (12). Transfection and overexpression of c-myc in these cells (209 myc cells) results in looser aggregates, but unchanged neuroendocrine expression (Ref. 5; Fig. 1F).

c-myc has striking effects on the growth of 209 cells. In normal serum, the doubling time of 209 myc cells (1.30 ± 0.13 days) is one-half of that of 209 cells (2.64 ± 0.19 days, average ± SEM; Fig. 2A). In these experiments, the cells have not been exposed to fresh medium during the growth curve, and the number of 209 myc cells is seen to peak and then decline after 6 days (Fig. 2A). In contrast, the slower growing 209 cells show a more prolonged plateau phase where cell loss is not as significant. This decline in the number for the 209 myc cells is due to a dramatic increase in apoptosis at the post-plateau region, as demonstrated by Hoechst staining which shows an increase in the apoptotic fraction of the 209 myc cells. For the 209 myc cells, the apoptotic fraction increases from 5.0 ± 0.9% during the log phase growth in full serum (normal serum) medium to 44.7 ± 9.0% on day 15 of the growth curve (average ± SD; compare Fig. 2, B-1 to B-3). Furthermore, cell cycle analysis shows an increase in nuclei in the sub-G0 region at this time (Fig. 2C). In contrast, Hoechst staining and FACS analysis demonstrates smaller increases in the apoptotic fraction for the 209 cells for each of the growth curves examined, averaging 5.5 + 2.6% during log phase growth in full serum and 26.8 + 5.7% in the post-plateau phase (compare Fig. 2, B-2 to B-4, and Fig. 2C). FACS analysis demonstrates that many of the 209 myc cells persist in the S-phase + G2-M fractions during the post-plateau period, unlike the 209 cells. This finding suggests that the 209 myc cells are continuing to progress through the cell cycle despite nutrient deprivation, unlike the parental 209 cells.

To more fully characterize the apparent effect of c-myc expression on apoptosis, we compared the behavior of 209 myc and parental 209 cells grown continuously in serum-deprived [low (0.1%) serum] medium. Under these conditions, the Hoechst stain assay demonstrates a striking increase in the apoptotic fraction of the 209 myc cells to 58.0 ± 10.3% for cells grown for 5 days in low serum (Fig. 3A). In contrast, the apoptotic fraction of the parent 209 cells increases significantly less, to only 17.4 ± 1.8% for cells grown for 5 days in low serum (Fig. 3A). Flow cytometric analysis
Fig. 2. Expression of c-myc in 209 cells increases proliferation during log phase growth and enhances post-plateau apoptosis. A, Growth curves in full serum show that the numbers of 209 myc cells (■■■) reach a more rapid peak and sustain a more pronounced subsequent decline under nutrient-deprivation conditions than parental 209 cells (△). The curves shown are representative of five sets, each curve done in triplicate, with identical findings. Data are mean averages of triplicate experiments. Bars, SE. B, Hoechst stain of 209 myc and 209 cells in log phase growth (B-1 and B-2, respectively) and at day 15 (much later in the post-plateau decline phase for the 209 myc cells than for the 209 cells; B-3 and B-4, respectively). The cells were grown in 5% serum which was not replaced during the experiment. Prominent apoptotic nuclear morphologies (fragmented and pyknotic nuclei) appear in the 209 myc cells in the post-plateau phase. For the 209 cells, there are less numerous apoptotic nuclei. ×40. Fields shown are representative from one of two growth curves, each assessed in duplicate and with similar findings. C, FACS analyses of cell cycle for the 209 and 209 myc cells at day 3 (log phase growth, - - - -) and at day 15 (post-plateau growth, -----). The cell number is displayed on the vertical axis, and the fluorescent intensity of emitted light (proportional to DNA content) is shown on the horizontal axis. Analyses shown are representative points from one of two growth curves, each done in duplicate and with similar findings. Note the striking sub-G0 peak for 209 myc cells in the post-plateau period. Also, note the retention of a G2-M peak in the post-plateau period as compared with a loss of this peak for 209 cells during this same period.

PKC-β2, but not PKC-β1, Transfection Enhances Homotypic Cell Aggregation and Increases the Doubling Time of the 209 SCLC Cell Line. To test the hypothesis that PKC-β mediates some of the effects of c-myc on SCLC cell phenotype and growth, the two isoforms of PKC-β (β1 and β2) were transfected into the 209 SCLC cell line. These two isoforms represent alternate splice products from a single gene (13, 14). Little is known about the comparative functions of these two species, although their divergent expression pattern in different tissues (15) and their individual responses to physiological stimulation (16) suggest that they mediate differing phenotypic effects. We used the rat PKC-β1 and the bovine PKC-β2 for these studies (see "Materials and Methods" for specifics of these genes and constructs).

Expression (Fig. 4, A and B) of the exogenous PKC-β1 from the Moloney murine leukemia viral long terminal repeats in the pBABE vector did not affect cell aggregation (Fig. 1, A and B) or growth (Fig. 4C). Results were identical when PKC-β1 was expressed from the same promoters in the PMV7 vector (data not shown).

The effects of transfecting PKC-β2 into the 209 cells differed dramatically from those of the PKC-β1 described above. PKC-β2 transfectected in the same pBABE vector, as
well as in the vector pcd-SRα behind the SRα promoter, led to a marked decline in cell selection efficiency in hygromycin, and the pooled population of those cells that did survive did not express PKC-β2 protein at levels greater than the barely detectable levels in the vector control cells (data not shown). These results suggested that transfecting PKC-β2 has negative growth consequences for the 209 cells. To explore this possibility further, we next expressed PKC-β2 from the MC10 vector which uses an inducible sheep metallothionein promoter. The expression of exogenous PKC-β2 RNA from this construct was detected in pooled, selected, noninduced MT-PKC-β2 transfectants by the RT-PCR TAG procedure (17), priming from a 3' sequence in the vector adjacent to the cloning site (Fig. 5A). These cells also express PKC-β2 protein at levels of 1.3–2.0 times that of the vector-transfected control cells (Fig. 5, B and C). Thus, as we and others have seen for the MC10 vector with other gene inserts (18), the MT is "leaky" in the 209 cells, and addition of zinc did not significantly alter PKC-β2 expression over that seen in the uninduced cells (data not shown). We therefore used the uninduced conditions in subsequent experiments to evaluate the effects of low-level PKC-β2 expression.

In contrast to the lack of phenotypic effects of PKC-β1 in 209 cells, the low-level PKC-β2 expression from the uninduced MT promoter resulted in an increased doubling time from 2.31 ± 0.26 days (average ± SEM) for the 209 MT vector control cells to 3.88 ± 0.6 days for the PKC-β2-expressing 209 MTB2 cells (Fig. 6A). Flow cytometry shows that this growth prolongation in the PKC-β2-expressing cells is a consequence of an increased fraction of cells in G0-G1 for each of the growth curves studied and a decreased fraction in the S-phase compartment. (Fig. 6B shows a representative study, and the average of these studies is graphically displayed to the right.) For the PKC-β2 transfectants compared with the vector controls, the percentage of cells in G0-G1 is identical at day 5 of growth. However, with further growth, the percentage of cells in G0-G1 consistently increases more rapidly for the PKC-β2 transfectants than for the vector controls, and the S-phase steadily decreases. The increased ratio of G0-G1:S-phase for the PKC-β2 transfectants versus vector control cells is statistically apparent for the average of these studies at day 15, although the representative study shown actually displays this trend at day 7 (Fig. 6B). Finally, three pieces of data confirm that the slowing of

Fig. 3. Quantitation of myc-induced apoptosis for cells grown under continuous nutrient-depleted conditions. Equal numbers of 209 myc and parental 209 cells were seeded and grown for 5 days in 0.1% serum. A. Hoechst stain demonstrates a larger fraction of apoptotic bodies for the 209 myc cells (shown visually in the left panels and quantitatively in the far right panel) ■, 209 myc cells; □, 209 cells. ×40. The fraction of apoptotic nuclei was counted for >200 cells for two to three separate growth curves, each in duplicate. Data represent means. Bars, SE. B. FACS analysis of the above cells at day 5 ——, 209 myc cells; ..., ..., 209 cells. The cell number is displayed on the vertical axis, and the fluorescent intensity of emitted light (proportional to DNA content) is shown on the horizontal axis. The results shown are representative from one of two growth curves, each done in duplicate and with similar findings. Note the striking sub-G0 peak for the 209 myc cells and the marked decrement of cells in G0-G1.
Fig. 4. Transfection of 209 cells with PKC-β1. A, Northern blot analysis of poly(A)⁺ RNA shows expression of the predicted 5-kb band from transfected PKC-β1 (from long terminal repeat to long terminal repeat, including a 2.1-kb PKC-β1 insert) as well as a 3.5-kb band which appears to represent an alternate splice transcript from the construct. B, Western blot analysis demonstrates 2.5 times greater expression of the 80-kDa PKC-β1 protein compared with cells transfected with vector alone. The doublet seen for both endogenous and exogenous PKC-β1 may represent a posttranslational modification of this signal transduction protein, such as by phosphorylation (71) or proteolytic cleavage (72). The equal loading of protein in each lane is verified by fast green stain of filter shown below (lane 3 on this blot is a molecular weight ladder). C, The expression of PKC-β1 in the 209 cells has no effect on cell growth. The growth curve shown is representative of five curves assessed by either MTT (two curves) or manual counting with trypan blue (three curves), each performed in triplicate (mean averages) and with similar findings. Bars, SE. 209 pBABE-PKC-β1 cells (▲) and 209 pBABE cells (▲).

growth associated with PKC-β2 transfection is not due to increased apoptosis. First, PKC-β2 expression does not increase the fraction of cells in the sub-G₀ compartment of the cell cycle (Fig. 6B). Furthermore, PKC-β2 expression does not result in an increased fraction of cells with an apoptotic nuclear morphology by Hoechst stain, nor a decrease in cell viability by trypan blue staining (data not shown).

The expression of PKC-β2 further differs from that of PKC-β1 in that it is associated with altered characteristics of the 209 cellular aggregates. The PKC-β2-transfected cells displayed enhanced homotypic cell aggregation compared with vector controls and parental cells of equal passage number (Fig. 1, C–E). Interestingly, as the transfected cells were observed over multiple passages, the cell aggregates loosened such that later passage of PKC-β2 cells was indistinguishable from that of vector controls. This loss of increased cell aggregation appeared to be independent of cell growth characteristics or of PKC-β2 expression, since both the slower growth rate and the PKC-β2 expression were preserved (data not shown). Other characteristics of 209 cells were not altered by PKC-β2, and the expression of neuroendocrine markers such as dopa decarboxylase (19) or human achaete scute homologue 1 (20) were not changed (data not shown).

Enhanced PKC-β2 Expression Decreases myc-induced Apoptosis in Low Serum without Affecting Growth or Phenotype of c-myc-expressing Cells in Normal Serum. The above data indicated that PKC-β2 transfection into 209 cells actually has phenotypic and growth effects which are the opposite of those seen with c-myc transfection, although c-myc transfection is associated with increased steady-state PKC-β expression in these cells (7). We therefore wondered whether the endogenous PKC-β expression modifies the consequences of c-myc expression in these cells. To address this question, we transfected the MT-PKC-β2 construct into the 209 myc cells to determine whether further increasing the level of PKC-β2 alters the effects of c-myc.

Expression of the transfected PKC-β2 in the 209 myc cells (209 myc-MTB2 cells) was verified, first, by the RT-PCR-TAG protocol using a 3′ primer derived from vector sequences (Fig. 7A), and, second, by priming across a restriction site present only in the bovine-derived exogenous PKC-β2 and not in the human gene (Fig. 7B). The latter study suggested that the exogenous PKC-β2 transcripts represent approximately one-half of the total PKC-β2 transcripts in the 209 myc-MTB2 cells. Finally, multiple analyses showed that PKC-β2 protein in the 209 myc-MTB2 cells is expressed 1.2–2.0 times higher than that of vector-transfected controls (Fig. 7C). This increased expression of PKC-β2 is not associated with alterations in the expression of the exogenous c-myc protein in these cells (Fig. 7C). The increased PKC-β2 expression has no morphological effect on 209 myc cells (Fig. 1, F–H), nor is there an effect on the neuroendocrine markers human achaete scute homologue 1 protein or dopa decarboxylase expression (data not shown).

Importantly, and unlike the situation for the parental 209 cells, PKC-β2 transfection into the 209 myc cells has no consistent effect on the log or on the post-plateau growth phases of cells in full (9.5%) serum (Fig. 8A). However, PKC-β2 transfection significantly alters the characteristics of the 209 myc cells grown in low (0.1%) serum. First, during a typical growth curve under such conditions, the number of PKC-β2-transfected cells peaks at a higher cell density and at a later time point than vector-transfected control cells (7 days versus 3–5 days, Fig. 8B). For these growth curves,
PKC-β2 Decreases c-myc Apoptosis

vector-transfected 209 myc cells and slightly lower S-phase and G2-M fractions (as seen for a representative curve in Fig. 9). These higher G1:S and the G1:G2 ratios for the PKC-β2 transfectants compared with the vector controls are more apparent for the refed cells surviving low serum growth (Fig. 9).

Discussion

Our data in this article indicate that the PKC-β2 and c-myc proteins can interact to modulate the phenotype of cultured SCLC cells. We first found that c-myc expression, alone, in these cells leads to loosening of the homotypic cell aggregates and increased cell proliferation in full serum medium. c-myc expression enhanced apoptosis in nutrient-depleted conditions, and this effect was associated with the continued cycling of c-myc-expressing cells. These findings are similar to those resulting from c-myc expression in other cell types (21).

The phenotypic and growth effects of PKC-β2 expressed alone in the 209 cells are the opposite of those resulting from myc expression. First, PKC-β2 expression increased homotypic cell aggregation of these cells, consistent with previous studies that show that phorbol ester-induced activation of PKC triggers compaction of the mouse embryo (22) and aggregation of SCLC cells (23) and human basophils (24). Second, PKC-β2 expression slowed cell growth in full serum medium. The ability of PKC-β2 to induce such a response varies with cell type (25–30), suggesting that multiple factors which may be cell-type specific must influence the effect of PKC on the cell cycle. The contrasting actions that we found for PKC-β2 and c-myc on 209 cell phenotype and proliferation are similar to findings for these proteins in the HL-60 human promyelocytic leukemia cell line. For the HL-60 cells, the monocytic differentiation and decreased proliferation induced by 1,25-dihydroxyvitamin D3 treatment is associated with, and likely mediated by, both increased expression of PKC-β (31–33) and decreased expression of c-myc (34).

We attempted to separate the actions of PKC-β2 from those of c-myc by increasing the expression of PKC-β2 in the 209 myc cells. We found, first, that when these PKC-β2-transfected 209 myc cells are grown in full serum medium, the phenotypic and mitogenic effects of c-myc predominate over any growth-slowing effects of PKC-β2, and these cells tolerated increased PKC-β2 protein without any phenotypic effects. Second, we found that the increased expression of PKC-β2 leads to a decrease in myc-induced apoptosis under nutrient-deprived conditions. This protective action of PKC has been demonstrated in other models of apoptosis (35–40).

Mechanisms by which myc-mediated apoptosis is altered may differ for different cell types and different regulatory factors. Among the ability of cytokines to abrogate myc-mediated apoptosis has been related to a decrease of the G1 checkpoint (41, 42). In contrast, the protective action of PKC-β2 in the 209 myc cells is associated with an augmentation of cells in G0-G1. In this regard, PKC-β2 in our studies may be functioning much like the growth regulatory factor interleukin 3 and the oncogenes v-src and activated raf. In a murine hematopoietic model, the protection from γ-irradiation-in-
duced apoptosis afforded by each of these factors has been linked to their ability to augment G₁ arrest (43).

Potential molecular mechanisms underlying our observations for the interaction between c-myc, PKC-β2, and the cell cycle remain to be determined. When we sought to determine the behavior of cellular factors that might mediate these results, we found that our cell system differs from other myc-expressing cells (44, 45) in that the relative levels of bcl-2 and bax do not appear to play roles in the apoptotic responses we observe (data not shown). However, we have not examined the phosphorylation of bcl-2 (46), nor have we evaluated the expression of other members of the bcl-2 family, including bcl-x, bak, and mcl-1, nor the expression of members of the interleukin-converting enzyme pathway, all of which may modulate the apoptotic response. Increasing p53 protein levels can mediate actions similar to those we observed with PKC-β2 transfection (47). However, we also could not correlate levels of p53 or p21^{CIP1/WAF1} proteins with the PKC-β2 effects in these cells (data not shown). PKC may stimulate DNA binding by p53 by phosphorylation of this protein (48, 49), and we have not examined this yet. Other potential nuclear phosphorylation targets of PKC-β2 that may mediate our results include the nuclear lamins (50) and other members of cell cycling and apoptotic pathways (42, 51), as well as myc itself (52–54). Finally, PKC-β2 may be acting through the mitogen-activated protein kinase cascade (55).

Because our earlier studies of PKC isoform expression in SCLC cells suggested a specific relationship between the expression of c-myc and the β isoforms of PKC (7), our current investigations have focused on the role of PKC-β in myc-expressing cells. We found that the expression of each of the PKC-β isoforms, β₁ and β₂, produce different phenotypic and growth effects in our cells. These findings suggest that our observations regarding the role of PKC-β2 in myc-induced apoptosis may be specific for this isoform. Whether other PKC isoenzymes may function similarly to PKC-β2 and suppress apoptosis in c-myc-expressing cells remains to be
PKC-\(\beta\) Decreases \(c\)-\(myc\) Apoptosis

In the multistep model of carcinogenesis, increased tumorigenicity is driven by the sequential acquisition of mutations that either enhance proliferation or decrease apoptosis. This concept is demonstrated by the following model of our SCLC transfection system. \(c\)-\(myc\) overexpression increases the proliferative capacity of these cells, but this positive effect would be balanced or even outweighed in the tumor cell population by the increased propensity of cells to undergo \(myc\)-induced apoptosis in growth factor-depleted conditions. \(c\)-\(myc\) may be tolerated in our transfected cells because of selection for those cells with augmented steady-state PKC-\(\beta 2\) expression which lessens \(myc\)-induced apoptosis.

\(c\)-\(myc\) expression increases the proliferative capacity of a classic SCLC cell line but also increases the apoptotic capacity of these cells. PKC-\(\beta 2\) decreases both \(G_0-G_1\) transit and \(myc\)-induced apoptosis in low serum. The nuclear pathways in which PKC-\(\beta 2\) participates are unknown, but are likely parallel or are the same as those used by \(c\)-\(myc\). In this capacity, the expression of PKC-\(\beta 2\) may be an indirect adaptation of the \(myc\)-expressing cells to augment population survival. Thus, the \(myc\)-PKC-\(\beta 2\) relationship we demonstrate may exemplify the multistep model of carcinogenesis. These data suggest how acquisition of both increased \(c\)-\(myc\) and PKC-\(\beta 2\) expression in SCLC tumors could select for cells likely to contribute to the inevitable relapse of this cancer after chemotherapy treatment and to the subsequent resistance to further drug intervention.

**Materials and Methods**

**Cell Culture.** NCI-H209 is a well-established SCLC cell line (13). The 209 \(myc\) cell line, expressing a full-length exogenous \(c\)-\(myc\) behind its own intact promoter, is a generous gift from Bruce Johnson (National Cancer Institute-Navy Medical Oncology Branch, Naval Hospital, Bethesda, MD; Ref. 4), and grows in neomycin-selective media. Unless otherwise specified, these cells were grown in RPMI 1640 with 9.5% fetal bovine serum (Sigma), 100 units/ml penicillin, and 100 \(\mu\)g/ml streptomycin (Life Technologies, Inc.) at 37°C incubators containing 5% CO\(_2\). For experiments in low serum, cells were grown in 0.1% FCS.

**Preparation of Constructs Used for Transfections: MT-PKC\(\beta 2\) (MT-\(\beta 2\), psra-\(pcd\)-PKC-\(\beta 2\) is an expression vector containing the full-length bovine PKC-\(\beta 2\) behind the SV40 early promoter and the R-U5 segment of human T-cell leukemia virus type 1 long terminal repeat (62), and was a generous gift from DNAX Research Institute of Molecular and Cellular Biology (Palo Alto, CA). The amino acid homology between bovine and human PKC-\(\beta 2\) is more than 98% (63). The MT-CB6 + vector is an inducible eukaryotic expression vector containing the pMT-1 sheep metallothionein promoter as well as a neomycin resistance gene (64, 65). To prepare the MT-\(\beta 2\) construct, the PKC-\(\beta 2\) insert was excised from psra-\(pcd\)-PKC-\(\beta 2\) by partial digestion with EcoRI. The ends of the complete 2.5-kb PKC-\(\beta 2\) were filled in, and the insert was ligated into the EcoRV site of the MT-CB6 + vector. The construct was verified using multiple restriction cuts and also by PCR using primers spanning the cloning site of the vector 3' to the insert (lower primer, 5'-TTCAACAGCCGCTCACCACCC-3') and into the 3' end of the insert (upper primer from aa 618 (13), 5'-CCATCCCACAGTCCTAACAC-3').

![Fig. 7. The expression of PKC-\(\beta 2\) in 209 \(myc\) cells transfected with the MT-PKC-\(\beta 2\) construct is demonstrated at the RNA and protein levels. A, RT-PCR using the TAG procedure (see "Materials and Methods") demonstrates a 530-bp fragment (open arrow) only in the lane containing the RT reaction (RT') for the 209 myc MTB2 cells. B, To differentiate further between the expression of endogenous versus exogenous PKC-\(\beta 2\) in the 209 myc cells, PCR was performed across a restriction site present only in the exogenously expressed bovine PKC-\(\beta 2\) (see "Materials and Methods"). Both the endogenous and exogenous PKC-\(\beta 2\) RT-PCR products can be seen as the 666-bp bands in the PvuII (−) lanes. Following PvuII restriction (+ lanes), the 666-bp band alone is seen in 209 myc MT cells (−) while new 318- and 348-bp restriction fragments are seen in the 209 myc MTB2 cells (two solid arrows, −→). The remaining 666-bp band in the myc MTB2 cells probably represents the nonrepressed endogenous PKC-\(\beta 2\) RT-PCR product. C, Western blot analysis demonstrates that the predicted 80-kDa PKC-\(\beta 2\) protein is more highly expressed in 209 myc cells transfected with PKC-\(\beta 2\) compared with those transfected with vector alone. Equal loading of these lanes was verified by cutting the membrane in half and simultaneously blotting with β-actin, which labels the predicted 45-kDa protein. In addition, uncharted expression of the transfected \(c\)-\(myc\) gene was confirmed in the MT versus the MTB2 209 myc cells by visualization of a strongly labeled band at 70 kDa (predicted size of a chimeric protein expressed from the construct 4 consisting of a full-length \(c\)-\(myc\) and neomycin resistance sequences) as compared with detection of the 110-kDa topoisomerase protein.
**pBABE-PKC-β1.** PMT-PKC-β1 is an expression vector containing the full-length rat brain PKC-β1 and was a generous gift from the Genetics Institute (Cambridge, MA). The rat PKC-β1 has previously been used to transfect and alter the growth of the human HT29 colon cancer cell line (25). pBABE(hygro) is a retroviral vector which expresses genes from the Moloney murine leukemia long terminal repeat and contains the hygromycin resistance gene behind a SV40 promoter (69). The PMT-PKC-β1 construct was restricted with XhoI, isolating the full-length PKC-β1 insert, which was initially subcloned into the XhoI site of the BlueScript vector (Stratagene), producing BS-β1. BS-β1 was then restricted with Smal, which cut into the insert 46 bp upstream from the ATG start site. BamHI linkers were added to this site, and the construct was subsequently restricted with BamHI and XhoI. The PKC-β1 insert was then cloned into the pBABE(hygro) vector cut with BamHI and SalI. This pBABE-β1 in the sense orientation was verified with multiple restriction cuts.

Rat brain PKC-β1 expressed in the PV7 vector (25, 67) was also used for these studies and was a generous gift from I. Bernard Weinstein (Columbia-Presbyterian Cancer Center, New York, NY).

**Cell Transfection.** The plasmids used for transfection were purified by cesium chloride centrifugation. Cells (6 x 10⁴) in a 10 cm plate were transfected by lipofection using 60 μg of Lipofect (Life Technologies, Inc.) and procedures suggested by the manufacturer, with the exception that the cells were kept in serum-free media for only 5 h. For the 209 myc cells, which were already neomycin resistant, insertion of the neomycin-selectable MT-β2 construct required cotransfection with the pBABE(hygro) vector, done at a 20:1 molar ratio of MT-β2:pBABE. Anti-biotic selection was initiated 2 days after lipofection. To derive pooled populations of stably transfected cells, cells transfected with constructs containing the neomycin resistance gene (MT, MTβ2, and c-myc) were grown in the presence of 0.4 mg/ml G418 (Sigma), and cells transfected with constructs containing the hygromycin resistance gene [pBABE(hygro)] were grown in the presence of 0.2 mg/ml hygromycin (Sigma).

**Analysis of Transient Expression in Transfected Cells.** Expression of transfected PKC-β1 was verified by Northern blot analysis. Polyadenylated RNA was isolated, and 10 μg of RNA were separated by gel electrophoresis on 1.5% agarose-formaldehyde gels, transferred to nylon membranes (Zeta-Probe; Bio-Rad), and hybridized exactly as reported previously (7). Probes were prepared as inserts of human PKC-β (American Type Culture Collection 59289), or human β-actin (a gift from Don Cleveland, Johns Hopkins University School of Medicine, Baltimore, MD). cDNA probes were oligolabeled with [a-32P]dCTP to a specific activity of approximately 10⁶ cpm/μg of DNA (68). Blots were stripped and reprobed as per the manufacturer’s protocol.

Expression of PKC-β2 was verified in transfected using RNA template-specific PCR (17). First, the MT-CB6 + vector was sequenced around the cloning site (Sequenase Version 2.0 kit; United States Biochemical). Second, a 20-bp unique sequence was selected from the region of the vector cloning site, 3' to the insert. A 25-bp nonsense TAG sequence was added to the 5' end of this sequence, yielding the downstreamprimer, 5'-GTTGCGTACCGAGGTTCGGCAATGTAAGCTCAGTCTGAGGCAGCAT-3'. This primer was used for the RT reaction, with 6 μg of RNA template. Third, the second-strand reaction and amplification were done from a 5′-μl aliquot directly of the RT reaction. This 50-μl reaction used 200 nM of each of the following primers: a downstream primer consisting of the 25-bp TAG sequence, 5′-GTTGCGTACCGAGGTTCGGCAATGTAAGCTCAGTCTGAGGCAGCAT-3′ and an upstream primer starting at aa 635, 5′-CATCAGCATCAAGGATCCTCAGTCTGGAGG-3′, in a buffer containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 100 μM deoxynucleotide triphosphate, 10% (v/v) DMSO, and 10 μM of each deoxynucleotide triphosphate. Thermal cycling started with a denaturation at 94°C for 5 min, at which time 1 unit of Tag polymerase was added, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 70°C for 2 min, and extension at 72°C for 2 min, and a final elongation step at 72°C for 6 min. These conditions yielded specific priming of a 530-bp fragment derived only from RNA and not from DNA, because no product was seen in the control reactions which had been run without the reverse transcriptase enzyme.

For the 209 myc cells, analysis of the expression of endogenous versus exogenous PKC-β2 expression took advantage of a Prull site located at aa 406 in the exogenous bovine PKC-β2, but not in the endogenous human PKC-β. First, a RT reaction was performed using random oligomers. Then a PCR reaction was performed identically as above, with the exception that the annealing temperature was 55°C. The upstream primer was taken from the PKC-β-specific V3 region: 5′-CCACC(G/A)GAAGG(C/A)AGT-
PKC-β2 Decreases c-myc Apoptosis

Fig. 9. FACS analyses of PKC-β2-transfected 209 myc cells grown in low serum. 209 myc MTB2 cells (—) and 209 myc MT cells (- - -). The cell number is displayed on the vertical axis, and the fluorescent intensity of emitted light (proportional to DNA content) is shown on the horizontal axis. The flow cytometric studies are from a single growth curve and show cells at log phase growth (day 3) in normal (9.5%) serum, cells grown in parallel for 5 days in low (0.1%) serum, and cells that survived 10 days of low serum growth and were refed and grown for 7 days in normal serum. The single growth curve shown is representative of four separate growth curve studies, each analyzed in duplicate and showing similar results. Arrowhead in the bottom FACS indicates the apex of the G0-G1, S-phase for the 209 myc MT cells. Note that by day 5 of low serum growth, the percentage of cells in G0-G1 is higher for the 209 myc MTB2 cells than for the 209 myc MT controls, whereas the percentage of cells in the S-phase and G2-M is lower. The ratio of cells in the G0-G1 to the S-phase or G2-M fractions increases for both 209 myc MTB2 and 209 myc MT cells surviving low serum growth, although to a significantly greater extent for the PKC-β2-transfected cells.

GAGG-3' (aa 290), and the downstream primer, 5'-CGGGGGCGATG-TAGTCGG-3' (aa 512), yielding an amplified product of 666 bp. Each 50-μl PCR reaction was run in quintuplicate, and then the products were combined, extracted with phenol-chloroform (pH 9) twice, followed by extraction with chloroform-isomyl alcohol, precipitated with ammonium acetate and ethanol, washed with 70% ethanol, and brought up to 20-μl volume. The entire product was then restricted with PvuII, and the fragments were visualized with ethidium bromide on a 2% agarose gel. When the the exogenous PKC-β2 was the template, PvuII restriction yielded 316- and 348-bp fragments; when the endogenous PKC-β was the template, only the unrestricted 666-bp product resulted.

Analysis of Protein Expression. Cells (1 × 10^6) were washed in ice-cold PBS and then solubilized by boiling in Laemmli buffer (69). Samples were resolved on 1% SDS-8% polyacrylamide gels, transferred 12 h at a constant 33 V in Tris-glycine with 10% methanol, as described previously (7). Filters were blocked with either TBST/1% BSA [50 mM Tris (pH 7.5), 0.15 M NaCl, 0.05% Tween 20, and 1% bovine serum albumin] or TTBS/5% Biotto [100 mM Tris (pH 7.5), 0.15 M NaCl, 0.1% Tween 20, and 5% nonfat dried milk] and hybridized. Primary antibodies used for these studies were affinity-purified rabbit polyclonal anti-PKC-β1 (Santa Cruz Biotechnology, Inc. sc-209), monoclonal anti-c-myc (C-33; Santa Cruz Biotechnology, Inc. sc-42), polyclonal human antihuman topoisomerase (Topogen, Inc. 2012), and monoclonal anti-β-actin (clone AC-15; Sigma A-5441).

Immunoprecipitation of PKC-β2 used a protocol supplied by Life Technologies, Inc. Cells (50 × 10^6) were washed twice in ice-cold PBS and then lysed in 250 μl of ice-cold buffer A [50 mM Tris (pH 7.5), 0.15 M NaCl, 1% volume Triton X-100, 25 μg/ml leupeptin, 25 μg/ml aprotinin, 2 mM EDTA, and 2 mM EGTA] with a chilled Dounce homogenizer followed by a 30-min incubation at 4°C. Cell debris was removed by a brief centrifugation and then cell lysate was incubated with 5 μg/ml of antibody for 2 h at 4°C. To confirm specificity, parallel reactions were run in which the PKC-β2 antibody had been preincubated with a 1:1 (v/v) ratio of peptide (Life Technologies, Inc.) as per the manufacturer's suggestions. Subsequently, both antibody and peptide control reactions were precipitated with 25 μl of protein A-agarose (Life Technologies, Inc.) for 1 h at 4°C, and the pellets were retrieved by brief centrifugation in a microfuge at 15,000 rpm. Pellets were washed twice with buffer A with 0.4 M NaCl and then boiled in Laemmli buffer for 5 min. Protein electrophoresis and Western blotting was continued as described above.

All filters were washed and incubated with appropriate secondary antibodies complexed to avidin-biotinylated-horseradish peroxidase (Amersham). Protein was detected by the enhanced chemiluminescence system (Amersham). The protein films were photographed onto discs (Maxxell MF 2HD) by the Eagle Eye System (Stratagene) and then quantitated using the ImageQuant program (Molecular Dynamics).

Growth Curves. Growth measurements were done using the MTT assay (Sigma) and measuring absorption at 570-nm wavelength following a 3-h incubation. For this assay, all cells were grown in phenol red-free media. On day 0, these cells were triturated into one to three cell aggregates in suspension, then placed in 24-well plates in triplicate, at concentrations of 5 × 10^4 cells/1 ml/well. For the cell lines used, we established a linear relationship between the MTT assay and the cell number. For each growth curve, cell doubling times were calculated as the log (2) divided by the slope of the log(MTT) versus time over the rapid phase of growth.

Flow Cytometric Analysis of Cell Cycle. Cell cycle progression was examined in 0.5 × 10^6 total cells by flow cytometry using the detergent-trypsin-propidium iodide protocol of Vindelov et al. (70). Analysis of the stained nuclei was performed on an Epics 752 flow cytometer (Coulter Corp.). DNA content of 10^5 cells was determined from the propidium iodide signal and by nuclear size forward angle light scatter, and debris was excluded by conventional scatter gating. Cell cycle distribution was assessed by integrating the area under the peaks using the Elite program (Coulter Corp.).

Analysis of Nuclear Morphology. Cells were cytopun at 700 rpm for 7 min onto Cytoslide microscope slides (Shandon, Inc.) and then fixed in methanol and stained with 0.1 μg/ml Hoechst dye 33342 (Sigma Chemical Co.). Nuclei were visualized by fluorescence microscopy using a Zeiss Axioskop microscope (Zeiss) with filter set 48/79/02. Apoptotic nuclei appeared to be condensed or fragmented and could be easily distinguished from normal nuclei.

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References


PKC-\(\beta_2\) Decreases c-myc Apoptosis


