Retinoblastoma Protein-dependent Growth Signal Conflict and Caspase Activity Are Required for Protein Kinase C-sigend Apoptosis of Prostate Epithelial Cells*

(Received for publication, March 6, 1997, and in revised form, July 3, 1997)

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Both protein kinase C and the retinoblastoma tumor suppressor protein have been linked to the regulation of cell growth and cell death, suggesting the differential roles these factors play in mediating cell fate. In some cells, protein kinase C-induced activation of the retinoblastoma protein results in G1 arrest. However, inducible overexpression and activation of the protein kinase Ca isoform or the addition of 12-O-tetradecanoylphorbol-13-acetate in the prostate epithelial cell line, LNCaP, resulted in apoptosis preceded by induction of p21 and dephosphorylation of the retinoblastoma protein. Consistent with a role for the retinoblastoma growth suppressor protein in protein kinase C-induced apoptosis, DU145 cells, which do not express functional retinoblastoma protein or LNCaP cells, which have been transfected with the retinoblastoma inhibitor, E1a, were resistant to apoptosis. LNCaP apoptosis was initiated by a unique conflict between the growth-suppressive activity of the retinoblastoma protein and growth-promoting mitogenic signals. Thus, when this conflict was prevented by serum depletion, apoptosis was suppressed. The caspase family of cysteine proteases is believed to encompass the execution machinery of mammalian apoptosis, and addition of the cell-permeable caspase inhibitor, Z-Val-Ala-Asp-fluoromethylketone, afforded nearly total protection from protein kinase C-signaled apoptosis. This protection correlated with the total loss of caspase activity as measured by the proteolytic cleavage of nuclear poly(ADP-ribose) polymerase. On the basis of these results, we propose that protein kinase C regulates a novel cell death pathway that is initiated by a cellular conflict between retinoblastoma growth-suppressive signals and serum mitogenic signals in proliferating prostate epithelial cells and that is executed by the caspase family of cysteine proteases.

Cell death is influenced by diverse environmental signals that include cytokines and steroids or the depletion of peptide growth factors and sex hormones in cells of the immune system, nervous system, and the epithelial component of various tissues (1, 2). Soluble molecules, however, are not exclusive in the initiation of an apoptotic response in cells. Suppression of apoptosis in anchorage-dependent cells requires their attachment to the extracellular matrix, disruption of which is sufficient to induce apoptosis. Consistent with this, we have presented evidence, in vitro and in vivo, that the loss of β1 integrin-dependent cell anchorage resulted in apoptosis of several epithelial cell lines requiring the retinoblastoma (Rb)

1 The abbreviations used are: Rb, retinoblastoma tumor suppressor protein; TPA, 12-O-tetradecanoylphorbol-13-acetate; PARP, nuclear poly(ADP-ribose) polymerase; cdk, cyclin-dependent kinase; PKC, protein kinase C; ZVAD-fmk, Z-Val-Ala-Asp-fluoromethylketone.

*This study was supported by Specialized Projects of Research Excellence in Prostate Cancer Grant F50 CA65606 (to M. L. D.) and DK/CA47650 (to C. T. P.) from the National Institutes of Health, by grants CA29524 and CA21181 from the American Cancer Society, and by the American Lung Association (to R. G. F.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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This paper is available on line at http://www.jbc.org
associated with the regulation of cell death as well (12, 13). Most PKC isoforms require the physiological activator diacylglycerol, which is derived from membrane phospholipids; for full activity, however, the phorbol ester, 12-O-tetradecanoylphorbol-13-acetate (TPA), or the macrocyclic lactone, bryostatin 1, will also activate PKC. Additionally, PKC activity also requires association with cellular membranes and/or cytoskeletal components to execute many of its physiological functions (15, 16).

PKC modulates signal transduction pathways that have been linked to both positive and negative regulation of the cell cycle and the initiation of apoptosis; however, the specialized roles of the individual isozymes and their physiological targets that modulate these events are not known. The response of a particular type of cell to PKC activators appears to depend not only on specific PKC isozyme expression but also on the status of the functional targets of PKC. Thus, growth regulation by PKC is likely mediated by the cell cycle machinery, but the regulation of cell death may require other, undefined components of the PKC signaling pathway.

By using an inducible overexpression system of PKCα in the present study, we examined the role of Rb in PKC-regulated apoptosis of LNCaP cells. Results from this study are the first to demonstrate that PKC-induced apoptosis was preceded by the rapid induction of p21 and Rb dephosphorylation and required Rb activity for cell death. Moreover, Rb could only induce apoptosis in opposition to serum mitogenic signals in proliferating LNCaP cells and that the execution of apoptosis was dependent on the activation of the caspase family of cysteine proteases. Taken together, the experimental evidence indicates that PKC and Rb are linked in a novel signal transduction pathway which in the presence of mitogenic signals culminates in caspase-regulated apoptosis.

MATERIALS AND METHODS

Cell Culture and Reagents—The cell lines LNCaP (17) and DU145 were propagated in RPMI 1640 medium or in minimum essential medium, respectively, supplemented with 10% fetal calf serum, 2 mM t-glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin (Life Technologies, Inc.). The cells were kept at 37 °C in a humidified atmosphere of 5% CO₂ and subcultured weekly. Chemicals for cell culture treatments, bryostatin 1, staurosporine, and TPA (LC Laboratories, B-6697, S-8451, and P-1680, respectively), were dissolved in 100% ethanol, aliquoted, and stored at −20 °C. The peptide Z-Val-Ala-Asp(fluoromethylketone) (ZVAD-fmk) (Enzyme Systems Products, FK-009) was dissolved in dimethyl sulfoxide. The final concentration of ethanol or dimethyl sulfoxide was <0.1%. Viability was followed by trypan blue exclusion (Life Technologies, Inc.) or by the colorimetric MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-terazolium, inner salt) assay (Promega).

Transfection of LNCaP Cells—The previously described cell lines LNK9 (vector control) and LNA17 are subclones of LNCaP cells in which expression of exogenous human PKCα is regulated by the presence or absence of tetracycline in the medium.2 These clones have stably incorporated plasmid pUHD 10-1, encoding a tetracycline-responsive transactivator protein, and plasmid pUHD 10-3/PKCα, from which human PKCα is expressed (clone LNA17) under control of a tetracycline-responsive promoter. For stable clones expressing E1a or E1a 928, the pCMV-E1a-12S or pCMV-E1a-12S-928 plasmids (3) were transfected into LNCaP cells and clones were isolated in 500 µg/ml of G418.

Western Blot Analysis—Expression of PKCα, Rb and p21 proteins was determined by Western blot analysis using the following antibodies: p21 (PharMingen; 15091A); PKCα (Life Technologies, Inc.; 13222-013); and Rb (PharMingen; 14001A). LNCaP cells and the PKCα transfectants were lysed in radioimmunoprecipitation buffer (50 mM Tris (pH 7.5), 120 mM NaCl, 0.5% Nonidet P-40, 40 µM phenylmethanesulfonyl fluoride, 5 µg/ml leupeptin, 5.5 µg/ml aprotinin). Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis using the NOVEX system, electroblotted to Hybrid nitrocellulose mem-

brane (Amersham), and blocked in 10% nonfat dry milk in TBST (10 mM Tris (pH 8.0), 150–500 mM NaCl, 0.1% Tween 20) for 1 h at room temperature, the primary antibodies were allowed to bind to 4 °C, overnight, in TBSTM (10 mM Tris (pH 8.0), 150–500 mM NaCl, 0.1% Tween 20, 2.5% nonfat dry milk). Appropriate horseradish peroxidase-conjugated secondary antibodies (Amersco) were incubated for 1 h at room temperature. Membranes were washed 3 times (10–15 min each at room temperature) following the primary and secondary antibody incubations. Immunoblots were developed with the ECL system, as described by the manufacturer (Amersham).

RESULTS

Regulation of p21 Expression and Rb Activation by PKC Regulators—We have previously demonstrated that depriving anchorage-dependent cells of matrix contact induced a novel apoptotic pathway dependent on Rb activity (3). We have also developed a model of TPA-induced apoptosis in Rb⁺/⁻ prostate cells that exhibits induction of PKCα expression and membrane translocation (12, 13). Taken together, these studies implicated an apoptotic mechanism uniquely regulated by Rb in epithelial cells that may be initiated by PKC signal transduction. To ascertain the functional significance of Rb in PKC-mediated apoptosis, we began by examining the response of Rb⁺/⁻ and Rb⁻/⁻ prostate epithelial cells to the PKC activator, TPA. The human prostate epithelial cell line, DU145, which does not express functional Rb (18), failed to undergo TPA-induced apoptosis as measured by the exclusion of trypan blue, terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling assay, and nuclear condensation (Fig. 1A and results not shown). At moderate concentrations of TPA, which induced apoptosis in 70% of the Rb⁺ LNCaP culture by 48 h, the DU145 cultures remained viable.

Rb is maintained in its active state through cdk inactivation achieved either by loss of the regulatory cyclins or by induction of the p21/p27 and p16 family of cdk inhibitors. To determine whether Rb is activated through increased cdk inhibitor expression or reduced cdk expression following TPA treatment, we examined p21, p27, and p16 protein levels following the addition of TPA to LNCaP cells. The cdk inhibitor p21, which inhibits both cyclin E-cdk2 and cyclin D1-cdk4/6, was shown to be strongly induced within 12 h of TPA addition in LNCaP cells and remained elevated throughout the 48-h time course (Fig. 1A). In contrast, no change in the level of the cdk inhibitors p27 or p16 was evident, whereas there were changes in the levels of cyclin D1 or E (data not shown). Increased p21 expression might be expected to lead to dephosphorylation and activation of Rb. In Fig. 1A, we show that activation of Rb does indeed follow the induction of p21 with significant conversion from hyper- to hypophosphorylated Rb by 12 h following TPA, coincident with the onset of apoptosis. By 24 h, Rb had been completely converted to the hypophosphorylated form correlating to 40% reduction in cell viability. The protein remained dephosphorylated at 48 h at which time cell viability had been reduced to 31%.

Since TPA is a potent activator of the PKC family (14), we wanted to confirm that PKC activity was associated with the induction of p21 and Rb activation. Staurosporine, a potent but nonspecific catalytic inhibitor of protein kinase C (19), and bryostatin 1, a specific activator and down regulator of PKC, have both been shown to prevent TPA-induced apoptosis (12, 13). Pretreatment of LNCaP cultures with staurosporine or bryostatin 1 prior to TPA addition not only inhibited apoptosis but also completely prevented p21 induction and Rb activation (Fig. 1B and data not shown).

E1a Inhibition of Rb Function Suppressed PKC-mediated Apoptosis—Our results using activators and inhibitors of PKC suggested that PKC recruits the Rb cell cycle control pathway to initiate LNCaP apoptosis. Since we had previously demon-

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2 J. E. Gschwend, Y. Wolny, W. R. Fair, and C. T. Powell, submitted for publication.
Fig. 1. TPA-induced apoptosis is preceded by induction of p21 and Rb activation. Cell survival was measured by the trypan blue exclusion assay. Protein extracts were analyzed by Western blot analysis employing p21- or Rb-specific antibodies. ppRb, hyperphosphorylated (inactive) form of Rb; pRb, hyperphosphorylated (active) form. A, cell survival of Rb− LNCaP cells and Western blot of LNCaP at 0, 12, 24, and 48 h following treatment with 10 nM TPA. B, LNCaP cells were either untreated (control), treated with 10 nM TPA (TPA), or pretreated for 90 min with 50 nM staurosporine prior to TPA treatment (TPA + STS), and cell count and protein extracts were obtained 48 h later. The line graph and columns represent the average and S.D. of duplicate viable cell counts expressed as a percentage of untreated control normalized to 100%.

Fig. 2. Functional inhibition of Rb by E1a transfection suppressed TPA-induced apoptosis. One × 10⁶ untransfected LNCaP cells (LNCaP), LNCaP cells expressing the 928 mutant (928) or LNCaP cells expressing adenoviral E1a (E1α) were treated with 10 nM TPA for 48 h, and the number of viable cells was obtained by trypan blue exclusion. Inset, Western blot for E1a and E1α-928 expression in LNCaP clones. The untransfected parental LNCaP cells (UT) and 293 cells are negative and positive controls for E1a expression, respectively. The columns represent the average and S.D. of duplicate viable cell counts expressed as a percentage of untreated control of each cell line normalized to 100%.

Stratified that expression of exogenous Rb was sufficient to induce apoptosis in LNCaP cells (3), we next wanted to determine whether functional inhibition of endogenous Rb could suppress apoptosis in these cells. To address this question, the viral oncogene E1a, which binds to and functionally inhibits Rb (20), was stably integrated into LNCaP cells, and the resulting transfectants were challenged with TPA. As shown in Fig. 2, LNCaP cells stably expressing E1a did not undergo apoptosis after addition of TPA. Western blot analysis of Rb in the E1a-LNCaP line demonstrated that Rb was dephosphorylated with normal kinetics following TPA treatment (data not shown), indicating that Rb function was not disrupted upstream in its regulatory pathway but following dephosphorylation in the presence of E1a as expected. Because E1a has cellular targets in addition to Rb, we used a control E1a expression vector with a mutation at nucleotide 928 that blocks interaction with Rb without disrupting interactions with the other proteins (21). In contrast to wild-type E1a, the Rb-specific E1a point mutant (E1α-928) did not block apoptosis in TPA-treated LNCaP cells, suggesting that the anti-apoptotic activity of E1a results from inhibition of Rb function alone.

Apoptosis-resistant LNCaP Cells Do Not Exhibit p21 Induction or Rb Dephosphorylation—In many cells, TPA-induced membrane translocation of PKC is followed by degradation and depletion of PKC cellular pools (22). In LNCaP cells, membrane translocation of PKCa following TPA treatment adds a new dimension for at least 36 h before down-regulation is observed, and TPA exposure longer than 48 h results in an apoptotic-resistant phenotype with undetectable levels of PKCa protein (19). If p21 and Rb reside in a PKC-regulated pathway, it is likely then that long term exposure of LNCaP to TPA should result in the emergence of apoptotic-resistant cells incapable of p21 induction or Rb activation. When LNCaP cells were cultured in TPA for 6 days, we observed an initial apoptotic response of 57% by day 2 (Fig. 3). Interestingly, by day 4 post-TPA treatment, the surviving cells had grown back to the original plating number and continued to proliferate out through day 6. Apoptosis of LNCaP cells correlated with the induction of p21 and Rb dephosphorylation at day 1. However, by day 2 the levels of p21 had begun to decline and Rb was just beginning to convert to the hyperphosphorylated form. By day 4, the cells were TPA insensitive and have begun to proliferate and p21 expression and Rb phosphorylation had returned to normal (Fig. 3). Thus, TPA sensitivity and the emergence of an apoptotic-resistant subpopulation correlated precisely with p21 levels and the activation state of Rb.

Inducible Overexpression and Activation of PKCa Resulted in p21 Induction, Rb Activation, and Apoptosis—Data from the PKC activation studies coupled to the observation that depletion of PKC resulted in a nonresponsive phenotype suggested that PKC has a regulatory role in cell cycle events leading to apoptosis. Because we previously identified PKCa as the predominant isotype associated with TPA-induced apoptosis (13), we next examined the effects of inducible PKCa overexpression in LNCaP cells. In two separate clones (LNa1, LNa17), which express exogenous PKCa under control of the
LNCaP cells, nonexpressing (Tet 
induction of p21. Bryostatin 1 by itself had no effect on cell 
a
the extremely high levels of exogenous PKC 

In the presence of tetracycline, expression of PKC 
a
resulted in a 30- and 50-fold induction of PKC 
a
represent the average and S.D. of duplicate viable cell counts.

For the lack of apoptosis in PKCa-over-

A Cellular Conflict between Rb Growth-inhibitory Signals 
and Serum Mitogenic Signals Triggers Apoptosis in LNCaP 
Cells—Cytokines and growth factors in the extracellular 
environment are critically important in determining cell survival 
(23). However, opposing growth-regulatory signals that arise 
in some cells, such as forced c-myc expression (growth promoting) 
in fibroblasts in the absence of serum growth factors (growth 
hibiting), precipitates a conflict resulting in a dysfunctional 
cell cycle and apoptosis (23). Because Rb transfection, which 
is growth inhibited, induced apoptosis of proliferating epithelial 
cells (3), we predicted the opposite scenario in our model: that 
PKCo-mediated Rb activation provides a negative signal for 
growth arrest in direct opposition to a positive mitogenic signal 
in proliferating LNCaP cells; and that this unique conflict 
results in apoptosis. If this conflict initiated apoptosis in 
PKCo-overexpressing cells, then removal of the mitogenic 
signal by depletion of serum growth factors might prevent apo-

Cells—

FIG. 3. TPA-resistant LNCaP cells do not exhibit p21 induction or Rb activation. Viable cell number was obtained at the indicated 
times from LNCaP cells cultured in the continuous presence of 10 nM 
TPA for 6 days. Cell extracts were also prepared from the same cultures 
and expression of p21 and Rb phosphorylation examined by Western 
blot analysis as indicated. ppRb, hyperphosphorylated (inactive) form of 
Rb; pRb, hypophosphorylated (active) form of Rb. Viable cell number 
was determined by trypan blue exclusion. The columns represent the 
average and S.D. of duplicate viable cell counts.

FIG. 4. Inducible overexpression of PKCa in the presence of the 
PKC activator, bryostatin 1, induces apoptosis concomitant with 
p21 induction and Rb activation. Cell viability and protein 
extracts were obtained from cultures of uninduced (Tet−) 
and induced (Tet+) PKCa-expressing clone LNa17 or vector only control 
line (LNGK9) in the absence (−) or presence (+) of bryostatin (Bry) for 48 h. 
Expression of PKCa, p21, and Rb was examined by Western blot analysis 
using specific antibodies as indicated. ppRb, hyperphosphorylated 
(inactive) form of Rb; pRb, hypophosphorylated (active) form. Cell 
viability was determined by trypan blue exclusion, and the columns 
represent the average and S.D. of duplicate viable cell counts expressed as a 
percentage of the Tet+/Bry− control normalized to 100%.

| Duration of treatment | 10% serum | Serum free | 10% serum + ZVAD-fmk |
|-----------------------|-----------|------------|---------------------|
| h                     | %         | %          | %                   |
| 24                    | 72 ± 0.3  | 73 ± 2.2   | 104 ± 4.6           |
| 48                    | 92 ± 3.5  | 67 ± 0.7   | 93 ± 4.8            |
| 72                    | 12 ± 0.8  | 61 ± 0.1   | 88 ± 5.3            |

TABLE I

Viability of LNa17 cells following activation of PKCa in either 10% 
serum, serum-free medium, or 10% serum + 100 μM ZVAD-fmk

Data are mean ± S.D. of duplicate, triplicate, or quadruplicate 
measurements of viability by trypan blue exclusion or the MTS assay. 
This is representative of at least three similar experiments.

3 M. L. Day et al., unpublished results.
Rb-mediated Apoptosis Is Carried Out by the Caspase Family of Cysteine Proteases—Our results have demonstrated that PKC-signalled apoptosis of LNCaP cells is triggered by an Rb-dependent conflict with mitogenic signals. Because a death-promoting role for Rb contradicts its traditional role in cell cycle regulation, we were compelled to identify the mechanism by which cell death was executed. Since the caspase family of cysteine proteases are thought to encompass the execution phase of programmed cell death, we next examined the role of caspases in Rb-mediated apoptosis. As shown in Table I, the cell-permeable caspase inhibitor ZVAD-fmk afforded nearly total protection from apoptosis in the bryostatin 1-treated PKCα-expressing line, LNa17. Due to the high level of protection by ZVAD-fmk pretreatment, we expected to see a strong correlation with the loss of caspase proteolytic activity. To this end, we examined the efficacy with which ZVAD-fmk might inhibit caspase function by analyzing proteolytic cleavage of the caspase substrate, PARP, by Western blot analysis using specific antibodies as indicated. ppRb, hyperphosphorylated (inactive) form of Rb; pRb, hypophosphorylated (active) form.

**FIG. 5.** Serum depletion inhibits cleavage of PARP in LNCaP cells. Protein extracts were obtained from LNa17 cells expressing activated PKCα following 72 h in serum-containing (+ Serum) or serum-depleted (~ Serum) medium at the times indicated. Rb phosphorylation and cleavage of PARP were examined by Western blot analysis using specific antibodies as indicated. ppRb, hyperphosphorylated (inactive) form of Rb; pRb, hypophosphorylated (active) form.

**FIG. 6.** PKC inhibitors, caspase inhibitors and Rb mutation prevent PARP cleavage in LNCaP cells. Protein extracts were obtained from LNa17 cells expressing activated PKCα in the absence or presence of 100 μM ZVAD-fmk or 50 μM staurosporine (STS) in serum-containing medium at the times indicated. Protein extracts were also obtained from DU145 cells treated with 10 nM TPA at the times indicated. Rb phosphorylation and cleavage of PARP were examined by Western blot analysis using specific antibodies as indicated. ppRb, hyperphosphorylated (inactive) form of Rb; pRb, hypophosphorylated (active) form. ND, not detected.

not lead to growth arrest, but in opposition to mitogenic signals in proliferating cells, resulted in apoptosis. This conflict-mediated apoptosis did not occur in Rb−/− prostate epithelial cells and was prevented by functional inhibition of Rb or removal of mitogenic signals. From our use of caspase inhibitors and examination of their activity, it is apparent that execution of this apoptotic pathway is mediated through this proteolytic pathway. Taken together, these results suggest that PKC signals a unique apoptotic pathway that is initiated by an Rb-dependent growth signal conflict in proliferating LNCaP cells and executed by the caspase proteases.

Programmed cell death is regulated by diverse cellular factors, some of which possess differential functions that may include the maintenance of cell growth and survival. Two such regulatory molecules, PKC and Rb, which traditionally are implicated in the transduction and execution of growth-regulatory and differentiation signals (26–29), have been linked to the regulation of apoptosis as well (3, 12, 13). Although much of the molecular function of PKC and Rb are known, specific PKC isoforms and the mechanism of Rb-mediated apoptosis that it signals are completely unknown. The current study was initiated based on the observation that the Rb−/− LNCaP cells undergo TPA-induced apoptosis associated with p21 induction and Rb activation and that DU145 cells, which exhibit p21 induction but lack functional Rb, do not. Therefore, it appears that the signal to decrease cdk activity through p21 is sent in TPA-treated Rb−/− DU145 cells, but they do not undergo apoptosis, perhaps because they lack Rb. In total, these results suggested that Rb activity is required for TPA-induced apoptosis. To investigate this further, we focused on this pathway in the Rb−/− LNCaP line. TPA-treated LNCaP cells did not exhibit loss of cyclin D1 or cyclin E expression; however, there was a dramatic increase in the level of p21. In contrast to p21 induction, there was little change in the level of the related inhibitor p27 or the cdk4/6 inhibitor p16. Therefore, it appeared that a PKC-mediated increase in p21 expression leads to a inhibition in G1 cdk activity, resulting in Rb hypophosphorylation and cell death.

The response of a particular type of cell to PKC activators appears to depend not only on the combination of PKC isoforms expressed in the cell but also on the expression and status of the functional targets of PKC. As an early "functional target of PKC, Raf-1 becomes phosphorylated (activated) by TPA in a variety of cell types, which exhibit various responses, such as proliferation, transformation, growth arrest, and differentiation. However, in LNCaP cells overexpressing
activated PKCα. Raf-1 is phosphorylated very early in a pathway that terminates in cell death. This finding suggests that Raf-1 phosphorylation by PKCα represents a putative signaling cascade leading to Rb hypophosphorylation and apoptosis. The association between PKC signal transduction and activation of Rb was further established by staurosporine-mediated inhibition of p21 induction and Rb activation. Coupled to the observation that this pathway was inhibited by the depletion of PKC, suggested a signal-transducing role for PKC in G1 regulation that in LNCaP cells results in apoptosis.

Because these studies provided correlative evidence but not definitive proof of PKC function in this pathway, we next examined this mechanism in inducible PKCα-overexpressing lines. The p21/Rb pathway was indeed activated following PKCα induction in the presence of the PKC activator, bryostatin 1, implicating this isoform in cell cycle regulation and apoptosis. The effects of inducible overexpression of PKCα have not been examined previously, and our results contrast with those of Ways et al., who, employing constitutive overexpression of PKCα in MCF-7 cells, showed that TPA and bryostatin 1-induced cell death was associated with G2–M accumulation, minimal p21 induction, and lack of Rb activation (30). Additionally, parental MCF-7 cells treated with TPA demonstrated G1 arrest associated with p21 induction and Rb activation and were mostly unaffected by bryostatin 1. The results in MCF-7 cells were complicated by the finding that constitutive overexpression of PKCα, in the absence of PKC activators, resulted in increased expression of PKCβ and decreased expression of PKCγ and p53 (31). None of these changes were observed in LNCaP cells following inducible overexpression of PKCα. Interestingly, Livneh et al. reported that ectopic expression of the epithelial-specific PKCγ isoform regulated a similar program in NIH 3T3 cells, resulting in p21 induction and Rb dephosphorylation (32). However, these PKCγ-expressing fibroblasts did not undergo apoptosis as do inducible PKCα-expressing LNCaP epithelial cells but remain G1 arrested.

Our observations along with those of other laboratories suggest that accumulation of hypophosphorylated Rb is a general response of PKC activity leading to G1 arrest in a variety of cell types (33). It has also been demonstrated in fibroblasts that disruption of integrin-mediated contact results in the induction of p21 and G1 cyclin inactivation leading to dephosphorylation of Rb and growth arrest (34, 35). As in fibroblasts, however, we have found that the same integrin-regulated pathway induced in several different epithelial cell lines, including prostate, resulted in p21 induction and Rb activation, but in contrast to growth arrest of fibroblasts, the epithelial cells underwent apoptosis (3). The present results using ectopic expression of PKCα demonstrate that PKC activation, like β1, integrin, initiates a signaling pathway that results in Rb hypophosphorylation and apoptosis of prostate epithelial cells. The evidence that Rb is central to this PKC-induced apoptotic pathway was confirmed by the finding that LNCaP clones, stably expressing the adenovirus E1a protein, prevented TPA-induced apoptosis. It therefore appears that activation of Rb, which leads to growth suppression in some cell types, uniquely regulates an apoptotic pathway in prostate epithelial cells and possibly other epithelial cells (3).

Because LNCaP cells respond to Rb activation by undergoing apoptosis, Rb itself must encompass some aspect of the cell death signal; however, the nature of such a signal is unknown. Apoptosis is thought to be a default pathway in cells receiving opposing cell proliferation signals. Evan et al. have published extensively on growth signal conflict-induced apoptosis using c-myc overexpression in fibroblasts deprived of serum growth factors (23). Although our findings demonstrate that conflicting growth signals induce apoptosis, it is due to the opposite scenario, where negative Rb growth-inhibitory signals precipitate an apoptotic conflict with positive mitogenic signals in proliferating prostate epithelial cells. To confirm this possibility, we found that if such a conflict was prevented in LNCaP cells, by removing the mitogenic signal (serum depletion) or eliminating the growth-inhibitory signal (Rb deletion in the DU145 cells or Rb inactivation by E1a transfection in LNCaP cells), then apoptosis does not occur.

We next set out to determine the mechanism through which apoptosis is executed by this Rb-mediated conflict. We began by investigating the interleukin-1β-converting enzyme-related proteases, which have been implicated in apoptosis of many diverse systems. When we used the cell-permeable caspase inhibitor, ZVAD-fmk, we observed total protection from apoptosis in the bryostatin 1-treated PKCα-expressing line, LNCaP. When we examined cleavage of the caspase substrate, PARP, we found it unaltered by 12 h of PKCα activation, which is the time when Rb is becoming activated, but that complete cleavage had occurred by 24 h, at which time Rb is fully activated, suggesting that Rb activation preceded activation of the caspases. In determining the order of events in this apoptotic pathway, we observed that inhibition of PKCα by staurosporine (initiation phase) not only inhibits Rb activation but also inhibits apoptosis and the associated cleavage of PARP. Coupled to the findings that ZVAD-fmk inhibited both apoptosis and PARP cleavage (execution phase) in LNCaP cells but did not inhibit Rb hypophosphorylation places Rb downstream of PKC yet upstream of caspase activation. Therefore, we postulate that the cellular conflict signal provided by Rb in proliferating cells is initiated by PKC and terminates in the recruitment of caspases to execute apoptosis.

Tumor suppression is manifest not only by cell cycle arrest but also through the initiation of cell death programs as well. The role of the p53 tumor suppressor gene product in apoptosis has been extensively documented in cells that are responding to DNA-damaging agents or chemotherapeutic agents or in cells that have a deregulated cell cycle (reviewed in Ref. 1). Although Rb has been shown to inhibit multiple p53-dependent apoptotic pathways (1, 23), accumulation of hypophosphorylated Rb leading to G1 arrest and apoptosis occurs in multiple p53-independent pathways as well (36, 37). Two such p53-independent pathways are induced by release of the lipid second messenger ceramide (38, 39) or by DNA-damaging agents (40), both of which result in the accumulation of hypophosphorylated Rb, G1 arrest, and apoptosis. Apoptosis also occurs normally in the androgen-dependent prostate epithelium of p53−/− mice following castration (41), indicating that this is a p53-independent process. p53 transcriptionally activates a number of different genes including p21 in response to DNA damage; however, p21 expression can occur independently of p53 function (42).

PKC and Rb modulate a variety of physiologic responses in cells ranging from the regulation of cell growth and differentiation to cell death. Therefore, it is likely that cell survival is dictated by a complex intracellular environment through which PKC signaling pathways are responding to common extracellular cues. Some of these PKC-regulated pathways terminate in the accumulation of hypophosphorylated Rb; however, depending on the type of cell and its environment, PKC and Rb activities may encompass differential roles, directing the cell toward quiescence, differentiation, or apoptosis. We maintain that the primary role of Rb in regulating cell cycle is to signal growth arrest; however, we suggest that it is the extracellular and intracellular mitogenic influences that dictates whether the cell will survive or die in response to that signal.
Acknowledgments—We thank D. Dean, V. Dixit, and K. Pienta for advise and comments and S. Frisch and M. Green for antibodies and plasmids.

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