Protein Kinase C δ Stimulates Apoptosis by Initiating G1 Phase Cell Cycle Progression and S Phase Arrest*

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Overexpression of protein kinase C δ (PKCδ) stimulates apoptosis in a wide variety of cell types through a mechanism that is incompletely understood. PKCδ-deficient cells are impaired in their response to DNA damage-induced apoptosis, suggesting that PKCδ is required to mount an appropriate apoptotic response under conditions of stress. The mechanism through which it does so remains elusive. In addition to effects on cell survival, PKCδ elicits pleiotropic effects on cellular proliferation. We now provide the first evidence that the ability of PKCδ to stimulate apoptosis is intimately linked to its ability to stimulate G1 phase cell cycle progression. Using an adensoviral-based expression system to express PKCα, -δ, and -ε in epithelial cells, we demonstrate that a modest increase in PKCδ activity selectively stimulates quiescent cells to initiate G1 phase cell cycle progression. Rather than completing the cell cycle, PKCδ-infected cells arrest in S phase, an event that triggers caspase-dependent apoptotic cell death. Apoptosis was preceded by the activation of cell cycle checkpoints, culminating in the phosphorylation of Chk-1 and p53. Strikingly, blockade of S phase entry using the phosphatidylinositol 3-kinase inhibitor LY294002 prevented checkpoint activation and apoptosis. In contrast, inhibitors of mitogen-activated protein kinase cascade failed to prevent apoptosis. These findings demonstrate that the biological effects of PKCδ can be extended to include positive regulation of G1 phase cell cycle progression. Importantly, they reveal the existence of a novel, cell cycle-dependent mechanism through which PKCδ stimulates cell death.

Protein kinase C (PKC) is a family of serine/threonine protein kinases composed of three subclasses consisting of the classical (α, β1, and γ), novel (δ, ε, η, and θ), and atypical (ζ and η/λ) PKC isoforms. PKC isoforms differ in their requirement for calcium and their responsiveness to the lipid second messenger diacylglycerol. The classical (calcium-dependent) and novel (calcium-independent) isoforms are responsive to diacylglycerol, whereas the atypical isoforms are diacylglycerol-insensitive (reviewed in Ref. 1). Most cells express multiple PKC isoforms that require distinct cofactors and exhibit unique intracellular localizations. Pharmacological inhibitors have been used to assess the roles of individual PKC isoforms. Much of what is known regarding the biological roles of PKCδ has been derived from studies using the PKCδ-selective inhibitor rottlerin, the specificity of which is in question. The isolation of cell lines overexpressing or lacking individual isoforms has been used to decipher the physiological roles of select PKC isoforms. A major limitation of this approach is that it does not readily distinguish between the effects of PKC activation versus the subsequent down-regulation of PKC expression. We opted to use adenoviruses to drive selective, modest increases in PKC activity and to provide substantial insight into the regulation of cellular survival by PKCδ (2–4). We selected thyroid epithelial cells for this analysis because alterations in PKC activity, expression, and structure (5–9) have been documented in human thyroid tumors. The results of this analysis revealed the existence of a novel mechanism through which PKCδ stimulates apoptosis and demonstrate a tight linkage between the effects of PKCδ on cell proliferation and cell death.

EXPERIMENTAL PROCEDURES

Reagents—The following antibodies were used: active caspase-3 (Asp-175), caspase-3 (Cell Signaling, Beverly, MA), phospho-H2AX (Ser-139) (Upstate, Charlottesville, VA), phospho-p53 (Ser-15) (Cell Signaling), cyclins A (C-19), and E (M-20) (Santa Cruz Biotechnology, Santa Cruz, CA), cyclin B1 (Cell Signaling), nPKCδ (C-20) (Santa Cruz Biotechnology), Cdk-2 (M2) (Santa Cruz Biotechnology), LY294002 and SB203580 were purchased from Sigma. Q-VD-OPh (QVD) and GF109203X were from Calbiochem (EMD Biosciences, La Jolla, CA). SP600125 was from Alexis Biochemicals (Lausen, Switzerland), and U0126 was from Promega (Madison, WI).

Cell Culture—Wistar rat thyroid cells were propagated in three hormone-containing growth medium (3H, containing thyroid-stimulating hormone, insulin, and serum) as described previously (10). Cells were rendered quiescent by starvation in Coon’s modified Ham’s F-12 medium devoid of thyroid-stimulating hormone and growth factors (basal medium) for 48–72 h.

Adenoviral Infection—Adenoviruses for PKCα, -δ (2, 3), and -ε and kinase-defective PKCδ(K376R) (11) were used. Quiescent cells were infected with adenoviruses for PKCα (2,000 or 10,000 particles (pl)/cell), PKCδ (5,000 p/cell), or PKCe (7,000 p/cell) in basal medium for 16 h (day 1 post-infection). Virus was removed, and the cells washed twice with DPBS containing 1% nonessential amino acids, and TPA was used, TPA (25 nM) was added at day 2 post-infection for 60 min, and the cells were washed and then incubated in basal medium

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for the times indicated. Inhibitors were added at the time of infection and again at day 1 post-infection.

**Flow Cytometry**—Floating and trypsinized cells were collected and pelleted at 1,000 rpm for 5 min at 4 °C. Cells were fixed in ice-cold MeOH overnight and resuspended in 250 μl each RNase (200 units/ml) and propidium iodide (0.1 mg/ml in 0.1% Triton X-100, 0.037 mg/ml EDTA) in phosphate-buffered saline. Cells were analyzed on a BD Biosciences FACScan and analyzed using CellQuest™ Pro software.

**DNA Laddering**—Floating and adherent cells were collected and resuspended in TE/Triton buffer (0.2% Triton X-100, 10 mM Tris, pH 8.0, 1 mM EDTA). Following incubation on ice for 10 min, an aliquot of the lysate containing total DNA was removed, and the remaining lysate was centrifuged at 14,000 × g for 15 min at 4 °C. Supernatant containing low molecular weight DNA was transferred to a fresh tube and treated with DNase-free RNase A (60 μg/μl) for 1 h at 37 °C. SDS (0.5%) and proteinase K (150 μg/ml) were added, and the samples were incubated for 1 h at 50 °C. DNA was precipitated by addition of 0.1 volume of 0.5 M NaCl and 1.0 volume isopropanol, followed by incubation on ice for 10 min. Following centrifugation, DNA was resuspended in TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA) and fractionated on 2% agarose gels.

**Immunostaining**—DNA synthesis was assessed by BrdUrd incorporation as described in Kupperman *et al.* (10). For active caspase-3 and γ-H2AX staining, cells were fixed in 3.7% formaldehyde/phosphate-buffered saline, permeabilized in 0.2% Triton X-100 for 2 min, and stained with primary and secondary antibodies for 1 h at 37 °C. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). Images were captured on a Zeiss Axiophot microscope fitted with a Hamamatsu ORCA-ER digital camera using Axiovision 4.2 software.

**Western Blotting**—Cells were washed in ice-cold phosphate-buffered saline and lysed in radioimmune precipitation assay buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 0.5% sodium deoxycholate, 1% Nonidet P-40, 0.1% SDS) plus protease inhibitors. Protein content in clarified lysates was assayed using the Bio-Rad DC protein assay. Incubations with primary antibodies were performed overnight at 4 °C, followed by incubation with horseradish peroxidase-conjugated secondary antibodies and detection via chemiluminescence.

**Kinase Assays**—For PKC assay, infected cells were scraped in isosonic buffer (50 mM Tris, pH 7.4) containing protease inhibitors, sonicated, and clarified by centrifugation. Protein concentration was determined, and samples were dispensed into 20-μl aliquots in triplicate. 30

**FIGURE 1. PKCα stimulates cell cycle progression in quiescent thyroid cells.** A, upper panel, cells were infected with PKCα (10,000 particles (p)/cell), PKCδ (5,000 p/cell), or PKCε (7,000 p/cell) (see “Experimental Procedures”) and harvested at days 3–5 post-infection (gray bars). Floating and adherent cells were collected and analyzed for cell cycle position by FACS analysis. Control (open bars) represents mock infected cells. Where indicated, TPA was used on day 2 post-infection (black bars) as described under “Experimental Procedures.” Results show one of three complete time-course experiments performed (two full time-course experiments were performed with PKCα plus TPA). Similar results with PKCδ (minus TPA) on days 2–4 have been observed in more than six experiments. Lower panel, parental cells were stimulated with TPA (25 nM) for 60 min, washed, and incubated in basal medium for 24, 48, and 72 h. TPA failed to stimulate cell cycle progression in parental cells. Two experiments were performed with similar results. B, cells were infected as described above and harvested at day 2 post-infection, and kinase activity was assessed using PKCα-pseudosubstrate peptide as substrate (12). The results of a representative experiment performed in triplicate are shown. Similar results were observed in three independent experiments. C, cells infected as described above were harvested at day 2, and PKC expression was assessed by Western blotting with PKCα-, δ-, and ε-specific antibodies.
μl of kinase reaction buffer (50 mM Tris, pH 7.4, 1 mM EGTA, 25 μM ATP, 7.5 mM MgAc, 10 μM PKCa pseudosubstrate peptide (12), 100 μg/ml phosphatidylserine, 1 μCi/sample [γ-32P]ATP, 1 μM TPA) was added, and the samples were incubated for 10 min at 30 °C. Reactions were terminated by spotting 25 μl of reaction mixture onto Whatman PE-81 paper. Papers were washed in 0.1M phosphoric acid, rinsed in Nonidet P-40, 2.5 mM EGTA, 1 mM NaF, 1mM ATP, 7.5 mM MgAc, 10 μM PKCa, and air dried prior to counting. Results are presented as counts per min (CPM)/μg of protein. For Cdk-2 assays, Cdk-2 was immunoprecipitated (Cdk-2 (M2) goat, Santa Cruz Biotechnology), washed in lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 2.5 mM EGTA, 1 mM NaF, 1 mM β-glycerophosphate) containing protease inhibitors, followed by kinase buffer (50 mM HEPES pH 7.5, 10 mM MgCl₂, 1 mM dithiothreitol) and in vitro kinase assays performed as described in Lewis et al. (13) using histone H1 as substrate.

**RESULTS**

**PKCδ Induces Aberrant Cell Cycle Progression**—Phorbol esters stimulate proliferation and impair differentiated function in thyroid epithelial cells. Because differentiated function is an important prognostic indicator for patients with thyroid tumors (14), the identity of the PKC isozymes responsible for these effects is important to ascertain. To elucidate the contributions of PKCa, -δ, and -ε, the phorbol ester-responsive isozymes expressed in Wistar rat thyroid cells (15), to the regulation of thyroid cell proliferation and function, we utilized adenoviruses to overexpress individual PKC isoforms. Quiescent Wistar rat thyroid cells were infected with adenoviruses expressing PKCa, -δ, or -ε at varying multiplicities of infection and kinase activity measured in cell lysates prepared at day 2 post-infection. Conditions were derived under which each isozyme exhibited a modest 2-fold increase over endogenous PKC activity, and could be selectively activated with a dose of TPA to which uninfected cells did not respond (2) (Fig. 1B and data not shown). Western blotting indicated that the PKC isoforms were substantially overexpressed under these conditions (Fig. 1C). Immunostaining experiments conducted with PKC-selective antibodies revealed that >95% of the cells were infected under these conditions (data not shown).

Flow cytometry analysis of propidium iodide-stained cells revealed that PKCδ selectively induced cell cycle entry. Expression of PKCδ decreased the proportion of G1 phase cells and significantly increased the number of S phase cells (Fig. 1A). The effects of PKCδ on S phase entry were similar in the presence and absence of TPA activation. Strikingly, the effects of PKCδ on cell cycle progression were not reproduced by PKCa or -ε even following expression at higher levels than PKCδ and in the presence or absence of phorbol ester treatment. At the concentration used, TPA failed to stimulate cell cycle progression in mock infected cells (Fig. 1A, lower panel). To corroborate these data, the effects of PKCδ on molecular markers of cell cycle progression were analyzed. PKCδ stimulated the expression of cyclins A and E, as well as Cdk-2, the catalytic partner for the G1 phase cyclins (Fig. 2A). Expression was increased beginning at day 2 post-infection and sustained over...
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FIGURE 4. PKCα selectively stimulates hypodiploid DNA content. Cells infected with PKCα, -δ, or -ɛ (gray bars) were treated with 25 nM TPA for 60 min on day 2 post-infection where indicated (black bars). Cells were fixed on days 3–5 post-infection, stained with propidium iodide, and analyzed for DNA content by flow cytometry. Control (open bars) represents mock infected quiescent cells. One representative time course from three performed with similar results is shown (two full time-course experiments were performed with PKCδ plus TPA).

4 days. The up-regulation of cyclins and Cdk-2 was functional, as evidenced by the sustained increase in G2 phase activity (Fig. 2B).

Intriguingly, PKCα-infected cells did not progress into G2,M (Fig. 1A), suggesting that these cells fail to complete the cell cycle. This is most clearly seen in Fig. 3A where PKCα stimulated a time-dependent increase in S phase cells (days 2–3) without a coordinate increase in G2 phase cells (days 2–5). This pattern was strikingly different from that observed following mitogenic (3H) treatment of quiescent cells (Fig. 3C). PKCα-infected cells arrested with a DNA content that was greater than that of G1 phase (3H, 30 h) cells and less than that of G2/M phase (3H plus nocodazole) cells. In agreement with these data, PKCα failed to stimulate cyclin B1 expression, a marker of G2 phase cells (Fig. 2A). Together, these results indicate that a modest increase in PKCα activity is sufficient to induce exit from quiescence, but not to stimulate cell proliferation. Rather, PKCα-expressing cells proceed through G1 phase and subsequently arrest in S phase.

PKCα-stimulated S Phase Arrest Is Followed by Apoptosis—The proportion of PKCα-infected S phase cells declined over time, commensurate with an increase in the proportion of cells containing hypodiploid DNA (Figs. 3A and 4). Despite their similar activities, PKCα and -ɛ did not stimulate hypodiploid DNA content in the presence or absence of TPA activation (Fig. 4). Similar to its effects on cell cycle progression, hypodiploid DNA content induced by PKCδ did not require TPA activation.

To document that the effects of PKCα on hypodiploid DNA content reflected apoptosis, caspase-3 cleavage was examined. Western blotting using an antibody that detects pro- and cleaved forms of caspase-3 revealed the presence of cleaved caspase-3 in lysates prepared from detached (floating) PKCα-infected cells (Fig. 5A). The abundance of intact pro-caspase-3 was markedly reduced in these cells. Longer exposures of the Western blot shown in Fig. 5A revealed the presence of cleaved caspase-3 in adherent cells. Control represents mock infected quiescent cells. Three experiments were performed with similar results. To confirm that apoptosis was initiated in adherent cells, we analyzed the expression of active caspase-3 using an antibody that selectively recognizes the cleaved form of caspase-3. Staining for active caspase-3 using an antibody that selectively recognizes the cleaved form of caspase-3 was first detected at day 2 post-infection and increased over time (Fig. 5B). The caspase-3 positive cells invariably exhibited an apoptotic nuclear morphology assessed by DAPI staining. Active caspase-3 staining was abolished following treatment with the pan-caspase inhibitor Q-VD-OPh (QVD) (Fig. 5B). Finally, PKCα stimulated QVD-sensitive DNA laddering, confirming that cell death was apoptotic (Fig. 5C).
PKCa is subject to caspase-dependent cleavage, generating a C-terminal fragment containing an intact kinase domain (16). DeVries et al. (17) reported that overexpression of the PKCa fragment resulted in its accumulation in the nucleus and the induction of apoptosis. Western blotting with a C-terminal-directed antibody failed to reveal the presence of significant amounts of the PKCa fragment in apoptotic thyroid cells (data not shown), hence we do not believe PKCa cleavage initiates apoptosis in these cells.

Given that apoptosis temporally follows the accumulation of cells in S phase, we reasoned that PKCa-infected cells die from S phase. Indeed, inhibition of apoptosis with QVD prevented the accumulation of cells with hypodiploid DNA and markedly increased the proportion of S phase cells (Fig. 3B). This result, together with the absence of cyclin B1 expression as well as of G2/M phase cells (Figs. 1 and 2), strongly suggests that expression of PKCa stimulates S phase arrest that culminates in apoptosis.

PKCa—PKCδ Stimulates the DNA Damage Response Pathway—We set out to confirm the effects of PKCa on S phase entry by monitoring effects on DNA synthesis. DNA synthesis was examined by incorporation of the thymidine analog bromodeoxyuridine (BrdUrd) into replicating DNA (10). Unlike mitogen-treated thyroid cells where BrdUrd labeling is uniform (13, 18, 19), BrdUrd localized to punctate foci in PKCa-infected cells (Fig. 6A). The significance of these foci remains to be determined; however, they are reminiscent of the foci observed following stalled replication and/or DNA damage (20, 21). This prompted us to investigate whether PKCa activated the DNA damage response pathway.

ATR-, ATM-, and DNA-dependent protein kinase (DNA-PK) are key mediators of the cellular response to DNA damage. These PI3K-like kinases are activated by replication stress and DNA double strand breaks. Activation of ATR/ATM results in the phosphorylation and activation of downstream substrates, notably Chk-1 and Chk-2. Stalled replication is a robust stimulator of ATR activity and culminates in the activating phosphorylation of Chk-1 on serine 345 (22). Overexpression of PKCa, but not PKCa, stimulated Chk-1 phosphorylation (Fig. 6B). Phosphorylation was maximal at day 2 post-infection, similar to the time course over which PKCa stimulated DNA synthesis (data not shown). To confirm the activation of cell cycle checkpoints, the effects of PKCa on p53 phosphorylation were examined. ATR and ATM phosphorylate p53 on serine 15 (23). PKCa stimulated p53 phosphorylation at this site, whereas PKCa failed to do so (Fig. 6C). Phosphorylation of p53 in PKCa-expressing cells was maximal at day 2 post-infection with significant phosphorylation also observed at day 3. To further document checkpoint activation, we assessed the effects of PKCa on the phosphorylation of histone H2AX (designated γ-H2AX), an indicator of DNA double strand breaks (24). Immunostaining experiments revealed an increase in γ-H2AX staining as early as day 2 post-infection (Fig. 6D).

Thus, γ-H2AX staining temporally coincided with BrdUrd incorporation and Chk-1 and p53 phosphorylation. In contrast, caspase-3 activation was first detected at day 3 post-infection (Fig. 5B), suggesting that it followed checkpoint activation. To eliminate the possibility that DNA cleavage itself induced checkpoint activation, the effects of PKCa on checkpoint activation were examined in the presence of the caspase inhibitor QVD. QVD had no effect on the ability of PKCa to stimulate Chk-1, p53, or H2AX phosphorylation (Fig. 6, B and C, and data not shown). These data support a model wherein PKCa stimulates replication stress, leading to checkpoint activation and ultimately, apoptosis.

PKCa—PKCδ Stimulated Apoptosis Requires Cell Cycle Entry—PKCa has been reported to activate JNK (25, 26) and to require p38 or ERK for induction of apoptosis (4, 27). Pharmacological inhibitors of JNK (SP600125), p38 (SB203580), and MEK1 (U0126) were used to determine whether PKCa-stimulated apoptosis requires activity of these MAPK family members. None of these inhibitors impaired DNA laddering induced by PKCa (Fig. 7A). Intriguingly, treatment with the pan-PKC inhibitor GF109203X also failed to impair DNA laddering. To further examine whether kinase activity was required for PKCa-stimulated apoptosis, the effects of kinase-deficient PKCa were examined. Following expression at levels similar to wild type PKCa (data not shown), kinase-deficient PKCa stimulated apoptosis as assessed by hypodiploid DNA content (Fig. 7B). Therefore, kinase activity is not strictly required for the effects of PKCa on apoptosis in thyroid cells. Indeed, kinase-dependent and -independent effects of PKCa on apoptosis have been previously reported (28, 29).

Given that apoptosis temporally follows a delay in S phase, we set out to determine whether inhibition of cell cycle entry would prevent apoptosis. Phosphatidylinositol 3-kinase (PI3K) activity is required for thy-
roid cell proliferation (19). Therefore, the PI3K inhibitor LY294002 was used to investigate the consequences of cell cycle inhibition on PKCδ-stimulated apoptosis. Unlike the other inhibitors tested, LY294002 abolished DNA laddering (Fig. 7A). FACS analysis confirmed that LY294002 blocked the accumulation of cells containing hypodiploid DNA and that this inhibitor prevented S phase entry (Fig. 7C). Consistent with the latter effect, LY294002 abolished DNA synthesis in PKCδ-expressing cells (Fig. 7D). PDK-1 phosphorylates PKCδ on T505, a modification that increases kinase activity. Because PDK-1 is downstream of PI3K, we considered the possibility that LY294002 inhibited PKCδ kinase activity. Pretreatment of PKCδ-infected cells with LY294002 (data not shown), or addition of LY294002 directly to in vitro kinase assays, did not impair PKCδ kinase activity (Fig. 7E). Additionally, LY294002 had no effect on PKCδ expression (Fig. 8A). Therefore, we speculate that the inhibitory effects of LY294002 on apoptosis are mediated predominantly through its ability to block S phase entry. In agreement with this notion, the JNK, MEK1, and p38 inhibitors were less effective than LY294002 in preventing S phase entry (Fig. 7C) and DNA synthesis (Fig. 7D). The PKC inhibitor GF109203X only partially reduced PKCδ-stimulated DNA synthesis.

To further establish that checkpoint activation arose as a consequence of delayed progress through S phase, the effects of LY294002 on PKCδ-stimulated Chk-1 and p53 phosphorylation were investigated. Interestingly, treatment with PI3K (LY294002), MEK1 (UO126), p38 (SB203580), or JNK (SP600125) inhibitors blocked or significantly impaired Chk-1 phosphorylation (Fig. 8A). However, these inhibitors differed in their ability to block PKCδ-induced p53 phosphorylation. Only the PI3K inhibitor (LY294002) completely inhibited p53 phosphorylation. Because LY294002 is known to inhibit ATR, ATM, and DNA-PK activity (30–33) its effects on Chk-1 phosphorylation were assessed. In contrast to its effects on PKCδ-stimulated Chk-1 phosphorylation, LY294002 failed to inhibit Chk-1 phosphorylation in response to aphidicolin (an inhibitor of replication, and positive control for ATR activation/Chk-1 phosphorylation) (Fig. 8B). Therefore, at the concentration used in these studies, LY294002 does not directly inhibit ATR activity. Cumulatively, these data suggest that replication stress in...
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FIGURE 8. LY294002 blocks checkpoint activation by PKCδ. A. PKCδ-infected cells were left untreated or treated with LY294002, UO126, SB203580, SP600125, or QUVID as described in legend to Fig. 7. Cells were harvested on day 2 post-infection and subjected to Western blotting for p-Chk1 (Ser-345), p-p53 (Ser-15), PKCδ, and actin. The inhibitors had no effect on PKCδ expression. Greater than five experiments were performed in which LY294002 effects on phosphorylation of Chk-1 were observed. Three experiments were performed with the inhibitors UO126, SB203580, SP600125, and QUVID. B. quiescent (Qu) Wistar rat thyroid cells were stimulated with 3H growth medium (22 h) followed by treatment with aphidicolin (10 μM, 1 h). Where indicated, LY294002 (15 μM) was added 1 h prior to aphidicolin. Lysates were subjected to Western blot analysis with antibodies to pChk-1 (Ser-345) and actin. Two experiments were performed with similar results.

FIGURE 9. Model for PKCδ-stimulated apoptosis. PKCδ stimulates entry into S phase, followed by S phase arrest, checkpoint activation, and apoptosis. S phase entry was inhibited by LY294002, suggesting that PI3K is required for the effects of PKCδ on G2 to S phase cell cycle progression. Blockade of S phase entry using LY294002 prevented checkpoint activation and apoptosis. The pan-caspase inhibitor QUVID blocked apoptosis and increased the proportion of S phase cells. Although not shown here, it is possible that additional pathways activated by PKCδ contribute to checkpoint activation and apoptosis.

Together with the protracted S phase observed in PKCδ-infected cells, prompted us to investigate whether PKCδ activated cell cycle checkpoints. ATR, ATM, and DNA-PK are activated by DNA damage and replication stress and play an essential role in the maintenance of genomic stability through their ability to halt cell cycle progression and facilitate DNA repair. Under conditions of replication stress or irreparable DNA damage, checkpoint kinases facilitate cell death. Overexpression of PKCδ, but not PKCα, stimulated Chk-1 and p53 phosphorylation, indicators of checkpoint activation. PKCδ-infected cells also exhibited phosphorylation of histone H2AX, a marker of DNA double strand breaks.

If apoptosis was a direct consequence of replication stress induced by S phase arrest, then inhibition of S phase entry would be expected to rescue PKCδ-expressing cells from apoptosis. Indeed, treatment with the PI3K inhibitor LY294002 blocked S phase entry, checkpoint activation, and apoptosis. In contrast, inhibitors of MAPK cascades did not prevent apoptosis. However, MEK1, JNK, and p38 inhibitors were much less effective than the PI3K inhibitor in blocking S phase entry in PKCδ-infected thyroid cells. Hence, our data strongly suggest that it is the accumulation of PKCδ-expressing cells in S phase that induces apoptosis and that apoptosis is a consequence of replication stress.

Whether checkpoint activation is essential for PKCδ-mediated apoptosis remains to be determined. As members of the phosphatidylinositol kinase family, ATM, ATR, and DNA-PK are inhibited by LY294002 (30–33). To ascertain whether LY294002 rescued apoptosis through the direct inhibition of checkpoint kinase activity, its effects on aphidicolin-stimulated Chk-1 phosphorylation were examined. At the concentration used in these studies, LY294002 blocked Chk-1 phosphorylation in response to PKCδ, but not that stimulated by aphidicolin. Nonetheless, we cannot exclude a role for other phosphatidylinositol kinases (i.e. ATM or DNA-PK) in the inhibition of apoptosis by LY294002. PKCδ associates with and inhibits the activity of DNA-PK (34), raising the possibility that PKCδ stimulates S phase entry and coordinates inhibits DNA repair, resulting in the accumulation of single-stranded DNA and checkpoint activation. Full-length PKCδ has been found in the nucleus (17), and we observed nuclear staining following overexpression of PKCδ in thyroid cells (data not shown). Therefore, PKCδ is in the appropriate cellular compartment to elicit effects on DNA synthesis and repair. Complexes containing PKCδ and c-abl, a protein-tyrosine kinase activated by oxidative stress and DNA damage, have also been reported (35, 36). PKCδ associates with many cellular proteins, including p73B (37), members of the phospholipid scramblase family PSL-1 (38) and PSL-3 (39), lamin B (40), and src family kinases (reviewed in Ref. 41). Complexes between PKCδ and mammalian target of rapamycin (mTOR), as well as PI3K, have also been described (42–44). It is possible that PKCδ-containing protein complexes elicit functional consequences on DNA repair and apoptosis, either through alterations in protein phosphorylation or through a scaffolding function. If PKCδ acts via the latter mechanism, this would explain why PKCδ-stimulated apoptosis is independent of PKCδ kinase activity in thyroid and other (29) cells.

At first glance, the ability of PKCδ to stimulate G1 phase cell cycle progression appears to conflict with numerous reports in the literature that PKCδ mediates growth suppression. However, there is increasing evidence that PKCδ positively regulates some aspects of cell cycle progression. Kitamura et al. (45) reported that serum stimulated the biphasic activation of PKCδ in fibroblasts. The second wave of PKCδ activity was temporally correlated with entry into S phase. Overexpression of PKCδ enhanced serum-stimulated DNA synthesis, whereas kinase-defective mutants impaired it, effects that were observed in both 3Y1 and NIH3T3 fibroblasts. In a separate study, overexpression of PKCδ led to increased E2F promoter activ-
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In 3Y1 fibroblasts (46). Therefore, accumulating evidence supports a conserved role for PKCδ in the positive regulation of early events in the cell cycle. Despite its requirement for serum-stimulated DNA synthesis, chronic overexpression of PKCδ mediated growth inhibition in 3Y1 cells (45). Similarly, transient expression of PKCδ decreased the proportion of M phase cells. In capillary endothelial cells, overexpression of PKCδ caused delayed exit from S phase (47), whereas in PKCδ-overexpressing CHO cells, treatment with phospholipase A2 stimulated G2/M arrest (48). Although apoptosis was not reported in these studies, apoptosis is a common end point of stalled cell cycle progression. PKCδ-expressing thyroid cells exhibited sustained increases in Cdk-2 expression and activity. This would be expected to result in the stabilization of E2F, an event known to trigger apoptosis. Moreover, replication stress stabilizes E2F via ATM-dependent phosphorylation, and DNA damage leads to the acetylation of E2F1 and its recruitment to apoptotic promotors (49). Together, these data indicate that PKCδ is capable of promoting G1 to S phase transition and either inhibits cell cycle progression on both cell proliferation and survival (50–54).

The failure of PKCδ (Fig. 9). This is likely to be a well conserved mechanism through which PKCδ regulates cell survival, given the reports that PKCδ regulates not only apoptosis, but also cell cycle progression. The signaling pathways through which PKCδ regulates apoptosis may vary by cell type; however, we suggest that the mediators of PKCδ effects on apoptosis are those that convey effects on cell proliferation. In thyroid epithelial cells, it is clear that the targets of PKCδ include members of the phosphatidylinositol kinase family, thereby identifying phosphatidylinositol kinases as downstream mediators of PKCδ effects. It will be important to investigate whether PKCδ plays a role in thyroid-stimulating hormone-driven proliferation, and/or downstream from thyroid oncogenes such as Ras and Ret indicate their effects on both cell proliferation and survival (50–54).

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Supporting Materials and Methods

Figure 1. PKCδ promotes cell cycle progression through inhibition of apoptosis. (A) Cell cycle distribution of 3Y1 cells overexpressing PKCδ (top) or control cells (bottom) (0.5 mg actin). (B) Cell cycle distribution of 3Y1 cells overexpressing PKCδ (top) or control cells (bottom) (0.5 mg actin).

Figure 2. PKCδ promotes cell cycle progression through inhibition of apoptosis. (A) Cell cycle distribution of 3Y1 cells overexpressing PKCδ (top) or control cells (bottom) (0.5 mg actin). (B) Cell cycle distribution of 3Y1 cells overexpressing PKCδ (top) or control cells (bottom) (0.5 mg actin).
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