Suppression of Apoptosis in the Protein Kinase Cδ Null Mouse in Vivo*

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Protein kinase C (PKC) δ is an essential regulator of mitochondrial dependent apoptosis in epithelial cells. We have used the PKCδ−/− mouse to ask if loss of PKCδ protects salivary glands against γ-irradiation-induced apoptosis in vivo and to explore the mechanism underlying protection from apoptosis. We show that γ-irradiation in vivo results in a robust induction of apoptosis in the parotid glands of wild type mice, whereas apoptosis is suppressed by greater than 60% in the parotid glands of PKCδ−/− mice. Primary parotid cells from PKCδ−/− mice are defective in mitochondrial dependent apoptosis as indicated by suppression of etoposide-induced cytochrome c release, poly(ADP-ribose) polymerase cleavage, and caspase-3 activation. Notably, apoptotic responsiveness can be restored by re-introduction of PKCδ by adenoviral transduction. Etoposide and γ-irradiation-induced activation of p53 is similar in primary parotid cells and parotid glands from PKCδ−/+ and PKCδ−/− mice, indicating that PKCδ functions downstream of the DNA damage response. In contrast, activation of the c-Jun amino-terminal kinase is reduced in primary parotid cells from PKCδ−/− cells and in parotid C5 cells, which express a dominant inhibitory mutant of PKCδ. Similarly, c-Jun amino-terminal kinase activation is suppressed in vivo in γ-irradiated parotid glands from PKCδ−/− mice. These studies indicate an essential role for PKCδ downstream of the p53 response and upstream of the c-Jun amino-terminal kinase activation in DNA damage-induced apoptosis in vivo and in vitro.

Apoptosis is a highly conserved and precisely regulated mode of cell death that is essential for the elimination of damaged cells from multicellular organisms. Inappropriate activation or inhibition of apoptosis contributes to the pathogenesis of cancer (1, 2) and autoimmune disease (3), and may exacerbate collateral damage to normal tissues in some therapeutic regimes. For instance, in the salivary gland, it is clear that apoptosis contributes to destruction of the salivary gland as a consequence of γ-irradiation for head and neck tumors, resulting in dramatic reduction in salivary gland function (4). Thus, strategies to suppress apoptosis in the surrounding non-cancerous tissue may dramatically affect the efficacy of treatment and the quality of life for many individuals.

Cell toxins, including DNA damaging drugs, organelle poisons, oxidative stress, and ionizing or ultraviolet irradiation, activate the intrinsic apoptotic program. Distinct forms of cell damage elicited by these agents initiates signaling cascades that converge at the mitochondria, resulting in cytochrome c release and subsequent caspase activation (5). Commitment to this pathway is controlled by pro- and anti-apoptotic signal transduction molecules including members of the Bcl-2 family of proteins (6), protein kinases such as Akt (7), mitogen-activated protein kinase family members, and specific PKCδ isoforms (8).

Members of the protein kinase C family have been shown to exhibit pro- and anti-apoptotic activities depending on the isoform and cellular context (9). PKCδ, a ubiquitously expressed isozyme, has been identified in many studies as a pro-apoptotic isoform. Apoptotic stimuli such as etoposide, ionizing and UV irradiation, and brefeldin A can induce caspase-mediated cleavage of PKCδ (10–13) releasing a COOH-terminal constitutively active kinase fragment that potently activates apoptosis (14). In many cell types, transfection of either the COOH-terminal kinase fragment (15, 16), or full-length PKCδ (13) is sufficient to induce apoptosis. Alternatively, pharmacological inhibition of PKCδ, or expression of a kinase-dead PKCδ mutant, can suppress cell toxin-induced death (11, 13). A role for PKCδ in apoptosis is supported by studies that show that smooth muscle cells isolated from PKCδ−/− mice are resistant to H2O2, UV irradiation, and TNFα-induced apoptosis (17). Recent studies have shown that PKCδ may modulate apoptosis through the regulation of signaling pathways such as JNK and STAT1, or by regulating the basal transcription of p53 (18–20).

Whereas a multitude of studies indicate a role for PKCδ in epithelial cell apoptosis in vitro, nothing is known about the contribution of PKCδ to apoptosis in vivo. Here we show that loss of PKCδ protects parotid salivary glands against γ-irradiation-induced apoptosis in vivo. Primary parotid epithelial cells isolated from PKCδ−/− mice are likewise resistant to a variety of apoptotic cell toxins, but the apoptotic response can be completely recovered by re-introduction of PKCδ. These studies demonstrate that PKCδ is required for efficient induction of apoptosis in vivo and in vitro, and suggest that manipulation of PKCδ expression or activation may be useful to therapeutically modulate the radiosensitivity of epithelial tissues.

EXPERIMENTAL PROCEDURES

Tissue Preparation—The PKCδ−/− mouse was generated by Dr. K. Nakayama at Kyushu University (21) and maintained at the University of Colorado Health Sciences Center in accordance with Laboratory Animal Care guidelines and protocols. Wild type littermates (PKCδ+/+) were used for all studies shown. For removal of salivary glands, 4–6-
week-old female C57Bl/6 PKC\(\delta^{+/+}\) and PKC\(\delta^{-/-}\) mice were deeply anesthetized using Avertin (0.4–0.6 mg/g; intraperitoneal) followed by exsanguination. The parotid and submandibular glands were removed and snap frozen in liquid nitrogen or fixed in 10% neutral buffered formalin and embedded in paraffin for histological analysis. Four-micrometer sections were cut from the paraffin-embedded tissue for staining with hemotoxylin and eosin. For tissue extracts, frozen glands were ground to a fine powder using a mortar and pestle in the presence of liquid nitrogen; gland powder was transferred to a pre-chilled microcentrifuge tube and 500 \(\mu\)l of gland lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 2 mM EDTA, 50 mM NaF, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 1 mM NaVO\(_3\), 0.5 mM diithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and Complete protease inhibitor mixture) was added. Extracts were vortexed and transferred to a pre-chilled Dounce homogenizer (7-ml Pyrex number 7727) and homogenized on ice. Extracts were then centrifuged at 13,000 \(\times\) g for 30 min at 4 °C, and the supernatant was stored at −80 °C.

**Analysis of Apoptosis in Vivo**—Four to 6-week-old female PKC\(\delta^{+/+}\) and PKC\(\delta^{-/-}\) mice were anesthetized as described and the head and neck regions were irradiated using a RS 2000 Biological Irradiator while the remainder of the body was shielded with lead. Parotid and submandibular glands were removed 24 h post-irradiation, fixed in 10% neutral buffered formalin, and embedded in paraffin for immunohistochemistry. Four-micrometer sections were cut from the paraffin-embedded tissue for immunohistochemistry for detection of activated caspase-3. Sections were heated to 60 °C and then re-hydrated, followed by blocking with avidin and biotin. Sections were incubated with anti-active caspase 3 (1:100; Cell Signaling Technology, Beverly, MA, number 9661) overnight at 4 °C. Endogenous peroxidase was quenched with 1% H\(_2\)O\(_2\). Following buffer rinses, sections were incubated in goat anti-rabbit secondary antibody (1:200; Elite PK6101, Vectastain, Burlingame, CA) for 30 min at room temperature. The ABC Reagent (Elite PK6101, Vectastain) was added and tissue sections were incubated for an additional 30 min at room temperature. The signal was detected with diaminobenzidine substrate (HK150–5L Biogenex, San Ramon, CA) for 30 min at room temperature. The ABC Reagent (Elite PK6101, Vectastain, Burlingame, CA) was added and tissue sections were incubated for an additional 30 min at room temperature. The signal was detected with diaminobenzidine substrate (HK150–5L Biogenex, San Ramon, CA) and diluted 1:1000. Rabbit polyclonal antibody directed against p53 was purchased from Cell Signaling Technology (Beverly, MA) and diluted 1:1000. Rabbit polyclonal antibodies directed against poly(ADP-ribose) polymerase (PARP), phospho-JNK, phospho-p53 (human serine 15, mouse serine 18), and phospho-STAT1 (serine 727) were purchased from Cell Signaling Technology (Beverly, MA) and diluted 1:1000. Rabbit polyclonal antibodies directed against

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**Caspase-3 Activity Assay**—Caspase-3 activity was quantified using the Biomol Quantzyme Colorimetric Assay Kit (BioMol, Plymouth Meeting, PA). Adherent and floating cells were collected and lysed in caspase lysis buffer supplemented with 0.1% Triton X-100, aprotinin (4 \(\mu\)g/ml), Pefabloc (0.5 mg/ml), and leupeptin (2 \(\mu\)g/ml) as previously described (11, 26). Caspase-3 activity in 30 \(\mu\)g of cell lysate was measured by cleavage of Ac-DEVD-p-nitroanilide colorimetric substrate, and absorbance at 405 nm was quantified in a microtiter plate reader (Molecular Devices, Sunnyvale, CA) at 10-min intervals for 7 h.

**Cytochrome c Release Assay**—Primary parotid cells were grown on coverslips in 6-well dishes. To assay for cytochrome c release, etoposide-treated cells were fixed in 2% paraformaldehyde for 15 min, followed by three washes in phosphate-buffered saline, and then a 30-min incubation in Blocking Buffer (phosphate-buffered saline, 10% fetal bovine serum, 0.2% Triton X-100). Cytochrome c primary monoclonal antibody (clone 6H2.B4, BD Pharmingen) was diluted (1:500) in 2% bovine serum albumin and 0.2% saponin and added to cells for 30 min at room temperature. Cells were then washed in Blocking Buffer three times for 15 min each, followed by a 30-min incubation in Alexa Fluor 488 Fluor goat anti-mouse (Molecular Probes, Eugene, OR) secondary antibody dilution of 1:200 in 2% bovine serum albumin and 0.2% saponin with the addition of 5 \(\mu\)g/ml 4’,6-diamidino-2-phenylindole dihydrochloride hydrate, or as the percent of the total number of GFP-positive cells in the adenoviral transduced cells.

**TUNEL Analysis and Cell Counts**—Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) was performed using In Situ Cell Death Detection TMR Red (Roche Molecular Biochemicals). Cells were counterstained with 5 \(\mu\)g/ml 4’,6-diamidino-2-phenylindole dihydrochloride hydrate (Sigma) for 30 min and stained cells were visualized using a fluorescent microscope. TUNEL-positive cells with condensed chromatin were scored as apoptotic. In samples transduced with adenoviruses expressing AdPKC\(\delta\)-GFP or AdGFP, only GFP positive cells that met the criteria of being apoptotic were scored. Three hundred to 1000 cells were scored for each variable per experiment and the number of apoptotic cells was expressed as a percentage of the total cell number in experiments where the cells were stained with TUNEL and 4’,6-diamidino-2-phenylindole dihydrochloride hydrate, or as the percent of the total number of GFP-positive cells in the adenoviral transduced cells.

**Immunoblot Analysis**—Immunoblot analysis was performed as previously described (11). Primary mouse parotid cells were prepared under sterile conditions as previously described (22–25). A 1% (v/v) cell suspension was seeded onto collagen-coated dishes (Falcon/BD Biosciences, Fairlawn, NJ) in a 1:1 mixture of Dulbecco’s modified Eagle’s medium/nutrient mixture F-12 supplemented with 10% fetal calf serum, 5 \(\mu\)g/ml transferrin, 1.1 \(\mu\)M hydrocortisone, 0.1 \(\mu\)M retinoic acid, 2.0 \(\mu\)M T3, 5 \(\mu\)g/ml insulin, 80 \(\mu\)g/ml epidermal growth factor (Collaborative Biomedical Products, Bedford, MA), 5 mM l-glutamine, 50 \(\mu\)g/ml gentamicin sulfate, and a trace element mixture. Cells grew to ~80% confluence in 5 days and were used at that time for experiments without further passage. Tissue culture reagents were obtained from BIOSOURCE (Rockville, MD) unless otherwise indicated.

**JNK Activity Assay**—Adherent and floating cells were collected and lysed in JNK buffer as previously described (26). Lysates were clarified at 12,500 \(\times\) g for 5 min, and the protein concentration was determined using the DC protein assay (Bio-Rad). A 100-\(\mu\)g volume of a 10% suspension of GST-c-Jun-(1–79) immobilized on agarose-glutathione beads (Sigma) was added to 300 \(\mu\)g of protein in a final volume of 1 ml and incubated for 2 h at 4 °C. Beads were then washed 3 times in HEPES Binding Buffer (26). The kinase assay was conducted by adding 40 \(\mu\)l of JNK kinase buffer (26) and incubating at 30 °C for 20 min. The reaction was terminated by addition of 2 \(\times\) sample buffer (Pierce) followed by boiling for 5 min. Reaction products were resolved on a 10% SDS-PAGE gel, stained with Bio-safe Coomassie (Bio-Rad) to assess the position of...
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GST-c-Jun-(1–79). Gels were then dried and subjected to autoradiography to determine the extent of GST-c-Jun-(1–79) phosphorylation. GST-c-Jun-(1–79) expression vector was kindly provided by Dr. Lynn Heasley (University of Colorado Health Sciences Center, Denver, CO). The expression and isolation of this fusion protein has been described previously (26).

Adenovirus Construction and Expression in Primary Parotid Cells—Generation and use of AdPKCδ has been described previously (13). To generate AdGFP and AdPKCδ-GFP, DNA encoding PKCδ-GFP or GFP was digested with XhoI and XbaI and ligated into the adenoviral shuttle vector, pShuttle-CMV, which had likewise been digested with XhoI and XbaI. DNA from all vectors was grown and isolated from DAM− cells because the XbaI site cannot be recognized within methylated DNA. Recombinant adenoviruses (AdGFP, AdPKCδ-GFP, and AdPKCδ) were prepared as described (27) and then titered in HEK293 cells based on GFP or E2 expression. Primary parotid cells were infected with AdPKCδ-GFP or AdGFP at a multiplicity of infection (focus forming units/cell) of 500, whereas parotid C5 cells were infected with AdPKCδ/H9254 because the XbaI site cannot be recognized within methylated DNA. Recombinant adenoviruses (AdGFP, AdPKCδ-GFP, and AdPKCδ) were prepared as described (27) and then titered in HEK293 cells based on GFP or E2 expression. Primary parotid cells were infected with AdPKCδ-GFP or AdGFP at a multiplicity of infection (focus forming units/cell) of 500, whereas parotid C5 cells were infected with AdPKCδ/H9254 at a multiplicity of infection of 200. Cells were infected in serum-free Dulbecco’s modified Eagle’s medium for 1 h with occasional shaking, after which the virus containing medium was aspirated and replaced with normal medium. Infection was allowed to proceed for 24 h before the addition of apoptotic agents.

Miscellaneous—Etoposide and breafeldin A were purchased from Sigma and dissolved in dimethyl sulfoxide. Recombinant mouse TNFα was purchased from Calbiochem. Cyclohexamide was purchased from Fischer Scientific—a UV cross-linker (Fischer Scientific model UVXL-1000) at a wavelength of 254 nm.

RESULTS

PKCδ−/− Mice Are Protected Against γ-irradiation-induced Apoptosis in Vivo—Our previous work with chemical and biological inhibitors indicated that PKCδ functions to promote apoptosis in salivary cells in vitro (11, 13). A significant cause of morbidity in patients treated with γ-irradiation for head and neck tumors is the loss of salivary gland function as a result of collateral damage to these glands (4, 28, 29). To determine whether genetic disruption of PKCδ can protect against salivary gland apoptosis in vivo, we have examined the response of the parotid and submandibular gland to γ-irradiation in PKCδ+/+ and PKCδ−/− mice. Given the possibility that PKCδ may contribute to salivary gland development, we examined the morphology of parotid and submandibular salivary glands isolated from 6-week-old PKCδ+/+ and PKCδ−/− mice. Histological analysis revealed no observable differences in glandular architecture, suggesting that PKCδ+/+ parotid and submandibular salivary glands develop normally (Fig. 1A). Loss of PKCδ could potentially result in compensatory changes in the expression of other PKC isoforms. To address this, we examined PKC isoform expression in parotid glands isolated from PKCδ+/+ and PKCδ−/− mice. As expected, PKCδ expression was undetectable in parotid glands from PKCδ−/− mice (Fig. 1B). Although some mouse to mouse variation was apparent, no consistent differences were observed in the expression of PKCa, β1, β2, e, η, and ζ isoforms in the parotid glands of PKCδ+/+ and PKCδ−/− mice (Fig. 1B). These data suggest that the phenotype observed in PKCδ−/− salivary glands is because of disruption of the PKCδ gene locus.

To determine whether genetic disruption of PKCδ protects against apoptosis in vivo, the head and neck region of PKCδ+/+ and PKCδ−/− mice was γ-irradiated and the parotid and submandibular salivary glands were surgically removed after 24 h for analysis of apoptosis. Tissue sections were stained with an anti-active caspase-3 antibody and counterstained with hematoxylin. Very little apoptosis (<0.1%) was observed in the untreated parotid glands of PKCδ+/+ or PKCδ−/− mice (Fig. 2, A and B). However, at a dose of 1 Gy, 16% of the cells in the parotid gland of PKCδ−/− mice were apoptotic, as shown by the presence of active caspase-3, as compared with 6% of the cells in the parotid gland of PKCδ−/− mice, indicating that the lack of PKCδ affords protection against apoptosis. A similar level of protection was observed in the parotid glands from PKCδ−/− mice when the irradiation dose was increased to 2 Gy (data not shown). A less profound, yet still significant, suppression of apoptosis was also observed in the parotid glands of PKCδ−/− mice at a dose of 5 Gy (Fig. 2, A and B). Apoptotic cells in both PKCδ+/+ and PKCδ−/− parotid glands primarily consisted of acinar epithelial cells, with little evidence of apoptosis in the ductal epithelial cells (Fig. 2A). In contrast to the parotid gland, the submandibular gland was resistant to γ-irradiation-induced apoptosis at the doses used (Fig. 2, C and D). At 5 Gy of γ-irradiation only 2% of submandibular epithelial cells from PKCδ+/+ mice were active caspase-3 positive, compared with 30% of parotid epithelial cells. Although the percentage of active caspase-3 positive cells was slightly lower in submandibular glands from PKCδ−/− mice at 5 Gy as compared with PKCδ+/+ mice, this difference was not significant. These results are consistent with our previous observation that submandibular gland epithelial cells are more resistant than parotid epithelial cells to genotoxin-induced apoptosis in vitro (26). Overall, these results indicate that PKCδ contributes to γ-irradiation-induced apoptosis in vivo in radiation-sensitive tissues such as the parotid gland.

Suppression of Apoptosis in PKCδ−/− Primary Parotid Cells: Reconstitution with PKCδ Restores Sensitivity to Etoposide—To further explore how genetic disruption of PKCδ protects cells from apoptosis, we analyzed apoptosis in primary cell cultures derived from the
parotid glands of PKC<sup>+/+</sup> and PKC<sup>-/-</sup> mice. Primary parotid cells were treated with etoposide, UV-C irradiation (UV), or brefeldin A for 24 h and DNA fragmentation was assayed by TUNEL. As seen in Fig. 3A, PKC<sup>-/-</sup> parotid cells were resistant to apoptosis induced by all cell toxins examined. The extent of suppression of apoptosis was similar with all agents tested (etoposide, 43%; brefeldin A, 44%; and UV, 36% suppressed). Similar experiments using primary parotid cells isolated from PKC<sup>+/+</sup> mice showed no suppression of apoptosis as compared with cells from PKC<sup>-/-</sup> mice, indicating that haploinsufficiency of PKC is not sufficient to suppress apoptosis (Fig. 3B). To ask if loss of PKC can suppress apoptosis initiated by death receptors, primary parotid cells were treated with TNFα + cyclohexamide. As seen in Fig. 3C, TNFα-induced apoptosis is reduced by >60% in parotid cells derived from PKC<sup>-/-</sup> mice as compared with PKC<sup>+/+</sup> mice. Taken together, these data indicate that in primary parotid epithelial cells, genetic disruption of PKC<sub>δ</sub> attenuates apoptosis induced both via the intrinsic and death receptor pathways.

Whereas resistance to apoptosis in PKC<sup>-/-</sup> parotid cells likely results from loss of PKC<sub>δ</sub> expression, to verify that apoptosis is directly dependent on PKC<sub>δ</sub>, we asked if re-expression of PKC<sub>δ</sub> is sufficient to restore the apoptotic response. PKC<sup>-/-</sup> and PKC<sup>-/-</sup> parotid cells were transduced with adenoviruses expressing either GFP (AdGFP) or a PKC<sub>δ</sub>-GFP (AdPKC<sub>δ</sub>-GFP) fusion protein. As shown in Fig. 3D, transduction of AdPKC<sub>δ</sub>-GFP into PKC<sup>-/-</sup> cells completely restores their apoptotic response to etoposide, whereas PKC<sup>-/-</sup> cells transduced with AdGFP remained resistant to etoposide-induced apoptosis. These data clearly show that reconstitution of PKC<sub>δ</sub>-<sup>-/-</sup> parotid cells with PKC<sub>δ</sub> is sufficient to restore apoptotic sensitivity.

FIGURE 2. PKC<sup>-/-</sup> mice are resistant to γ-irradiation-induced apoptosis in vivo. The head and neck region of PKC<sup>+/+</sup> and PKC<sup>-/-</sup> mice was treated with 1 or 5 Gy of γ-irradiation and the mice were sacrificed after 24 h. Panels A and C, parotid (A) and submandibular (C) salivary glands were surgically removed and stained for anti-active caspase-3. Between 2 and 8 mice were analyzed for each dose; representative pictures are shown. Arrows show examples of anti-caspase-3 positive cells. Panels B and D, the percentage of active caspase-3 from parotid glands (B) or submandibular glands (D) was quantified as described under “Experimental Procedures.” White bars, PKC<sup>+/+</sup> mice; black bars, PKC<sup>-/-</sup> mice. Asterisks indicate that differences are statistically significant from PKC<sup>+/+</sup> (p < 0.05). UT, untreated.
PKCδ Functions Downstream of p53-regulated DNA Damage Signaling—The DNA damage response in most cells requires the p53 protein that mediates cell cycle arrest, activates DNA repair, and regulates the expression of pro-apoptotic genes, such as Bax (30, 31). Upon DNA damage, p53 is phosphorylated on serine 18 in the mouse, resulting in stabilization of the protein and increased transcriptional activity. To determine whether p53 activation is abrogated in PKCδ−/− parotid cells, we analyzed p53 serine 18 phosphorylation, p53 protein accumulation, and the expression of p53 target genes in PKCδ−/− and PKCδ−/− cells exposed to etoposide. Phosphorylation of p53 on serine 18 was apparent by 1 h of etoposide treatment in both cell types and correlated with an increase in total p53 protein (Fig. 4A). Quantification of phospho-p53 from three similar experiments showed no significant differences between PKCδ−/+ and PKCδ−/− cells (Fig. 4B). Transcriptional targets of activated p53 include the CDK inhibitor p21, which induces cell cycle arrest, and the pro-apoptotic Bcl-2 family member, Bax. As shown in Fig. 4A, etoposide induces the expression of both proteins within 2–4 h in primary parotid cells derived from PKCδ−/+ mice. The expression of p21 and Bax were also induced in parotid cells derived from PKCδ−/− mice, albeit the level of induction was consistently, but not significantly lower, perhaps reflecting the fact that multiple pathways regulate the expression of these genes (Fig. 4A). Likewise, DNA microarray analysis shows no significant difference in Bax mRNA expression in parotid glands from PKCδ−/+ and PKCδ−/− mice exposed to γ-irradiation for 4 h, however, p21 mRNA expression is reduced by 36% in parotid glands from PKCδ−/− mice as compared with PKCδ−/+ mice at this time point (p < 0.05).

To determine whether PKCδ is required for p53 regulation in vivo, we isolated parotid glands from γ-irradiated PKCδ−/+ and PKCδ−/− mice and analyzed p53 phosphorylation and protein accumulation. As shown in Fig. 4C, phosphorylation of p53 was detectable 30 min following γ-irradiation and accumulation of the p53 protein was dramatically increased after 4 h of γ-irradiation in parotid glands isolated from both PKCδ−/+ and PKCδ−/− mice. In line with our results in primary parotid
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from the mitochondria initiates caspase activation in apoptotic cells via the intrinsic pathway. In the experiment shown in Fig. 5A, we analyzed the subcellular localization of cytochrome c in etoposide-treated cells by immunofluorescence microscopy. In untreated PKCδ+/+ and PKCδ−/− cells, cytochrome c shows punctuate staining, indicative of mitochondrial sequestration of cytochrome c. However, after 6 h of etoposide treatment, 75% of the PKCδ+/+ cells have a diffuse cytosolic pattern of cytochrome c staining, indicating release of cytochrome c into the cytosol (Fig. 5, A and B). Cytochrome c release was dramatically suppressed in etoposide-treated PKCδ−/− cells with only 28% of the cells exhibiting diffuse cytochrome c staining (Fig. 5, A and B). Caspase-3 activation was also suppressed in etoposide-treated PKCδ−/− cells (Fig. 5C), as was the cleavage of PARP, a well characterized caspase substrate (Fig. 5D). Quantification of the PARP cleavage fragment by densitometry shows that PARP cleavage and caspase-3 activation are suppressed to a similar extent in PKCδ−/− cells (46 vs. 40%). Taken together, these results demonstrate that PKCδ is required for apoptotic events that occur downstream of DNA damage signaling, but upstream of cytochrome c release at the mitochondria.

**JNK Activation Is Suppressed in Primary Parotid Cells and Parotid Glands Lacking PKCδ—** Whereas the role of JNK in apoptosis appears to vary between cell types, in general, sustained activation of JNK correlates with promotion of apoptosis (32, 33). We have previously shown that treatment of parotid C5 cells with etoposide results in sustained activation of JNK and that PKCδ contributes to activation of the JNK pathway in these cells (11, 26). Here we show that JNK activation in response to etoposide is suppressed in an established parotid cell line (parotid C5) transduced with an adenovirus that expresses dominant inhibitory PKCδ (AdPKCδKD) (Fig. 6A), and in primary parotid cells from PKCδ−/− mice (Fig. 6B). Fig. 6A shows that treatment of parotid C5 cells with etoposide results in sustained activation of JNK, and that the maximal induction of JNK activity is decreased by >50% in cells expressing PKCδKD. Likewise, JNK activation in etoposide-treated primary parotid cells from PKCδ−/− mice is suppressed up to 20% as compared with cells from PKCδ+/+ mice (Fig. 6B). Notably, JNK activation in primary parotid cells appears to be more transient than in the parotid C5 cells, with the level of activated JNK decreasing by 8 h after etoposide treatment in both PKCδ−/− and PKCδ+/+ cells (Fig. 6B). Our data indicate that JNK activation in vivo following γ-irradiation also requires PKCδ. In the experiment shown in Fig. 6C, the salivary glands of PKCδ+/+ and PKCδ−/− mice were γ-irradiated in vivo and JNK activity was determined in tissue lysates prepared from these glands. As seen here, JNK activation in vivo occurs rapidly following γ-irradiation and returns to nearly untreated levels after 4 h. JNK phosphorylation in γ-irradiated PKCδ−/− parotid glands is markedly suppressed (65% at 1 h) as compared with γ-irradiated glands from PKCδ+/+ mice (Fig. 6C). These results show that JNK activation is disrupted in vitro and in vivo in cells and tissue lacking PKCδ and suggest that JNK signaling functions downstream of PKCδ in apoptotic signal transduction.

Our data supports previous studies that show that PKCδ contributes to the activation of JNK in apoptotic cells (11, 19, 34). It is clear, however, that additional signal transduction pathways must contribute to propagation of the apoptotic signal. In particular we have previously shown that etoposide induces a 2–3-fold increase in STAT1 phosphorylation at serine 727 in HeLa cells, and that STAT1 phosphorylation at serine 727 in these cells is dependent on PKCδ activity (18). Here we have analyzed STAT1 serine 727 phosphorylation in etoposide-treated PKCδ−/− primary parotid cells and in vivo in γ-irradiated PKCδ−/− parotid tissue. Basal phosphorylation of STAT1 was detected in both PKCδ+/+ and PKCδ−/− cells, however, treatment with etoposide

**FIGURE 4. Regulation of p53 in PKCδ+/+ parotid cells in vitro and PKCδ−/− parotid tissue in vivo.** Panel A, primary parotid cells from PKCδ+/+ and PKCδ−/− mice were treated with 200 μM etoposide for the indicated number of hours and cells were harvested. Panel B, quantification of phospho-p53. The graph is the average of three separate experiments. Panel C, PKCδ−/− (white bars) and PKCδ+/+ (black bars) mice were irradiated with a 5 Gy dose of γ-irradiation for the indicated number of hours. Parotid salivary glands were then surgically removed and protein lysates were prepared for immunoblot analysis. Panels A and C, immunoblots were probed with anti-phospho-p53 (serine 15), anti-total p53, anti-Bax, or anti-p21 as indicated. All experiments were repeated 3 or more times with similar results.

cells, no differences were observed in p53 phosphorylation and protein accumulation between irradiated PKCδ+/+ and PKCδ−/− parotid glands. These data indicate that DNA damage-induced activation of p53 is not altered in PKCδ−/− mice in vitro and in vivo, implying that PKCδ is required for apoptosis downstream of the DNA damage response.

**Apoptosis Is Suppressed at the Mitochondria in Primary Parotid Cells Isolated from PKCδ−/− Mice—** Our results indicate that the DNA damage response is intact in PKCδ−/− parotid cells. We next addressed the possibility that events at, or downstream of the mitochondria, are suppressed in PKCδ−/− parotid epithelial cells. Release of cytochrome c...
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revealed no consistent increase in phosphoserine 727 STAT1 in either cell type (Fig. 7A). In contrast, STAT1 serine 727 phosphorylation increased rapidly in γ-irradiated parotid glands, however, analysis of four experiments showed no consistent differences in STAT1 serine 727 phosphorylation between parotid glands isolated from PKCδ+/+ and PKCδ−/− mice (Fig. 7B). Taken together, these data provide in vitro and in vivo evidence that STAT1 phosphorylation is not exclusively regulated by PKCδ in apoptotic cells. STAT1 phosphorylation at serine 727 is thought to be important for the transcription of pro-apoptotic genes (35). Hence, the basal serine 727 phosphorylation observed in primary cells may be sufficient to support expression of these genes. In response to γ-irradiation, STAT1 serine 727 phosphorylation may be regulated by protein kinases in addition to PKCδ.

DISCUSSION

The availability of a PKCδ−/− mouse model provides a clean genetic system in which to fully explore the function of PKCδ in promoting apoptosis. In the present studies we show that genetic disruption of the PKCδ gene protects the parotid salivary gland against γ-irradiation-induced apoptosis in vivo. Reintroduction of PKCδ into PKCδ−/− primary parotid cells restores their apoptotic capability, demonstrating that PKCδ is sufficient to specifically control the ability of these cells to undergo apoptosis. Our studies show that DNA damage-induced activation of p53 is normal in vitro and in vivo in the absence of PKCδ, however, PKCδ−/− cells are defective in downstream components of the apoptotic pathway including DNA fragmentation, cytochrome c release, and caspase activation. We also show that PKCδ is required for death receptor-induced apoptosis; presumably this reflects a role for PKCδ in amplification of this pathway via the mitochondria. Importantly, our in vitro and in vivo studies indicate that JNK activation is an important downstream signal transduction component of PKCδ in apoptotic cells. Taken together, our findings indicate that PKCδ is required for the efficient induction of salivary epithelial cell apoptosis in vivo and in vitro.

A problem common to radiation therapy of tumors is the unintended destruction of the surrounding radiosensitive normal tissue. Radiation treatment of head and neck tumors frequently causes permanent damage to the major salivary glands, resulting in severe salivary gland hypo-

FIGURE 5. Cytochrome c release, caspase-3 activation, and PARP cleavage are suppressed in PKCδ−/− primary salivary cells. Primary parotid cells from PKCδ−/− and PKCδ+/+ mice were treated with 200 μm etoposide for 6 and 8 h (A and B) or with 50, 100, and 200 μm etoposide for 18 h (C and D). Panel A, immunofluorescence of cytochrome c (green) in untreated (UT) primary parotid cells and cells treated with 200 μm etoposide as indicated. Cells were counterstained with 4',6-diamidino-2-phenylindole dihydrochloride hydrate (blue) to visualize the nucleus. Panel B, the release of cytochrome c from the mitochondria shown in panel A was quantified as described under “Experimental Procedures.” Asterisks indicate that differences are statistically significant from PKCδ−/− (p < 0.05) PKCδ+/+, white bars; PKCδ−/−, black bars. Panel C, primary parotid cells from PKCδ−/− (white diamonds) or PKCδ+/+ (black squares) mice were treated with the indicated dose of etoposide for 24 h. Caspase-3 activity was assayed using a colorimetric assay and Ac-DEVD-p-nitroaniline as a substrate, as described under “Experimental Procedures.” Panel D, primary parotid cells were treated as described in panel C and cleavage of PARP was detected as described under “Experimental Procedures.” The arrow indicates the position of the 89-kDa PARP cleavage fragment.
Recent studies on PKCδ mice have identified diverse roles for this signaling molecule in control of immunity (21, 38), apoptosis (17), and cell migration (39, 40). Our findings show that loss of PKCδ in primary salivary cells suppresses apoptosis induced from a variety of cell toxins that generate specific forms of cellular stress such as DNA damage, death receptor ligation, protein cross-linking, and inhibition of membrane trafficking pathways. This suggests that PKCδ functions at a point within the apoptotic pathway downstream of the initial sensor events, but upstream of the convergence of these diverse signaling pathways. To address this experimentally, we analyzed the abundance of phospho- and total p53, and the expression of p53 target genes, in primary parotid cells and parotid glands isolated from PKCδ<sup>−/−</sup> and PKCδ<sup>+/−</sup> mice. The p53 tumor suppressor is a critical regulator of DNA damage-induced apoptosis and its stability and basal transcriptional activity has been shown to be controled by PKCδ (20). Although both p53 protein and phospho-p53 are induced by etoposide and γ-irradiation (Fig. 4), no changes in p53 phosphorylation or protein abundance were observed in PKCδ<sup>−/−</sup> primary parotid cells or PKCδ<sup>−/−</sup> parotid glands. Etoposide increased the expression of the p53-regulated target genes, p21 and Bax, in both PKCδ<sup>−/−</sup> and PKCδ<sup>+/−</sup> cells, although the level of expression of these proteins in PKCδ<sup>−/−</sup> parotid cells appears to be slightly decreased. This may be explained by the fact that other signaling pathways, including members of the STAT family and the checkpoint control protein Rad9, contribute to the expression of these proteins (41–44). Whereas our studies suggest that PKCδ functions downstream of the DNA damage response, targets of PKCδ in apoptotic cells include proteins involved in the DNA damage response.

![Image](https://example.com/image.png)
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such as Rad9, c-Abl, DNA-dependent protein kinase, and p73β (12, 45–47). Thus, PKCα may contribute to the DNA damage response via p53 independent mechanisms.

We have previously proposed that PKCα functions by integrating stress signals induced from wide ranging stimuli at a point upstream of the mitochondria. To address this specifically, we analyzed mitochondrial events as well as downstream apoptotic events in PKCα−/− and PKCα−/− cells. Cytochrome c release is the initial event that we find to be defective in PKCα−/− cells, suggesting that PKCα is required for apoptotic signal transduction at, or prior to, the level of the mitochondria. This is in agreement with previous studies from our laboratory and others that have reported that PKCα is required for mitochondrial membrane depolarization and cytochrome c release in various cell systems (13, 17, 48–50). Likewise, we find that caspase-3 activation, PARP cleavage, and DNA fragmentation are all suppressed in PKCα−/− cells, supporting our previous observations using dominant negative kinases and chemical inhibitors of PKCα (11, 13). PARP cleavage and phosphatidylinerse exposure on the cell surface are also partially inhibited in PKCα−/− smooth muscle cells (17).

We hypothesize that activated PKCα interacts with downstream signaling cascades to regulate the apoptotic machinery. Indeed, in apoptotic cells phosphorylation of JNK, extracellular signal-regulated kinase, and other protein kinases, in addition to PKCα, may contribute to the DNA damage response via p53 independent mechanisms.

PKCα phosphorylates at serine 727 in both PKCα+/+ and PKCα−/− primary parotid cells, etoposide does not further increase phosphorylation at this site. Hence, basal phosphorylation of STAT1 at serine 727 may be sufficient to drive the expression of pro-apoptotic genes in these cells. In contrast to this, γ-irradiation induces similar levels of STAT1 serine 727 phosphorylation in PKCα+/+ and PKCα−/− parotid glands. This indicates that the precise control of STAT1 activation may be variably regulated in response to specific forms of DNA damage. In response to γ-irradiation, STAT1 activation may be required to drive the expression of specific subsets of apoptotic genes that are critical for mediating the response to this type of DNA stress. Furthermore, because loss of PKCα does not alter γ-irradiation-induced STAT1 activation, our data suggests that multiple protein kinases, in addition to PKCα, may control STAT1 activation in response to this agent.

Execution of the apoptotic pathway is presumably under the control of multiple regulatory circuits that combine to determine the magnitude of the apoptotic response. Hence, loss of PKCα does not ablate the apoptotic response, but consistently results in a 40–60% suppression of apoptosis both in vitro and in vivo. Interestingly, the magnitude of suppression of apoptosis in PKCα−/− primary parotid cells appears to be independent of the particular target, an observation we made previously for agents that induce the intrinsic pathway (13). This suggests that PKCα functions to regulate the apoptotic machinery rather than damage response pathways. Elucidation of how PKCα regulates the apoptotic machinery, as well as identification of its targets will be critical for the design of targeted therapeutic approaches to modulate the function of PKCα in apoptotic cells.

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