Inhibition of protein kinase C (PKC) activity in transformed cells and tumor cells containing activated p21\textsuperscript{RAS} results in apoptosis. To investigate the pro-apoptotic pathway induced by the p21\textsuperscript{RAS} oncoprotein, we first identified the specific PKC isozyme necessary to prevent apoptosis in the presence of activated p21\textsuperscript{RAS}. Dominant-negative mutants of PKC, short interfering RNA vectors, and PKC isozyme-specific chemical inhibitors directed against the PKC\(\delta\) isozyme demonstrated that PKC\(\delta\) plays a critical role in p21\textsuperscript{RAS}-mediated apoptosis. An activating p21\textsuperscript{RAS} mutation, or activation of the phosphatidylinositol 3-kinase (PI3K) Ras effector pathway, increased the levels of PKC\(\delta\) protein and activity in cells, whereas inhibition of p21\textsuperscript{RAS} activity decreased the expression of the PKC\(\delta\) protein. Activation of the Akt survival pathway by oncogenic Ras required PKC\(\delta\) activity. Akt activity was dramatically decreased after PKC\(\delta\) suppression in cells containing activated p21\textsuperscript{RAS}. Conversely, constitutively activated Akt rescued cells from apoptosis induced by PKC\(\delta\) inhibition. Collectively, these findings demonstrate that p21\textsuperscript{RAS}, through its downstream effector PI3K, induces PKC\(\delta\) expression and that this increase in PKC\(\delta\) activity, acting through Akt, is required for cell survival. The p21\textsuperscript{RAS} effector molecule responsible for the initiation of the apoptotic signal after suppression of PKC\(\delta\) activity was also determined to be PI3K. PI3K (p110\textsuperscript{CAAX}, where AA is alphatic amino acid) was sufficient for induction of apoptosis after PKC\(\delta\) inhibition. Thus, the same p21\textsuperscript{RAS} effector, PI3K, is responsible for delivering both a pro-apoptotic signal and a survival signal, the latter being mediated by PKC\(\delta\) and Akt. Selective suppression of PKC\(\delta\) activity and consequent induction of apoptosis is a potential strategy for targeting of tumor cells containing an activated p21\textsuperscript{RAS}. 

The ras oncogene family is among the most commonly mutated group of genes in human cancer. Its protein products code for three closely related p21\textsuperscript{RAS} proteins, including H-Ras, K-Ras, and N-Ras. p21\textsuperscript{RAS} proteins are localized in the inner plasma membrane, bind GDP and GTP, and possess an intrinsic GTPase activity. p21\textsuperscript{RAS} proteins function as plasma membrane-bound guanine nucleotide-binding proteins and act as molecular switches, thereby regulating signal transduction pathways for hormones, growth factors, and cytokine receptors (1). Several downstream effector proteins of p21\textsuperscript{RAS} have been identified that bind preferentially to p21\textsuperscript{RAS} in the GTP-bound state, including Raf, phosphatidylinositol 3-kinase (PI3K), and a family of GDP-GTP exchange factors for the Ras small GTPases (Ral-GDS). Raf proteins, which are proto-oncogene-encoded serine/threonine kinases, activate the MEK-ERK signaling pathway. PI3K activation results in the activation of the anti-apoptotic serine/threonine kinase Akt, among other molecules. Other p21\textsuperscript{RAS} targets include the GTPase-activating proteins, p120\textsuperscript{GAP} and neurofibromin (2). p21\textsuperscript{RAS} proteins were shown to influence proliferation, differentiation, transformation, and apoptosis by relaying mitogenic and growth signals from the membrane into the cytoplasm and the nucleus.

Specific point mutations localized in codons 12, 13, 59, 61, 63, 116, 117, and 146 can lock the p21\textsuperscript{RAS} protein in the active GTP-bound state and permit stimulation of downstream signaling cascades in the absence of extrinsic p21\textsuperscript{RAS} activation. Ras mutations can be found in human malignancies with an overall frequency of 20%. A particularly high incidence of ras gene mutations has been reported in malignant tumors of the pancreas (80–90%, K-ras), in colorectal carcinomas (30–60%, K-ras), in non-melanoma skin cancer (30–50%, H-ras), and in hematopoietic neoplasia of myeloid origin (18–30%, K- and N-ras) (3).

In addition to its central involvement in cell proliferation, recent studies indicate that the presence of an activated p21\textsuperscript{RAS} protein sensitizes transformed or malignant cells to apoptotic stimuli (4–9). Various signaling pathways have been proposed for this pro-apoptotic activity. Chou et al. (10) reported activated p21\textsuperscript{RAS} can cause apoptosis in transformed murine fibroblast cells through activation of the transcription factor NF\(\kappa\)B. Another study suggested that the p21\textsuperscript{RAS}/MAPK pathway is involved in Ras-specific apoptosis (11). The latter study also found that activating p21\textsuperscript{RAS} mutations increased colon cancer cell sensitivity to 5-fluorouracil-induced apoptosis through the negative regulation of gelsolin expression (12). Our previous studies demonstrated that suppression of protein kinase C (PKC) activity in cells expressing activated p21\textsuperscript{RAS} rapidly
induces apoptosis via FADD/caspase-8 signaling (9). We also found that reactive oxygen species are necessary as downstream effectors of the Ras-mediated apoptotic response to PKC inhibition (7).

There are at least 12 PKC isoforms that are classified into three subfamilies according to the structure of the N-terminal regulatory domain, which determines their sensitivity to the second messengers Ca\(^{2+}\) and diacylglycerol (13). Despite the high degree of homology, however, there is a surprising degree of nonredundancy. Thus, individual PKC isoforms mediate different and unique cellular functions in different cell types and different tissues (14). PKC\(\theta\) belongs to the subfamily of novel isoforms (PKC\(\theta\), PKC\(\epsilon\), PKC\(\theta\), and PKC\(\eta\)), which are insensitive to Ca\(^{2+}\). PKC\(\theta\) is widely regarded as having pro-apoptotic properties (15–17). Caspase activation mediates cleavage of PKC\(\alpha\), which results in release of the active catalytic domain (18, 19). In addition, PKC\(\theta\) activity is known to initiate a number of pro-apoptotic signals, such as increased expression and stability of p53 (20, 21), mitochondrial cytochrome c release (22, 23), and c-Ab1 activation (24). But recent studies have also shown that PKC\(\theta\) can protect cells against apoptotic stimuli under certain conditions (25). PKC\(\theta\) has been reported to regulate B-lymphocyte survival (26). Knock-out experiments have shown that PKC\(\theta\)-deficient mice have a severely deregulated immune system and develop autoimmune disease (27, 28). Thus, PKC\(\theta\) activation can serve as a pro-apoptotic signal, or as a survival signal, to determine cell fate.

This study examines the mechanism of apoptotic signaling induced by the p21\(^{RAS}\) oncprotein. We found that PKC\(\theta\) plays a critical role in suppressing p21\(^{RAS}\)-mediated apoptosis, and selective inhibition of this isozyme initiates apoptosis in cells containing activated p21\(^{RAS}\). Our data further demonstrate that unregulated Ras activity, through activation of the downstream effector PI3K, up-regulates PKC\(\theta\) expression and subsequently activates Akt, generating an anti-apoptotic effect and protecting against Ras-mediated apoptosis.

**EXPERIMENTAL PROCEDURES**

**Plasmids and Reagents**—The activated Ras effectors, Raf-1 (Raf-22W), PI3K (p110\(^{CAAX}\)), Rlf (Rlf-C\(^{CAAX}\)), and Raf-GDS (RaIA-28N, dominant-negative) were cloned into the pBabe puro vector (29), and the H-Ras effector loop mutants, Ser-35, Gly-37, and Cys-40 (double mutations), and H-Ras V12 single mutant (V12) were cloned into the pSG5 vector (30). These vectors were kindly provided by C. Counter (Duke University) and J. Downward (Medical Research Council, UK). The pEGFP-PKC\(\theta\)-KR vector (dominant-negative PKC\(\theta\)) (31) was kindly provided by Dr. D. Kufe (Dana-Farber Cancer Institute). pEF\(\alpha\)-a-Vak and pEF\(\alpha\)-c-Akt were kindly provided by Dr. Geoffrey Cooper (Boston University). The GST-NORE CT and FLAG-MST1 CT vectors were generously provided by Dr. J. Avruch (Massachusetts General Hospital).

The chemical inhibitors used in this study specific to PKC isoforms, PI3K, p21\(^{RAS}\), and MAPK are listed in Table 1. All inhibitors were dissolved in dimethyl sulfoxide for use, and their effects were measured relative to dimethyl sulfoxide (vehicle)-treated controls. The concentration of all inhibitors was optimized to produce greater than 90% inhibition of target molecule activity.

**Cell Culture and Treatment**—NIH/3T3 and Balb cell lines were obtained from the ATCC (Manassas, VA). NIH/3T3-Ras cells were produced by stable transfection of v-Harvey Ras and selected and maintained in 0.5 mg/ml of geneticin (Invitrogen). Ki-v-Ras-Balb (KBalb) cells were produced by stable infection of Balb with retroviral vector stocks containing v-Kirsten Ras, and were selected and maintained with geneticin. The human pancreatic tumor lines Hs 766s, BxPC-3, and MIA PaCa-2 were obtained from the ATCC. All cell lines were maintained in Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen) supplemented with 2 mM L-glutamine, 100 units/ml penicillin, and 100 \(\mu\)g/ml streptomycin (Invitrogen). Media were additionally supplemented with 10% donor calf serum (NIH/3T3, NIH/3T3-Ha-v-Ras, Balb/3T3, Ki-v-Ras-Balb, and Balb-myc) or 10% fetal bovine serum (BxPC-3, MIA PaCa-2). Cells were cultured at 37 °C and 5% CO\(_2\).

**Cell Proliferation Assay**—Cell proliferation was assessed using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Roche Applied Science). The number of viable cells growing in a single well on a 96-well microtiter plate was estimated by adding 10 \(\mu\)l of MTT solution (5 mg/ml in phosphate-buffered saline (PBS)). After 4 h of incubation at 37 °C, the stain was diluted with 100 \(\mu\)l of dimethyl sulfoxide. The optical densities were quantified at a test wavelength of 550 nm and a reference wavelength of 630 nm on a multiwell spectrophotometer.

**siRNA Knockdown of PKC\(\theta\) and PKC\(\alpha\)**—siRNA duplexes for PKC\(\theta\) (siRNAs) were obtained from Qiagen (Valencia, CA). The siRNA sequences for targeting PKC\(\theta\) were PKC\(\theta\)-siRNA-1 (5'-GAUGAAGGAGGCGCUAGTT-3') and PKC\(\theta\)-siRNA-2 (5'-GGCUGAGUUCGCGUGGA-CTT-3') (32). The corresponding scrambled siRNAs were used as negative control. These siRNA sequences were also cloned into the pRNA6.1-Neo vector with a GFP tag according to the manufacturer’s instructions (GenScript, Piscataway, NJ). siRNA for PKC\(\alpha\) (PKC-PKC\(\alpha\)-V6) was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Transfection of siRNA (oligonucleotide) was performed using 50 \(\mu\)M PKC\(\theta\) siRNA or the same amount of scrambled siRNA and Lipofectamine 2000 (Invitrogen), according to the manufacturer’s instructions. Transfection of plasmid-based siRNA vectors was carried out using the same method. PKC\(\theta\) protein levels were determined by immunoblot analysis.

**Assay of PKC\(\alpha\) and PKC\(\theta\) Kinase Activities**—PKC\(\alpha\) and PKC\(\theta\) activities were measured with an assay kit (Upstate Cell Signaling). After 2 days of treatment with inhibitors, cells were lysed in 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Triton X-100, 20 mM MgCl\(_2\), 5 mM EGTA, 1 mM orthovanadate, 50 \(\mu\)g/ml phenylmethylsulfonyl fluoride, and 3 \(\mu\)g/ml aprotinin. PKC\(\alpha\) and PKC\(\theta\) were immunoprecipitated from 200 \(\mu\)g of protein extracts as described above. Immunocomplexes were washed three times with the kinase buffer (20 mM Tris-HCl, 10 mM MgCl\(_2\), pH 7.5) and then incubated with a PKC-specific peptide substrate, \([\gamma\text{-}^{32}\text{P}]\text{ATP, and inhibitors of cAMP-dependent kinase and calmodulin kinase for 10 min at 30 °C.}^{32}\text{P} \) incorporated into the substrate was separated from residual


**RESULTS**

Selective Down-regulation of PKCδ Induces Apoptosis in Cells with Activated p21RAS—To begin determination of whether a specific PKC isozyme is responsible for suppression of Ras-mediated apoptosis, six isozyme-specific or nonspecific PKC inhibitors were used to suppress PKCa, PKCα/β, PKCβ1/2, PKCδ, or general PKC activity (Table 1). Optimal maximal effective concentrations of each inhibitor (>90% inhibition of relevant enzyme activity) were determined in pilot experiments (Fig. 1A or data not shown). The elevated activity of p21RAS in KBalb, MIA PaCa-2, and NIH/3T3-Ras cell lines compared with their counterpart cell lines (Balb, BxPc-3, and NIH/3T3, respectively) was confirmed by an activated p21RAS pulldown assay (Fig. 1B). Cells were treated for 48 h, and cell proliferation was quantitated by an MTT assay. Among the isoyme-specific inhibitors, rottlerin dramatically and specifically decreased the proliferation of both MIA PaCa-2 and NIH/3T3-Ras cells (both of which express a mutant, activated p21RAS protein), compared with the corresponding Hs766T cells and NIH/3T3 cells (which contain wild-type p21RAS) (Fig. 1C and D). The pan-PKC inhibitor bisindolylmaleimide I and the diacylglycerol antagonist 1-O-hexadecyl-2-O-methyl-rac-glycerol also strongly suppressed the growth of the cells containing activated p21RAS, consistent with our previous studies, while having no significant effect on most of the cell lines that contained a wild-type p21RAS (7, 8). In contrast, rottlerin modestly but consistently stimulated proliferation of those cell lines expressing wild-type p21RAS (Balb and Hs766T cells). Cytofluorometric analysis of propidium iodide-stained nuclei showed that rottlerin caused 24–35% apoptosis (manifested as hypodiploid nuclei) in activated Ras-containing cell lines after 60 h of treatment, whereas the matched cells containing wild-type p21RAS displayed only 12–14% apoptosis (Fig. 1E). PKCδ expression and activity were analyzed in the rottlerin-treated cells to confirm suppression of activity.

To confirm inhibition of PKCδ activity by rottlerin, both in vivo and in vitro kinase assays were performed. For the in vivo assays, cells were treated with rottlerin at 20 μM for 48 h and then lysed, and PKCδ was immunoprecipitated by a specific anti-PKCδ antibody. The incorporation of [γ-32P]ATP into a PKC substrate peptide (QKRPSQRSKYL) by the immunoprecipitates was quantitated. Exposure to rottlerin, at the concentrations used in the apoptosis studies described above, blocked greater than 85% of the PKCδ activity in all of the cell lines tested, except Balb (Fig. 2A). In contrast, rottlerin produced only a slight, statistically insignificant decrease in PKCα activity (Fig. 2B). For the in vitro kinase assay, rottlerin was added to immunopurified PKCδ protein and incubated for 4 h, and PKCδ kinase activities were assayed using the artificial substrate. Rottlerin directly inhibited 55–90% of PKCδ activity relative to the corresponding cells containing wild-type p21RAS (Fig. 2, A and B).

**TABLE 1**

| Name            | Concentration | Target  | Vendor           |
|-----------------|---------------|---------|------------------|
| FPT III         | 100 p21RAS    | PKCδ    | Calbiochem       |
| Rottlerin       | 20            | PKCδ    | Calbiochem       |
| LY294002        | 10            | PI3K    | Calbiochem       |
| PD98059         | 0.5 p21RAS    | MAPK    | Calbiochem       |
| Saliogel        | 5             | PKCδ    | Calbiochem       |
| G06976          | 0.3           | PKCα/β  | Calbiochem       |
| Bisindolylmaleimide I | 10 | pan-PKC | Calbiochem |
| LY245321        | 0.01 PKCβ1 and β2 | AK Scientific, Inc. |
| HMGα            | 120           | pan-PKC | Calbiochem       |

* FPT III is [E]2-oxo-2-[[3,7,11-trimethyl-2,6,10-dodecatrienyl]oxy]aminomethylphosphonic acid (2,2-dimethyl-1-oxopropoxy)methyl ester, Na; HMG is 1-O-hexade- cyl-2-O-methyl-rac-glycerol.
Similarly, total levels of PKCδ protein were elevated in the cell lines containing activated p21<sup>RAS</sup> relative to the corresponding lines containing wild-type p21<sup>RAS</sup> (Fig. 2D). The data obtained with the chemical inhibitors of PKC isozymes are consistent with PKCδ being the relevant PKC target for Ras-mediated apoptosis.

Unexpectedly, exposure to rottlerin for 48 h suppressed PKCδ protein levels in all cell lines tested (Fig. 2D), whereas the levels of other PKC isozymes, including PKCa, -β, -η, and -θ, were not changed by rottlerin (Fig. 2E). The magnitude of suppression of PKCδ levels by rottlerin in the cells containing activated p21<sup>RAS</sup> (3–5-fold) approached the magnitude of the suppression of PKCδ activity in these cells by rottlerin (6–10-fold). Thus, although we can demonstrate direct inhibition of PKCδ activity by rottlerin in <i>in vitro</i> assays, the marked suppression in PKCδ activity observed after 48 h <i>in vivo</i> may be because of suppression of isozyme protein levels as well as direct inhibition of enzyme activity.

As the specificity of chemical kinase inhibitors is never absolute, we employed two specific genetic techniques to suppress PKCδ activity. At 48 h after transfection of each of two PKCδ-specific hairpin vectors targeted at different PKCδ sequences into matched pairs of cell lines, BxPc-3/MIA PaCa-2 and NIH/3T3/NIH/3T3-Ras, immunoblot analysis demonstrated that expression of PKCδ protein was significantly diminished by transfection with the PKCδ-siRNA-2 vector. In contrast, the PKCδ-siRNA-1 vector did not produce significant knockdown of PKCδ protein. After 60 h of treatment with rottlerin, cells were fixed and stained with propidium iodide, and the apoptotic (hypodiploid) fractions (M1 fractions) were evaluated by flow cytometry. Counts refers to cell numbers; FL2-H is a log scale of fluorescence channels. Similar results were observed in three independent experiments. Bis-1, bisindolylmaleimide I; HMG, 1-O-hexadecyl-2-O-methyl-rac-glycerol.
Cells that took up the vector DNA were identified by green fluorescence. TUNEL-positive cells stained red. Superimposition displayed transfected, apoptotic cells as yellow (Fig. 3, C and D). For cells transfected with the PKCδ hairpin vector, 40–50% cells were undergoing apoptosis at the 48-h time point, whereas cells transfected with the control scrambled hairpin vector displayed a frequency of apoptosis of less than 10% (Fig. 3H).

Similar results were obtained when a dominant-negative, kinase-dead PKCδ mutant protein was used as an alternative method of blocking specifically PKCδ activity in cells expressing an activated p21RAS or wild-type p21RAS (Fig. 3, E and F). Transfection of NIH/3T3-Ras cells with the dominant-negative PKCδ vector produced a 30–40% fraction of cells with a hypo-diploid (apoptotic) DNA content, compared with a less than 5–10% apoptotic fraction in cells expressing a wild-type p21RAS (Fig. 3H). Transfection of the empty vector as a control generated no significant apoptosis above background levels. The induction of apoptosis by the competitive expression of dominant-negative PKCδ with a single-base mutation, rendering it catalytically inactive, also demonstrates that it is the kinase activity of PKCδ that is required for the survival of cells expressing activated p21RAS, rather than a noncatalytic function of the molecule. We studied the effects of knockdown of PKCα (via expression of a PKCα-siRNA) as a specificity control. Forty-eight hours after transfection of PKD-PKCαV6, the levels of PKCα protein were significantly decreased in both NIH/3T3 and NIH/3T3-Ras cells (Fig. 3B). Analysis of apoptosis demonstrated that PKCα inhibition by PKCα-siRNA induced apoptosis in ~20% of both NIH/3T3 and NIH/3T3-Ras cells, with no selective toxicity for cells containing an activated p21RAS (Fig. 3, G and H). This finding was consistent with results of the MTT.
assay (Fig. 1, C and D). Collectively, the data demonstrate that the PKCδ isozyme plays a critical survival role in p21RAS-induced apoptosis.

**PKCδ Inhibition Induces Mitochondrial Apoptotic Pathways in Cells Expressing an Activated p21RAS—**To further characterize the molecular mechanisms of p21RAS-mediated apoptotic pathways, we investigated the influence of mitochondrial apoptotic signaling when PKCδ activity is suppressed by assay of caspase-3 and caspase-9 activation. Immunoblot analysis demonstrated that exposure to rottlerin activated both pro-caspase-3 and pro-caspase-9 exclusively in the cells expressing activated p21RAS (Fig. 4, A and B). For caspase-3, the full-length protein (35 kDa) and the large cleavage fragment (17 kDa) were detected (Fig. 4A); three activation fragments from caspase-9 zymogen (35, 17, and 10 kDa) were detected (Fig. 4B).

The PI3K Ras Effector Pathway Is Sufficient to Sensitize Cells to Apoptosis by PKCδ Inhibition—Although previous studies have demonstrated that either constitutive expression of activated p21RAS or acute increases in endogenous p21RAS activity stimulate apoptosis following inhibition of PKC activity in multiple types and lineages of cells, the roles of specific p21RAS downstream effectors in the process have never been determined. In general, three major effector pathways activated by Ras have been defined as follows: Raf1/MAPK, Ral-GDS, and PI3K. To begin to address this question, p21RAS effector loop mutants, consisting of the activating Ras mutation (V12) and a second mutation (Ser-35, Gly-37, or Cys-40) were employed. The three RasV12 mutants (Ser-35, Gly-37, or Cys-40) differ in their ability to bind to p21RAS effectors (Raf, Ral-GEFs, and the p110 subunit of PI3K, respectively) (29). Cells were treated with 20 μM rottlerin for 60 h and subjected to flow cytometric analysis. All three p21RAS downstream effector-loop mutants stimulated apoptosis to some extent after inhibition of PKCδ, although expression of the C40 mutant consistently generated the greatest amount of apoptosis (data not shown).

The Ras effector loop mutants are not completely specific in their activation of a single Ras effector pathway, and each activates all three pathways to some degree. To more clearly identify the downstream effector of p21RAS relevant to Ras-mediated apoptosis, we activated single effector pathways using expression vectors for activated PI3K (p110CAAX), Raf (Raf-22W), and Raf-GEF (RIF-C) into NIH/3T3 cells. The dominant-negative RalA-22N vector was used as control. Constitutive activation of the PI3K pathway (transfection of p110CAAX) was capable of inducing apoptosis after PKCδ inhibition (apoptotic frequency 38.22%). In contrast, the other Ras effectors

**FIGURE 3. Effect of PKCδ inhibition on the viability of cells expressing activated p21RAS.** A, immunoblot analysis of PKCδ, PKCa, or β-actin expression in NIH/3T3-Ras and MIA PaCa-2 cells transfected with PKCδ siRNA-1 or -2. A total of 50 nM of PKCδ siRNAs was used. Transfections with a scrambled siRNA or with vehicle alone were used as negative controls. In all experiments, transfections with scrambled siRNA or with vehicle alone yielded identical results, and in subsequent experiments only the scrambled siRNA transfection control is shown. B, knockdown activity of PKCδ siRNA or scrambled siRNA (control) on NIH/3T3 (lanes 1 and 2) and NIH/3T3-Ras cells (lane 3) evaluated by immunoblotting cell lysates for PKCδ or β-actin. NIH/3T3-Ras (C) and MIA PaCa-2 (D) were transfected with pRNA-U6-1.1-GFP scrambled siRNA hairpin vector (control) or pRNA-U6-1.1-GFP-PKCδ siRNA-2 hairpin vector. After 72 h, apoptosis was detected by TUNEL assay. NIH/3T3 and BxPc-3 were used as control cell lines, respectively. Shown are ×200 magnifications. MIA PaCa-2 cells (E) and NIH/3T3-Ras cells (F) were transfected with a dominant-negative, kinase-dead PKCδ vector. After 72 h, apoptosis was detected by TUNEL assay. BxPc-3 and NIH/3T3 were used as control cell lines (respectively). Shown are ×200 magnifications. G, NIH/3T3 and NIH/3T3-Ras cells were co-transfected with PKD-PKCδ siRNA and pEGFP or scrambled siRNA and pEGFP. After 72 h, apoptotic cells were assayed by TUNEL reagent. H, quantitation of apoptotic cells in the transfected populations, with error bars indicating S.D. Top panel, PKCδ siRNA; middle panel, PKCδ-KR; bottom panel, PKCa siRNA. Data shown are representative of at least three independent experiments.

Raf and Raf-GEF induced little apoptosis in response to PKCδ suppression (Fig. 4A).

To independently confirm that the downstream p21RAS effector PI3K plays a critical role in Ras-mediated apoptosis, a PI3K-specific inhibitor (LY294002) and a MAPK inhibitor (PD98095) were employed. In dose-ranging studies, we evaluated concentrations of LY294002 and PD98095 from 1.0 to 60 μM, assaying for efficiency of target enzyme inhibition and for toxicity. Even at 60 μM concentrations of LY294002 or PD98095, only modest and nonstatistically significant levels of apoptosis were observed. At 10 μM concentrations, there were no signs of toxicity or apoptosis (data not shown). Their effectiveness in suppressing the relevant target pathways at 10 μM concentrations was confirmed by immunoblot (Fig. 5, B and C). An NIH/3T3 cell line stably expressing p110 CAAX was pretreated with LY294002 (10 μM) for 30 min, and then rottlerin

**FIGURE 4. Inhibition of PKCδ induces the cleavage of caspase-3 and caspase-9 in cells expressing activated p21RAS.** All the cells were treated with 20 μM rottlerin for 60 h. Thereafter, cells were harvested, and cell lysates were prepared and subjected to immunoblot analysis to detect the levels of caspase-3/cleaved caspase-3 and caspase-9/cleaved caspase-9. A representative blot from duplicate experiments, producing similar results, is shown.
(20 μM) was added for 60 h. In the presence of the PI3K inhibitor, the apoptosis induced by rottlerin decreased nearly 50% (Fig. 5D). Similarly, in KBalb cells, LY294002 blocked 50% of the apoptosis induced by PKCδ inhibition (Fig. 5E). In cells that contained an activated p21RAS, but not those with activation of the single PI3K effector pathway, the MAPK inhibitor PD98095 also suppressed p21RAS-dependent apoptosis to some extent, but to a substantially lesser degree than did PI3K inhibition. Collectively, these data demonstrate that the pro-apoptotic signal manifested during Ras-mediated apoptosis following PKCδ inhibition is mediated mainly through the downstream effector PI3K, although the MAPK effector pathway may contribute to some extent in this process.

The Anti-apoptotic Akt Ras Effector Pathway Is Regulated by PKCδ—One direct downstream target of the PI3K pathway are the members of the Akt kinase family, which have well characterized pro-survival and anti-apoptotic activities, mediated through regulation of the mitochondrial apoptotic machinery and the expression of genes involved in this process. To determine the role of Akt in p21RAS-mediated apoptosis, we first examined Akt levels and activity in three paired cell lines with wild-type or mutant Ras alleles. These cells were treated with rottlerin for 48 h or left untreated. Akt activity, as assessed by quantitation of serine 473-phosphorylated Akt, was down-regulated 50% by rottlerin in all three cell lines expressing activated Ras (Fig. 6A). In contrast, no significant changes in levels of p-Akt were induced in the cell lines expressing wild-type Ras. Exposure to rottlerin also suppressed total Akt levels to a modest extent in some cell lines, but this effect was independent of the presence of activated p21RAS. If the suppression of Akt activity by PKCδ inhibition in activated Ras-containing cells was responsible for the resulting apoptosis, enforced expression of activated Akt should be able to effect rescue from apoptosis. MIA Paca-2 and NIH/3T3-Ras were co-transfected with a PKCδ hairpin vector (which efficiently suppressed PKCδ levels in these experiments by at least 85%, see Fig. 3A for an example) and a constitutively activated Akt (vAkt) expression vector, or a cAkt expression vector as a control. Akt activity in these...
PKCδ in Balb cells as it was in Balb cells containing a mutated, activated p21^RAS (KBalb cells) (e.g. 62% suppression in Balb compared with 83% suppression in KBalb). Accordingly, the effects of PKCδ down-regulation on p-AKT levels cannot be directly compared between Balb and KBalb cells in these studies. Collectively, these studies indicate that activation of Akt is required for survival of cells expressing activated Ras, that PKCδ is required for the activation of Akt by Ras, and that induction of Ras-mediated apoptosis by suppression of PKCδ is effected through interference with this anti-apoptotic Ras effector pathway.

A pro-apoptotic p21^RAS effector, NORE1 (33), which binds to Ras-GTP and is a member of the RASS family of tumor suppressors, has been described by Eckfeld et al. (34). To determine whether activation of this Ras effector pathway plays any role in Ras-mediated apoptosis induced by suppression of PKCδ, we employed vectors expressing the fragment of NORE1, which binds to its apoptotic effector MST1 (amino acids 358–413), or the C-terminal noncatalytic segment of MST1 (amino acids 307–487) as glutathione S-transferase fusion peptides, each of which has been demonstrated to block formation of the NOR-MST1 apoptotic complex (33). Neither peptide attenuated the apoptosis induced by suppression of PKCδ (data not shown).

p21^RAS Up-regulates PKCδ Protein Levels Post-transcriptionally—We observed that cell lines expressing an activated p21^RAS invariably expressed substantially more PKCδ protein than did their wild-type p21^RAS–expressing counterparts (Fig. 7A), suggesting that p21^RAS activity may up-regulate PKCδ protein expression as well as activity (Fig. 2, D and E). To test this hypothesis, NIH/3T3 cells were transfected with pSG5-H-(V12)Ras, and PKCδ protein levels were assayed over time. PKCδ protein levels increased rapidly after transfection, reaching peak levels at 18 h (Fig. 7B). In contrast, transfection with the empty pSG5 hairpin vectors had no effect on PKCδ protein levels (33). Neither peptide attenuated the suppression of PKCδ protein expression by suppression of PKCδ (data not shown).

PKCδ and Activated p21^RAS

FIGURE 6. Regulation of AKT activity by PKCδ. A, treatment with rottlerin reduces Akt activity in cells expressing activated Ras. All cells were treated with 20 μM rottlerin for 60 h in 96-well plates. Total Akt and p-Akt levels were assayed using a SuperArray Case ELISA kit (*, p < 0.005), +, treated with 20 μM rottlerin; −, treated with vehicle (control). B, pAkt protein levels in MIA PaCa-2 and NIH/3T3-Ras cells, assayed by immunoblotting with an antibody specific for phosphorylated serine 473 Akt, or for total Akt, or for pAkt expression was dramatically decreased when p21^RAS expression was knocked down by PKCδ siRNA in KBalb cells (Fig. 6F). For reasons not yet elucidated, we found PKCδ siRNA was consistently not as effective in down-regulating PKCδ in Balb cells as it was in Balb cells containing a mutated, activated p21^RAS (KBalb cells) (e.g. 62% suppression in Balb compared with 83% suppression in KBalb). Accordingly, the effects of PKCδ down-regulation on p-AKT levels cannot be directly compared between Balb and KBalb cells in these studies. Collectively, these studies indicate that activation of Akt is required for survival of cells expressing activated Ras, that PKCδ is required for the activation of Akt by Ras, and that induction of Ras-mediated apoptosis by suppression of PKCδ is effected through interference with this anti-apoptotic Ras effector pathway.

A pro-apoptotic p21^RAS effector, NORE1 (33), which binds to Ras-GTP and is a member of the RASS family of tumor suppressors, has been described by Eckfeld et al. (34). To determine whether activation of this Ras effector pathway plays any role in Ras-mediated apoptosis induced by suppression of PKCδ, we employed vectors expressing the fragment of NORE1, which binds to its apoptotic effector MST1 (amino acids 358–413), or the C-terminal noncatalytic segment of MST1 (amino acids 307–487) as glutathione S-transferase fusion peptides, each of which has been demonstrated to block formation of the NOR-MST1 apoptotic complex (33). Neither peptide attenuated the apoptosis induced by suppression of PKCδ (data not shown).

p21^RAS Up-regulates PKCδ Protein Levels Post-transcriptionally—We observed that cell lines expressing an activated p21^RAS invariably expressed substantially more PKCδ protein than did their wild-type p21^RAS–expressing counterparts (Fig. 7A), suggesting that p21^RAS activity may up-regulate PKCδ protein expression as well as activity (Fig. 2, D and E). To test this hypothesis, NIH/3T3 cells were transfected with pSG5-H-(V12)Ras, and PKCδ protein levels were assayed over time. PKCδ protein levels increased rapidly after transfection, reaching peak levels at 18 h (Fig. 7B). In contrast, transfection with the empty pSG5 vector had no effect on PKCδ protein levels. Conversely, when p21^RAS activity, or PI3K activity, in KBalb and NIH/3T3-Ras cells was inhibited by the Ras inhibitor (E,E)-[2-oxo-2-[(3,7,11-trimethyl-2,6,10-dodecatrienyloxy)amino]ethyl]phosphonic acid, (2,2-dimethyl-1-oxoproxy)methyl ester, Na or the PI3K inhibitor LY294002, respectively, PKCδ protein
PKCδ and Activated p21^{RAS}

levels fell (Fig. 7C). This regulation of PKCδ by p21^{RAS} is not at the level of transcription, as PKCδ transcript levels, assessed by quantitative RT-PCR, did not vary as a function of p21^{RAS} activity (data not shown).

To determine which p21^{RAS} effector pathway mediated the up-regulation of PKCδ protein, p110^{CAAX}, Raf-22w, RIF CAAAX, and RalA-28N expression vectors were each stably transfectioned into two different cell lines, and PKCδ protein levels were quantitated (Fig. 8A). The p110^{CAAX} expression vector was consistently the most potent inducer of PKCδ expression in both Balb and NIH/3T3 cells, although activation of each of the other two effector pathways could also induce PKCδ protein expression to a variable extent. In agreement, the C40 p21^{RAS} effector loop mutant, which activates predominantly the PI3K pathway, was the most potent of the effector mutants for induction of PKCδ protein levels (Fig. 8B). The expression of other isoforms of PKC (\(-\alpha, -\theta,\) \(-\beta,\) and tubulin were not changed by expression of p21^{RAS} or PI3K (Fig. 8, C and D). Similarly, levels of p53, cyclin D1, and p16 were not changed by expression of the p21^{RAS} or PI3K (data not shown).

**DISCUSSION**

The p21^{RAS} family members include critical molecular switches transducing signals to diverse downstream pathways, ultimately controlling such processes as proliferation, cytoskeletal integrity, apoptosis, cell adhesion, and cell migration (35–38). p21^{RAS} and Ras-related proteins are frequently deregulated in cancers, leading to invasion and metastasis and enhancement of survival by activation of anti-apoptotic pathways. Paradoxically, several studies have demonstrated that enforced, high level expression of oncogenic p21^{RAS} can induce a permanent growth arrest in normal cells, mimicking natural senescence (39, 40). Activation of the Raf-1/MEK/p38MAPK pathway is thought to be essential for oncogenic p21^{RAS}-induced senescence (40, 41). Additionally, inactivation of the ARF/p53 tumor suppressor pathway in mouse fibroblasts and skin keratinocytes, or inactivation of the p16/Rb tumor suppressor pathway in human fibroblasts, can bypass p21^{RAS}-induced senescence, suggesting that cellular fate resulting from oncogenic p21^{RAS} signaling is dependent upon the cellular context and the integration of tumor suppressor signals (39, 42). Our laboratory and others have established p21^{RAS} as a modulator of apoptosis in transformed cells and malignant cells and also in normal cells. Through FADD, caspase 8, and downstream effector reactive oxygen species, p21^{RAS} sensitizes cells to apoptosis induced by PKC inhibition (7, 43, 44). However, the specific molecular mechanism by which oncogenic p21^{RAS} mediates apoptosis in...
the setting of inhibition of PKC activity remains incompletely understood. As different PKC isozymes may have opposing functions with respect to cellular proliferation, differentiation, and apoptosis, a more complete understanding of this process required identification of the specific PKC isozyme required for survival of cells expressing activated p21RAS, along with elucidation of the specific p21RAS downstream effectors evoking the apoptotic outcome.

In this study, we demonstrate that PKCδ activity is required to prevent the induction of apoptosis in cells expressing activated p21RAS. It is noteworthy that p21RAS activity, and in particular activation of the PI3K pathway, up-regulates PKCδ protein levels, thus positively reinforcing an anti-apoptotic, protective response to p21RAS dysregulation in the cell. Conversely, when this induction is prevented by siRNA knockdown of PKCδ, programmed cell death is initiated. Pro-apoptotic activity engendered by activated PKCδ has been described in a number of other systems (45), and isoform differences in the proapoptotic effects of Ras have been reported, with K-Ras sensitizing cells to γ-irradiation-induced apoptosis but H-Ras exerting a protective effect (46). Activated K-Ras has been reported to sensitize cells to pro-apoptotic effects of PKC agonists through phosphorylation of serine residue 181 of p21RAS and its translocation to intracellular membranes (47). K-Ras, but not H-Ras, was reported to bind to and potentially inactivate or sequester the anti-apoptotic proteins Bcl-2 and Bcl-XL (48). In contrast to these observations of isotype-specific differences in p21RAS functions, we have previously reported, and confirm herein, that both activated K-Ras and H-Ras sensitize cells to apoptosis induced by inhibition of PKCδ activity, which prevents their obligate activation of the Akt survival pathway.

PKCδ has been reported to both positively and negatively regulate apoptotic programs (49–52). These findings have generated conflicting hypotheses as to the role of PKCδ in the control of cell proliferation and survival. The normal phenotype of PKCδ-null mice demonstrates that PKCδ is not required for appropriate control of cell proliferation during normal development (28, 53). In contrast, PKCδ may be recruited during cellular transformation and become necessary for one or more components of the malignant phenotype. Inhibition of PKCδ was reported to suppress the metastatic potential of breast cancer cells (54) and to reduced their survival (55). Similar results were reported using non-small cell lung cancer cells (56). Our findings support this hypothesis. We find that PKCδ functions as a survival signal in a variety of cells with dysregulated activation of p21RAS. PKCδ expression is up-regulated in response to p21RAS activity, primarily through PI3K activation, and is required for the survival of these cell lines. However, PKCδ is not required for the survival or proliferation of the counterparts of these cell lines containing wild-type p21RAS, whether or not they are transformed, and indeed suppression of PKCδ actually leads to a small but reproducible increase in the proliferation or normal cells.

Activated Akt is a well established survival factor, exerting anti-apoptotic activity by suppressing the release of cytochrome c from the mitochondria (57). Activated Akt also inactivates the pro-apoptotic factors BAD and procaspase-9 by direct phosphorylation (58). A number of reports have suggested relationships between PKCδ activity and Akt activation. One recent study showed that PKCδ contributes to the phosphorylation of Ebp1 in PC12 cells (59). Phosphorylation of Ebp1 is required for its association/interaction with active nuclear Akt, and the Akt-Ebp1 complex mediates an anti-apoptotic effect in intact cells. A very recent study suggested that both PKCδ and PKCe can negatively regulate Akt activity in mouse keratinocytes (60), although this study utilized only chemical inhibitors lacking absolute specificity. Whereas PKCα has been shown to promote the dephosphorylation and inactivation of Akt in prostate cancer cells (61), another study demonstrated that phosphorylation of PKCδ protects glioma cells from TRAIL-induced apoptosis by activation of Akt (62). In our study, we prove that Akt activity can be regulated by PKCδ (Fig. 6F) and that cells in which PKCδ has been selectively suppressed by treatment with PKCδ-siRNA can be rescued from apoptosis by enforced expression of a constitutively activated Akt (Fig. 6C). These data suggest that oncogenic p21RAS protein, through PI3K and PKCδ, induces Akt activity, initiating an anti-apoptotic signaling cascade that is required for their survival. However, whether PKCδ alone is the major regulator of Akt activity under all conditions remains to be elucidated, as the levels of p-Akt were only slightly changed after knocking down PKCδ in cells containing wild-type (normal) Ras (Fig. 6F).

There are two possible explanations for the differences observed on PKCδ actions of Akt between normal cells and those containing activated p21RAS as follows: the first is the relative differences in efficiency of transient transfection, and the second is that the robust regulation of Akt activity by PKCδ requires the involvement of an activated p21RAS protein.

It is noteworthy that the PI3K effector pathway, in addition to generating the anti-apoptotic signal mediated through PKCδ and activation of Akt (and up-regulation of PKCδ levels and activity), is also responsible for the pro-apoptotic signal delivered in Ras-transformed cells, which is uncovered and made manifest by inhibition of PKCδ. We show that isolated activation of the PI3K pathway is sufficient to render cells susceptible Ras-mediated apoptosis and, conversely, that inhibition of the PI3K pathway is sufficient to protect cells from apoptosis initiated by suppression of PKCδ.

In conclusion, this study significantly extends our understanding of the mechanism underlying p21RAS-mediated apoptosis by identifying the molecules required to redirect p21RAS signaling from a proliferative/transforming outcome toward instead an apoptotic fate. Furthermore, elucidation of the particular PKC isozyme necessary for survival of cells transformed by p21RAS suggests that selective suppression of PKCδ activity, and the consequent induction of apoptosis, is a potential strategy for targeting of tumor cells containing an activated p21RAS GTPase.

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