Ras Triggers Ataxia-telangiectasia-mutated and Rad-3-related Activation and Apoptosis through Sustained Mitogenic Signaling*

Aphrothiti J. Fikaris, Aurélia E. Lewis†, Adili Abulaiti, Oxana M. Tsygankova, and Judy L. Meinkoth‡

From the Department of Pharmacology, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104

Genetic evidence indicates that Ras plays a critical role in the initiation and progression of human thyroid tumors. Paradoxically, acute expression of activated Ras in normal rat thyroid cells induced deregulated cell cycle progression and apoptosis. We investigated whether cell cycle progression was required for Ras-stimulated apoptosis. Ras increased CDK-2 activity following its introduction into quiescent cells. Apoptotic cells exhibited a sustained increase in CDK-2 activity, accompanied by the loss of CDK-2-associated p27. Blockade of Ras-induced CDK-2 activity and S phase entry via overexpression of p27 inhibited apoptosis. Inactivation of the retinoblastoma protein in quiescent cells through expression of HPV-E7 stimulated cell cycle progression and apoptosis, indicating that deregulated cell cycle progression is sufficient to induce apoptosis. Ras failed to induce G1 phase growth arrest in normal rat thyroid cells. Rather, Ras-expressing thyroid cells progressed into S and G2 phases and evoked a checkpoint response characterized by the activation of ATR. Ras-stimulated ATR activity, as evidenced by Chk1 and p53 phosphorylation, was blocked by p27, suggesting that cell cycle progression triggers checkpoint activation, likely as a consequence of replication stress. These data reveal that Ras is capable of inducing a DNA damage response with characteristics similar to those reported in precancerous lesions. Our findings also suggest that the frequent mutational activation of Ras in thyroid tumors reflects the ability of Ras-expressing cells to bypass checkpoints and evade apoptosis rather than to simply increase proliferative potential.

Ras is perhaps best known for its ability to induce malignant transformation through the stimulation of cell proliferation, invasion, metastasis, angiogenesis, genomic instability, and cell survival (1–3). Recent evidence has emerged that Ras also plays a prominent role in triggering apoptosis (4). Although several reports have identified downstream intermediates through which Ras stimulates apoptosis in acute settings (5–8), few studies have assessed whether Ras-induced apoptosis is a consequence of deregulated cell cycle progression, particularly in cell types where Ras elicits a mitogenic response.

Point mutations (most commonly codons 12 and 61) in H-, K-, and N-Ras are frequent in human thyroid tumors (9–13). Ras is believed to play roles in the initiation and progression of thyroid tumors, based on the high frequency of Ras mutations in benign adenomas and in advanced tumors (9, 14–17). Interestingly, in contrast to the induction of G1 phase growth arrest observed in primary human diploid or murine embryonic fibroblasts (18), retroviral-mediated expression of activated Ras stimulates sustained proliferation in primary human thyrocytes, cells that normally fail to proliferate in vitro (19, 20). The molecular mechanism through which Ras stimulates proliferation rather than growth arrest remains to be established but may be related to the inability of Ras to increase the expression of p16INK4a in human thyroid cells (20).

Normal rat thyroid cells share many features with human thyrocytes, including thyroid-stimulating hormone-dependent cell proliferation and differentiation. Continuous lines of rat thyroid cells have been used extensively to model Ras transformation in thyroid cells (21). Inducible expression of activated Ras in rat thyroid PC-CL3 cells (22) or infection of Wistar rat thyroid cells with an adenovirus expressing activated Ras (23) stimulated cell cycle progression as expected but also induced apoptosis, a result difficult to reconcile with the high frequency of Ras mutations in human thyroid tumors. The ability of Ras to acutely stimulate cell proliferation and cell death in the same cell is reminiscent of the effects of the Myc oncoprotein (24). Although unrestrained proliferation is a potent inducer of apoptotic and DNA damage cascades (25–27), Myc stimulates proliferation and apoptosis through distinct signaling pathways (28). In this report, we investigated whether Ras independently regulates cell cycle progression and apoptosis. Strikingly, unlike the situation for Myc, Ras engages the apoptotic pathway through effects on the cell cycle.

EXPERIMENTAL PROCEDURES

Cell Culture and Adenoviruses—Wistar rat thyroid cells were cultured in Coon’s modified Ham’s F-12 medium supplemented with bovine thyroid-stimulating hormone (1 milliunit/ml), insulin (10 μg/ml), calf serum (5%), and transferrin (5 μg/ml) as previously described (29). The cells were grown to 70% confluence and rendered quiescent by starvation in hormone, growth factor, and serum-free basal medium for 48 h. The quiescent cells were mock infected (given basal medium)
Ras Induces Cell Cycle-dependent Apoptosis

or infected with adenoviruses for 16 h, the virus was removed, and the cells were maintained in basal medium for varying times. The morning after infection (16 h after the addition of virus) is referred to as day 1 post-infection, and the cells were collected in 24-h intervals thereafter (days 1–4). Where indicated QVD3 (number 551476; Calbiochem, La Jolla, CA) was added at the time of infection and on day 1 post-infection.

Replication-defective adenoviruses expressing HA-tagged human H-Ras (cellular Ras or RasG12, activated Ras or RasV12, activated Ras impaired in membrane association or RasV12A186, and a Ras effector domain mutant, RasV12G37) were constructed using the AdEasy Vector System (Q-Biogene, Carlsbad, CA). HA-RasV12A186 was created by mutating cysteine 186 to alanine in the CAAX box of RasV12 using the QuikChange site-directed mutagenesis kit (Stratagene). The pShuttle vector containing an HA tag was kindly provided by Dr. M. Kazanietz (Department of Pharmacology, University of Pennsylvania). Ras cDNAs were amplified from pDCR-Ras vectors kindly provided by Dr. M. White (Department of Cell Biology, University of Texas Southwestern Medical Center) and cloned into EcoRV and Xhol sites of HA-pShuttle. Following recombination with adenoviral DNA in BJ5183 cells, recombinants were transfected into QBI-293A cells, plaque-purified, and propagated as per the manufacturer. Titrations were performed in Wistar rat thyroid cells to derive conditions sufficient to infect >90% of the cells. RasV12 (or RasL61) was infected at 5000 particles/cell (unless otherwise noted), and the other Ras mutants were infected at doses that resulted in equal levels of Ras expression. The RasL61 adenovirus was a kind gift from Dr. J. Nevins (Howard Hughes Medical Institute, Duke University) and used as previously described (23). The p27 adenovirus was a generous gift from Dr. P. Seth (Department of Medicine, Northwestern University) and used at 10,000–20,000 particles/cell. The HPV-E7 adenovirus was a kind gift from Dr. M. Herlyn (Wistar Institute, Philadelphia, PA) and used at 5000 particles/cell.

Immunostaining—Cells plated on coverslips were fixed in 3.7% formaldehyde in phosphate-buffered saline for 20 min, permeabilized in 0.5% Nonidet P-40, and incubated with primary and secondary antibodies for 1 h at 37 °C. The nuclei were stained with DAPI (Sigma). The images were captured using a Zeiss Axioskop microscope fitted with a Hamamatsu ORCA-ER digital camera and analyzed using Zeiss-Axiovision 4.2 software. Equal exposure times and magnifications were used for images within an experiment. At least 200 cells/condition/experiment were counted from random fields. Immunostaining of HA-Ras was assessed using HA ascites generously provided by Dr. J. Field (Department of Pharmacology, University of Pennsylvania). Cleaved caspase-3 was examined using the Asp-175 antibody that reacts only with the active, cleaved form of caspase-3 (Cell Signaling, Beverly, MA; number 9661). The percentage of active caspase-3-positive cells was determined by counting the number of DAPI-positive nuclei that reacted with the active caspase-3 antibody. The cells were scored in a blinded fashion by two observers.

DNA Laddering—Cells harvested in 10 mM Tris (pH 8.0), 1 mM EDTA, 0.2% Triton X-100 were incubated on ice for 10 min. A sample of total DNA was recovered, and the remaining cell homogenate was pelleted at 14,000 × g for 15 min at 4 °C. The supernatant was removed, and low molecular weight DNA was isolated as described in (30). Low molecular weight DNA was analyzed by electrophoresis on 2% agarose gels and imaged using GelDoc XR and Quantity One 4.5.2 software (Bio-Rad).

Western Blotting—The cells were disrupted in radioimmunoprecipitation assay buffer plus protease inhibitors and clarified by centrifugation, and protein was quantified by Bio-Rad DC Protein Assay as in Ref. 31. Equal amounts of protein were electrophoresed on 12% SDS-PAGE gels. The membranes were probed with primary antibodies overnight at 4 °C and with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 1 h at room temperature. The proteins were detected via chemiluminescence using the FUJI LAS-3000 system. Caspase-3 (number 9662; used for all Western blot experiments), phospho-Chk1 (Ser345) (number 2341), phospho-p53 (Ser15) (number 9284), phospho-Rb (Ser307–311) (number 9308), cyclin B1 (number 4135), and phosphor-ERK (Thr202/Tyr204) (number 9101) antibodies were from Cell Signaling (Beverly, MA). CDK-2 (M2, sc-163), cyclin A (C-19, sc-596), HPV16-E7 (ED17, sc-6981), actin (C-11, sc-1615), and ERK2 (C-14, sc-154) antibodies were from Santa Cruz Biotechnology, Inc. The p27 antibody (number 610241) was from BD Pharmingen (San Jose, CA). The pan-Ras antibody (Ab-4) (number OP41) was from Calbiochem (La Jolla, CA).

CDK-2 Activity—CDK-2 activity was measured in vitro using histone H1 as substrate as described in Ref. 31. When analyzed in detached cells, pooled cells from four 100-mm plates were used to precipitate CDK-2 (sc-163G; Santa Cruz) from equal amounts of cell protein as in total and adherent cells.

DNA Synthesis—DNA synthesis was examined by BrdUrd incorporation as described in Ref. 31. BrdUrd was added for 4 h at the times indicated, and the cells were fixed and stained with sheep anti-BrdUrd (Biosign, Int.), fluorescein isothiocyanate anti-sheep IgG, and DAPI. The number of BrdUrd-positive cells is expressed as the percentage of the total nuclei (mean ± S.E.).

FACS Analysis for DNA Content—Detached and adherent cells were collected, fixed, treated with 200 units/ml RNase, stained with 0.1 mg/ml propidium iodide, and subjected to FACS analysis as described in Ref. 30.

RESULTS

Ras-induced Apoptosis Is Caspase-mediated—We previously reported that acute expression of H-RasL61 induced cell cycle progression and apoptosis in Wistar rat thyroid cells, a continuous line of rat thyroid cells (23). The goal of the present studies was to assess the potential of Ras to engage cell death pathways through effects on the cell cycle. Adenoviruses expressing HA-tagged cellular (RasG12), activated (RasV12), and activated mutant Ras impaired in membrane localization (RasV12A186)

3 The abbreviations used are: QVD, N-(2-quinoilyl)valeryl-aspartyl-(2,6-difluorophenoxymethyl)ketone; HA, hemagglutinin; Rb, retinoblastoma; DAPI, 4′,6-diamidino-2-phenylindole; BrdUrd, bromodeoxyuridine; FACS, fluorescence-activated cell sorter; ATM, ataxia-telangiectasia-mutated; ATR, ATM and Rad-3-related.
were generated (see "Experimental Procedures"). Conditions were derived where more than 90% of the cells expressed Ras on day 4 post-infection (RasV12 shown in Fig. 1A). DAPI staining of nuclei revealed marked alterations in nuclear size and structure as well as a substantial decrease in cell number over time in RasV12-infected cells (Fig. 1A, DAPI). To confirm that cell loss reflected apoptosis, RasV12-infected cells were analyzed for DNA fragmentation and caspase activation, hallmarks of apoptotic cell death. As expected, RasV12-infected cells exhibited DNA laddering (Fig. 1, B and C). Cell surface Annexin V staining, a marker expressed on early apoptotic cells, was observed beginning at day 3 post-infection (data not shown). Importantly, expression of similar levels of RasG12 or RasV12A186 failed to stimulate DNA laddering (Fig. 1B).

Western blotting using an antibody that recognizes both intact procaspase-3 (33 kDa) and cleaved, active caspase-3 (17 kDa) revealed procaspase-3 cleavage in cells infected with RasV12, but not RasG12 or RasV12A186 (Fig. 1D). These data were corroborated using an antibody that specifically recognizes cleaved caspase-3 in immunostaining experiments (Fig. 1F). Inclusion of the pan caspase inhibitor QVD blocked procaspase-3 cleavage as expected (Fig. 1, E and F) and prevented DNA laddering (Fig. 1C). QVD also blocked procaspase-3 cleavage and DNA laddering stimulated by RasL61 (data not shown). These results extend our previous studies by showing that caspase-mediated apoptosis is a specific response to the expression of active, membrane-localized Ras.

Ras Stimulates Unrestrained CDK-2 Activity in Detached Cells—To determine whether cells destined to die entered the cell cycle, effects on CDK-2 activity, a marker of G1-S phase cell cycle progression, were assessed. CDK-2 was immunoprecipitated from total cell lysates prepared from RasL61- or RasV12-infected cells, and kinase activity was measured in vitro. Both RasL61 and RasV12 (Fig. 2, A and C), but not RasG12 (Fig. 2C) or RasV12A186 (not shown), stimulated CDK-2 activity. CDK-2 activity was increased on day

FIGURE 1. Active, membrane-localized Ras induces caspase-dependent apoptosis. A, quiescent cells were mock infected or infected with an adenovirus expressing RasV12 (see “Experimental Procedures”) and analyzed for Ras expression at days 1–4 post-infection via HA immunostaining (top row). Similar results were obtained following infection with viruses expressing RasG12 and RasV12A186 (see “Experimental Procedures”) and using a pan-Ras antibody (data not shown). The nuclei were stained with DAPI (bottom row). Shown are representative images from one of three time course experiments. B–D and F, cells were mock infected (m) or infected with viruses expressing RasV12, RasG12, or RasV12A186. Where indicated, QVD was used at 50 μM. B and C, low molecular weight DNA was prepared from cells harvested at day 4 post-infection and analyzed for DNA laddering. A representative experiment is shown from three experiments that compared RasV12 and RasG12 and two experiments that compared RasV12 and RasV12A186. Three experiments using QVD were performed. D and E, total cell lysates from cells harvested at day 4 post-infection were subjected to Western blotting with an antibody that detects both inactive (pro) and cleaved (active) forms of caspase-3. Short (s) and long (l) exposures are shown to illustrate procaspase-3 and active caspase-3 cleavage products on the same blot. Equal protein loading was confirmed by Western blotting for actin. The effects of RasV12 on pro-caspase-3 cleavage were reproduced in more than five experiments. The inability of RasG12 and RasV12A186 to induce procaspase-3 cleavage was seen in three and two experiments, respectively. E, RasV12 was infected at 40K particles/cell in this experiment to highlight effects on procaspase-3 cleavage. QVD was used at 50 μM. Two experiments were performed using these conditions, and two experiments were performed using RasV12 (5K particles/cell) and QVD at 50 μM with similar results. F, summary of two immunostaining experiments performed on days 2 and 4 post-infection using an antibody specific for the cleaved (active) form of caspase-3. The percentage of active caspase-3 positive cells (means ± S.E.) is shown. A third experiment analyzed on day 5 post-infection yielded similar results.
Ras Induces Cell Cycle-dependent Apoptosis

2 post-infection (data not shown), a time that preceded apoptosis as assessed by the presence of floating cells or the induction of cell surface Annexin V, suggesting that Ras-induced CDK-2 activity culminates in apoptosis. To directly determine whether CDK-2 activity was increased in dying cells, CDK-2 activity was assessed in extracts prepared from separated populations of adherent versus detached cells. Both adherent (attached) and detached cells displayed high CDK-2 activity (Fig. 2A, top panel). Detached but not adherent cells exhibited procaspase-3 cleavage (Fig. 2B), indicating that the detached population contained apoptotic cells. ERK activity was increased in adherent and detached cells, documenting that CDK-2 activity in both populations was due to RasV12 (Fig. 2B). In further support of this, deprivation of attachment by trypsin-EDTA release failed to induce CDK-2 activity, and plating cells on agarose-coated dishes failed to induce apoptosis (32). Most importantly, CDK-2 activity was not impaired by

4 A. J. Fikaris, unpublished data.

FIGURE 2. Detached cells exhibit high CDK-2 activity and cleavage of cyclin A and p27. A, CDK-2 activity in CDK-2 (left) versus IgG (right) precipitates prepared from mock (m) versus RasL61-infected cells from total (tot), attached (att), and detached (det) cells harvested at day 4 post-infection (top row). One representative experiment of four performed using the CDK-2 antibody is shown. A single control experiment using IgG was performed. Equal amounts of CDK-2 were precipitated (WB: CDK-2), and Western blots for bound cyclin A and p27 are shown. B, cell lysates from attached and detached cells harvested on day 4 were subjected to Western blotting for procaspase-3 cleavage and activated ERK (phosphorylated; ERK-P). Three experiments were performed with similar results. C, CDK-2 activity was assessed as described for A in cells infected with RasG12 versus RasV12 in the absence (−) and presence (+) of QVD (50 μM). Equal amounts of CDK-2 were precipitated (WB: CDK-2); D, cell lysates from attached versus detached RasL61-infected cells harvested on day 4 were subjected to Western blotting for cyclin A and p27. Total cell lysates from mock infected cells were analyzed similarly. Short (s) and long (l) exposures are shown to illustrate expression of intact proteins and cleavage products (arrows). More than five experiments were performed with similar results. IP, immunoprecipitation; WB, Western blot.

QVD (Fig. 2C). Taken together, these data indicate that RasV12 stimulates CDK-2 activity in adherent cells that subsequently detach and that CDK-2 activity does not arise as a consequence of cell detachment or caspase-mediated cleavage events.

To investigate the molecular basis for the high CDK-2 activity in detached cells, CDK-2 precipitates were analyzed for the presence of associated proteins. A modest but reproducible decrease in CDK-2-associated cyclin A was observed in detached cells (Fig. 2A, third panel). Furthermore, although p27 was associated with CDK-2 in adherent cells, it was not present in CDK-2 precipitates prepared from detached cells (Fig. 2A, bottom panel). Further examination of p27 levels revealed the presence of p27 cleavage products selectively in lysates prepared from detached cells (Fig. 2D, lower panels). Similar effects were observed for cyclin A (Fig. 2D). Cleavage of cyclin A to a nondegradable fragment (33) has been reported to play a role in apoptosis (34). Cleavage of cyclin-dependent kinase inhibitors p27 and p21 in apoptotic cells has also been reported (35). These data provide two potential mechanisms through which RasV12 could enforce CDK-2 activity in dying cells, through removal of p27 from cyclin-CDK complexes and/or via cleavage of cyclin A.

Ras Activates the Replication Stress Checkpoint—To ensure proper passage through the cell cycle and avoid the generation of genetically aberrant progeny, cells have in place strict surveillance mechanisms. In response to DNA damage
and/or replication stress, checkpoint proteins delay cell cycle progression to facilitate repair, induce growth arrest, or stimulate apoptosis (36). Ataxia-telangiectasia-mutated (ATM) and ATM and Rad-3-related (ATR) kinases are the pivotal responders to DNA damage and replication stress, respectively. Once activated, these phosphatidylinositol 3-kinase-like kinases phosphorylate and activate downstream effectors including the Chk1/2 kinases and p53 (36–38). Because CDK-2 activity was sustained over days in RasV12-infected cells, we analyzed whether this response was sensed as replication stress.

Using an antibody that specifically recognizes Chk1 phosphorylated at serine 345 by ATR (Chk1p-Ser345) (39), the effects of RasV12 on ATR activity were investigated. Chk1 phosphorylation was first apparent at day 2 and increased through day 4 post-infection, effects that were selective for membrane-localized, activated Ras (Fig. 3A). To corroborate these data, a second marker of ATR (and ATM) activity, phosphorylation of p53 at serine 15 (p53p-Ser15) (40), was examined. RasV12 also stimulated serine 15 phosphorylation on p53 (Fig. 3A). The time course for Chk1 and p53 phosphorylation was similar to that for CDK-2 activity and preceded the induction of apoptosis. Importantly, QVD failed to impair Chk1 or p53 phosphorylation, eliminating the possibility that checkpoint activation was secondary to caspase-mediated DNA cleavage (Fig. 3B). These data suggest that sustained CDK-2 activity induced by Ras is sensed as replication stress and leads to apoptosis.

To document that apoptosis was not an artifact of high levels of Ras expression (5), the dose response for Ras-stimulated cell cycle progression, checkpoint activation, and apoptosis was compared. Importantly, the effects of RasV12 on cyclins A and B1 expression, procaspase-3 cleavage, and Chk1/p53 phosphorylation exhibited a similar response at each dose (Fig. 3C). Remarkably, even when expressed at levels similar to endogenous Ras in these cells, RasV12 induced cell cycle progression, checkpoint activation, and apoptosis. These data strongly suggest that the effects of Ras on cell cycle progression and apoptosis are intricately linked.

Cell Cycle Progression Is Required for Checkpoint Activation and Apoptosis—To determine whether apoptosis was a consequence of sustained CDK-2 activity, the cyclin-dependent kinase inhibitor p27 was overexpressed. Conditions were derived where p27 was expressed at levels sufficient to block RasV12-stimulated CDK-2 activity (Fig. 4A). Under these conditions, p27 blocked RasV12-stimulated DNA synthesis (Fig. 4, B and C) and entry into the S phase (Fig. 4D). Furthermore, p27 blocked Rb phosphorylation at serines 807/811, sites of G1 cyclin/CDK activity, and prevented the induction of cyclins A and B1, effects that were induced selectively by active, membrane-localized Ras (Fig. 4E and not shown).

RasV12 on ATR activity were investigated. Chk1 phosphorylation was first apparent at day 2 and increased through day 4 post-infection, effects that were selective for membrane-localized, activated Ras (Fig. 3A). To corroborate these data, a second marker of ATR (and ATM) activity, phosphorylation of p53 at serine 15 (p53p-Ser15) (40), was examined. RasV12 also stimulated serine 15 phosphorylation on p53 (Fig. 3A). The time course for Chk1 and p53 phosphorylation was similar to that for CDK-2 activity and preceded the induction of apoptosis. Importantly, QVD failed to impair Chk1 or p53 phosphorylation, eliminating the possibility that checkpoint activation was secondary to caspase-mediated DNA cleavage (Fig. 3B). These data suggest that sustained CDK-2 activity induced by Ras is sensed as replication stress and leads to apoptosis.

To document that apoptosis was not an artifact of high levels of Ras expression (5), the dose response for Ras-stimulated cell cycle progression, checkpoint activation, and apoptosis was compared. Importantly, the effects of RasV12 on cyclins A and B1 expression, procaspase-3 cleavage, and Chk1/p53 phosphorylation exhibited a similar response at each dose (Fig. 3C). Remarkably, even when expressed at levels similar to endogenous Ras in these cells, RasV12 induced cell cycle progression, checkpoint activation, and apoptosis. These data strongly suggest that the effects of Ras on cell cycle progression and apoptosis are intricately linked.

Cell Cycle Progression Is Required for Checkpoint Activation and Apoptosis—To determine whether apoptosis was a consequence of sustained CDK-2 activity, the cyclin-dependent kinase inhibitor p27 was overexpressed. Conditions were derived where p27 was expressed at levels sufficient to block RasV12-stimulated CDK-2 activity (Fig. 4A). Under these conditions, p27 blocked RasV12-stimulated DNA synthesis (Fig. 4, B and C) and entry into the S phase (Fig. 4D). Furthermore, p27 blocked Rb phosphorylation at serines 807/811, sites of G1 cyclin/CDK activity, and prevented the induction of cyclins A and B1, effects that were induced selectively by active, membrane-localized Ras (Fig. 4E and not shown).
Co-infection of p27 with RasV12 decreased the proportion of progression and apoptosis, an activated Ras mutant containing a point mutation within the effector loop, RasV12G37 (41), was utilized. When expressed acutely, RasV12G37 failed to induce the expression of cyclins A and B1 (Fig. 5E). Interestingly, RasV12G37 also failed to induce apoptosis, as assessed by procaspase-3 cleavage (Fig. 5E). Taken together, these data suggest that cell cycle entry is an obligate prerequisite to apoptosis.

To determine whether checkpoint activation induced by Ras resulted from replication stress, we examined whether the blockade of cell cycle progression impaired checkpoint activation. Co-infection of p27 with RasV12 inhibited Chk1 and p53 phosphorylation (Fig. 5F). These data indicate that checkpoint activation requires cell cycle entry, supporting the notion that checkpoint activation and apoptosis arise as consequences of replication stress. In further support of this idea, RasV12 stimulated cell cycle progression, checkpoint activation, and apoptosis in a second line of rat thyroid cells (PC-C13 cells), and these effects were blocked by overexpression of p27 (data not shown).

Rb Deregulation Is Sufficient to Stimulate Replication Stress and Apoptosis in Thyroid Cells—Given that RasV12-induced apoptosis appeared to arise as a consequence of sustained mitogenic signaling, we explored whether forced cell cycle progression induced by human papillomavirus type 16-E7 (HPV-E7) protein was sufficient to stimulate apoptosis. HPV-E7 deregulates the cell cycle through its ability to bind and inactivate Rb family proteins and the Cip/Kip family of CDK inhibitors (42). Expression of HPV-E7 in quiescent thyroid cells (Fig. 6A, lower panel) stimulated Rb phosphorylation, the expression of cyclins A and B1 (Fig. 6A, upper panels), and cell cycle progression into S phase (Fig. 6B) as early as day 2 post-infection. HPV-E7 also increased the proportion of cells with hypodiploid DNA content and stimulated caspase-mediated DNA fragmentation and procaspase-3 cleavage (Fig. 6, C–E) with a time course that was delayed compared with its effects on cell cycle progression. Importantly, HPV-E7 induced checkpoint activation over a time course similar to that for cell cycle progression (Fig. 6F). These data demonstrate that forced cell cycle progression is sufficient to stimulate checkpoint activation and apoptosis in thyroid cells.

**DISCUSSION**

Ras elicits pleiotropic effects on cell proliferation and cell survival. For example, in collaboration with mutations that inactivate tumor suppressor genes and confer telomere maintenance, Ras transforms primary human cells, where it is believed to provide sustained mitogenic signals (43, 44). In contrast, overexpression of activated Ras alone induced G1 phase growth arrest in primary human and murine cells (18, 45). When expressed from its endogenous promoter in mice, Ras failed to induce proliferation or growth arrest in many tissues (46). However, when targeted to the lung, GI tract (47) or pancreas (48), Ras induced hyperplasia. High intensity Ras signaling in many cell types induced apoptosis (5). Given the variable nature of Ras effects on cell proliferation and survival, the precise contributions of Ras to cellular transformation have been difficult to decipher. Ras plays an essential role in the initiation and progression of human thyroid tumors, which exhibit frequent activating mutations in Ras (49, 50) and B-Raf (51). RET/PTC oncogenes, another frequent alteration in human thyroid
Ras Induces Cell Cycle-dependent Apoptosis

When expressed in quiescent thyroid epithelial cells, Ras stimulated cell cycle entry that was temporally followed by apoptosis. CDK-2 activity, a marker of G1 and S phase cell cycle progression, was increased in apoptotic cells, suggesting that Ras-expressing cells perish as a consequence of sustained mitogenic signaling. As evidence for this, the induction of CDK-2 activity preceded apoptosis and was not attenuated by caspase inhibitors. These data argue that sustained CDK-2 activity is the cause rather than a consequence of apoptosis. In further support of this notion, overexpression of p27 at levels sufficient to block CDK-2 activity and cell cycle progression induced by Ras prevented apoptosis. Furthermore, forced cell cycle progression induced by the expression of HPV-E7 was sufficient to stimulate apoptosis. Although this does not exclude other pro-apoptotic effects of Ras, it does indicate that Ras-induced apoptosis in thyroid cells can be explained by its ability to deregulate cell cycle progression. There is an established literature that links CDK-2 activity to apoptosis (55–57), and in a number of instances, blockade of CDK-2 activity has been shown to suppress cell death (58–60). The data reported here demonstrate that Ras engages the apoptotic pathway through effects on CDK-2 activity, or via downstream targets of CDK-2 activity, in thyroid cells. This is not surprising in that the induction of apoptosis by deregulated cell cycle progression is an essential innate mechanism of tumor suppression (61). Interestingly, however, the effects of Ras in thyroid epithelial cells differ from other instances where oncogenes initiated cell cycle progression and apoptosis. In primary keratinocytes, Ras stimulated G1 phase cell cycle arrest followed by apoptosis (45). By contrast, a substantial proportion of Ras-infected thyroid cells progress into the S and G2 phases, suggesting that apoptosis arises through a distinct mechanism.

tumors, elicit many of their effects through Ras and B-Raf (52). We previously reported that acute expression of activated Ras stimulates cell cycle progression and apoptosis in normal rat thyroid cells (23). Here we present evidence that these effects are intricately related and that expression of activated Ras is sufficient to induce checkpoint activation and replication stress, similar to that observed in precancerous lesions in humans (53, 54).

When expressed in quiescent thyroid epithelial cells, Ras stimulated cell cycle entry that was temporally followed by apoptosis. CDK-2 activity, a marker of G1 and S phase cell cycle progression, was increased in apoptotic cells, suggesting that Ras-expressing cells perish as a consequence of sustained mitogenic signaling. As evidence for this, the induction of CDK-2 activity preceded apoptosis and was not attenuated by caspase inhibitors. These data argue that sustained CDK-2 activity is in these cells. Acute activation of Myc in quiescent cells induced cell cycle entry and apoptosis. Although inhibition of Myc-induced CDK-2 activity impaired cell cycle progression but not apoptosis (28), inhibition of Ras-induced CDK-2 activity impaired both in thyroid cells. Significantly, the effects of Ras on cell cycle progression, checkpoint activation (see below), and apoptosis were seen following exposure of activated Ras at levels similar to those of endogenous Ras in thyroid cells. Hence, the observed effects are highly unlikely to reflect artifacts associated with overexpression.

Defects in cell cycle transit have been causally linked to the activation of DNA damage and apoptotic cascades (25–27). The protracted progress of Ras-infected thyroid cells through the S and G2 phases prompted us to investigate whether Ras activated S and/or G2 phase cell cycle checkpoints (36–38). ATR was activated in Ras-infected cells as evidenced by activat-
Ras Induces Cell Cycle-dependent Apoptosis

Inhibiting phosphorylations of Chk1 and p53. Checkpoint activation was not a consequence of DNA cleavage during apoptosis, because caspase inhibitors did not prevent Ras-induced ATR activity. On the other hand, co-expression of p27 with Ras blocked ATR activity strongly suggesting that checkpoint activation arises as a consequence of replication stress. We attempted to determine whether blockade of checkpoint activation rescued Ras-infected cells from apoptosis. Unfortunately, inhibition of ATR activity using caffeine (62) impaired cell cycle progression, as did the Chk1 inhibitors, SB-218078 (63) and Go-6976 (64). Variable results were obtained using an adenovirus expressing D130A dominant negative Chk1 (39). In some experiments, expression of dominant negative Chk1 blocked procaspase-3 cleavage, an indicator of apoptosis, without affecting cell cycle progression. However, in other experiments, dominant negative Chk1 potentiated apoptosis, effects that may be due to the fact that ATR and Chk1 play essential roles in normal cells (65). Nonetheless, recent findings indicate that replication stress is a critical determinant to tumor formation (53, 54). Early precursor lesions, but not normal tissues, exhibit markers of a DNA damage response, including activation of ATR and phosphorylation of p33, effects remarkably similar to those elicited by Ras in thyroid cells. These findings imply that defects in surveillance pathways constitute a strong selective pressure for tumor formation by Ras. With respect to this, it is intriguing that Ras was recently reported to induce the bypass of radiation-induced G2 and M phase cell cycle checkpoints (66).

The mechanism through which deregulated cell cycle progression, or sustained CDK-2 activity, is sensed as aberrant in thyroid cells is unclear. Ras induces the expression of p19ARF in many cells (67). ARF functions as a rheostat, sensing the strength of mitogenic signals and inducing apoptosis when those signals exceed a critical threshold (68). Curiously, we were unable to detect p19ARF in Ras-expressing thyroid cells. High levels of cyclin E-associated CDK-2 activity have been shown to phosphorylate Rb on serine 567, leading to the complete release of E2F (25). Deregulation of E2F up-regulates the transcription of caspases (69) and induces apoptosis via the induction of pro-apoptotic proteins PUMA, Noxa, Bim, and Hrk/DP5 (70); the down-regulation of survival signals (27, 71); or the induction of DNA double strand breaks (72), or ATM, Nbs1, and Chk2 (73). ATR and ATM phosphorylate and stabilize E2F (74), providing a means through which Ras could reinforce CDK-2-mediated release of E2F. Strikingly, far less p27 was associated with CDK-2 in detached compared with adherent thyroid cells, an effect that would contribute to sustained CDK-2 activity in response to Ras. Also, p27 and cyclin A were cleaved in detached thyroid cells, similar to previous reports of cyclin A (33, 34) and p27 (35) cleavage during apoptosis. Hence, there may be multiple mechanisms through which Ras enforces CDK-2 activity, a signal that when unopposed, induces apoptosis.

These data suggest a refinement in our working model for how Ras contributes to thyroid epithelial cell transformation and perhaps to the transformation of other cells. Rather than solely stimulating cell division, sustained mitogenic signaling by Ras culminates in checkpoint activation and apoptosis. Hence, the frequent mutational activation of Ras in human thyroid tumors is likely to rely on the ability of Ras to mediate the bypass of cell cycle checkpoints and apoptosis in addition to its ability to stimulate cell proliferation.

Acknowledgments—We thank Dr. T. D. Halazonetis (Wistar Institute, Philadelphia, PA) for critical evaluation of this manuscript. We are also grateful to G. Prendergast for technical assistance.

REFERENCES

1. Campbell, P. M., and Der, C. J. (2004) Semin. Cancer Biol. 14, 105–114
2. Malumbres, M., and Barbacid, M. (2003) Nat. Rev. Cancer 3, 459–465
3. Giehl, K. (2005) Biol. Chem. 386, 193–205
4. Cox, A. D., and Der, C. J. (2003) Oncogene 22, 8999–9006
5. Joneson, T., and Bar-Sagi, D. (1999) Mol. Cell. Biol. 19, 5892–5901
6. Chou, C. K., Liang, K. H., Tseng, C. C., Huang, G. C., Chuang, J. I., Chang, T. Y., and Liu, H. S. (2005) Life Sci. 78, 1823–1829
7. Tanaka, Y., Nakayamada, S., Fujimoto, H., Okada, Y., Umehara, H., Kataoka, T., and Minami, Y. (2002) J. Biol. Chem. 277, 21446–21452
8. Khochkhatchev, A., Rabizadeh, S., Xavier, R., Nedwidek, M., Chen, T., Zhang, X. F., Seed, B., and Avruch, J. (2002) Curr. Biol. 12, 253–265
9. Lemoine, N. R., Mayall, E. S., Wylie, F. S., Farr, C. J., Hughes, D., Padua, R. A., Thurston, V., Williams, E. D., and Wyndham-Thompson, D. (1988) Cancer Res. 48, 4459–4463
10. Namba, H., Gutman, R. A., Matsuo, K., Alvarez, A., and Fagin, J. A. (1990) J. Clin. Endocrinol. Metab. 71, 223–229
11. Suarez, H. G., du Villard, J. A., Severino, M., Caillou, B., Schummer, M., Tubiana, M., Parmentier, C., and Monier, R. (1990) Oncogene 5, 565–570
12. Nikiforova, M. N., Lynch, R. A., Biddinger, P. W., Alexander, E. K., Dorn, G. W., 2nd, Tallini, G., Kroll, T. G., and Nikiforov, Y. E. (2003) J. Clin. Endocrinol. Metab. 88, 2318–2326
13. Vasko, V., Ferrand, M., Di Cristofaro, J., Carayon, P., Henry, J. F., and de Micco, C. (2003) J. Clin. Endocrinol. Metab. 88, 2745–2752
14. Namba, H., Rubin, S. A., and Fagin, J. A. (1990) Mol. Endocrinol. 4, 1474–1479
15. Lemoine, N. R., Mayall, E. S., Wylie, F. S., Williams, E. D., Goyms, M., Stringer, B., and Wyndham-Thomas, D. (1989) Oncogene 4, 159–164
16. Karga, H., Lee, J. K., Vickery, A. L., Jr., Thor, A., Gaz, R. D., and Jameson, J. L. (1991) J. Clin. Endocrinol. Metab. 73, 832–836
17. Garcia-Rosten, G., Zhao, H., Camp, R. L., Pollan, M., Herrero, A., Pardo, J., Wu, R., Carcangiu, M. L., Costa, J., and Tallini, G. (2003) J. Clin. Oncol. 21, 3226–3235
18. Serrano, M., Lin, A. W., McCurrach, M. E., Beach, D., and Lowe, S. W. (1997) Cell 88, 593–602
19. Bond, J. A., Wylie, F. S., Rowson, J., Radulescu, A., and Wyndham-Thomas, D. (1994) Oncogene 9, 281–290
20. Jones, C. J., Kipling, D., Morris, M., Hepburn, P., Skinner, J., Bounacer, A., Wu, R., Carcangiu, M. L., Costa, J., and Tallini, G. (2003) J. Clin. Oncol. 21, 3226–3235
21. Meinkoth, J. L. (2004) Cancer Treat. Res. 122, 131–148
22. Shirokawa, J. M., Elisei, R., Knauf, J. A., Hara, T., Wang, J., Saavedra, H. I., and Fagin, J. A. (2000) Mol. Endocrinol. 14, 1725–1738
23. Cheng, G., Lewis, A. E., and Meinkoth, J. L. (2003) Mol. Endocrinol. 17, 450–459
24. Pelengaris, S., Khan, M., and Evan, G. (2002) Nat. Rev. Cancer 2, 764–776
25. Harbour, J. W., and Dean, D. C. (2000) Genes Dev. 14, 2393–2409
26. Melkручans, W., and Schlegel, R. (1995) J. Cell. Biochem. 58, 160–174
27. Dimova, D. K., and Dyson, N. J. (2005) Oncogene 24, 2810–2826
28. Rudolph, B., Saffrich, R., Zicker, J., Henglein, B., Muller, R., Ansorge, W., and Eilers, M. (1996) EMBO J. 15, 3065–3076
29. Kupperman, E., Woolford, D., Wen, W., and Meinkoth, J. L. (1996) Endocrinology 137, 96–104
30. Santiago-Walker, A. E., Pikaris, A. J., Kao, G. D., Brown, E. J., Kazanietz, M. G., and Meinkoth, J. L. (2005) J. Biol. Chem. 280, 32107–32114
31. Lewis, A. E., Pikaris, A. J., Prendergast, G. V., and Meinkoth, J. L. (2004) J. Biol. Chem. 279, 32107–32114
Ras Induces Cell Cycle-dependent Apoptosis

G. J. Bigner, D. D., Palmieri, G., Cossu, A., Flanagan, A., Nicholson, A., Ho, I. W., Leung, S. Y., Yuen, S. T., Weber, B. L., Seigler, H. F., Darrow, T. L., Paterson, H., Marais, R., Marshall, C. J., Wooster, R., Stratton, M. R., and Futreal, P. A. (2002) *Nature* 417, 949–954

Melillo, R. M., Castellone, M. D., Guarino, V., de Falco, V., Ciriafici, A. M., Salvatore, G., Caiazzo, F., Basolo, F., Giannini, R., Ruhoffner, M., Orntoft, T., Fusco, A., and Santoro, M. (2005) *J. Clin. Investig.* 115, 1068–1081

Bartkova, J., Horejsi, Z., Kaeberlein, M., Zieger, K., Goldberg, P., Sehested, M., Nesland, J. M., Lucas, C., Orntoft, T., and Bartek, J. (2005) *Nature* 434, 864–870

Gorgoulis, V. G., Vassiliou, L. V., Karakaidos, P., Zacharatos, P., Kotsinas, A., Lolioglou, T., Venere, M., Ditullio, R. A., Jr., Kastrinakis, N. G., Levy, B., Kletsas, D., Yoneta, A., Herlyn, M., Kittas, C., and Halazonetis, T. D. (2005) *Nature* 434, 907–913

Meikrantz, W., Gisselbrecht, S., Tam, S. W., and Schlegel, R. (1994) *Proc. Natl. Acad. Sci. U. S. A.* 91, 3754–3758

Shi, L., Chen, G., He, D., Bosc, D. G., Litchfield, D. W., and Greenberg, A. H. (1996) *J. Immunol.* 157, 2381–2385

Gil-Gomez, G., Berns, A., and Brady, H. J. (1998) *EMBO J.* 17, 7209–7218

Zhou, B. B., Li, H., Yuan, J., and Kirschner, M. W. (1998) *Proc. Natl. Acad. Sci. U. S. A.* 95, 6785–6790

Park, D. S., Morris, E. J., Padmanabhan, J., Shelanski, M. L., Geller, H. M., and Greene, L. A. (1998) *J. Cell Biol.* 143, 457–467

Wang, J., and Walsh, K. (1996) *Science* 273, 359–361

Lowe, S. W., and Lin, A. W. (2000) *Carcinogenesis* 21, 485–495

Sarkaria, J. N., Busby, E. C., Tibbetts, R. S., Roos, P., Taya, Y., Karnitz, L. M., and Abraham, R. T. (1999) *Cancer Res.* 59, 4375–4382

Jackson, J. R., Gilmartin, A., Imburgia, C., Winkler, J. D., Marshall, L. A., and Roshak, A. (2000) *Cancer Res.* 60, 566–572

Kohn, E. A., Yoo, C. J., and Eastman, A. (2003) *Cancer Res.* 63, 31–35

Brown, E. J., and Baltimore, D. (2000) *Genes Dev.* 14, 397–402

Knauf, J. A., Ouyang, B., Knudsen, E. S., Fukasawa, K., Babcock, G., and Fagin, A. J. (2006) *J. Biol. Chem.* 281, 3800–3809

Sherr, C. J. (2001) *Nat. Rev. Mol. Cell. Biol.* 2, 731–737

Lowe, S. W., and Sherr, C. J. (2003) *Curr. Opin. Genet. Dev.* 13, 77–83

Noble, Z., Polakoff, J., Davaluri, R. V., McCurrach, M. E., Jacobs, M. D., Narita, M., Zhang, M. Q., Lazebnik, Y., Bar-Sagi, D., and Lowe, S. W. (2002) *Nat. Cell Biol.* 4, 859–864

Hershko, T., and Ginsberg, D. (2004) *J. Biol. Chem.* 279, 8627–8634

Stanelle, J., and Putzer, B. M. (2006) *Trends Mol. Med.* 12, 177–185

Howard, T. M., and Kowalik, T. F. (2006) *Oncogene* 25, 746–755

Rogoff, H. A., Pickering, M. T., Frame, F. M., Debatis, M. E., Sanchez, Y., Jones, S., and Kowalik, T. F. (2004) *Mol. Cell. Biol.* 24, 2968–2977

Sears, R. C., and Nevin, J. R. (2002) *J. Biol. Chem.* 277, 11617–11620

NOVEMBER 17, 2006 • VOLUME 281 • NUMBER 46

JOURNAL OF BIOLOGICAL CHEMISTRY 34767