Oncogenic RAS Induces Accelerated Transition through G2/M and Promotes Defects in the G2 DNA Damage and Mitotic Spindle Checkpoints*

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Activating mutations of RAS are prevalent in thyroid follicular neoplasms, which commonly have chromosomal losses and gains. In thyroid cells, acute expression of HRASV12 increases the frequency of chromosomal abnormalities within one or two cell cycles, suggesting that RAS oncoproteins may interfere with cell cycle checkpoints required for maintenance of a stable genome. To explore this, PCCL3 thyroid cells with conditional expression of HRASV12 or HRASV12 effector mutants were presynchronized at the G1/S boundary, followed by activation of expression of RAS mutants and release from the cell cycle block. Expression of HRASV12 accelerated the G2/M phase by ~4 h and promoted bypass of the G2 DNA damage and mitotic spindle checkpoints. Accelerated passage through G2/M and bypass of the G2 DNA damage checkpoint, but not bypass of the mitotic spindle checkpoint, required activation of mitogen-activated protein kinase (MAPK). However, selective activation of the MAPK pathway was not sufficient to disrupt the G2 DNA damage checkpoint, because cells arrested appropriately in G2 despite conditional expression of HRASV12,S35 or BRAFV600E. By contrast to the MAPK requirement for radiation-induced G2 arrest, RAS-induced bypass of the mitotic spindle checkpoint was not prevented by pretreatment with MEK inhibitors. These data support a direct role for the MAPK pathway in control of G2 progression and regulation of the G2 DNA damage checkpoint. We propose that oncogenic RAS activation may predispose cells to genomic instability through both MAPK-dependent and independent pathways that affect critical checkpoints in G2/M.

Human tumors, including those of the thyroid (1–3), arise from a single transformed cell. Despite their clonal origin, cells from advanced carcinomas are often genetically heterogeneous. Tumor cell variability is thought to result from genomic instability. Clonal heterogeneity in turn tends to predict a poor outcome and resistance to therapy. This is also the case in thyroid cancer. In thyroid tumors, the nature of the oncogenic events involved in the initiation of the neoplastic clone may determine the likelihood of genomic instability occurring at a later stage. Among the various forms of thyroid neoplasia, follicular adenomas and carcinomas are commonly aneuploid, whereas abnormalities in chromosome number are comparatively less frequent in papillary thyroid carcinomas. Mutations of all three RAS genes are found in benign and malignant follicular neoplasms and in follicular variant papillary thyroid carcinomas and are believed to be one of the early steps in thyroid tumor formation (4–9). By contrast, rearrangements of the tyrosine kinase receptor gene RET (rearranged in transfection; RET/PTC) are only found in papillary thyroid carcinomas, which are commonly diploid and less aggressive. A possible explanation for this is that activating mutations of RAS, but not of RET, promote tumor progression in part by decreasing genomic stability. Consistent with this is the observation that acute expression of HRASV12 increases the frequency of micronuclei, an accepted indicator of chromosomal instability, whereas expression of RET/PTC does not (10).

The rate of spontaneous mutations acquired during the natural life span of a cell is exceedingly low (11, 12). This suggests that one of the early genetic disruptions involved in tumor development may confer cells with a “mutator” phenotype (13, 14) and hence a predisposition to the accumulation of additional abnormalities. Indeed, germ line mutations in genes such as p53, ATM (ataxia telangiectasia mutated), and BRCA1/2 that are involved in DNA repair and regulation of cell cycle checkpoints are found in cancer susceptibility syndromes. Although proteins encoded by p53, ATM, and BRCA1/2 have numerous functions, progression to the malignant state in these cancer syndromes is likely to be at least caused in part by genomic instability. Although not as widely appreciated, oncoproteins such as RAS have also been proposed to promote tumor progression through induction of genomic instability. For example, Finney and Bishop (15) reported that replacement of a normal Hras gene with an activated mutant Hras by homologous recombination in rat1 fibroblasts is not in itself sufficient to induce transformation but rather requires secondary changes such as gene amplification events, including amplification of the mutant Ras allele. This study supports the concept that RAS may serve as a mutator gene under physiological conditions, because the mutant HRAS protein in these experiments was expressed under the control of its own promoter. The ability of activated RAS to promote chromosomal instability is also supported by studies demonstrating that expression of the human HRAS oncogene in p53-null cells leads to premature entry of cells into the S phase, increased permissivity for gene amplification, and generation of aberrant chromosomes within a single cell cycle (16–20). Oncogenic RAS has also been shown to produce chromosome aberrations in rat mammary carcinoma cells (21), rat prostatic tumor cells (22), and a human colon carcinoma cell line (23). The demonstration by Agapova et al. (20) that expression of activated HRAS promotes bypass of G2 DNA damage checkpoint in p53 mutant cells suggests that oncogenic RAS-induced genomic instability may potentially be due to a relaxation of this checkpoint. The effectors downstream of RAS that are required for this effect have not been fully elucidated. Activation by RAS of the Ral guanine nucleotide exchange factor is responsible for dampening the G2 arrest induced by ethyl methanesulfonate in p53-deficient

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MDAH041 fibroblasts (24). On the other hand, activation of the RAS downstream effectors MEK2 3 and ERK are required for exit from DNA damage-induced G2 cell cycle arrest (25) and the transition from G2 into M (26, 27), respectively.

A role for the MEK/ERK pathway in G2/M is further supported by the observation that activated ERK associates with the mitotic apparatus of somatic mammalian cells (28, 29). ERK was also reported to associate with kinetochores during early prophase, but this association was not apparent at later stages of mitosis. Both ERK and its activator MEK localize to the mitotic spindle from prophase through anaphase and to the midbody during cytokinesis. Furthermore, activated ERK was found to associate with the spindle microtubule motor CENP-E during mitosis (29) and is capable of regulating microtubule dynamics during mitosis (30). These results strongly support a role for MEK and ERK in regulating the progression of cells through G2 and mitosis, suggesting that inappropriate activation of these RAS effectors in cells expressing oncogenic RAS could potentially disrupt the orderly transition of these cells through these latter cell cycle stages, which are critical for maintaining genomic integrity.

We previously showed that acute expression of HRASV12 increases the frequency of chromosome misalignment, multiple spindle formation, centrosome amplification, and generation of micronuclei within the first few cell cycles after activation in rat thyroid PCCL3 cells (31). These cells are not transformed and have wild type p53 genes. Rapid induction of these chromosomal abnormalities by RAS is consistent with a disruption of progression of cells through G2/M and/or alternation of the integrity of critical checkpoints needed to ensure genomic stability. Here we investigated this possibility by presynchronizing PCCL3 thyroid cells with conditional expression of HRASV12 or HRASV12 effector mutants at the G1/S boundary, followed by activation of expression of RAS mutants and release from the G1 block into a radiation-induced G2 arrest or a nocodazole-activated mitotic checkpoint. This allowed us to follow the progression of cells through G2/M as well as the G2 DNA damage and mitotic spindle checkpoints and explore the contribution of RAS effectors to this effect.

### MATERIALS AND METHODS

**Cell Lines**—The well differentiated rat thyroid cell line PCCL3 was propagated in H4 complete medium, which consisted of Coon's modification of Ham's F-12 medium containing 5% fetal bovine serum, glutamine (286 μg/ml), apo-transferrin (5 μg/ml), hydrocortisone (10 nM), insulin (10 μg/ml), thyrotropin (10 μIU/ml), penicillin, and streptomycin. The following cell lines have been previously described: rTα, PCCL3 cells stably expressing the reverse tetracycline transactivator rTα (32); Ras-25, PCCL3 cells with doxycycline (dox)-induced expression of HRASV12 or HRASV12 effector mutants at the G1/S boundary, followed by activation of expression of RAS mutants and release from the G1 block into a radiation-induced G2 arrest or a nocodazole-activated mitotic checkpoint. This allowed us to follow the progression of cells through G2/M as well as the G2 DNA damage and mitotic spindle checkpoints and explore the contribution of RAS effectors to this effect.

3 The abbreviations used are: MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; PKC, phosphatidylinositol 3-kinase; BrdUrd, bromodeoxyuridine; DAPI, 4',6-diamidino-2-phenylindole; dox, doxycycline; PI, propidium iodide; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline; Gy, gray(s); FACS, fluorescence-activated cell sorter.

pTK-hygro using Lipofectamine 2000 (Invitrogen), and clones were selected based on the absence of expression under basal conditions and strong induction by dox.

**Reagents**—FITC-conjugated anti-BrdUrd IgG was purchased from Pharmigen (San Diego, CA). Antibodies to phospho-MEK1/2 (sc-7995), MEK2 (sc-524), ERK1 (sc-94), phospho-ERK1/2 (sc-7383), HRAS (SC-520), cyclin B1 (SC-245), HDAC1 (SC-6298), and glyceraldehyde-3-phosphate dehydrogenase (SC-20357) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and anti-phospho-MEK1/2 (9121S) goat polyclonal IgG was from Cell Signaling Technology (Beverly, MA). Thymidine, nocodazole, 4',6-diamidino-2-phenylindole (DAPI), propidium iodide, thyrotropin, insulin, apo-transferrin, and hydrocortisone were purchased from Sigma, and PD98059 and wortmannin were from Calbiochem. Coon's modification of Ham's F-12 medium was from Irvine Scientific (Irvine, CA). Fetal bovine serum, penicillin-streptomycin, and glutamine were purchased from Invitrogen.

**Monitoring Cell Cycle Progression by FACS Cell Synchronization**—To synchronize cells in G1/S, cells were plated into 60-mm tissue culture dishes at ~50% confluence in H4 medium and incubated at 37 °C in 5% CO2 for 24 h. The medium was then replaced with fresh H4 medium containing 4 μM thymidine, and the cells were incubated for 14 h. The cells were then washed twice with PBS, H4 medium was added, and the cells were incubated for 9 h. The medium was then replaced with fresh H4 medium containing 4 μM thymidine, and the cells were incubated in the absence or presence of 1 μg/ml dox for 14 h. The cells were released by washing twice with PBS and then adding fresh H4 medium containing 10 μM BrdUrd with or without 1 μg/ml dox. After 2 h the cells were washed with PBS to remove BrdUrd, and fresh H4 medium with or without 1 μg/ml dox was added. To induce the G2 DNA damage checkpoint cells were irradiated 4 h after release cells with 10 Gy of x-rays or 15 Gy of γ-rays (Faxitron cabinet x-ray irradiator or Cesium irradiator, respectively). To induce the mitotic spindle checkpoint, nocodazole was added 5 h after release from the G1/S block to a final concentration of 0.4 μg/ml. At the indicated times after release, the cells were washed with PBS, harvested by trypsinization, fixed in 5 ml of cold (−20 °C) 70% ethanol, and incubated overnight at 4 °C.

**Cell Cycle Analysis**—The fixed cells were pelleted by centrifugation and resuspended in 50 μl of 0.85% NaCl. To denature DNA, 2 ml of 2 M HCl was added, and the cells were incubated for 20 min at room temperature. The cells were then pelleted by centrifugation, resuspended in 1 ml of 0.1 M sodium borate (pH 8.6), and washed one time with PBS. The cells were then resuspended in 10 μl of FITC-conjugated anti-BrdUrd IgG (Pharmigen, San Diego, CA) and incubated for 1 h at room temperature with mixing. Five hundred microliters of propidium iodide (PI) staining solution (50 μg/ml PI, 50 μg/ml RNase A) was added, and the number of BrdUrd-positive cells in S, G2/M, and G1 was determined by FACS analysis using a Coulter EPICS XL flow cytometer (Miami, FL) at an excitation range of 488 nm (argon laser) and a 525 BP filter for FITC and 620 BP for propidium iodide.

**Monitoring Cell Cycle Progression by Manually Counting Mitotic Cells**—The cells were synchronized in G1/S with thymidine as described above, except BrdUrd was not added to the releasing medium. Where indicated nocodazole was added 5 h after release to a final concentration of 0.4 μg/ml to induce the mitotic spindle checkpoint. At the indicated times the cells were harvested by trypsinization, washed with PBS, spotted onto a microscope slide, and fixed by incubating with ethanol/acetic acid (19:1) for 20 min at room temperature. The cells were then incubated for 5 min with PBS containing 0.2% Triton X-100, PBS, and then PBS containing 2 μg/ml DAPI. The slides were washed and mounted in Vectorshield (Vector Lab-
oratories, Burlingame, CA). The number of mitotic cells (characterized by condensed chromosome structures observed in prophase through telophase) and nonmitotic cells was determined by manually counting using a fluorescent microscope from a sufficient number of randomly selected fields to obtain at least 5,000 cells.

**Western Blotting**—The cells were synchronized in G1/S with thymidine as described above. At the indicated times the cells were harvested, washed with cold PBS, resuspended in buffer A (20 mM Tris-HCl pH 7.4, 135 mM NaCl, 2 mM EDTA, 1% Triton X-100, 25 mM ß-glycerophosphate, 10% glycerol, 1 mM sodium orthovanadate, sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml pepstatin, 10 µg/ml aprotonin, 10 µg/ml E-64), and incubated for 20 min on ice. The cells were lysed by repeatedly passing through a 16-gauge needle. The lysates were collected, and protein concentrations were determined using Coomassie protein assay as directed by manufacturer (Pierce).

**Nuclear Fractionation**—The cells were collected, washed twice with PBS, and resuspended in buffer B (10 mM Tris, pH 7.4, 2 mM MgCl₂, 1
mm EDTA, 1 mm sodium orthovanadate, 10 mm sodium fluoride, 1 mm phenylmethylsulfonyl fluoride, 10 μg/ml pepstatin, 10 μg/ml aprotinin, 10 μg/ml E-64). The cells were incubated for 15 min on ice, passed through a 16-gauge needle, and centrifuged at 5,000 × g for 8 min at 4 °C. The supernatant was collected (cytosolic fraction), and the protein concentrations were determined using Coomassie. The pellets were washed twice with buffer B, suspended in SDS-PAGE gel loading buffer (10% SDS, 25% glycerol, 0.1% bromphenol blue, 0.75 M Tris-HCl, pH 8.8), incubated for 5 min at 95 °C, and loaded on to an SDS-polyacrylamide gel. Immunoblotting was performed as previously described (36).

**RESULTS**

**HRASV12 Expression Accelerates Transition through G2/M**—To examine the effects of activated RAS on progression through G2/M, we used Ras-25 cells, PCCL3 cells with dox-inducible expression of HRASV12. Effects of HRASV12 on G2/M were determined by first synchronizing cells at the G1/S boundary by double thymidine treatment and then inducing expression of HRASV12 by the addition of dox for 14 h prior to release. Cell cycle progression was then monitored as described under “Material and Methods.” PCCL3 cells expressing HRASV12 had an ~2-h delay in progression through S phase (Fig. 1) but had an accelerated transition through G2/M because they entered the next G1 ~2 h sooner. Thus, expression of HRASV12 resulted in a net acceleration of G2/M of ~4 h. The abnormal duration of G2/M is illustrated by the flattened G2/M peak seen between 8–12 h after release (Fig. 1A, upper panels). Treatment of the parental line, rtTA (PCCL3 cells that only express the reverse tetracycline transactivator), with dox did not significantly affect the progression of cells through S or G2/M (Fig. 1A, lower panels). Acute expression of HRASV12 did not affect the ability of cells to synchronize at the G1/S border or to release from the double thymidine block (data not shown), indicating that the differences were not due to the effects of HRASV12 on cell synchronization. To confirm the accelerated transition through G2/M, the cells were collected, and the mitotic cells were quantified after release from the double thymidine block by staining with DAPI and visual counting of cells with condensed chromosomes (Fig. 1C). The decreased number of cells in mitosis after RAS activation points to an asynchronous passage of cells through mitosis, consistent with an abbreviated G2 and/or less likely, of the mitotic phase (Fig. 1C). A time course of cyclin B1 kinase activity in cells treated as described for Fig. 1C demonstrates lower levels of kinase activity between 8 and 12 h after release in cells expressing HRASV12 (Fig. 1D). The decrease in kinase activity corresponded closely with cyclin B1 immunoreactivity, indicating no intrinsic effect of HRASV12 on kinase activity (Fig. 1E). These results are consistent with a reduced number of cells in G2/M at any of the sampled time points, presumably because of a more rapid transit through these stages in cells expressing HRASV12. Acute HRASV12 Expression Promotes Exit from the DNA Damage and Mitotic Spindle Checkpoints—Next we determined whether inappropriate activation of HRAS resulted in abnormalities of either the DNA damage or mitotic spindle checkpoints, which could also contribute to the chromosomal abnormalities we observed after acute expression of HRASV12. To determine the effects of HRASV12 expression on the G2 DNA damage checkpoint, Ras-25 cells were synchronized and released as in Fig. 1A, except that 4 h after release they were exposed to 15 Gy of ionizing radiation. The cells were harvested at the indicated times, and the cell cycle stage of the BrdUrd-positive cells was determined by FACS analysis. As shown in Fig. 2, irradiated cells that did not express HRASV12 remained in G2/M for ~4 h longer than unirradiated cells. In the presence of dox there was a more rapid entry of BrdUrd-positive cells into the next G1, indicating that the irradiation-induced G2 arrest was partially overcome by expression of HRASV12 (Fig. 2).

We next explored the effects of HRASV12 expression on the mitotic spindle checkpoint. Nocodazole, a microtubule disruptor that arrests cells in metaphase, was added 5 h after release from the double thymidine block (a time point when most cells were in S phase) to activate the mitotic checkpoint. The cells were harvested at the indicated times, smeared on glass slides, and stained with DAPI, and mitotic cells were counted manually. In the absence of dox, nocodazole induced an accumulation of Ras-25 cells in mitosis that peaked at 15 h after release and then declined slightly over the next 15 h. In cells expressing HRASV12, nocodazole induced a peak of mitotic cells at 13 h, which rapidly declined over the next 10 h (Fig. 3A). Cyclin B1, which is rapidly degraded as cells exit mitosis, had reduced activity (not shown) and was expressed at lower levels and declined earlier in HRASV12-expressing cells despite the continued presence of nocodazole (Fig. 3B). In parental rtTA cells, nocodazole produced an accumulation of cells in mitosis that peaked at 15 h and did not decline for more than 30 h (data not shown). Compared with parental cells, Ras-25 cells without dox exited the nocodazole-induced mitotic arrest slightly faster. This is possibly due to slight leakiness of HRASV12 in the absence of dox. The decline in the number of mitotic cells and the decrease in cyclin B1 levels and kinase activity indicate that HRASV12 expression promoted bypass of the nocodazole block. However, cells did not undergo cytokinesis but moved into the next G1 with a 4N DNA content (data not shown). To confirm that differences in cell viability were not responsible for the HRASV12-induced changes, we confirmed that mitosis-arrested cells underwent cytokinesis after the removal of nocodazole. Thus, cells expressing HRASV12 underwent cytokinesis and returned to a 2N DNA content within 2 h after removal of nocodazole, whereas in the absence of dox cells required ~3.5 h to recover from mitotic arrest (Fig. 3C). The ability of cells to undergo cytokinesis and return to a 2N DNA content confirms their viability and the faster recovery from the microtubule disruption seen in the HRASV12-expressing cells, which is consistent with decreased sensitivity to the nocodazole-induced checkpoint.

**Activated MEK and ERK Increase in the Nucleus in Late S and G2/M**—Recent reports demonstrating that MEK and ERK are involved in the normal progression of cells through G2/M (26, 27) suggest that inappropriate activation of this pathway may at least in part be responsible for the acceleration observed in cells expressing HRASV12. To further investigate the role of MEK/ERK in G2/M transition, we monitored the phosphorylation status of MEK and ERK through the cell cycle. To do this Ras-25 cells were synchronized by double thymidine as described for Fig. 1 (C and D), and the cells were harvested at the indicated times after release. The cell lysates were prepared from nuclear and cytosolic fractions and Western blotted for total and phosphorylated MEK and ERK. In the presence of dox there was a marked increase in nuclear phospho-ERK and phospho-MEK levels begin-
ning at 6 h and peaking at 8 h after release (corresponding to late S and early G2). In the absence of dox there was a modest increase in nuclear phospho-ERK and phospho-MEK levels, which began at 6 h (Fig. 4, A and B). The increase in nuclear phospho-ERK and phospho-MEK levels in both the absence and presence of dox was associated with higher total MEK and ERK. This suggests that the increase in phospho-ERK and phospho-MEK in late S and early G2 is likely at least in part due to increased nuclear import or retention of MEK and ERK. Cytosolic phospho-ERK and phospho-MEK were also markedly increased in HRASV12-expressing cells, which cannot be accounted for by changes in total cytosolic MEK or ERK (Fig. 4, C and D).

**Inhibition of the MAPK Pathway Retards G2/M Progression and Prevents G2/M Acceleration by Acute Expression of HRASV12 in PCCL3 Cells**—To explore the effects of MAPK on cell cycle progression, we treated rT3 cells with 35 or 70 μM PD98059 beginning at the release from a double thymidine block. Treatment with the MEK inhibitor resulted in a modest dose-dependent delay in exit from S phase and a

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**Figure 2.** Acute RAS activation promotes exit from the DNA damage checkpoint. Ras-25 cells were synchronized in G1/S and released as described for Fig. 1A. Four hours after release, the cells were exposed to 15 Gy of x-ray radiation (Faxitron cabinet irradiator) and maintained in culture until harvested for FACS analysis of BrdUrd-positive cells at the indicated times. A, in the absence of dox, radiated cells were delayed in G2. Expression of HRASV12 (+ dox) resulted in more rapid exit from the G2 block (see 14-h FACS profile). B, impact of oncogenic RAS expression on the percentage of BrdUrd-positive cells entering the next G1 following radiation-induced DNA damage. A greater number of irradiated cells entered G1 compared with those not expressing the oncoprotein. The data points represent the means of a single experiment performed in duplicate. Similar results were obtained in two additional experiments.
significant prolongation of G2/M, consistent with a requirement for MEK/ERK in the normal transit through the latter stage of the cell cycle (Fig. 5, A and B), as reported by others in fibroblasts (25, 27). The effects of PD98059 on G2/M are reciprocal to those seen after acute expression of HRASV12. Indeed, pretreatment of Ras-25 cells with PD98059 reversed entirely the HRASV12-mediated acceleration of G2/M (Fig. 5, C and D).

Activation of MEK and PI3K Are Required for HRASV12-induced Bypass of the G2 DNA Damage, but Not the Mitotic Spindle Checkpoint—Treatment of cells with PD98059 (added with dox 14 h prior to release) slowed the entry of irradiated cells expressing HRASV12 into the next G1 (Fig. 6A). Wortmannin had similar effects (Fig. 6B). We confirmed that the concentration of wortmannin used (300 nM) blunted RAS-induced activation of PI3K without affecting radiation-induced phosphorylation of p53 at Ser-15 by ATM (not shown). These data suggest that activation of MEK and PI3K are required for HRASV12-induced bypass of the G2 DNA damage checkpoint. To determine whether the HRASV12-induced bypass of the nocodazole-induced mitotic phase arrest was also dependent on activation of MEK and/or PI3K, Ras-25 cells were treated as in Fig. 3A except that PD98059 or wortmannin were added 14 h prior to release, and the number of mitotic cells was counted at the indicated times. The addition of PD98059 or wortmannin did not restore the mitotic spindle checkpoint in the HRASV12-expressing cells (Fig. 6, B and C), suggesting that neither of these pathways alone is required for the effect. However, activation of MEK/ERK pathways by doxycycline-induction of BRAFV600E allowed cells to partially escape the nocodazole-induced mitotic phase arrest (Fig. 6D).

Activation of Single RAS Effector Pathways Is Not Sufficient to Promote Bypass of the G2 DNA Damage Checkpoint—To determine whether HRASV12-induced activation of the MEK/ERK pathway was sufficient to promote bypass of the G2 DNA damage checkpoint, we developed PCCl3 cells with dox-inducible expression of HRASV12,S35, an effector mutant that preferentially activates the MEK/ERK pathway. We also tested the role of the RAL-GDS and the PI3K pathways using PCCl3 cells with dox-inducible expression of HRASV12,G37 and HRASV12,C40, respectively. Western blots probed for phospho-AKT, phospho-ERK, or HRAS demonstrated similar expression of HRAS and selective activation of MEK/ERK pathway by HRASV12,S35 and of the PI3K-akt pathway by HRASV12,C40 (Fig. 7A). As shown in Fig. 7B, expression of HRASV12,S35, HRASV12,G37, or HRASV12,C40 was not sufficient to promote bypass of the G2 DNA damage checkpoint. This suggests that activation of MEK/ERK in combination with an additional RAS effector pathway may be necessary for bypass of the G2 DNA damage checkpoint seen in HRASV12-expressing cells. Alternatively, the slightly lower activation of MEK/ERK seen after HRASV12,S35 expression as compared with HRASV12 may not have been sufficient to disrupt the checkpoint. To address this possibility we used the previously described PC-BRAFV600E-6 cell line (PCCl3 cells with dox-inducible expression of BRAFV600E (34), a constitutively active mutant of BRAF) that has a slightly greater dox-induced activation of MEK/ERK than Ras-25 cells (data not shown). Expression of BRAFV600E did not promote bypass of the G2 DNA damage checkpoint, confirming that activation of the MEK/ERK pathway is not sufficient to disrupt this checkpoint.

DISCUSSION

Mutations that constitutively activate RAS proteins are characteristic of many human cancers (reviewed in Refs. 37–39), including those arising from thyroid follicular cells (4–9). The aberrant activation of RAS proteins has been implicated in facilitating virtually all aspects of the malignant phenotype of the cancer cell, including cellular proliferation, invasion and metastasis, inhibition of apoptosis, and angiogenesis (reviewed in Ref. 40–43). Additionally, RAS oncoproteins may also contribute to oncogenesis through a decrease in genetic stability (16–23). Although much is known regarding the mechanisms by which aberrant RAS activation promotes uncontrolled proliferation by promoting entry in the S phase and increasing cell survival, less is known regarding how RAS promotes genetic instability.

We previously demonstrated that acute expression of HRASV12 increases the frequency of chromosome misalignment, multiple spindle formation, centrosome amplification, and generation of micronuclei within the first few cell cycles after activation in rat thyroid PCCL3 cells (31). Because these changes suggest problems in the G2/M phase of the cell cycle, here we manipulated the timing of expression of HRASV12 at defined points in the cell cycle to explore the effects of activated RAS on progression through G2/M. This demonstrated that expression of activated HRAS accelerates the G2/M phase of the cell cycle by ∼4 h. Furthermore, the HRASV12-induced acceleration was blunted by inhibition of MEK, indicating that MEK activity is required for this effect. A role for the MAPK pathway in regulating cell cycle progression during G2 and M is supported by a number of recent studies (25, 27–29, 44–47). Proteins in the MAPK pathway may regulate organelle disassembly and mitotic structures during G2/M phase transitions. For example, active ERK localization to the mitotic kinetochore may regulate proteins
involved in chromosome segregation during metaphase to anaphase transition (28, 29).

A predisposition to gain or lose whole chromosomes in colorectal cell lines has been linked to abnormalities in the mitotic checkpoint (48), which is activated by the presence of unattached kinetochores (49). Given the key role for RAS in mitotic spindle assembly in fission yeast (50) and the fact that the majority of spindle checkpoint genes are highly conserved between higher and lower eukaryotes, it not surprising that expression of oncogenic RAS relaxes the mitotic spindle checkpoint. To our knowledge this is the first report demonstrating that expression of HRASV12 promotes bypass of this important checkpoint. In Schizosaccharomyces pombe, RAS1, a RAS homolog, signals through two major pathways that appear to regulate distinct functions: mating (through Byr2, functionally analogous to RAF) and cytoskeletal organization (through SCD1, a putative guanine nucleotide exchange factor activating CDC42, functionally analogous to a RHO-like GTPase). SCD1 in turn forms a complex with MOE1 and localizes to the spindle during mitosis (51). Double mutants (MOE1 with RAS1 or SCD1) accumulate in early mitosis and have severe spindle assembly defects, suggesting that the RAS1-SCD1 pathway is required for mitotic functions. Interestingly, SCD1 overexpression also results in spindle damage (52). These data point to potential mechanisms by which constitutively activated RAS could disrupt chromosomal stability.

The response to DNA damage is an essential surveillance system to maintain genomic integrity. The ATM kinase is a central transducer of this response. Lack of a functional ATM kinase, such as occurs in the ataxia telangiectasia syndrome, results in chromosomal instability and a predisposition to cancer. Recently, DNA damage has been shown to activate ERK through events downstream of ATM (53, 54). Moreover, ERK appears to be required for appropriate function of the DNA damage checkpoint, because cells are unable to recover in a timely fashion from ionizing radiation-induced G2 arrest when MEK2 activation is blocked (25). The apparent roles for MEKs and ERK in the G2 checkpoint suggest that this checkpoint could be affected by expression of constitutively active RAS. Indeed, the irradiation-induced arrest of PCCL3 thyroid cells in G2 was relaxed by expression HRASV12. In contrast to HRASV12-induced bypass of the mitotic spindle checkpoint, bypass of the G2 DNA damage checkpoint was dependent on activation of MEK/ERK pathway, because the checkpoint was restored with the addition of the MEK inhibitor PD98059. However, we cannot fully rule out the possibility that in the presence of nocodazole the MEK inhibitor blocks cells in G2 and that this is responsible for the decrease in mitotic
 cells rather than a bypass of the checkpoint. However, activation of the MEK/ERK pathway was not sufficient for this effect. Thus, G2 arrest was not dampened by dox-inducible expression of HRASV12,C35, BRAFV600E, or MEK1 S217E/S221E. These results, together with the lack of G2 checkpoint bypass following conditional expression of the RAS effector mutants HRASV12,C37 and HRASV12,C40, which selectively activate RAL-GDS and PI3K, respectively, indicate that signaling through any single RAS effector is not sufficient to compromise the G2 DNA damage checkpoint. In this respect, our data differs from that of Agapova et al. (24). Whereas they also noted that expression of mutant HRAS in human MDAH041 fibroblasts and Saos-2 osteosarcoma cells attenuated G2 arrest following DNA damage, they observed that signaling via RAL-GDS could account for this effect. The explanation for the discrepancy is not clear. However, there are experimental differences that could be responsible: 1) The cell lines used by Agapova et al. are p53-deficient, whereas PCCL3 cells are not (31). 2) The two studies used different DNA damaging stimuli (radiation versus an alkylating agent or a topoisomerase inhibitor), which could signal G2 arrest through different pathways. Indeed, ATM and ATR (ATM- and RAD3-related), two

FIGURE 5. PD98059 slows progress of PCCL3 cells through G2/M. Ras-25 and rtTA cells were synchronized in G1/S and released as described for Fig. 1A, except that where indicated 35 or 70 μM PD98059 was added to releasing medium. At the indicated times, the cells were harvested, fixed, and stained with FITC-conjugated anti-BrdUrd IgG and PI. FACS analysis was used to determine the number of BrdUrd-positive cells at each stage of the cell cycle. A, FACS analysis of BrdUrd-labeled rtTA cells shows that PD98059 evoked a concentration-dependent prolongation of G1/M and delayed exit from mitosis. B, corresponding FACS histograms from experiment shown in A. C, FACS analysis of BrdUrd-labeled Ras-25 cells shows 70 μM PD98059 inhibition of Ras-induced acceleration through G1/M. D, corresponding FACS histogram from experiment shown in C. Similar results were obtained in an additional independent experiment.
P38-like kinases that initiate the signaling process in response to DNA damage, are differentially activated by different types of DNA damaging agents (55, 56). Finally, there may be tissue and/or species difference in the effector pathways required.

Because an effector mutant that selectively activates more than one RAS effector pathway is not available, it was not possible to investigate further which RAS effectors are sufficient to promote bypass of the G2 DNA damage checkpoint. However, the observation that the addition of either wortmannin or PD98059 prevented the HRASV12-induced DNA damage checkpoint, would disable this protective mechanism. Conceivably early oncogenic events, such as RAS mutations, may under some conditions also promote functional defects in the DNA damage and mitotic checkpoints and thus favor tumor progression.

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