Ras Mediates Radioresistance through Both Phosphatidylinositol 3-Kinase-dependent and Raf-dependent but Mitogen-activated Protein Kinase/Extracellular Signal-regulated Kinase Kinase-independent Signaling Pathways

Theresa M. Grana, Elena V. Rusyn, Hong Zhou, Carolyn I. Sartor, and Adrienne D. Cox


ABSTRACT

Cells transformed by the oncogenic small GTPase, Ras, display a radioresistant phenotype in response to ionizing radiation (IR). To determine the mechanisms by which Ras mediates radioresistance in epithelial cells, we assessed the importance of three major survival pathways that can be activated by Ras [phosphatidylinositol 3-kinase (PI3-K) > Akt, nuclear factor kB (NF-kB), and Raf > mitogen-activated protein kinase/extracellular signal-regulated kinase kinase (MEK) > extracellular signal-regulated kinase] as necessary or sufficient for Ras-mediated radioresistance in matched pairs of RIE-1 rat intestinal epithelial cells expressing oncogenic Ras or empty vector (RIE-Ras and RIE-vector). Inhibiting PI3-K with LY294002 sensitized RIE-1 cells to IR in a dose-dependent manner, indicating that PI3-K is necessary for radioresistance, whereas inhibition of NF-kB with the super-repressor Iskbe had little effect on survival. Expression of either the constitutively active catalytic subunit of PI3-K, p110caax, or the Raf effector domain mutant 12V/40C, which retains binding to PI3-K but is impaired in binding to other Raf effectors, was sufficient to confer partial radioresistance. Expression of either a constitutively active form of the serine/threonine kinase Raf-I or the Ras effector domain mutant 12V/35S, which retains binding to Raf but is impaired in binding to other Raf effectors, was also sufficient to confer partial radioresistance. Surprisingly, however, even complete inhibition of MEK activity by using U0126 resulted in no change in post-IR survival whatsoever. Thus, whereas Raf contributes to Ras-mediated radioresistance, this is accomplished through a MEK-independent pathway. Taken together, these results indicate that multiple pathways, including both PI3-K-dependent and Raf-dependent but MEK-independent signaling, are required for Ras-mediated radioresistance in epithelial cells. Finally, we demonstrate that Ras-mediated radioresistance can be uncoupled from Ras-mediated transformation, in that PI3-K is required for radioresistance but not transformation, whereas MEK and NF-kB are required for transformation but not radioresistance in RIE-1 epithelial cells.

INTRODUCTION

The goal of radiation therapy in cancer treatment is to ensure that tumor cells die outright or, at a minimum, become incapable of proliferation (i.e., of reproductive or clonogenic survival). Therefore, understanding the properties of tumor cells that increase or decrease their responsiveness to radiation is key to improving radiation therapy. The cellular response to radiation is complex: the balance between death, arrest, and survival is tipped by the presence or absence of signaling through specific pathways whose identities are not yet clear. Signaling from the small GTPase, Ras, has been under intense investigation in the past decade due to the importance of Ras as a regulated switch mediating pathways that control transcriptional activation of genes governing such critical and diverse cellular functions as proliferation, differentiation, and apoptosis (1, 2). Oncogenic mutations in Ras occur frequently in many types of cancer, and oncogenically mutated Ras can also confer radioresistance (3–10).

Evidence for Ras-mediated radioresistance has been demonstrated both in rodent model systems, including NIH 3T3 fibroblasts, REF fibroblasts, rat rhabdomyosarcomas, and other cell types (4–7, 9), and in human tumor cell lines including EJ Ras-transformed bladder carcinoma, DLD-1 colon carcinoma, and HT1080 fibrosarcoma (8, 10). However, overexpression of Ras or the presence of activated Ras does not increase radioresistance in all contexts (11–13). Nevertheless, blocking Ras activity by using pharmacological inhibitors or anti-Ras neutralizing antibodies or by genetic loss of the activated ras allele from a tumor cell line has been shown to increase radiosensitivity in certain contexts (9, 10, 14, 15). Thus, the preponderance of evidence suggests that the activity of oncogenic Ras induces radioreistance and that interfering with signaling from Ras increases radiosensitivity.

Whereas oncogenic Ras can clearly tip the balance toward survival and radioresistance, the signaling pathways by which this is accomplished are not fully understood and are likely to be cell type dependent. The fact that oncogenic transformation and radioresistance can be uncoupled is shown by the inability of the transforming oncogenes Myc and Abl to confer radioresistance (7). Therefore, although several signaling pathways downstream of Ras have been fairly well delineated, and their contribution to Ras-mediated transformation has been well studied, it remains unclear which of these pathways also contribute to Ras-mediated radioresistance, particularly in epithelial cells, from which the majority of human cancers arise. Likely candidates include the proliferative pathway Raf-MEK-ERK, the survival pathways PI3-K-Akt and NF-kB, and stress response pathways involving JNK and p38 MAPKs.

Several of these Ras-mediated signaling pathways are up-regulated in response to IR. For example, the proliferative Raf-ERK kinase cascade is activated by IR (2–5 Gy) in laryngeal squamous carcinoma cells, in U937 human myeloid leukemia cells, and in MCF-7 and MDA-MB-231 mammary carcinoma cells (16–19). The stress-response MAPK, JNK, has been reported to be activated in NIH 3T3, Jurkat, and U937 cells in response to very high doses of IR (20–100 Gy; Refs, 20–22) and also after a more clinically relevant 2-Gy dose in MDA-MB-231 mammary carcinoma cells and A431 squamous carcinoma cells (19, 23). Another stress response MAPK, p38, has also been shown to be activated in a variety of cell types, including

The abbreviations in use are: MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; SAK, stress-activated protein kinase; PI3-K, phosphotidylinositol 3-kinase; JNK, Jun NH2-terminal kinase; phospho, phosphorylated; IR, ionizing radiation; NF-kB, nuclear factor kB; GFP, green fluorescent protein; TBS/T, 50 mM Tris, 150 mM NaCl, and 1% (v/v) Tween 20; REF, rat embryo fibroblast.

Received 12/26/01; accepted 5/8/02.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported by NIH Grants CA67771, CA76092 (to A. D. C.), and CA83733 (to C. I. S.). T. M. G. was supported by a NIH Cancer Cell Biology Training Grant.

2 To whom requests for reprints should be addressed, at Department of Radiation Oncology, University of North Carolina at Chapel Hill, Campus Box 7312, Chapel Hill, NC 27599-7512. E-mail: cox@rad onc.unc.edu.
293 human embryonic kidney cells, SW480 colon carcinoma cells, HeLa cervical carcinoma cells, and U2OS osteosarcoma cells (24–26), and such activation has been proposed to increase radiation-induced apoptosis. Finally, IR has been shown to activate NF-κB gene expression in several cell types as well (27, 28).

Whether these Ras-regulated pathways that are altered by IR also contribute to Ras-mediated increases in radiation survival remains unclear. Antisense Raf increased radiosensitivity and caused tumor regression in irradiated SQ-20B laryngeal carcinoma-bearing athymic nude mice (29, 30), but whether this pathway specifically mediates radioresistance downstream of oncogenic Ras was not examined. Some studies have shown that inhibition of MEK causes radiosensitization, whereas others have shown that MEK is dispensable for radioresistance in several carcinoma cell lines (31, 32).

We examined the activation of and requirement for several Ras-mediated signaling pathways in response to IR, using the RIE-1 rat intestinal epithelial model system. To isolate specific signaling pathways in the radiosponse phenotype, we used pharmacological inhibitors as well as ectopic expression of effector domain mutants of Ras and of downstream Ras effectors. Our results suggest that PI3-K is a major contributor, but not the sole contributor, to Ras-mediated radioresistance and that a novel MEK-independent pathway downstream of Raf also plays an important role. Furthermore, our results also demonstrate specific ways in which signaling to Ras-mediated radioresistance can be uncoupled from Ras-mediated transformation.

MATERIALS AND METHODS

Molecular Constructs. The pBABEpuro vector is described in Ref. 33. pBABE-H-ras(12V), N-ras(12D), K-ras(12V), H-ras(12V,35S), H-ras(12V,37G), and H-ras(12V,40C) constructs were a gift from Aidan Coffey, Vanderbilt University, Nashville, TN) were maintained in monolayer culture in DMEM-H (Life Technologies, Inc., Gaithersburg, MD) supplemented with 5% fetal bovine serum, 50 units/ml penicillin, and 50 mg/ml streptomycin at 37°C in a humidified atmosphere of 90% air and 10% CO2.

Cells. RIE-1 rat intestinal epithelial cells (Ref. 39; a gift of Robert J. Coffey, Vanderbilt University, Nashville, TN) were maintained in monolayer culture in DMEM-H (Life Technologies, Inc., Gaithersburg, MD) supplemented with 5% fetal bovine serum, 50 units/ml penicillin, and 50 mg/ml streptomycin at 37°C in a humidified atmosphere of 90% air and 10% CO2. Cells were transfected with the pBABE plasmids using Fugene (Boehringer Mannheim, Indianapolis, IN) according to the manufacturer’s instructions, and stable cell lines were established by selection in 2 μg/ml puromycin (Life Technologies, Inc.).

Clonogenic Survival Curve Assays. RIE-1 cells stably expressing Ras or empty vector were plated at low density and irradiated with graded, single doses from a Co60 Theratron irradiator (Atomic Energy of Canada, Limited) at a dose rate of 100 cGy/min. The culture medium was changed immediately after IR. After 2 weeks of incubation, samples were fixed in methanol/acetic acid (3:1, v:v) and stained in 1% crystal violet, and the number of viable colonies (defined as those colonies containing ≥50 cells) per dish was counted. Surviving fraction was calculated from the number of colonies formed in the irradiated dishes compared with the number formed in the unirradiated control, where plating efficiency is defined as the percentage of cells plated that form colonies, and surviving fraction = number of colonies formed/(number of cells plated × plating efficiency). To assure that plating efficiency was not affected by pharmacological inhibitors, we counted the number of colonies formed on control, inhibitor-treated but mock-irradiated dishes. The lack of effect of inhibitors on plating efficiency was expected because the cells are plated the day before irradiation and are not exposed to inhibitors until immediately before and during irradiation. Each point on the survival curve represents the mean surviving fraction from at least two dishes. The curve was fitted to the linear quadratic model \( S = e^{-\alpha D + \beta D^2} \) using a macro for KaleidaGraph, kindly supplied by Dr. Eric Bernhard (Department of Radiation Oncology, University of Pennsylvania) and originally based on a similar program developed by Dr. Michael Joiner, who was then at the Gray Laboratory, Northwood, United Kingdom.

Clonogenic Survival in the Presence of Pharmacological Inhibitors. RIE-1 cells were incubated with pharmacological inhibitors of PI3-K (LY294002; Biomol, Plymouth Meeting, PA), MEK (U0126; Alexis, San Diego, CA), or p38 MAPK (SB203580; Calbiochem, San Diego, CA) for 25 min and then exposed to a single dose of 7 Gy of IR. The concentrations of inhibitors used are shown in the figure legends. Inhibitors were removed 10 min after irradiation through replacement of the culture medium. Cells were cultured for 14 days before fixation, staining, and counting. The number of colonies formed in inhibitor-treated dishes was compared with the number of colonies formed in the vehicle (DMSO)-treated dishes. The absolute surviving fraction in each case was normalized to that of a “no drug-no irradiation” control.

Western Blotting. Protein expression levels and phosphorylation status were determined by Western blot analysis. RIE-1 cells stably expressing H-Ras(12V) or empty vector were starved for 24 h in 0.5% serum, exposed to a single dose of 7 Gy of IR, and then lysed at various times as shown. For inhibitor treatment, cells were starved for 24 h in 0.5% fetal bovine serum, treated with an inhibitor or an equivalent amount of vehicle (DMSO) for 30 min, and then exposed to 7 Gy of IR. Cells were lysed in Triton X-100 lysis buffer [50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1% (v/v) Triton X-100, 10% (v/v) glycerol, 1 mM sodium orthovanadate, 10 mM p-nitrophenyl phosphate, 20 mM β-glycerophosphate, 0.5 mM Pefabloc, 5 μg/μl leupeptin, and 10 μg/ml aprotonin] at 55 min after drug treatment (25 min after IR). Protein concentration in the lysates was determined using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA). Twenty μg of protein were loaded for each sample. Proteins were separated by SDS-PAGE on 10% SDS, 15% acrylamide low cross-linker gels for anti-ERK blots (40) and on Bio-Rad Criterion precast gels 12.5% Tris-HCl for all other probes. Separated proteins were electroblotted onto polyvinylidene difluoride membranes and blocked in TBS/T containing 5% nonfat powdered milk. Primary and secondary antibodies were added sequentially. After washing with TBS/T, the blots were developed using SuperSignal chemiluminescent substrate (Pierce, Rockford, IL).

Antibodies. Primary antibodies were all rabbit polyclonal antibodies diluted 1:1,000 in Tris-buffered saline/Tween/5% BSA and were as follows: anti-phospho-Akt (specific for Akt phosphorylated at serine 473), anti-Akt, anti-phospho-p38 MAPK, and anti-p38 MAPK (Cell Signaling, Beverly, MA); anti-ERK (sc-94; Santa Cruz Biotechnology, Santa Cruz, CA); and anti-phospho-JNK and anti-JNK (Promega, Madison, WI). For all primary antibodies, the secondary antibody used was horseradish peroxidase-conjugated antirabbit IgG (Amersham, Arlington Heights, IL) diluted 1:30,000 in TBS/T + 5% nonfat milk.

RESULTS

Ras Confers Resistance to Radiation in RIE-1 Epithelial Cells. Activated Ras has been shown to confer radioresistance to the majority of cell types examined. Many of these have been of fibroblast origin, whereas most human tumors arise from cells of epithelial origin. To determine whether Ras also induces radioresistance in RIE-1 rat intestinal epithelial cells, standard clonogenic survival assays were carried out using RIE-1 cells stably transformed by oncogenic H-Ras(12V) (designated RIE-Ras) and compared with survival in matched control RIE-1 cells expressing only empty vector (RIE-vector). As shown in Fig. 1, for each dose given, RIE-Ras cells consistently survived better than RIE-vector cells, demonstrating that Ras does confer radioresistance in RIE-1 cells. For our additional studies with inhibitors, we chose a dose of 7 Gy because, as shown in Fig. 1, this dose produces a modest amount of cell death (surviving fractions of between approximately 0.2 and 0.5) and also falls on the exponential portion of the survival curve.
IR Alters the Phosphorylation Status of the Serine/Threonine Kinases Akt and ERK1/ERK2, but not the JNK or p38 SAPKs. In many cell types, Ras up-regulates both the PI3-K-Akt survival pathway and the Raf\textgreater MEK\textgreater ERK proliferative/survival pathway in response to serum stimulation. To determine whether Ras also alters signaling to the PI3-K or ERK pathways in RIE-1 cells in response to radiation, we exposed RIE-Ras and RIE-vector cells to 7 Gy of IR and examined the phosphorylation status of downstream kinases at various time points by Western blot analysis.

The Raf\textgreater MEK\textgreater ERK pathway has been shown to enhance survival after a variety of cellular stresses, but the nature of its response to IR has not been established. Because the level of phosphorylation of the PI3-K downstream target Akt can be used as an indirect measure of PI3-K activity, we determined the levels of phospho-Akt after IR. We observed that these are responsive to IR in a biphasic manner (Fig. 2A): immediately after IR, phospho-Akt levels drop below the basal level in both RIE-Ras and RIE-vector cells. They begin to rise again at 30 min and 10 min in RIE-vector and RIE-Ras cells, respectively. Phospho-Akt levels rise to near-basal levels at 60 min and then decrease again by the 2 h time point, suggesting that phosphorylation of Akt is dynamically regulated after IR. In response to IR, RIE-Ras cells down-regulate phospho-Akt to a lesser extent than RIE-vector cells. RIE-Ras cells display higher levels of phospho-Akt than RIE-vector cells both basally and in response to IR, indicating that the PI3-K\textgreater Akt survival pathway may contribute to Ras-mediated radioresistance.

The Raf\textgreater MEK\textgreater ERK pathway also has been implicated in downstream responses to IR in some contexts. Therefore, we assessed activation of this pathway after IR in RIE-1 cells by Western blotting for the phosphorylated, activated forms of ERK1 and ERK2. As expected, phospho-ERK levels were higher basally in RIE-Ras cells than in RIE-vector cells. Increases in phospho-ERK were seen as early as 10 min after IR in RIE-Ras cells and 15 min after IR in RIE-vector cells (Fig. 2B). Phospho-ERK levels began to drop by 4 h after IR in RIE-vector cells but remained higher than the basal level in RIE-Ras cells for at least 4 h. IR activated ERK1 and ERK2 in both RIE-Ras and RIE-vector cells, with higher levels of activation in RIE-Ras cells, indicating that the Raf\textgreater MEK\textgreater ERK pathway may also be important for radioresistance.

Ras also regulates the JNK and p38 MAPKs. Because JNK and p38 are up-regulated in response to many cellular stresses, including UV radiation, they might also be up-regulated in response to IR. Therefore, we examined the phosphorylation status of JNK and p38 MAPK after IR (Fig. 2, C and D, respectively). We detected no phosphorylation of either JNK or p38 MAPK in response to IR, although we could demonstrate that these SAPKs can be phosphorylated in RIE-1 cells in response to the cellular stressor anisomycin (Fig. 2, C and D). Therefore, JNK and p38 MAPKs can be phosphorylated in RIE-1 cells but are not phosphorylated after IR under these conditions.

Signaling through PI3-K Is Necessary for Ras-mediated Radioresistance in RIE-1 Epithelial Cells. To determine which signaling pathways are necessary for Ras-mediated radioresistance in RIE-1 epithelial cells, we used pharmacological inhibitors of specific signaling pathways downstream of Ras. Western blot analysis was used to determine the effectiveness of the inhibitors at the concentrations used, and clonogenic survival assays of colony formation after 7 Gy...
RalGDS and PI3-K signaling pathways. The effector domain mutant RalGDS is not involved in conferring radioresistance but that additional activities are required. Survival of RIE-37G cells was similar to that of RIE-vector cells, implying that RalGDS is not involved in radioresistance in this context. We conclude that Ras uses multiple signaling pathways to confer radioresistance and that no one pathway can be singled out as solely sufficient for Ras-mediated radioresistance by this method.

We therefore also used a complementary approach by stably expressing constitutively activated forms of PI3-K and Raf-1 in RIE-1 cells and analyzing the consequences to radioresistance via clonogenic assays. Both a constitutively active form of PI3-K, p110α-CAAX, and a constitutively active form of Raf-1, ΔRaf22W, conferred radioresistance intermediate to that of RIE-Ras versus RIE-vector (Fig. 4). These results are in agreement with those of the Raf effector domain mutants and demonstrate that both the PI3-K and Raf pathways are partially sufficient for Ras-mediated radioresistance in this context.

**Signaling through MEK Is Not Necessary for Ras-mediated Radioresistance.** Because both RIE-35S and RIE-Raf cells display a phenotype of intermediate radioresistance and because Ras signaling to ERK is up-regulated in response to IR, we expected that the complete pathway of Raf→MEK→ERK would mediate radioresistance in RIE-1 cells. However, to our surprise, the MEK inhibitor U0126 did not affect survival in clonogenic assays despite complete inhibition of ERK phosphorylation. Because MEK phosphorylates and activates ERK, inhibition of MEK activity by U0126 should block in signaling to Raf and PI3-K. The effector domain mutant H-Ras(12V,40C) retains signaling through PI3-K but is impaired in signaling to Raf and RalGDS. To determine which signaling pathways were sufficient for Ras-mediated radiation resistance, RIE-1 cell lines stably expressing each of these effector domain mutants, RIE-H-Ras(12V,35S) (RIE-35S), RIE-H-Ras(12V,37G) (RIE-37G), and RIE-H-Ras(12V,40C) (RIE-40C), were established and analyzed for radioresponse in clonogenic survival assays. Both RIE-35S and RIE-40C conferred radioresistance intermediate between that of RIE-vector and RIE-Ras cells at lower doses of radiation (1–6 Gy), whereas at higher doses (10–15 Gy), cells expressing the effector domain mutants were not more radioresistant than RIE-vector cells (data not shown). These results indicate that Ras activation of either Raf (i.e., RIE-35S) or PI3-K (RIE-40C) may be partially sufficient to confer radioresistance but that additional activities are required. Survival of RIE-37G cells was similar to that of RIE-vector cells, implying that RalGDS is not involved in radioresistance in this context. We conclude that Ras uses multiple signaling pathways to confer radioresistance and that no one pathway can be singled out as solely sufficient for Ras-mediated radioresistance by this method.

![Fig. 3. Constitutively activated PI3-K and Raf are each partially sufficient to confer radioresistance compared with constitutively activated Ras. Radiation survival curves for stable RIE-1 cell lines expressing empty vector or constitutively activated H-Ras(12V), PI3-K (p110α-CAAX), or Raf-1 (ΔRaf22W) were obtained by the same procedures as described in the Fig. 3 legend and outlined in “Materials and Methods.” The ability of both PI3-K and Raf to induce similar clonogenic survival intermediate between that of Ras and vector indicates that neither of these Ras effectors can substitute completely for Ras signaling.](cancerres.aacrjournals.org/article-fig4.jpg)
Ras-mediated radioresistance, we blocked NF-κB translocation to the nucleus under these conditions (data not shown). We then performed clonogenic survival assays in the presence or absence of Ad-SR-IκB (Fig. 7). In contrast to inhibition of the PI3-K survival pathway by LY294002, which resulted in decreases in survival of >40% (Fig. 3B), inhibition of NF-κB resulted in only a small decrease in clonogenic survival. Indeed, the presence of the negative control adenovirus created a greater difference in survival than did the additional expression of the inhibitory SR-IκB. This was true regardless of whether the negative control adenovirus expressed only GFP (as in Fig. 7) or only lacZ (data not shown). Thus, we conclude that NF-κB plays only a minor role in Ras-mediated radioresistance in these cells.

Each Ras Isoform Confers Greater Radiation Resistance than Vector Only. Nearly all of the literature on Ras-mediated radioresistance focuses on H-Ras-transformed cells. The original study of Ras-mediated radioresistance found that H-, N-, and K-ras all induced radioresistance (3), but this study was done in NIH 3T3 fibroblasts, and it was unclear whether similar results would be observed in epithelial cells, from which most human cancers arise. In addition, one

phosphorylation of ERK. As expected, U0126 completely blocked phosphorylation of ERK1 and ERK2 (Fig. 5A) at both 1 and 10 μM in both RIE-Ras and RIE-vector cells. However, when cells were treated with 1 or 10 μM U0126 and irradiated with 7 Gy, the surviving fraction was not altered (Fig. 5B). Similar results were seen with the MEK inhibitor PD98059 (data not shown). Therefore, although Ras signaling through Raf is partially sufficient, and Ras signaling to ERK is up-regulated upon IR, MEK activation is not necessary for Ras-induced radioresistance in RIE-1 cells. Thus, a novel Raf-dependent, MEK-independent pathway mediates radioresistance in this epithelial cell type.

p38 MAPK Activity Is Also Not Necessary for Ras-mediated Radioresistance. Although we did not observe p38 MAPK activation after IR, it was still possible that basal levels of p38 MAPK activity were required. Thus, we tested whether inhibition of p38 MAPK activity with SB203580 altered radiation survival of RIE-vector or RIE-Ras cells. As shown in Fig. 6, p38 MAPK inhibition did not significantly alter clonogenic survival after IR. Although the levels of phospho-p38 MAPK in response to IR were too low to examine an effect of SB203580, the inhibitor could block p38 MAPK activity in response to anisomycin treatment (data not shown). Therefore, Ras does not mediate radioresistance by up-regulating p38 MAPK.

Inhibition of the NF-κB Survival Pathway Only Weakly Impairs Postirradiation Survival. Another major survival pathway downstream of Ras is that mediated by the antiapoptotic transcription factor NF-κB. To determine the relative importance of NF-κB in Ras-mediated radioresistance, we blocked NF-κB activation by use of an adenovirus expressing the super-repressor SR-IκBα, a nongravable mutant form of inhibitory κB (S32A/S36A) that locks NF-κB in the cytoplasm. Electrophoretic mobility shift assays for the presence of NF-κB in nuclear lysates of RIE-Ras and RIE-vector cells in the presence or absence of Ad-SR-IκB confirmed that the super-repressor blocked NF-κB translocation to the nucleus under these conditions (data not shown).
study in a melanoma cell line showed that the presence of an oncogenic form of N-Ras could make cells more sensitive to IR, rather than more resistant (45). Therefore, we compared the relative radioresistance of a matched panel of RIE-1 epithelial cells transformed by oncogenic H-, N-, and K-Ras. In clonogenic survival assays, we observed that each of the three oncogenic Ras isoforms conferred increased survival over RIE-vector cells (Fig. 8). H-ras-transformed RIE cells were the most radioresistant at the clinically relevant dose of 2 Gy, with the remaining Ras isoforms showing intermediate resistance. Thus, all three major isoforms of Ras can confer radioresistance to RIE-1 epithelial cells. This finding is consistent with the ability of all three Ras isoforms to activate both PI3-K and Raf.

**DISCUSSION**

**Ras Makes RIE-1 Cells More Resistant to IR.** We observed that transformation by each of the activated Ras isoforms, H-Ras(12V), N-Ras(12D), and K-Ras(12V), makes RIE-1 epithelial cells more resistant to radiation. Conflicting reports from other groups led us to investigate the ability of each of the Ras isoforms to enhance radiation resistance. Most previous studies have focused on H-Ras; several have shown that the activated forms of H-Ras and K-Ras mediate radioresistance in both transformed rodent and human tumor cell lines (3–8). However, that is not invariably the case; in some circumstances, oncogenic Ras has even been reported to radiosensitize cells (11–13). We show here that each of the activated Ras isoforms does mediate radioresistance in RIE-1 epithelial cells. DLD-1 colorectal carcinoma and HT1080 fibrosarcoma cell lines bearing oncogenically mutated K- and N-ras, respectively, were recently reported to be radiosensitive compared with their variant daughter cell lines, in which oncogenic ras had been deleted (10). These results are in agreement with our data showing that each Ras isoform possesses activity that enhances cell survival after IR.

**Activation of the PI3-K Survival Pathway Is a Critical Component of Ras Signaling That Is Both Necessary and Partially Sufficient for Radioresistance.** The PI3-K survival-associated signaling pathway allows cells to better tolerate a variety of cellular insults, including serum deprivation, extracellular matrix detachment, and DNA damage (46–50). Ras is known to activate the PI3-K>Akt pathway, but it was not clear whether PI3-K would play a role in Ras-mediated radioresistance because PI3-K is thought to promote survival by suppressing apoptosis (51). For most cells exposed to IR, death while attempting to divide, i.e., clonogenic death, rather than apoptotic death, is the predominant mechanism of cell killing (52). Now we demonstrate that PI3-K also is a critical component that is both necessary and partially sufficient for Ras-mediated radioresistance in RIE-1 epithelial cells.

Using clonogenicity (colony formation) as an end point, we show here that blocking PI3-K activity with the inhibitor LY294002 sensitized RIE-Ras cells to IR, i.e., that PI3-K is necessary for radioresistance. The observation that PI3-K is partially sufficient for radioresistance is supported here by two different lines of evidence. First, the Ras effector domain mutant 40C that retains binding to PI3-K but is impaired in binding to other Ras effectors confers partial radioresistance. Second, expression of a constitutively activated form of PI3-K, p110α-CAAX, confers partial radioresistance. These results are consistent with the ability of Ras to use PI3-K as an immediate downstream effector target and to up-regulate PI3-K activity to increase cellular survival.

In RIE-Ras cells, the PI3-K inhibitor LY294002 reduced PI3-K activity but failed to return it to the basal level seen in RIE-vector cells. This is consistent with the lesser effect of LY294002 on the clonogenic survival of RIE-Ras cells and also supports the idea that Ras up-regulates PI3-K to enhance radioresistance. PI3-K-related kinase family members such as ATM, ATR, and DNA-PK are not inhibited at a concentration of 1 μM LY294002 as used here, nor are these proteins known to be activated by Ras. The observation that 1 μM LY294002 significantly decreased the survival for RIE-vector cells indicates that PI3-K is a major target of radiosensitization by the kinase inhibitor LY294002. It is also possible that Ras may activate additional, LY294002-insensitive isoforms of PI3-K that also contribute to the PI3-K activity remaining after LY294002 treatment and thereby contribute to radioresistance.

While this manuscript was in preparation, Gupta et al. (53) showed that blockade of PI3-K with LY294002 radiosensitizes Ras-transformed REFs and two human tumor cell lines containing activated ras alleles. Furthermore, expression of a constitutively active PI3-K in REFs increased radiation resistance. Our studies were carried out with epithelial cells, confirming and extending the finding that Ras uses PI3-K to enhance radiation resistance to multiple cell types. Indeed, PI3-K may play an even greater role in epithelial cells than in fibroblasts because in our study PI3-K inhibition resulted in decreased survival in both RIE-Ras and RIE-vector epithelial cells, whereas Gupta et al. (53) observed that PI3-K inhibition decreased survival in Ras-transformed REFs, but not in normal REFs.

**Akt Phosphorylation Is Regulated Dynamically after IR.** Unexpectedly, we observed that phospho-Akt levels were regulated in a dynamic manner after irradiation, including a dramatic drop in phospho-Akt in as short a time as 5 min after IR. The rapid kinetics of changes in Akt phosphorylation after IR have not been reported previously, and they suggest the existence of an IR-regulated Akt-phosphatase. To date, no phosphatases that regulate Akt activity have been reported. It is not clear whether Akt is a necessary downstream component of the PI3-K activity that mediates radioresistance, but Akt phosphorylation is restored more rapidly and to a greater extent in RIE-Ras cells than in RIE-vector cells. Additional studies will be required to determine both the mechanism of rapid down-regulation of phospho-Akt and its role in radioresistance.

**The NF-κB Survival Pathway Is Not Critical for Ras-mediated Radioresistance in RIE-1 Cells.** NF-κB is a major survival pathway that is activated downstream of Ras. Although Ras requires NF-κB for transformation and IκBα blocks Ras focus formation (36), we show here that inhibition of NF-κB by use of the super-repressor IκBα had relatively little effect on post-IR survival in RIE-Ras or RIE-vector cells. A previous study showed that SR-IκBα moderately decreased...
clonogenic survival in HT1080 fibrosarcoma cells after a single 10-Gy treatment, although it is not known whether the presence of the oncogenic ras mutation (N-Ras61K) modulated this response (54). Furthermore, SR-IκBε only modestly decreased survival in several human tumor cell lines, whereas inhibition of NF-κB by use of the 26S proteasome inhibitor PS-341, which blocks the activity of both NF-κB and other proteins also regulated by proteosomal degradation, displayed somewhat better radiosensitizing activity (55). However, this effect was independent of ras mutation status. Taken together, these results suggest that NF-κB alone is not a significant contributor to Ras-mediated radioresistance, although proteosome inhibition can impair survival in a non-Ras-dependent manner.

The SAPKs JNK and p38 MAPK Do Not Play a Role in Ras-mediated Radioresistance. The SAPKs JNK and p38 MAPK are activated in response to multiple cellular stresses including tumor necrosis factor, osmotic stress, and heat shock (56, 57). Therefore, we expected that both JNK and p38 MAPK would also be activated in response to IR. However, neither JNK nor p38 MAPK phosphorylation was up-regulated by IR in either RIE-Ras or RIE-vector cells, and the p38 MAPK inhibitor SB203580 did not alter clonogenic survival of RIE-1 cells. These results were surprising because the SAPKs are responsive to DNA-damaging agents and are up-regulated by UV radiation (24). However, it has been shown recently that SAPKs are not always up-regulated by IR (26, 58), and evidence exists that there are cell type differences in SAPK induction (24). In addition, SAPKs are most often reported to be activated at doses of IR that are much higher (20–100 Gy) than the daily doses used for radiotherapy (2–3 Gy) and in our studies (1–7 Gy). Because neither JNK nor p38 MAPK was phosphorylated in response to IR in RIE-1 cells, and inhibition of p38 MAPK did not alter radiation survival, we conclude that Ras does not induce radioresistance through the SAPKs in RIE-1 cells.

Raf but not MEK Plays a Critical Role in Ras-mediated Radioresistance in RIE-1 Cells. Ras activates the Raf>MEK>ERK signal transduction pathway to promote cell proliferation (59) and to play a role in cell survival in some contexts (60–62). However, RIE-1 epithelial cells with MEK activity transiently blocked using the MEK inhibitors U0126 or PD98059 did not show any changes in clonogenic survival after irradiation. There are conflicting reports in the literature concerning the consequences of MEK inhibitor treatment on radioresistance. Others using the same MEK inhibitors have likewise reported that MEK activity is not necessary for radioresistance of REFs, bladder carcinoma cells, or squamous cell carcinoma cells (31, 32, 53). However, long-term treatment with the MEK inhibitors PD98059 and U0126 has been shown to radiosensitize A431 squamous carcinoma cells and MDA-MB-231 mammary carcinoma cells (19, 63). These distinctions may be due to cell type differences or to differences in MEK inhibitor exposure. These inhibitors reduce proliferation and thus may block colony formation in the absence or presence of radiation, as opposed to specifically radiosensitizing cells. Long-term treatment may also inhibit autocrine loops that regulate PI3-K activity and/or the activity of other molecules involved in radioresistance.

The lack of effect of MEK inhibitors on radioresistance has generally been interpreted to mean that the Raf>MEK>ERK kinase cascade is not relevant to radioresistance, thereby leading to a potentially confusing situation wherein the role of Raf in mediating radioresistance is unclear. It is certainly possible that cell type and context are the simple and final determinants of a requirement for Raf and that these vary greatly. Another, more unifying option may be that although MEK itself and thus the Raf>MEK>ERK cascade do not mediate radioresistance, the Raf-1 kinase plays a significant role but with different downstream consequences than MEK activation. Such a model could resolve the apparent discordance between the ability of Raf antisense constructs to radiosensitize cells (29, 30) and the lack of ability of MEK inhibitors to do the same thing.

We show here in two ways that Raf is partially sufficient for Ras-mediated radioresistance in RIE-1 epithelial cells. Both the Ras effector domain mutant 35S, which retains signaling through Raf but is impaired in signaling through other Ras effectors, and a constitutively active form of Raf, Δ22W, were able to induce radioresistance in RIE-1 epithelial cells. In addition, the basal levels of phosphorylated, active forms of ERK were increased in RIE-Ras cells compared with RIE-vector cells, and these were further increased in both cell lines by IR. Thus, Ras-mediated radioresistance proceeds at least in part through Raf. These results are in agreement with previous studies that have shown that Raf is up-regulated in response to IR in laryngeal squamous carcinoma cells (17) and that antisense Raf reduces survival in the same cell type (29, 30), although none of these reports concerns Raf regulation of radiosensitivity in the context of signaling downstream of Ras activation.

MEK-independent Signaling Downstream of Raf. Because we were able to block MEK activity without altering clonogenic survival, we concluded that MEK activity at the time of IR exposure does not play a role in Ras-mediated radioresistance of RIE-1 cells. Thus, although Raf is partially sufficient for Ras-mediated radioresistance, the signaling pathway downstream of Raf does not proceed via the conventional Raf>MEK>ERK kinase cascade, and instead, an unidentified non-MEK effector of Raf is critical in the context of RIE-Ras and radioresistance.

Several types of studies have suggested that Raf mediates both MEK-dependent and -independent signaling pathways. First, although both parental Ras and the 35S Ras effector domain mutant that retains Raf activation can inhibit myogenesis, blocking MEK with PD98059 fails to block this effect (64). Thus, Raf signaling can be Raf dependent but MEK independent. Second, a mutant of activated Raf engineered to disrupt MEK1/2 binding ([RafBXB(T481A)]) uncouples Raf from growth and morphological transformation but not from NF-κB or p90 S6 kinase activation (65). Thus, Raf signaling can be both MEK dependent and independent. Third, a murine genetic knockout of Raf-1 that has been restored with a Raf mutant (340F/341F) that fails to activate MEK nevertheless causes ERK phosphorylation and activation (66). Thus, Raf can activate ERK in a MEK-independent manner. Our present results suggest that a MEK-independent pathway downstream of Raf is also important in mediating radioresistance. Additional investigations will be required to determine the components of such a pathway.

Uncoupling of Ras-mediated Radioresistance and Transformation. Transformation by some oncogenes such as Abl and Myc is not sufficient to confer radioresistance (7, 9), but the signaling pathways that permit transformation but fail to induce radioresistance have not yet been identified for these oncogenes. We demonstrate here for the first time and in three distinct ways that the Ras-mediated radioresistant phenotype can be uncoupled from that of Ras-mediated transformation. First, although we show here that PI3-K is necessary and partially sufficient for Ras-mediated radioresistance in RIE-1 cells, inhibition of PI3-K with LY294002 does not impair Ras-induced soft agar growth or protect against suspension-induced cell death (anoikis) in RIE-1 cells (35), nor does activated PI3-K confer resistance to anoikis in this cell type (35). These results demonstrate that PI3-K is important for Ras-mediated radioresistance but not for Ras-mediated transformation in RIE-1 epithelial cells. Conversely, MEK is not necessary for Ras-mediated radioresistance in RIE-1 cells, as shown here by the complete lack of effect of MEK inhibition on postradiation survival, whereas it is crucial for Ras-mediated transformation in this same cell type, as shown by the ability of a MEK inhibitor to reverse Ras-mediated growth and morphological transformation (67). Finally,
like MEK. NF-κB plays a more important role in Ras-mediated transformation than in Ras-mediated radiosensitivity in RIE-1 cells. Although we show here that inhibition of NF-κB with the SR-1xκBα does little to alter post-IR survival, the same procedure has been shown to block Ras-induced morphological transformation (36). Therefore, Ras uses distinct effector pathways to mediate distinct biological consequences.

In conclusion, we have provided new evidence that Ras mediates radiosensitivity in RIE-1 epithelial cells through both PI3-K and a Raf-dependent pathway. The role of each of these Ras effector pathways in mediating radiosensitivity may be uncoupled from its role in mediating transformation. Our results support the use of inhibitors of both the PI3-K and Raf effectors as possible radiosensitizers and reinforce the importance of providing such inhibition at the level of the immediate downstream effectors of these Ras signaling pathways.

ACKNOWLEDGMENTS

We thank Drs. Eric Bernhard and Mike Joiner for the KaleidaGraph macros used in plotting the survival curves, Dr. Aidan McFall for sharing many reagents, Dr. Elaine M. Zeman for many helpful discussions, and Dr. Elaine M. Zeman and Patricia L. Joyce for critical reading of the manuscript.

REFERENCES


Ras Mediates Radioresistance through Both Phosphatidylinositol 3-Kinase-dependent and Raf-dependent but Mitogen-activated Protein Kinase/Extracellular Signal-regulated Kinase Kinase-independent Signaling Pathways

Theresa M. Grana, Elena V. Rusyn, Hong Zhou, et al.


Updated version  Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/62/14/4142

Cited articles  This article cites 65 articles, 37 of which you can access for free at:
http://cancerres.aacrjournals.org/content/62/14/4142.full.html#ref-list-1

Citing articles  This article has been cited by 27 HighWire-hosted articles. Access the articles at:
/content/62/14/4142.full.html#related-urls

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.