Mitogen-activated Protein Kinase Kinase 2 Activation Is Essential for Progression through the G2/M Checkpoint Arrest in Cells Exposed to Ionizing Radiation*

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An increasing body of evidence suggests that mitogen-induced activation of the RAF/ERK signaling pathway is functionally separate from the stress-induced activation of the SEK/JNK/p38 signaling pathway. In general, stress stimuli strongly activate the p38s and the JNKs while only weakly activating ERK1 and ERK2. However, a number of independent groups have now shown that the RAF/ERK signaling pathway is strongly activated by ionizing radiation. In this work, we examine this paradox. We show that both mitogen-activated protein (MAP) kinase kinase 1 (MEK1) and MAP kinase kinase 2 (MEK2) are activated by ionizing radiation. Blockage of this activation through the use of dominant negative MEK2 increases sensitivity of the cell to ionizing radiation and decreases the ability of a cell to recover from the G2/M cell cycle checkpoint arrest. Blocking MEK2 activation does not affect double-strand DNA break repair, however. Although MEK1 is activated to a lesser extent by ionizing radiation, expression of a dominant negative MEK1 does not affect radiation sensitivity of the cell, the G2/M checkpoint of the cell, or double-strand break repair. Because ionizing radiation leads to a different cell cycle arrest (G2/M arrest) than that typically seen with other stress stimuli, and because we have shown that MEK2 can affect G2/M checkpoint kinematics, these results provide an explanation for the observation that the MEKs can be strongly activated by ionizing radiation and only weakly activated by other stressful stimuli.

Double-strand DNA breaks (dsbs)1 are recognized as key components of the initiation of multi-step carcinogenesis. A number of cancer predisposition genes and oncogenes exert their effects through the cell signaling pathways initiated by dsbs. For example, the cancer predisposition genes ATM and DNA-PK are kinases that are activated by dsbs. Cells with mutations in these two genes show a decreased ability to repair dsbs, and it is thought that this inability leads to the genomic instability that is characteristic of cancers containing ATM or DNA-PK mutations (Refs. 1–3 and reviewed in Refs. 4 and 5). Whereas ATM and DNA-PK are required for the signaling initiated by dsbs, the cancer predisposition gene BRCA2 is required for the actual repair of dsbs. Cells that lack BRCA2 show decreased ability to survive ionizing radiation (6–8), and this inability to repair dsbs is central to BRCA2-initiated carcinogenesis (8–10). Several oncogenes also have similar effects on dsb signaling and dsb repair. The c-abl proto-oncogene has been shown to be activated upon the formation of dsbs via an interaction with DNA-PK (12–15), and the retroviral oncogene, FBR v-fos, has been shown to inhibit the response of the cell to ionizing radiation (16). All of these findings implicate dsb formation in the initiation of carcinogenesis.

Much of the work implicating dsb formation in the initiation of carcinogenesis has focused on the signaling pathways induced by dsbs (e.g. ATM/DNA-PK) and on the repair of those dsbs (e.g. BRCA2). Less is known about the signaling induced by the agent causing the dsb. The most commonly used agent to induce dsbs is ionizing radiation. Whereas dsbs are the most prominent effect of ionizing radiation exposure, ionizing radiation also causes lipid peroxidation, glutathione depletion, and protein oxidation (reviewed in Refs. 17 and 18). Thus, ionizing radiation activates the stress response pathway of the cell in a manner that is not necessarily dependent on the formation of dsbs (19).

Ionizing radiation will activate JNK (20), p38, SEK (21), and NF-κB (22), and it will induce the immediate early genes, c-jun, c-fos, and egr-1 (23–27). Although it is not surprising that ionizing radiation activates the stress response pathway, it is unexpected that the typically growth-responsive MAP kinase cascade (reviewed in Refs. 28–30) is also activated by ionizing radiation. Although exceptions exist (31), an increasing body of evidence suggests that the stress response pathway is functionally separate from the growth-responsive MAP kinase pathway (11, 32–36). The stress response pathway (SEK, JNK, and p38) is poorly activated by mitogens, whereas the MAP kinase pathway is generally poorly activated by stressful stimuli (UV radiation, osmotic stress, etc.) (11, 32–36). However, c-ras, c-RAF, ERK1, and ERK2 have all been shown by independent groups to be activated by ionizing radiation (37–41). It is unknown how activation of the MAP kinase pathway affects cellular survival in response to ionizing radiation, but the fact that activation has been seen so consistently suggests that activation of the MAP kinase cascade could be important for response of the cell to ionizing radiation.

The activation of the MAP kinase pathway by the stress...
stimulus of ionizing radiation could be due to the fact that dsbs lead to different cell cycle checkpoint control than what is normally seen for DNA-damaging agents (reviewed in Refs. 42 and 43). Repair of dsbs follows two pathways, nonhomologous recombinational repair (occurring in G1) and homologous recombination repair (44–47). Homologous recombinational repair occurs in G2, the time point when homologous, undamaged double-strand DNA is present to serve as a template for correct repair. Because the damaged DNA strand has a template for repair, homologous recombinational repair leads to fewer mutations than nonhomologous repair (44, 47, 48). Therefore, the G2/M checkpoint is essential for the proper repair of DNA damaged by ionizing radiation. Recently, the MAP kinase pathway has been implicated in G2/M cell cycle regulation. In Xenopus oocytes, MAP kinase activity has been shown to be necessary for progression through G2 (49–51). The MEK1 and MEK2 activator, c-mos, has also been shown to be necessary for progression through G2 (52), and in mouse oocytes, MAP kinase becomes activated at metaphase and localizes to the microtubule-organizing centers (53). Because the MAP kinase pathway is required for G2/M progression in a number of systems and because ionizing radiation leads both to activation of the MAP kinase pathway and to G2/M arrest, it is possible that ionizing radiation activates the MAP kinase cascade to exert an effect on G2/M checkpoint control.

To address these questions, we focused on MEK1 and MEK2, two components of the MAP kinase cascade. These two proteins are phosphorylated in vitro by RAF (54–55) and can both phosphorylate ERK1 and ERK2 (30). MEK1 and MEK2 are approximately 80% homologous and are very similar in size (MEK1 = 45 kDa; MEK2 = 46 kDa). They differ in the first 30 amino acids of the N terminus and in a proline-rich region that is only found in MEK1 (56, 57). In this work, we find that both MEK1 and MEK2 are specifically activated by ionizing radiation in a variety of cell lines. We show that cells that express dominant negative MEK2 show radiation hypersensitivity that is not seen in cells which equivalently express dominant negative forms of MEK1. Expression of dominant negative MEK2 leads to a slightly delayed G2/M arrest upon ionizing radiation exposure and a substantial inability to progress through the G2/M arrest upon recovery from that radiation exposure. Finally, we show that the effect of MEK2 on radiation sensitivity can be reversed by forcing G2 progression through the use of pharmacological agents. These data imply that ionizing radiation activates the MAP kinase cascade in a specific manner to maintain G2/M checkpoint fidelity.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—HeLa cells, NIH 3T3-L1 cells, and BzPC-3 cells were obtained from the American Type Tissue Collection (ATCC) and grown in Dulbecco’s modified Eagle’s media supplemented with 10% fetal bovine serum (Sigma), 2 mM L-glutamine (Sigma), and 1× antimycotic/antibiotic (Sigma). HBL100 cells were obtained from the ATCC and grown in McCoy’s medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum. Serum starvation was performed in Dulbecco’s modified Eagle’s media supplemented with 0.5% fetal bovine serum, 2 mM L-glutamine, and 1× antimycotic/antibiotic for 24 h followed by incubation in 0.1% fetal bovine serum for 24 h. Metabolic labeling was performed by incubating the cells in methionine-free media (Life Technologies, Inc.) for 30 min, followed by the addition of methionine-free media supplemented with 50 μCi of [35S]methionine for 3 h. Stably transfected cell lines were generated by transfecting HeLa cells at 80% confluence with 20 μg of indicated construct per ml of PEF4 (Inovotrogen), pREP4-K97A MEK1, pREP4-S222A MEK1, pREP4-R101A MEK2, pREP4-wt MEK2, pREP4-S222E MEK1) using the calcium phosphate precipitation method. Cells were split 1:3 the following day. Two days after transfection, selection was begun in 500 μg/ml hygromycin B (Life Technologies, Inc.). Three weeks later, approximately 1000 colonies were pooled and shown to express the desired protein by Western blotting. All cell lines were used within 2 weeks of establishment or within 3 weeks following liquid nitrogen thawing.

Plasmids—The plasmids used in this work are the gifts of Edwin Krebs, University of Washington, Seattle (K97A MEK1, S222A MEK1); Natalie Ahn, University of Colorado, Boulder (pCML-MEK2); and Kun-I Chen, University of Michigan, Ann Arbor (pGEX-MEK2). The K97A MEK and S222A MEK plasmids and the MEK1 vector pCDNA3.1 (Invitrogen). To subclone these genes into the pREP4 vector (Invitrogen), pCDNA-K97A MEK1, pCDNA-S222A MEK1, and pREP4 were digested with XhoI and HindIII (Life Technologies, Inc.). The K97A MEK1 fragment and the S222A MEK1 fragment was then ligated into pREP4. To generate the dominant negative K101A MEK2 construct, the MEK2 construct was digested with XhoI and HindIII (Life Technologies, Inc.) and ligated into pGEM4Z (Promega). Pgem4Z-MEK2 was then digested with PstI (Life Technologies, Inc.) to generate a 400-bp fragment containing the region to be mutated. This 400-bp PstI fragment was then ligated into pGEM7Z-400-bp MEK2 construct was then digested with BanII and NcoI (Life Technologies, Inc.), and an oligonucleotide containing the sequence 5′-CCAGGCGGCGTATCCACCTGCAGATAAGCCG-3′ was ligated into the digested MEK2. This oligonucleotide sequence contains a 2-base pair replacement (in bold) designed to mutate Lys-101 to alanine and a single base pair replacement (in italics) designed to insert an XhoI site without changing the amino acid sequence. The altered 400-bp fragment was then sequenced to ensure that the desired mutation was present and in frame. The plasmid containing the K101A MEK2 construct was then digested with PstI and the mutated 400-bp fragment was reinserted into pGEM4Z-MEK2. This plasmid was then sequenced to ensure that the mutation was present and in frame. In addition, in vitro translation of the protein gave a 46-kDa fragment that could be immunoprecipitated with an antibody to MEK2. The pGEM4Z-K101A MEK2 fragment was then digested with BanII and inserted into the pREP4 vector. All pREP4 plasmids used for transfections were purified by ultracentrifugation using CsCl gradients prior to transfection.

Immunoprecipitations and Kinase Assays—Immunoprecipitations and kinase assays were performed using a method described previously with slight modifications (58). Cells were washed twice in phosphate-buffered saline (PBS) and harvested by scraping. Cells were pelleted by centrifugation at 2000 rpm and resuspended in PBS followed by another 2000 rpm centrifugation. Cells were then lysed in Lysis Buffer (20 mM Tris (pH 7.5), 0.27 mM sucrose, 1 mM sodium orthovanadate (Sigma), 10 mM sodium β-glycerophosphate (Sigma), 0.5 μM okadaic acid (Life Technologies, Inc.), 50 mM NaF (Sigma), 5 mM sodium pyrophosphate (Sigma), 1% Triton X-100, 0.1% β-mercaptoethanol, 1 mM benzamidine (Sigma), 0.2 mM phenylmethylsulfonylfluoride (Sigma), 5 μg/ml leupeptin (Sigma), and 2 μg/ml aprotinin (Sigma)). The suspension was then sonicated briefly and centrifuged at 14,000 rpm at 4 °C for 10 min. The supernatant was then extracted. Protein concentration was determined using the Bio-Rad protein assay. 100 μg of total protein was then incubated with 10 μl of the given antibody (anti-N-terminal MEK1 or anti-N-terminal MEK2, and total volume was adjusted to 700 μl using Lysis Buffer) for 2 h at 4 °C. Protein A/G (Santa Cruz Biotechnology) was then added for 1 h. The suspension was then centrifuged, and the precipitate was washed five times with Lysis Buffer (the 2nd and 3rd lysis buffer contained 0.5 μM NaCl). For the metabolic labeling experiment, 2× SDS-PAGE buffer was added. The suspension was boiled and electrophoresed on a 10% SDS-PAGE gel. The gel was then dried and subjected to autoradiography. For the kinase assays, the precipitate was then washed an additional three times in Kinase Buffer (50 mM Tris (pH 7.5), 0.05% Brij 35 (Sigma), 0.1% β-mercaptoethanol, 0.5 μM okadaic acid (Life Technologies, Inc.), 0.27 mM sodium orthovanadate, and 10 mM magnesium chloride). After the washes, Kinase Buffer was added to the immunoprecipitate such that the total volume was 50 μl. 25 μl of this solution was added to a microcentrifuge tube containing 2.5 mg of OXT-K71A ERK1 (Upstate Biotechnology, Inc.) and incubated at 30 °C for 5 min. The kinase reaction was then initiated by the addition of 10 μl of 0.5 mM ATP supplemented with 1 μl of [γ-32P]ATP (NEN Life Science Products, 3000 μCi/ml) and allowed to incubate for 10 min at 30 °C. The reaction was terminated by the addition of 2× SDS-PAGE Sample Buffer followed by boiling. 10 μl of the kinase reaction was then electrophoresed through a 7% polyacrylamide gel, dried, and subjected to autoradiography. Counting was performed by passing the gel through a phosphor imager and using the Packard Electronic Autoradiography Instant Imager and comparing activity of the sample with the activity of the unstained sample.

Western Blotting—Cells were washed twice with PBS and then harvested by scraping. Cells were centrifuged at 2000 rpm, rinsed again with PBS, and centrifuged at 2000 rpm. The pellet was then resus-
pended in Lysis Buffer and allowed to sit on ice for 10 min. The suspension was then sonicated. The suspension was centrifuged at 14,000 rpm for 10 min, and the pellet was discarded. Western blotting was then performed as described previously (58). Briefly, protein concentration was standardized using the Bio-Rad Protein Assay. Equal amounts of protein were subjected to polyacrylamide gel electrophoresis (10% gel). Lysates were transferred to nitrocellulose filters (Amersham Pharmacia Biotech) for 1 h at 40 V. To ensure equal loading of protein and equal transfer efficiency, the membrane was stained with Ponceau S prior to blocking. The membrane was blocked using 5% nonfat dry milk, 0.3% Tween 20 in Tris-buffered saline. After blocking and subsequent washing, the blot was exposed to a 1:1000 dilution of the given antibody overnight at 4 °C. The blot was then washed extensively with Tris-buffered saline, 0.3% Tween 20 before being exposed to a horse-radish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology) at a dilution of 1:2500 at room temperature for 1 h. Bands were visualized using the ECL detection system (Amersham Pharmacia Biotech) using the manufacturer’s instructions. Antibodies used were anti-phosphorylated MEK1/2 (New England Biolabs), anti-nonphosphorylated MEK1/2 (New England Biolabs), anti-N-terminal MEK1 (Santa Cruz Biotechnology), and anti-N-terminal MEK2 (Santa Cruz Biotechnology).

Irradiation and Cell Survival Assays—Cells were irradiated using a 137 Cs source at a dose rate of 2.35 Gy/min. Colony forming assays were performed as described previously (16). Briefly, cells were plated at 500 cells/dish and irradiated at a given dose. After irradiation, cells were returned to the 37 °C incubator. They were refed fresh media every 2 days. After 14 days, the colonies were fixed in ethanol and stained with crystal violet. Colonies were counted manually. Relative survival was determined by comparing colony number of cells irradiated at a given dose to the colony number of unirradiated cells plated at the same density and cultured for the same amount of time.

For cellular survival following treatment with vanadate, approximately 2000 cells of each cell line (vector-only cells, K97A MEK1 cells, and K101A MEK2 cells) were plated 12 h prior to the commencement of the experiment. These cells were then treated with 50 μM vanadate (Sigma) for 8 h prior to irradiation. 16 h after irradiation with 5 Gy, cells were washed extensively and refed with normal media. All cell lines showed a strong G1 arrest by flow cytometry analysis. Three weeks later, cell survival was quantitated as described above (survival was compared with cells treated with vanadate in the same manner, but not irradiated). For cellular survival following treatment with caffeine, approximately 2000 cells of each cell line studied were plated as above. The cells were irradiated with 5 Gy and then treated with 2 mM caffeine (Calbiochem) 24 h after irradiation. 32 h after irradiation, flow cytometry showed recovery from G2 arrest in K101A MEK2 cells (relative to irradiated/no caffeine cells) and no difference between irradiated vector-only cells and K97A MEK1 cells either treated or untreated with caffeine (32 h after irradiation, vector-only cells and K97A MEK1 cells show normal cell cycle distribution; therefore, treatment with caffeine at 24 h has no effect on the cell cycle profile). Survival was measured as described above.

DNA Repair Assays—Equal cell numbers were embedded into agarose plugs as described previously (8). The plugs were then exposed to the indicated doses of ionizing radiation at 4 °C. To allow the cells to repair damaged DNA, the cell plugs were then cultured for the indicated period. The plugs were then digested overnight in a solution containing 10 mM Tris (pH 7.4), 20 mM NaCl, 50 mM EDTA, and 1 mg/ml proteinase K (Life Technologies, Inc.) at 50 °C. The plugs were then embedded in a 0.7% agarose gel and subjected to pulsed field gel electrophoresis at 3 V/cm, 45-s pulse time at 14 °C for 48 h (CHEF II system, Bio-Rad). Southern blotting was then performed using random prime labeled HeLa cell genomic DNA as a probe. Quantitation of repair was performed using the Packard Eclipsen Electric Autoradiography Instant Imager.

Flow Cytometry—Cells were plated to 40% confluency on 100-mm plates. 18 h later, the cells were exposed to 5 Gy ionizing radiation (except for the unexposed plate). At the indicated time point, the cells were trypsinized, centrifuged, and washed 2× with PBS, 1× 106 cells were then suspended in 1 ml of PBS and then reinfected with 5 μg/ml propidium iodide. 1 ml of Vindelov’s solution (10 mM Tris (pH 8.0), 10 mM NaCl, 0.7 units/ml RNase, 50 μg/ml propidium iodide, 0.1% Nonidet P-40) was then added, and the cells were allowed to incubate in the dark at 4 °C overnight. Flow cytometry was performed using a Becton Dickinson FACs Caliber using the Vanderbilt University Flow Cytometry Core. Data acquisition software is CellQuest Version 3.1. Excitation was with 488 nm air-cooled argon-ion laser at 15 milliwatts. Emission was collected at 585/42 nm band pass filter at a flow rate of 12 ± 3 μl/min. 10,000 events were measured per experiment.

RESULTS

MEK1 and MEK2 Are Activated by Ionizing Radiation—The MAP kinase kinases (MEKs) are a central component of the growth-response signaling pathway (reviewed in Refs. 29 and 30). Whereas a great deal of work has centered on their role in transmitting mitogenic signals, there is an increasing body of evidence that the MEKs could play a role in the response of the cell to the stress of ionizing radiation (37–39).Activation of MEK1 and MEK2 involves phosphorylation upon conserved serine residues (Ser-218 and Ser-222 on MEK1, Ser-222 and Ser-226 on MEK2; see Refs. 55 and 60). To test the activation of MEK1 and MEK2 upon ionizing radiation exposure, an antibody that specifically recognizes phosphorylated MEK1 and MEK2 was obtained. HeLa cells were serum-starved and exposed to increasing doses of ionizing radiation. As a control, one plate of cells was mock-irradiated (0 Gy). 10 min after exposure, lysates were generated, and Western blotting was performed using the antibody directed against either phosphorylated MEK1 or phosphorylated MEK2. Under these conditions, we were unable to distinguish between the similarly sized MEK1 and MEK2. However, the Western blot shown in the upper panel of Fig. 1 shows that in HeLa cells, the 45-kDa MEK1 and/or the 46-kDa MEK2 are phosphorylated in response to physiological doses of ionizing radiation (upper blot, Fig. 1). To determine whether this activation of MEK1 and MEK2 is generalizable over cell lines, the same experiment was performed in a human non-transformed breast cell line (HBL100), in a human pancreatic adenocarcinoma cell line (BxPC-3), and in a mouse pre-adipocyte cell line (NIH 3T3-L1, the phosphorylated sites recognized by the antibody are conserved in mouse MEK1 and MEK2 and human MEK1 and MEK2; see Refs. 56 and 57). In all of these cell lines either MEK1 or MEK2 were phosphorylated in response to ionizing radiation and showed various dose responses (Fig. 1, bottom three blots). The * in Fig. 1 refers to a 32-kDa cross-reacting band that is seen in all cell lines and that is unresponsive to serum starvation or ionizing radiation. This cross-reactive band can be used to standardize for equal protein loading (Fig. 1). In addition, Western blots using antibody against total MEK1 or total MEK2 indicate that ionizing radiation does not lead to increased total MEK protein in the time course of this experiment (data not shown). Thus, these experiments show that phosphorylation of the MEKs in response to ionizing radiation is a general effect of ionizing radiation exposure.

To show that MEK1 and MEK2 are not only phosphorylated in response to ionizing radiation, but are activated as well, IP kinase assays were performed. Antibodies directed against the non-conserved regions of MEK1 and MEK2 (N terminus) were used to limit cross-reactivity between the two proteins, and catalytically inactive ERK1 (K71A ERK1) was used as a substrate. Because MEK1 is downstream of Ras (30), as a positive control, activity of the MEKs from HeLa cells transformed with v-ras were compared with activity of the MEKs from HeLa cells transfected with empty vector. Fig. 2A shows that MEK1 and MEK2 have much higher activity in cells transformed with v-ras, while these antibodies perform appropriately in IP kinase assays. HeLa cells were then serum-starved and exposed to various doses of ionizing radiation. As a positive control for increased kinase activity, cells were restimulated with 20% serum (Fig. 2B, far left lane), MEK1 shows a slight increase in activity at both 2.5 and 10 Gy, whereas MEK2 is strongly activated by 10 Gy of ionizing radiation (Fig. 2B). The same experiment was then performed using an IP-depletion strategy aimed at eliminating the remaining MEK1 and MEK2. Cells
**Role of MEK2 in G2/M Checkpoint Recovery**

**Fig. 1.** MEK1 and MEK2 are phosphorylated in response to ionizing radiation. Cells were serum-starved for 2 days and then exposed to the different doses of ionizing radiation. 10 min after exposure, the cells were lysed, and equal amounts of lysates were electrophoresed. Western blotting was performed using an antibody that recognizes active, phosphorylated MEK1 (Ser-218 and Ser-222 phosphorylation) or MEK2 (Ser-222 and Ser-226 phosphorylation). The cell lines used to show MEK phosphorylation in response to ionizing radiation were (top to bottom) as follows: HeLa cells, a human cervical cancer cell line; HB1-100 cells, a human non-transformed breast epithelial cell line; BxPC-3 cells, a human pancreatic adenocarcinoma cell line and NIH 3T3-L1 cells, a mouse pre-adipocyte cell line. MEK1 runs as a 45-kDa band and MEK2 runs as a 46-kDa band and are indistinguishable in this assay. The * refers to a 32-kDa band that cross-reacts with the antibody to MEK1 but is not seen with the antibody to MEK2.

**Blot = Phosphorylated MEK1/2**

Overexpression of Dominant Negative MEK2, but Not Dominant Negative MEK1, Increases Sensitivity of the Cell to Ionizing Radiation—To determine whether the activation of MEK1 and MEK2 is significant for the response of the cell to ionizing radiation, dominant negative forms of these two proteins were used. Two dominant negative forms of MEK1 were obtained (60). The first, K97A MEK1, replaces a lysine in the ATP-binding domain, with an alanine, such that the kinase cannot bind ATP to transfer the phosphate. The second, S222A MEK1, replaces a serine, which is essential for activation, with an alanine, such that full activation cannot be achieved (generous gifts of Edwin Krebs, University of Washington, Seattle). These two dominant negative constructs have been previously shown to slow cell growth and inhibit activation by EGF and serum stimulation (60). In addition, as a control for overexpression of MEK1, a constitutively active form of MEK1 (S222E MEK1) was also obtained (gift of Edwin Krebs, University of Washington, Seattle). We subcloned these three constructs into pREP4 (Invitrogen). This vector replicates episomally in HeLa cells and contains the hygromycin resistance gene driven by the cytomegalovirus promoter. The constructs were transfected into HeLa cells, selected for 3 weeks in hygromycin, and approximately 1000 clones were pooled. Because activity of dominant negative proteins is dependent on their expression relative to wild-type protein, we wanted to show expression of the dominant negative constructs in these cells relative to wild-type MEK1 expression. Lysates were generated and Western blots were performed (with equivalent lysate protein concentration) using an antibody against MEK1. This antibody will recognize both endogenous wild-type protein and overexpressed dominant negative protein. Fig. 3A shows that relative to cells stably transfected with vector only, both K97A MEK1 cells and S222A MEK1 cells have greatly overexpressed dominant negative MEK1 (Fig. 3A). In addition, the constitutively active S222E MEK1 is also expressed to a high degree (Fig. 3A). To show that these constructs affect the activity of MEK1 in response to ionizing radiation, IP kinase assays were performed using exposed and unexposed cells. The cells containing the stably transfected empty vector show the 1.6–1.8-fold activation of MEK1 upon exposure to ionizing radiation, whereas the cells containing the dominant negative constructs showed no activation upon exposure to ionizing radiation (Fig. 3B). In addition, the S222E MEK1 cell line shows elevated basal activity which can be increased approximately 1.4-fold upon ionizing radiation exposure (Fig. 3B).

To test the K97A MEK1 and the S222A MEK1 dominant negative effects of constructs on cell survival in response to ionizing radiation, colony forming assays were performed. This assay measures the ability of a cell to survive ionizing radiation exposure and to proliferate following ionizing radiation exposure (16). Approximately 500 cells were plated per ionizing radiation dose. The plates were exposed to the indicated dose of ionizing radiation, and colony formation was scored 2 weeks later. The S222A MEK1 cell line had slightly decreased survival at both 4 and 6 Gy of ionizing radiation, whereas the K97A MEK1 cell line only showed slightly decreased survival at 6 Gy of ionizing radiation (Fig. 3C). Neither of these cell lines showed significant difference from the S222E MEK1 cell line (Fig. 3C), so the dominant negative MEK1 constructs have little effect on HeLa cell survival in response to ionizing radiation.

Because MEK2 is activated in IP kinase assays to a greater extent than MEK1, it is possible that a dominant negative form of MEK2 would have a larger effect on the ability of HeLa cells to survive ionizing radiation exposure. To test the effect of a
dominant negative MEK2, we mutated wild-type MEK2 (57, 61) (generous gift of Natalie Ahn, University of Colorado, Boulder) to K101A MEK2. This mutation was designed to be analogous to the K97A MEK1 mutation, as this region of MEK2 is highly conserved with MEK1 (56, 57). By analogy, the K101A MEK2 mutation should render MEK2 unable to bind ATP to transfer the phosphate to its substrate. Both the dominant negative K101A MEK2 and wild-type MEK2 were subcloned into the pREP4 vector and transfected into HeLa cells. After 3 weeks of hygromycin selection, approximately 1000 colonies were pooled. Western blots were performed (equivalent protein concentrations of lysates) using an antibody that recognizes both endogenous, wild-type MEK2 and transfected, dominant negative MEK2. Fig. 4A shows that K101A MEK2 and wt MEK2 are highly expressed relative to cells stably transfected with vector alone (Fig. 4A). To show that expression of the dominant negative MEK2 leads to decreased MEK2 activation in response to ionizing radiation, IP kinase assays were performed on these cells using kinase-inactive ERK1 as a substrate. The cells containing only the empty vector show a 4–6-fold up-regulation of MEK2 activity in response to ionizing radiation, whereas the K101A MEK2 cells show no activation in response to ionizing radiation (Fig. 4B). In addition, expression of wild-type MEK2 shows higher basal MEK2 activity that can be increased only slightly in response to ionizing radiation (Fig. 4B).

Colony forming assays were then performed to determine the influence of MEK2 on the cell's survival response to ionizing radiation. 500 cells were plated 12 h prior to radiation exposure. Cells were exposed to the given dose, and colony formation was scored 2 weeks later. At low doses of ionizing radiation, the K101A MEK2 cells do not show decreased cell survival, but at higher doses of radiation (>3 Gy), these cells are significantly more radiosensitive (Fig. 4C). Since cells containing wild-type MEK2 show no differences in survival relative to the vector-only cells, the decreased survival of cells expressing dominant negative MEK2 is not simply an overexpression phenomenon. Thus, whereas both MEK1 and MEK2 are activated in response to ionizing radiation, only expression of dominant negative MEK2 leads to radiosensitivity.

\textbf{Dominant Negative MEK2 Has No Effect on Double-strand DNA Repair but Leads to Defective G2/M Checkpoint Control—}
Two explanations are possible for the effect of MEK2 on radiation survival. First, MEK2 may influence DNA repair. Ionizing radiation causes double-strand DNA breaks, and a number of proteins that cause radiation hypersensitivity do so by not allowing efficient double-strand break repair (62). In addition, the MEK activator, c-mos, has been shown to influence cellular genomic stability (63). For these reasons, the effect of MEK2 on double-strand break repair was tested using the K101A MEK2, K97A MEK2, and vector-only cell lines. Equal numbers of cells were embedded in agarose plugs and exposed to 10 Gy of ionizing radiation. These cells were then allowed to repair the damaged DNA for 30, 60, or 120 min or not allowed to repair (0 min) the damage. After the plugs were digested overnight with proteinase K, they were embedded in a 0.7% agarose gel and subjected to pulse-field electrophoresis. Southern blotting was then performed, and percent repair was quantitated. A representative experiment is shown in Fig. 5A. Under these electrophoretic conditions, the damaged, unrepaired DNA migrates as a discrete band, whereas the undamaged or repaired DNA barely migrates out of the agarose plug. The results of three independent experiments are quantitated in Fig. 5. There are no significant differences in the abilities of these three cell lines...
to repair double-strand DNA breaks (Fig. 5). This result is expected because the K101A MEK2 survival curve (Fig. 4C) shows a shoulder at low doses of ionizing radiation, implying the ability to repair sublethal DNA damage (3).

Since the MAP kinase pathway has been shown to be necessary for progression through G2/M (50, 52), a second explanation for the effect of dominant negative MEK2 on ionizing radiation sensitivity might be a dysregulated G2/M cell cycle checkpoint. In HeLa cells, the G2/M checkpoint shows the greatest arrest due to ionizing radiation damage (48). To determine the effect of MEK2 on the G2/M checkpoint, the dominant negative K101A MEK2 cell line was used. As controls, the vector-only cell line, the K97A MEK1 cell line and the S222A MEK1 cell line were also used. Asynchronously growing cells were exposed to 5 Gy ionizing radiation; the difference in survival between the K101A MEK2 cell line and the vector-only cell line is maximal at this dose. At the indicated time points after exposure, cells were harvested and stained with propidium iodine, and flow cytometry was performed. The vector-only cells and the K97A MEK1 cells show G2 arrest 12 h after exposure with recovery 24 h after exposure (Fig. 6, vector-only cells are shown in upper panels and K97A MEK1 cells are shown in middle panels). In contrast, the K101A MEK2 cells show G2/M arrest. Full arrest eventually occurs after approximately a 5–7-h delay (Fig. 6). However, the major cell cycle dysfunction in the K101A MEK2 cell line is an inability to recover from G2/M arrest. At both the 24- and 36-h time points, significant numbers of K101A MEK2 cells are still arrested in G2 (Fig. 6). At these time points, both the vector-only cells and the K97A MEK2 cells have recovered and show relatively nor-
mal cell cycle profiles (Fig. 6). The S222A MEK1 cell line showed similar profiles to the K97A MEK1 dominant negative was made. Both the wild-type MEK2 gene and the K101A MEK2 gene were transected into HeLa cells independently. After 3 weeks of hygromycin selection (pREP4 vector), approximately 1000 colonies from each transfection were pooled. Again, a separate control cell line containing only the vector was also made. Lysates from the three cell lines (K101A MEK2 cells, vector-only cells, and wild-type MEK2 cells) were generated, and Western blotting was performed using an antibody that recognizes the N terminus of MEK2. Both the wild-type MEK2 cells and the K101A MEK2 cells overexpress MEK2 as shown by this blot. B, to test whether the stably transfected K101A MEK2 blocked MEK2 activation in response to ionizing radiation, IP kinase assays were performed. Two serum-starved plates of cells were used. One was exposed to 10 Gy ionizing radiation and the other was not exposed. Phosphorylation of kinase-dead (K71A ERK2) was then quantitated. Each data point was performed in triplicate. Counts/min phosphate transferred per mg of substrate is shown in a graph. C, survival of the cells stably expressing the given MEK2 construct was measured using the colony forming assay. Again, relative survival refers to the survival relative to an unirradiated plate of cells from the same cell line plated on the same day at the same density. Each experiment was performed in triplicate. Relative survivals and S.E.s are graphed. All cell lines were used within 3 weeks of generation or within 2 weeks of thawing.

FIG. 4. Cells that express dominant negative MEK2 are hypersensitive to ionizing radiation. A, a site-directed MEK2 mutant (K101A MEK2) designed to mimic the K97A MEK1 dominant negative was made. Both the wild-type MEK2 gene and the K101A MEK2 gene were transected into HeLa cells independently. After 3 weeks of hygromycin selection (pREP4 vector), approximately 1000 colonies from each transfection were pooled. Again, a separate control cell line containing only the vector was also made. Lysates from the three cell lines (K101A MEK2 cells, vector-only cells, and wild-type MEK2 cells) were generated, and Western blotting was performed using an antibody that recognizes the N terminus of MEK2. Both the wild-type MEK2 cells and the K101A MEK2 cells overexpress MEK2 as shown by this blot. B, to test whether the stably transfected K101A MEK2 blocked MEK2 activation in response to ionizing radiation, IP kinase assays were performed. Two serum-starved plates of cells were used. One was exposed to 10 Gy ionizing radiation and the other was not exposed. Phosphorylation of kinase-dead (K71A ERK2) was then quantitated. Each data point was performed in triplicate. Counts/min phosphate transferred per mg of substrate is shown in a graph. C, survival of the cells stably expressing the given MEK2 construct was measured using the colony forming assay. Again, relative survival refers to the survival relative to an unirradiated plate of cells from the same cell line plated on the same day at the same density. Each experiment was performed in triplicate. Relative survivals and S.E.s are graphed. All cell lines were used within 3 weeks of generation or within 2 weeks of thawing.

Role of MEK2 in G₂/M Checkpoint Recovery

To determine whether an inability to arrest in G₂ or an inability to recover from G₂ arrest is responsible for the radiosensitivity of the K101A MEK2 cell line, pharmacological manipulation was performed. Vanadate has been shown to inhibit dephosphorylation of CDC2, thereby leading to an arrest in G₂ (64). K101A MEK2 cells were exposed to vanadate for 8 h before ionizing radiation exposure. 16 h after radiation exposure, cells were washed extensively, refed with normal growth media, and colony forming assays were performed. Fig. 7A shows that treatment of K101A MEK2 cells with ionizing radiation and vanadate leads to an increased G₂ arrest when compared with K101A MEK2 cells treated with ionizing radiation alone (Fig. 7A, middle panels). This forced G₂ arrest does recover radioresistance in the K101A MEK2 cell line, as vanadate-treated K101A MEK2 cells show similar radiosensitivity to untreated K101A MEK2 cells (Fig. 7B).

To study recovery from the G₂/M arrest, caffeine was used. Caffeine is typically regarded as an agent that sensitizes cells to ionizing radiation (65, 66). Treatment of cells with caffeine prior to irradiation abolishes the G₂ arrest and leads to radiosensitivity (65, 66). We used caffeine in a slightly different
manner. By exposing K101A MEK2 cells to caffeine 24 h after ionizing radiation exposure, we were able to use caffeine to force the K101A MEK2 cells to recover from the G2/M arrest in a timely manner. Fig. 7A shows that irradiated, non-caffeine-treated K101A MEK2 cells have significant numbers of cells arrested in G2 32 h after ionizing radiation exposure. When these cells are treated with caffeine 24 h after irradiation, the cells show a normal cell cycle distribution 8 h later (Fig. 7A, right panels). Thus, caffeine can be used to force recovery from the G2/M arrest in otherwise terminally arrested K101A MEK2 cells. This forced G2/M arrest recovery reverses the radiosensitivity of K101A MEK2 cells upon ionizing radiation (Fig. 7B), whereas treatment of vector-only cells or K97A MEK1 cells at these time points with either caffeine or vanadate has no appreciable effect on cell survival (Fig. 7B). Therefore, the radiosensitivity of cells that express dominant negative MEK2 is most likely due to an inability to recover from G2 arrest and not due to an inability to arrest in G2 in a timely manner.

DISCUSSION

The RAF/ERK signaling pathway is generally regarded to be strongly responsive to mitogenic signals and only weakly responsive to stressful signals (11, 32, 36). The only stressful stimulus that strongly activates the MAP kinase pathway is ionizing radiation (37–41). Whereas a number of cell stressors and DNA-damaging agents cause a G1/S arrest, physiologic doses of ionizing radiation cause a G2/M arrest (42). In this work, we have studied the possibility that ionizing radiation activates the RAF/ERK signaling pathway to influence G2/M checkpoint kinetics. We have shown that both MEK1 and MEK2 are activated in response to ionizing radiation (Figs. 1 and 2). This activation has functional significance as dominant negative MEK2 is essential for cell survival in response to ionizing radiation (Fig. 4). We have also shown that expression of dominant negative MEK2 leads to a delayed induction of the G2/M cell cycle checkpoint and a vastly decreased ability to recover from that checkpoint once it is established (Fig. 6).
Finally, we show using pharmacological means that it is the faulty recovery from the G2/M arrest and not the delayed G2/M arrest that is responsible for the radiosensitivity in cells that express dominant negative MEK2 (Fig. 7).

Previous work on activation of the MAP kinase pathway by ionizing radiation focused on the activation of the components of the pathway and not on the cellular effects of that activation. We have carried that work a step further by showing activation of MEK1 and MEK2 at doses (2.5 Gy) that are likely to be physiologically relevant. In addition, we have focused on the effect that activation of these kinases has on cell survival and cell cycle checkpoint control. Although there is a pharmacological agent (PD98059) that inhibits activation of MEK1 (IC50 = 5 μM) and MEK2 (IC50 = 50 μM) (54, 67), the doses required to completely inhibit MEK2 activity (100 μM) were toxic to HeLa cells. For this reason, dominant negative MEK1 and MEK2 constructs were employed. The dominant negative constructs allowed us to selectively block MEK1 and MEK2 activity, respectively. Our results show that the inhibition of MEK2 activation causes cells to become both radiosensitive and checkpoint-defective. These defects in cells expressing dominant negative MEK2 suggest that MEK activation by ionizing radiation is a specific component of the stress response of the cell and not a nonspecific effect of cellular stress as previously suggested (41).

The importance of the MAP kinase pathway in all phases of cell cycle regulation is being increasingly recognized. The MEK inhibitor, PD98059, has been shown to reverse the nerve growth factor-induced G1 arrest in fibroblasts (68). In addition, expression of a constitutively active form of MEK1 induces differentiation in PC12 cells (69) and in megakaryocytes (70). These findings indicate that in some cell lines, MEK activity leads to G0/G1 arrest. In other instances, however, such as upon growth factor stimulation and upon cellular transformation by v-ras and v-raf, MEK activity is necessary for progression through G1 (67, 71). Inhibition of MEK activity in pre-adipocytes also enhances adipocyte differentiation (72). Constitutively active MEK1 will also transform some cell lines (71). These findings imply that MEK activity helps the cell progress through G1. Therefore, the data surrounding the activity of MEK in G1 cell cycle regulation is largely contradictory. The effect of MEK on G1 cell cycle regulation is largely dependent on the circumstance and cell line tested.

Recently, the MAP kinase pathway has also been implicated in G2 cell cycle regulation. In Xenopus oocytes, MAP kinase activity has been shown to be necessary for progression through G2, possibly by inhibiting an inhibitor of cyclin B/CDC2 kinase activity (49–51). c-mos (an activator of MEK1 and MEK2) has been shown to be essential for progression through G2 (52). In addition, there is mounting circumstantial
Evidence that the MAP kinase pathway plays an essential role in meiosis. In Caenorhabditis elegans, activation of the MAP kinase pathway is necessary for meiotic cell cycle progression (73), and in mouse oocytes, MAP kinase becomes activated at metaphase and becomes localized to the microtubule-organizing centers in meiotic maturation (53). Because the repair of double-strand DNA breaks seen during physiological crossing-over during meiosis is similar to the repair of ionizing radiation-induced double-strand DNA breaks (74), activation of the MAP kinase cascade may lead to similar effects in meiosis and DNA repair. To this point, however, the MEKs have not been implicated in the maintenance of stress-induced cell cycle checkpoints or in progression through those checkpoints. In this article, we have shown that cells that express dominant negative MEK2 have a defective G2/M arrest. Although these cells eventually arrest at levels seen in controls, these cells take slightly longer (approximately 5–7 h longer in asynchronous cells) to fully enter G2 arrest. In addition, these cells take remarkably longer to recover from G2 arrest (Fig. 6). Because our cell survival assays require the cells to recover from the insulating agent and to proliferate after that recovery, we are not measuring only cell death but instead measuring both cell death and proliferation capacity following recovery. Thus, a dysfunctional recovery from G2 arrest would manifest in our assay by showing increased cell death and decreased proliferation capacity, leading to decreased colony formation. We show through the use of caffeine to force recovery from G2 arrest that the delay in recovery from radiation-induced G2 arrest mediates the radiosensitivity of the K101A MEK2 cells. Activation of MEK2 by ionizing radiation likely influences the progression through the G2/M checkpoint. Because ionizing radiation causes a G2/M arrest, this could provide an explanation for the finding that the RAF/ERK pathway is activated by ionizing radiation but not activated by other stress stimuli.

FIG. 7. Delayed recovery from the G2 arrest leads to radiosensitivity in the K101A MEK2 cell line. A, flow cytometry was performed to monitor G2 arrest after irradiation and treatment of the cells with the various pharmacological agents. For left profile, cell cycle profile of asynchronously growing K101A MEK2 cells. Middle profile, the timing of the experiment is shown in the upper panel. Below this is cell cycle profile of K101A MEK2 cells irradiated with 5 Gy and harvested 16 h later. Full arrest has not occurred at this time point. The bottom panel shows the cell cycle profile of K101A MEK2 cells pretreated with vanadate followed by irradiation with 5 Gy. Cells treated with vanadate plus irradiation in this manner show a significantly increased G2 arrest relative to K101A MEK2 cells treated with only irradiation. For right panels, the upper panel shows the time course of the experiment. Below this panel is a cell cycle profile of K101A MEK2 cells irradiated with 5 Gy and harvested 32 h later. These cells show a significant number still arrested in G2. The bottom panel shows the cell cycle profile of K101A MEK2 cells irradiated with 5 Gy and treated with caffeine 24 h following irradiation. Eight hours following caffeine treatment (32 h following irradiation), cells were harvested. Caffeine treatment leads to a recovery from the G2 arrest and allows a cell cycle profile which is now indistinguishable from asynchronously growing cells. B, survival of cells treated with 5 Gy ionizing radiation and either caffeine or vanadate in the time course described above. Neither caffeine nor vanadate lead to a change in cell survival in the vector-only cells or the K97A MEK1 cells. Vanadate treatment (forced G2/M arrest) had no effect on K101A MEK2 cell survival, whereas caffeine treatment (forced recovery from G2/M arrest) leads to significantly increased survival.

Role of MEK2 in G2/M Checkpoint Recovery
Acknowledgments—We thank Edwin Krebs (University of Washington, Seattle), Natalie Ahn (University of Colorado, Boulder), and Kun-Liang Guang (University of Michigan, Ann Arbor) for their generous gifts of plasmids. We thank Marilyn Thompson and Michael Perlman for critical comments on the manuscript and Philip Browning, Steve Hann, David Miller, and P. Anthony Weil for helpful discussions concerning the data.

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