# Opposing Roles of c-Jun $NH_2$-Terminal Kinase and p38 Mitogen-Activated Protein Kinase in the Cellular Response to Ionizing Radiation in Human Cervical Cancer Cells

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## Abstract

**Exposure of cells to ionizing radiation induces activation of multiple signaling pathways that play critical roles in determining cell fate. However, the molecular basis for cell death or survival signaling in response to radiation is unclear at present. Here, we show opposing roles of the c-jun $NH_2$-terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPK) pathways in the mitochondrial cell death in response to ionizing radiation in human cervical cancer cells. Ionizing radiation triggered Bax and Bak activation, Bcl-2 down-regulation, and subsequent mitochondrial cell death. Inhibition of JNK completely suppressed radiation-induced Bax and Bak activation and Bcl-2 down-regulation. Dominant-negative forms of stress-activated protein kinase/extracellular signal-regulated kinase kinase 1 (SEK-1)/mitogen-activated protein kinase kinase-4 (MKK-4) inhibited JNK activation. Radiation also induced phosphoinositide 3-kinase (PI3K) activation. Interestingly, inhibition of PI3K effectively attenuated radiation-induced mitochondrial cell death and increased clonogenic survival. Inhibition of PI3K also suppressed SEK-1/MKK-4 and JNK activation, Bax and Bak activation, and Bcl-2 down-regulation. In contrast, inhibition of p38 MAPK led to enhanced Bax and Bak activation and mitochondrial cell death. RacN17, a dominant-negative form of Rac1, inhibited p38 MAPK activation and increased Bax and Bak activation. Exposure of cells to radiation also induced selective activation of c-Src among Src family kinases. Inhibition of c-Src by pretreatment with Src family kinase inhibitor PP2 or small interfering RNA targeting of c-Src attenuated radiation-induced p38 MAPK and Rac1 activation and enhanced Bax and Bak activation and cell death. Our results support the notion that the PI3K-SEK-1/MKK-4-JNK pathway is required for the mitochondrial cell death in response to radiation, whereas the c-Src-Rac1-p38 MAPK pathway plays a cytoprotective role against mitochondrial cell death. (Mol Cancer Res 2008;6(11):1718–31)**

## Introduction

Exposure of cells to ionizing radiation results in the simultaneous activation or down-regulation of multiple signaling pathways that play critical roles in cell type–specific control of survival or death in response to ionizing radiation. Improved understanding of the mechanisms involved in cell death and survival in response to radiation may ultimately afford novel strategies of intervention in specific signal transduction pathways to favorably alter the therapeutic efficacy of human malignancy treatments.

Mitogen-activated protein kinases (MAPK) transduce signals from the cell membrane to the nucleus in response to a variety of different stimuli and participate in various intracellular signaling pathways that control a wide spectrum of cellular processes, including growth, differentiation, and stress responses. MAPKs include extracellular signal–regulated kinase (ERK), c-Jun $NH_2$-terminal kinase (JNK), and p38 MAPK. The ERK pathway, activated by mitogenic stimuli such as growth factors, cytokines, and phorbol esters, plays a major role in regulating cell growth, survival, and differentiation (1-5). In contrast, JNK and p38 MAPK are weakly activated by growth factors, but respond strongly to stress signals including tumor necrosis factor, interleukin-1, ionizing and UV irradiation, hyperosmotic stress, and chemotherapeutic drugs (6-11). Activation of these kinases is strongly associated with apoptotic cell death induced by stress stimuli. Many reports suggested that JNK or p38 MAPK induces apoptosis by regulating the translocation of Bax from the

Received 1/18/08; revised 7/2/08; accepted 7/10/08.
**Grant support:** Basic Atomic Energy Research Institute, Nuclear R&D Programs, Ministry of Science and Technology of Korea.
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doi:10.1158/1541-7786.MCR-08-0032

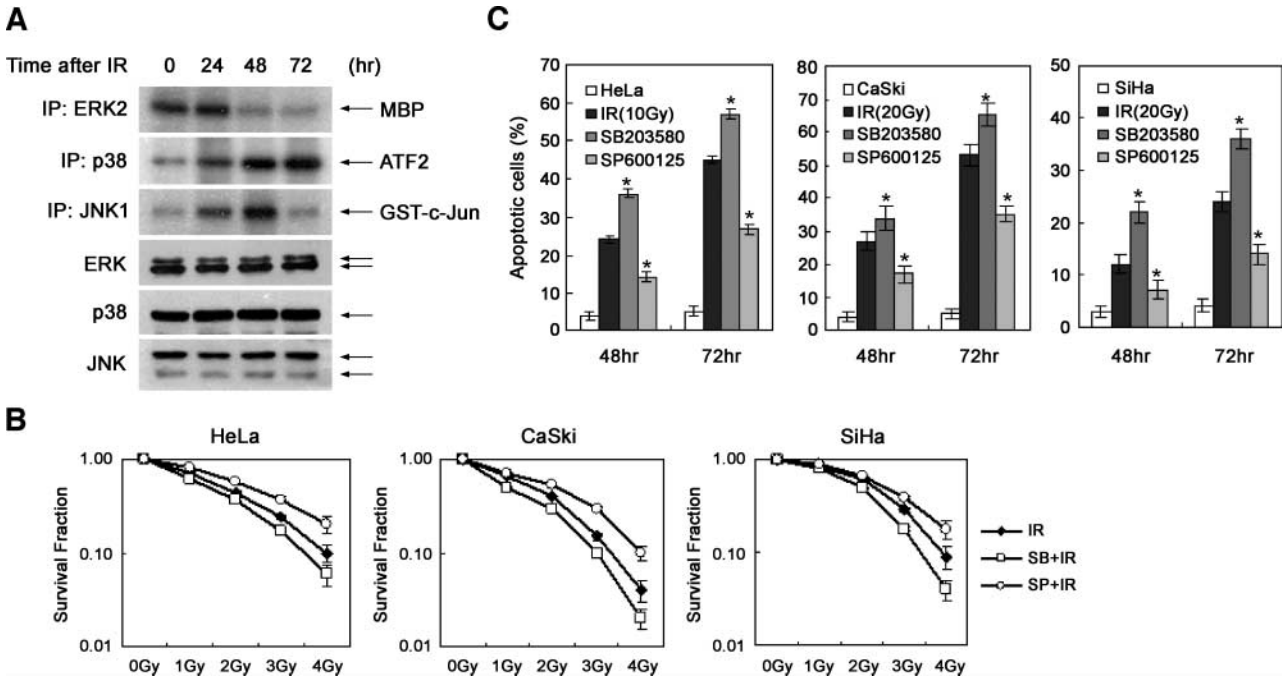



**FIGURE 1.** MAPKs are implicated in the regulation of apoptosis in human cervical cancer cells. **A.** Regulation of MAPK activity after radiation treatment. HeLa cells were exposed to 10 Gy of γ-radiation. After 24, 48, and 72 h, immune complex kinase assays and Western blot analysis with anti-ERK, anti-p38 MAPK, and anti-JNK antibodies were done. Myelin basic protein (*MBP*), activating transcription factor-2 (*ATF-2*), and glutathione *S*-transferase-c-Jun (*GST-c-Jun*) were used as substrates for ERK, p38 MAPK, and JNK, respectively. **B.** Effects of MAPK inhibition on radiation-induced cell survival. Treatment with p38 MAPK inhibitor, SB203580, or JNK inhibitor, SP600125, in three human cervical cancer cell lines (HeLa, CaSki, and SiHa) and analysis of their effects on clonogenic survival. **C.** Effects of MAPK inhibition on radiation-induced cell death. HeLa cells were exposed to 10 Gy and CaSki and SiHa cells to 20 Gy of γ-radiation in the presence of p38 MAPK inhibitor, SB203580, or JNK inhibitor, SP600125. After 48 and 72 h, cell death was determined by flow cytometric analysis. Columns, mean of three independent experiments; bars, SE. *, $P < 0.05$.

cytoplasm to the mitochondria in response to various stimuli (12-14). These results are consistent with the fact that JNK and/or p38 MAPK acts at early step before dysfunction of mitochondria and caspase activation in several cell types (15-17). In contrast, recent observations showed that p38 MAPK is a critical regulator of cell survival and proliferation in response to cisplatin, camptothecin, doxorubicin, UV irradiation, and repetitive low-grade oxidative stress (18-20). Furthermore, other reports showed that p38 MAPK signaling is essential for the maintenance of a transformed cell phenotype in human malignancy (21, 22). However, the fundamental link between MAPK signaling and cell survival or apoptotic cell death machinery, especially in response to radiation, is yet to be clearly defined.

Phosphoinositide 3-kinase (PI3K) is a lipid kinase that mediates several cellular responses in both physiologic and pathophysiologic states. Recent studies provide evidence that PI3K is involved in apoptosis, in contrast to its well-known inhibitory effects on cell death reported earlier. For example, PI3K is positively implicated in apoptosis induction in response to various stimuli, including hypoxia (23), arsenic compounds (24), glucose deprivation (25), and serum withdrawal (26). These findings indicate that PI3K activates two functionally opposite pathways, depending on cell type, nature of stimulus, and activities of other signaling pathways. Whereas the contribution of PI3K to cell survival is extensively characterized, its role and precise mechanisms in apoptotic cell death regulation remain largely unknown.

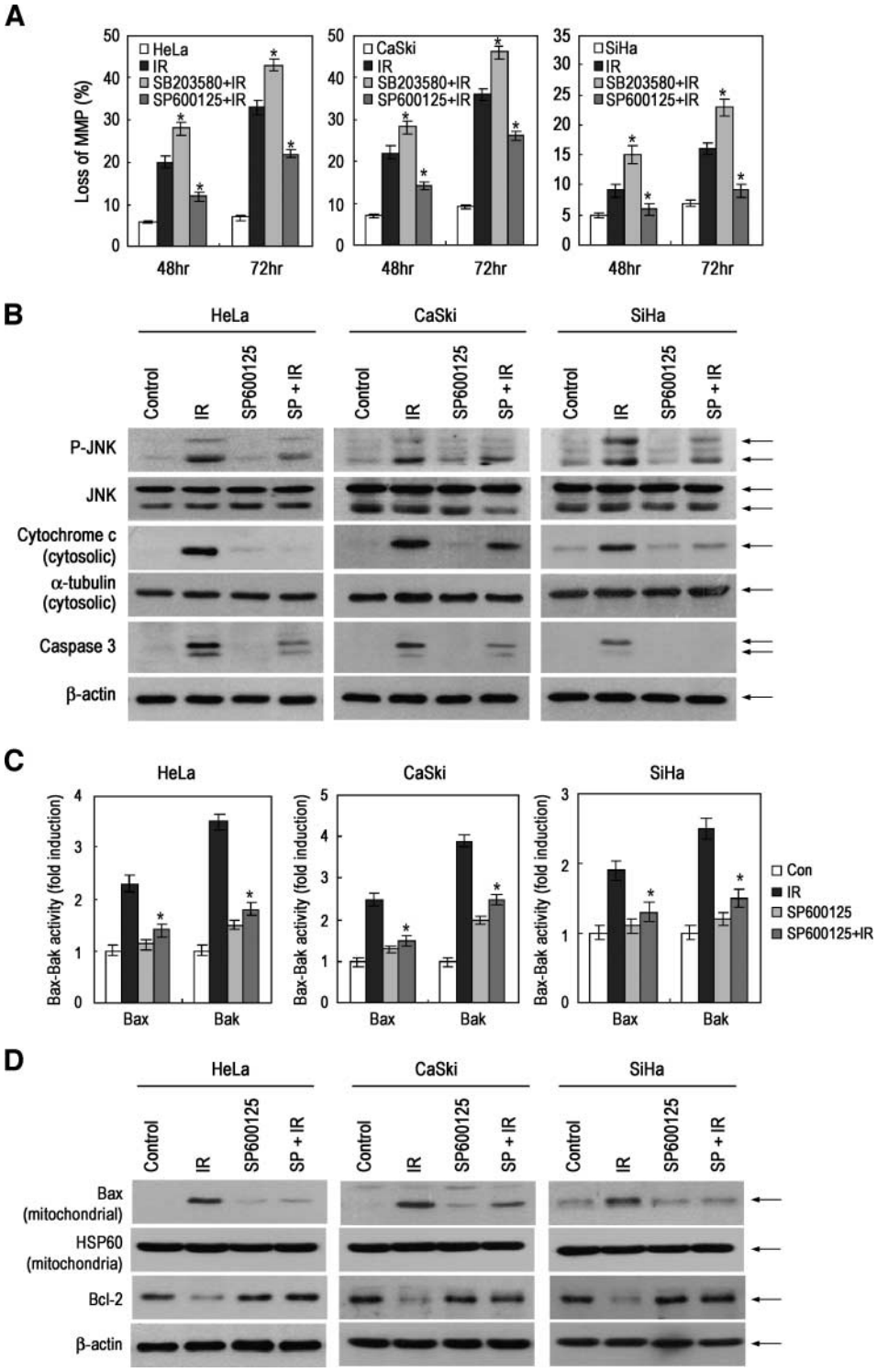
In this report, we show that the JNK and p38 MAPK signaling pathways play opposite roles in the cellular response to ionizing radiation of human cervical cancer cells. The PI3K-stress-activated protein kinase/extracellular signal-regulated kinase kinase 1 (SEK-1)/mitogen-activated protein kinase kinase-4 (MKK-4)-JNK pathway is shown to be essential for stimulating mitochondrial cell death in response to radiation, whereas the c-Src-Rac1-p38 MAPK pathway plays a cytoprotective role against mitochondrial cell death. Elucidation of the regulatory mechanisms of cell death and cell survival in response to radiation should further facilitate technical advancements in radiation cancer therapy and drug development.

## Results

### *MAPK Involvement in Apoptosis of Human Cervical Cancer Cells in Response to Ionizing Radiation*

MAPKs are implicated in the regulation of apoptotic cell death in response to various stimuli. To confirm the potential involvement of MAPK in ionizing radiation–induced cell death, we initially measured changes in MAPK activity after radiation treatment using an immune complex kinase assay (Fig. 1A). Irradiation of cells led to a dramatic increase in p38 MAPK and JNK activities (Fig. 1A). JNK and p38 MAPK

activities were apparent at 24 hours and peaked at 48 hours after irradiation. The total MAPK cellular levels remained constant. To further determine the involvement of p38 MAPK or JNK in radiation response, we used specific inhibitors of these kinases in experiments with three human cervical cancer cell lines (HeLa, CaSki, and SiHa) and analyzed their effects on clonogenic survival and apoptotic cell death in response to radiation. As shown in Fig. 1B, clonogenic survival assay revealed that the JNK inhibitor SP600125 significantly reduced radiation sensitivity in human cervical cancer cells, whereas the p38 MAPK inhibitor SB203580 enhanced radiation response. Moreover, pretreatment with



SP600125 clearly attenuated radiation-induced apoptotic cell death, but SB203580 enhanced cell death (Fig. 1C). Our findings indicate that JNK plays a role in radiation-induced apoptotic cell death, whereas p38 MAPK acts as a cell survival factor in response to radiation. In addition, exposure of cells with radiation caused a marked reduction of ERK2 activity (Fig. 1A). Moreover, overexpression of ERK2 significantly attenuated radiation-induced cell death (Supplementary Fig. S1), suggesting that down-regulation of ERK is also required for human cervical cancer cell death in response to radiation treatment.

### *JNK Mediates Bax and Bak Activation and Bcl-2 Down-Regulation in Response to Ionizing Radiation*

To further investigate the role of JNK in radiation-induced mitochondrial pathway of apoptosis, we pretreated the JNK inhibitor SP600125 and analyzed its effects on mitochondrial membrane potential. SP600125 markedly suppressed radiation-induced mitochondrial membrane potential loss (Fig. 2A), cytochrome *c* release from mitochondria, and caspase-3 activation (Fig. 2B). Because Bcl-2 family proteins are crucial in mitochondrial apoptotic cell death pathways (27), we analyzed whether JNK is involved in stimulation of Bax or Bak activity and changes in Bcl-2 status in response to radiation. Inhibition of JNK by SP600125 attenuated radiation-induced conformational changes of Bax and Bak (Fig. 2C) and subsequent mitochondrial translocation of Bax (Fig. 2D). Moreover, inhibition of JNK suppressed radiation-induced down-regulation of Bcl-2 (Fig. 2D). These results imply that JNK mediates Bax and Bak activation and Bcl-2 down-regulation in human cervical cancer cells in response to ionizing radiation.

### *SEK-1/MKK-4 Involvement in JNK Signaling during Radiation-Induced Apoptosis*

Recent studies show that SEK-1/MKK-4 is involved in JNK signaling during apoptosis progression (28). Accordingly, we examined whether SEK-1/MKK-4 participates in ionizing radiation–induced JNK activation and subsequent mitochondrial cell death. Following irradiation of cells, SEK-1/MKK-4 activity was evident at 24 hours and peaked at 48 hours (Fig. 3A). Ectopic expression of dominant-negative SEK-1/MKK-4 efficiently blocked radiation-induced cell death (Fig. 3B). Moreover, dominant-negative SEK-1/MKK-4 significantly repressed JNK activation (Fig. 3C), conformational changes of Bax and Bak (Fig. 3D), translocation of Bax to mitochondria, and down-regulation of Bcl-2 (Fig. 3C). Inhibition of SEK-1/MKK-4 additionally led to attenuation of radiation-induced mitochondrial membrane potential loss (Fig. 3E), cytochrome *c* release from mitochondria, and caspase-3 activation (Fig. 3F). In addition, clonogenic survival assay revealed that inhibition of SEK-1/MKK-4 by small interfering RNA (siRNA) targeting significantly reduced radiation response in human cervical cancer cells (Fig. 3G). Based on these findings, we propose that SEK-1/MKK-4 is an upstream regulator of JNK in mitochondrial cell death triggered by ionizing radiation.

### *PI3K Functions in JNK-Mediated Mitochondrial Cell Death*

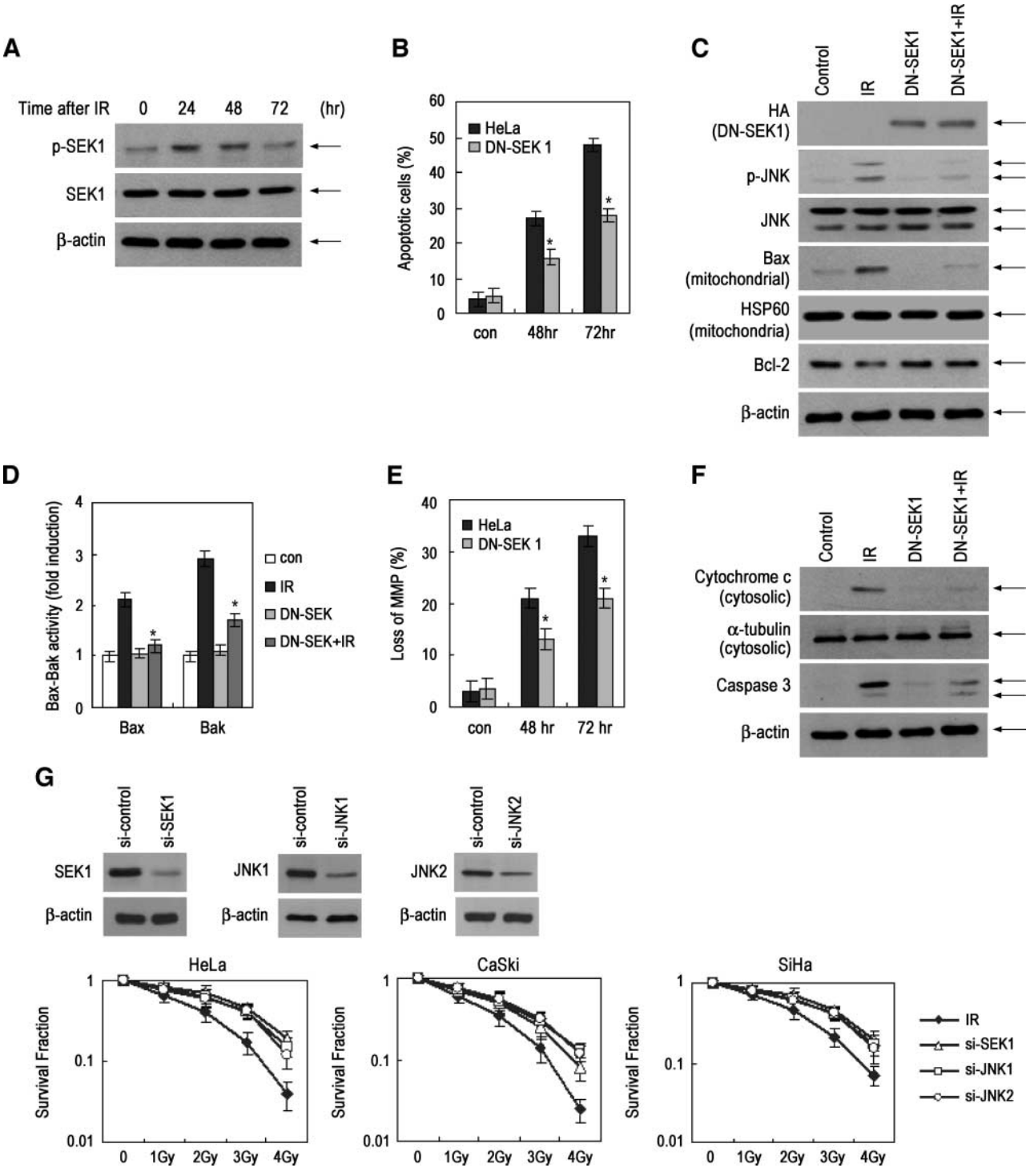
Considerable evidence shows that PI3K enhances cell survival in response to various cellular stress conditions. In contrast, recent studies provide evidence that PI3K is involved in apoptosis. Thus, we investigated whether PI3K is activated and involved in cervical cancer cell response to radiation. Exposure of cells to radiation induced marked activation of PI3K, which was apparent at 24 hours and peaked at 48 hours (Fig. 4A). Interestingly, inhibition of PI3K by pretreatment with LY294002 or siRNA targeting of p110 or p85 resulted in a marked decrease in radiation-induced apoptotic cell death of human cervical cancer cells (Fig. 4C). Moreover, inhibition of PI3K by pretreatment with LY294002 or siRNA targeting of p110 or p85 increased clonogenic survival of human cervical cancer cells (Fig. 4B). LY294002 effectively inhibited radiation-induced Bax and Bak activation (Fig. 4D), Bax translocation to mitochondria, and down-regulation of Bcl-2 (Fig. 4E), and significantly attenuated mitochondrial membrane potential loss (Fig. 4F), cytochrome *c* release from mitochondria, and caspase-3 activation (Fig. 4G). Moreover, radiation-induced SEK-1/MKK-4 and JNK activation were also inhibited, whereas ERK and p38 MAPK were not affected (Fig. 4H). These results suggest that PI3K is responsible for radiation-induced mitochondrial cell death through activation of the SEK-1/MKK-4-JNK signaling pathway.



**FIGURE 2.** JNK activation is required for mitochondrial dysfunction–mediated apoptosis in response to ionizing radiation in human cervical cancer cells. **A.** Effects of MAPK inhibition on radiation-induced mitochondrial transmembrane potential loss. HeLa cells were exposed to 10 Gy and CaSki and SiHa cells to 20 Gy of γ-radiation in the presence of p38 MAPK inhibitor, SB203580, or JNK inhibitor, SP600125. After 48 and 72 h, the mitochondrial transmembrane potential was determined as retention of $DiOC_6(3)$ by flow cytometry. Columns, mean of three independent experiments; bars, SE. *, $P < 0.05$. **B.** Effects of JNK inhibition on radiation-induced cytochrome *c* release. HeLa cells were exposed to 10 Gy of γ-radiation and CaSki and SiHa cells to 20 Gy of γ-radiation in the presence or absence of the JNK inhibitor SP600125. After 48 h, the cytosolic fraction was subjected to Western blot analysis with anti–cytochrome *c* and anti–α-tubulin antibodies. Total cell extracts were subjected to Western blot analysis with anti–caspase-3 and anti–β-actin antibodies. α-Tubulin and β-actin were used as the cytosolic marker protein and loading control, respectively. **C.** Effects of inhibition of JNK on Bax and Bak activation. HeLa cells were exposed to 10 Gy of γ-radiation and CaSki and SiHa cells to 20 Gy of γ-radiation in the presence or absence of SP600125. After 48 h, activity-related modulation of Bax or Bak was determined by flow cytometric analysis with specific antibodies recognizing the $NH_2$-terminal epitopes of the proteins, as described in Materials and Methods. Columns, mean of three independent experiments; bars, SE. *, $P < 0.05$. **D.** JNK activation is essential for stimulating Bax and Bak activity as well as down-regulation of Bcl-2. HeLa cells were exposed to 10 Gy of γ-radiation and CaSki and SiHa cells to 20 Gy of γ-radiation in the presence or absence of SP600125. After 48 h, mitochondrial fractions were subjected to Western blot analysis with anti-Bax and anti-HSP60 antibodies. HSP60 was used as a mitochondrial marker protein. Total cell extracts were subjected to Western blot analysis with anti–Bcl-2 and anti–β-actin antibodies. β-Actin was used as the loading control.

*Confirmation of the Antiapoptotic Role of p38 MAPK following Irradiation*

Because suppression of p38 MAPK enhanced radiation sensitivity in cervical cancer cells, we further investigated whether p38 MAPK plays antiapoptotic role against mitochondrial cell death in response to radiation. Inhibition of p38 MAPK by pretreatment with SB203580 or forced expression of a dominant-negative form of p38 MAPK led to enhancement of radiation-induced Bax and Bak activation (Fig. 5A) and mitochondrial membrane potential loss (Fig. 5B) but did not affect Bcl-2 down-regulation (Fig. 5C). Moreover, radiation-induced cytochrome *c* release and caspase-3 activation were further stimulated by SB203580 treatment (Fig. 5C). Conversely, overexpression of wild-type (WT) p38 MAPK led to a



reduction in radiation-induced Bax and Bak activation (Fig. 5D), mitochondrial membrane potential loss (Fig. 5E), cytochrome *c* release, caspase-3 activation (Fig. 5F), and apoptotic cell death (Fig. 5G). These results clearly indicate that p38 MAPK plays a cytoprotective role against mitochondrial cell death induced by ionizing radiation.

### *Rac1 Acts as an Upstream Regulator of p38 MAPK in Survival Signaling of Cells following Irradiation*

Rac1, a well-characterized upstream regulator of p38 MAPK, is activated by various types of cellular stress. We further examined whether Rac1 is involved in ionizing radiation–induced p38 MAPK activation. Following irradiation, Rac1-Pak binding in cells was dramatically increased, indicative of Rac1 activation (Fig. 6A). Ectopic expression of RacN17, a dominant-negative form of Rac1, significantly decreased p38 MAPK activation induced by radiation (Fig. 6B). Moreover, inhibition of Rac1 was associated with effective stimulation of radiation-induced Bax and Bak activation (Fig. 6C), mitochondrial membrane potential loss (Fig. 6D), and apoptotic cell death (Fig. 6E). These results support the theory that Rac1 acts as an upstream regulator of p38 MAPK in the survival response to ionizing radiation.

### *c-Src Is Involved in Activation of the Rac1-p38 MAPK Signaling Pathway*

Recent studies show that Src family kinases are associated with MAPK activation in response to genotoxic and oxidative stress. Thus, we investigated whether Src family kinases are involved in the radiation response of human cervical cancer cells. Among Src family kinases, c-Src was selectively activated in response to radiation treatment (Fig. 7A). Inhibition of c-Src activity by pretreatment of cells with the PP2 clearly attenuated radiation-induced Rac1 and p38 MAPK activation (Fig. 7B). Moreover, PP2 enhanced Bax and Bak activation (Fig. 7C), mitochondrial membrane potential loss (Fig. 7D), and cell death (Fig. 7E) induced by radiation. Consistently, siRNA targeting studies also clearly revealed that inhibition of c-Src suppressed radiation-induced Rac1 and p38 MAPK activation (Fig. 7F) and enhanced mitochondrial membrane potential loss (Fig. 7G) and cell death (Fig. 7H). Our data imply that c-Src kinase plays a cytoprotective role in mitochondrial cell death in response to ionizing radiation through stimulation of the Rac1-p38 MAPK pathway.

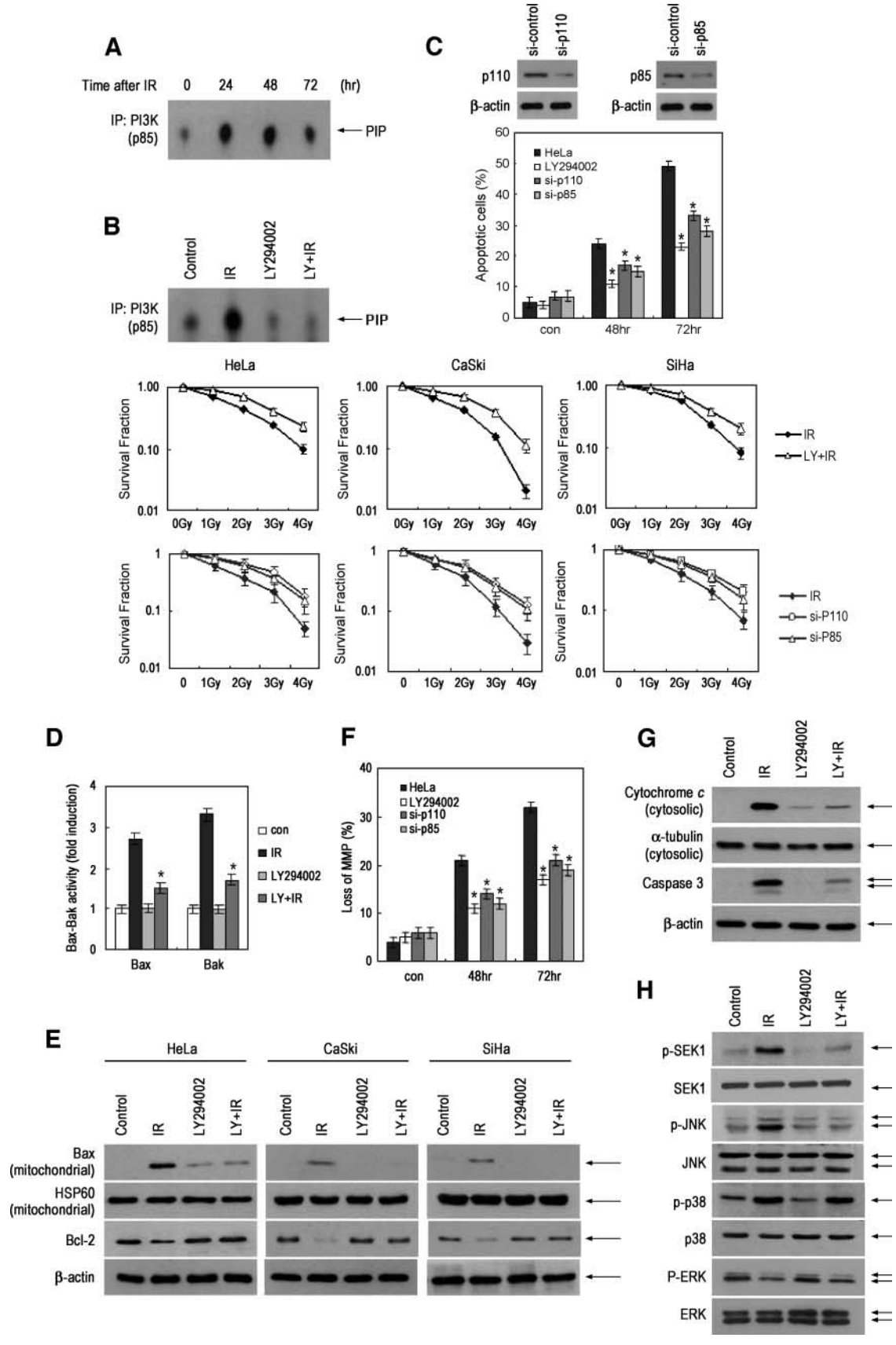
## Discussion

Intracellular signaling molecules play important roles in determining the radiation response of tumor cells. However, the basis for a link between MAPK signaling and cell survival or cell death machinery, especially in response to radiation, is yet to be clearly defined. In this investigation, we elucidate regulatory mechanisms of cell survival and cell death in response to ionizing radiation in human cervical cancer cells. Our data show that the PI3K-SEK-1/MKK-4-JNK pathway is essential for mitochondrial cell death in response to radiation, whereas the c-Src-Rac1-p38 MAPK pathway plays a cytoprotective role against radiation-induced mitochondrial cell death.

Several reports provide evidence that JNKs function as proapoptotic kinases in response to various stimuli (6-11). The JNK pathways activate caspases and may also target other factors implicated in mitochondrial apoptotic cell death regulation, including p53, Bcl-2, and Bax (27). Indeed, we found that JNK influences the mitochondrial death pathway because its inhibition leads to a failure in cytochrome *c* and apoptosis-inducing factor release and subsequent caspase activation triggered by ionizing radiation. Potential targets of JNK-mediated release of cytochrome *c* and apoptosis-inducing factor should therefore be present in cells. Possible mechanisms accounting for JNK signaling leading to mitochondrial membrane potential loss are activation of Bax and Bak (29) and down-regulation of Bcl-2 (30). We found that radiation induces Bax and Bak activation and Bcl-2 down-regulation in human cervical cancer cells. Moreover, inhibition of JNK led to complete attenuation of both results induced by radiation. Accordingly, we propose that JNK is a critical mediator of Bax and Bak activation and Bcl-2 down-regulation, resulting in the release of cytochrome *c* and apoptosis-inducing factor and subsequent cell death in response to ionizing radiation in human cervical cancer cells.

It has been well known that PI3K triggers cytoprotective events in response to diverse stimuli (31-33). Interestingly,

**FIGURE 3.** SEK-1/MKK-4 acts upstream of JNK during radiation-induced apoptosis. **A.** Activation of SEK-1/MKK-4 after irradiation. HeLa cells were exposed to 10 Gy of γ-radiation. After 24, 48, and 72 h, proteins were subjected to Western blot analysis with anti–phospho-SEK, anti-SEK, and anti–β-actin antibodies. β-Actin was used as the loading control. **B.** Role of SEK-1/MKK-4 in radiation-induced cell death. HeLa cells were exposed to 10 Gy of γ-radiation in the presence of dominant-negative forms of SEK. After 48 and 72 h, cell death was determined by flow cytometric analysis. Columns, mean of three independent experiments; bars, SE. *, $P < 0.05$. **C.** SEK-1/MKK-4 is located upstream of JNK. HeLa cells were exposed to 10 Gy of γ-radiation in the presence dominant-negative forms of SEK. After 48 h, total cell extracts were subjected to Western blot analysis with anti–phospho-JNK, anti-JNK, anti–Bcl-2, and anti–β-actin antibodies. Western blotting of mitochondrial fractions was done with anti-Bax and anti-HSP60 antibodies. HSP60 and β-actin were used as the mitochondrial marker protein and loading control, respectively. **D.** Quantitative analysis of Bak and Bax activation in cells overexpressing dominant-negative forms of SEK. HeLa cells were exposed to 10 Gy of γ-radiation in the presence of dominant-negative SEK. After 48 h, activity-related modulation of Bax or Bak was determined by flow cytometric analysis using specific antibodies recognizing $NH_2$-terminal epitopes of the proteins, as described in Materials and Methods. Columns, mean of three independent experiments; bars, SE. *, $P < 0.05$. **E.** Effect of SEK-1/MKK-4 inhibition on radiation-induced mitochondrial transmembrane potential loss. HeLa cells were treated with 10 Gy of γ-radiation in the presence of dominant-negative forms of SEK. After 48 and 72 h, the mitochondrial transmembrane potential of cells was determined as retention of $DiOC_6(3)$ added during the last 30 min of treatment, using flow cytometry. Columns, mean of three independent experiments; bars, SE. *, $P < 0.05$. **F.** Dominant negative forms of SEK attenuated radiation-induced cytochrome *c* release from mitochondria. HeLa cells were treated with 10 Gy of γ-radiation in the presence of dominant-negative forms of SEK-1. After 48 h, the cytosolic fraction obtained was subjected to Western blot analysis with anti–cytochrome *c* and anti–α-tubulin antibodies. Total cell extracts were subjected to Western blot analysis with anti–caspase-3 and anti–β-actin antibodies. α-Tubulin and β-actin were used as the cytosolic marker protein and loading control, respectively. **G.** Effects of SEK-1 inhibition on radiation-induced cell survival. Inhibition of SEK-1, JNK1, or JNK2 by siRNA targeting in three human cervical cancer cell lines (HeLa, CaSki, and SiHa) and analysis of their effects on clonogenic survival.



however, in this study we found that PI3K is critically required for the radiation-induced apoptotic cell death. Exposure of cervical cancer cells with ionizing radiation induced PI3K activity, and inhibition of PI3K effectively attenuated radiation-induced cell death, suggesting that PI3K is responsible for the enhancement of radiation sensitivity in cervical cancer cells. Our findings coincide with recent reports that PI3K is positively implicated in apoptotic response to various stimuli, including hypoxia (23), arsenic compounds (24), glucose deprivation (25), and serum withdrawal (26). Moreover, we provide further evidence that PI3K is associated with activation of the SEK-1/MKK-4-JNK pathway in response to radiation. Exposure of cervical cancer cells to radiation induced SEK-1 and JNK, and inhibition of PI3K effectively attenuated radiation-induced SEK-1 and JNK. In addition, in normal fibroblasts, ionizing radiation induced activation of the PI3K-SEK-1/MKK-4-JNK pathway (Supplementary Fig. S2A), and inhibition of PI3K or JNK in these normal cells attenuated radiation-induced cell death (Supplementary Fig. S2B). However, we failed to find PI3K, SEK-1/MKK-4, and JNK activation after relatively low-dose (2 Gy) irradiation (Supplementary Figs. S2A and S3A), which did not cause apoptotic cell death (Supplementary Fig. S3B). These results indicate that PI3K is positively implicated in SEK-1/MKK-4-JNK–mediated mitochondrial cell death in response to ionizing radiation.

The ERK pathway plays a major role in regulating cell growth and survival (1-5). We found here that exposure of cervical cancer cells with radiation caused a marked reduction of ERK activity. Moreover, overexpression of ERK significantly attenuated radiation-induced apoptotic cell death, suggesting that down-regulation of ERK is involved in cervical cancer cell death in response to radiation.

Many reports suggested that p38 MAPK induces apoptotic cell death in response to various stimuli (15-17). In contrast, in this study we showed that p38 MAPK played a role in cell survival response against radiation-induced mitochondrial cell death. We showed that introduction of p38 MAPK inhibitors enhanced radiation-induced Bax and Bak activation and subsequent mitochondrial cell death. Conversely, overexpression of p38 MAPK attenuated Bax and Bak activation and mitochondrial cell death. This is in good agreement with the recent observations showing that p38 MAPK is a critical regulator for cell survival and proliferation in response to cisplatin, camptothecin, doxorubicin, UV irradiation, and repetitive low-grade oxidative stress (18-20). These results clearly indicate that p38 MAPK plays a cytoprotective role against mitochondrial cell death induced by ionizing radiation.

It is well established that the Rac1-p38 MAPK pathway is activated by various types of membrane-associated cellular signals (34, 35). We provided further evidence that Rac1 is involved in cell survival in response to radiation. Inhibition of Rac1 activity with RacN17 attenuated p38 MAPK activation and significantly enhanced Bax and Bak activation and mitochondrial cell death, suggesting that Rac1 acts as an upstream regulator of p38 MAPK in cell survival response to ionizing radiation.

We further show that c-Src is essential for the activation of Rac1 and p38 MAPK in human cervical cancer cells. Our data clearly show that c-Src kinase is activated in response to radiation and that inhibition of c-Src leads to suppression of Rac1-p38 MAPK signaling in human cervical cancer cells. Moreover, inhibition of c-Src effectively enhanced radiation-induced Bax and Bak activation and mitochondrial cell death. Based on these findings, we conclude that c-Src kinase is associated with activation of the Rac1-p38 MAPK signaling pathway in cell survival response to radiation.

In summary, we show that the PI3K-SEK-1/MKK-4-JNK signaling pathway is critically involved in the mitochondrial cell death in response to ionizing radiation, whereas the c-Src-Rac1-p38 MAPK signaling pathway plays a cytoprotective role against mitochondrial cell death. Improved understanding of the mechanisms involved in cell death and cell survival in response to radiation should allow the development of novel

**FIGURE 4.** PI3K activation is required for stimulation of the SEK-1-JNK signaling pathway and subsequent cell death. **A.** Activation of PI3K in response to radiation. HeLa cells were exposed to 10 Gy of γ-radiation. After 24, 48, and 72 h, total cell extracts were immunoprecipitated with anti-PI3K antibody (anti-p85), and PI3K assay was done on the immune complexes. A representative autoradiogram of the PI3K assay is shown, and the position of phosphatidylinositol phosphate (*PIP*) is indicated. **B.** Effect of the PI3K inhibitor LY294002 on radiation-induced cell death. HeLa cells were exposed to 10 Gy of γ-radiation in the presence or absence of LY294002. After 48 h, total cell extracts were immunoprecipitated with anti-PI3K antibody (anti-p85), and PI3K assay was done on the immune complexes. A representative autoradiogram is shown, and the position of phosphatidylinositol phosphate is indicated. Inhibition of PI3K by pretreatment with LY294002 or siRNA targeting of p110 or p85 in three human cervical cancer cell lines (HeLa, CaSki, and SiHa) and analysis of their effects on clonogenic survival. **C.** Effect of PI3K inhibition on radiation-induced cell death. HeLa cells were exposed to 10 Gy of γ-radiation in the presence or absence of LY294002 or siRNA of p110 or p85. After 48 and 72 h, cell death was determined by flow cytometric analysis. Columns, mean of three independent experiments; bars, SE. *, $P < 0.05$. **D.** Effects of PI3K inhibition on Bax and Bak activation. HeLa cells were exposed to 10 Gy of γ-radiation in the presence or absence of LY294002. After 48 h, activity-related modulation of Bak or Bax was determined by flow cytometric analysis using specific antibodies recognizing the $NH_2$-terminal epitopes of these proteins, as described in Materials and Methods. Columns, mean of three independent experiments; bars, SE. *, $P < 0.05$. **E.** Effect of PI3K inhibition on radiation-induced Bax translocation and Bcl-2 down-regulation. HeLa cells were exposed to 10 Gy of γ-radiation and CaSki and SiHa cells to 20 Gy of γ-radiation in the presence or absence of LY294002. After 48 h, total cell extracts were subjected to Western blot analysis with anti–Bcl-2 and anti–β-actin antibodies. Western blotting of mitochondrial fractions was done with anti-Bax and anti-HSP60 antibodies. HSP60 and β-actin were used as the mitochondrial marker protein and loading control, respectively. **F.** Effect of PI3K inhibition on radiation-induced mitochondrial transmembrane potential loss. HeLa cells were exposed to 10 Gy of γ-radiation in the presence or absence of LY294002 or siRNA of p110 or p85. After 48 and 72 h, the mitochondrial transmembrane potential was determined as retention of $DiOC_6(3)$ added during the last 30 min of treatment, using flow cytometry. Columns, mean of three independent experiments; bars, SE. *, $P < 0.05$. **G.** Effect of PI3K inhibition on radiation-induced cytochrome *c* release from mitochondria. HeLa cells were exposed to 10 Gy of γ-radiation in the presence or absence of LY294002. After 48 h, the cytosolic fraction was subjected to Western blot analysis with anti–cytochrome *c* and anti–α-tubulin antibodies. Western blotting of the total cell extracts was done using anti–caspase-3 and anti–β-actin antibodies. α-Tubulin and β-actin were used as the cytosolic marker protein and loading control, respectively. **H.** Effect of PI3K inhibition on activation of the SEK-1-JNK signaling pathway. HeLa cells were exposed to 10 Gy of γ-radiation in the presence or absence of LY294002. After 48 h, proteins were subjected to Western blot analysis with anti–phospho-SEK, anti–phospho-JNK, anti-JNK, anti–phospho-p38 MAPK, anti–p38 MAPK, anti–phospho-ERK, and anti-ERK antibodies.

therapeutic strategies by specific intervention in the cellular signal transduction pathways activated in response to radiation treatment, thus augmenting the therapeutic efficacy of cancer treatment.

## Materials and Methods

### *Materials*

Monoclonal antibody to cytochrome *c* was obtained from PharMingen, and polyclonal antibodies to phospho-ERK, p38 MAPK, SEK-1/MKK-4, PTEN, Bcl-2, Bax, α-tubulin, and HSP60 were purchased from Santa Cruz. β-Actin, L-α-phosphatidylinositol, and enolase were purchased from Sigma. Polyclonal antibodies to caspase-3, PARP, ERK, JNK, phospho-p38 MAPK, phospho-JNK, and phospho-SEK were obtained from Cell Signaling Technology. Polyclonal antibodies to p85, activating transcription factor-2 protein, myelin basic protein protein, glutathione *S*-transferase-c-Jun protein, and Pak-conjugated agarose were purchased from Upstate Biotechnology, Inc. The MAPK/ERK kinase inhibitor (PD98059), p38 MAPK inhibitor (SB203580), JNK inhibitor (SP600125), and PI3K inhibitor (LY294002) were obtained from Calbiochem.



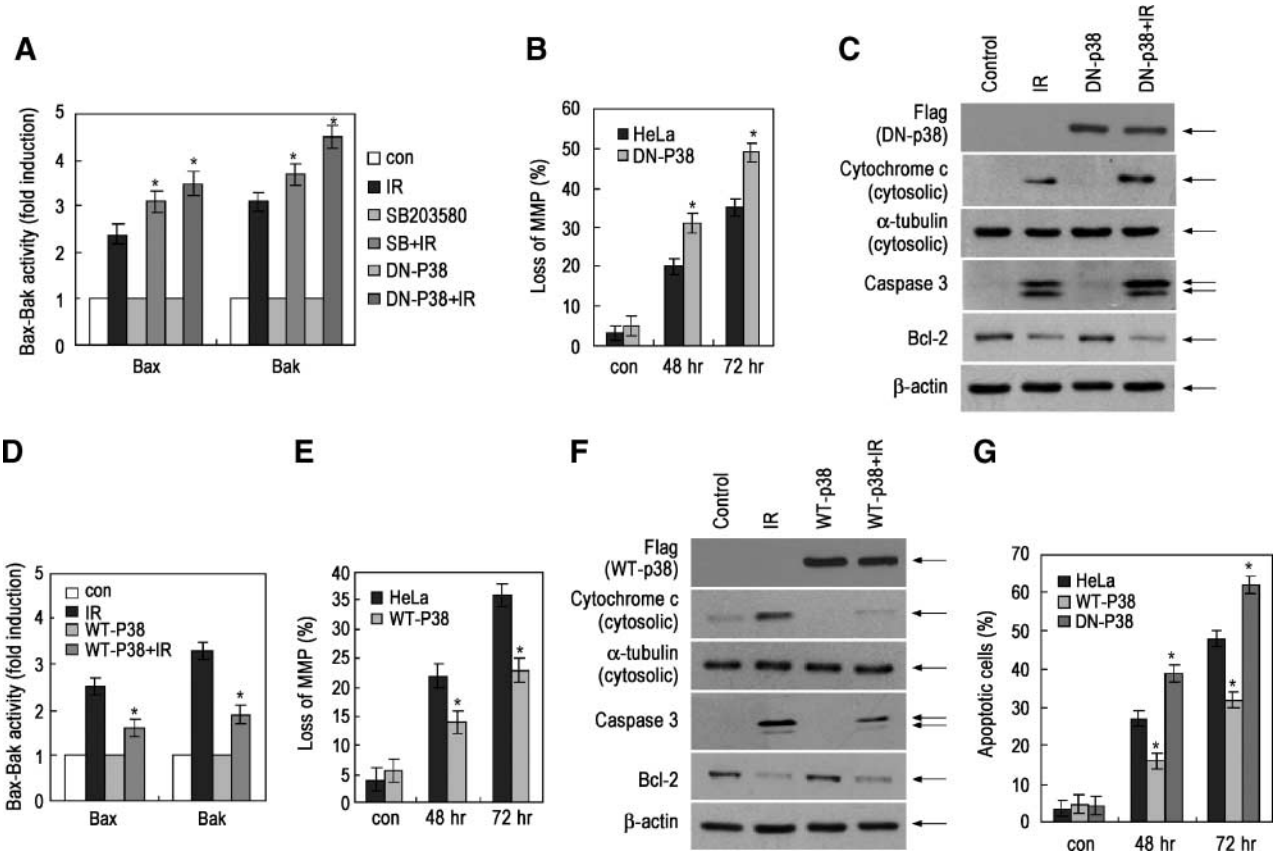



**FIGURE 5.** p38 MAPK plays a cytoprotective role against radiation-induced apoptosis in human cervical cancer cells. **A.** Effects of p38 MAPK inhibition on Bax and Bak activation in response to radiation. HeLa cells were exposed to 10 Gy of γ-radiation in the presence or absence of SB203580 or overexpression of dominant-negative (*DN*) p38 MAPK. After 48 h, activity-related modulation of Bak or Bax was determined by flow cytometric analysis using specific antibodies recognizing the $NH_2$-terminal epitopes of these proteins, as described in Materials and Methods. Columns, mean of three independent experiments; bars, SE. *, $P < 0.05$. **B.** Effects of overexpression of dominant-negative p38 MAPK on radiation-induced mitochondrial transmembrane potential loss. HeLa cells overexpressing dominant-negative p38 MAPK were exposed to 10 Gy of γ-radiation. After 48 and 72 h, the mitochondrial transmembrane potential was determined as retention of $DiOC_6(3)$ using flow cytometry. Columns, mean of three independent experiments; bars, SE. *, $P < 0.05$. **C.** Effects of overexpression of dominant-negative p38 MAPK on radiation-induced cytochrome *c* release. HeLa cells overexpressing dominant-negative p38 MAPK were exposed to 10 Gy of γ-radiation. After 48 h, cytosolic fractions were subjected to Western blot analysis with anti–cytochrome *c* and anti–α-tubulin antibodies. Western blotting of total cell extracts was done using anti–caspase-3, anti-Bcl2, and anti–β-actin antibodies. α-Tubulin and β-actin were used as the cytosolic marker protein and loading control, respectively. **D.** Effects of p38 MAPK on Bax and Bak activation in response to radiation. HeLa cells overexpressing WT-p38 MAPK were exposed to 10 Gy of γ-radiation. After 48 h, activity-related modulation of Bak or Bax was determined by flow cytometric analysis using specific antibodies recognizing the $NH_2$-terminal epitopes of these proteins, as described in Materials and Methods. Columns, mean of three independent experiments; bars, SE. *, $P < 0.05$. **E.** Effects of overexpression of p38 MAPK (WT-p38 MAPK) on radiation-induced mitochondrial transmembrane potential loss. HeLa cells overexpressing WT-p38 MAPK were exposed to 10 Gy of γ-radiation. After 48 and 72 h, the mitochondrial transmembrane potential was determined as retention of $DiOC_6(3)$ using flow cytometry. Columns, mean of three independent experiments; bars, SE. *, $P < 0.05$. **F.** Effects of overexpression of WT-p38 MAPK on radiation-induced cytochrome *c* release. HeLa cells overexpressing WT-p38 MAPK were exposed to 10 Gy of γ-radiation. After 48 h, cytosolic fractions were subjected to Western blot analysis with anti-cytochrome *c* and anti–α-tubulin antibodies. Western blotting of total cell extracts was done with anti-caspase-3, anti-Bcl2, and anti–β-actin antibodies. α-Tubulin and β-actin were used as the cytosolic marker protein and loading control, respectively. **G.** Effects of overexpression of WT-p38 MAPK or dominant-negative p38 MAPK on radiation-induced cell death. HeLa cells overexpressing WT-p38 MAPK or dominant-negative p38 MAPK were exposed to 10 Gy of γ-radiation. After 48 and 72 h, cell death was determined by flow cytometry. Columns, mean of three independent experiments; bars, SE. *, $P < 0.05$.

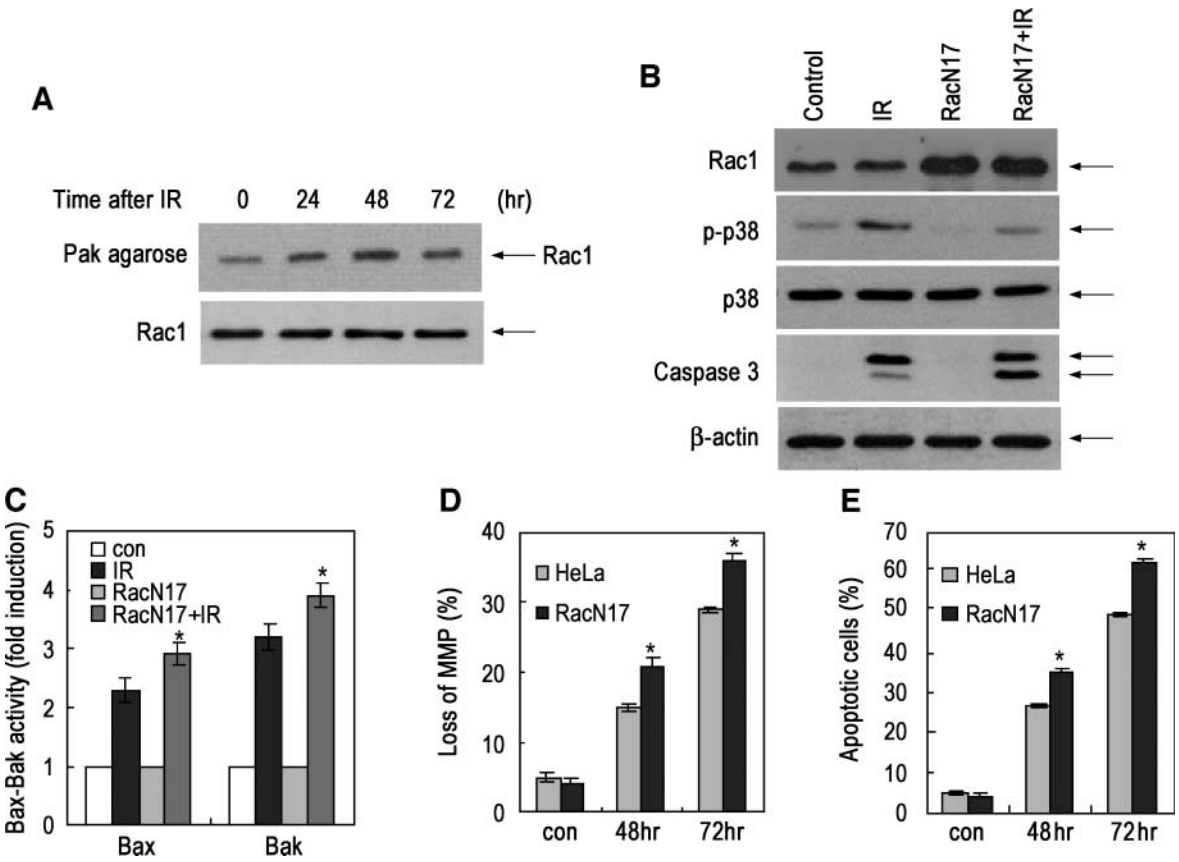



**FIGURE 6.** Rac1 is an upstream activator of p38 MAPK in survival signaling of cells following radiation. **A.** Analysis of interactions between Rac1 and Pak in HeLa cells at 24, 48, and 72 h following exposure to 10 Gy of γ-radiation, as detected by Western blot analysis with anti-Rac1 antibody after incubation with Pak-conjugated agarose. Expression of Rac1 in cells exposed to 10 Gy of γ-radiation was detected by Western blot analysis with anti-Rac1 antibody. **B.** Effect of a dominant-negative form of Rac1 (RacN17) on radiation-induced p38 MAPK phosphorylation. Western blot analysis of phospho-p38 MAPK and caspase-3 activation after exposure of HeLa cells overexpressing RacN17 to 10 Gy of γ-radiation. β-Actin was used as the loading control. **C.** Effects of a dominant-negative form of Rac1 (RacN17) on Bax and Bak activation in response to radiation. HeLa cells were exposed to 10 Gy of γ-radiation in the presence or absence of RacN17. After 48 h, activity-related modulation of Bak or Bax was determined by flow cytometric analysis using specific antibodies recognizing the $NH_2$-terminal epitopes of these proteins, as described in Materials and Methods. Columns, mean of three independent experiments; bars, SE. *, $P < 0.05$. **D.** Effect of a dominant-negative form of Rac1 (RacN17) on radiation-induced mitochondrial transmembrane potential loss. HeLa cells were exposed to 10 Gy of γ-radiation in the presence or absence of RacN17. After 48 and 72 h, the mitochondrial transmembrane potential of cells was determined as retention of $DiOC_6(3)$ using flow cytometry. Columns, mean of three independent experiments; bars, SE. *, $P < 0.05$. **E.** Effect of a dominant-negative form of Rac1 (RacN17) on radiation-induced apoptosis. HeLa cells were exposed to 10 Gy of γ-radiation in the presence or absence of RacN17. After 48 and 72 h, cell death was determined by flow cytometric analysis. Columns, mean of three independent experiments; bars, SE. *, $P < 0.05$.

### *Cell Culture and Transfection*

Human cervical carcinoma cell lines (HeLa, CaSki, and SiHa) were obtained from the American Type Culture Collection. HeLa and CaSki cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum, whereas SiHa cells were cultured in MEM supplemented with 10% fetal bovine serum and nonessential amino acids. Media were supplemented with 100 units/mL penicillin and 100 μg/mL streptomycin, and all cells were incubated at 37°C in 5% $CO_2$. Cells were transfected with indicated cDNA plasmid or control plasmid using Lipofectamine PLUS reagent (Invitrogen) by following the procedure recommended by the manufacturer.

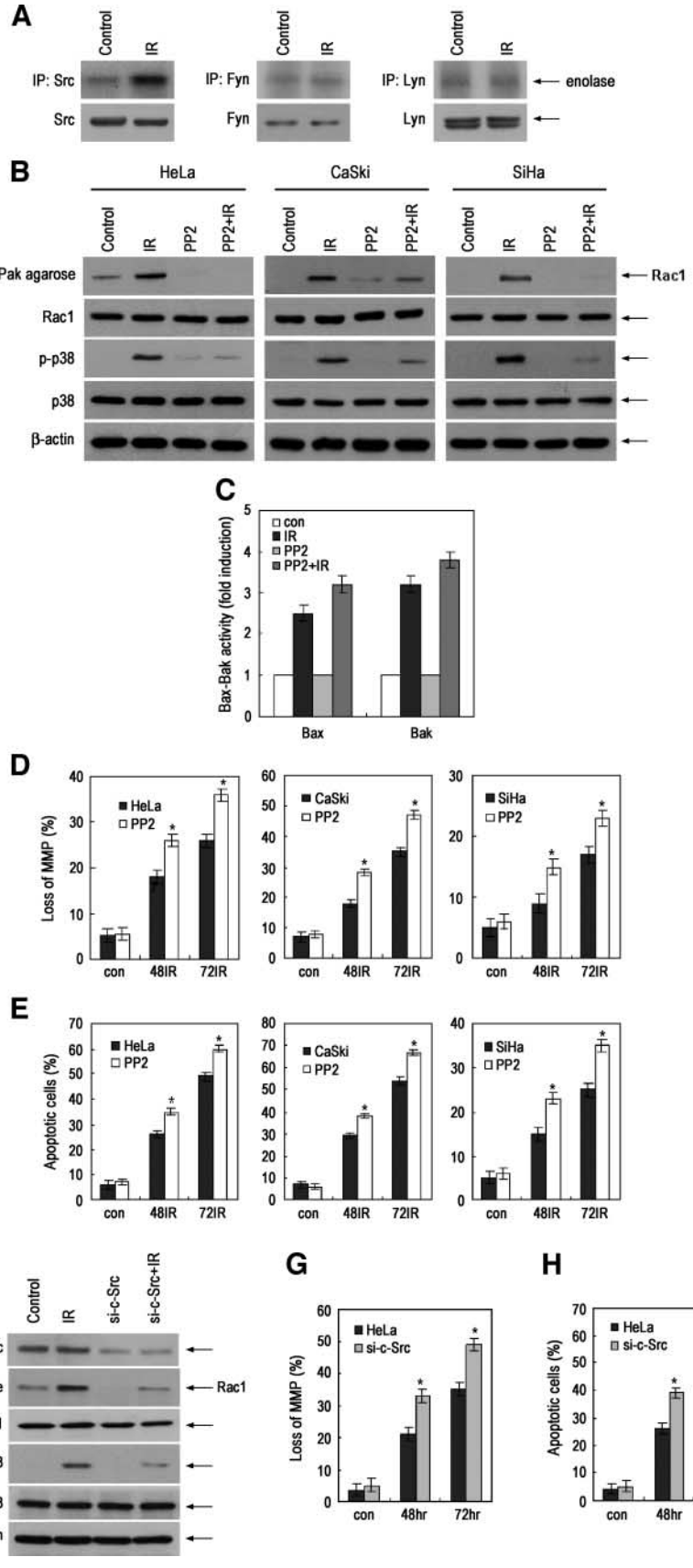
### *Clonogenic Survival Assay*

Cells were plated in triplicate into 60-mm culture dishes at a density of $5 \times 10^2$ per dish and exposed to range of doses of γ-radiation at 0 to 4 Gy. After 10 d of incubation, the culture medium was decanted and the colonies were fixed in methanol. Colonies were stained with 0.4% trypan blue dye and the number of colonies containing >5 mm cells was counted. The fraction of cells surviving irradiation was normalized to the surviving fraction of the corresponding untreated control.

### *siRNA Transfection*

RNA interferences of SEK-1, JNK1, JNK2, p110, and c-Src were done using 21-bp (including a 2-deoxynucleotide overhang) siRNA duplexes purchased from Ambion. siRNA targeting of p85 was done using 21-bp siRNA duplexes purchased from Santa Cruz. The sense strand nucleotide sequences were, for SEK-1, GGACAGAAGUGGAAA-UAUUTT; JNK1, GACCUAAAUAUGCUGGAUATT; and p110, GACAAUGAAUUAAGGATT.

A control siRNA specific for green fluorescent protein (CCACTACCTGAGCACCCAG) was used as the negative



control. Cells were plated on 100-mm dishes at 50% confluency, and siRNA duplexes (50 nmol/L) were introduced into cells using Lipofectamine 2000 (Invitrogen) by following the procedure recommended by the manufacturer.

### *Quantification of Cell Death*

Fluorescence-activated cell sorting analysis using propidium iodide staining detects cell death by means of the dye entering the cells along with changes in the target cell membrane and DNA damage. For the cell death assessment, cells were plated in 60-mm dishes at a density of $2 \times 10^5$ per dish and treated with radiation the next day. At indicated time points, cells were harvested by trypsinization, washed in PBS, and then incubated in propidium iodide (2.5 μg/mL) for 5 min at room temperature. Then, cells (10,000 per sample) were analyzed on a FACScan flow cytometer using Cell Quest software.

### *Irradiation*

Cells were plated in 35-, 60-, or 100-mm dishes and incubated at 37°C under humidified 5% $CO_2$-95% air in culture medium until 70% to 80% confluent. Cells were then exposed to γ-rays with $^{137}$Cs γ-ray source (Atomic Energy of Canada, Ltd.) with a dose rate of 3.81 Gy/min.

### *Western Blot Analysis*

Western blot analysis was done as previously described (36). Briefly, cell lysates were prepared by extracting proteins with lysis buffer [40 mmol/L Tris-HCl (pH 8.0), 120 mmol/L NaCl, 0.1% NP40] supplemented with protease inhibitors. Proteins were separated by SDS-PAGE and transferred onto a nitrocellulose membrane. The membrane was blocked with 5% nonfat dry milk in TBS and incubated with primary antibodies for 1 h at room temperature. Blots were developed with a peroxidase-conjugated secondary antibody and proteins visualized by enhanced chemiluminescence procedures (Amersham) using the manufacturer's protocol.

### *Measurement of Mitochondrial Membrane Potential*

Mitochondrial membrane potential was determined as retention of the mitochondria-specific dye $DiOC_6(3)$ (Molecular Probes, Inc.). Cells were loaded with 30 nmol/L $DiOC_6(3)$ during the last 30 min after radiation treatment. After medium removal, cells were washed twice with PBS and the relative amount of retained $DiOC_6(3)$ was measured by flow cytometric analysis.

### *Preparation of Cytosolic and Mitochondrial Protein Fractions for Measuring Cytochrome c and Bax Translocation*

Cells were collected and washed twice in ice-cold PBS, resuspended in S-100 buffer [20 mmol/L HEPES, pH 7.5, 10 mmol/L KCl, 1.9 mmol/L $MgCl_2$, 1 mmol/L EGTA, 1 mmol/L EDTA, a mixture of protease inhibitors], and incubated on ice for 20 min. Homogenization was done with a Dounce glass homogenizer and a loose pestle (Wheaton) for 70 strokes. Homogenates were spun at $1,000 \times g$ to remove unbroken cells, nuclei, and heavy membranes. The supernatant was recentrifuged at $14,000 \times g$ for 30 min to collect the mitochondria-rich (pellet) and cytosolic (supernatant) fractions. The mitochondria-rich fraction was washed once with extraction buffer, followed by final resuspension in lysis buffer [150 mmol/L NaCl, 50 mmol/L Tris-Cl (pH 7.4), 1% NP40, 0.25% sodium deoxycholate, 1 mmol/L EGTA] containing protease inhibitors for Western blot analysis.

### *Flow Cytometric Analysis of Bax and Bak Activation*

Bax- and Bak-associated conformational changes were analyzed as described previously (37). Briefly, cells were fixed with 0.25% paraformaldehyde for 5 min and incubated with antibodies that recognized the $NH_2$-terminal epitopes of Bax (clone 6A7; PharMingen) or Bak (AM03. TC100; Oncogene Research Products) diluted 1:100 in PBS containing digitonin (100 g/mL) and 5% fetal bovine serum at 4°C for 30 min. Cells were washed thrice with

**FIGURE 7.** c-Src signaling plays a survival role in protecting cells from radiation-induced apoptosis in human cervical cancer cells. **A.** Activation of Src family kinases in response to radiation. Src family kinases were detected using the immune complex kinase assay with anti–c-Src, anti-fyn, or anti-lyn antibodies. Enolase was used as a substrate for Src family kinases. **B.** Effect of c-Src inhibition by pretreatment with PP2 on PI3K and Rac activation, and phosphorylation of p38 MAPK in HeLa, CaSki, and SiHa cells. HeLa cells were exposed to 10 Gy and CaSki and SiHa cells to 20 Gy of γ-radiation in the presence or absence of the c-Src inhibitor PP2. Analysis of interactions between Rac1 and Pak in HeLa, CaSki, and SiHa cells at 48 h after exposure to γ-radiation, as detected by Western blot analysis with anti-Rac1 antibody after incubation with Pak-conjugated agarose. After 48 h, total cell extracts were subjected to Western blot analysis with anti–phospho-p38 MAPK, anti–p38 MAPK, and anti–β-actin antibodies. **C.** Effects of c-Src inhibition by pretreatment with PP2 on Bax and Bak activation. HeLa cells were exposed to 10 Gy of γ-radiation in the presence or absence of the c-Src inhibitor PP2. After 48 h, activity-related modulation of Bak or Bax was determined by flow cytometric analysis using specific antibodies recognizing the $NH_2$-terminal epitopes of these proteins, as described in Materials and Methods. Columns, mean of three independent experiments; bars, SE. *, $P < 0.05$. **D.** Effect of inhibition of c-Src by PP2 pretreatment on radiation-induced mitochondrial transmembrane potential loss. HeLa cells were exposed to 10 Gy of γ-radiation and CaSki and SiHa cells to 20 Gy of γ-radiation in the presence or absence of PP2. After 48 and 72 h, mitochondrial transmembrane potential of cells was determined as retention of DiOC6(3) added during the last 30 min of treatment using flow cytometry analysis. Columns, mean of three independent experiments; bars, SE. *, $P < 0.05$. **E.** Effect of inhibition of c-Src by PP2 pretreatment on radiation-induced apostosis. HeLa cells were exposed to 10 Gy of γ-radiation and CaSki and SiHa cells to 20 Gy of γ-radiation in the presence or absence of PP2. After 48 and 72 h, cells death was determined by flow cytometric analysis. Columns, mean of three independent experiments; bars, SE. *, $P < 0.05$. **F.** Effect of c-Src inhibition by siRNA targeting on Rac activation and phosphorylation of p38 MAPK in HeLa cells. Analysis of interactions between Rac1 and Pak in Hela cells at 48 h after exposure to γ-radiation, as detected by Western blot analysis with anti-Rac1 antibody after incubation with Pak-conjugated agarose. After 48 h, total proteins were subjected to Western blot analysis with anti–phospho-p38 MAPK, anti–p38 MAPK, and anti–β-actin antibodies. **G.** Effect of c-Src inhibition by siRNA targeting on radiation-induced mitochondrial transmembrane potential loss. HeLa cells were exposed to 10 Gy of γ-radiation. After 48 and 72 h, the mitochondrial transmembrane potential of cells was determined as retention of DiOC6(3) added during the last 30 min of treatment using flow cytometry analysis. Columns, mean of three independent experiments; bar, SE. *, $P < 0.05$. **H.** Effect of c-Src inhibition by siRNA targeting on radiation-induced apostosis. HeLa cells were exposed to 10 Gy of γ-radiation. After 48 and 72 h, cell death was determined by flow cytometric analysis. Columns, mean of three independent experiments; bars, SE. *, $P < 0.05$.

PBS and incubated with a FITC-conjugated antimouse antibody (diluted 1:200) in PBS at 4°C for 30 min. After washing thrice with PBS, cells (10,000 per sample) were analyzed on a FACSCalibur flow cytometer using CellQuest software.

### *Immune Complex Kinase Assay*

Cell lysates were incubated with primary antibody, and immune complexes were collected on protein A-Sepharose beads and resuspended in a kinase assay mixture containing [$\gamma$-$^{32}$P]ATP and substrate. Proteins were separated on SDS-polyacrylamide gels, and bands detected by autoradiography.

### *PI3K Assay*

Cells were lysed in Tris-HCl (20 mmol/L, pH 8.0), 137 mmol/L NaCl, 1 mmol/L $MgCl_2$, 1 mmol/L $CaCl_2$, 1% NP40, 10% glycerol, 2 mmol/L sodium orthovanadate, 10 μg/mL aprotinin, 10 μg/mL leupeptin, and 1 mmol/L phenylmethylsulfonyl fluoride. Lysates were clarified, and equal amounts of the lysate proteins (400 μg) were immunoprecipitated with an antibody against the p85 subunit of PI3K (Upstate Biotechnology). Immune complexes were washed twice with 1% NP40, 1 mmol/L sodium orthovanadate, and PBS (pH 7.4); twice with 100 mmol/L Tris-HCl (pH 7.5), 500 mmol/L LiCl, and 1 mmol/L sodium orthovanadate; and twice with 150 mmol/L NaCl and 50 mmol/L Tris-HCl (pH 7.2). Kinase reactions were initiated by adding 5 mg/mL L-$\alpha$-phosphatidylinositol (Sigma) in 20 mmol/L HEPES (pH 7.4), 5 mmol/L $MnCl_2$, 10 μmol/L ATP, 10 μCi of [$\gamma$-$^{32}$P]ATP, and 2.5 mmol/L EGTA. After 20-min incubation, reactions were quenched by adding 1 mol/L HCl. Phospholipids were extracted with a 1:1 mixture of chloroform/methanol and separated by TLC.

### *Statistical Analysis*

Statistical analyses were done using Student's $t$ test. The data were expressed as mean ± SE derived from at least three independent experiments. Differences were considered significant at $P < 0.05$.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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