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Activation of Bak and Bax through c-Abl-Protein Kinase Cδ-p38 MAPK Signaling in Response to Ionizing Radiation in Human Non-small Cell Lung Cancer Cells*

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Intracellular signaling molecules and apoptotic factors seem to play an important role in determining the radiation response of tumor cells. However, the basis for the link between signaling pathway and apoptotic cell death machinery after ionizing irradiation remains still largely unclear. In this study, we showed that c-Abl-PKCδ-Rac1-p38 MAPK signaling is required for the conformational changes of Bak and Bax during ionizing radiation-induced apoptotic cell death in human non-small cell lung cancer cells. Ionizing radiation induced conformational changes and subsequent oligomerizations of Bak and Bax, dissipation of mitochondrial membrane potential, and cytochrome *c* release from mitochondria. Small interference (siRNA) targeting of Bak and Bax effectively protected cells from radiation-induced mitochondrial membrane potential loss and apoptotic cell death. p38 MAPK was found to be selectively activated in response to radiation treatment. Inhibition of p38 MAPK completely suppressed radiation-induced Bak and Bax activations, dissipation of mitochondrial membrane potential, and cell death. Moreover, expression of a dominant negative form of protein kinase Cδ (PKCδ) or siRNA targeting of PKCδ attenuated p38 MAPK activation and conformational changes of Bak and Bax. In addition, ectopic expression of RacN17, a dominant negative form of Rac1, markedly inhibited p38 MAPK activation but did not affect PKCδ activation. Upon stimulation of cells with radiation, PKCδ was phosphorylated dramatically on tyrosine. c-Abl-PKCδ complex formation was also increased in response to radiation. Moreover, siRNA targeting of c-Abl attenuated radiation-induced PKCδ and p38 MAPK activations, and Bak and Bax modulations. These data support a notion that activation of the c-Abl-PKCδ-Rac1-p38 MAPK pathway in response to ionizing radiation signals conformational changes of Bak and Bax, resulting in mitochondrial activation-mediated apoptotic cell death in human non-small cell lung cancer cells.

Sensitivity of tumor cells to radiation is a critical determinant of the probability of local control and ultimately of cure of cancers by radiation therapy. Thus, one approach to improving the outcome of radiation therapy depends on determining which factors lead to tumor cell sensitivity to therapy. It has been shown that many factors affect susceptibility of tumor cells to ionizing radiation. Among them, intracellular signaling molecules and apoptotic factors seem to play an important role in determining the intrinsic radiosensitivity of tumor cells (1, 2).

The Bcl-2 family proteins constitute critical control points in the intrinsic apoptotic pathway. Anti-apoptotic members of the Bcl-2 family, such as Bcl-2, Bcl-XL, Bcl-w, and Mcl-1 act primarily to preserve mitochondrial membrane potential and suppress the release of apoptotic cell death-activating factors such as cytochrome *c* and apoptosis-inducing factor (3, 4). In contrast, pro-apoptotic members of the Bcl-2, such as Bax, Bak, Bid, Bad, Bok, and Bim mainly induce the release of pro-apoptotic mediators by causing mitochondrial dysfunction, which in turn activate the initiator caspase-9 (5, 6). These proteins are subdivided into "multidomain" pro-apoptotic proteins (Bax or Bak) and "BH3-only" proteins (Bid, Bim, and Bok). BH3-only proteins, which act as sensors of cellular stress, are activated by transcriptional up-regulation and/or post-translational modification following an apoptotic stimulus (7). Once activated, these proteins induce the activation of Bax and/or Bak. As a consequence, Bax and Bak form oligomeric pores leading to the release of apoptogenic factors from mitochondria into the cytosol (8, 9). However, Bax or Bak forms a homo-oligomerized multimer in response to stress signals without activations of BH-3 only proteins by yet unknown mechanisms resulting in downstream mitochondrial dysfunction and subsequent cytochrome *c* release (10, 11). Recently it has been demonstrated that p38 MAPK[3] induces apoptosis by regulating the conformational activation of Bax and subsequent mitochondrial relocalization of Bax in response to various stimuli, including UVB, cisplatin, taxol, and nocodazole (12–14). These results are consistent with the fact that p38 MAPK acts at early step(s) prior to dysfunction of mitochondria and caspase activation in several cell types (15–17). However, the precise mechanism of p38 MAPK-mediated conformational activation of Bax still remains to be elucidated.

PKCδ, a member of the novel PKC subfamily, is actively involved in apoptotic cell death in a stimulus or in a tissue type-dependent manner (18); it regulates the expression and function of apoptotic-related pro-

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[3] The abbreviations used are: MAPK, mitogen-activated protein kinase; PKC, protein kinase C; MEK, mitogen-activated protein kinase kinase; PARP, poly(ADP-ribose) polymerase; z-VAD-fmk, benzyloxycarbonyl-Phe-Val-Ala-Asp (Ome)-fluoromethylketone; $DiOC_6(3)$, 3,3′-dihexylocarbocyanine iodide; siRNA, small interfering RNA; JNK, c-Jun N-terminal kinase; ERK, extracellular signal-regulated kinase; FACS, fluorescence-activated cell sorting; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; GST, glutathione *S*-transferase.

teins. Activation of PKCδ was accompanied by subsequent activation of all three MAPK cascades (19). Other reports suggested that PKCδ is associated with a rapid increase in JNK and p38 MAPK activity but ERK were not significantly activated (20, 21). Similar to other PKCs, activation of PKCδ stimulates its translocation to cellular membrane and increases its serine/threonine kinase activity. In several model systems, tyrosine phosphorylation is involved in regulating PKCδ activity with resultant increase or decrease in kinase activity depending on the cell type and phosphorylation sites (22, 23). Signaling through the insulin-like growth factor-1, epidermal growth factor, platelet-derived growth factor receptor, or exposure to H_2O_2 increases both tyrosine phosphorylation and catalytic activity of PKCδ (24, 25). Tyrosine phosphorylation was also observed in response to etoposide in glioma cells, and this phosphorylation was essential for the apoptotic effect of PKCδ (26). In contrast, tyrosine phosphorylation of PKCδ in Ras- or v-Src-transformed cells results in inactivation of PKCδ catalytic function and causes rapid degradation of PKCδ (27, 28). However, little is known about the identity of tyrosine kinases that directly phosphorylate PKCδ and about tyrosine phosphorylated PKCδ-mediated apoptotic cell death pathways.

In the present study, we provide new evidence that radiation induces mitochondrial activation-mediated apoptotic cell death through conformational changes of pro-apoptotic proteins Bax and Bak, and that c-Abl-PKCδ-Rac1-p38 MAPK signaling is essential for the apoptotic modulations of Bak and Bax. The molecular signaling pathways involved in the initiation of apoptotic cell death in response to ionizing radiation that we elucidated in this study will guide the development of novel strategies in radiation therapy of cancer.

EXPERIMENTAL PROCEDURES

Materials—Anti-PKCα, -β1, -δ, and -ζ and anti-HSP60 and c-Abl antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-MAPK, anti-phospho-MAPK (P202/Y204), anti-p38 MAPK, anti-phospho-p38 MAPK, and anti-phospho-JNK polyclonal antibodies were purchased from New England Biolabs (Beverly, MA). β-Actin was from Sigma. Polyclonal antibody to caspase 3 and monoclonal antibodies to PARP and cytochrome *c* were obtained from BD Pharmingen (San Diego, CA). Anti-phosphotyrosine antibody, MBP protein, and Pak-conjugated agarose were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). z-VAD-fmk, z-DEVD-fmk, PD98059, SB203580, and SP600125 were obtained from Calbiochem (San Diego, CA).

Cell Culture and Transfection—Non-small cell lung cancer cells (NCI-H460 and NCI-H1299) were grown in RPMI 1640 supplemented with 10% fetal bovine serum, glutamine, HEPES, and antibiotics at 37 °C in a humidified incubator with a mixture of 95% air and 5% CO_2. The cells were transfected with dominant negative forms of p38 MAPK, ERK, JNK, or Rac (RacN17) cloned into pcDNA3.1 vector, or dominant negative forms of PKC isozymes (PKCα, -β, -δ, and -ζ-KR) cloned into the pHANE vector using Lipofectamine PLUS reagent (Invitrogen) by following the procedure recommended by the manufacturer.

siRNA Transfection—RNA interferences of Bak, PKC isozymes, and c-Abl were performed using 21-bp (including a 2-deoxynucleotide overhang) siRNA duplexes purchased from Ambion (Austin, TX). siRNA targeting of Bax was performed using 23-bp siRNA duplexes purchased from New England Biolabs (Beverly, MA). The sense strand nucleotide sequence for Bak siRNA was GGAUUCAGCUAUUCUGGAAdTdT. Bax siRNA was AACATGGAGCTGCAGAGGATGAdTdT, PKCα siRNA was GGACAUAUCAAAAUUGCUGdTdT, PKCβ siRNA was GGAAGGCGAGUACUUCAAUdTdT, PKCδ siRNA was GGCCAAGGUGUUGAUGUCUdTdT, PKCζ siRNA was GGCCUCAUCAUUCAUGUUUdTdT, and c-Abl siRNA was GGUCCAUCUCGCUGAGAUAdTdT. A control siRNA specific to the green fluorescent protein DNA sequence CCACTACCTGAGCACCCAG was used as a negative control. For transfection, non-small cell lung cancer cells were plated in 10-cm dishes at 30% confluency, and siRNA duplexes (200 nM) were introduced into the cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendations.

Irradiation—Cells were plated in 3.5-, 6-, or 10-cm dishes and incubated at 37 ° C under humidified 5% CO_2-95% air in culture medium until 70–80% confluent. Cells were then exposed to γ-rays with ^{137}Cs γ-ray source (Atomic Energy of Canada, Ltd., Canada) with a dose rate of 3.81 Gy/min.

Quantification of Cell Death—FACS 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, the cells were plated in 60-mm dish with cell density of $2' \times 10^5$cells per dish and treated with radiation the next day. At indicated time points, cells were harvested by trypsinization, washed in phosphate-buffered saline, 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 FACSscan flow cytometer, using Cell Quest software.

Measurement of Mitochondrial Membrane Potential—Mitochondrial membrane potential was determined by the retention of mitochondrial-specific dye $DiOC_6$ (3). Cells were loaded with 30 nM $DiOC_6$ (3) during the last 30 min of radiation treatment. After removal of the medium, the cells were washed twice with phosphate-buffered saline, and the concentration of retained $DiOC_6$ (3) was measured using flow cytometer (BD Biosciences).

Isolation of Cytosolic and Mitochondrial Fractions—Cells were collected and washed twice in ice-cold phosphate-buffered saline, and were resuspended in S-100 buffer (20 mM HEPES (pH 7.5), 10 mM KCl, 1.9 mM $MgCl_2$, 1 mM EGTA, 1 mM EDTA, and a mixture of protease inhibitors) and incubated on ice for 20 min. After 20-min incubation on ice, the cells were homogenized with a Dounce glass homogenizer with a loose pestle (Wheaton, Millville, NJ) for 70 strokes. Cell homogenates were spun at $1000 \times g$ to remove unbroken cells, nuclei, and heavy membranes. The supernatant was respun at $14{,}000 \times g$ for 30 min to collect the mitochondria-rich (the pellet) and the cytosolic (the supernatant) fractions.

Western Blot Analysis—Cells were solubilized with lysis buffer (120 mM NaCl, 40 mM Tris (pH 8.0), 0.1% Nonidet P-40) and boiled for 5 min, and an equal amount of protein (30 μg/well) was analyzed on 7.5–15% SDS-PAGE. After electrophoresis, proteins were transferred onto a nitrocellulose membrane. The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline and then incubated with primary antibodies for 1 h at room temperature. Blots were developed by peroxidase-conjugated secondary antibody, and proteins were visualized by enhanced chemiluminescence (ECL) procedures (Amersham Biosciences) according to the manufacturer's recommendations.

Flow Cytometric Analysis of Bak and Bax Activation—Bak- or Bax-associated conformational changes were assessed as previously described (11). Briefly, after fixation (0.25% paraformaldehyde, 5 min) and washing, cells were incubated for 30 min in the presence of digitonin (100 μg/ml) with antibodies recognizing N-terminal epitopes of Bak (AM03TC100, Oncogene Research Products) or Bax (clone 6A7, BD Pharmingen). After incubation with a fluorescein isothiocyanate-conjugated anti-mouse antibody for 30 min, cells (10,000 per sample) were analyzed on a FACSCalibur flow cytometer, using Cell Quest software.

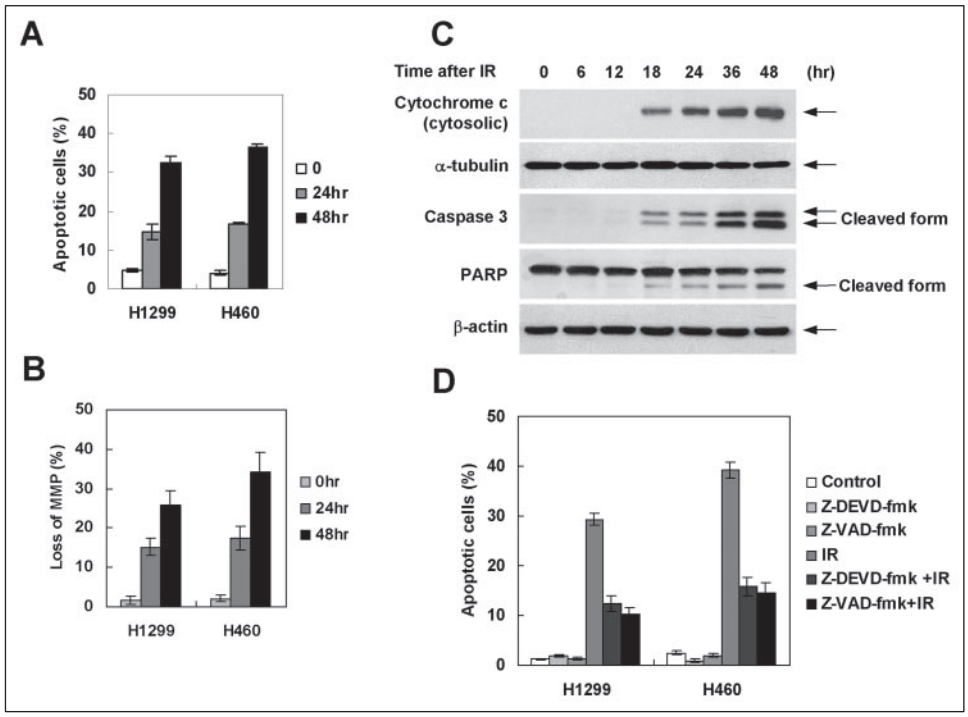

FIGURE 1. **Ionizing radiation induces mitochondrial dysfunction-mediated apoptotic cell death in human non-small cell lung cancer cells.** *A*, quantitative analysis of the time-dependent cell death after ionizing radiation (10 Gy) in human non-small cell lung cancer cells. Cell death was determined by flow cytometric analysis as described under "Experimental Procedures." *B*, loss of mitochondrial membrane potential after irradiation. Mitochondrial transmembrane potential of cells was determined by retention of $DiOC_6$ (3) added during the last 30 min of irradiation. After removal of the medium, the amount of retained $DiOC_6$ (3) was measured by flow cytometry. *C*, analysis of the cytochrome *c* release from the mitochondria, caspase 3 activation, and PARP cleavage after irradiation in NCI-H1299 cells, as detected by Western blot analysis with cytosolic fraction or total cell lysates. α-Tubulin was used as a cytosolic marker protein. *D*, flow cytometric analysis of the ionizing radiation-induced cell death in the presence of the pan-caspase inhibitor (z-VAD-fmk, 30 μM) or caspase 3 specific inhibitor (z-DEVD-fmk, 30 μM).

Cross-linking of Bak and Bax Proteins—Cells were permeabilized at room temperature with 0.015–0.02% digitonin for 1–2 min in isotonic buffer A (10 mM HEPES, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, pH 7.4) containing protease inhibitors. The permeabilized cells were shifted to 4 °C, scraped, and collected into centrifuge tubes. The supernatants (digitonin/cytosol) were collected after centrifugation at 15,000 × *g* for 10 min at 4 °C. The pellet was further extracted with ice-cold lysis buffer (2% CHAPS in buffer A containing protease inhibitors) for 60 min at 4 °C to obtain membrane fraction. Cells permeabilized with digitonin or membranes extracted with CHAPS were incubated with cross-linker (dissuccinimidyl suberate with linker lengths of 11.4 Å) on a head-to-head rocker for 30 min at room temperature. After quenching the unreacted cross-linkers with 1/10 volume of 2 M Tris-HCl (pH 7.4), cells or extracts were incubated for another 30 min at room temperature with rocking. After cross-linking, membranes were extracted with 2% CHAPS in buffer A and mixed with a non-denaturing loading buffer before SDS-PAGE (29).

Immunoprecipitation—Solubilized extracts (100–500 μg) in lysis buffer were precleared with protein A-Sepharose and the resultant supernatants were incubated with primary antibody (2 μg/ml) at 4 °C for 4 h. Immunoprecipitates were collected by incubating with protein A-Sepharose for 1 h, followed by centrifugation for 2 min at 4 °C. The pellets were washed with lysis buffer five times. The immunoprecipitates dissolved in SDS-sample buffer were analyzed by Western blotting as described above.

Immune Complex Kinase Assay—Proteins from 300 μg of cell extracts were immunoprecipitated with primary antibody (2 μg/ml) at 4 °C for 4 h. The immunoprecipitates were washed twice kinase reaction buffer (50 mM HEPES (pH 7.5), 10 mM $MgCl_2$, 1 mM dithiothreitol, 2.5 mM EGTA, 1 mM NaF, 0.1 mM Na_3VO_4, and 10 mM β-glycerophosphate) and then resuspended in 20 μl of kinase reaction buffer. The kinase assay was initiated by adding 20 μl of kinase reaction buffer, containing 10 μg of substrate and 2 μCi of [γ-^{32}P]ATP (ICN). The reactions were carried out at 30 °C for 30 min and terminated by adding SDS sample buffer, and the mixtures were boiled for 5 min. The reaction products were analyzed by SDS-PAGE and autoradiography.

RESULTS

Ionizing Radiation Induces Mitochondrial Dysfunction-mediated Apoptotic Cell Death in Human Non-small Cell Lung Cancer Cells—To investigate kinetics of the apoptotic cell death induced by ionizing irradiation in human non-small cell lung cancer cells, we treated two different cell lines (NCI-H1299 and NCI-H460) with 10-Gy radiation for various amount of time and analyzed induction of the cell death by flow-cytometry. Fig. 1*A* shows that there is a time-dependent increase of apoptotic cells, reaching over 30% of both cells at 48 h after irradiation. To evaluate the contribution of the mitochondrial pathway to the induction of apoptosis by ionizing radiation, we examined changes in mitochondria membrane potential and release of cytochrome *c* into the cytosol. Treatment of cells with radiation induced a significant disruption of mitochondrial membrane potential (Fig. 1*B*) and release of cytochrome *c* to the cytosol (Fig. 1*C*). Radiation also caused activation of caspase-3 and cleavage of poly ADP-ribose polymerase (PARP) along with the cytochrome *c* release into the cytosol (Fig. 1*C*), in agreement with previous studies suggesting that caspase-3 activation follows cytochrome *c* release (30, 31). Requirement of caspase activities for radiation-induced apoptosis was examined by using a broad-spectrum caspase inhibitor, z-VAD-fmk, and a caspase 3-specific inhibitor, z-DEVD-fmk. These caspase inhibitors were able to attenuate radiation-induced apoptotic cell death (Fig. 1*D*) as well as activation of caspases (data not shown). These results indicate that mitochondrial dysfunction-mediated cytochrome *c* release and subsequent activation of caspases are involved in the process of radiation-induced apoptotic cell death in human non-small cell lung cancer cells.

Radiation-induced Apoptotic Cell Death Involves Alterations in the Conformation of Bak and Bax Proteins—Because it has been shown that the pro-apoptotic Bcl-2 family members Bak and Bax are crucial to the mitochondrial dysfunction-mediated apoptotic cell death pathways (32), we investigated whether radiation treatment induces activation of Bak or Bax. We first analyzed activity-related conformational changes of Bak and Bax by flow cytometric analysis with antibodies recognizing N-terminal epitopes of Bak or Bax. As shown in Fig. 2*A*, ionizing irra-

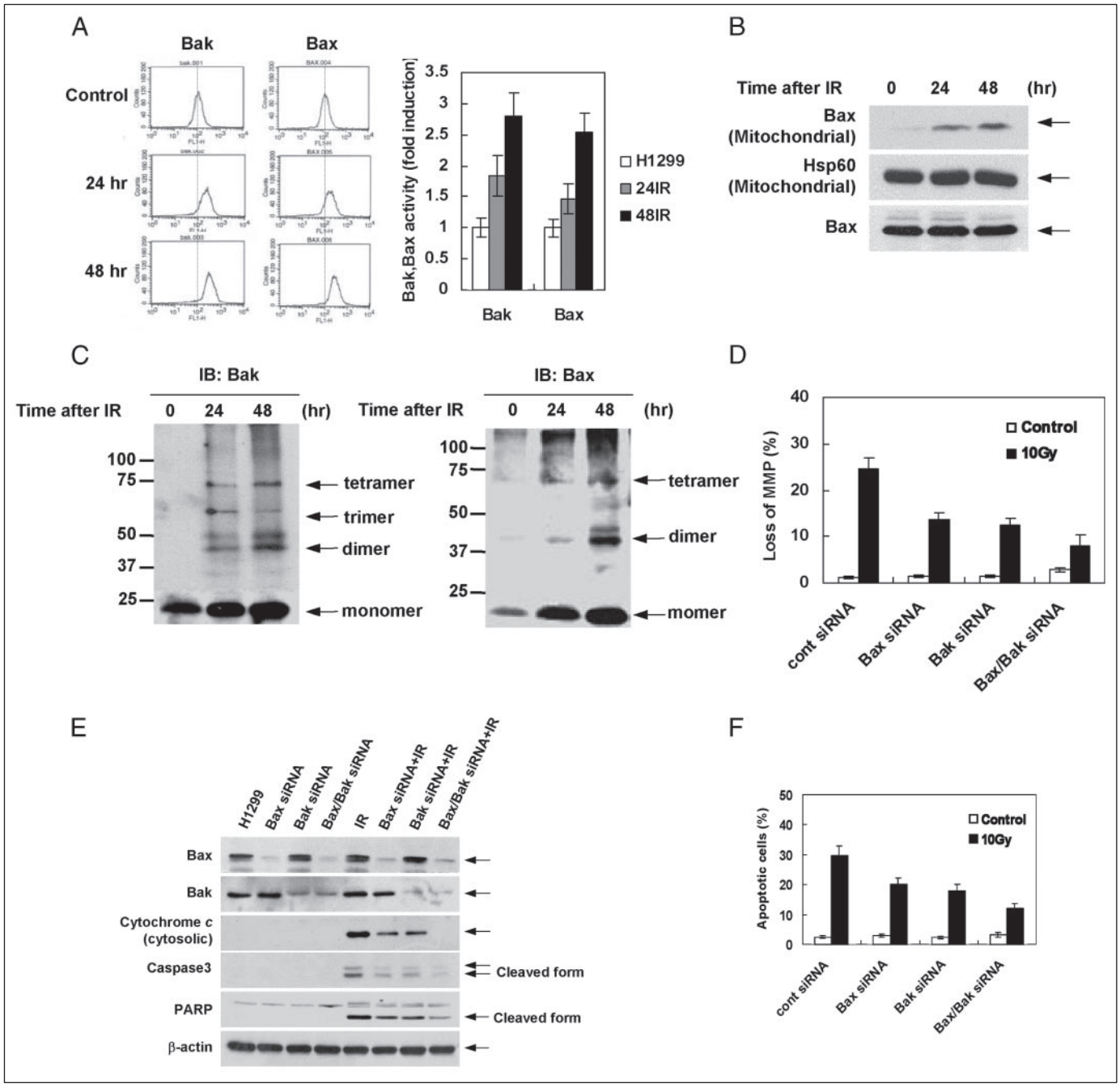

FIGURE 2. **Activity-related modulation of Bax and Bak proteins during radiation-induced cell death in human non-small cell lung cancer cells.** *A*, quantitative analysis of the activations of Bak and Bax after ionizing radiation (10 Gy). Activity related modulations of Bak and Bax were determined by flow cytometric analysis using specific antibodies recognizing N-terminal epitopes of Bak or Bax as described under "Experimental Procedures." *B*, analysis of the Bax translocation by mitochondrial fractionation in NCI-H1299 cells 24 and 48 h after irradiation (10 Gy). HSP60 was used as a mitochondria marker protein. *C*, analysis of Bax and Bak oligomerization in mitochondrial fractionation of NCI-H1299 cells 24 and 48 h after irradiation (10 Gy), as described under "Experimental Procedures." *D*, analysis of Bax- and Bak-siRNA effect on cytochrome *c* release by cytosolic fractionation, caspase 3 activation, and PARP cleavage in NCI-H1299 cells 36 h after irradiation (10 Gy), as detected by Western blot analysis with cytosolic fraction and total cell lysates. A non-related control siRNA that targeted the green fluorescent protein was used as a control. β-Actin was used as a loading control. *E*, analysis of mitochondrial membrane potential in control-siRNA- and Bax- and/or Bak-siRNA-transfected NCI-H1299 cells after irradiation (10 Gy). Mitochondrial membrane potential of cells was determined by retention of $DiOC_6$ (3) added during the last 30 min of irradiation (36 h) with a flow cytometry. *F*, quantitative analysis of the apoptotic cell death in control-siRNA- and Bax- and/or Bak-siRNA-transfected NCI-H1299 cells after irradiation (10 Gy). Cell death was determined by flow cytometric analysis as described under "Experimental Procedures."

diation resulted in activity-related modulations of both Bak and Bax, seen as a shift to the right in the resulting histogram. We also observed redistribution of Bax from cytosol to the mitochondria without changing the total protein expression levels of Bax after ionizing irradiation (Fig. 2*B*). To identify oligomerization of Bak and Bax, we fractionated membrane fraction followed by chemical cross-linking with disuccinimidyl suberate. Western blot analysis of cross-linked proteins shows that both Bak and Bax were oligomerized into dimmers and higher in multiples of ~23 and ~21 kDa, the monomers, respectively (Fig. 2*C*), suggesting that these newly formed complexes of Bak and Bax are homo-oligomers. In addition, small interfering RNA (siRNA) targeting of the Bak or Bax significantly attenuated radiation-induced dissipation of mitochondrial membrane potential (Fig. 2*D*), cytochrome *c* release and subsequent caspase activation (Fig. 2*E*), and cell death (Fig. 2*F*). The

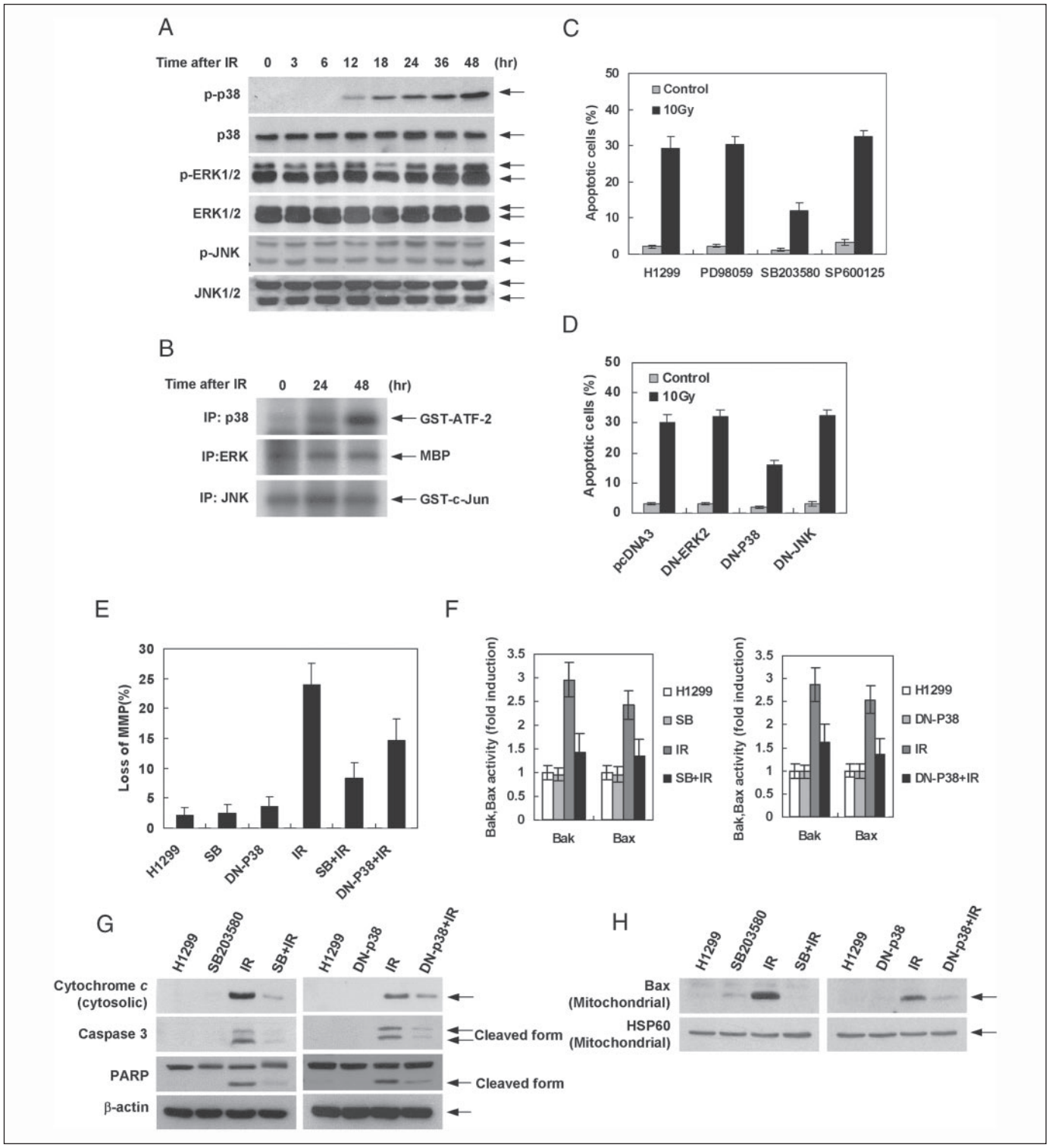

FIGURE 3. **p38 MAPK activation is required for the ionizing radiation induced conformational changes of Bak and Bax and apoptotic cell death.** *A*, analysis of MAP kinases activation in NCI-H1299 cells 3, 6, 12, 18, 24, 36, and 48 h after ionizing irradiation (10 Gy), as detected by immunoblot analysis with anti-phospho-p38 MAPK, -phospho-ERK, or -phospho-JNK antibodies, or anti-p38 MAPK, -ERK, or -JNK antibodies. *B*, analysis of MAPK activation in NCI-H1299 cells 24 and 48 h after ionizing irradiation (10 Gy), as detected by immune complex kinase assay with anti-p38 MAPK, -ERK, or -JNK antibodies. GST-ATF-2, myelin basic protein (*MBP*) and GST-c-Jun were used as substrates for p38 MAPK, ERK, or JNK, respectively. *C*, quantitative analysis of the cell death in NCI-H1299 cells 36 h after radiation (10 Gy) in the presence or absence of MEK/ERK inhibitor, PD98059 (30 μM), p38 MAPK inhibitor, SB203580 (25 μM), or JNK inhibitor, SP600125 (5 μM). *D*, quantitative analysis of the cell death in NCI-H1299 cells 36 h after radiation (10 Gy) in cells expressing dominant negative forms of ERK, p38 MAPK, or JNK. *E*, analysis of mitochondrial membrane potential 36 h after irradiation in the presence of p38 MAPK inhibitor SB203580 (25 μM) or overexpression of dominant negative forms of p38 MAPK. Mitochondrial transmembrane potential of cells was determined by retention of $DiOC_6$ (3) added during the last 30 min of irradiation (36 h) with a flow cytometry. *F*, analysis of the cytochrome *c* release from the mitochondria, caspase 3 activation, and PARP cleavage after irradiation in NCI-H1299 cells in the presence of p38 MAPK inhibitor SB203580 (25 μM) or overexpression of dominant negative forms of p38 MAPK, as detected by Western blot analysis with cytosolic fractions or total cell lysates. β-Actin was used as a loading control. *G*, quantitative analysis of the activations of Bak and Bax after ionizing radiation (10 Gy) in NCI-H1299 cells in the presence of p38 MAPK inhibitor SB203580 (25 μM) or overexpression of dominant negative forms of p38 MAPK. Activity-related modulations of Bak and Bax were determined by flow cytometric analysis using specific antibodies recognizing N-terminal epitopes of Bak or Bax as described under "Experimental Procedures." *H*, analysis of the Bax translocation by mitochondrial fractionation in NCI-H1299 cells 24 and 48 h after irradiation (10 Gy) in the presence of p38 MAPK inhibitor SB203580 (25 μM) or overexpression of dominant negative forms of p38 MAPK. HSP60 was used as a mitochondria marker protein.

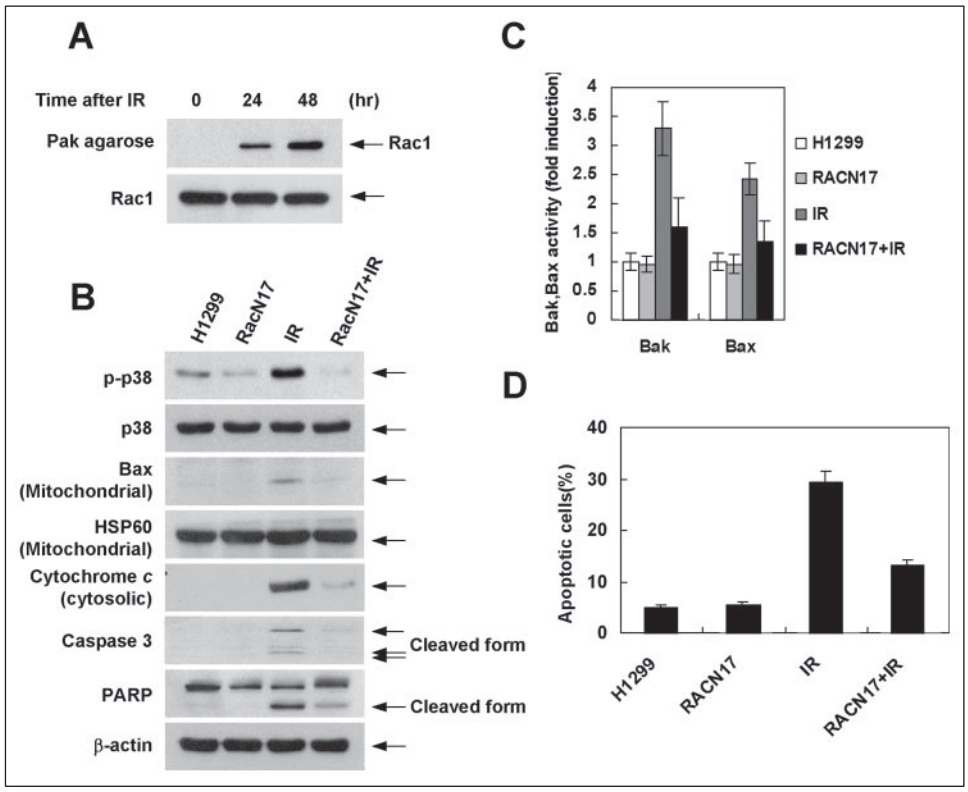

FIGURE 4. **Rac1 involves p38 MAPK activation during radiation-induced apoptotic cell death.** *A*, analysis of interaction between Rac and Pak in NCI-H1299 cells 24 and 48 h after irradiation (10 Gy), as detected by Western blot analysis with anti-Rac antibody after incubation with Pak-conjugated agarose. Expression level of Rac after irradiation (10 Gy) was detected by Western blot analysis with anti-Rac antibody. *B*, analysis of the phospho-p38 MAPK, Bax translocation to the mitochondrial membrane, cytochrome *c* release from the mitochondria, caspase 3 activation, and PARP cleavage after irradiation in NCI-H1299 cells overexpressing dominant negative forms of Rac, RacN17, as detected by Western blot analysis with cytosolic fraction or total cell lysates. β-Actin was used as a loading control. *C*, quantitative analysis of the activations of Bak and Bax after ionizing radiation (10 Gy) in NCI-H1299 cells overexpressing dominant negative forms of Rac, RacN17. Activity related modulations of Bak and Bax were determined by flow cytometric analysis using specific antibodies recognizing N-terminal epitopes of Bak or Bax as described under "Experimental Procedures." *D*, quantitative analysis of the apoptotic cell death in after irradiation (10 Gy) in NCI-H1299 cells overexpressing dominant negative forms of Rac, RacN17. Cell death was determined by flow cytometric analysis as described under "Experimental Procedures."

cells simultaneously transfected with Bax siRNA and Bak siRNA show a more dramatic attenuation of the aforementioned radiation-induced phenomena than the cells treated with either siRNA alone (Fig. 2*F*). These results indicate that radiation-induced mitochondrial dysfunction-mediated apoptotic cell death in human non-small cell lung cancer cells is mediated by the conformational changes and subsequent oligomerization of pro-apoptotic Bcl-2 family members Bax and Bak.

Activation of p38 MAPK Is Required for Radiation-induced Conformational Changes of Bak and Bax and Apoptotic Cell Death—To investigate a potential involvement of MAPKs in ionizing radiation-induced apoptotic cell death, we first analyzed the activation status of ERK1/2, JNK, and p38 MAPK by immunoblot analysis with antibodies specific to the phosphorylated form of these kinases. Treatment of cells with radiation resulted in a dramatic increase of the phosphorylated form of p38 MAPK in both NCI-H1299 and NCI-H 460 cell lines (Fig. 3*A* and data not shown), indicating its activation in non-small cell lung cancer cells. However, the levels of the phosphorylated form of ERK1/2 and JNK did not alter over the time course examined in both cell lines after irradiation. In addition, the ERK1/2 was constitutively phosphorylated in control in both cell lines. The total cellular level of MAPKs also remained constant. Immune complex kinase assay also clearly revealed that p38 MAPK was selectively activated in response to ionizing radiation, consistent with phosphorylation of p38 MAPK (Fig. 3*B*). To further determine whether selective activation of p38 MAPK is required for the radiation-induced apoptosis, we pre-treated cells with PD98059, an MEK/ERK-specific inhibitor, SB203580, a p38 MAPK-specific inhibitor, or SP600125, a JNK-specific inhibitor, or transfected cells with dominant negative forms of ERK, p38 MAPK, or JNK and analyzed its effect on radiation-induced apoptotic cell death. As shown in Fig. 3 (*C* and *D*), inhibition of p38 MAPK by treatment of SB203580 or forced expression of a dominant negative form of p38 MAPK markedly suppressed radiation-induced apoptotic cell death. However, inhibition of ERK and JNK did not affect radiation-induced cell death. Because it has been shown that p38 MAPK acts at early step(s) prior to dysfunction of mitochondria and caspase activation during apoptotic cell death in several model systems (15, 16, 17), we investigated whether p38 MAPK is involved in the radiation-induced mitochondrial dysfunction and cytochrome *c* release. Inhibition of p38 MAPK by treatment with SB203580 or by ectopic expression of a dominant negative form of p38 MAPK effectively blocked the loss of mitochondrial membrane potential (Fig. 3*E*), cytochrome *c* release to the cytosol and caspase activation (Fig. 3*F*) seen after irradiation. We further studied whether p38 MAPK activation is required for the activity-related modulations of Bax and Bak. Inhibition of the p38 MAPK completely suppressed the radiation-induced conformational changes of both Bak and Bax (Fig. 3*G*) and subsequent mitochondrial translocation of Bax (Fig. 3*H*). These results indicate that p38 MAPK acts as an important mediator of the apoptotic conformations of Bak/Bax and subsequent mitochondrial dysfunction during radiation-induced apoptotic cell death.

Rac1 Is Required for the p38 MAPK Activation during Radiation-induced Apoptotic Cell Death—Recently, it has been shown that Rac1 is involved in various MAPKs signaling during apoptotic cell death progression (15, 33). We examined whether Rac1 is involved in ionizing radiation-induced p38 MAPK activation and cell death. Treatment of cells with radiation dramatically increased Rac1-PAK binding, indicating radiation-induced Rac1 activation (Fig. 4*A*). Moreover, ectopic expression of RacN17, a dominant negative Rac1, significantly repressed radiation-induced Bak and Bax activation (Fig. 4*C*) and cytochrome *c* release as well as p38 MAPK activation (Fig. 4*B*). In addition, inhibition of Rac1 efficiently blocked radiation-induced apoptotic cell death (Fig. 4*D*). Conversely, introduction of the constitutively active form of Rac1 (RacV12) enhanced radiation-induced p38 MAPK activation, Bak and Bax activation, and apoptotic cell death (data not shown). These results suggest that Rac1 is an upstream regulator of p38 MAPK during radiation-induced apoptotic cell death.

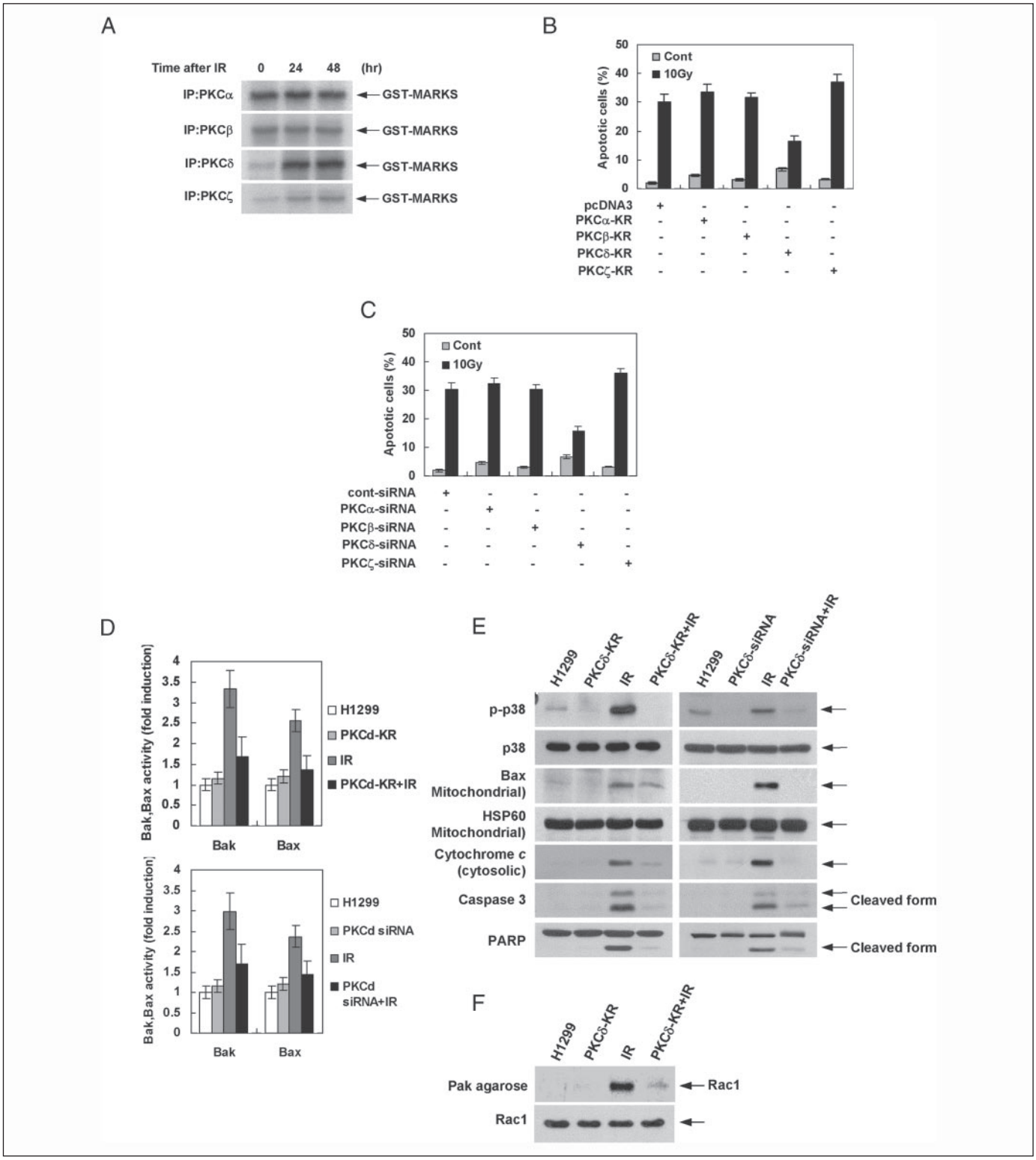

FIGURE 5. **PKCδ locates upstream of Rac1 and p38 MAPK during radiation-induced apoptotic cell death.** *A*, analysis of activations of PKC isozymes in NCI-H1299 cells 24 and 48 h after ionizing irradiation (10 Gy), as detected by immune complex kinase assay with anti-PKCα, PKC*β*, PKCδ, or PKC*ζ* antibodies, as described under "Experimental Procedures." GST-MARKS was used as substrate for PKC isoforms. *B*, quantitative analysis of the apoptotic cell death at 36 h after irradiation (10 Gy) in NCI-H1299 cells overexpressing dominant negative forms of PKC isoforms, PKCα-KR, PKC*β*-KR, PKCδ-KR, or PKC*ζ*-KR. Cell death was determined by flow cytometric analysis. *C*, quantitative analysis of the apoptotic cell death after irradiation (10 Gy) in control-siRNA- and PKCα-, PKC*β*-, PKCδ-, or PKC*ζ*-siRNA-transfected NCI-H1299 cells. A non-related control siRNA that targeted the green fluorescent protein was used as a control. Cell death was determined by flow cytometric analysis. *D*, analysis of the activations of Bak and Bax in NCI-H1299 cells overexpressing dominant negative forms of PKCδ or in cells transfected with PKCδ-siRNA at 36 h after ionizing radiation (10 Gy). Activity related modulations of Bak and Bax were determined by flow cytometric analysis using specific antibodies recognizing N-terminal epitopes of Bak or Bax as described under "Experimental Procedures." *E*, analysis of the p38 MAPK activation, Bax translocation, cytochrome *c* release, caspase 3 activation, and PARP cleavage in NCI-H1299 cells overexpressing dominant negative forms of PKCδ (PKCδ-KR) or in control-siRNA- and PKCδ-siRNA-transfected cells 36 h after irradiation (10 Gy), as detected by Western blot analysis with anti-phopho-p38 MAPK, -p38 MAPK, -Bax, -cytochrome *c*, -caspase 3, or -PARP antibodies. *F*, analysis of interaction between Rac1 and Pak in NCI-H1299 cells overexpressing dominant negative forms of PKCδ (PKCδ-KR) 36 h after irradiation (10 Gy), as detected by Western blot analysis with anti-Rac antibody after incubation with Pak-conjugated agarose. Expression level of Rac1 after irradiation (10 Gy) was detected by Western blot analysis with anti-Rac1 antibody.

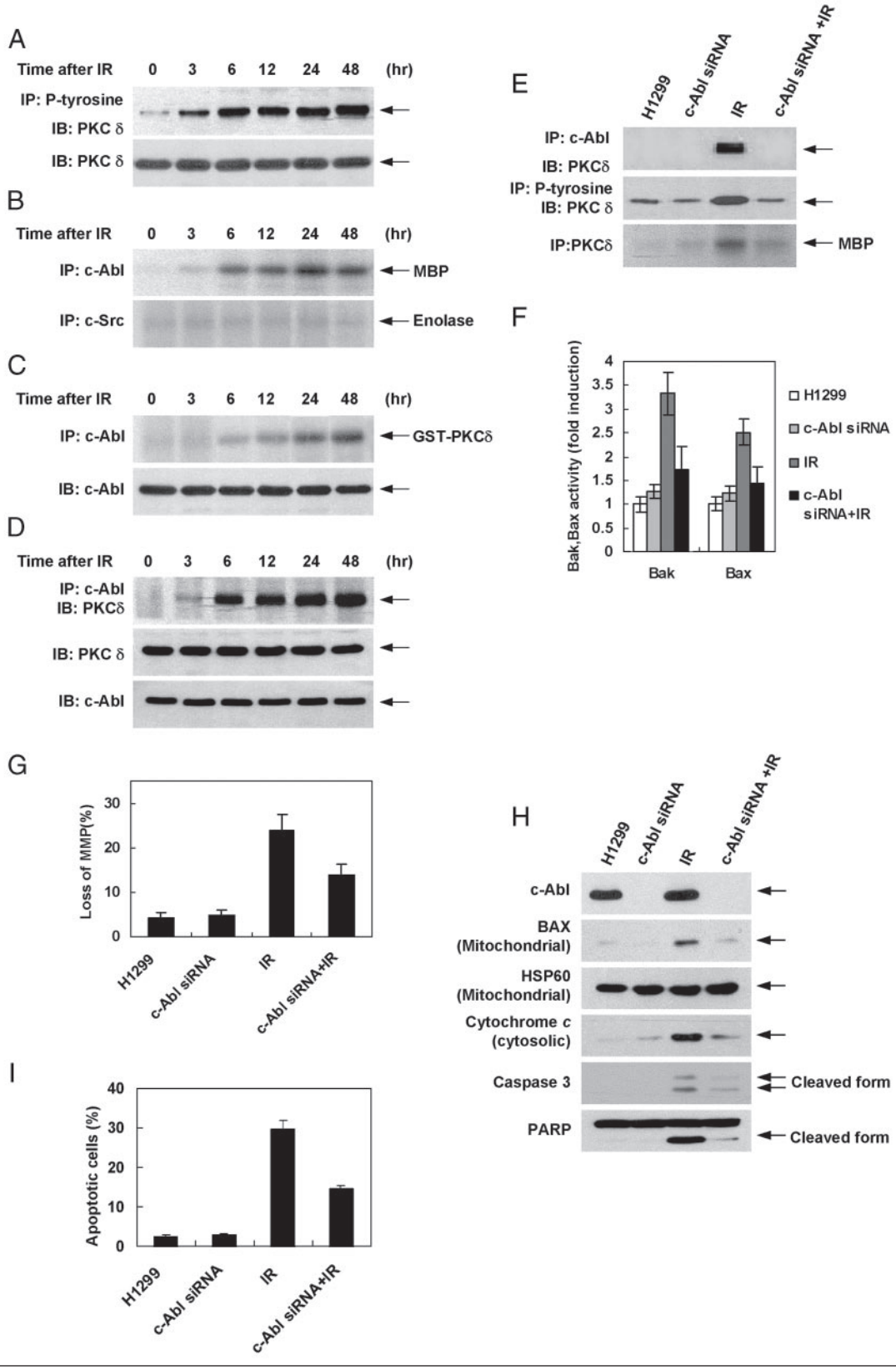

FIGURE 6. **Tyrosine phosphorylation of PKCδ by c-Abl kinase during ionizing radiation-induced apoptotic cell death.** *A*, cell lysates at 3, 6, 12, 24, 48 h after irradiation were immunoprecipitated with anti-phospho-tyrosine antibody and immunoblotted with anti-PKCδ antibody. The total level of PKCδ during the time course was detected by Western blot analysis with anti-PKCδ antibody. *B*, activities of c-Abl and c-Src were detected by immune complex kinase assay with anti-c-Abl and -c-Src antibodies, as described under "Experimental Procedures." Myelin basic protein (*MBP*) or enolase was used as substrate for c-Abl or c-Src, respectively. *C*, PKCδ phosphorylation by c-Abl was detected by immune complex kinase assay with anti-c-Abl antibody using GST-PKCδ as a substrate. *D*, analysis of interaction between c-Abl and PKCδ in NCI-H1299 cells after irradiation. Cell lysates at 3, 6, 12, 24, and 48 h after irradiation were immunoprecipitated with anti-c-Abl antibody and immunoblotted with anti-PKCδ antibody. *E*, analysis of interaction between c-Abl and PKCδ, and tyrosine phosphorylation and kinase activity of PKCδ after irradiation (10 Gy) in control-siRNA- or c-Abl-siRNA-transfected NCI-H1299 cells. A non-related control siRNA that targeted the green fluorescent protein was used as a control.

Activation of PKCδ Is Necessary for the Mitochondrial Dysfunction-mediated Apoptotic Cell Death in Response to Ionizing Radiation—PKC is a well known upstream regulator of the mitogen-activated protein kinases and plays important roles in a variety of cellular functions, including apoptosis (34, 35). To address the question of a potential involvement of PKC in radiation-induced apoptotic cell death, we first investigated the activation status of PKC isozymes by immune complex kinase assay. As shown in Fig. 5*A*, exposure of cells with ionizing irradiation resulted in increase of the PKCδ and PKCζ activities. However, levels of the activity of PKCα and PKCβ did not alter over the time course examined. To further determine whether PKCδ and/or PKCζ activities are required for the radiation-induced apoptotic cell death, we transfected cells with dominant negative forms of various PKC isozymes (α, β, δ, and ζ) and analyzed their effects on radiation-induced mitochondrial dysfunction and apoptotic cell death. As shown in Fig. 5*B*, cells overexpressing a dominant negative form of PKCδ selectively attenuated radiation-induced cell death. However, expression of dominant negative forms of other PKC isozymes (α, β, and ζ) failed to suppress radiation-induced cell death. In addition, siRNA targeting of PKCδ, but not other PKC isozymes, selectively attenuated apoptotic cell death induced by radiation (Fig. 5*C*). Furthermore, forced expression of a dominant negative form of PKCδ or siRNA targeting of PKCδ effectively suppressed conformational changes of Bak and Bax (Fig. 5*D*), mitochondrial translocation of Bax, mitochondria membrane potential loss, cytochrome *c* release, and caspase 3 activation as well as p38 MAPK activation seen after irradiation (Fig. 5*E*). In addition, dominant negative forms of PKCδ clearly attenuated radiation-induced Rac1-PAK binding (Fig. 5*F*). These findings suggest that activation of PKCδ is necessary for the p38 MAPK-mediated progression of apoptotic cell death pathway, and that Rac1 lies between PKCδ and p38 MAPK signaling during ionizing irradiation-induced apoptotic cell death.

Tyrosine Phosphorylation of PKCδ by c-Abl Is Required for Radiation-induced Apoptotic Cell Death—Because tyrosine phosphorylation is involved in regulating PKCδ activity, and diverse signals are associated with phosphorylation of PKCδ on tyrosine, we examined whether ionizing radiation can induce tyrosine phosphorylation of PKCδ. To detect phosphorylation of PKCδ on tyrosine, anti-phospho-tyrosine precipitates were analyzed by immunoblot analysis with anti-PKCδ. As shown in Fig. 6*A*, exposure of cells to radiation dramatically induced phosphorylation of PKCδ on tyrosine. Tyrosine phosphorylation of PKCδ was apparent within 6 h and remained elevated until 48 h after radiation exposure. The total level of PKCδ did not change during the time course. Because it has been shown that c-Abl or c-Src kinase is associated with tyrosine phosphorylation of PKCδ in response to genotoxic and oxidative stress (36, 37), we next investigated whether c-Abl or c-Src activity can be stimulated by ionizing radiation. Fig. 6*B* shows the activation of c-Abl in response to radiation in NCI-H1299 cells. The c-Abl activation was observed within 6 h and sustained until 48 h after irradiation, consistent with tyrosine phosphorylation of PKCδ. However, we failed to detect c-Src activation over the time course examined after irradiation (Fig. 6*B*). We further examined whether c-Abl can directly phosphorylates PKCδ in response to radiation. Direct phosphorylation of PKCδ by c-Abl was determined by immune complex kinase assay using GST-PKCδ as a substrate. As shown in Fig. 6*C*, PKCδ phosphorylation by c-Abl was gradually increased in response to radiation. Moreover, the interaction of c-Abl with PKCδ was also markedly increased in a time dependent fashion (Fig. 6*D*). To further examine whether c-Abl is necessary for the induction of PKCδ-mediated apoptotic cell death pathway, we transfected cells with c-Abl-siRNA and its effects on radiation-induced mitochondrial dysfunction and apoptotic cell death. siRNA targeting of c-Abl clearly attenuated radiation-induced interaction between c-Abl and PKCδ, tyrosine phosphorylation, and activation of PKCδ (Fig. 6*E*). Moreover, inhibition of c-Abl by transfection of c-Abl-siRNA attenuated p38 MAPK activation, apoptotic conformation of Bak and Bax (Fig. 6*F*), mitochondrial membrane potential loss (Fig. 6*G*), cytochrome *c* release, caspase activation (Fig. 6*H*), and apoptotic cell death (Fig. 6*I*) seen after irradiation. These results suggest that activation of c-Abl kinase is essential for the activation of PKCδ and subsequent mitochondrial dysfunction-mediated apoptotic cell death progression in response to ionizing radiation.

DISCUSSION

Intracellular signaling molecules and apoptotic factors seem to play an important role in determining the radiation response of tumor cells. The molecular mechanism by which apoptotic cell death occurs in response to ionizing radiation has been widely explored but not precisely deciphered. In this study, we demonstrate that ionizing radiation induces mitochondrial activation-mediated apoptotic cell death in non-small cell lung cancer cells through activity-related conformational changes of pro-apoptotic proteins Bax and Bak, and that c-Abl-PKCδ-Rac1-p38 MAPK signaling pathway is essential for the induction of apoptotic conformation of Bak and Bax.

Multidomain pro-apoptotic members of the Bcl-2 family, Bax and Bak can facilitate mitochondrial dysfunction-mediated apoptosis, in response to various stimuli, such as cisplatin, etoposide, TRAIL (tumor necrosis factor-related apoptosis-inducing ligand), or arsenic trioxide, through homo-oligomerization dependent on their activity-related conformational changes (11, 38, 39). We found that ionizing radiation-induced apoptotic cell death involves induction of the apoptotic conformation and subsequent oligomerization of Bak and Bax. It has been suggested that BH3-only proteins, such as Bid or Bim, are activated by transcriptional up-regulation and/or post-translational modification following an apoptotic stimulus and induce the activation of Bax and/or Bak (7–9). However, we failed to detect to any changes in Bid and Bim in non-small lung cancer cells after irradiation (data not shown), suggesting that undefined mechanisms other than Bid or Bim activation can be involved in radiation-induced conformational activation of Bak and Bax.

It has been suggested that p38 MAPK is positively implicated in induction of apoptosis in response to various stress signals, including tumor necrosis factor-α, interleukin-1, UV irradiation, hyperosmotic stress, and chemotherapeutic drugs (15, 40–42). Consistent with these findings, we also found that ionizing radiation induces selective activation of p38 MAPK and that inhibition of p38 MAPK effectively attenuated radiation-induced apoptotic cell death. Moreover, inhibition of p38 MAPK completely inhibited radiation-induced conformational

F, quantitative analysis of the activations of Bak and Bax in NCI-H1299 cells overexpressing c-Abl-siRNA 36 h after ionizing radiation (10 Gy). Activity-related modulations of Bak and Bax were determined by flow cytometric analysis using specific antibodies recognizing N-terminal epitopes of Bak or Bax as described under "Experimental Procedures." *G*, mitochondrial membrane potential in NCI-H1299 cells overexpressing control-siRNA- or c-Abl-siRNA 36 h after ionizing radiation (10 Gy). Mitochondrial transmembrane potential of cells was determined by retention of $DiOC_6$ (3) added during the last 30 min of irradiation with a flow cytometry. *H*, analysis of the c-Abl expression, Bax translocation, cytochrome *c* release, caspase 3 activation, and PARP cleavage in NCI-H1299 cells overexpressing control-siRNA- or c-Abl-siRNA 36 h after irradiation (10 Gy), as detected by Western blot analysis with anti-c-Abl, -Bax, -cytochrome *c*, -caspase 3, or -PARP antibodies. *I*, quantitative analysis of the apoptotic cell death at 36 h after irradiation (10 Gy) in NCI-H1299 cells overexpressing control-siRNA- or c-Abl-siRNA. Cell death was determined by flow cytometric analysis.

changes of Bak and Bax, mitochondrial membrane potential loss, and cytochrome *c* release, suggesting that activation of p38 MAPK is essential for the induction of apoptotic conformation of Bak and Bax, resulting in mitochondrial activation-mediated apoptotic cell death. These findings are consistent with previous studies demonstrating that NH_2 and COOH termini of Bax are rich in glycine and hydroxyl amino acids such as serine and threonine (43, 44), which are potential targets of the p38 MAPK, and are critical for regulating the subcellular distribution of Bax (45). Phosphorylation of either terminus may cause conformational change, facilitating oligomerization of Bax. However, in contrast to Bcl-2 and Bad, evidence for Bax and Bak phosphorylations are rare. Alternatively, it is conceivable that, although Bax and Bak are not directly phosphorylated, the phosphorylation of Bax- and Bak-binding protein may facilitate apoptotic conformation of Bax and Bak.

It is well established that the Rac1-p38 MAPK pathway is activated by various types of membrane-associated cellular signals induced by many apoptosis-inducing agents (15, 33). In this study, we provided further evidence that Rac1 is involved in the radiation-induced apoptotic cell death. Inhibition of Rac1 activity with expression of RacN17, a dominant negative form of Rac1, significantly reduced radiation-induced p38 MAPK activation and Bak and Bax activations, suggesting that Rac1 acts as an upstream regulator of p38 MAPK during radiation-induced apoptotic cell death. This is in good agreement with the recent studies showing that the Rac-p38 MAPK-Bax translocation pathway plays an essential role in the apoptosis induced by UV irradiation (46) or phytosphingosine treatment (40).

PKCδ, a member of the novel PKC subfamily, is actively involved in apoptosis induced by a variety of stimuli, including UV light, ionizing radiation, cisplatin, etoposide, and doxorubicin (47, 48). In this study, we provided further evidence that exposure of cells with ionizing radiation results in an increase of PKCδ activity, which plays a critical role in radiation-induced apoptotic cell death. We showed that inhibition of PKCδ effectively attenuated apoptotic cell death induced by radiation, but inhibition of other PKC isozymes (PKCα, PKCβ, and PKCζ) did not. Activation of PKCδ was accompanied by subsequent activation of all three MAPK cascades (19). Other reports suggest that PKCδ is associated with a rapid increase in JNK and p38 MAPK activity, but ERK are not significantly activated (20, 21). However, we found that PKCδ is selectively associated with p38 MAPK activation in response to radiation. Inhibition of PKCδ effectively attenuated p38 MAPK signaling. In addition, inhibition of PKCδ attenuated radiation-induced Rac1 activation. These results suggest that activation of PKCδ is necessary for the radiation-induced progression of apoptotic cell death in non-small cell lung cancer cells and that PKCδ is located upstream of Rac1-p38 MAPK signaling in response to radiation. In line with this observation, overexpression of PKCδ in prostate cancer cells was shown to markedly enhance the apoptosis-inducing effect (49, 50). Our previous report also demonstrated that PKCδ overexpression increased radiosensitivity in normal fibroblasts through Rac1-p38 MAPK signaling pathway (51).

PKCδ is normally activated by diacylglycerol produced from receptor-mediated hydrolysis of inositol phospholipids (37). However, PKCδ is also phosphorylated on tyrosine residue upon diverse stimulation of the cells with a concomitant increase or decrease in enzymatic activity (52). The relation between tyrosine phosphorylation of PKCδ and its catalytic activation appears to depend on cell type and phosphorylation site (53). In cells transformed with Ras or v-Src, Src phosphorylates PKCδ on tyrosine, inactivates PKCδ catalytic function, and causes rapid degradation of PKCδ (27). However, upon stimulation of various cells with phorbol ester, growth factors, or hormones, PKCδ is phosphorylated on a tyrosine residue and catalytically activated (54, 55). Tyrosine phosphorylation of PKCδ also occurs in response to various apoptotic stimuli, including H_2O_2, UV radiation, etoposide, and ceramide (56, 48, 57, 58). Similarly, we also observed a dramatic increase in tyrosine phosphorylation of PKCδ after ionizing irradiation. Previous reports demonstrated that, in response to H_2O_2 and phorbol 12-myristate 13-acetate, Src family kinases promote tyrosine phosphorylation of PKCδ (54, 56). However, we failed to find activation of Src family kinases after irradiation in non-small cell lung cancer cells. Moreover, inhibition of Src with transfection of Src-siRNA did not affect PKCδ phosphorylation, catalytic activation, and cell death (data not shown). Instead, we found that ionizing radiation-induced PKCδ phosphorylation on tyrosine is associated with c-Abl tyrosine kinase. c-Abl was suggested to be activated in response to genotoxic and oxidative stress, and activated c-Abl interacts with PKCδ in response to both stimuli for the activation of PKCδ (48, 57). We also found that c-Abl directly interacts and phosphorylates PKCδ in response to ionizing radiation. Moreover, siRNA targeting of c-Abl effectively attenuated radiation-induced PKCδ and p38 MAPK activations and subsequent apoptotic cell death. These results suggest that tyrosine phosphorylation of PKCδ, through direct interaction with c-Abl kinase, results in catalytic activation of PKCδ and promotes radiation-induced mitochondrial activation-mediated apoptotic cell death progression.

In conclusion, we investigated ionizing radiation-induced signaling pathways leading to mitochondrial dysfunction-mediated apoptotic cell death that plays an important role in determining the sensitivity of non-small cell lung cancer cells to the ionizing radiation. We demonstrated that p38 MAPK-mediated activation of pro-apoptotic proteins Bak and Bax promotes ionizing radiation-induced apoptotic cell death in human non-small cell lung cancer cells and that tyrosine phosphorylation of PKCδ by c-Abl is responsible for p38 MAPK activation in response to ionizing radiation. We believe that the attempt of dissecting the specific signal transduction pathways involved in the initiation of apoptotic cell death in cancer cells in response to ionizing radiation, like the one in this study, will guide the development of novel strategies in radiation therapy of cancer.

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