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Inhibition of Nuclear Factor-κB Activity Is Involved in E1A-mediated Sensitization of Radiation-induced Apoptosis*

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The adenoviral E1A protein has been implicated in the potentiation of apoptosis induced by various external stimuli, but the exact mechanism of that potentiation is not clear. In this study, we compared the sensitivity to ionizing γ-irradiation of E1A transfectants with that of parental cells in a human ovarian cancer cell line (SKOV3.ip1); we found that the E1A transfectants became sensitive to radiation-induced apoptosis. Recently, activation of the transcription factor nuclear factor-κB (NF-κB) has been shown to play a key role in the anti-apoptotic pathway of radiation-induced apoptosis. In an attempt to determine whether NF-κB was involved in the E1A-mediated sensitization of radiation-induced apoptosis, we found that radiation-induced activation of NF-κB occurred in the parental cells but was blocked in the E1A transfectants. Furthermore, parental cells co-transfected with NF-κB and E1A were better protected from undergoing apoptosis upon irradiation than those transfected with E1A alone. Thus, our results suggest that inhibition of NF-κB activation by E1A is a plausible mechanism for E1A-mediated sensitization of radiation-induced apoptosis.

In the past, E1A was considered to be an oncogene, because it immortalizes primary rodent cells and transforms them in cooperation with a second oncogene such as E1B or activated *ras* (1), but no evidence has been found to suggest that an oncogenic role exists for E1A in human cancers. Recent findings indicate that E1A can function as an anti-oncogene to suppress transformation, metastasis, and tumorigenicity as demonstrated previously (2–6). E1A also has been reported to increase cellular susceptibility to apoptosis, particularly under the conditions of serum starvation or high cell density (7). Furthermore, E1A-expressing derivatives of primary mouse embryo fibroblasts were shown to undergo rapid apoptosis following treatment with ionizing radiation or with several chemotherapeutic compounds (8). Apoptosis is an active physiological process that can be triggered by a wide variety of cellular stresses, including DNA damage, ultraviolet (UV) radiation, ionizing radiation, heat shock, and oxidative stress, as well as by extracellular stimuli acting through the cell-surface receptors (9). The mechanisms by which E1A sensitizes cells that undergo apoptosis in response to various stimuli are not clear.

Many apoptotic stimuli, including tumor necrosis factor-α, ionizing radiation, and several chemotherapeutic compounds, activate NF-κB,[1] a pleiotropic transcription factor that regulates many genes, such as immunoreceptors, cytokines, viruses, and others (10). Recently, NF-κB has been shown to have an anti-apoptotic role, blocking apoptosis induced by tumor necrosis factor-α, ionizing radiation, or the chemotherapeutic agent daunorubicin (11–14).

In light of our observation and reported findings, we hypothesized that NF-κB may play a protective role in the E1A-mediated sensitization of cells to apoptotic stimuli. Thus, we tested E1A transfectants of human cancer cell lines for their sensitivity to ionizing γ-irradiation. The cells transfected with E1A were highly sensitive to radiation-induced apoptosis based on *in vitro* growth studies, DNA fragmentation assays, and fluorescence-activated cell sorter analysis. In addition, we found that NF-κB inactivation is required in the E1A-mediated sensitization of radiation-induced apoptosis.

MATERIALS AND METHODS

Cell Lines and Cultures—The SKOV3.ip1 cell line was generated from ascites developed in *nu/nu* mouse by administering an intraperitoneal injection of SK-OV-3, a human ovarian carcinoma cell line obtained from the American Type Culture Collection (6). Cells were grown in Dulbecco's modified Eagle's medium/F-12 (Life Technologies, Inc.) supplemented with 10% fetal bovine serum. The ip1-E1A2 and ip1-Efs cells were established by transfecting the SKOV3.ip1 cells with E1A-expressing pE1A plasmid DNA and pE1A-dl343 containing a 2-base pair frameshift deletion in the E1A coding region, respectively. The transfectants were grown under the same conditions, except that G418 (800 μg/ml) was added to the culture medium. Cells were irradiated with a ^{137}Cs source emitting a fixed dose rate of the indicated Gy (Irradiator model 0103, U. S. Nuclear Corp.).

In Vitro Growth Rate Analysis—The *in vitro* growth rates of the cell lines were assessed by counting the cells using a Coulter counter or by MTT assay (15). For MTT assays, cells (3×10^3/well) were plated in 96-well culture plates in 0.2 ml of culture medium and allowed to adhere for 2 h; 20 μl of MTT was then added to each well. Cells were cultured for an additional 2 h, and 100 μl of lysis buffer (20% sodium dodecyl sulfate (SDS) in 50% *N,N*-dimethylformamide, pH 4.7) was added. The cells were incubated overnight, and absorbance at 570 nm was measured. For cell counting, samples of 4×10^4 cells with or without γ-radiation were plated in triplicate in a six-well plate. After different time intervals, the cells were harvested and counted using Coulter counter.

Immunoblotting—Immunoblot analysis was performed as described previously (6). A monoclonal antibody against the E1A proteins, M58 (PharMingen), was used.

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[1] The abbreviations used are: NF-κB, nuclear factor-κB; PBS, phosphate-buffered saline; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; RelA, a p65 subunit of NF-κB; Gy, gray(s).

[^{3}H]Thymidine Incorporation Assay—The assay was carried out as described previously (6). Briefly, the cells were plated at a confluence of 80% in 150-mm Petri dishes. After irradiation, the cells were trypsinized, plated in 96-well plates (3×10^3 cells/well), and incubated for 8 h with 1 μCi of [^{3}H]thymidine. The radioactivity of the cells was determined with a scintillation counter.

DNA Fragmentation Analysis—The analysis was carried out as described previously (16). Approximately 2×10^6 cells were lysed in 0.3 ml of NTE buffer (100 mM NaCl, 10 mM Tris-Cl, 5 mM EDTA, pH 8.0) containing 0.5% SDS. The lysate was heated at 65 °C for 10 min to inactivate nucleases and was digested overnight with 1 mg/ml of proteinase K at 50 °C. The lysate was then centrifuged at 55,000 $\times$ *g* for 30 min at 4 °C to remove the chromosomal DNA. The supernatant was digested with 100 μg/ml RNase for 30 min at 37 °C followed by extraction of phenol and chloroform. The DNA was then precipitated with ethanol and dissolved in TE buffer. An equal amount of DNA from each sample was analyzed on a 1.5% agarose gel.

Propidium Iodide Staining and Flow Cytometry Assay—Samples of 2×10^6 cells were collected, washed once with phosphate-buffered saline (PBS), and fixed with 70% ice-cold ethanol overnight. After fixation, cells were washed with PBS to remove residual ethanol, pelleted, and resuspended in PBS containing 50 μg/ml of propidium iodide (Sigma). The staining was performed at 4 °C for at least 30 min, and samples were analyzed using an Epics Profile flow cytometer (Coulter) in the core facility at The University of Texas M. D. Anderson Cancer Center.

Electrophoretic Mobility-shift Assays—Cell extracts were prepared as described previously (19). Briefly, cells were scrapped into 1.5 ml of cold PBS, pelleted for 10 s, and resuspended in 100 μl of cold buffer A (10 mM HEPES-KOH, pH 7.9, at 4 °C, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride) by flicking the tube. The cells were allowed to swell on ice for 10 min, then vortexed for 10 s. Samples were centrifuged for 10 s, and the supernatant fraction was discarded. The pellet was resuspended in 20–100 μl of cold buffer C (20 mM HEPES-KOH, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride) and incubated on ice for 20 min for high salt extraction. Cellular debris was removed by centrifugation for 2 min at 4 °C, and the supernatant fraction containing DNA-binding proteins was stored at −70 °C. The nuclear extract (5 μg of protein) was incubated with 1 μg of poly(dI-dC) (Pharmacia Biotech Inc.) on ice for 20 min, and a ^{32}P-labeled double-stranded oligonucleotide containing the κB site from the human immunodeficiency virus was added (20). Binding of the probe was carried out at room temperature for 20 min. The resulting complexes were resolved in 4% nondenaturing polyacrylamide gel. The mutant κB competitor was a double-stranded oligonucleotide containing mutations in the κB site from human immunodeficiency virus (20).

Transient Transfection—Expression vectors for RelA (pMT2T) (17) and transin-*lacZ* (a *lacZ* gene driven by a transin promoter that does not have NF-κB binding sites) were used in this study. 1.5×10^5 cells in a 35-mm well were transfected with 2.2 μg of total DNA (RelA or vector, 1.2 μg; pukE1A, 0.4 μg; transin-*lacZ*, 0.6 μg) using lipofectAMINE (Life Technologies, Inc.) as described in the manufacturer's protocol. The cells were divided into two sets 32 h after transfection; one set received 10 Gy of γ-irradiation, while the other set was used as a nonirradiated control. The cells were cultured for 48 h after irradiation and then stained for β-galactosidase-expressing cells as described previously (18). The blue cells were counted and normalized against the number of blue cells in the nonirradiated set.

RESULTS AND DISCUSSION

To test whether E1A can sensitize cells to radiation-induced changes during growth, *in vitro* growth rate analysis was first performed using an MTT assay. We used a human ovarian cancer cell line derivative, SKOV3.ip1, that was stably transfected with either wild-type Ad5 E1A (ip1-E1A2) or an E1A frameshift Efs mutant (ip1-Efs) (6). When the cells were irradiated, the E1A transfectants showed a more significant decrease in growth rate than the parental or Efs transfectants did when they were irradiated under the same conditions during a 3-day period (Fig. 1*A*). An optimal radiation dose of 5 Gy was chosen after the cells were irradiated with varying doses (data not shown). In the absence of radiation, the growth rates of the parental and Efs transfectants were similar, and the growth rate of the E1A transfectant was slightly lower (6). The changes

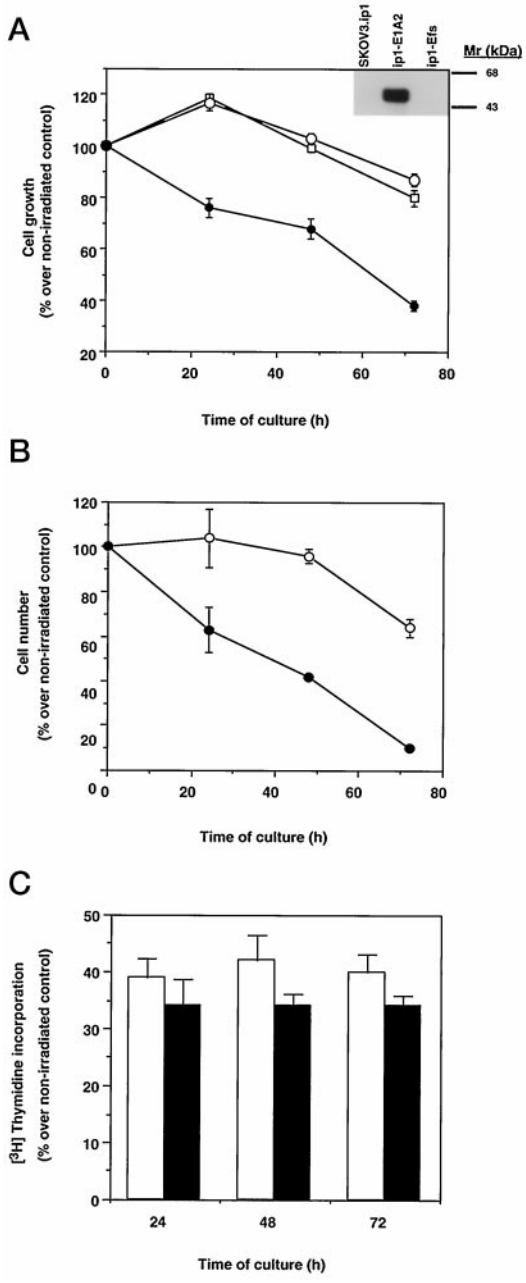

FIG. 1. **Ip1-E1A2 cells are susceptible to radiation-induced retardation of growth and cell number without changes in their DNA replication rate.** *A*, SKOV3.ip1, ip1-Efs, and ip1-E1A2 cells (3×10^3/well) were plated in 96-well culture plates in 0.2 ml of culture medium. Cells were allowed to adhere for 2 h (time 0), the growth rate was then analyzed before and after irradiation (5 Gy) by MTT assay at the indicated time intervals as described under "Materials and Methods." The cell lysates were prepared and analyzed by immunoblotting for the presence of E1A (*inset*) as described under "Materials and Methods." Symbols: □, ip1-Efs; ○, SKOV3.ip1; ●, ip1-E1A2. *B*, SKOV3.ip1 and ip1-E1A2 cells (4×10^4/well) both with and without γ-irradiation (5 Gy) were plated in six-well culture plates in 3 ml of culture medium. Cells were allowed to adhere overnight, and the cells were counted at the indicated time intervals. Symbols: ○, SKOV3.ip1; ●, ip1-E1A2. *C*, SKOV3.ip1 and ip1-E1A2 cells were plated at a confluence of 80% in 150-mm Petri dishes. After irradiation (5 Gy), the cells were trypsinized and plated into 96-well plates (3×10^3 cells/well), and 1 μCi of [^{3}H]thymidine in 20 μl of medium was added to each well for 8 h before the indicated time points. Incorporation of [^{3}H]thymidine was determined as described under "Materials and Methods." The values, expressed as percentages, have been normalized against the corresponding nonirradiated control cells. The *bars* indicate the standard deviation of triplicate samples. The experiment was repeated at least once with similar results. Symbols: □, SKOV3,ip1; ■, ip1-E1A2.

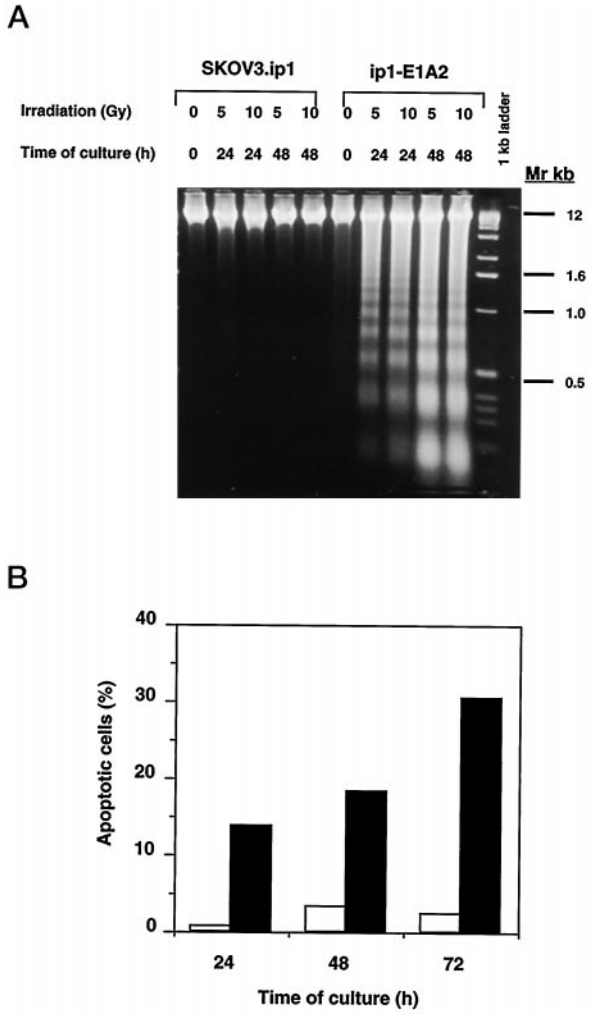

FIG. 2. **Ionizing radiation induces DNA fragmentation and apoptosis in ip1-E1A2 cells but not in SKOV3.ip1 cells.** *A*, cells were irradiated (0, 5, or 10 Gy) and cultured for the indicated period of time and the DNA was extracted and analyzed by gel electrophoresis. *B*, SKOV3.ip1 and ip1-E1A2 cells were irradiated at a dose of 5 Gy, and quantitation of apoptotic cells, expressed as a percentage, was done at the time intervals shown using flow cytometric analysis of the sub-G_1 phase. Symbols: □, SKOV3.ip1; ■, ip1-E1A2.

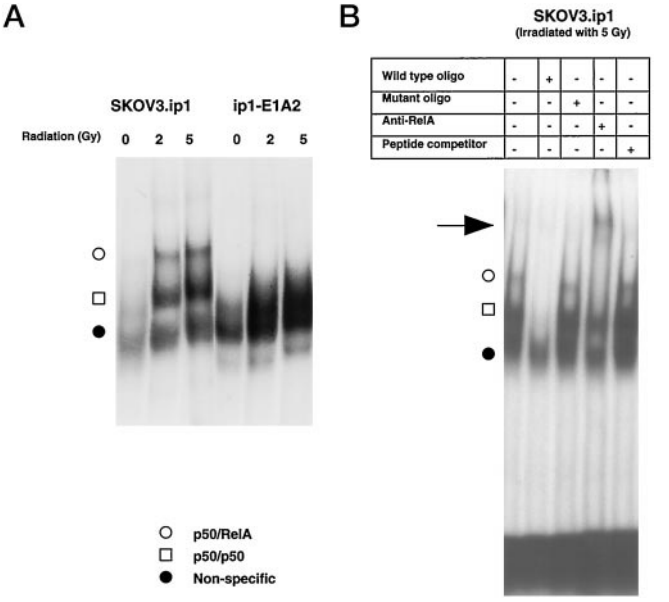

FIG. 3. **E1A inhibits the activation of NF-κB induced by ionizing radiation.** *A*, equal amounts of nuclear extracts from SKOV3.ip1 and ip1-E1A2 cells (before and after irradiation with the indicated dose rates) were subjected to electrophoretic mobility-shift analysis of NF-κB. *B*, nuclear extracts from irradiated SKOV.ip1 cells were incubated in both the presence and absence of wild-type NF-κB and a mutant oligonucleotide. An antibody to RelA (SC-109, Santa Cruz) was also incubated under the same conditions in both the presence and absence of a peptide competitor (SC-109 P). Electrophoretic mobility-shift analysis was carried out as described under "Materials and Methods." These are representative experiments; the same results were found in other experiments. The post-irradiation nuclear extracts were prepared 2.5 h after irradiation.

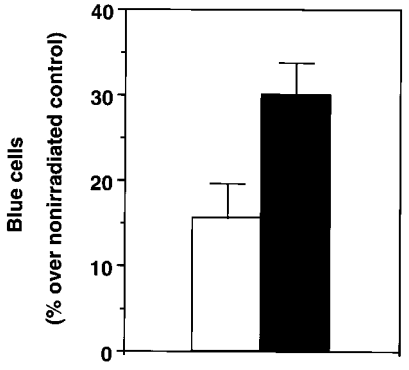

FIG. 4. **NF-κB partially rescues ip1-E1A2 cells from radiation-induced apoptosis.** SKOV3.ip1 cells were transfected with the E1A expression vector and cotransfected with a control vector or an NF-κB expression vector; *lacZ* cotransfection was used for tracking the viable cells (see "Materials and Methods"). After transfection, the cells were divided and plated into two dishes. One set was irradiated with 10 Gy, and the other served as a control. After 48 h, the cells were stained for β-galactosidase (blue), and the results were expressed as a percentage over the nonirradiated control. Symbols: □, E1A + vector; ■, E1A + NF-κB.

in growth upon radiation were confirmed in another assay by counting the cells before and after irradiation during the same period of time (Fig. 1*B*). As a control, we also checked the expression of E1A in these cell lines by immunoblot analysis using a monoclonal antibody (M58); the results showed that the ip1-E1A2 cells expressed E1A but the parental (SKOV3.ip1) and mutant (ip1-Efs) cells did not (*inset*, Fig. 1). These results suggest that the introduction of E1A into SKOV3.ip1 cells significantly facilitates the radiation-induced retardation of cell growth. However, measurement of DNA synthesis rates using the [^{3}H]thymidine incorporation assay revealed no significant difference between the parental and E1A-transfected cells after irradiation when the radioactive counts were normalized against the counts in the nonirradiated control (Fig. 1*C*). Presumably, the net cell-growth rate depends on a fine balance between the cell-replication rate and the cell-death rate. The fact that cell replication requires DNA synthesis and that the effect of radiation on DNA synthesis does not change significantly between E1A transfectants and parental cells (Fig. 1*C*) indicates that the effect of radiation on cell replication cannot account for the differential response to radiation between E1A transfectants and parental cells (Fig. 1, *A* and *B*). Therefore, a change in cell death is most likely to be the major cause of the differential response to radiation during cell growth between the E1A transfectants and the parental cells (Fig. 1, *A* and *B*). To study the apoptotic changes, cells were irradiated and analyzed by DNA fragmentation analysis. Indeed, the E1A-transfected cells showed dramatic changes in DNA fragmentation after irradiation for 24 h, but the parental cells (SKOV3.ip1) did not exhibit DNA fragmentation even after irradiation for 48 h at doses of 5 or 10 Gy (Fig. 2*A*), which is reminiscent of the often-seen resistance to radiotherapy among cancer patients. The 180–200-base pair DNA fragments

and the multiples of them that were observed in this study represent a characteristic fragmentation pattern attributed to apoptotic phenomena (21, 22). To quantitate the apoptotic changes observed in E1A-transfected cells upon irradiation, we measured the sub-G_1 population of cells by flow cytometry. As shown in Fig. 2*B*, more than 30% of the E1A-transfected cells showed the apoptotic peak observed at 72 h after irradiation, while the apoptotic population in the parental cells was less than 5% during the same period. A similar but more profound trend was also observed with the administration of a higher dose of 10 Gy (data not shown). Thus, our results are consistent with the notion that E1A can sensitize cells to radiation-induced apoptotic changes.

Ionizing radiation is known to induce the activation of RelA (p65), a subunit of NF-κB in many systems (14, 23, 24), but the involvement of ionizing radiation in the E1A-mediated susceptibility of cells to radiation has not been examined. Therefore, we analyzed SKOV3.ip1 cells and their E1A transfectants for RelA DNA binding activity before and after irradiation. The results indicate that ionizing radiation induces NF-κB DNA binding activity in parental cells but not in E1A-transfected cells (Fig. 3*A*). Increasing the radiation dose to 10 Gy did not activate RelA in the E1A-transfected cells, and there was no further increase in activation in the parental cells (data not shown). The activated complex induced by radiation in the SKOV3.ip1 cells was abolished in the presence of a wild-type oligonucleotide, but a mutant oligonucleotide failed to displace the active complex, indicating the specificity of the DNA binding activity (Fig. 3*B*). As reported previously (13, 25), the two specific DNA-protein complexes probably represent the RelA and p50 heterodimers and the p50 homodimer, respectively. To identify and confirm the presence of the RelA subunit of NF-κB, which is usually present in the activated complex as a heterodimeric partner of the p50 subunit, in SKOV3.ip1 cells induced by radiation, we performed a mobility-shift analysis in the presence of a polyclonal Re1A antibody (SC-109, Santa Cruz). The results of the analysis showed a supershifted complex that disappeared in the presence of a peptide competitor (SC-109P) (Fig. 3*B*). Thus, the slower of the two specific complexes should represent the RelA and p50 heterodimers, and the faster complex is most likely p50 and the p50 homodimer, the function of which is not yet clear. The E1A-mediated inactivation of the radiation-induced NF-κB activity shown in Fig. 3 was also confirmed when two other pairs of cell lines, a breast cancer cell line (MDA-MB-435), a mouse melanoma cell line (k1735), and their E1A transfectants were examined (data not shown). Taken together, these results indicate that E1A is capable of inhibiting RelA activation induced by radiation.

Because RelA has recently been shown to be associated with anti-apoptotic activity, we question whether inactivation of RelA by E1A is required for E1A-mediated apoptosis in response to radiation. If this is indeed the case, transient overexpression of RelA would be expected to counteract and rescue the radiation-induced apoptotic changes in the presence of E1A. To test this possibility, we transiently transfected the E1A and *lacZ* genes into SKOV3.ip1 cells both with and without the RelA expression plasmid, irradiated the cells, and analyzed the surviving cells by staining the cells with 5-bromo-4-chloro-3-indol-β-D-galactoside. This approach, which uses the number of β-galactosidase staining cells as an index of the surviving cells, has been used routinely by others to demonstrate the protective role of RelA against stress-induced apoptosis. The results show that RelA indeed can prevent at least part of the E1A transfectants from undergoing apoptosis upon irradiation (Fig. 4). Taken together, our results strongly suggest that RelA plays a role in protecting cells from ionizing radiation-induced apoptosis, and the E1A-mediated inactivation of RelA contributes significantly to the E1A-mediated sensitization of radiation-induced apoptosis.

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