The p65 subunit of nuclear factor-κB is a molecular target for radiation sensitization of human squamous carcinoma cells

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Abstract

The transcription factor nuclear factor-κB (NF-κB) is activated in response to various stimuli including ionizing radiation. Disruption of NF-κB activation by mutant forms of the NF-κB inhibitor IκB-α or by proteasome inhibitors enhances both sensitivity to radiation and radiation-induced apoptosis. Human squamous carcinoma SCC-35 cells stably expressing a fragment (residues 1 to 84) of human p65 have been shown to exhibit down-regulation of both endogenous p65 mRNA and its protein. The mutant protein also inhibited radiation-induced NF-κB activation by preventing the proteolysis of IκB-α. This resulted in enhancement of cellular radiosensitivity and radiation-induced apoptosis. The NH_2-terminal region of p65 is thus a potential molecular target for disruption of NF-κB activation and sensitization of tumors to radiotherapy. [Mol Cancer Ther 2004;3(6):693–8]

Introduction

The transcription factor nuclear factor-κB (NF-κB) contributes to cell differentiation, cell proliferation, and cell death and plays a prominent role in the immune response in mammals (reviewed in ref. 1). NF-κB consists of homodimers or heterodimers of the Rel family members NF-κB1 (p50), NF-κB2 (p52), c-Rel, RelA (p65), and RelB (2). These Rel family proteins each contain a Rel homology domain at their NH_2 terminus, which includes both a conserved DNA binding domain and a dimerization domain. In resting cells, NF-κB is sequestered in the cytoplasm as a result of its interaction with inhibitory IκB proteins (3). Exposure of cells to tumor necrosis factor-α (TNF-α), phorbol esters, or DNA-damaging agents induces the phosphorylation, ubiquitination, and degradation of IκB-α resulting in the translocation of NF-κB to the nucleus and consequent transcriptional activation of its target genes (1). NF-κB also regulates expression of the IκB-α gene, thereby contributing to an autoregulatory feedback mechanism (2). Exposure of cells to ionizing radiation (IR) induces NF-κB activation by a signaling pathway that includes ataxia telangiectasia mutated gene (4), the product of this gene is mutated in individuals with ataxia telangiectasia, a human genetic disease exhibiting extreme sensitivity to IR. We and others have therefore investigated the potential for sensitization of cancer cells to radiation through interruption of NF-κB signaling (reviewed in ref. 5). Although direct strategies for inhibition of NF-κB have resulted in enhancement of apoptosis, the effects of such strategies on cellular radiosensitivity have been difficult to ascertain (6). In most studies, vector-mediated expression of mutant IκB-α or chemical proteasome inhibitors, which interfere with IκB-α degradation, has been used to regulate the activation of NF-κB in response to genotoxic stress (reviewed in ref. 5). Although such small molecule inhibitors have been shown to exert an antitumor effect with *in vivo* xenografts, including those of human breast, head and neck, pancreatic, and prostate cancer (7, 8), concerns remain with regard to the nonspecific effects of these compounds. To examine the effects of direct modulation of NF-κB activation in human head and neck squamous carcinoma cells, we have now applied an approach that targets the p65 subunit of NF-κB. We thus transfected cells with a vector encoding a mutant p65 that contains only a portion of the DNA binding domain and lacks both nuclear localization signal and transcriptional activation domain. The effects of expression of this mutant on cellular radiosensitivity were determined.

Materials and Methods

Cell Culture, Transfection, and Clonal Selection

Human head and neck squamous carcinoma SCC-35 cells have been previously characterized as resistant to radiation based on clinical and radiobiological parameters (9). The cells were cultured in 10-cm^2 dishes and maintained in DMEM supplemented with 10% fetal bovine serum and 2 mmol/L L-glutamine. They were passaged routinely at 80% to 90% confluence and checked for *Mycoplasma* contamination at 3-month intervals. Cells in the exponential phase of growth were transfected with 5 μg

Received 11/18/03; revised 3/24/04; accepted 4/13/04.

Grant support: National Cancer Institute grants CA68023 (M. Jung) and CA45408 and CA74175 (A. Dritschilo and M. Jung).

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of plasmid DNA encoding the p65 mutant [p65(1-84 aa)] with the use of LipofectAMINE (5 μg/mL; Life Technologies, Inc., Rockville, MD). G418-resistant cells were subcloned and maintained in complete medium containing G418 (400 μg/mL).

Construction of the p65 Mutant Expression Vector

The 250-bp fragment of human p65 cDNA that encodes amino acids 1 to 84 was amplified by PCR with the full-length cDNA as template (10) and the primers H65F (5′-GGCCATGGACGAACTGTTCCC) and H65R (5′-GGAGGGTCCTTGGTGACCAG). The amplified fragment was subcloned into the eukaryotic expression vector pCR3.1 (Invitrogen, San Diego, CA), which confers resistance to G418, and the sequence of the insert was confirmed by automated DNA sequencing. Plasmid DNA was prepared by CsCl density gradient centrifugation in the presence of ethidium bromide.

Northern Blot Analysis and Quantitative Real-time PCR

Total RNA was isolated from cells with the use of RNAzol B (Tel-Test) and subjected to electrophoresis on a 1% agarose gel containing formaldehyde. The separated molecules were transferred to a nitrocellulose membrane, cross-linked to the membrane by exposure to UV, and subjected to hybridization with an [α-^{32}P]dATP-labeled p65 cDNA probe for 16 hours. The membrane was washed and subjected to autoradiography. For quantitative analysis, real-time PCR was carried out using total RNA, primers (p65, p50, or glyceraldehyde-3-phosphate dehydrogenase), and TaqMan One-Step PCR Master Mix Reagents Kit according to the manufacturer's protocol (Applied Biosystems, Foster City, CA). Three replicates were run for each sample. Negative controls with no RNA template were included in every analysis, and all the samples were normalized with glyceraldehyde-3-phosphate dehydrogenase. The results were analyzed using the amplification curves and threshold cycle collected from real-time PCR data analysis. The fold change between genes was calculated using the average threshold cycle of the samples and the following formula:

$$2^{\text{threshold cycle of gene of interest} - \text{threshold cycle of control gene}}$$

Immunoblot Analysis

After exposure to γ-radiation (with a ^{137}Cs J.L. Shepherd Mark I laboratory irradiator) or TNF-α (10 ng/mL; Life Technologies), cells were lysed in a solution containing 50 mmol/L Tris-HCl (pH 7.5), 100 mmol/L NaCl, 0.1% NP40, 1 mmol/L phenylmethylsulfonyl fluoride, 10 mg/mL leupeptin, 1 mg/mL aprotinin, and 1 mg/mL pepstatin. The lysate was centrifuged at 12,000 × *g* for 20 minutes, and the protein concentration of the resulting supernatant was determined with a BCA protein assay kit (Pierce Chemical Co., Rockford, IL). Alternatively, cells were homogenized for preparation of cytosolic and nuclear fractions. The cytosolic fraction in EB [10 mmol/L HEPES (pH 7.9), 10 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 2 mmol/L DTT, 0.1 mmol/L EDTA, 0.1 mmol/L EGTA, and protease inhibitors] containing 0.1% NP40 was isolated by centrifugation at 25,000 × *g* for 1 minutes, and the resulting pellet was resuspended in a nuclear extraction. The extracted nuclear proteins were isolated by centrifugation at 50,000 × *g* for 30 minutes. Equal amounts of protein samples were fractionated by SDS-PAGE on a 10% gel, and the separated proteins were transferred to a nitrocellulose membrane. The membrane was exposed for 1 hour to 5% nonfat dried milk in PBS and probed with antibodies to p65 or IκB-α phosphorylated on Ser^{32} (Santa Cruz Biotechnology, Santa Cruz, CA) or with antibodies specific for poly(ADP-ribose) polymerase (PARP; Enzyme Synthesis, Dublin, CA). Immune complexes were detected with horseradish peroxidase–conjugated secondary antibodies and an enhanced chemiluminescence kit (Amersham Pharmacia Biotech, Little Chalfont, United Kingdom). Equal loading and transfer of proteins were confirmed by immunoblot analysis with antibodies to β-actin (Amersham Pharmacia Biotech) or by staining the membrane with Ponceau S stain.

Luciferase Reporter Gene Assay

Cells were subjected to transient transfection by incubation for 16 hours with the NF-κB–specific luciferase reporter plasmid pNF-κB-Luc (5 μg/mL; Santa Cruz Biotechnology) and a β-galactosidase vector (New England Biolabs, Beverly, MA) in the presence of Lipofectin (Life Technologies). After exposure to IR (20 Gy) or TNF-α (10 ng/mL), the cells were lysed for 6 hours in reporter lysis buffer (Promega, Madison, WI) and the lysate was centrifuged for 2 minutes at 14,000 × *g*. The cell extracts were assayed for β-galactosidase and luciferase activities.

Radiation Survival Assay

Radiation sensitivity was determined as described previously (11). In brief, exponentially growing cells were plated in T-25 flasks and exposed to graded doses of radiation with a dose accuracy of ±5%. Physics support for calibration and quality assurance was provided for all experimental protocols. Colony counts were fitted to single-hit multitarget and linear quadratic models (12).

Determination of Apoptotic Index

Adherent and floating cells were collected at various times after exposure to γ-radiation and were stained with acridine orange and ethidium bromide. About 600 cells (~100 cells per field) were scored for aberrant chromosome organization by fluorescence microscopy. The apoptotic index was determined as the percentage of cells with nuclei exhibiting characteristics of apoptosis.

Measurement of Caspase-3 Activity

The activity of caspase-3 was assayed fluorometrically with the specific fluorogenic substrate MOCAc-DEV-DAPK(DNP)-NH_2 (Peptide Institute, Osaka, Japan). Cell extracts (60 μg of protein) were incubated for 1 hour at 37°C with 600 nmol/L substrate in a reaction mixture (500 μl) containing 10 mmol/L HEPES-NaOH (pH 7.4), 40 mmol/L β-glycerophosphate, 50 mmol/L NaCl, 2 mmol/L $MgCl_2$, 5 mmol/L EGTA, 1 mmol/L DTT, 2 mmol/L ATP,

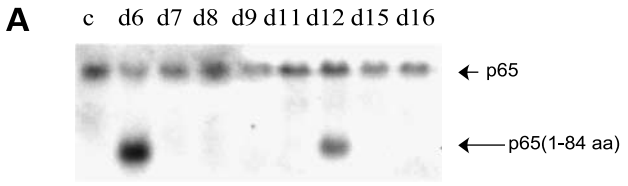
10 mmol/L creatine phosphate, and 50 μg/mL creatine kinase. The fluorescence of the cleaved substrate was measured with a Hitachi F-4500 spectrophotometer (Hitachi Instruments, Inc., San Jose, CA) at excitation and emission wavelengths of 328 and 393 nm, respectively.

Results

Effects of p65 Mutant on the Expression of Endogenous p65

The NF-κB heterodimer composed of p50 and p65 subunits is abundant in most cell types. The p65 subunit contains a DNA binding domain in its NH_2-terminal region (residues 1 to 300), a nuclear localization signal in its central region (residues 301 to 304), and a transcriptional activation domain in its COOH-terminal region (2). Deletion of the DNA binding domain of p65 has been shown previously to interfere with NF-κB function (13).

To investigate the effects of targeting p65 as a means of disrupting NF-κB activation, we transfected radioresistant human squamous carcinoma SCC-35 cells with an expression vector encoding amino acids 1 to 84 of human p65. The resulting G418-resistant colonies were isolated and analyzed for expression of p65(1-84 aa) and endogenous p65. Northern blot analysis revealed that the cell clones contained both endogenous p65 mRNA and transcripts corresponding to mutant p65 (Fig. 1A); however, the amount of p65 mRNA was reduced compared with that in the parental SCC-35 cells. To further quantitate expression levels, real-time PCR assays were performed using total RNAs with primers specific for p65, p50, and glyceraldehyde-3-phosphate dehydrogenase. Differences in p65 expression levels in those cells compared with that in controls were normalized by glyceraldehyde-3-phosphate dehydrogenase and tabulated in Fig. 1A. Data show ~2-fold reduction of p65 expression levels in clonal cells; however, no significant changes were observed in p50 expression. Of these clonal cells, the line d6, which contained the largest amount of p65(1-84 aa) mRNA, was characterized further.



mRNA Expression (fold)				
Name/Accession	*Clone 6*	*Clone 11*	*Clone 12*	*Pool*
NF_kB (p65)/L19067	-2.4	-2.0	-2.3	-1.2
NF_kB (p50)/M58603	+1.05	+1.19	+1.07	+1.0

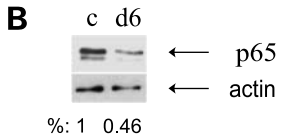


Figure 1. Effects of mutant p65(1-84 aa) on the abundance of endogenous p65 mRNA and protein in SCC-35 cells. **A,** Total RNA was isolated from parental SCC-35 cells and clonal lines of SCC-35 cells harboring an expression vector for p65(1-84 aa) (*d6* to *d16*). The RNA was subjected to Northern blot analysis with [α-^{32}P]-labeled p65 cDNA probe. *Small arrow* and *large arrow,* positions of the transcripts corresponding to endogenous p65 and to p65(1-84 aa), respectively. Quantitative mRNA expression levels of p65 and p50 were obtained by performing real-time PCR using total RNAs of control, pooled, and clonal cells. Expressions of p65 and p50 messages were normalized based on glyceraldehyde-3-phosphate dehydrogenase expression, and the normalized expression levels in these cells transfected with mutant p65 were expressed as fold changes compared with the corresponding value for control cells. **B,** Extracts prepared from parental SCC-35 cells and d6 cells were subjected to immunoblot analysis with antibodies to p65. *Arrow,* position of endogenous p65. Actin expression was used as a loading control.

Immunoblot analysis with antibodies to p65 revealed that the amount of endogenous p65 protein in d6 cells was reduced to ~46% compared with that in the parental SCC-35 cells (Fig. 1B). The equal loading was confirmed by actin expression. These data demonstrate that expression of p65(1-84 aa) resulted in a marked reduction in the amounts of endogenous p65 mRNA and protein, suggesting that the mutant functions as a modulator of NF-κB function. We speculate that mutant p65 may modulate endogenous p65 activities through competition on NF-κB–mediated gene expression, including p65 transcription.

Effects of p65(1-84 aa) on Cellular Sensitivity to IR

Impaired regulation of NF-κB in certain cell types is associated with intrinsic cellular radiosensitivity (5). To examine the role of NF-κB in determining cell sensitivity to radiation, we performed clonogenic survival assays after exposure of SCC-35 and d6 cells to graded doses of γ-radiation (Fig. 2). The sensitivity of d6 cells to IR was greater than that of the parental SCC-35 cells. According to the single-hit multitarget model (12), D_0 values were determined to be 1.9 Gy (with $\alpha = 0.16$ and $\beta = 0.02$) for SCC-35 cells and 1.2 Gy (with $\alpha = 0.11$ and $\beta = 0.06$) for d6 cells. Because of the potential pitfalls of clonal heterogeneity associated with determination of radiation sensitivity in a clonal cell population, we also performed survival experiments with d12 cells. As shown in Fig. 2, we obtained similar results ($D_0 = 1.1$ Gy), suggesting that the observed p65(1-84 aa)–induced increase in radiation sensitivity is not simply due to clonal selection.

Effects of p65(1-84 aa) on the Activation of NF-κB

Signal-induced phosphorylation and degradation is an indicator of NF-κB activation. We therefore next determined the effect of p65(1-84 aa) expression on radiation-induced phosphorylation and subsequent degradation of IκB-α in the cytoplasm. Immunoblot analysis with antibodies specific for IκB-α phosphorylated on Ser^{32} revealed that phosphorylation of IκB-α on this residue was apparent within 30 minutes of irradiation of SCC-35 cells and thereafter decreased (Fig. 3A). However, in d6 cells, IR induced an increase in the phosphorylation of IκB-α that was also apparent at 30 minutes but which remained markedly increased at 3 hours. In parallel, IR gradually decreased the amount of unphosphorylated IκB-α within

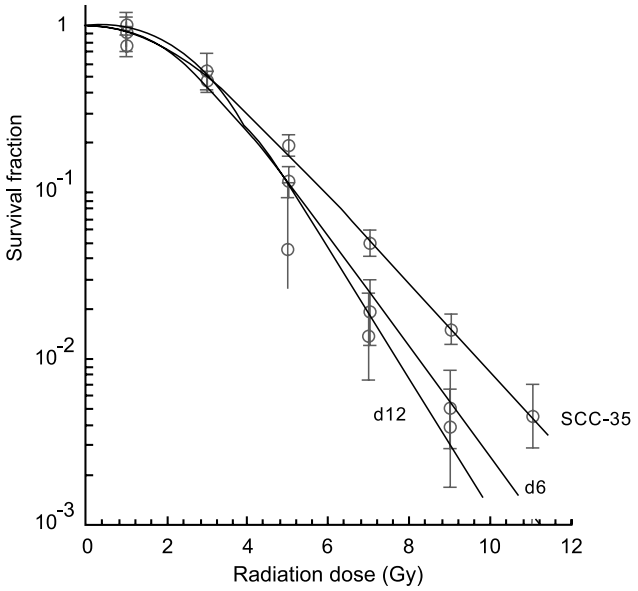


Figure 2. Effect of p65(1-84 aa) expression on cell survival after irradiation. SCC-35, d6, and d12 cells in the exponential phase of growth were exposed to the indicated doses of γ-radiation. Clonogenic survival was determined by counting the number of colonies containing >50 cells after 2 weeks of growth. The surviving fraction is shown in a semi-logarithmic plot against radiation dose. *Points,* means from triplicate flasks from two to three independent experiments; *bars,* SE.

1 hour and increased within 3 hours in control cells. However, irradiation resulted in a decrease in the amount of this protein in d6 cells; the abundance of IκB-α in untreated control cells was greater than that in untreated d6 cells (Fig. 3A). These data thus show that p65(1-84 aa) interfered with the proteolysis of phosphorylated IκB-α, suggesting that the phosphorylation of this protein is not sufficient for its dissociation from NF-κB. This conclusion is consistent with the previous observation that amino acids 1 to 101 of p65 are required for its binding to IκB-α (14). We therefore propose that p65(1-84 aa) interferes with the inhibitory function of IκB-α by binding to this protein in both cytoplasm and nucleus.

To determine whether p65(1-84 aa) inhibits the activation of NF-κB, we transfected d6 cells with the NF-κB–specific luciferase reporter vector pNF-κB-Luc and examined the effect of IR or TNF-α on luciferase activity. The stimulatory effects of these agents on NF-κB activity were reduced by a factor of 2 to 3 in d6 cells compared with those apparent in SCC-35 cells (Fig. 3B). Expression of p65(1-84 aa) thus inhibited radiation-induced NF-κB activation albeit less quantity than that by TNF-α.

Effects of p65 Mutant on Radiation-Induced Apoptosis

Activation of NF-κB contributes to the protection of many cell types from apoptotic cell death induced by DNA-damaging agents. However, under certain conditions and in certain cell types, activation of NF-κB induces apoptosis (5). Given that clonogenic survival analysis provides a measure of all types of cell death, we next determined the effect of p65(1-84 aa) expression on radiation-induced apoptosis. Radiation induced a dose-dependent increase in the percentage of both SCC-35 and d6 cells as well as d12 cells (data not shown) characterized as apoptotic 48 hours after irradiation (Fig. 4A). However, whereas the maximum proportion of apoptotic cells was 8% (evident at 20 Gy) for SCC-35 cells, confirming that apoptosis is not a major pathway of cell death in these cells, the maximum apoptotic index was ~14% for clonal cells. The baseline apoptotic index for both cell lines was <1%. The time courses of the changes in the apoptotic index induced by 5 Gy of γ-radiation revealed that a substantial increase in this parameter was apparent at 24 hours in clonal cells but not until 48 hours in SCC-35 cells (Fig. 4B).

The nuclear enzyme PARP, which plays an important role in the repair of DNA damage and in maintenance of genomic integrity (15), is specifically cleaved between its NH_2-terminal DNA binding domain and its multifunctional COOH-terminal domain by caspase-3 or a caspase-3–like protease early during the execution phase of apoptosis (16). We therefore examined the effects of mutant p65 on both PARP cleavage and caspase-3 activity. Exposure of d6 cells

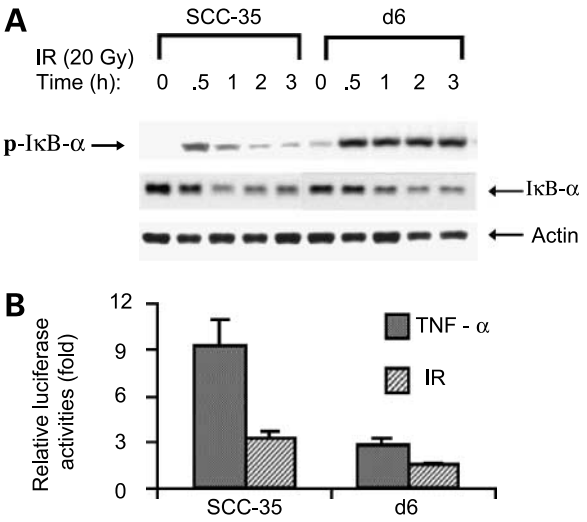


Figure 3. Effects of p65(1-84 aa) expression on various aspects of NF-κB activation. **A,** Effects of mutant p65(1-84 aa) on the radiation-induced changes in the abundance of Ser^{32}-phosphorylated IκB-α in the cytoplasm. SCC-35 and d6 cells were exposed to 20 Gy of IR, and at the indicated times thereafter, cytosolic fractions were prepared and subjected to immunoblot analysis with antibodies specific for Ser^{32}-phosphorylated IκB-α (*p-IκB-α*) or unphosphorylated IκB-α, respectively. The membrane was also reprobed with antibodies to β-actin. *Time 0,* cells were subjected to sham irradiation. **B,** Effects of p65(1-84 aa) on the IR-induced or TNF-α–induced increase in the transactivation activity of NF-κB. SCC-35 and d6 cells were transfected with the NF-κB–specific luciferase reporter plasmid pNF-κB-Luc and a β-galactosidase expression vector. The cells were subsequently exposed to 20 Gy of IR or incubated with TNF-α (10 ng/mL), and after 6 hours, cell extracts were prepared and assayed for luciferase and β-galactosidase activities. Luciferase activity was normalized based on β-galactosidase activity, and the normalized luciferase activity of treated cells was expressed as fold increase compared with the corresponding value for untreated cells. *Columns,* means from three independent experiments; *bars,* SE.

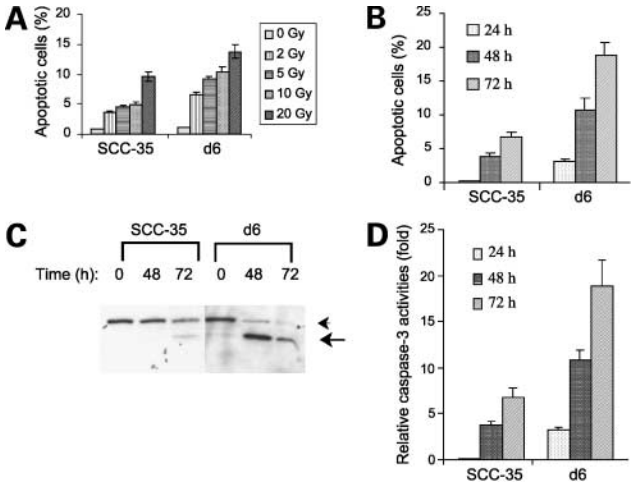


Figure 4. Effects of p65 (1-84 aa) expression on radiation-induced apoptosis. **A** and **B,** Effects of p65(1-84 aa) on the dose-response relation and time course of the radiation-induced increase in the apoptotic index. SCC-35 and d6 cells were either exposed to the indicated doses of IR and examined after 48 hours (**A**) or exposed to 5 Gy of IR and examined at the indicated times thereafter (**B**). *Columns,* means from three independent experiments; *bars,* SE. **C,** Effect of p65(1-84 aa) on IR-induced PARP cleavage. Cells were exposed to 5 Gy of IR, and at the indicated times thereafter, cell extracts were prepared and subjected to immunoblot analysis with antibodies to PARP. *Arrowhead* and *arrow,* uncleaved and cleaved PARP, respectively. **D,** Effect of p65(1-84 aa) on the IR-induced activation of caspase-3. Cells were exposed to 5 Gy of IR and, at the indicated times thereafter, cell extracts were prepared and assayed for caspase-3 activity. *Columns,* means from three independent experiments expressed as fold increase relative to the activity of untreated cells; *bars,* SE.

to γ-radiation at doses as low as 2 Gy resulted in PARP cleavage, whereas PARP cleavage in SCC-35 cells required much larger doses of IR (>20 Gy; data not shown). Exposure to 5 Gy of γ-radiation induced a substantial increase in PARP cleavage within 48 hours in d6 cells, whereas cleavage of this enzyme remained barely detectable at 72 hours in SCC-35 cells (Fig. 4C). Similarly, IR (5 Gy) induced an earlier and more pronounced activation of caspase-3 in d6 cells than in SCC-35 cells (Fig. 4D). Together, these observations suggest that caspase-3 contributes to the cleavage of PARP during IR-induced apoptosis in SCC-35 cells, that NF-κB protects these cells from apoptosis, and that expression of p65(1-84 aa) potentiates IR-induced apoptosis in the tumor cells.

Discussion

Cellular responses to IR include the activation of signaling pathways that lead to the expression of survival or death factors. In general, activation of NF-κB plays an important role in cellular survival after exposure to stressful stimuli such as IR. Proteins that contribute to radiation-induced NF-κB signaling therefore provide potential targets for sensitization of tumor cells to radiation. Furthermore, disruption of NF-κB activation by expression of IκB-α mutants has been shown to result in an increase in the radiosensitivity of human tumor cells in a cell type–specific manner (reviewed in ref. 5). Our data show that the radiation-resistant SCC-35 human tumor cells become sensitized to IR as a result of inhibition of NF-κB activation.

Crystallographic studies have identified the sites of physical interaction between NF-κB (p50-p65) and IκB-α (17-19). The ankyrin repeat 6 and an adjacent partial proteolytic signal sequence of IκB-α interact with the NH_2-terminal immunoglobulin-like motif of the Rel homology domain of p65, including residues Tyr^{20}, Glu^{22}, Glu^{49}, Arg^{50}, His^{181}, Arg^{246}, and Arg^{294}. In addition, ankyrin repeats 1 and 3 of IκB-α interact with sequences encompassing the nuclear localization signal of p65. We have now shown that expression of a NH_2-terminal fragment (residues 1 to 84) of p65 both blocks the degradation of IκB-α after its stimulus-induced phosphorylation and disrupts NF-κB activation, thereby increasing the sensitivity of radiation-resistant human squamous carcinoma cells to radiation-induced cell death.

Phosphorylation of IκB proteins and their subsequent degradation is an important mechanism of NF-κB activation. Rapid resynthesis of these proteins results in their entry into the nucleus and removal of NF-κB from its DNA binding sites (2, 17). Thus, IκB proteins have been considered potential molecular targets for modulation of NF-κB activation; indeed, such modulation has been achieved by expression of mutant forms of IκB (1). Our data now show that expression of the NH_2-terminal portion of p65 comprising amino acids 1 to 84 results in down-regulation of both endogenous p65 protein and its mRNA. IκB-α was phosphorylated within 30 minutes of exposure to IR in both parental SCC-35 cells and cells expressing mutant p65. However, whereas subsequent degradation of IκB-α was apparent within 1 hour of irradiation in the parental cells, no such degradation was detected for up to 3 hours in d6 cells. These results suggest that the NH_2 terminus of p65 is a potential molecular target for disruption of NF-κB activation through inhibition of IκB-α degradation. They also suggest that the degradation of phosphorylated IκB-α requires an interaction with additional sequences of p65, such as the nuclear localization signal or transcriptional activation domain, or additional events such as a conformation change or phosphorylation of p65 (20).

Although both necrotic and apoptotic mechanisms of cell death may contribute to antitumor therapy, it is important to understand the role of each to tailor treatment to specific tumor. In most cells, NF-κB mediates cell survival signals, protecting cells from apoptosis. Thus, when NF-κB activation is impaired, as has been achieved by the expression of IκB-α mutants (5), cells undergo apoptosis. A previous study in which NF-κB was targeted by viral gene therapy was performed with cells that were highly sensitive to apoptotic death (21).

Activation of caspases results in the proteolysis of intracellular proteins that contribute to the maintenance of cellular integrity. PARP is a prominent substrate of caspase-3 during apoptosis. We have now shown that the

increase in the sensitivity of SCC-35 cells to IR-induced apoptosis achieved by expression of mutant p65 was accompanied by an increased extent of PARP cleavage and caspase-3 activation, indicating the importance of caspase-3 as a downstream protease during IR-induced apoptosis. These data also suggest that NF-κB contributes to the regulation of caspase-3 and PARP.

In summary, our data demonstrate that the mutant p65 functions as a modulator of NF-κB in three ways (1). Expression of mutant p65(1-84 aa) results in down-regulation of both endogenous p65 mRNA and its protein possibly through transcriptional regulation or mRNA stability (2). Mutant p65 interferes with the inhibitory function of IκB-α by blocking proteolysis of phosphorylated IκB-α after cell irradiation, suggesting that dissociation and proteolysis of IκB-α require additional events. We speculate that the binding of mutant p65(1-84 aa) to IκB-α may thus stabilize the phosphorylated form of IκB-α (3). Mutant p65 expression results in increases of cellular radiosensitivity and radiation-induced apoptosis. Therefore, disruption of radiation-induced signal transduction through NF-κB represents a means of achieving cellular radiosensitization and may provide a basis for new anti-tumor therapeutic strategies.

Acknowledgments

We thank S.M. Ruben for providing p65 cDNA, J. Tuturea and N. Zaer for technical assistance, and E. North for help in preparation of the manuscript.

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