A novel ionizing radiation-induced signaling pathway that activates the transcription factor NF-κB

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The signaling pathway through which ionizing radiation induces NF-κB activation is not fully understood. IκB-α, an inhibitory protein of NF-κB mediates the activation of NF-κB in response to various stimuli, including cytokines, mitogens, oxidants and other stresses. We have now identified an ionizing radiation-induced signaling pathway that is independent of TNF-α. IκB-α degradation is rapid in response to TNF-α induction, but it is absent in response to ionizing radiation exposure in cells from individuals with ataxia-telangiectasia (AT). Overexpression of wild-type ATM, the product of the gene defective in AT patients, restores radiation-induced degradation of IκB-α. Furthermore, phosphorylation of IκB-α by immunoprecipitated ATM kinase is increased in control fibroblasts and transfected AT cells following ionizing radiation exposure. These data provide support for a novel ionizing radiation-induced signaling pathway for activation of NF-κB and a molecular basis for the sensitivity of AT patients to oxidative stresses.

Keywords: ionizing radiation; NF-κB; IκB-α; ataxia-telangiectasia (AT); ATM

Introduction

The nuclear transcription factor kappa B (NF-κB) plays an integral role in signal transduction pathways induced by cytokines, mitogens, ionizing radiation, or other agents (Sen and Baltimore, 1986; Baeuerle and Baltimore, 1988; Brach et al., 1991; Brown et al., 1995; Brockman et al., 1995; Traenckner et al., 1995; DiDonato et al., 1996; Whitside et al., 1995). NF-κB is maintained in the cytoplasm bound to inhibitor proteins, the IκBs. Various stimuli activate a large number of distinct signaling pathways that result in activation of NF-κB by regulating its interaction with IκB-α. Most studies of NF-κB activation have focused on cytokine-induced signaling (Sen and Baltimore, 1986; Baeuerle and Baltimore, 1988; Brown et al., 1995; Brockman et al., 1995; Traenckner et al., 1995; DiDonato et al., 1996; Whitside et al., 1995). Two distinct biochemical mechanisms have been described for cytokine-induced pathways (Brown et al., 1995; Brockman et al., 1995; Traenckner et al., 1995; DiDonato et al., 1996; Whitside et al., 1995; Imbert et al., 1996). Exposure of cells to TNF-α or interleukine-1 (IL-1) induces the phosphorylation of IκB-α on Ser32 and Ser36 and its subsequent ubiquitin-mediated degradation (Brown et al., 1995; Chen et al., 1996). The free form of NF-κB then translocates to the nucleus and activates the transcription of various genes important in cell survival or death (Beg and Baltimore, 1997; Wang et al., 1997; Van Antwerp et al., 1997). Alternatively, IκB-α may be phosphorylated on Tyr42, resulting in dissociation from NF-κB without subsequent proteolysis (Imbert et al., 1996).

Several protein kinases that mediate IκB-α phosphorylation have been described (Barroga et al., 1995; Baeuerle and Baltimore, 1996; Cao et al., 1996; Malinin et al., 1997; DiDonato et al., 1997; Regnier et al., 1997), consistent with the existence of distinct NF-κB-mediated signal transduction pathways. Recent reports have shown the identification of a high molecular mass IκB kinase complex (Chen et al., 1996) and the isolation of two Ikk kinases (Mercurio et al., 1997; Woronicz et al., 1997; Lee et al., 1997; Zandi et al., 1997). These kinases phosphorylate Ser32 and Ser36 on IκB-α in response to TNF-α or IL-1 treatment; however, the complete mechanisms of IκB kinase activation remain to be elucidated.

Exposure of cells to ionizing radiation results in the generation of oxygen free radicals, oxidative damage to cellular components, and induction of DNA strand breaks (Teoule, 1987). Failure to repair such DNA damage may result in cell death (Ward, 1988). Ionizing radiation also induces signal transduction pathways, resulting in the activation of transcription factors that regulate cell growth, DNA damage repair, and proliferation (Brach et al., 1991; Fuks et al., 1993; Hallahan et al., 1991).

The ionizing radiation-induced NF-κB activation has been reported following small and large doses (Brach et al., 1991; Mohan and Meltz, 1994). However, the radiation specific signaling pathways is not known. Previously, we have reported that the regulation of NF-κB is impaired in cells from patients with the complex human genetic disease, ataxia-telangiectasia (AT), which exhibits exquisite sensitivity to ionizing radiation and abnormalities in cellular regulation processes (Jung et al., 1995; Shafman et al., 1995; 1997; Baskaran et al., 1997). The mutated ATM gene found in AT patients (Savitsky et al., 1995a,b) is believed to be involved in a number of functions that lead to neurological, immunological and radiobiological dysfunction (Gatti et al., 1991). We have also shown that immunoprecipitated ATM phosphorylates IκB-α in vitro (Jung et al., 1997). To investigate the signaling pathway for ionizing radiation-induced NF-κB activation, we have used AT fibroblasts as a model

system. We report here that ionizing radiation-induced signaling pathway for NF-κB activation is independent of that mediated by TNF-α treatment. Furthermore, our data shows that wild type ATM is involved in the ionizing radiation-induced NF-κB activation.

Results and Discussion

TNF-α-induced NF-κB activation

We first examined the integrity of the signaling pathway responsible for TNF-α-induced NF-κB activation in AT fibroblasts. Immunoblot analysis revealed TNF-α-induced phosphorylation of IκB-α within 5 min and its degradation within 15 min in AT (AT5BIVA) cells (Figure 1a). Similar results were obtained with control MRC5CV1 fibroblasts. Immunoblot analysis also showed that translocation of the p65 subunit of NF-κB to the nucleus was apparent within 30 min of exposure of AT or control fibroblasts to TNF-α (Figure 1b). It was also confirmed by using immuno-cytochemical analysis (data not shown). Electrophoretic mobility-shift assays (EMSAs) performed with nuclear extracts and a synthetic κB oligonucleotide probe demonstrated that TNF-α increased NF-κB binding activity in nuclear extracts of control and AT5BIVA fibroblasts within 30 min (Figure 1c); similar results were obtained with AT cells (AT3BIVA and AT4BIVA) derived from other patients. The specificity of binding activity was demonstrated by 'supershift' analysis with antibodies to p65 and p50 subunits of NF-κB. Phorbol ester (phorbol 12-myristate 13-acetate) and okadaic acid also each induced degradation of IκB-α in AT fibroblasts (data not shown). Thus, the signaling pathways responsible for NF-κB activation in response to TNF-α, phorbol ester, or okadaic acid appear intact in AT cells.

Ionizing radiation-induced NF-κB activation in AT

Next, we exposed control and AT cells to 20 Gy of ionizing radiation and measured the amount of IκB-α levels at various times during the subsequent 5 h. Degradation of IκB-α was apparent during the first 2 h in control cells, after which newly synthesized IκB-α protein began to appear (Figure 2a). In contrast, the amount of IκB-α remained constant during the 5 h post-radiation in AT cells, consistent with our previous observation that NF-κB is not activated in response to ionizing radiation in AT cells (Jung *et al.*, 1995). EMSA analysis revealed that ionizing radiation induced marked increase in NF-κB binding activity after 1 and 3 h in control cells, whereas AT cells showed a reduced extent of constitutive NF-κB binding activity that was not substantially increased after exposure to ionizing radiation (Figure 2b). The specificity of the NF-κB binding activity was confirmed by supershift analysis with antibodies to p65 or to the transcription factor E2F-1 (nonspecific control). Whereas ionizing radiation induced translocation of p65 to the nucleus within 1 h in control cells, no such translocation was evident in AT cells (Figure 2c). To measure the activity of NF-κB in intact cells, we transfected control and AT cells with a plasmid DNA

containing the chloramphenicol acetyltransferase (CAT) reporter gene under the control of the NF-κB-sensitive long terminal repeat of human immunodeficiency virus (HIV-LTR). Relative CAT activity in cell extracts was increased ∼14-fold 2 h after exposure of whole cells to ionizing radiation, whereas irradiation of AT cells had a negligible effect on CAT activity (Figure 2d). In addition, treatment of control cells with cycloheximide, an inhibitor of protein synthesis, before irradiation revealed that the half-life of IκB-α in such cells is ∼1 h (Figure 3a and b).
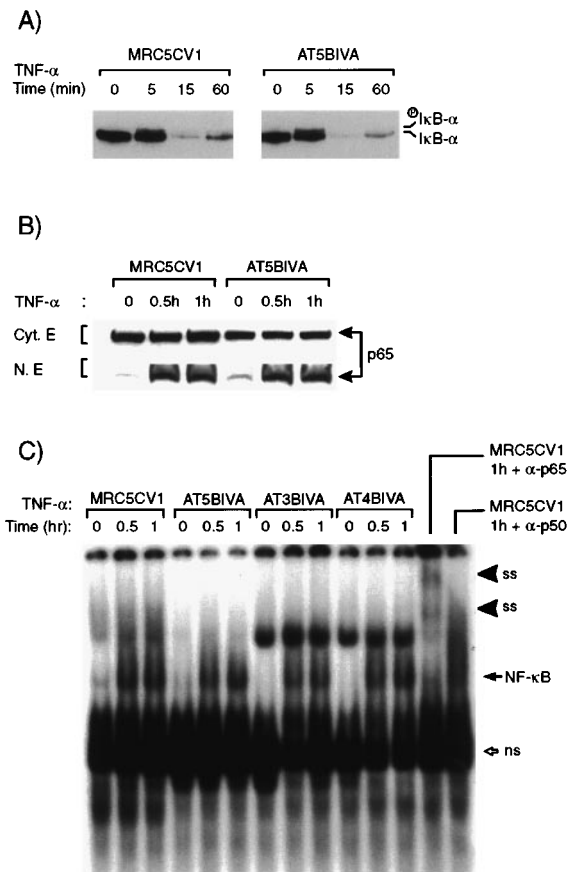

Figure 1 Effects of TNF-α on the phosphorylation and proteolysis of IκB-α (**a**) and nuclear translocation (**b**) and binding activity of NF-κB in normal human (MRC5CV1) and AT fibroblasts. (**a**) Control and AT (AT5BIVA) cells were incubated with TNF-α (10 ng/ml) for the indicated times (time-zero cells were not exposed to TNF-α), after which total cell extracts (20 μg of protein) were fractionated by SDS-polyacrylamide gel electrophoresis (PAGE) and the separated proteins were transferred to a nitrocellulose membrane and subjected to immunoblot analysis with antibodies to IκB-α (Santa Cruz Biotech.). The phosphorylated (^{P}) and unphosphorylated forms of IκB-α are indicated. (**b**) Control and AT cells were exposed to TNF-α for the indicated times, after which cytosolic (Cyt) and nuclear extract (NE) fractions were prepared and subjected to immunoblot analysis with antibodies to the p65 subunit of NF-κB. Control and AT (AT5BIVA, AT3BIVA, AT4BIVA) cells were treated with TNF-α for the indicated times, after which nuclear extracts were prepared and subjected to EMSAs with a ^{32}P-end-labeled b oligonucleotide probe (Santa Cruz Biotechnology). The positions of free probe (open arrow) and the NF-κB-probe complex (solid arrow) are indicated. The specificity of the complex formed with the extract of control cells treated with TNF-α for 60 min is indicated (arrowheads) by 'super-shifting' in the presence of antibodies to p65 or to p50 subunits of NF-κB (last two lanes, respectively)

Effects of tyrosine kinase and proteosome inhibitors on IκB-α proteolysis

Given that phosphorylation of Tyr42 of IκB-α is associated with a nondegradative mechanism of NF-κB activation (Imbert *et al.*, 1996), we investigated whether the apparent lack of IκB-α proteolysis in AT cells is attributable to this alternative mechanism of activation. Immunoprecipitates of IκB-α from irradiated cells were subjected to immunoblot analysis with antibodies to phosphotyrosine. Tyrosine phosphorylation of IκB-α was not detected in control cells during the 3 h period immediately after irradiation (data not shown). In addition, pretreatment of control cells with staurosporine (Imbert *et al.*, 1996) and genistein (data not shown), inhibitors of protein tyrosine kinases, did not inhibit IκB-α degradation induced by ionizing radiation; these inhibitors had no effect on IκB-α abundance in irradiated or nonirradiated AT cells (Figure 3c). Thus, phosphorylation of Tyr42 of IκB-α does not appear to contribute to ionizing radiation-induced signaling in control or AT fibroblasts. In contrast, pretreatment of control cells with the proteosome inhibitor IAT3170 prevented the degradation of IκB-α in response to ionizing radiation; the inhibitor had no effect on the amount of IκB-α in irradiated AT cells (Figure 3c).

Inhibition of ionizing radiation-induced IκB-α degradation by N-acetylcysteine

Because exposure of cells to ionizing radiation results in the generation of oxygen free radicals, we also examined the effects of antioxidants in control cells. N-acetylcysteine (NAC), which is a source of cysteine for replenishing intracellular glutathione and counteracts the effects of reactive oxygen intermediates, reduced the extent of ionizing radiation-induced IκB-α degradation, whereas the antioxidant pyrrolidine-dithiocarbamate (PDTC) (up to 200 μM) did not (Figure 3d). These data suggest that the ionizing radiation-induced reactive oxygen intermediates scavenged by NAC are preferentially associated with the ionizing radiation-mediated signaling pathway for degradation of IκB-α.

Phosphorylation of IκB-α by ATM immuno complex

Several mitogen- and cytokine-inducible protein kinases responsible for IκB-α degradation have been identified. These include casein kinase II, the 90-kD ribosomal S6 kinase (p90[rsk1]) and Ikk-1 and 2 (or α and β) (Barroga *et al.*, 1995; Baeuerle and Baltimore, 1996; Cao *et al.*, 1996; Malinin *et al.*, 1997; DiDonato *et al.*, 1997; Regnier *et al.*, 1997; Mercurio *et al.*, 1997;
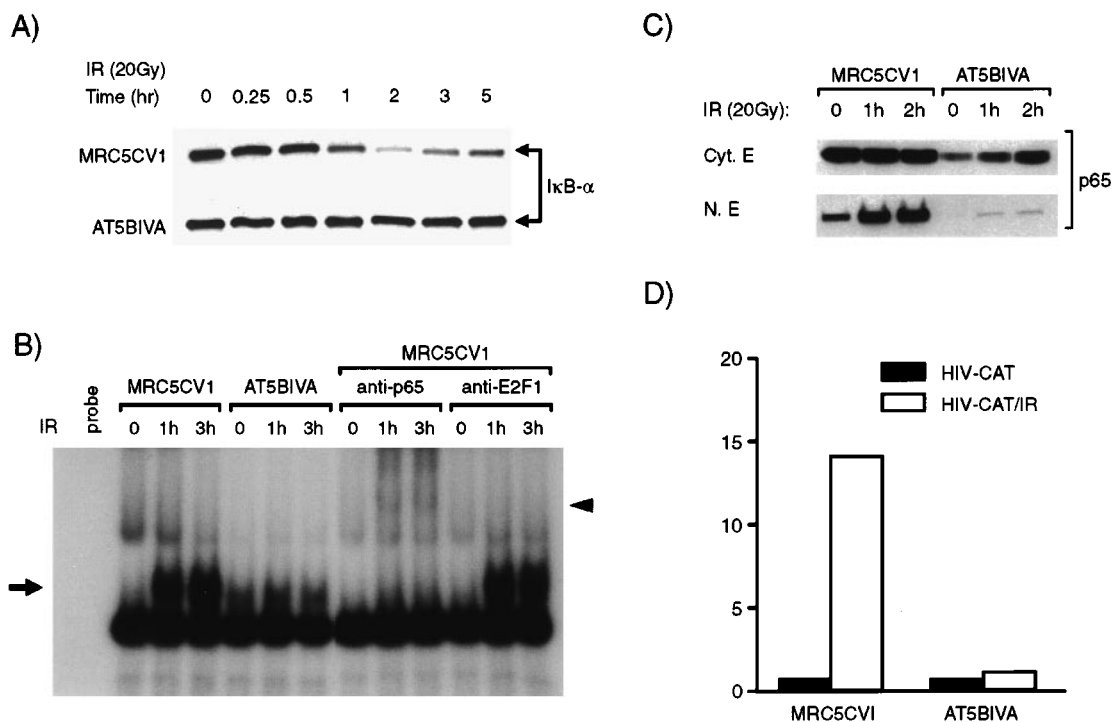

Figure 2 Effects of ionizing radiation on IκB-α degradation (**a**) and on the binding activity (**b**), nuclear translocation (**c**) and transactivation (**d**) of NF-κB in control and AT fibroblasts. (**a**) Cells were exposed to 20 Gy of γ-radiation and, at the indicated times after irradiation (time-zero cells were not exposed to ionizing radiation), total cell extracts were subjected to immunoblot analysis with antibodies to IκB-α as described in Figure 1a). (**b**) Nuclear extracts of cells were prepared at the indicated times after irradiation and subjected to EMSA analysis with a ^{32}P-labeled b oligonucleotide as in Figure 1c. A 'probe' lane contains no cell extracts. Supershift analysis was performed with the extracts from control cells and antibodies to p65 or to E2F-1. Arrow and arrowhead indicate specific NF-κB-probe complexes in the absence or presence of antibodies to p65, respectively. Nuclear extract and cytosolic fractions prepared from cells at the indicated times after irradiation were subjected to immunoblot analysis with antibodies to p65 as in Figure 1b). (**d**) Cells were transfected with an HIV-CAT reporter construct and, after 48 h, irradiated. CAT activity was determined in cell extracts prepared 0 or 2 h after irradiation. Data are expressed relative to the activity of each cell type at time 0 and are means of three separate experiments

Woroncz et al., 1997; Lee et al., 1997; Zandi et al., 1997). We have also previously shown that immuno-precipitated ATM functions as a protein kinase and is capable of phosphorylating IκB-α in vitro (Jung et al., 1997). Therefore, we determined whether ATM kinase activity is inducible by ionizing radiation with the use of an in vitro kinase assay consisting of immunopre-cipitated ATM protein from irradiated cells and a glutathione S-transferase (GST)-IκB-α fusion protein as the substrate (Figure 4a) (Jung et al., 1997). Exposure of control cells to ionizing radiation resulted, 30 min later, in an approximately threefold increase in the phosphorylation of GST-IκB-α mediated by immunoprecipitated ATM, whereas no such effect was apparent with AT cells (Figure 4a). These observations thus suggest that the defect in ionizing radiation-induced signaling through NF-κB is directly attributable to the functional status of the ATM (mutated in AT) gene product in AT cells.
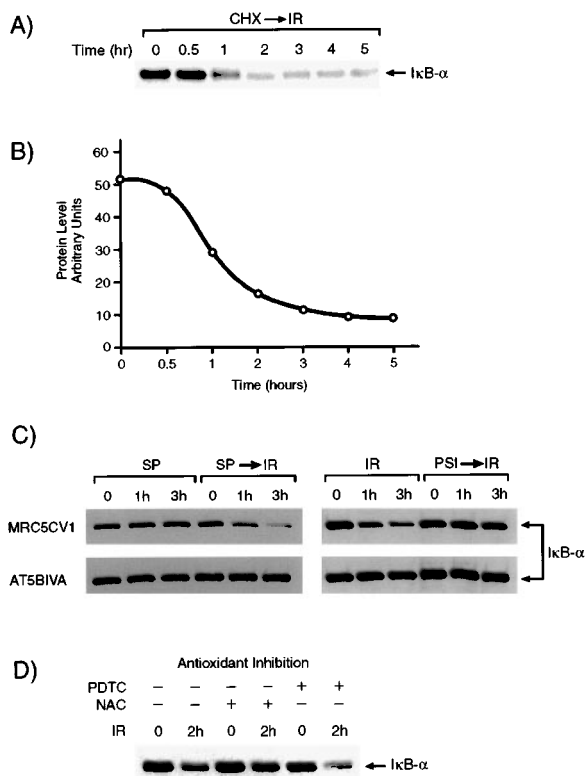

A)

CHX → IR

Time (hr) 0 0.5 1 2 3 4 5   ← IκB-α

B)

C)

SP   SP → IR   IR   PSI → IR
0 1h 3h 0 1h 3h   0 1h 3h 0 1h 3h

MRC5CV1

AT5BIVA   } IκB-α

D)

Antioxidant Inhibition
PDTC − − − − + +
NAC   − − + + − −
IR   0 2h 0 2h 0 2h   ← IκB-α

Figure 3 (a) Determination of half-life of IκB-α in irradiated control cells. Cells were pretreated with cycloheximide (10 μg/ml) for 30 min before exposure to 20 Gy of ionizing radiation. At the indicated times after irradiation, total cell extracts were prepared and subjected to immunoblot analysis with antibodies to IκB-α as described in Figure 1a. (b) Quantitation of the data as shown in (a) by scanning densitometry. Control or AT cells were exposed to ionizing radiation (IR) or sham radiation (SR) in the presence of 20 μM of staurosporine (SP), after which total cell extracts were prepared at the indicated times and subjected to immunoblot analysis with antibodies to IκB-α (left panel). Alternatively, cells were subjected to irradiation in the absence or presence of the proteosome inhibitor (PSI) IAT3170 (10 μM), with cell extracts prepared at the indicated times after irradiation and subjected to immunoblot analysis with antibodies to IκB-α (right panel). (d) Control cells were exposed to ionizing radiation in the absence or presence of the antioxidants PDTC (200 μM) or NAC (30 μM), after which cell extracts were prepared at the indicated times and subjected to immunoblot analysis for IκB = α

Expression of the full-length ATM cDNA in AT

We next transfected AT cells with a vector containing the full-length ATM cDNA under the control of a methallothionine-inducible promoter (pMAT-1) (Zhang et al., 1997). Exposure of these cells (AT5BIVA/pMAT-1) to ionizing radiation in the presence of CdCl₂ resulted in a pattern of IκB-α degradation similar to that observed in control cells (Figure 4b). An in vitro kinase assay was performed with GST-IκB-α as substrate and recombinant ATM im-munoprecipitated from AT5BIVA/pMAT-1 cells with antibodies to its histidine-tag. Exposure of the trans-fected cells to ionizing radiation resulted, 30 min later, in an increase in the phosphorylation of GST-IκB-α mediated by immunoprecipitated ATM (Figure 4c).

Recent reports have shown identification of Iκks that are cytokine-responsive IκB kinases and are mediated through TNFR-2 and NIK (Malinin et al., 1997; DiDonato et al., 1997; Regnier et al., 1997; Mercurio et al., 1997; Woroncz et al., 1997; Lee et al., 1997; Zandi et al., 1997). Whether Ikk kinases are directly involved in the phosphorylation of IκB-α or require additional proteins is not clear. Our results suggest an involvement of such factors as intermedi-ates, however, we report here that ATM is another important protein kinase associated with the ionizing radiation-induced NF-κB activation. Further investi-gations into ATM interactions with NIK and Iκk will be needed to resolve these questions.

Taken together, our data provide evidence for a previously uncharacterized ionizing radiation-induced signaling pathway that leads to NF-κB activation. This pathway is defective in AT cells and the defect is corrected by the expression of wild-type ATM. These observations support a model for ionizing radiation-induced signaling that include ATM-mediated phos-phorylation of IκB-α for NF-κB activation.

Materials and methods

Cell culture, plasmids and transfections

MRC5CV1 is a normal fibroblast cell line immortalized by using SV40 T-antigen, kindly provided by Dr CF Arlett. AT fibroblasts are derived from ataxia telangiectasia patients. Cells were maintained in modified Eagle's medium (MEM) supplemented with 10% (MRC5CV1) or 20% (AT cells) FBS. Cell growth was in 5% CO₂ at 37°C. Cells were determined to be free of mycoplasma infection by testing at 3-month intervals. Plasmid DNAS, pMAT-1, containing the full length ATM cDNA as described in detail in the previous report (Zhang et al., 1997), and pHIV-CAT, containing the long terminal repeat of HIV linked to the cDNA of chloramphenicol transferase (CAT), were introduced to cells by using Lipofectine (GIBCO), as recommended by the manufacturer. Stably transfected cells were generated as described (Jung et al., 1995).

Immunoblotting and immunoprecipitation

After treatment of cells with either TNF-α (10 ng/ml), γ-ray (20 Gy), PSI/IAT3170 (10 μM), SP (20 μM), cyclohex-imide (10 μg/ml), NAC (30 mM), PDTC (200 μM), for the indicated times, cells were collected and lysed with TNN buffer (40 mM Tris-Cl, 120 mM NaCl, 0.5% NP-40, 20 mM

NaF, 20 mM β-glycerophosphate, 500 μM Na₃VO₄, 2 μg/ml aprotinin, 2 μg/ml pepstatin, 2 μg/ml leupeptin, 1 mM PMSF, pH 7.6) on ice for 20 min. Total cell extracts (20 μg of protein) were fractionated by SDS–polyacrylamide gel electrophoresis (PAGE) and the separated proteins were transferred to a nitrocellulose membrane and subjected to immunoblot analysis with antibodies to IκB-α (Santa Cruz Biotech.). The protein products were visualized with enhanced chemiluminescence (Amersham). Equal loading was determined by using anti-actin. AT5BIVA cells stably transfected with pMAT-1 plasmid DNA were exposed to ionizing radiation in the presence or absence of 10 μM CdCl₂. For immunoprecipitation, the cell lysates were subjected to centrifugation at 14 500 r.p.m. for 20 min at 4°C and the supernatants were incubated for 2 h at 4°C with 1 μg of anti-NF-κB (p65) or IκB-α polyclonal

antibody. The immune complex was collected on protein A-Sepharose (Pharmacia) and washed five times with TNN lysis buffer prior to boiling in SDS sample buffer. Immunoprecipitated proteins were separated on SDS–polyacrylamide gels and transferred to nitrocellulose and immunoblotted with specific antibodies.

Immune complex kinase assay

Kinase activity was assayed in kinase buffer (20 mM Tris-Cl, 10 mM MgCl₂, 20 mM β-glycerophosphate, 100 μM Na₃VO₄, 2 mM DTT, 20 μM ATP, 10 μg/ml aprotinin, 2 μg/ml leupeptin, pH 7.5) with 3 μCi [γ-³²P]ATP at 37°C for 30 min. His-ATM immune complexes were prepared as described in immunoprecipitation and washed in kinase buffer before determining kinase activity (Jung *et al.*,
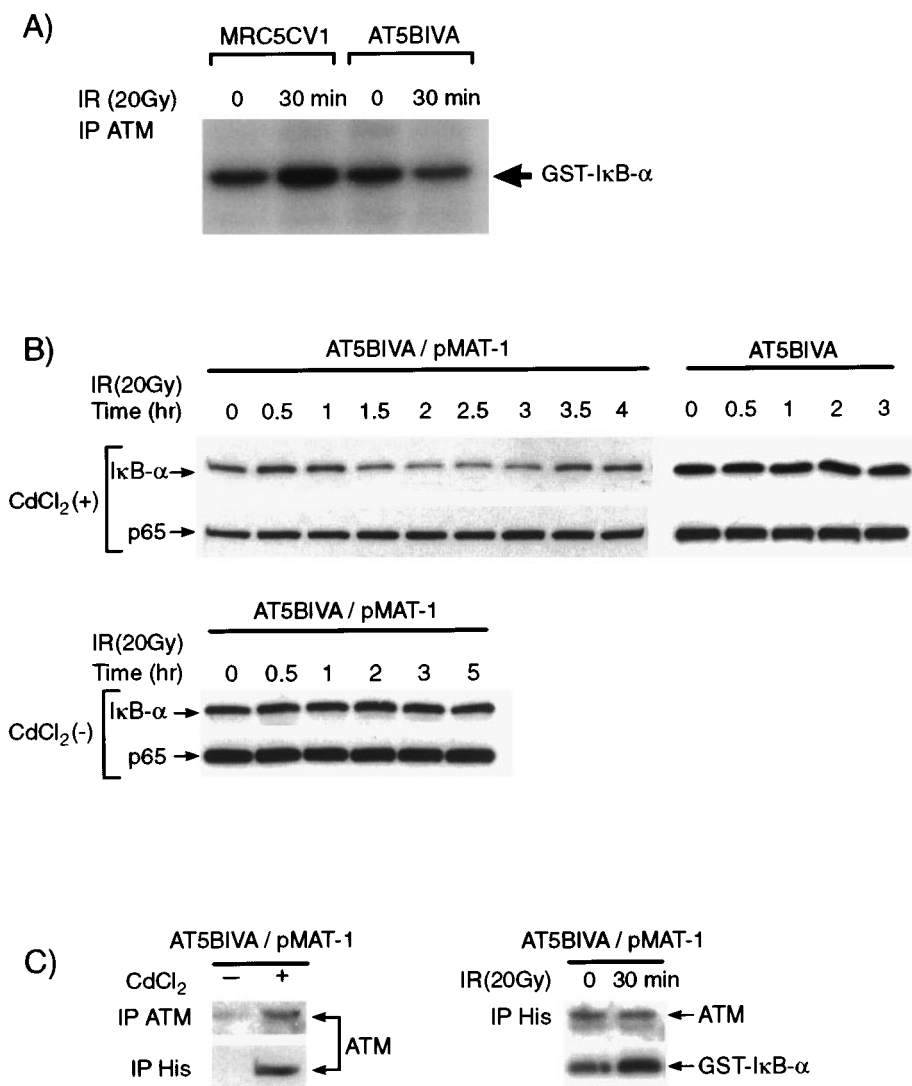

Figure 4 Effects of ionizing radiation on ATM kinase activity. (a) Control and AT cells were exposed to 20 Gy of ionizing radiation and, at the indicated times, cell extracts were subjected to immunoprecipitation with antibodies to ATM. The immunoprecipitates were then incubated for 30 min at 37°C with [γ-³²P]adenosine triphosphate and GST-IκB-α, after which the phosphorylation of IκB-α was analysed by SDS–PAGE and autoradiography. (b) AT5BIVA cells stably transfected (or not) with pMAT-1 plasmid DNA were exposed to ionizing radiation in the presence or absence of 10 μM CdCl₂, after which, at the indicated times, cell extracts were subjected to immunoblot analysis with antibodies to IκB-α or to p65. (c) AT5BIVA cells transfectd with pMAT-1 were incubated for 16 h in the absence or presence of 10 μM CdCl₂, after which cell extracts were subjected to immunoprecipitation (IP) with antibodies to ATM or to the His tag of recombinant ATM, and the immunoprecipitates were analysed by SDS–PAGE (left panel). Alternatively, the transfected cells were exposed to ionizing radiation and, at the indicated times, cell extracts were subjected to immunoprecipitation with antibodies to the His tag. The immunoprecipitates were then subjected either to immunoblot-analysis with antibodies to ATM (upper right panel) or to the *in vitro* kinase assay with GST-IκB-α (lower right panel)

1997). GST-IκB-α protein was used as a substrate as described (Jung *et al.*, 1997). Samples were analysed by 10% SDS – polyacrylamide gel and autoradiography.

NF-κB activity measurements

Nuclear extracts were prepared and subjected to electrophoric mobility shift assay (EMSA) as described (Jung *et al.*, 1995). Supershift analysis was performed with the extracts from control cells and antibodies to p65 or to E2F-1. For *in vivo* NF-κB function, reporter gene activity was measured after 48 h post-transfection with the CAT Assay System (Promega). Data are expressed relative to the activity of each cell type at time 0 and are means of three separate experiments.

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