ATM Is Required for IkB Kinase (IKK) Activation in Response to DNA Double Strand Breaks*

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Following challenge with proinflammatory stimuli or generation of DNA double strand breaks (DSBs), transcription factor NF-κB translocates from the cytoplasm to the nucleus to activate expression of target genes. In addition, NF-κB plays a key role in protecting cells from proapoptotic stimuli, including DSBs. Patients suffering from the genetic disorder ataxia-telangiectasia, caused by mutations in the ATM gene, are highly sensitive to inducers of DSBs, such as ionizing radiation. Similar hypersensitivity is displayed by cell lines derived from ataxia-telangiectasia patients or Atm knockout mice. The ATM protein, a member of the phosphatidylinositol 3-kinase (PI3K)-like family, is a multifunctional protein kinase whose activity is stimulated by DSBs. As both ATM and NF-κB deficiencies result in increased sensitivity to DSBs, we examined the role of ATM in NF-κB activation. We report that ATM is essential for NF-κB activation in response to DSBs but not proinflammatory stimuli, and this activity is mediated via the IκB kinase complex. DNA-dependent protein kinase, another member of the PI3K-like family, P38k itself, and c-Abl, a nuclear tyrosine kinase, are not required for this response.

The transcription factor NF-κB is activated by a broad array of stress-related signals, including proinflammatory cytokines and DNA damage (1–3). In most cells, NF-κB is sequestered in the cytoplasm in an inactive form because of tight association with its inhibitors, the IκBs. Activation of NF-κB is achieved through signal-induced phosphorylation of IκB at specific amino-terminal serine residues by the IκB kinase (IKK)1 complex. This phosphorylation triggers IκB degradation via the ubiquitin-proteasome pathway, resulting in NF-κB translocation into the nucleus (4). The IKK complex consists of two highly homologous kinase subunits (IKKα and IKKβ) and a nonenzymatic regulatory component, IKKγ/NEMO (5, 6). NF-κB plays a critical role in cellular protection against a variety of apoptotic stimuli, including DNA damage (7–11), and inhibition of NF-κB leads to radiosensitization (12). Acute radiosensitivity is a hallmark of the human genetic disorder ataxia-telangiectasia (A-T) (13). This pleiotropic disease is also characterized by progressive cerebellar degeneration, immunodeficiency, and extreme predisposition to lymphoreticular malignancies (14). The gene responsible for A-T, ATM, encodes a multifunctional serine/threonine protein kinase, which is a member of the phosphatidylinositol 3-kinase (PI3K)-like family of large proteins (see Refs. 15–17; reviewed in Ref. 18). Like A-T patients, Atm-null mice are extremely sensitive to ionizing radiation (IR) (19). Similar hypersensitivity to a variety of DSB-inducing agents (20, 21), including the topoisomerase inhibitors etoposide (22) and camptothecin (CPT) (23), is observed in cultured cells isolated from A-T patients or Atm-deficient mice.

ATM appears to mediate the response of NF-κB to DSBs, since NF-κB activation by IR and CPT is reduced or abolished in A-T cells (24, 25). The DNA-dependent protein kinase (DNA-PK), another member of the PI3K-like family of protein kinases, has also been implicated in the activation of NF-κB following exposure to IR (26). We analyzed the response of the NF-κB pathway to IR and a radiomimetic agent, neocarzinostatin (NCS), in ATM-deficient murine tissues and human cell lines. Our results clearly indicate that functional ATM is required for the activation of the NF-κB pathway by agents that induce DSBs but not by proinflammatory stimuli. We also show that downstream of ATM, the activation of NF-κB requires a functional IKK complex. Two other protein kinases involved in cellular responses to DNA damage, c-Abl and DNA-PK, are not required for the activation of NF-κB by IR. P38k itself is also not likely to be involved in this pathway.

EXPERIMENTAL PROCEDURES

Cell Culture and Cell Extracts—Human embryonic kidney 293 cells were maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum. Cell extracts were prepared as described previously (2). Human lymphoblasts were grown in RPMI with 10% fetal bovine serum and lysed in lysis buffer (10 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40) in the presence of protease and phosphatase inhibitors.

Mice and Tissue Extracts—Atm−/−, DNA-PKcs−/−, Ku70−/−, and Ku80−/− mice were described previously (27–30). Adult mice were
exposed to 20 Gy of ionizing radiation and injected with LPS (5 μg/g) or mock-treated. After 75 min mice were sacrificed, and organs were obtained. Protein extracts were isolated from liver or kidney in tissue extraction buffer (50 mM Heps, pH 7.5, 300 mM NaCl, 1% Triton X-100, 10% glycerol, 1 mM dithiothreitol) containing protease and phosphatase inhibitors.

**Electrophoretic Mobility Shift Assay (EMSA)**—EMSA was performed as described previously (2). Briefly, 10 μg of cell extract or 40 μg of tissue extract were incubated with 2 μg of poly(dI-dC) and 5,000–10,000 pm of 32P-labeled oligonucleotide probes. After 30 min at room temperature, the samples were analyzed on native 5% polyacrylamide gel. For supershift EMSA, protein extracts were incubated with 0.5 μg of anti-p65 NF-κB (C-20; Santa Cruz Biotechnology) or anti-p50 NF-κB (NLS; Santa Cruz Biotechnology) antibodies for 20 min, after which the labeled oligonucleotide was added, and incubation was continued for another 20 min. EMSA was also performed in the presence of an excess of unlabeled r oligonucleotide or AP-1 oligonucleotide to show the specificity of DNA binding.

**Kinase Assay**—Immune-complex IKK assays were performed with 250 μg of mouse tissue extracts that were immunoprecipitated with anti-IKKα antibody (M-280; Santa Cruz Biotechnology) and protein-A beads. IKK kinase assays from human cell cultures were carried out with 500 μg of whole cell extracts, using anti-IKKα antibody (B78–1; Pharmingen) and protein A/G-agarose beads. IKK kinase reactions were carried out as described (2). The presence of equal amounts of IKKα in the kinase reactions was verified by immunoblotting.

**Immunoblotting**—For the analysis of IκBα degradation, cell extracts were electrophoresed on 10% polyacrylamide gels and blotted onto polyvinylidene difluoride membranes. A rabbit polyclonal anti-IκBα antibody (M-280; Santa Cruz Biotechnology) and protein-A/G-agarose beads. IKK kinase reactions from human cell cultures were carried out as described (2). The presence of equal amounts of IKKα in the kinase reactions was verified by immunoblotting.

**RESULTS**

To examine the involvement of PI3K-like kinases in activation of NF-κB in response to IR, we first tested the effect of the microbial metabolite wortmannin. At low nanomolar concentrations, wortmannin inhibits the lipid kinase activity of PI3K. At higher concentrations, this drug also inhibits PI3K-like protein kinases, including ATM and DNA-PK (31). Pretreatment of human embryonic kidney 293 cells with wortmannin at concentrations higher than 20 μM inhibited IR-induced NF-κB DNA binding, IκBα degradation, and IKK activation (Fig. 1). It had no effect, however, on tumor necrosis factor (TNF)-induced NF-κB or IKK activation. Similar results were obtained with HeLa cells (data not shown). It should be noted that at 2 μM, a concentration fully sufficient for inhibition of PI3K activity, wortmannin had no effect on the activation of NF-κB by IR. Thus, one or more members of the PI3K-like protein family, but not PI3K itself, are involved in IR-induced NF-κB activation.

We further investigated the involvement of ATM in the NF-κB response to IR using wild type (wt) and Atm−/− mice. Intraperitoneal injection of LPS activated NF-κB DNA binding activity in the liver of both wt and Atm−/− mice. Exposure to IR, however, induced NF-κB DNA binding activity in wt but not in Atm−/− mice (Fig. 2A). Kidney extracts yielded similar results (Fig. 2B). IR-elicted NF-κB binding activity contain p65 and p50 NF-κB proteins in the liver and p50 in the kidney, as revealed by antibody supershift experiments. These results indicate that in vivo, ATM is required for the activation of NF-κB in response to IR but not for induction of this pathway by LPS. To determine whether ATM is also required for IR-induced IKK activation, IKK kinase activity was analyzed in liver protein extracts derived from wt and Atm−/− mice. LPS treatment resulted in activation of IKK in both types of mice, whereas IR lead to activation of IKK in wt but not Atm−/− mice (Fig. 2C). These observations clearly demonstrate that in vivo, ATM is required for IR-induced activation of the entire IKK-NF-κB pathway.

Similar studies were carried out in mice deficient for each of the three components of the DNA-PK holoenzyme: the catalytic subunit (DNA-PKcs), and the DNA-binding regulatory subunit, the Ku heterodimer (Ku70/Ku80). Unlike Atm−/− mice, the induction of NF-κB DNA binding activity in response to either IR or LPS treatment was intact in livers and kidneys of DNA-PKcs−/−, Ku70−/−, and Ku80−/− mice (Fig. 3). Normal NF-κB induction in response to IR was also detected in c-Abl-null mice (Fig. 4). These results indicate that DNA-PK and c-Abl are not required for the IR-induced activation of the NF-κB pathway in vivo.

The role of ATM in IKK activation and IκBα degradation was also studied in human lymphoblasts. The responses to IR and to the radiomimetic drug NCS were defective in A-T lymphoblasts (Fig. 5, A and B), whereas the response to phorbol 12-myristate 13-acetate (PMA) was intact in these cells (Fig. 5C). These results further support the above observations, indicat-
ing that a functional ATM is required for the induction of IKK by DSBs. It is noteworthy that the DSB-induced activation of IKK in A-T lymphoblasts appears reduced and delayed but not completely abolished. This pattern is reminiscent of other signaling pathways mediated by ATM and reflects the involvement of other kinases, possibly ATR, in the late response to DSBs (reviewed in Ref. 18).

**Fig. 2.** ATM is required for IR-induced NF-κB and IKK activation. Wild type (+/+), and Atm-null (−/−) mice were left untreated (Un), exposed to 20 Gy of IR, or injected with LPS (5 μg/g). Protein extracts were isolated 75 min later from liver (A) or kidney (B) and used in EMSA to measure NF-κB DNA binding activity. Equal loading is demonstrated by NF-1 binding activity. EMSA was also performed after incubation of protein extracts with anti-p65 or anti-p50 antibodies to examine the protein composition of NF-κB, or in the presence of an excess of unlabeled AP-1 or κB oligo to demonstrate the specificity of DNA binding. Because NF-1 activity was not detectable in kidney extracts, equal loading was examined by immunoblotting with anti-c-Jun NH2-terminal kinase 1 antibody (JNK1).

**Fig. 3.** DNA-PK is not required for IR-induced NF-κB activation. Wild type (+/+), DNA-PKcs−/− (PK−/−), Ku70−/−, and Ku80−/− mice were treated with IR or LPS as described for Fig. 2. Protein extracts were isolated from liver (A) or kidney (B) and used in EMSA to measure NF-κB DNA binding activity. Un, untreated; JNK1, c-Jun NH2-terminal kinase 1.

**Fig. 4.** c-Abl is not required for IR-induced NF-κB activation. Wild type (+/+), and c-Abl−/− (−/−) mice were exposed to IR as described for Fig. 2. Protein extracts were isolated from liver or kidney and used in EMSA to measure NF-κB DNA binding activity (upper panel). Equal protein loading was examined by immunoblotting with anti-c-Jun NH2-terminal kinase 1 antibody (lower panel).
FIG. 5. ATM is required for IR- and NCS-induced IκBα degradation and IKK activation in human lymphoblasts. Protein extracts were obtained from A-T and control lymphoblasts at different time points after exposure to 20 Gy of IR (A), 250 ng/ml NCS (B), or 10 ng/ml PMA (C). Degradation of IκBα was assessed by immunoblotting (top panels). Tubulin served as a loading control. IKK kinase activity was measured by immunocomplex kinase assay using glutathione S-transferase-IκBα (GST-IκBα) as a substrate (bottom panels).
of IKK were completely abolished in the IKKγ/NEMO-deficient cells in response to either NCS (Fig. 6A) or PMA (Fig. 6B). These results indicate that IKKγ/NEMO is essential for activation of this pathway also in response to DSBs.

**DISCUSSION**

Our results clearly demonstrate that ATM is essential for activation of the entire NF-κB pathway by DSBs in both cultured human cells and mouse tissues, including IKK activation, IκBα degradation, and induction of NF-κB DNA binding activity. ATM is not required for activation of this pathway by proinflammatory stimuli, such as TNF, PMA, or LPS.

The activity of c-Abl, a nuclear tyrosine kinase, is stimulated by IR in an ATM-dependent manner (33, 34). In addition, ATM interacts with c-Abl and phosphorylates it 

ATM was suggested to directly phosphorylate IκBα itself in vitro on its carboxyl terminus (38). However, the physiological significance of this observation is not clear. In vitro phosphorylation by DNA-PK on residues in the carboxyl terminus of IκBα enhanced its ability to associate with NF-κB and to inhibit NF-κB DNA binding properties (39), suggesting a role for DNA-PK in down-regulation of NF-κB and termination of the NF-κB response. Direct phosphorylation of IκBα by ATM, if occurring in vivo, might play a similar role.

The vast majority of the signals that induce activation of the latent NF-κB complex originate at ligand-receptor interactions on the cell membrane and require IKK activation. However, the activation of NF-κB by two types of DNA-damaging agents, short wavelength UV and IR, was found to proceed through distinct mechanisms (2). NF-κB activation in response to UV radiation is neither mediated through damage to nuclear DNA (40), nor does it depend on IKK activation or amino-terminal IκBα phosphorylation, although it still involves proteasome-mediated IκBα degradation (2, 41). Unlike UV radiation, exposure to IR leads to activation of IKK and subsequent induction of phosphorylation-dependent IκBα degradation (2). A recent study shows that activation of NF-κB in response to DNA-damaging agents versus proinflammatory stimuli (see Refs. 2 and 24 and Fig. 5).

The mechanism by which DSBs activate NF-κB is still not entirely clear. The data presented here point to a critical role...
for ATM in DSB-induced NF-κB activation. The involvement of the IKK complex in this response is further supported by our observation that mutant cells lacking IKKγ/NEMO are unable to activate this pathway in response to NCS. ATM is primarily a nuclear protein that plays a crucial role in the rapid and efficient induction of multiple signaling pathways in response to DSBs, leading to repair of DNA damage and activation of cell cycle checkpoints and cellular stress responses (18). Its involvement in the response of NF-κB to DSBs suggests that the initial nuclear signaling events in this pathway are triggered by ATM. It is unclear at this point how this signal then translocates to the cytoplasm to activate the IKK complex.

Impaired activation of NF-κB leads to enhanced apoptosis in response to IR (10, 12, 43) and other agents that induce DSBs, such as CPT (42). It is still not clear how the absence of ATM leads to the hypersensitivity to DSBs exhibited by A-T cells. It appears that defective activation of cell cycle checkpoints does not necessarily contribute to this aspect of the A-T phenotype. It has been suggested that a subtle defect in DSB repair may underlie this increased sensitivity (44). However, it is also conceivable that a defect in the induction of anti-apoptotic genes responsive to NF-κB contributes to enhanced cell death of A-T cells in response to DSBs.

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