**ATM Gene Product Phosphorylates IκB-α**

**Mira Jung,** Alexei Kondratyev, Sung A. Lee, Alexandre Dimtchev, and Anatoly Dritschilo

Division of Radiation Research, Departments of Radiation Medicine and Microbiology, Vincent T. Lombardi Cancer Center, Georgetown University Medical Center, 9700 Reservoir Road NW, Washington, DC 20007.

**Abstract**

The recently cloned ATM gene is mutated in patients with ataxia telangiectasia, but its biological functions remain to be experimentally determined. Structural analysis has revealed ATM sequence similarities to the catalytic domains of phosphatidylinositol 3-kinase and other members of this family of yeast and mammalian proteins. Rabbit polyclonal antibodies raised against polypeptide regions unique to the COOH terminus and to the NH2 terminus of the published ATM sequence confirm ATM as M, ~350,000 protein in normal cells, which is missing in AT cells. Immunoprecipitated protein(s) is capable of phosphorylating IκB-α in an in vitro kinase assay. However, we did not observe a phosphatidylinositol 3-kinase or a DNA-dependent protein kinase function by ATM immunoprecipitates. These data support a protein kinase activity for ATM and suggest a role in NF-κB activation.

**Introduction**

ATM is a human autosomal recessive disease that is characterized by a variety of clinical and cellular defects, including cerebellar degeneration, immune deficiency, and extreme radiation sensitivity (1). Recent cloning of the ATM gene (mutated in AT) shows that it spans size from 4.5 to 12 kb containing an open reading frame of 9168 bp encoding a M, 350,000 protein (2, 3). Multiple mutations have been identified that result in ATM protein truncations (2, 3). However, the protein product and functions of ATM have yet to be reported.

It has been speculated that ATM plays a role in signal transduction. Analysis of the sequence has revealed homology to a family of yeast and mammalian proteins that include PI-3 kinase, DNA-PK, and protein kinase (2). A mouse knockout model has been reported, confirming deficiencies in neurological function, T lymphocyte maturation, and G1 checkpoints, as well as displaying extreme sensitivity to γ-irradiation (4). Expression of recombinant full-length ATM, which would allow for direct determination of protein functions, has proven difficult, and to date, there is no report of success.

In this paper, we describe the characterization of highly specific antibodies against ATM. We have used these antibodies to immunoprecipitate proteins for examination in in vitro kinase assays to determine the possible biochemical functions of ATM.

**Materials and Methods**

**Cell Lines.** MRC5CV1 is a normal fibroblast cell line immortalized by using SV40 T-antigen. AT29RM is derived from a patient, an AT homozygote, expressing a truncated ATM gene product. MRC5CV1 and AT29RM cells were kindly provided by Drs. C. F. Arlett (University of Sussex, Falmer, Brighton, UK) and Yossi Shiloh (Tel Aviv University, Ramat Aviv, Israel), respectively. Cells were maintained in modified Eagle’s medium for MRC5CV1 and in RPMI 1640 for AT29RM, supplemented with 10% or 20% fetal bovine serum. Cell growth was in 5% CO2 at 37°C. Cells were determined to be free of Mycoplasma infection by testing at 3-month intervals.

**Antibody Generation and Western Analysis.** The anti-ATMC and anti-ATMN antibodies were raised in rabbits against a polypeptide either from the COOH terminus (amino acids 2991–3005; α-ATMC) or from the NH2 terminus of ATM (amino acids 35–55; α-ATMN), respectively. Antibodies were purified by using peptide-coupled affinity columns. Total cell extracts were prepared and denatured by boiling for 5 min in SDS-polyacrylamide sample buffer. One hundred fifty μg of total cell extract were electrophoresed on SDS/5% PAGE and electrotransferred to Immobilon-P membrane (Millipore, Bedford, MA). The membrane was then incubated with 5% nonfat dry milk in PBS for 1 h at room temperature and incubated overnight at 4°C with α-ATMC or α-ATMN (1 μg/ml). The protein products were visualized with enhanced chemiluminescence (Amersham). α-DNA-PK (1 μg/ml) was used to determine a high molecular mass of protein in addition to pretranslated molecular markers.

**PI-3 Kinase Assay.** MRC5CV1 cells were treated with 50 ng/ml of PDGF(A/B) (Boehringer Mannheim) for 10 min after pretreatment with or without 100 μM Wortmannin (Sigma) for 30 min. Cell extracts were then precipitated with α-p85 (Transduction Lab) or α-ATMC. PI-3 kinase assays were performed as described by Whitman et al. (5). The phosphorylation cocktail was performed at 25°C for 80 min in 50 μl of reaction solution containing 50 μM ATP, 10 mM MgCl2, 5 μCi [γ-32P]ATP (DuPont New England Nuclear, Boston, MA), and 1 μg of λ-phosphatidylinositol phosphatase (Fika, Buchs, Switzerland). Phospholipids were resolved on oxidized Silica gel 60 TLC plates (Merek), followed by autoradiography.

**GST-fusion Protein Constructions.** The GST fusion genes were generated using PCR and were fused, in frame, between EcoRI and NotI restriction sites of the pGEX-4T-1 plasmid vector (Pharmacia). These constructs contain the COOH terminus (amino acids 198–302; GST-1κB-a-C) of 1κB-α and the NH2 terminus (amino acids 1–68) of p53. The constructs were confirmed by sequencing. The plasmids were transformed into E. coli strain BL21 cells (Stratagene) for protein expression.

**DNA-dependent PK Assay.** α-ATMC or α-DNA-PK immunoprecipitates from MRC5CV1, obtained 15 min after irradiation (5 Gy) were resuspended in kinase buffer (10 mM Tris (pH 7.4), 100 mM NaCl, and 1 mM EDTA) containing either 100 ng of SPI (Promega) or 500 ng of GST-p53 protein and 5 μCi of [γ-32P]ATP (3000 Ci/mmol). The reaction was performed in the absence or presence of linear double-stranded DNA. Purified DNA-PK complex (Promega) was used as a control. The samples were then analyzed by electrophoresis on 4–15% gradient SDS-polyacrylamide gel, followed by autoradiography.

**In Vitro Protein Kinase Assay.** Cells were lysed in ATM kinase buffer, as described elsewhere, containing 1% 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate and 1% phenylmethylsulfonyl fluoride. Immunoprecipitation was carried out for 4 h at 4°C using α-ATMC (1 μg/ml). Polypeptides (1 μg) used in generating each of the antibodies were added to extracts before kinase assays. Immunoprecipitates were washed three times with lysis buffer and twice with the ATM kinase buffer (20 mM HEPES-NaOH, 10 mM MgCl2, and 10 mM MnCl2 (pH 7.4)). The reaction was performed for 15 min at 37°C in the presence of 2.5 μCi of [γ-32P]labeled ATP, 50 μM ATP, and 1 μg of each substrate. The samples were analyzed on 10–20% SDS-PAGE, dried, and autoradiographed.
Results and Discussion

Characterization of Antibodies against ATM. The ATM gene has been identified and predicted as a Mr 350,000 protein. However, the ATM gene product has not been characterized, albeit predicted as a PI-3 kinase-like or a cell cycle regulatory factor based on the structural similarities (2). To determine the biochemical function of ATM, rabbit polyclonal antibodies were developed against polypeptides selected from the published sequence of the COOH terminus (α-ATMC) and to the NH₂ terminus (α-ATMN) of ATM. These specific sequences show no homology to known mammalian proteins searched through the GenBank data base. Western analyses were performed using cell extracts from “normal” human fibroblasts (MRC5CV1) and AT29RM, derived from a patient, an AT homozygote (6). AT29RM contains a deletion of 175-nucleotides at 4437 (amino acid 1488), resulting in a truncated gene product. To circumvent the ambiguity of the location of such a large molecular weight protein, we first determined the location of a large protein by using a SDS/5% polyacrylamide gel and probing with an antibody against DNA-PK, the molecular weight of which has been reported as Mr ~460,000 (7). The same membrane was then probed with α-ATMC. As shown in Fig. 1, both antibodies, α-ATMC and α-ATMN, recognized a specific protein, Mr ~350,000, only in MRC5CV1. However, α-ATMN recognizes a Mr ~180,000 protein in AT29RM. This observation is consistent with the report that a deletion of ATM was present in AT29RM, resulting in a truncated ATM. It is interesting that we have consistently observed double bands of ATM in MRC5CV1. It is possible that more than one ATM gene product is present in these cells, possibly through altered splicing. Furthermore, we have observed that the expression level of DNA-PK is much lower in AT cells compared with that of normal cells.

Functional Analyses of ATM Gene Product. Previously noted sequence homologies with known proteins have been used to place ATM into a family of genes with 60–70% similarity in the PI-3 kinase domain. This suggests a role in signal transduction as a phospholipid protein kinase (2). Phosphorylated phosphatidylinositol phospholipids serve as intracellular second messengers in signaling pathways that are stimulated by PDGF and are inhibited by Wortmannin (5, 8, 9).

Cellular extracts prepared from PDGF-treated cells with or without pretreatment by Wortmannin were precipitated with antibody against the p85 subunit of PI-3 kinase. As shown in Fig. 2A, the PI-3 kinase activity was induced by PDGF(A/B), and this activation was inhibited by Wortmannin. However, immunoprecipitates using α-ATMC did not phosphorylate PIs. Furthermore, testing other phospholipids (PI-4-P and PI-4,5-P) as substrates (Fig. 2B) confirmed that ATM protein does not phosphorylate any of these phospholipids.
ATM Functions as a Protein Kinase. Protein phosphorylation is a general mechanism for the regulation of intracellular events in response to stimuli. Can the ATM gene product function as a protein kinase capable of phosphorylating proteins important in signal transduction? We have reported previously that regulation of transcriptional activation of NF-κB is aberrant in AT fibroblasts (14). NF-κB is maintained in the cellular cytoplasm in a complex with IkB-α, a protein inhibitor of this transcription factor (15, 16). IkB-α masks the nuclear localization signal of NF-κB, but in response to stimuli, such as cytokines, ionizing radiation, and reactive oxygen intermediates, IkB-α becomes phosphorylated and undergoes proteolysis (17–21). The unbound NF-κB then translocates to the nucleus and activates transcription of various genes (22).

The COOH terminus of IkB-α has shown to be constitutively phosphorylated, whereas the NH₂ terminus contains signal-induced phosphorylation sites (18, 20). To determine whether ATM functions as a protein kinase, we constructed and used a fusion protein between GST and the COOH terminus of IkB-α (amino acids 198–302; GST-IkBα-C) as a substrate for phosphorylation by immunoprecipitated ATM. GST-IkBα-C was phosphorylated with immunoprecipitates from MRC5CVI cell extract (Fig. 4) by using either α-ATMC or α-ATMN antibodies. Similar data were obtained by using the GST-IkBα intact (data not shown). The phosphorylation signals were significantly decreased when polypeptides, which were initially used for immunizing rabbits for antibody generation, were added to the cell extracts. When α-ATMC immunoprecipitate was used, the phosphorylated signal was also observed in extracts from AT29RM cells, but

ATM also shows strong functional homology to DNA-PK (2, 3). This protein participates in the cellular responses to DNA double-strand breaks as a complex consisting of Ku70, Ku80, and DNA-PKcs (10). Ku proteins bind to the DNA breaks and activates DNA-PKcs, phosphorylating a variety of proteins in vitro, including p53 (10). This predicted function for ATM is particularly associated with the observed radiation sensitivities of mammalian mutants lacking components of DNA-PK. Both XRS-5 mutant rodent cells, which lack Ku80 protein, and severe combined immunodeficiency disease mice, which lack DNA-PKcs function, are extremely sensitive to killing by ionizing radiation (11–13). However, recent studies have shown that DNA-PK activities are normal in AT cells (13).

To test whether ATM is DNA-PK-like, we used GST-p53 and SP1 as substrates for phosphorylation and cell extracts from MRC5CV1 15 min after irradiation (5 Gy). As shown in Fig. 3, immunoprecipitated ATM protein did not phosphorylate the test substrates in the presence or absence of double-stranded DNA fragments, whereas purified DNA-PK did (Lane 2 in a and b; Ref. 7).
at a lower intensity, although the same concentrations of substrate were added to each reaction. However, the phosphorylation of IκBα was not observed when α-ATMN was used with cell extracts from AT29RM, which contains a truncated ATM. Therefore, we cannot rule out the possibility that α-ATMC antibodies may also precipitate another protein kinase. It is known that Casein kinase II phosphorylates IκBα at the COOH terminus, but probe with antibodies to Casein kinase II confirmed its absence (data not shown; Ref. 21). These data support protein kinase activity and substrate specificity for the ATM gene products using the two different antibodies. Furthermore, these antibodies were found to be useful in immunoprecipitation, which permitted the systematic analysis of the postulated functions of the ATM gene product(s).

In summary, we have shown that antibodies specific to the NH2 and COOH termini of ATM recognized Mf ~350,000 protein, which is not present in AT cells. The in vitro kinase analyses using immunoprecipitated protein show that there are no PI-3 kinase or DNA-PK activities. However, protein kinase activity is present in cell extracts from human normal fibroblasts. IκB-α serves as a substrate for phosphorylation by immunoprecipitated protein, whereas p53 and SP1 do not. Therefore, we interpret these data to support protein kinase activity for ATM, with specificity for IκB-α. This is consistent with a signal transduction model (Fig. 5) that places ATM upstream of NF-κB/IκB-α in a signaling cascade.

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References

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