The Product of the ATM Gene Is a 370-kDa Nuclear Phosphoprotein

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Neuronal degeneration, gonadal abnormalities, and immune deficiency are some of the major manifestations of the hereditary disease ataxia telangiectasia, which is caused by mutations in a single gene, designated ATM. Here we show that the product of the ATM gene is a 370-kDa nuclear phosphoprotein. Because ATM knockout mice recapitulate the clinical symptoms of the human disease, we have examined ATM gene expression in mice. In mouse embryos at gestation day 13.5, ATM mRNA is expressed ubiquitously, with high levels detected in the nervous system and lung. Elevated ATM mRNA levels were also found in the thymus of mouse embryos at gestation day 18.5, a time when V(D)J recombination is occurring. In adult mice, ATM protein was detected in all tissues examined and was present at elevated levels in the testis, spleen, and thymus. The ATM expression pattern and the nuclear localization of the ATM protein are consistent with the proposed function of ATM in the activation of cell cycle checkpoints, DNA repair, and genetic recombination.

Ataxia telangiectasia (AT)1 is a rare autosomal recessive disease that affects multiple organs (1). Clinical manifestations of AT include neurological disorders, cancer predisposition, humoral and cellular immune deficiencies, thymic atrophy, abnormal gonadal development, diabetes, and dilated blood vessels (telangiectases). At the microscopic level, individuals with AT have neuronal degeneration in the cerebellum, the brain stem, dorsal root ganglia, spinal cord, and peripheral nerves, as well as reduced numbers of germ cells and mature lymphocytes (2–4). The tumors that occur most frequently in individuals with AT are leukemia and lymphomas of T cell origin, and to a lesser extent, of B cell origin (5). It is estimated that 0.5–1% of the general population may be heterozygotes for a mutation of the AT gene (6). These people may be at increased risk of cancer, including cancer of the breast (6, 7).

Cell lines established from patients with AT exhibit higher rates of apoptosis after exposure to x-rays or radiomimetic drugs (8, 9). Cells carrying one mutant AT gene allele are also radiosensitive, albeit not as much as cells carrying two mutant AT gene alleles (9). Unlike normal cells, AT cells do not temporarily halt cell cycle progression after ionizing irradiation (8, 10), indicating a defect in DNA damage-activated checkpoints. Because AT cells exhibit a delay in the induction of p53 and p21 after ionizing radiation (11–13), the ATM gene product may use the p53 pathway as one of its effectors. In addition, AT cells may have a defect in DNA repair that is unrelated to cell cycle checkpoint abnormalities (14). Taken together, these studies indicate that the product of the AT gene may be involved in cell cycle checkpoints and DNA repair. Aberrant cell cycle progression after DNA damage and lack of DNA repair may contribute to the genomic instability and increased sensitivity of AT cells to irradiation.

The gene responsible for AT, ATM (mutated in AT), was mapped to chromosome 11q22–23 (15) and was later cloned (16, 17). The open reading frame of ATM encodes a protein of 3056 amino acids with a calculated molecular weight of about 350,000. Sequence homology searches indicate that ATM falls into a class of proteins that are related to the catalytic subunit of phosphatidylinositol (PI) 3-kinase (18). This group of proteins includes TEL1, MEC1, TOR1, and TOR2 of the budding yeast Saccharomyces cerevisiae, RAD3 of the fission yeast Sacccharomyces pombe, MEI-41 of Drosophila melanogaster, and in humans the catalytic subunit of the DNA-dependent protein kinase (DNA-PKcs) and FRAP. A region of ATM adjacent to the putative kinase domain encompassing approximately 1,000 amino acids also shares significant homology to MEI-41, MEC1, TEL1, RAD3, and DNA-PKcs. These sequence homologies appear to reflect functional homology because many of the PI 3-kinase-related proteins are, like the ATM gene product, involved in DNA repair, cell cycle control, and recombination.

Mice carrying germline mutations of ATM have been created by homologous recombination (19). The ATM knockout mice consistently score low in neurological tests, have reduced numbers of functional T cells, and are infertile due to abnormalities in germ cell maturation. Because the ATM knockout mice exhibit many of the symptoms that are characteristic of the AT disease in human, mice provide a good model to study the function of ATM and the pathology of AT. In this study, we have identified the ATM protein and examined ATM expression in mouse tissues. We show that ATM is a nuclear, 370-kDa phosphoprotein that is widely expressed in adult mouse tissues.

**EXPERIMENTAL PROCEDURES**

**Antibodies and Cell Lines—**Antibodies against DNA-PKcs (20) were kindly provided by Tom Shenk (Princeton University). The antibody against β-actin was from Sigma. The anti-RbAp48 antibody was described earlier (21). Cell lines GM02184, GM03189, GM0332, and GM03395 were obtained from Human Genetic Mutant Cell Repository (Camden, NJ). All other cell lines used in this study were obtained from American Type Culture Collection (Rockville, MD). All cells were grown as suggested by the providers.

Production of Anti-ATM Antibodies—Reverse transcription-polymerase chain reaction was performed to obtain two cDNAs encompassing human ATM cDNA sequences 2940–4536 and 7731–9168, respectively. After confirmation of their identity with ATM sequence (17) by DNA

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1The abbreviations used are: AT, ataxia telangiectasia; PI, phosphatidylinositol; DNA-PKcs, DNA-dependent protein kinase catalytic subunit; GST, glutathione S-transferase; DAPI, 4',6-diamidino-2-phenylindole.
sequencing, the two cDNA were fused in-frame with the sequence encoding glutathione S-transferase (GST). The fusion proteins were overexpressed in *Escherichia coli* and purified by affinity chromatography. Mouse polyclonal antisera specific for the fusion proteins and hryidoma cell lines were generated according to standard procedures (22).

**ATM Protein Analysis**—Cells or tissues were resuspended in ice-cold EBC buffer (0.5% Nonidet P-40, 50 mM Tris, pH 7.6, 120 mM NaCl, 1 mM EDTA, and 1 mM β-mercaptoethanol) containing 1 mM of phenylmethanesulfonyl fluoride, aprotinin, leupeptin, Na_3_VO_4_, and 50 mM of NaF, and lysed by sonication. The lysates were cleared of insoluble materials by centrifugation at 12,000 × g for 10 min at 4 °C. Protein concentrations were determined by the method of Bradford (23). Unless specified otherwise, immunoprecipitations, including all washes, were performed with BCA buffer as described previously (24). In immunoblot analysis of ATM, proteins were separated by electrophoresis using 5% denaturing polyacrylamide gels and immune complexes in the blot were detected by enhanced chemiluminescence (Amersham Corp.). Procedures for subcellular fractionation were performed as described previously (24).

In immunostaining experiments, cells were fixed with neutral 4% paraformaldehyde at room temperature for 10 min. After washing with phosphate-buffered saline, the cells were permeabilized with 0.3% Triton in TBST (50 mM Tris, pH 7.6, 150 mM NaCl, and 0.05% Tween 20) for 10 min. The cells were then blocked with 4% milk in TBST for 2 h and subsequently incubated with anti-ATM antibodies and an fluorescent isothiocyanate-conjugated anti-mouse antibody. Nuclear staining for 4',6-diamidino-2-phenylindole (DAPI) was performed by including DAPI (0.5 μg/ml) in the secondary antibody.

To metabolically label cells with [32P]orthophosphoric acid for the analysis of ATM phosphorylation, 1 × 10⁷ cells in log growing phase were washed once with phosphate-free RPMI 1640 medium (Life Technologies, Inc.) and incubated in 3 ml of phosphate-free RPMI 1640 supplemented with 10% dialyzed fetal calf serum for 30 min at 37 °C. 1.5 mCi of [32P]orthophosphoric acid (10 mCi/ml and 337 TBq/mmol; DuPont NEN) was then added to the cells. The cells were collected 90 min later and lysed. After immunoprecipitation, half of the immune complexes were incubated with 10 units of shrimp alkaline phosphatase (Boehringer Mannheim) in 100 μl of 50 mM Tris, pH 7.6, and 1 mM MgCl₂ for 30 min at 37 °C. All immune complexes, with or without phosphatase treatment, were analyzed by immunoblotting and autoradiography.

In *Situ* Hybridization—To obtain probes specific for mouse ATM mRNA, a ZAP cDNA library from postnatal day 2 mouse brains was screened using a human ATM DNA probe (18). A cDNA, which encompasses nucleotides 9191–9620 of the mouse ATM open reading frame (25) and approximately 200 nucleotides 3' to the termination codon, was used as template for *in vitro* transcription. [32P]-Labeled sense and antisense RNA probes for mouse ATM were made using T3 and T7 RNA polymerase (Promega), respectively. In *in situ* hybridization was performed essentially as described by Cox et al. (26).

**RESULTS**

**ATM Is a 370-kDa Protein**—Human ATM amino acid sequences 980-1512 and 2577–3056 (16) were each fused to GST, and the resultant fusion proteins, designated GST-ATM-N and GST-ATM-C, respectively, were used to raise polyclonal antisera in mice. In immunobLOTS, the antisera raised against GST-ATM-C, but not the preimmune serum, were detected by immunoblotting with the anti-GST-ATM-C antisera. Additionally, the anti-GST-ATM-N antiserum and two monoclonal antibodies generated from mice immunized with GST-ATM-N or preimmune serum or two anti-ATM-C monoclonal antibodies (2B3 and 2C1), proteins in the immune complexes were then analyzed by immunoblotting using the anti-GST-ATM-C antisera.

**ATM Is a 370-kDa Protein**—In immunoblotting experiments, the majority of ATM was detected in the nuclear fraction (Fig. 2A). Similar results were obtained with subcellular fractions from another human B cell line, Raji (data not shown).

We also examined the subcellular localization of ATM by...
staining cells with a monoclonal antibody (2C6) against ATM (Fig. 2, B–E). The human WI-38 fibroblasts, which expresses ATM as determined by immunoblotting (data not shown), was stained by 2C6. The staining by this antibody was predominantly nuclear. In contrast, the AT fibroblasts (GM03395) were not stained by 2C6. Thus, by subcellular fractionation and staining of cells with an ATM-specific antibody, ATM was found to be primarily a nuclear protein.

**ATM Is Phosphorylated in Vivo**—In immunoblots, we occasionally observed ATM in at least two forms with a slight difference in electrophoretic mobility. To test if ATM could be a phosphorylated protein, we metabolically labeled GM02184 and GM03189 cells, which are derived from an apparently normal individual and from an individual with AT, respectively, using [32P]orthophosphoric acid. Cell lysates were immunoprecipitated with anti-GST-ATM-C or GST-ATM-N antiserum in RIPA buffer. Half of the immune complexes were incubated with alkaline phosphatase. Proteins in the immune complexes were then analyzed by immunoblotting with the GST-ATM-C antiserum. B, autoradiography of the same blot shown in A. Lane 1, immunoprecipitation with the GST-ATM-N antiserum, GM02184 cells; lane 2, immunoprecipitation with the GST-ATM-C antiserum, GM02184 cells; lane 3, identical to lane 1 except that the precipitates were treated with phosphatase; lane 4, identical to lane 2 except that precipitates were treated with phosphatase; lane 5, immunoprecipitation with the GST-ATM-C antiserum, GM03189 cells. Immunoblotting was performed using the subcellular fractions from the 5 × 10^6 cells and antibodies specific for GST-ATM-C and RbAp48. To detect GST, the subcellular fractions from the 5 × 10^6 Akata cells were mixed with glutathione-agarose beads. Proteins that did not bind to the beads were removed by extensive washing with EBC buffer. The bound GST was analyzed by denaturing gel electrophoresis followed by Coomassie Blue staining. B–E, DAPI staining and immunostaining with 2C6, same field as in B, AT fibroblasts, 2C6; C, same field as in B, DAPI; D, AT fibroblasts, 2C6; E, same field as in D, DAPI.

**ATM Gene Expression in Mouse**—ATM knockout mice have neurological abnormalities, defective gonads, and impaired T cell development (19). To provide a basis for the observed tissue-restricted phenotype of the ATM knockout mice, we examined ATM mRNA expression pattern in mouse embryos and ATM protein levels in different adult mouse tissues. By in situ hybridization, ATM mRNA was found in all parts of the embryos at gestation day 10.5, 13.5, and 18.5 (Fig. 4, A–D, and data not shown). Elevated levels of ATM mRNA were observed throughout the nervous system of the mouse embryos (Fig. 4A). Similar levels of ATM mRNA were detected in the ventricular and migration zones of the brain which correspond to dividing and differentiating neurons, respectively. In addition to neuronal tissues, lung also had an elevated level of ATM mRNA. The hybridization signal in liver may not reflect the level of ATM mRNA, because the ATM sense probe, used as a negative control, generated as much signal as the antisense probe (Fig. 4C). At gestation day 18.5, elevated levels of ATM mRNA were also seen in thymus in addition to the nervous system (Fig. 4D).

In adult mice, ATM protein was found in all tissues examined (Fig. 4E). Thymus, spleen, and testis contained higher levels of ATM than liver, lung, brain, and kidney. The presence of elevated levels of ATM mRNA in the developing nervous system and ATM protein in adult testis and thymus is consistent with the finding that ATM mutations in mouse and man preferentially affect those tissues.

**DISCUSSION**

By using two antisera raised against different parts of ATM, we identified the ATM gene product as a protein of 370 kDa, a
molecular mass that is close to the calculated molecular weight of 350,000 from the ATM cDNA (16). This 370-kDa protein was absent in three AT cell lines, all of which exhibit typical AT cell phenotypes (14, 29, 30). Although the specific mutations of ATM in the three AT cell lines are unknown, earlier studies indicate that most ATM mutations in individuals with AT would result in mutant ATM gene products with large deletions or truncations (31, 32), which are unlikely to be detected by an antiseraum raised against the C terminus of ATM. The absence of the 370-kDa protein in the three AT cell lines examined is in agreement with the spectrum of ATM mutations identified to date.

The nuclear localization and phosphorylation of ATM is reminiscent of DNA-PKcs, a protein that is related to ATM by its primary structure and function. The PI 3-kinase domain and its adjacent region, spanning approximately 1,000 amino acids, of DNA-PKcs and ATM exhibit homology (16, 27). Functionally, DNA-PKcs is important for V(D)J recombination, repair of DNA double-strand breaks, and cell survival after DNA damage (33). The nuclear localization of ATM is consistent with the notion that ATM acts in a signal transduction pathway that is activated by damaged DNA and a DNA repair pathway. The observation of phosphorylated forms of ATM is intriguing. Although the significance of ATM phosphorylation is unknown, we speculate that phosphorylation may be one mechanism by which ATM function is regulated. In this regard, it has been shown that phosphorylation of DNA-PKcs inhibits its protein kinase activity and disrupts its association with the Ku antigen in vitro (20, 34). Although DNA-PKcs deficiency does not lead to a cell cycle checkpoint defect (35), DNA-PKcs and ATM might employ a similar strategy for their function based on their sequence homology and the presence of phosphorylated forms of DNA-PKcs and ATM. In preliminary experiments, we have detected ATM in a high molecular weight complex, suggesting that ATM is associated with other cellular factors. Perhaps, one or more ATM-associated factors could regulate ATM function using a scheme similar to the one employed by Ku for regulating DNA-PKcs (36).

ATM appears to be ubiquitously expressed. ATM mRNA is present in all human and adult mouse tissues examined (17, 25). In situ hybridization shows that ATM mRNA was expressed throughout the whole mouse embryo. ATM protein was found in all adult mouse tissues and in all cell lines used in this study, with the exception of three lines derived from individuals with AT. Additionally, ATM has been detected in the human B cell line Bjab, T cell line Molt4, the retinoblastoma cell line Weri27, the monkey kidney cell line CV1, mouse embryonic fibroblasts, and the teratocarcinoma cell line P19, and in the rat neuronal cell line PC12 (data not shown). This ubiquitous expression pattern of ATM underlies its essential roles in signal transduction that feeds the cell cycle checkpoints (10).

Although all tissues expressed ATM mRNA and protein, the steady state levels of ATM mRNA and ATM protein in different tissue types were not the same. In adult mice, thymus, spleen, and testis had elevated levels of ATM. Although the relative ATM level was not high in adult brains, ATM mRNA level was elevated in the developing brains of mouse embryos. Consistent with this, we have found higher levels of ATM protein in brains of mouse embryos and newborn mice compared with brains of adult mice (data not shown). The ATM knockout mice have reduced numbers of germ cells and mature T cells, are prone to thymic tumors, and repeatedly score low in neurological tests (19). Thus, with the exception of the spleen, mouse tissues that express relatively high levels of ATM mRNA or protein correspond to the tissues that are affected by inactivation of the ATM gene. Although individuals with AT also develop B cell lymphomas in addition to T cell lymphomas (5), no tumors originating from the spleen, an histologically heterogeneous tissue, have been reported in the ATM knockout mice. It is noteworthy to point out that we observed higher levels of ATM in the thymus of 8- or 16-day-old mice compared with the thymus of 75-day-old mice (data not shown). It is possible that the preferential manifestation of the consequences of ATM mutation in mouse thymus over spleen may result from the importance of ATM function in the thymus early in life, as suggested by the elevated level of ATM expression in the thymus of young animals, coupled with the early death of mice because of malignant thymic lymphomas.

The identification of the ATM gene product and determina-

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tion of its expression pattern will facilitate future biochemical characterization of ATM. Whether and how phosphorylation of ATM affects its function will be studied. In addition, because most ATM mutations identified to date result in truncation or deletion of the protein, detection of the intact ATM protein may be an efficient approach to screen for ATM mutations.

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