Purification and Characterization of ATM from Human Placenta

A MANGANESE-DEPENDENT, WORTMANNIN-SENSITIVE SERINE/THREONINE PROTEIN KINASE

(Received for publication, October 1, 1999, and in revised form, December 28, 1999)

Doug W. Chan‡‡, Seong-Cheol Son‡‡, Wesley Block‡‡, Ruiqiong Ye‡, Kum Kum Khanna‡‡, Marc S. Wold‡‡, Pauline Douglas‡, Aaron A. Goodarzi‡, Jennifer Pelley‡, Yoichi Tayas§§, Martin F. Lavin** and Susan P. Lees-Miller¶¶

From the ‡Department of Biological Sciences, University of Calgary, Calgary, Alberta T2N 1N4, Canada, the **Queensland Institute of Medical Research, PO Royal Brisbane Hospital, Brisbane, Queensland 4029, Australia, the ‡‡Department of Biochemistry, University of Iowa College of Medicine, Iowa City, Iowa 52242, and the §§National Cancer Center Research Institute, Tsukiji 5-1-1, Chuo-ku, Tokyo 104, Japan

ATM is mutated in the human genetic disorder ataxia telangiectasia, which is characterized by ataxia, immune defects, and cancer predisposition. Cells that lack ATM exhibit delayed up-regulation of p53 in response to ionizing radiation. Serine 15 of p53 is phosphorylated in vivo in response to ionizing radiation, and antibodies to ATM immunoprecipitate a protein kinase activity that, in the presence of manganese, phosphorylates p53 at serine 15. Immunoprecipitates of ATM also phosphorylate PHAS-I in a manganese-dependent manner. Here we have purified ATM from human cells using nine chromatographic steps. Highly purified ATM phosphorylated PHAS-I, the 32-kDa subunit of RPA, serine 15 of p53, and Chk2 in vitro. The majority of the ATM phosphorylation sites in Chk2 were located in the amino-terminal 57 amino acids. In each case, phosphorylation was strictly dependent on manganese. ATM protein kinase activity was inhibited by wortmannin with an IC50 of approximately 100 nM. Phosphorylation of RPA, but not p53, Chk2, or PHAS-I, was stimulated by DNA. The related protein, DNA-dependent protein kinase catalytic subunit, also phosphorylated PHAS-I, RPA, and Chk2 in the presence of manganese, suggesting that the requirement for manganese is a characteristic of this class of enzyme.

Ataxia telangiectasia is a human disorder characterized by ataxia, immunodeficiency, cell cycle checkpoint defects, and predisposition to cancer (1–3). The gene that is mutated in this disorder, ATM,2 encodes a nuclear polypeptide of approximately 350 kDa, which shares amino acid homology in its COOH terminus with the phosphatidylinositol-3 (PI-3) kinase family of proteins (4). Other members of this family include the catalytic subunit of DNA-dependent protein kinase (DNA-PKcs), FRAP, ATR, and the yeast gene products Mec-1, Tel-1, and Rad3 (for review, see Refs. 5 and 6). Highly purified DNA-PKcs has weak inherent DNA-stimulated serine/threonine protein kinase activity that is enhanced by interaction with DNA-bound Ku (7–12). DNA-PKcs does not have PI-3 kinase activity (13), and it is likely that, like DNA-PK, ATM also behaves as a serine/threonine protein kinase. Indeed, several reports have shown that immunoprecipitates of ATM from whole cells have serine/threonine protein kinase activity toward PHAS-I and p53 in vitro (14, 15). In immunoprecipitation assays, ATM protein kinase activity was absolutely dependent on the presence of manganese, and activity toward p53 and PHAS-I was not stimulated by DNA (14, 15). In contrast, others have reported that immunoprecipitated ATM has DNA-dependent kinase activity toward the 32-kDa subunit of single-stranded DNA-binding protein, RPA (16). A recent report has shown that, in crude extracts, ATM binds to DNA that has been damaged by irradiation with x-rays (17). These sometimes conflicting results underscore the need for biochemical characterization of ATM. Although recombinant ATM protein has been expressed in baculovirus (18, 19), the very low protein yield has made direct biochemical study very difficult.

Cells that lack ATM are defective in their ability to activate DNA damage response pathways that result in cell cycle arrest at both G1/S and G2/M. ATM acts upstream of p53 and is required for the activation of p53 and the subsequent up-regulation of the cyclin-dependent kinase inhibitor, p21 (for review, see Refs. 1–3). In addition, ATM is required for Chk2-dependent cell cycle arrest at G2/M (20), and Chk2 is phosphorylated in vivo in an ATM-dependent manner in response to ionizing radiation (20, 21). Recombinant ATM (18, 19) and immunoprecipitates of ATM from DNA-damaged cells (14, 15) phosphorylate p53 on serine 15, and ATM can interact directly with p53 (22). Serine 15 of p53 is phosphorylated in vivo in response to ionizing radiation (23), and phosphorylation at serine 15 plays an important role in the ionizing radiation-induced damage response pathway (for reviewed, see Refs. 24 and 25). However, the precise role of ATM in the activation of p53 remains to be determined. Serine 15 of p53, which occurs in the amino acid sequence PLSQE is also phosphorylated, in vitro, by DNA-PK (26), suggesting that ATM and DNA-PK recognize similar sequences in their target proteins.

1 The abbreviations used are: ATM, ataxia-telangiectasia mutated; PI-3, phosphatidylinositol-3; DNA-PKcs, DNA-dependent protein kinase catalytic subunit; DNA-PK, DNA-dependent protein kinase; DTT, dithiothreitol; GST, glutathione S-transferase; Gy, gray; RPA, replication protein, A.
Here we describe the purification of ATM from normal human tissue. The final protein fraction contains a polypeptide of approximately 350 kDa which cross-reacts with various antibodies to ATM but not to DNA-PKcs. The ATM-containing fraction phosphorylates serine 15 of p53, Chk2, the 32-kDa subunit of RPA, and PHAS-I in a strictly manganese-dependent manner. The protein kinase activity of purified ATM toward p53, PHAS-I, and Chk2 was not stimulated by double-stranded DNA or by Ku. Phosphorylation of RPA was stimulated by calf thymus DNA and calf thymus DNA in the presence of M13 single-stranded circular plasmid DNA, but not by irradiated plasmid DNA. We also show that the related protein, DNA-PKcs, is active in the presence of manganese in the absence of Ku, suggesting that a requirement for manganese may be characteristic of the PI-3 kinase family of enzymes.

**EXPERIMENTAL PROCEDURES**

**Protein Purification**—ATM was purified from human placenta following a similar procedure to that described previously for DNA-PKcs and Ku (9). All steps utilizing low pressure chromatography were performed at 4 °C. Chromatographic steps using the Biologic Protein Purification system (Bio-Rad) were performed at room temperature. All buffers (except buffer E, below) contained 0.2 mM phenylmethylsulfonyl fluoride, 0.1 mM benzamidine, 1 μM pepstatin, and 0.1 mM DTT. The presence of ATM and DNA-PKcs was monitored at all stages of purification by Western blotting using a rabbit polyclonal antibody to ATM, 4BA (22), or a rabbit polyclonal antibody, DPK1 to DNA-PKcs (8). Antibodies to the amino-terminal, Rad3 and carboxyl-terminal domains of ATM (27) were used in preliminary studies.

The initial stages of purification were similar to those described previously for the purification of DNA-PKcs and Ku (9). Human placenta was obtained from the Foothills Hospital, Calgary, in accordance with the safety and ethical requirements of the university. Freshly obtained human placenta was homogenized in buffer containing 0.5 M salt buffer and 10 mM magnesium chloride as described previously (9), and ATM was precipitated by the addition of ammonium sulfate to 40% saturation. After dialysis into buffer B (50 mM Tris-HCl, pH 8, 5 mM glycerol, 0.2 mM EDTA) containing 100 mM KCl, the sample was applied to a 5 × 30 cm column of DEAE-Fast Flow Sepharose (Amersham Pharmacia Biotech) equilibrated in the same buffer and eluted with buffer B containing 1 M KCl. In preliminary preparations, the protein sample was dialyzed into buffer B containing 100 mM KCl and applied to a 5 × 15 cm column of SP-Fast Flow Sepharose (Amersham Pharmacia Biotech). However, subsequently, this step was replaced by a 5 × 15 cm column of phenyl-Sepharose, Fast Flow (Amersham Pharmacia Biotech), equilibrated in buffer B containing 1 M KCl. ATM was eluted with buffer B containing 50 mM KCl. This column provided better yields of ATM protein than the SP-Sepharose column because of higher binding capacity for ATM. Also, ATM was separated from the majority of the DNA-PK because under these conditions, DNA-PKcs and Ku70/80 did not bind to the phenyl-Sepharose column. The ATM-containing fractions from the phenyl-Sepharose column were dialyzed into buffer B containing 100 mM KCl, applied to a 5 × 5 cm column of MacroPrep DEAE (Bio-Rad), and eluted with buffer B containing 100 mM KCl and

**Fig. 1.** Panel A, silver-stained 10% SDS-polyacrylamide gel of fractions from nickel chelation column. ATM was applied to a 5-ml HiTrap chelation Sepharose column as described under “Experimental Procedures.” 10-μl aliquots of fractions 1–26 (eluted with a 75-min linear gradient of 0–150 mM ammonium chloride and 0–150 mM imidazole in buffer containing 500 mM NaCl) were run on 10% SDS-acrylamide gels and silver stained. P represents the precolumn sample, and F is the flow-through fraction. The positions of molecular mass markers are shown on the left. The arrows indicate the presence of two polypeptides of approximately 300 and 350 kDa in the ATM-containing fractions. Panel B, Western blot for fractions from nickel chelation column. 10 μl of the fractions shown in panel A were analyzed on 8% SDS-polyacrylamide gels, transferred to nitrocellulose, and probed with antibody 4BA to ATM. No cross-reaction with antibodies to DNA-PKcs was observed (data not shown). The positions of molecular mass markers are shown on the left. Panel C, protein kinase activity in fractions from nickel chelation column. 3 μl of the precolumn (P), flow-through (F), and fractions 3, 5, 7, 9, 11, and 13 from the nickel chelation column (panel A) were incubated with PHAS-I (0.25 μg) under assay conditions in the presence of 10 mM manganese chloride. Fraction 9 was also assayed in the presence of 10 mM magnesium chloride (*), as described under “Experimental Procedures.” The corresponding autoradiogram is shown. Panel D, silver stain of concentrated ATM from the last Mono Q column. Fractions 3–15 from the nickel chelation column were pooled and applied to a Mono Q HR5/5 column equilibrated in buffer B (100 mM KCl plus 0.02% Tween 20). The column was eluted as described under “Experimental Procedures.” Too little ATM was present in these fractions to be detected by silver stain, therefore ATM-containing fractions were identified by Western blot, and peak fractions were pooled and concentrated using a Centricon microconcentrator. The final yield of ATM was 500 μl. 15 μl of purified ATM was analyzed by silver staining of a 10% SDS-polyacrylamide gel (lane 1). Known concentrations of DNA-PKcs were included to estimate the protein concentration. Lane 2 contained sample buffer alone; lanes 3–6 contained 5, 10, 20, or 50 ng of DNA-PKcs, respectively.
100 mM magnesium chloride. A similar step involving elution of DEAE-anion exchange resin using magnesium chloride was used for the purification of DNA-PKcs and Ku from HeLa cells (28) and placenta (9). Fractions containing ATM were then dialyzed into buffer B containing 100 mM KCl and passed over single-stranded DNA-cellulose (Sigma), either in batch mode or by column chromatography (1.5 × 4-cm column). Unlike DNA-PKcs and Ku, in our hands, ATM did not bind to either single-stranded DNA-cellulose or double-stranded DNA-cellulose. ATM from the flow-through fractions of the DNA-cellulose column was then applied to a 5-ml heparin HiTrap column (Amersham Pharmacia Biotech), equilibrated in buffer B containing 100 mM KCl and 0.02% Tween 20. This and all subsequent steps were performed on a Biologic Protein Purification system. ATM was eluted with a linear gradient of buffer B containing 100 mM KCl and 0.02% Tween 20, to buffer B containing 1× KCl and 0.02% Tween 20 over 75 min. 1-mL fractions were collected. ATM eluted at approximately 200 mM KCl, whereas DNA-PKcs applied to the same column under the same conditions eluted at approximately 300–400 mM KCl. ATM-containing fractions were pooled and dialyzed into buffer C (10 mM sodium phosphate, 50 mM KCl, 5% glycerol, pH 7.2). The sample was applied to an Econo-Pac hydroxyapatite cartridge (Bio-Rad) at a flow rate of 0.7 ml/min. The column was washed with buffer C before applying a 32-ml linear gradient of increasing buffer D (400 mM sodium phosphate, 50 mM KCl, 5% glycerol, pH 6.8). ATM eluted at approximately 20% buffer D, corresponding to approximately 90 mM NaCl. Fractions containing ATM were pooled and dialyzed into buffer B containing 100 mM KCl and 0.02% Tween 20 and applied to Mono Q HR5/5 fast protein liquid chromatography column that had been equilibrated in the same buffer. Bound proteins were eluted with a linear gradient of buffer B containing 100 mM KCl, 0.02% Tween 20, and MgCl₂ at a flow rate of 1 ml/min. The magnesium concentration was increased at 1 mM/min. ATM eluted off the Mono Q column between 22 and 30 mM MgCl₂. In preliminary preparations, ATM-containing fractions off the Mono Q column were applied to ATP-Sepharose, gamma-phosphate linked (Upstate Biotechnology Inc.) that had been equilibrated in buffer E (25 mM Hepes, pH 7.5, 1 mM DTT, 60 mM MgCl₂, 0.5 mM phenylmethylsulfon fluoride) containing 150 mM NaCl and eluted with buffer E containing 1× NaCl. In subsequent preparations ATM from the Mono Q step was dialyzed into buffer F (50 mM Tris-HCl, pH 8.0, 5% glycerol, without EDTA) containing 100 mM NaCl and applied to a 5-ml HiTrap chelation Sepharose column (Amersham Pharmacia Biotech) that had been loaded with nickel sulfate according to the manufacturer's recommendations. The column was equilibrated in buffer F containing 100 mM NaCl and after sample loading was washed sequentially with 15 ml of buffer F containing 100 mM NaCl followed by 15 ml of buffer F containing 500 mM NaCl. ATM was eluted with a linear gradient of buffer F containing 500 mM NaCl to buffer F containing 500 mM NaCl, 150 mM imidazole, and 150 mM ammonium chloride at 1.5 ml imidazole and ammonium chloride per minute. ATM-containing fractions were dialyzed and applied onto a Mono Q HR5/5 column that had been equilibrated in buffer B containing 30% buffer B and eluted with a gradient of buffer B containing 1× KCl and 0.02% Tween 20. ATM-containing fractions were desalted and concentrated on a Centricon 100 microconcentrator (Amicon), washed with buffer B containing 100 mM KCl, 0.02% Tween 20, 0.5 mM phenylmethylsulfon fluoride, 1 mM DTT, and stored in aliquots at −80 °C.

**Protein Kinase Assays—** ATM kinase assays contained 25 mM Tris-HCl, pH 8.0, 50 mM KCl, 5% glycerol, 0.5 mM DTT, 5 μCi of [γ-³²P]ATP, 10 μl cold ATP, 10 mM MnCl₂, 0.25–0.5 μg of PHAS-1 (Stratagene) or other substrate as indicated, and 2–4 ng of purified ATM. The reactions were incubated at 30 °C for 30 min, stopped with SDS sample buffer, and analyzed by SDS-polyacrylamide gel electrophoresis on 15% acrylamide gels. Gels were stained with Coomassie Blue, destained extensively, dried, and exposed to Fuji x-ray film with intensifying screen at −80 °C.

**DNA-PK purification** was performed and assayed as described previously (9, 40). Sonicated calf thymus DNA was prepared as described previously (28). Irradiated pUC18 plasmid DNA was prepared as described previously (29). Single-stranded M13 circular plasmid DNA was obtained from Life Technologies, Inc.

**Western Blotting**—ATM and DNA-PKcs were analyzed on 8% SDS-polyacrylamide gels, as described previously (9). The antibodies used for detection of ATM were a rabbit polyclonal to the Rad3 domain of ATM (Oncogene Scientific, ATB-3, PC116) and a mouse monoclonal antibody, DO1, obtained from Oncogene Scientific. The rabbit polyclonal antibody to DNA-PKcs (DKP1) was as described previously (8). A rabbit polyclonal antibody specific for serine 15 of p53 was as described previously (22).

**RESULTS**

The cDNA sequence of ATM predicts a polypeptide of approximately 350 kDa with amino acid similarity to the PI-3 kinase family of proteins in the carboxyl-terminal domain. Attempts to express active ATM in baculovirus have not been very successful, probably because of the large size of the protein. To date, most of the information regarding the properties of ATM has been obtained from immunoprecipitates. Because immunoprecipitates may contain contaminating or interacting proteins and because antibody binding could interfere with the biochemical properties of the putative protein, we considered it important to purify the protein by biochemical means to characterize the biochemical properties of ATM fully. Here we have used conventional biochemical techniques to purify ATM from human placenta. We previously described a procedure to purify the related protein DNA-PK from the same source (9) and therefore used these procedures as a guide to purify ATM. Because immunoprecipitates of ATM phosphorylate p53 on serine 15 and because the same residue is phosphorylated by DNA-PK in vitro, it was important to separate DNA-PK from ATM completely during the purification procedure.

The presence of ATM and DNA-PKcs was determined by Western blot at each stage of the purification, and fractions containing ATM were judiciously pooled. ATM was precipitated by 40% ammonium sulfate and bound to DEAE-Sepharose anion exchange matrix. DNA-PKcs and Ku proteins were also found in the same fractions. However, most of the DNA-PKcs and Ku ATM moved from ATM by binding with the DNA-PKcs and Ku buffer B containing 1× KCl. Under these conditions, most of the DNA-PKcs and Ku 1× KCl did not bind to the column, whereas ATM binding was and was eluted with buffer B containing 50 mM salt. Partially purified ATM that was eluted from DEAE by magnesium-containing buffer did not bind to either double-stranded or single-stranded DNA-cellulose resin. In contrast, DNA-PKcs and Ku bound tightly to both resins, again providing a means for separation of DNA-PK from ATM.

**Table I**

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonium sulfate precipitation</td>
<td>4179 mg</td>
</tr>
<tr>
<td>DEAE 1× eluate</td>
<td>3349</td>
</tr>
<tr>
<td>Phenyl-Sepharose, 50 mM eluate</td>
<td>92</td>
</tr>
<tr>
<td>DEAE, magnesium eluate</td>
<td>40</td>
</tr>
<tr>
<td>DNA-cellulose flow</td>
<td>22</td>
</tr>
<tr>
<td>Heparin HiTrap pooled fractions</td>
<td>8</td>
</tr>
<tr>
<td>Hydroxyapatite, pooled fractions</td>
<td>1.2</td>
</tr>
<tr>
<td>Mono Q, magnesium elution, pooled fractions</td>
<td>0.1</td>
</tr>
<tr>
<td>Nickel chelation HiTrap, pooled fractions</td>
<td>ND</td>
</tr>
<tr>
<td>Mono Q, salt elution, pooled fractions</td>
<td>0.001</td>
</tr>
</tbody>
</table>

**Protein yields during purification of ATM**

ATM was purified as described under “Experimental Procedures,” starting with approximately 300 g of placenta. Protein concentrations for steps up to the first Mono Q FPLC column were determined using the Bio-Rad protein assay and bovine serum albumin as standard. The concentration of ATM in the final step was estimated by comparison with known amounts of DNA-PKcs protein on silver-stained gels (see Fig. 1B); ND, not determined.
**Purification of Human ATM**

**FIG. 2. Phosphorylation of PHAS-I by purified ATM.** Panel A, approximately 2 ng of ATM was assayed for phosphorylation of PHAS-I (0.25 µg/reaction) in the presence of 10 mM MnCl₂ (lanes 1-3 and 5) or 10 mM MgCl₂ (lane 4). Wortmannin was added to the sample in lane 3 to 0.5 µM. Sonicated calf thymus DNA was added to the sample in lane 5 to a final concentration of 10 µg/ml. Lane 1 contained PHAS-I, manganese, and ATP but not ATM. Samples were analyzed on 15% SDS-polyacrylamide gels and exposed to x-ray film overnight at −80 °C with intensifying screens. Panel B, ATM-mediated phosphorylation of PHAS-I was assayed in the presence of 10 mM manganese chloride, with no other additions (lane 1), with the addition of 10 µg/ml calf thymus DNA (lane 2), or with 20 ng of Ku in the absence (lane 3) or presence (lane 4) of 10 µg/ml calf thymus DNA. Also shown is Ku assayed in the absence of added ATM, both in the absence (lane 5) and presence (lane 6) of added DNA.

DNA-PKcs also eluted at different salt conditions on heparin HiTrap chromatography, again providing for maximum separation of the two proteins.

In preliminary preparations, the final step of the purification of ATM was binding to ATP-Sepharose (gamma-phosphate linked). However, very little ATM was recovered from this column, and despite various elution strategies, the majority of the protein remained bound to the ATP-Sepharose beads. Preliminary experiments indicated that ATM bound to chelation Sepharose resin that had been loaded with nickel, copper, or zinc but not with magnesium or manganese. Chelation Sepharose was therefore used in place of the ATP-Sepharose column. ATM-containing fractions were applied to a nickel-loaded Hi Trap chelation Sepharose column and eluted with a gradient of ammonium chloride and imidazole in buffer B containing 500 mM NaCl. Two polypeptides of approximately 350 and 300 kDa were observed on silver-stained SDS-polyacrylamide gels (Fig. 1A, upper arrow and lower arrow, respectively). ATM was detected by Western blotting in fractions 3–21 (Fig. 1B). The upper band in the Western blot (Fig. 1B) comigrated with the upper band in the silver stained gel (Fig. 1A, upper arrow). The lower band in the Western blot ran slightly ahead of the 200-kDa marker and is likely a breakdown product of ATM. The ATM-containing fractions were assayed for their ability to phosphorylate PHAS-I, a substrate that has been shown previously to be phosphorylated by immunoprecipitates of ATM in the presence of manganese (14, 15). Manganese-dependent phosphorylation of PHAS-I was observed in the ATM-containing fractions (Fig. 1C). ATM-containing fractions were pooled and applied to a Mono Q fast protein liquid chromatography column and eluted with a salt gradient. The ATM-containing fractions were concentrated and desalted as described under “Experimental Procedures.” The final yield of ATM was estimated by comparison with known amounts of DNA-PKcs on silver-stained gels (Fig. 1D). The major band in the sample migrated at 350 kDa, coincident with ATM in Western blots. The polypeptide at approximately 300 kDa comigrated with the ATM breakdown product on Western blot. We estimate that approximately 2 µg of highly purified ATM was obtained from one placenta (Table I). In contrast, up to 500 µg of DNA-PKcs and 1 mg of Ku70/80 heterodimer could be purified from the same amount of starting material, suggesting that ATM is present in far less abundance than DNA-PKcs in human tissues. In preliminary studies, we examined rat liver and bovine testis as potential sources of ATM; however, poor antibody recognition and/or low abundance made purification of ATM from non-human sources impractical.

The highly purified ATM fraction was next assayed for its ability to phosphorylate a number of protein substrates in the presence of either magnesium or manganese as metal ion. ATM activity was also assayed in the presence of the PI-3 kinase inhibitor, wortmannin. Because the kinase activity of the related protein, DNA-PKcs, is modulated by DNA and the Ku70/80 heterodimer, ATM was also assayed in the presence of both sonicated calf thymus DNA and Ku. Phosphorylation of PHAS-I was completely dependent on the presence of manganese (Fig. 2A) and was inhibited by wortmannin (Fig. 2A, lane 3) with an IC₅₀ of approximately 100 nM (data not shown). The addition of sheared calf thymus DNA did not stimulate PHAS phosphorylation (Fig. 2A, lane 5). Phosphorylation of PHAS was also not affected by the addition of purified Ku heterodimer, in the presence or absence of DNA (Fig. 2B).

Serine 15 of p53 is phosphorylated in vivo in response to ionizing radiation (23). Further, previous studies indicated that immunoprecipitated ATM can, in the presence of manganese, phosphorylate p53 on serine 15 (14, 15, 22). We therefore examined whether purified ATM could also phosphorylate p53 in vitro. Phosphorylation of p53 by highly purified ATM was absolutely dependent on manganese, was inhibited by wort-

---


3 W. Block and S. P. Lees-Miller, unpublished observations.

4 W. Truong and S. P. Lees-Miller, unpublished observations.
magnesium chloride instead of manganese chloride; lane 2 contained protein substrate but no panel substrate in the presence of 10 mM manganese chloride; (Chk2(D57N)) (lacks the amino-terminal 57 amino acids, Chk2(D57N)) (panel C). Assay conditions were as described under “Experimental Procedures.” For panels A and B, lane 1 in each panel contained protein substrate but no ATM; lane 2 contained ATM plus protein substrate in the presence of 10 mM manganese chloride; lane 3, as lane 2 but with 0.5 μM wortmannin; lane 4, as lane 2 but with 10 mM magnesium chloride instead of manganese chloride; lane 5, as lane 2 but with 10 μg/ml sonicated calf thymus DNA. Panel C, Chk2(1–222) (lanes 1 and 3) or Chk2(D57N) (lanes 2 and 4) was incubated under assay conditions as described under “Experimental Procedures” either without (lanes 1 and 2) or with (lanes 3 and 4) 2 ng of purified ATM. The positions of Chk2(1–222) and Chk2(D57N) are indicated on the right. Lane 5 contained ATM alone. The corresponding autoradiogram is shown.

Phosphorylation of p53 and Chk2 by purified ATM. Approximately 2 ng of purified ATM was incubated with 0.5 μg of either recombinant GST-tagged p53 (amino acids 1–40) (panel A), recombinant GST-tagged Chk2 (amino acids 1–222) (panel B), or recombinant GST-tagged Chk2 that lacks the amino-terminal 57 amino acids, (Chk2(D57N)) (panel C). Assay conditions were as described under “Experimental Procedures.” For panels A and B, lane 1 in each panel contained protein substrate but no ATM; lane 2 contained ATM plus protein substrate in the presence of 10 mM manganese chloride; lane 3, as lane 2 but with 0.5 μM wortmannin; lane 4, as lane 2 but with 10 mM magnesium chloride instead of manganese chloride; lane 5, as lane 2 but with 10 μg/ml sonicated calf thymus DNA. Panel C, Chk2(1–222) (lanes 1 and 3) or Chk2(D57N) (lanes 2 and 4) was incubated under assay conditions as described under “Experimental Procedures” either without (lanes 1 and 2) or with (lanes 3 and 4) 2 ng of purified ATM. The positions of Chk2(1–222) and Chk2(D57N) are indicated on the right. Lane 5 contained ATM alone. The corresponding autoradiogram is shown.

The serine/threonine protein kinase Chk2 is phosphorylated in vivo in an ATM-dependent manner in response to ionizing radiation (20, 21). Because ATM phosphorylates p53 at serine 15 in the context PL5SQE, this suggests that, like DNA-PK, ATM protein kinase also recognizes serines that are followed by glutamine. Human Chk2 contains several putative ATM phosphorylation sites at amino acids 19, 26, 28, 33, 35, 50, 68, 357, and 432. The amino-terminal 222 amino acids of Chk2 were expressed as a GST construct and assayed for phosphorylation by purified ATM. Recombinant Chk2 was phosphorylated by ATM in a manganese-dependent manner (Fig. 3B). Phosphorylation was inhibited by wortmannin and was not stimulated by the addition of sonicated calf thymus DNA (Fig. 3B, lanes 3 and 5, respectively). Because the majority of the potential ATM phosphorylation sites are located in the amino-terminal 60 amino acids, we tested the ability of purified ATM to phosphorylate a GST construct in which the amino-terminal 57 amino acids of Chk2 had been deleted (Chk2(D57N)). ATM phosphorylated Chk2(D57N) weakly, consistent with the major in vitro phosphorylation sites in Chk2 being located between amino acids 1 and 57 (Fig. 3C).

A previous study has shown that immunoprecipitates of ATM phosphorylate the 32-kDa subunit of RPA in a DNA-dependent manner (16). RPA is also phosphorylated by DNA-PK at amino-terminal SQ sites (32, 33), and the yeast homolog of ATM, Mec-1, is required for the phosphorylation of RPA in yeast (34). We therefore assayed for the ability of purified ATM to phosphorylate both recombinant RPA (32- and 14-kDa subunits) and heterotrimeric RPA (32-, 14-, and 70-kDa subunits). The 32-kDa subunit of RPA (RPA32) was phosphorylated in the context of the recombinant protein, and phosphorylation was modestly, but consistently stimulated by the addition of sonicated calf thymus DNA (Fig. 4A). Phosphorylation was manganese-dependent and was inhibited by wortmannin (Fig. 4A). In contrast, the 32-kDa subunit of RPA in the context of the purified heterotrimeric protein was less strongly phosphorylated by purified ATM under the conditions used in this assay (Fig. 4B). RPA binds to single-stranded DNA and is required for DNA replication in vivo (for review, see Ref. 35). RPA is also required for nucleotide excision repair and enhances the binding of XPA protein to UV-damaged DNA (36). Gately et al. (16) have reported that phosphorylation of RPA32 by immunoprecipitates of ATM was stimulated by single-stranded closed circular DNA (MI3 DNA) in the presence of sonicated calf thymus DNA (calf thymus DNA). A recent report has shown that ATM binds preferentially to irradiation-damaged DNA-cellulose compared with undamaged DNA-cellulose and that this interaction does not require DNA-PKcs or Ku (17). Taking all of these findings into account, we postulated that RPA might act as a DNA-targeting subunit for purified ATM, in a manner similar to targeting of DNA-PKcs to DNA ends by the Ku heterodimer. RPA heterotrimer was therefore incubated with purified ATM either in the absence of DNA (Fig. 4C, lane 1) or in the presence of plasmid DNA that had been irradiated with 0, 10, 25, 50, or 100 Gy (Fig. 4C, lanes 2–6, respectively). The concentration of DNA used was shown previously to give maximum activation of DNA-PK (29). No stimulation of ATM activity toward RPA heterotrimer was observed (Fig. 4C). The
However, in each case the addition of highly purified Ku heterotrimer greatly enhanced the phosphorylation of all substrates tested (Fig. 5, lane 3 in each panel). However, in the presence of manganese, PHAS-I, Chk2, and RPA32 were highly phosphorylated by DNA-PKcs in the absence of Ku (Fig. 5, lane 5) and phosphorylation was partially DNA-dependent (Fig. 5, lanes 5–8). In contrast, a construct containing amino acids 1–40 of p53 was phosphorylated poorly by DNA-PKcs in the presence of manganese, regardless of the presence of Ku or DNA (Fig. 5).

**DISCUSSION**

We have developed a biochemical procedure to purify ATM from normal human tissue. The final product is highly enriched for ATM; however, we cannot exclude the possibility that trace amounts of contaminating proteins remain in the sample. It was not possible to purify ATM further because of the extremely low yields of protein obtained. We show that highly purified ATM has manganese-dependent serine/threonine protein kinase activity toward p53, RPA 32-kDa subunit, and PHAS-I, consistent with the reported properties of immunoprecipitated ATM (14–16). Like DNA-PK, ATM phosphorylates p53 on serine 15 in an “SQ motif,” suggesting that both protein kinases have similar recognition motifs for phosphorylation.

We also show that purified ATM phosphorylates Chk2, which is phosphorylated in vitro in an ATM-dependent manner (20, 21). Moreover, we show that the majority of the in vitro phosphorylation sites in the amino-terminal 222 amino acids of Chk2 are located in the first 57 amino acids, a region that contains six potential ATM phosphorylation sites at serines 19, 28, 33, 35, and threonine 26.

Purified ATM kinase activity was inhibited by wortmannin with an IC$_{50}$ of approximately 100 nM, which is similar to that of the Ku heterotrimer.

---

**Fig. 4. Phosphorylation of RPA by purified ATM.** Panel A, 0.5 μg of recombinant His-tagged RPA (32- and 14-kDa subunits) was incubated either alone (lane 1) or with ATM (lanes 2–5) in the presence of 10 mM manganese chloride (lanes 1–3 and 5) or 10 mM magnesium chloride (lane 4), as described in Fig. 3A. Wortmannin was added (to 0.5 μM final concentration) to the sample in lane 3. Panel B, lanes were exactly as in panel A, except they contained 0.5 μg of purified heterotrimeric RPA instead of recombinant RPA32/14. The position of migration of RPA14 was observed. Panel C, 0.5 μg of purified RPA heterotrimer was incubated with ATM (2 ng) in the presence of 10 mM manganese chloride in the presence of various types of DNA (final concentration 0.5 μg/ml). Lane 2, closed circular plasmid DNA; lane 3, plasmid DNA irradiated at 10 Gy; lane 4, plasmid DNA irradiated at 25 Gy; lane 5, plasmid DNA irradiated at 50 Gy; and lane 6, plasmid DNA irradiated at 100 Gy. Lane 1 contained an equivalent volume of 10 mM Tris-HCl and 1 mM EDTA, pH 8.0. Lane 7 contained ATM, heterotrimeric RPA, and sonicated calf thymus DNA (50 μg/ml). Lane 8 contained ATM, RPA, sonicated DNA (50 μg/ml), plus M13 plasmid DNA (20 μg/ml). Only the portion of the autoradiogram corresponding to RPA32 is shown.
found previously for ATM in immunoprecipitation kinase assays (14, 15). Wortmannin is a relatively specific inhibitor of the PI-3 kinase family, inhibiting the lipid kinase activity of p110 PI-3 kinase at low nM levels (37) and DNA-PK kinase activity with an IC50 of about 200 nM (13). In contrast, millimolar concentrations of wortmannin are required to inhibit serine/threonine protein kinases that are more closely related to the catalytic subunit of cAMP-dependent protein kinase (38). These data argue against the presence of any contaminating protein kinases in the ATM preparation.

Although DNA-PKcs and ATM are both members of the PI-3 kinase family of proteins, neither purified DNA-PKcs (13), immunoprecipitated ATM (14, 15), nor recombinant ATM (data not shown) has detectable kinase activity toward phosphatidylinositol. Likewise, we have not detected phosphatidylinositol kinase activity for ATM purified by this procedure (data not shown).

ATM was found not to bind to DNA-cellulose resin during the purification procedure. The addition of calf thymus DNA did not enhance the ATM-induced phosphorylation of PHAS-I, p53, or Chk2 in vitro; however, a modest stimulation of phosphorylation of the 32-kDa subunit of recombinant ATM was observed consistently with the addition of sheared calf thymus DNA. The DNA binding properties of RPA have been assigned to the 70-kDa subunit (35). Because DNA appears to stimulate RPA phosphorylation in the absence of the 70-kDa subunit, our results suggest that either the recombinant 32- and 14-kDa subunits of RPA have weak DNA binding properties themselves or that DNA enhances the activity of ATM toward RPA. The authors of this previous study speculated that secondary structure within the single-stranded DNA circle promoted phosphorylation of RPA32 by DNA-PK (34). The serine/threonine kinase activity of purified ATM is absolutely dependent on manganese. Similar results were found in ATM immunoprecipitation kinase assays (14, 15). The requirement for manganese is unlikely to be caused by a peculiarity of the substrate used because phosphorylation of PHAS-I, Chk2, RPA, and GST-p53 constructs by purified ATM all

![Fig. 5. Phosphorylation by DNA-PK in the presence of manganese.](image)

<table>
<thead>
<tr>
<th></th>
<th>DNA:</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Ku−</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>DNA:</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>PHAS-I</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p53</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chk2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RPA32</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Mg2+</th>
<th>Mn2+</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA:</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>PHAS-I</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>p53</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Chk2</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>RPA32</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>
showed an absolute requirement for manganese. Significantly, the p110 subunit of PI-3 kinase also shows manganese-dependent serine/threonine kinase activity (42), suggesting that manganese may be required for serine/threonine protein kinase activity of all PI-3 kinase family members. Remarkably, the protein kinase activity of DNA-PKcs toward several substrates including PHAS-I, RPA32, and Chk2, was also stimulated by manganese. Under the conditions used in these assays, DNA-dependent phosphorylation of PHAS-I and RPA 32 by DNA-PKcs in the presence of manganese was approximately equal to that of DNA-PKcs plus Ku in the presence of magnesium, suggesting that for these substrates, the presence of manganese can bypass the need for Ku. However, manganese did not support phosphorylation of hsp90 (28), various p53 GST fusion proteins, or a synthetic peptide (26) derived from p53 by DNA-

REFERENCES

Purification and Characterization of ATM from Human Placenta: A MANGANESE-DEPENDENT, WORTMANNIN-SENSITIVE SERINE/THREONINE PROTEIN KINASE
Doug W. Chan, Seong-Cheol Son, Wesley Block, Ruiqing Ye, Kum Kum Khanna, Marc S. Wold, Pauline Douglas, Aaron A. Goodarzi, Jennifer Pelley, Yoichi Taya, Martin F. Lavin and Susan P. Lees-Miller

doi: 10.1074/jbc.275.11.7803

Access the most updated version of this article at http://www.jbc.org/content/275/11/7803

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 42 references, 25 of which can be accessed free at http://www.jbc.org/content/275/11/7803.full.html#ref-list-1