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ATM protein purified from vaccinia virus expression system: DNA binding requirements for kinase activation

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Abstract

The ataxia-telangiectasia mutated (ATM) gene product plays a role in responding to double stand DNA breaks. Some biochemical studies of ATM function have been hampered by lack of an efficient expression system and abundant purified ATM protein. We report the construction of a vaccinia virus expressing ATM, vWR-ATM, which was used to produce large amounts of functional FLAG-tagged ATM protein (FLAG-ATM) in HeLa cells. Kinase activity of the purified FLAG-ATM was dependent on manganese and inhibited with wortmannin. Using the FLAG-ATM recombinant protein, GST-p53 serine 15 phosphorylation increased in the presence of damaged DNA. PHAS-1 phosphorylation was found to be DNA independent. Purified FLAG-ATM was recovered in the autophosphorylated form, as demonstrated by phosphorylation of ATM serine 1981. As shown by atomic force microscopy, FLAG-ATM bound to linear DNA both at broken ends and in mid-strands. Vaccinia virus is the most efficient ATM expression system described to date.

Keywords: Ataxia-telangiectasia; ATM; Vaccinia virus; Purification; Expression; Protein kinase; Atomic force microscopy; DNA; Recombinant protein

Ataxia-telangiectasia (A-T) cells display cell cycle checkpoint defects, chromosomal instability, and sensitivity to ionizing radiation (IR) [1]. The ataxia-telangiectasia mutated (ATM) gene encodes a 370-kDa protein kinase that is involved in the response to double-stranded breaks and the initiation of DNA repair. ATM is a member of a family of large protein kinases containing a C-terminal domain homologous to the phosphatidylinositol 3-kinase domain [2,3]. Proteins in this family play a role in cell cycle checkpoint or DNA damage repair. Other proteins in this family include Rad 3, Tel1, Mec1p, Mei-41, Rad 50, and DNA-PK. ATM targets many substrates for phosphorylation, such as p53, NBS1, Chk2, FANCD2, H2AX, BRCA1, and SMC1 [4]. ATM protein is undetectable by immunoblotting in nuclear extracts of cells from most A-T patients [5].

Overexpression of ATM is difficult due to the instability of the cDNA and the large protein size [6]. Two laboratories independently expressed ATM using baculovirus, but failed to obtain a significant ATM protein yield following purification [7,8]. Overexpression of ATM in insect cells resulted in only a fraction of

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recombinant protein in the soluble portions of cell preparations, while the majority associated with cellular membranes [7]. From 100 ml of infected insect cells, only 20 ng of ATM was recovered [8]. Expression of other recombinant proteins is often in milligram quantities.

Purification of endogenous ATM protein results in low yields due to low expression levels. Smith et al. [9] used a series of chromatography steps to purify endogenous ATM from 50 g of HeLa nuclear extract and obtained low amounts of ATM. Goodarzi and Lees-Miller [10] purified 30 μg of ATM from 300 L of HeLa cells. Chan et al. [11] purified ATM from a human placenta, resulting in approximately 2 μg of protein. In contrast, purification of DNA-PKcs, using a similar protocol, resulted in 500 μg of protein [12]. Rhodes et al., [13] transfected HEK 293T cells with a FLAG-ATM expression plasmid and purified the tagged ATM using an anti-FLAG affinity column; they purified 1 μg of protein from a 225 cm^2 flask that was seeded with 8×10^6 cells 2 days prior to purification (0.16 g of cells).

Vaccinia virus is a member of the poxvirus family, a group of large DNA viruses. Until 1972, vaccinia virus was widely used as a live vaccine against smallpox. Today, vaccinia is predominantly used as a tool to help identify targets of immune responses in microbial infections. Reports in the early 1980s introduced the use of vaccinia as a vector for transient expression of foreign genes [14,15]. Advantages for using vaccinia virus as an expression system include: (1) expression of large DNA insertions, (2) infectability of a wide host range, including most mammalian and avian cells, (3) cytoplasmic transcription, (4) retention of infectivity after insertion of foreign DNA, (5) high levels of protein expression, and (6) proper transport, secretion, processing, and posttranslational modification. There is also growing interest in investigating vaccinia virus in cancer immunotherapies [16,17] and HIV regulation [18].

We report the construction of vWR-ATM, a recombinant vaccinia virus manipulated to express functional FLAG-tagged ATM (FLAG-ATM), and the consistent recovery of approximately 30 μg of purified FLAG-ATM from 8×10^6 vWR-ATM-infected HeLa cells. This was used to document manganese-dependent, DNA-stimulated kinase activity of the purified FLAG-ATM. The protein was recovered in its autophosphorylated form. By direct visualization using atomic force microscopy (AFM), we observed ATM protein binding to linear DNA both at the DNA ends and internally.

Materials and methods

Cell culture and irradiation. CV-1 tk- cells were maintained in DME (Hyclone, Logan, UT) supplemented with 10% fetal calf serum (Hyclone, Logan, UT). HeLa cells were maintained in DMEM (Cellgro, Herndon, VA) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT) and 1% penicillin/streptomycin/glutamine (Invitrogen, Carlsbad, CA). A-T lymphoblastoid cells, L3, were maintained in RPMI (Cellgro, Herndon, VA) supplemented with 15% fetal bovine serum and 1% penicillin/streptomycin/glutamine. All cells were grown in a humidifying incubator at 37 °C with 5% CO_2. Cells treated with irradiation were exposed to 2 Gray of ionizing radiation (IR). Cells infected with vaccinia virus were returned to 37 °C after infection, until lysis.

Construction of pSCAT. pFT-YZ5, a baculovirus construct containing the full-length ATM cDNA, was generously donated by Y. Shiloh [7]. Sequences coding for the FLAG (DYKDDDDK) and hexahistidine (6× His) epitopes flank the 5′ end of the ATM coding sequence. A double digestion using *Sal*I and *Kpn*I restriction enzymes (New England Biolabs, Beverly, MA) released the entire ATM coding sequence and both tags. This resulted in a 4 kb piece containing FLAG, His, and the 5′ half of ATM, and a 5.7 kb fragment for the remaining 3′ half of ATM. The 5′ ATM fragment was inserted into the vaccinia vector pSC65 at the *Sal*I and *Kpn*I sites, producing pSC-5ATM. The 3′ ATM piece was ligated into pSC-5ATM at the *Kpn*I site and checked with restriction enzymes for insertion in the correction orientation. DNA sequencing was performed to ensure the integrity of all ligation sites. The final construct, pSCAT, was approximately 16.6 kb (Fig. 1A). ATM expression was driven by a synthetic early/late compound vaccinia virus promoter that allows for protein expression throughout the entire viral life cycle [19]. The 5′ and 3′ halves of the thymidine kinase (tk) gene were positioned outside the promoter and ATM cDNA, forming a recombination cassette. All plasmids were grown in MAX DH5α cells (Invitrogen, Carlsbad, CA) at 30 °C.

Construction of recombinant ATM vaccinia virus, vWR-ATM. Construction of recombinant virus was previously described [20]. Briefly, CV-1 tk- cells were infected with the WR strain of vaccinia virus (ATCC VR 1354) at a multiplicity of infection (MOI) of 0.1 pfu/cell for 2 h, followed by transfection of pSCAT using lipofectin (Invitrogen, Carlsbad, CA). After 48 h, cells were collected, resuspended in 1 ml Optimem (Invitrogen, Carlsbad, CA), sonicated, and plaqued on tk- cells to undergo selection for homologous recombination. Homologous recombination between the ATM cassette and the tk gene in the wildtype vaccinia virus resulted in integration of the ATM cDNA sequence into the genome. Repeated plaquing was performed until a purified virus was obtained. Different virus populations were tested for ATM expression. Recombinant vaccinia virus expressing full-length ATM is designated vWR-ATM. Recombinant ATM expressed by vWR-ATM is referred to as FLAG-ATM.

Immunoblot analysis and in vivo kinase assays of FLAG-ATM. Cell lysates were prepared using lysis buffer (20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 0.5% Triton X-100, 5% glycerol, 5 μg aprotinin (Sigma, St. Louis, MO), 5 μg leupeptin (Calbiochem–Novabiochem, San Diego, CA), and 1 mM PMSF (Sigma, St. Louis, MO)), incubated on ice, and cleared by centrifugation. Samples were electrophoresed on 5% or 7% denaturing polyacrylamide gels, transferred onto a nitrocellulose membrane (Osmonics, Westborough, MA), and incubated with the appropriate antibodies. Proteins were visualized using enhanced chemiluminescence (ECL; Amersham Biosciences, Piscataway, NJ). Densitometry readings were measured using Molecular Analyst System (Bio-Rad, Hercules, CA).

Cytoplasmic extracts of 1×10^6 vWR-ATM-infected L3 cells were analyzed by immunoblotting for ATM expression. Samples were collected every 4 h after infection, for 24 h. Blots were incubated with anti-ATM (Novus, Littleton, CO) or anti-FLAG M2 (Sigma, St. Louis, MO) antibodies. To observe in vivo p53 serine 15 phosphorylation, vWR-ATM-infected L3 cells were irradiated with 2 Gray IR at each timepoint collected and lysed 15 min later. Lysates were sonicated to prepare whole cell extracts and analyzed by immunoblotting. Blots were incubated with an anti-phospho-p53 serine 15 antibody (Cell Signaling, Beverly, MA) and anti-nibrin (Novus, Littleton, CO).

FLAG-ATM purification. Approximately 8×10^6 HeLa cells were infected with vWR-ATM at an MOI = 5 for 32 h. Cells were lysed with 2 mL lysis buffer (20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 0.5% Triton X-100, 5% glycerol, 5 μg aprotinin (Sigma, St.

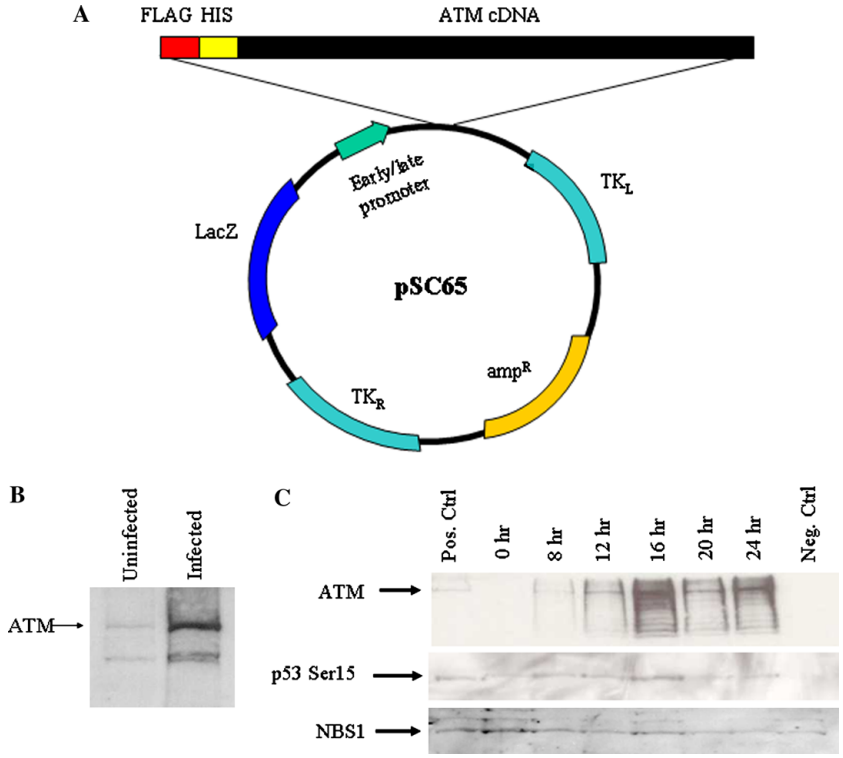

Fig. 1. (A) The pSCAT plasmid, containing full-length ATM cDNA within the pSC65 vaccinia vector. Full-length ATM cDNA, with FLAG and His tags, was subcloned into the pSC65 vector downstream of the early/late tandem promoter. Right and left halves of the thymidine kinase (tk) gene, situated on either side of ATM, direct the sites for homologous recombination, between pSCAT and the genome, to the tk gene of vaccinia. LacZ expression was used in identifying recombinant viruses that underwent complete (double) recombination. (B) Comparison of endogenous and recombinant ATM expression in infected and uninfected CV-1 cells. CV-1 cells were infected for 36 h with vWR-ATM. Whole cell lysates for uninfected and infected cells were analyzed by immunoblot analysis, incubating with anti-ATM. Densitometry quantified recombinant ATM levels at 8-fold over endogenous ATM levels. (C) FLAG-ATM expression and function was observed in L3 cells infected by vWR-ATM. Infection was examined over a 24-h period in 4-h intervals. ATM expression was observed 8 h after infection (top panel, lane 3), reaching maximal levels at 16 h (top panel, lane 5). Infection of L3 with a control construct of recombinant vaccinia virus produced no protein recognized by the ATM antibody (top panel, lane 8). Phosphorylation of p53 at Ser15 was observed from 8 h through 24 h (middle panel, lanes 3–7) with peak activity at 16 h. No phosphorylation was seen when cells were infected with the control construct (middle panel, lane 8). NBS1 protein levels were compared as a loading control (bottom panel). NBS1 and p53 levels were low due to a low viable cell count in a whole cell lysate preparation.

Louis, MO), 5 μg leupeptin (Calbiochem–Novabiochem, San Diego, CA), and 1 mM PMSF (Sigma, St. Louis, MO)), incubated for 15 min on ice, and cleared by centrifugation. NaCl concentration was increased to 350 mM for purification. Cytoplasmic extract was aliquoted into three fractions and each was incubated with 200 μl packed FLAG M2 affinity resin (Sigma, St. Louis, MO) for 2 h with constant agitation, allowing the FLAG-ATM protein to bind to the resin. Bound resin was collected by centrifugation, washed twice with lysis buffer, twice with 100 mM Tris, 0.5 M LiCl, and again with lysis buffer. One milligram per milliliter of FLAG peptide (Sigma, St. Louis, MO) was incubated with 200 μl bound resin on a rocker for 1 h to elute FLAG-ATM by peptide competition. Sequential resin binding of the same lysate was performed to deplete lysate of FLAG-ATM. Eluates were collected and concentrated using a Microcon YM-100 centrifugal filter (Millipore, Bedford, MA) in 20 mM Hepes, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM DTT, and 1 mM EDTA buffer. All purification steps were performed at 4 °C. Immunoblot analysis was performed to monitor recovery of FLAG-ATM protein during the purification procedure, incubating blots with anti-ATM. Purified FLAG-ATM was run on an acrylamide gel and silver stained to examine the purity of the sample. Protein concentration was measured by amino acid analysis. FLAG-ATM was analyzed using micro-liquid chromatography tandem mass spectrometry (μLC-MSMS)[21] to confirm ATM purification and identity.

FLAG-ATM in vitro kinase assays and phosphatase reactions. In vitro kinase assays were performed in kinase buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 10 mM $MnCl_2$, 10 mM $MgCl_2$, 1 mM DTT, 5 μg aprotinin, 5 μg leupeptin, 1 mM PMSF, and 25 nM microcystin) with 2 μl of purified FLAG-ATM, and 2 μg of either PHAS-1 (Stratagene, La Jolla, CA) or GST-p53 (Santa Cruz Biotechnology, Santa Cruz, CA) as the substrate. One hundred nanograms of sonicated sheared salmon sperm DNA (Stratagene, La Jolla, CA), DNA plasmid, or no DNA was pre-incubated with ATM for 3 min on ice. Upon addition of 20 μCi [^{33}P-γ]ATP (3000 Ci/mmol, Perkin–Elmer, Wellesley, MA) and 6.7 μM ATP, the kinase reactions were incubated at 30 °C for 15 min and stopped with SDS sample buffer. The radioactive reactions were electrophoresed on a SDS–PAGE gel, dried, and exposed to film. Twenty-five nanomolar wortmannin (Sigma, St. Louis, MO) was pre-incubated with ATM for 30 min at room temperature in inhibition reactions. Non-radioactive reactions, analyzed by immunoblotting, contained 13.3 μM ATP and were analyzed as previously described, incubating immunoblots with a phosphospecific p53 Ser15 antibody (Cell Signaling, Beverly, MA) or anti-ATM antibody. In phosphatase reactions, purified FLAG-ATM was incubated with 4 U of Protein Phosphatase 1 (New England Biolabs, Beverly, MA) in PP1 buffer and incubated at 30 °C for 1 h. Phosphorylation of serine 1981 of purified FLAG-ATM was observed by incubating immunoblots with anti-ATM protein kinase pS1981 (Rockland Immunochemicals, Gilbertsville, PA).

Atomic force microscopy visualization of ATM. For atomic force microscopy (AFM), all reactions were performed in 50 mM Hepes, pH 7.5, 150 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, and 0.1 mM EDTA. Ten microliter reactions contained a 1:5 dilution of FLAG-ATM and 1 μg/ml of a gel purified DNA fragment generated by restriction digestion of p6NPS-3 with *Eco*RV, resulting in the generation of blunt-ended linear 1236 bp DNA molecule. Reactions were incubated for 8 min at 30 °C, after which Hepes-buffered EM grade glutaraldehyde (Electron Microscopy Sciences, Fort Washington, PA) was added to a final concentration of 0.1% and incubation was continued at room temperature for at least 5 min prior to mounting. Samples were mounted by introduction of undiluted reactions to freshly cleaved mica (Ted Pella, Redding, CA) immediately followed by rinsing through a graded ethanol series using 20%, 40%, 80%, and 100% ethanol. Images were collected using a Digital Instruments NanoScope IIIa AFM in TappingMode (Veeco Metrology Group, Veeco, Santa Barbara, CA). For image analysis, random 2 μm square fields were collected and scored for the presence of DNA fragments, subdivided as either ATM bound or ATM unbound.

Results

FLAG-ATM expression and purification

HeLa cells were infected with vWR-ATM for 36 h to compare the ATM protein levels between endogenous and viral expression. Immunoblot analysis of uninfected and infected whole cell lysates showed approximately an 8-fold increase of ATM protein levels in the infected cells (Fig. 1B).

Expression and function of FLAG-ATM were examined over 24 h, using an ATM-deficient human lymphoblastoid cell line (L3), infected with vWR-ATM. L3 cells have a homozygous 103 C > T mutation, resulting in no detectable protein by immunoblotting. One million vWR-ATM infected L3 cells were collected every 4 h, exposed to 2 Gray IR, and lysed after 15 min. Immunoblot analysis of cell extracts from 0 to 24 h timepoints showed that ATM was detectable by 8 h, peaked at 16 h, and slightly decreased in later timepoints (Fig. 1C, top panel). ATM expression was not seen when L3 cells were infected with a recombinant vaccinia virus expressing a protein other than ATM, indicating that the presence of ATM was due to infection by the vWR-ATM virus (Fig. 1C, top panel, lane 8). Analysis with an antibody specific for phosphorylated p53 serine15 showed that IR-induced p53 phosphorylation was observed from 8 through 24 h after infection (Fig. 1C, middle panel). Induction of p53 serine15 phosphorylation in L3 cells signified a regain of function in an otherwise kinase deficient A-T cell line, indicating vWR-ATM expression of a functional ATM protein.

FLAG-ATM was purified using FLAG-M2 affinity resin from lysates of vWR-ATM infected HeLa cells. Peptide competition was used to elute FLAG-ATM. Samples at different elution steps were analyzed by immunoblotting, using anti-ATM antibody to monitor FLAG-ATM throughout the procedure (Fig. 2A). The

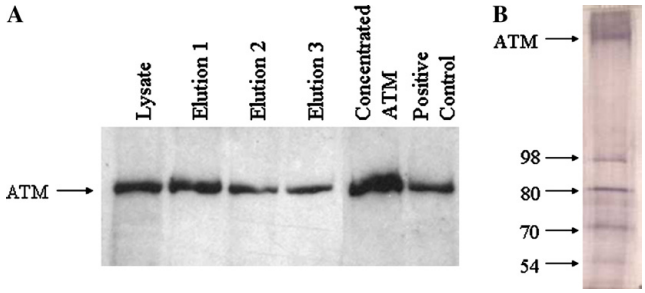

Fig. 2. (A) Immunoblot analysis of different fractions obtained from FLAG-ATM purification, using an anti-ATM antibody. Cytoplasmic protein from vWR-ATM infected HeLa cells (lane 1) was incubated with FLAG M2 resin. Peptide competition using FLAG peptide eluted FLAG-ATM from resin. Sequential elutions were performed, on the same lysate, with fresh FLAG M2 resin (lanes 2, 3, and 4). FLAG-ATM was pooled and concentrated (lane 5). Purified protein size was confirmed when compared with the positive control, ATM from a normal cell line (lane 6). (B) Concentrated purified FLAG-ATM was electrophoresed on a polyacrylamide gel and silver stained to visualize protein in sample. FLAG-ATM protein was observed at the appropriate molecular weight. FLAG-ATM was confirmed by immunoblotting (data not shown). Contaminants from 55 to 100 kDa were also noted.

presence of FLAG-ATM in the concentrated eluate was confirmed (Fig. 2A, lane 5). Analysis of blots using anti-FLAG antibody produced the same results (data not shown). Silver stain of a denaturing acrylamide gel loaded with purified FLAG-ATM showed the presence of full-length ATM, as well as other proteins ranging from 55 to 100 kDa (Fig. 2B). Peptides detected by tandem mass spectrometry confirmed ATM isolation and identity in the eluates (Table 1). The peptides were positioned in varying locations along the ATM sequence. Heat shock protein 70 (HSP70) was also identified as being present in the sample.

Purified ATM protein is functional

FLAG-ATM in vitro kinase assays containing PHAS-1 as a substrate showed comparable phosphorylation levels between reactions with or without DNA (Fig. 3A, lanes 1 and 2). ATM activity was inhibited by wortmannin pretreatment of FLAG-ATM (Fig. 3A, lanes 3 and 4), suggesting that function was retained after purification and that this kinase activity was blocked by an ATM inhibitor. FLAG-ATM activity, using PHAS-1, was comparable with or without DNA.

ATM activation is manganese-dependent

To examine the metal ion requirements of the purified protein's kinase activity, in vitro kinase reactions were performed using buffers containing 10 mM Mg^{2+}, 10 mM Mn^{2+} or neither ions. DNA was used to observe

Table 1
ATM peptides identified by mass spectrometry[a,b]

Amino acid location	Peptide sequence	ATM domain
33–41	LIRDPETI	p53 binding site
77–90	IAKPNVSASTQASR	p53 binding site
1110–1126	ALPLKLQQTAFENAYLK	Partial coverage leucine zipper
1993–2010	SKEETGISLQDLLLEIYR	FAT
2590–2604	QSSQLDEDRTEAANR	Between FAT and PI3 kinase
2764–2790	SGVLEWCTQTVPIGEFLVNNEDGAHKR	PI3 kinase

[a] Numbering according to native ATM sequence (Q13315).
[b] Only peptides that gave unique matches to experimental tandem mass spectrometry data (against the entire human proteome) were included (Sequest Xcorr >2 for peptides 1–3; Sonar scores of 0.05, 2.5, and 2.9 for peptides 4–6).

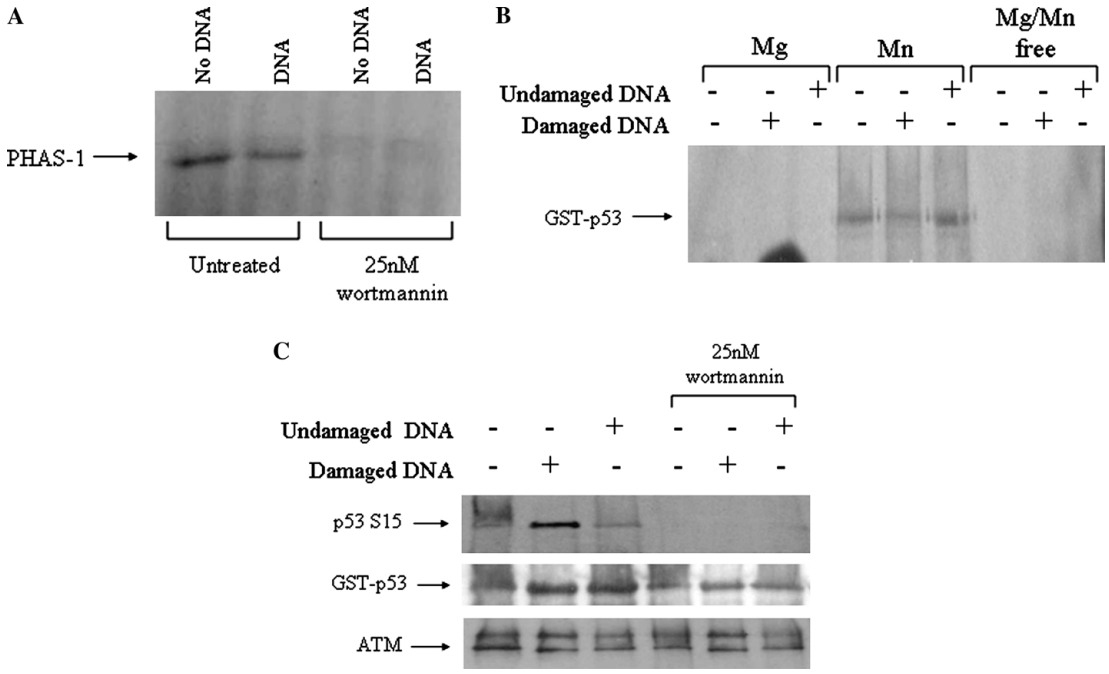

Fig. 3. (A) In vitro kinase assay using FLAG-ATM kinase and PHAS-1 substrate showed phosphorylation in reactions with and without DNA (lanes 1 and 2). Both reactions were inhibited by wortmannin (lanes 3 and 4). (B) FLAG-ATM phosphorylated GST-p53 in manganese-containing kinase buffer, in the presence or absence of DNA (lanes 4–6). No phosphorylation of GST-p53 was observed in magnesium and ion-free kinase reactions (lanes 1–3 and 7–9). Two micrograms of GST-p53 was added to all reactions. (C) GST-p53 was phosphorylated by FLAG-ATM at Ser15 in in vitro kinase reactions containing damaged DNA (top panel, lane 2). Weak phosphorylation was also noted in the absence of DNA (lane 1) or in the presence of undamaged DNA (lane 3). Phosphorylation in all reactions was inhibited by wortmannin (top panel, lanes 4–6). Blot was stripped and re-probed with anti-p53 to show loading of substrate (middle panel). ATM levels show that comparable amounts of FLAG-ATM were added to each reaction (bottom panel).

DNA activation of ATM kinase activity; sonicated sheared salmon sperm represented DNA with double strand break damage while plasmid DNA represented undamaged DNA, with no breaks. Reactions in the manganese kinase buffer produced phosphorylation of GST-p53 by FLAG-ATM, regardless of DNA content (Fig. 3B, lanes 4, 5, and 6). Reactions in the magnesium and magnesium/manganese free buffers did not phosphorylate GST-p53 (Fig. 3B, lanes 1–3 and 7–9), suggesting that FLAG-ATM kinase activity is dependent on manganese. FLAG-ATM also exhibited kinase activity in the presence and absence of DNA.

ATM activation exhibits DNA influence in p53 kinase reactions

In vitro kinase reactions with GST-p53 were performed in the presence and absence of DNA. Immunoblotting of the FLAG-ATM kinase reactions, using a phospho-p53 serine15 antibody, showed that both reactions without damaged DNA contained comparable phosphorylation levels (Fig. 3C, top panel, lanes 1 and 3). However, a significant increase in serine 15 phosphorylation was observed in the presence of damaged DNA (Fig. 3C, top panel, lane 2). Pretreatment of

FLAG-ATM with wortmannin prior to the kinase reactions inhibited phosphorylation (Fig. 3C, top panel, lanes 4–6). Reactions containing no FLAG-ATM exhibited no serine 15 phosphorylation (data not shown); therefore, phosphorylation was dependent on FLAG-ATM activity under the conditions of the assay.

Purified FLAG-ATM is already autophosphorylated on S1981

When purified FLAG-ATM was tested with a phospho-specific antibody for ATM serine 1981, before and after phosphatase treatment, it was clear that the purified protein was already activated (Fig. 4A). ATM levels showed equal loading in both lanes.

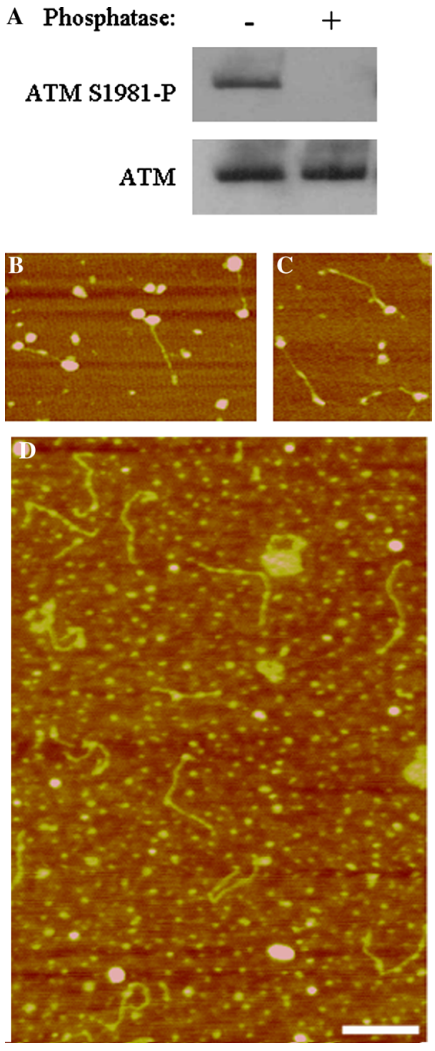

Fig. 4. (A) FLAG-ATM was treated (+) with phosphatase and analyzed by immunoblotting for autophosphorylation, using phosphoserine antibody to Ser 1981. Only the untreated (−) FLAG-ATM showed autophosphorylation at S1981 (top panel, lane 1). Reactions contained equal amounts of FLAG-ATM (bottom panel). (B,C) AFM images reveal abundant DNA–FLAG-ATM complexes in reactions containing mock phosphatase-treated FLAG-ATM and linear DNA. (D) Reactions containing linear DNA and phosphatase-treated FLAG-ATM exhibited markedly reduced DNA binding. Bar in (D) represents 200 nm for (B–D).

Atomic force microscopy of purified ATM shows DNA binding

To examine the DNA binding behavior of FLAG-ATM, in either the activated or deactivated form (with or without phosphorylation of serine 1981), we used AFM, following incubation with a blunt-ended linear DNA (Figs. 4B–D). Reactions containing FLAG-ATM and linear DNA were chemically fixed using glutaraldehyde after an 8-min incubation at 30 °C. Following fixation, reactions were mounted on freshly cleaved mica substrates and visualized by AFM. Images were scored for the presence of FLAG-ATM bound and unbound DNA molecules. FLAG-ATM bound DNA species were further characterized with respect to the location of FLAG-ATM at either internal positions or DNA termini (Table 2). In the absence of phosphatase treatment, 44% of the scored DNA molecules were found to carry particles with a size and visual appearance consistent with FLAG-ATM. Of the DNA molecules scored as FLAG-ATM bound, 38% were bound by FLAG-ATM on at least one DNA end. Phosphatase-treated FLAG-ATM preparations exhibited reduced DNA binding activity with only 20% of the DNA fragments displaying FLAG-ATM association; 48% of those associations were at DNA ends. A two-tailed test revealed the significant difference ($p < 0.001$) in DNA binding between phosphatase-treated FLAG-ATM and mock phosphatase-treated protein. While DNA binding was, overall, reduced by phosphatase treatment, FLAG-ATM/DNA complexes formed by either phosphatase-treated or -untreated FLAG-ATM displayed no significant difference with respect to whether binding took place at ends or mid-strand ($p > 0.2$). These data suggest that those FLAG-ATM molecules that retain DNA binding properties following phosphatase treatment associate with linear DNA in a manner similar to that of untreated FLAG-ATM and may, therefore, represent a population of the phosphatase-treated proteins that evaded dephosphorylation.

Table 2
FLAG-ATM bound to DNA using atomic force microscopy

Phosphatase treatment	Total DNA scored	Total FLAG-ATM bound to DNA	FLAG-ATM bound to DNA ends	Unbound DNA
No Treatment	238	105 (44%)	38%	133
Treatment	275	56 (20%)	48%	219
		($p < 0.001$)	($p > 0.2$)	

Discussion

Successful expression of FLAG-ATM with vWR-ATM makes the vaccinia viral system a novel technique for producing large quantities of ATM protein. Viral ATM has been expressed 8-fold over endogenous levels (Fig. 1B). The viral genome can incorporate and express large pieces of foreign DNA; the ATM coding sequence is over 9 kb. Equally important is cytoplasmic transcription. The vaccinia DNA genome contains no introns, thereby circumventing any idiosyncrasies of splicing due to cryptic splice sites, and performs transcription outside of the host nucleus. Endogenous ATM is predominantly nuclear although some cytoplasmic protein is found [22,23]. Although the majority of the recombinant ATM protein was cytoplasmic, FLAG-ATM was found in the nucleus as well (data not shown), most likely due to saturation in the nucleus. We used this in our favor because it allowed for gentle lysis without the use of sonication or other potentially harmful disruption methods that would result in damage to such a large protein.

Purification of FLAG-ATM using the FLAG M2 affinity resin was the most successful technique of several methods evaluated. However, other protein contaminants were also present. From 8×10^6 cells, we purified about 30 μg of FLAG-ATM, judging from amino acid analysis. Tandem mass spectrometry also identified high levels of HSP 70, a eukaryotic chaperone protein involved in protein folding and trafficking. This may be one of the contaminants present in the silver stain (Fig. 2B).

Infection of HeLa cells with vWR-ATM and purification of FLAG-ATM can be scaled up for production of large amounts of ATM. The live virus infects virtually 100% of cells, reaching maximum efficiency in a given number of cells. A major disadvantage of using the vaccinia virus as an overexpression system is the lack of stable ATM expression. We are unable to produce a constant supply of protein from infected cells because, as part of the virus life cycle, the host cell dies in 48 h. Re-infection of a new population of host cells with vWR-ATM is necessary for each round of protein production.

Purified FLAG-ATM exhibited manganese-dependent kinase activity and phosphorylation of PHAS-1 and GST-p53 targets, as previously reported [11,24,25]. Interestingly, FLAG-ATM kinase activity was significantly stronger in the presence of damaged DNA in the GST-p53 reactions. Smith et al. [9] observed similar results when the purified endogenous ATM from HeLa nuclear extracts showed binding to a DNA cellulose column, binding to DNA ends using AFM, and increased kinase activity with 5 ng of sheared DNA. In another report, endogenous ATM exhibited kinase activity that was activated by charged biological molecules [10]. DNA influenced ATM phosphorylation of replication factor a (RPA) [26]. ATM also exhibits a 3-fold binding increase when double-stranded DNA cellulose is irradiated [27]. ATM binds tightly to chromatin and is resistant to extraction after neocarzinostatin treatment, providing ex vivo evidence of ATM's association with DNA or chromatin [28].

The impact of DNA on ATM kinase activity has been controversial. DNA-stimulated kinase activity was not observed by Chan et al. [11] who purified ATM from human placenta. Kozlov et al. [29] also reported no DNA-induced increased activity in immunoprecipitated ATM. The discrepancies of DNA influence on ATM kinase activity between the different ATM preparations may be due to factors or proteins co-purified with ATM during the isolation process. The autophosphorylated state of purified ATM may also be different for placenta-purified ATM and our vaccinia-expressed ATM. Bakkenist and Kastan [30] proposed that inactive ATM is complexed as a homodimer and released as active monomers after autophosphorylation of both molecules on serine 1981. Phosphorylation of serine 1981 in purified FLAG-ATM supports the observed kinase activity in reactions without damaged DNA.

Discrepancies in DNA-stimulated kinase activity were also observed in ex vivo kinase reactions using endogenous ATM kinase. Canman et al. [24] and Banin et al. [25] both reported that DNA had no influence on ATM phosphorylation of p53 in vivo. Previous reports using ATM and DNA provide image-based evidence of an ATM–DNA complex [9,31]. Our observations of significant differences in the DNA binding properties of FLAG-ATM with and without phosphatase treatment suggest an important role for the phosphorylation state on ATM interactions with DNA. Serine 1981-phosphorylated FLAG-ATM displayed robust DNA binding, as determined by direct AFM visualization of the complexes formed by the protein in the presence of linear plasmid DNA. FLAG-ATM treated with phosphatase, however, exhibited significantly reduced DNA binding properties using the same AFM-based analysis. These data suggest that the phosphorylation state may be responsible for modulating interactions with DNA in vivo and that DNA modulated kinase activity may be a result of ATM phosphorylation-dependent competency to associate with DNA.

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