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ATP Activates Ataxia-Telangiectasia Mutated (ATM) *in Vitro*

IMPORTANCE OF AUTOPHOSPHORYLATION*

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Ataxia-telangiectasia Mutated (ATM), mutated in the human disorder ataxia-telangiectasia, is rapidly activated by DNA double strand breaks. The mechanism of activation remains unresolved, and it is uncertain whether autophosphorylation contributes to activation. We describe an *in vitro* immunoprecipitation system demonstrating activation of ATM kinase from unirradiated extracts by preincubation with ATP. Activation is both time- and ATP concentration-dependent, other nucleotides fail to activate ATM, and DNA is not required. ATP activation is specific for ATM since it is not observed with kinase-dead ATM, it requires Mn^{2+}, and it is inhibited by wortmannin. Exposure of activated ATM to phosphatase abrogates activity, and repeat cycles of ATP and phosphatase treatment reveal a requirement for autophosphorylation in the activation process. Phosphopeptide mapping revealed similarities between the patterns of autophosphorylation for irradiated and ATP-treated ATM. Caffeine inhibited ATM kinase activity for substrates but did not interfere with ATM autophosphorylation. ATP failed to activate either A-T and rad3-related protein (ATR) or DNA-dependent protein kinase under these conditions, supporting the specificity for ATM. These data demonstrate that ATP can specifically induce activation of ATM by a mechanism involving autophosphorylation. The relationship of this activation to DNA damage activation remains unclear but represents a useful model for understanding *in vivo* activation.

Neurodegeneration, immunodeficiency, genome instability, and cancer susceptibility represent the major hallmarks of the human genetic disorder ataxia-telangiectasia (A-T)[1] (1, 2). Extreme sensitivity to radiation in patients undergoing radiotherapy for cancer and in cells in culture are also characteristic of this disease (3–5). The exact basis of the radiosensitivity in A-T cells remains unknown but appears to be related to the failure to repair residual chromosomal breaks (6, 7) or activation of the structural maintenance of chromosomes (SMC1) protein in response to DNA damage, which is ATM-dependent and implicated in modulating radiosensitivity (8, 9). In A-T cells, faulty DNA damage recognition is also accompanied by defective cell cycle checkpoint activation, which is likely to contribute to the genetic instability and cancer susceptibility in this syndrome (10, 11).

The gene defective in A-T, *ATM* (A-T mutated), encodes a protein that is a member of the phosphatidylinositol 3-kinase (PI3-kinase) family (12). This group includes the catalytic subunit of DNA-dependent protein kinase (DNA-PKcs), A-T and rad3-related protein (ATR), and proteins in different organisms responsible for DNA damage recognition and cell cycle control (13). Although there is some evidence for regulation of ATM at the transcriptional and translational levels (14), it is primarily activated as a pre-existing protein by ionizing radiation and other agents that give rise to double strand breaks in DNA, suggesting that post-translational modification may be important in its activation (15–17). Changes in abundance of ATM protein in response to radiation have not been observed (18). Once activated, ATM phosphorylates a host of substrates, primarily involved in recognizing DNA damage or signaling this damage to cell cycle checkpoints (19), but there is also evidence for non-DNA damage activation (20). Among the ATM substrates involved in DNA damage recognition are Nbs1, defective in the Nijmegen breakage syndrome (21–23), BRCA1 (24, 25), Rad51 (26), and BLM, defective in Bloom's syndrome (27). ATM also plays a central role in the activation of cell checkpoints in response to radiation damage (10, 11). It is of some interest that this regulation occurs at multiple levels for a single checkpoint. In the case of the G_1/S checkpoint, activated ATM phosphorylates p53 on serine 15 (15–17), it also phosphorylates and activates Chk2 to in turn phosphorylate p53 on serine 20 (28), which may contribute to its stabilization at least in some cell types, and by phosphorylating mdm2 on serine 395 (29), it further ensures stabilization and activation of p53 to induce p21/WAF1 and the G_1/S checkpoint (30, 31). ATM phosphorylates Chk2, Nbs1, SMC1, and BRCA1 to achieve activation of the S phase checkpoint in parallel pathways (8, 9, 32). Finally, Chk2 and BRCA1 are also substrates for ATM in the G_2/M checkpoint (33).

Although multiple substrates and pathways controlled by ATM have been identified, it remains unclear as to how double strand breaks in DNA activate its kinase activity. In the case of the related PI3-kinase, DNA-PKcs, it is recruited to DNA strand breaks and activated by the Ku70/Ku80 heterodimer

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[1] The abbreviations used are: A-T, ataxia-telangiectasia; ATM, ataxia-telangiectasia mutated; ATR, ataxia-telangiectasia and Rad3-related; DNA-PK, DNA-dependent protein kinase; DNA-PKcs, DNA-PK catalytic subunit; BLM, defective in Bloom's Syndrome; Nbs1, defective in Nijmegen breakage syndrome; GST , glutathione *S*-transferase; Gy, gray; PI3-kinase, phosphatidylinositol 3-kinase; mTOR, mammalian target of rapamycin; AMP-PNP, 5′-adenylylimidodiphosphate.

(34). Intriguingly, inositol hexakisphosphate stimulates DNA-PK-dependent non-homologous DNA end joining *in vitro*, and this appears to be achieved by specific interaction with Ku70/Ku80 (35). Another member of the PI3-kinase family mTOR is altered in its activity by small molecules, acting as a sensor of ATP concentration (36). Protein kinase activity can also be regulated by autophosphorylation, which has been observed with DNA damage signaling PI3-kinases (37). However, only DNA-PK autophosphorylation was demonstrated to have an effect on the functional activity of the enzyme in a DNA-dependent manner (38). Autophosphorylation causes inactivation of DNA-PK kinase activity and disruption of the DNA-PKcs-Ku complex. Subsequently, it was shown that phosphatase treatment activated DNA-PK (39). Autophosphorylation has also been observed with ATM immunoprecipitated from irradiated control cells and with ATM expressed in baculovirus (25, 40), and there is evidence using ^{32}P-labeling *in vivo* to support this (23, 41), but its importance to ATM function has not been addressed. The putative autophosphorylation sites on ATM were suggested based on consensus motifs expected to be recognized by ATM in oriented peptide libraries (42). These include Ser-2941, Ser-1635, and Ser-440, but phosphorylation of these sites *in vivo* has not been reported. To investigate the activation of ATM and the possible involvement of autophosphorylation in this process, we have established an *in vitro* system involving immunoprecipitation of ATM and activation by ATP.

EXPERIMENTAL PROCEDURES

Materials and Reagents—Cell culture medium RPMI 1640 was from Invitrogen. Fetal bovine serum was from JRH Biosciences. Microcystin LR was from Biomol Research Laboaratories, Inc. (Sapphire Bioscience). λ-Protein phosphatase (λ-PPase) was from New England Biolabs. ATP, ADP, AMP, NADP, 5′-adenylylimidodiphosphate (AMP-PNP), GTP, and okadaic acid were from Sigma. Wortmannin and caffeine were from Fluka Chemie AG. Protein G-Sepharose 4 fast flow, protein A-Sepharose CL-4B, and Redivue [γ-^{32}P]ATP (specific activity ~3000 Ci/Mmol) were from Amersham Biosciences. Western Lightning chemiluminescence reagent was from PerkinElmer Life Sciences. Anti-mouse and anti-rabbit antibodies conjugated with peroxidase were from Silenus/Chemicon Australia. For ATM Western blotting, ATM 2C1 monoclonal antibody (GeneTex) was used. For ATR Western blotting and immunoprecipitations, ATR PA1–450 antibody (Affinity Bioreagents) was employed. Western blotting and immunoprecipitations for DNA-PK were performed with Ab-2 monoclonal antibody (Oncogene Research Products). All other reagents were from standard suppliers or as indicated in the text.

Cell Lines and Irradiation—Lymphoblastoid cell lines established from normal (C3ABR) and A-T patients (L3 and AT1ABR) were used. We acknowledge Yosef Shiloh, Tel Aviv, Israel for the L3 cell line that fails to produce ATM protein due to a homozygous truncation mutation (C103T). AT1ABR is an A-T cell line homozygous for the 7636del9 mutation that produces near full-length ATM protein that is kinase-dead (25). The Lymphoblastoid cell lines were cultured in RPMI 1640 medium with 10% fetal calf serum, 100 units/ml penicillin (Invitrogen), and 100 units/ml streptomycin (Invitrogen). All irradiations were performed at room temperature using an IBL437C irradiator (2.4 Gy/min, Compagnie ORIS Industrie).

Antibody Production—Construction of GST fusion proteins containing fragments of ATM protein was described previously (17). GST-ATM2 fusion protein (ATM amino acids 250–522) was expressed according to standard protocol (43). Full-length GST-ATM2 was isolated by preparative SDS-PAGE, eluted from gel slices, and used for sheep immunization according to standard protocols. Antibodies were purified from collected sheep sera by two consecutive affinity chromatography steps. GST-Affi-Gel 15 and GST-ATM2-Affi-Gel 15 affinity columns were synthesized according to the manufacturer's instructions (Bio-Rad). Firstly, serum diluted 1/10 in phosphate-buffered saline was applied to a GST-Affi-Gel 15 column to deplete it from anti-GST antibodies. Secondly, serum was passed through a GST-ATM2-Affi-Gel 15 column, and ATM-specific antibodies were eluted, dialyzed against phosphate-buffered saline containing 0.05% sodium azide, and concentrated by ultrafiltration on Ultrafree-4 BioMax 30K centrifugal filter (Millipore). Affinity-purified antibodies were used in all subsequent immunoprecipitations and kinase assays.

Immune Complex Protein Kinase Assays—Cells were collected by diluting with an equal volume of ice-cold phosphate-buffered saline containing 1 mM Na_3VO_4 and 1 mM NaF followed by centrifugation. After a brief wash with ice-cold phosphate-buffered saline, cells were lysed immediately for use in immune complex kinase assays. ATM, ATR, and DNA-PK kinase assays were performed as described (16, 44, 45) with modifications. 0.2% Tween 20 lysis buffer was modified by addition of 1 mM Na_3VO_4, 1 mM NaF, 10 mM Na_2MoO_4, 20 mM β-glycerophosphate, 5 μM microcystin-LR, 5 nM okadaic acid, 5 μg/ml each of aprotinin, leupeptin, and pepstatin, 1 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol. Cells were lysed by rotating for 45 min at 4 °C followed by centrifugation at 13,000 × *g* for 10 min. Cell lysates were precleared by constant mixing for 1 h with protein A/G-Sepharose. 1 mg of precleared lysate was used for immunoprecipitation with ATM, ATR, or DNA-PK antibodies (see above) overnight with constant mixing. Immune complexes were absorbed onto protein G-Sepharose and washed. After two washes with ice-cold lysis buffer, one wash with high salt buffer (lysis buffer with 0.5 M NaCl), and two washes with basic kinase buffer, immunocomplexes were subjected to procedures outlined below or used directly in kinase reactions. All washing buffers contained 1 mM Na_3VaO_4, 1 mM NaF, and 1 mM phenylmethylsulfonyl fluoride. The immunoprecipitates were resuspended in 30 μl of kinase buffer as described (44), and kinase reaction was carried out for 30 min at 30 °C either for autophosphorylation or for substrate phosphorylation of 1 μg of GST-p53 $_{1-44}$. Linear reaction conditions were established for ATM kinase assays. The reaction was stopped by addition of 5 μl of 6× SDS gel loading buffer. The reaction products were separated by SDS-PAGE. A biphasic gel system similar to one described by Ziv *et al.* (46) was used. The top part of the separating gel (5%) was used for immunoblot analysis of ATM, ATR, or DNA-PK to give an accurate estimation of the amount of kinase present in the reaction. The bottom part of the gel (12%) was stained with colloidal Coomassie G-250 to visualize the amount of p53 substrate present in the kinase reaction. Gels were dried onto a Whatmann 3 MM filter paper and exposed to an x-ray film (Fuji Photo Film Co., Ltd.) with an intensifying screen at −80 °C. Radioactivity was quantitated by excising ATM and p53 bands from the gel and counting the incorporated ^{32}P by liquid scintillation counting.

Activation and Inhibition of Kinase Activities—To prephosphorylate ATM prior to kinase reaction, washed protein G-bound ATM immunocomplexes were incubated with 1 mM ATP (or concentrations as indicated in the figure legends) in the following phosphorylation buffer: 20 mM Tris-HCl (pH 7.5), 20 mM $MgCl_2$, 10 mM $MnCl_2$, 20 mM β-glycerophosphate, 1 mM Na_3VO_4, 20 μM microcystin. Reactions were incubated for 30 min at 30 °C, washed three times in ice cold basic kinase buffer to remove the ATP, and then directly used in kinase assays as described above. Treatment of ATM immunocomplexes with ADP, AMP, NADP, AMP-PNP, GTP, dNTPs, and polyADP ribose was performed as outlined for ATP. Wortmannin was dissolved in Me_2SO to make a 20 mM solution, which was stored in aliquots at −80 °C. Wortmannin was used at 1 μM concentration in the reaction conditions described above. Caffeine was prepared fresh as a 100 mM solution in water. ATM immunocomplexes were incubated on ice with a range of concentrations of caffeine as indicated in phosphorylation buffer for 15 min and then subjected to the reaction conditions described above.

Phosphatase Treatment of Immune Complexes—Protein G-bound ATM immunocomplexes were additionally washed twice with basic phosphatase buffer (50 mM Tris-HCl, pH 7.5, 5 mM dithiothreitol, 2 mM $MnCl_2$) and then incubated with 100 units of λ-protein phosphatase (New England Biolabs) for 30 min at 30 °C. The reaction was stopped by addition of ice-cold basic kinase buffer containing 10 mM Na_3VoO_4 and 10 mM NaF. Phosphatase-treated immune complexes were washed twice with basic kinase buffer and used in kinase assays as described above.

Tryptic Phosphopeptide Mapping—Protein G-bound ATM-immunocomplexes from unirradiated, irradiated, and ATP-treated samples were subjected to the autophosphorylation reaction conditions described above. ^{32}P-labeled ATM was separated by 5% SDS-PAGE and visualized by autoradiography. The ATM band was excised and subjected to in-gel digestion using trypsin (sequencing grade, Promega). Peptides were extracted, and two-dimensional phosphopeptide mapping on thin-layer cellulose plates was performed as described (47) using electrophoresis at pH 1.9 in the first dimension followed by chromatography in buffer containing *n*-butanol/pyridine/acetic acid/water (15:10:3:12). Plates were exposed to x-ray film with an intensifying screen at −70 °C to detect phosphopeptides.

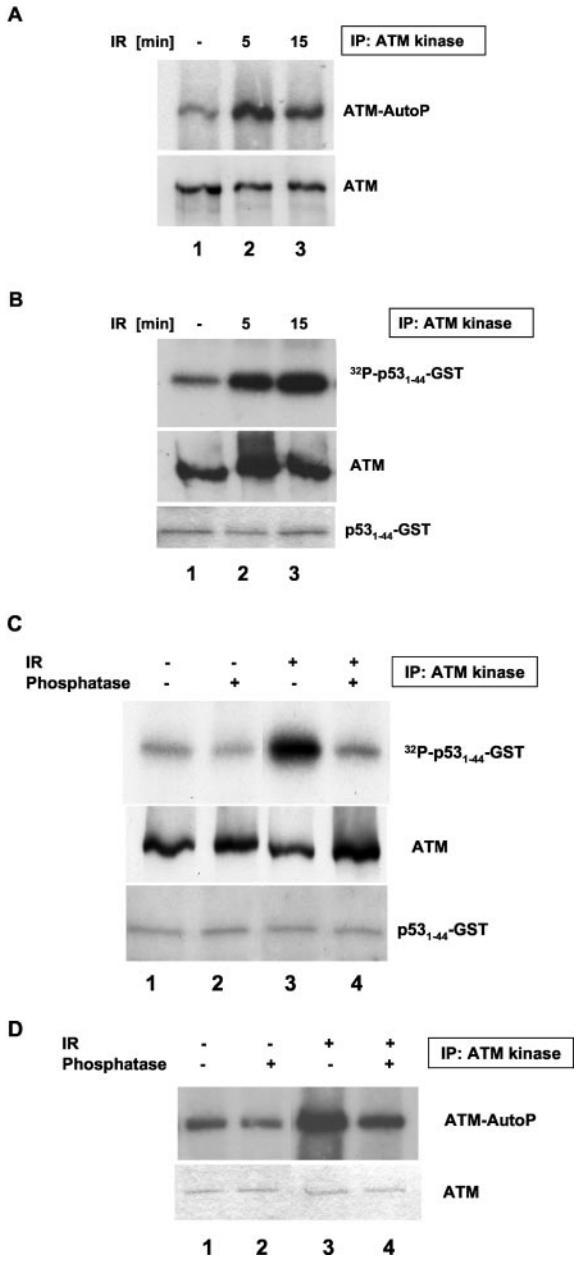

FIG. 1. **Radiation-induced activation of ATM.** *A*, radiation-induced increase in ATM autophosphorylation *in vitro*. ATM was immunoprecipitated from unirradiated and irradiated (5- and 15-min incubation) control C3ABR cells, and autophosphorylation was determined by incubation with 10 μCi of [γ-^{32}P]ATP, separation on 5% SDS-PAGE followed by transfer onto a nitrocellulose membrane, and autoradiography. The same membrane was immunoblotted with an anti-ATM antibody to determine ATM loading. *IR*, 10 Gy of ionizing radiation. *IP*, immunoprecipitate. *ATM-AutoP*, autoradiogram of radioactive phosphate incorporated into ATM. *ATM*, Western blot of the amount of ATM protein in the autophosphorylation reaction. *^{32}P-p53$_{1-44}$-GST*, autoradiogram of radioactive phosphate incorporated into the substrate peptide. *p53$_{1-44}$-GST*, Coomassie-stained gel indicating the amount of substrate in the reaction. *B*, radiation-induced increase in ATM kinase activity *in vitro*. Immunoprecipitated ATM was used in a standard kinase reaction in the presence of 10 μCi of [γ-^{32}P]ATP using GST-p53$_{1-44}$ as substrate. *ATM*, Western blot of the amount of ATM protein in the kinase reaction. As shown in *C*, phosphatase treatment abrogates ATM kinase activity *in* vitro. Immunoprecipitated ATM was incubated in phosphatase buffer or phosphatase buffer containing 100 units of λ-phosphatase for 30 min at 30 °C. After subsequent removal and inactivation of phosphatase by extensive washing with kinase buffer containing phosphatase inhibitors, ATM was subjected to a standard kinase reaction in the presence of 10 μCi of [γ-^{32}P]ATP using GST-p53$_{1-44}$ as substrate. As shown in *D*, phosphatase treatment affects ATM autophosphorylation *in vitro*. Immunoprecipitated ATM was incubated in phosphatase buffer or phosphatase buffer containing 100 units of λ-phosphatase for 30 min at 30 °C. After subsequent removal and inactivation of phosphatase by extensive washing with kinase buffer containing phosphatase inhibitors, ATM was subjected to autophosphorylation reaction in the presence of 10 μCi of [γ-^{32}P]ATP.

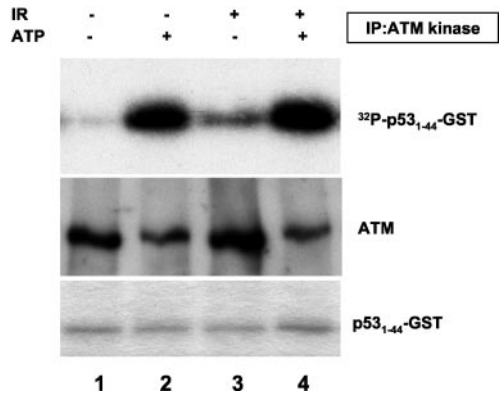

FIG. 2. **Preincubation of ATM from C3ABR cells with ATP causes a marked increase in ATM kinase activity *in vitro*.** Immunoprecipitated ATM was activated by incubation for 30 min at 30 °C in kinase buffer with and without 1 mM unlabelled ATP. After removal of ATP by extensive washing with kinase buffer, ATM was subjected to a standard kinase reaction using GST-p53$_{1-44}$ as substrate. *IP*, immunoprecipitate. *^{32}P-p53$_{1-44}$-GST*, autoradiogram of radioactive labeled substrate. *ATM*, Western blot of the amount of ATM protein in the kinase reaction. *p53$_{1-44}$-GST*, Coomassie-stained substrate in the reaction.

RESULTS

ATM Undergoes Autophosphorylation Post-irradiation—We initially determined whether ATM undergoes autophosphorylation in response to ionizing radiation using immunoprecipitation with anti-ATM antibody. Autophosphorylation of ATM was observed by 5 min after irradiation, and this did not appear to increase further up to 15 min after irradiation (Fig. 1*A*). Indeed, no increase in autophosphorylation was observed up to 1 h after irradiation (results not shown). On average, the extent of this increase was 2-fold as determined by scintillation counting of the ATM bands. As expected, ATM kinase activity toward p53$_{1-44}$ substrate increased in parallel, with time (Fig. 1*B*). No change in the amount of ATM protein occurred under these conditions. These data, together with previous results showing that ATM is a phosphoprotein (41), would predict that phosphatase treatment would abolish ATM kinase activity. The results in Fig. 1*C* demonstrate that when ATM is immunoprecipitated from irradiated cell extracts and subsequently treated with phosphatase, most of its kinase activity is lost (*lanes 3* and *4*). This is not an artifact due to residual phosphatase since addition of ^{32}P-labeled p53 to this incubation did not result in any release of free ^{32}P (results not shown). We also observed a decrease in the capacity of ATM to autophosphorylate after pretreatment with phosphatase (Fig. 1*D*). Thus it appears that phosphorylation status of ATM is critical to its kinase activity.

ATP Activates ATM Kinase—Previous data have revealed that when inactive forms of various protein kinases are incubated with unlabelled ATP, they became activated by mechanisms involving autophosphorylation (48–50). Since we have shown in Fig. 1 that autophosphorylation appears to be important for ATM activation, we rationalized that ATM protein, immunoprecipitated from unirradiated cell extracts, had the potential to be activated by significantly higher concentrations of ATP than those utilized to measure ATM kinase under standard conditions. Immunoprecipitated ATM protein from unirradiated extracts was incubated with unlabelled ATP (1

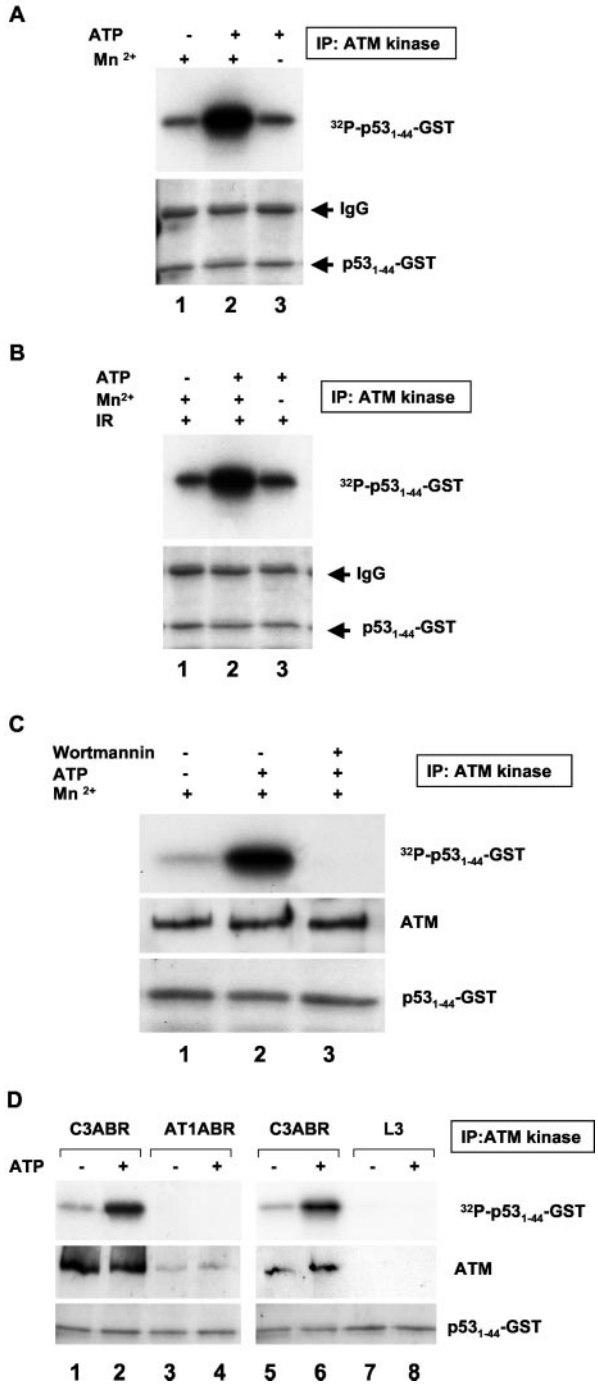

FIG. 3. **Specific activation of ATM kinase by ATP from C3ABR cells.** As shown in *A*, ATM kinase activation by ATP from unirradiated extracts is Mn^{2+}-dependent. Immunoprecipitated ATM was activated by incubation for 30 min at 30 °C in kinase buffer containing 1 mM unlabelled ATP in the presence or absence of Mn^{2+}. After removal of ATP by extensive washing with kinase buffer, ATM was subjected to a standard kinase reaction using GST-$p53_{1-44}$ as substrate. *IP*, immunoprecipitate. *^{32}P-$p53_{1-44}$-GST*, autoradiogram of radioactive labeled substrate. *$p53_{1-44}$-GST*, Coomassie-stained substrate in the reaction. As shown in *B*, ATM kinase activation by ATP in irradiated cell extracts is Mn^{2+}-dependent. ATM immunoprecipitated from cell extracts prepared form irradiated cells was activated by incubation for 30 min at 30 °C in kinase buffer containing 1 mM unlabelled ATP in the presence or absence of Mn^{2+}. After removal of ATP by extensive washing with kinase buffer, ATM was subjected to a standard kinase reaction using GST-$p53_{1-44}$ as substrate. *IR*, 10 Gy of ionizing radiation. *ATM*, Western blot of the amount of ATM protein in the kinase reaction. As shown in *C*, ATM kinase activation by ATP is inhibited by wortmannin. Immunoprecipitated ATM was incubated for 30 min at 30 °C in kinase buffer or kinase buffer containing 1 μM wortmannin and/or 1 mM ATP. After washing with kinase buffer, ATM was subjected to a normal kinase reaction using GST-$p53_{1-44}$ as substrate. As shown in *D*, ATM kinase activation by ATP is absent in A-T cell lines. Cell extracts were prepared from control (*C3ABR*) and two A-T cell lines, L3 not expressing ATM protein and AT1ABR expressing near full-length mutated ATM. Immunoprecipitates were activated by incubation for 30 min at 30 °C in kinase buffer containing 1 mM unlabelled ATP. After removal of ATP by extensive washing with kinase buffer, ATM was subjected to a standard kinase reaction using GST-$p53_{1-44}$ as substrate.

mM) for 30 min at 30 °C followed by washing out excess ATP and carrying out ATM kinase activity by standard assay. Under these conditions, we observed a dramatic increase in ATM kinase activity after preincubation with unlabelled ATP (Fig. 2).

The amount of ATM kinase activity exceeded that observed with ATM from irradiated extracts, 10-fold as compared with ~2-fold (Fig. 2, *lanes 2* and *3*). A further increase in kinase activity was also observed where ATM from irradiated extracts was preincubated with unlabelled ATP, and the overall extent of activation was approximately the same in both cases (Fig. 2, *lanes 2* and *4*). These results suggest that after irradiation, only a proportion of ATM protein is activated, but in the presence of excess ATP *in vitro*, all of the molecules are activated.

Activation Is ATM-specific—It is well established that *in vitro* ATM kinase activity has a requirement for Mn^{2+} (15, 16) and that low concentrations of the PI3-kinase inhibitor wortmannin inhibit ATM kinase (45). The results in Fig. 3*A* show that preincubation with ATP activates ATM kinase from unirradiated cell extracts in the presence of $MnCl_2$, but in its absence, no activation is observed.

Additional activation of ATM from irradiated extracts was seen with ATP, and again, this was Mn^{2+}-dependent (Fig. 3*B*). Wortmannin, when included in the preincubation step with ATP, completely abolished activity (Fig. 3*C*). No activation was obtained when ATP was incubated with immunoprecipitates from an A-T cell line expressing mutant protein (Fig. 3*D*, *lanes 3* and *4*). This A-T cell line has an in-frame 9-nucleotide deletion (7636del9) upstream from the kinase domain of ATM and expresses a less stable mutant form of ATM (*middle panel*). A second A-T cell line, L3, homozygous for a truncating mutation at nucleotide 103 and not producing ATM protein, also failed to show a response to ATP (Fig. 3*D*, *lanes 7* and *8*), supporting specificity for ATM in this activation. To define in more detail the dependence for ATP in ATM activation, we carried out ATP concentration and time course experiments. The results in Fig. 4*A* demonstrate that ATP concentrations of 50 μM and above activate ATM kinase after a 30-min preincubation. Activation was evident after a 5-min preincubation with 1 mM ATP, reaching a maximum by 30 min (Fig. 4*B*). Comparison is made with the effect of radiation (*lanes 1* and *2*)

Preincubation with ATP for 30 min followed by an increasing time of incubation in [γ^{32}P[ATP led to a gradual increase in p53 substrate phosphorylation (Fig. 4*C*). To determine whether ATM activation required ATP hydrolysis, immunoprecipitated ATM was preincubated with a non-hydrolyzable ATP analogue, AMP-PNP. In contrast to preincubation with ATP, AMP-PNP failed to activate ATM kinase toward p53 substrate (Fig. 5, *lane 4*). Substitution of ATP with GTP in the preincubation reaction did not cause any significant change in the level of ATM kinase activity (data not shown). Only ADP, dATP, and TTP had any appreciable effect on activity, and these were considerably less effective than ATP (Fig. 5). Addition of sonicated DNA to the preincubation step or to the incubation mix did not alter the kinase activity (results not shown). In addition, DNase treatment of the ATM immunoprecipitates did not prevent ATP activation of ATM (results not shown).

ATM Is Activated by Autophosphorylation in Vitro—To fur-

ther confirm that autophosphorylation is an inherent part of ATM activation, we carried out successive treatments with ATP and phosphatase with immunoprecipitated ATM from irradiated and unirradiated extracts. As observed above, ATP activated ATM kinase from unirradiated extracts, and subsequent phosphatase treatment abolished this capacity for activation (Fig. 6*A*, *lanes 3* and *4*). However, it was possible to restore activity after phosphatase treatment and washing away excess phosphatase by incubating again with ATP prior to carrying out the kinase assay (Fig. 6*A*, *lane 5*). This was also the case for immunoprecipitates from irradiated extracts (Fig. 6*A*, *right panel*). Increasing ATP concentrations over the range 0–2 mM failed to show a change in ATM autophosphorylation either for irradiated or for unirradiated immunoprecipitates (Fig. 6*B*). This can be explained by having different levels of activation of ATM at different ATP concentrations. In the less activated case, at lower ATP preincubation concentrations, more sites would be available for ^{32}P incorporation in the subsequent autophosphorylation, whereas at higher ATP concentrations, fewer sites would be unphosphorylated, but the enzyme would have more activity. The end result would represent little change in incorporation at the different ATP concentrations. To demonstrate that autophosphorylation was indeed part of the mechanism of activation, we carried out tryptic phosphopeptide mapping. For ATM from irradiated cells, at least seven phosphopeptides (*circled*) were evident after tryptic digestion, indicating that there are multiple phosphorylation sites on ATM (Fig. 6*C*). It is clear that when ATM kinase is activated by ATP, substantial overlap occurs between the two phosphorylation patterns (Fig. 6*C*). Notably absent in the ATP activated sample are phosphopeptides 3 and 7. It should be noted that ATM isolated from unirradiated cells has low basal activity, and this is reflected in appearance of some of these phosphopeptides. In essence, these data reveal that autophosphorylation appears to be essential for ATM kinase activity at least *in vitro*.

Previous results reveal that preincubation of cells with caffeine, a compound that overrides cell cycle checkpoints and radiosensitizes cells by inhibiting both ATM and ATR, did not affect radiation-induced ATM kinase when subsequently assayed *in vitro* (44). These data were interpreted to mean that the pathway leading to ATM activation in irradiated cells is insensitive to caffeine and that this activation does not involve autophosphorylation. These results appear to be at odds with the ATP-induced autophosphorylation of ATM observed here. To address this, we preincubated ATM immunoprecipitates from unirradiated cells with caffeine, prior to incubation with unlabelled ATP and subsequent washing out before measuring kinase activity. When caffeine (1 mM) was added with unlabelled ATP in the preincubation step to immunoprecipitates, it failed to interfere with activation of ATM (Fig. 7*A*). A small stimulation of activity was observed with caffeine alone. However, when caffeine was added to the kinase reaction with p53 substrate, as expected, it inhibited substrate phosphorylation by ATM (Fig. 7*B*). Caffeine concentrations up to 4 mM failed to interfere with *in vitro* activation (results not shown). It is evident from the results in Fig. 7*C* that preincubation of cells with caffeine did not interfere with the activation of ATM kinase, as observed by Sarkaria *et al.* (44). Again caffeine on its own had some stimulatory activity. Incubation of ATM immunoprecipitates with 1 mM caffeine in the kinase reaction had only a minimal effect on autophosphorylation (results not shown). These data suggest that although caffeine inhibits ATM kinase activity, it fails to interfere with autophosphorylation of ATM, thus distinguishing between the two types of activity.

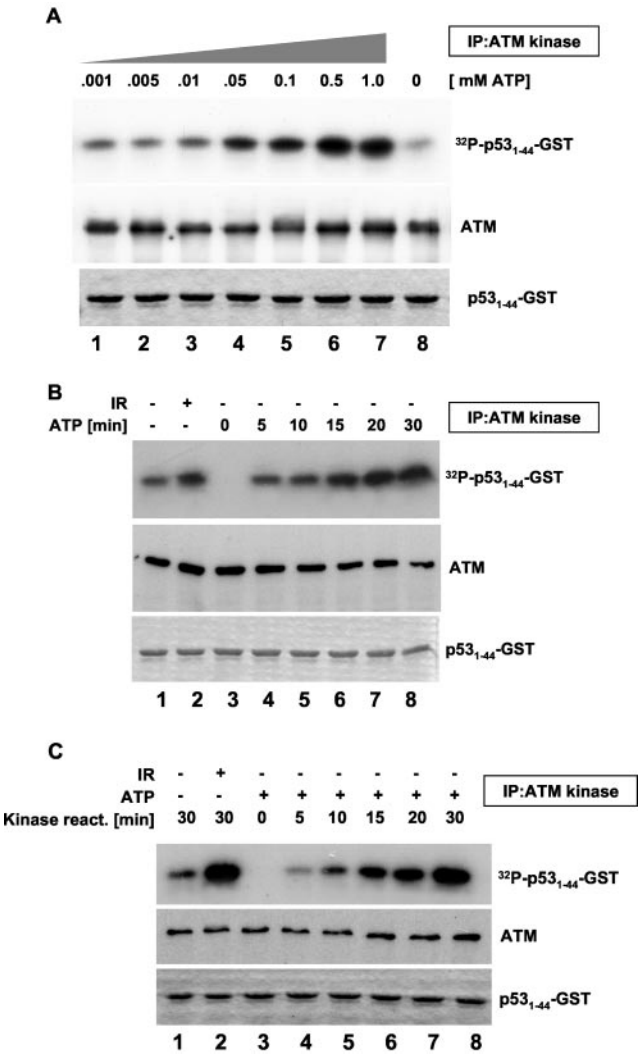

FIG. 4. **Concentration and time-dependent ATM activation by ATP.** *A*, effect of ATP concentration on activation. Immunoprecipitated ATM was incubated for 30 min at 30 °C in kinase buffer containing different concentrations of unlabelled ATP as indicated. After removal of ATP by extensive washing with kinase buffer, ATM was subjected to a standard kinase reaction using GST-p53$_{1-44}$ as substrate. *IP*, immunoprecipitate. *ATM*, Western blot of the amount of ATM protein in the autophosphorylation reaction. *^{32}P-p53$_{1-44}$-GST*, autoradiogram of radioactive phosphate incorporated into the substrate peptide. *p53$_{1-44}$-GST*, Coomassie-stained gel indicating the amount of substrate in the reaction. *B*, time course of ATP preincubation for activation. Immunoprecipitated ATM was incubated for different time intervals as indicated at 30 °C in kinase buffer containing 1 mM unlabelled ATP. After removal of ATP by extensive washing with kinase buffer, ATM was subjected to a standard kinase reaction using GST-p53$_{1-44}$ as substrate. *Lanes 1* and *2* depict ATM kinase activity in immunoprecipitates from unirradiated or irradiated samples without ATP preincubation conditions. *IR*, 10 Gy of ionizing radiation. As shown in *C*, immunoprecipitated ATM was activated by incubation for 30 min at 30 °C in kinase buffer containing 1 mM unlabelled ATP. After removal of ATP by extensive washing with kinase buffer, ATM was subjected to a standard kinase reaction for various time intervals as indicated using GST-p53$_{1-44}$ as substrate.

Since small molecules have also been shown to alter the autophosphorylation capacity and activity of other PI3-kinase family members (35, 36, 38, 39) we determined whether ATP activation might extend to DNA-PK and ATR *in vitro*. Incubation of ATP with immunoprecipitates of DNA-PK and ATP failed to significantly activate these kinases (Fig. 8, *A* and *B*), indicating that this was not some nonspecific effect capable of stimulating other family members because they possessed a protein kinase domain and an ATP-binding region.

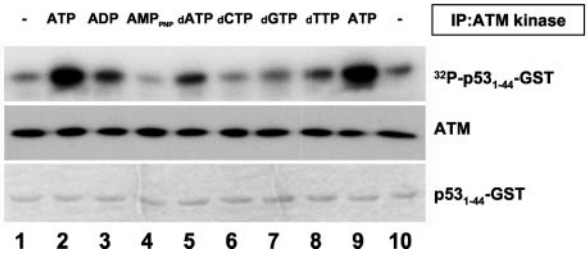

FIG. 5. **Activation of ATM by ATP analogues and other nucleotides.** Immunoprecipitated ATM was incubated for 30 min at 30 °C in kinase buffer containing ATP or other nucleotides as indicated. After removal of ATP by extensive washing with kinase buffer, ATM was subjected to a standard kinase reaction using GST-p53$_{1-44}$ as substrate. *^{32}P-p53$_{1-44}$-GST*, autoradiogram of radioactive phosphate incorporated into the substrate peptide. *ATM*, Western blot of the amount of ATM protein in the kinase reaction. *p53$_{1-44}$-GST*, Coomassie stained gel indicating the amount of substrate in the reaction.

DISCUSSION

The data described here provide good evidence that ATM can be activated *in vitro* by an autophosphorylation mechanism to phosphorylate downstream substrates such as p53. When immunoprecipitated from unirradiated cell extracts, ATM has a low basal level of kinase activity that is markedly enhanced by prior incubation with unlabelled ATP. The extent of ATM activation exceeds that observed in irradiated samples, suggesting that ATP is titrating out all the ATM activity. Further enhancement of ATM activation by ATP in immunoprecipitates from irradiated cells by ATP supports this. Previous data reveal that ATM is only activated 2- to 3-fold above the basal level by exposure to radiation doses as high as 10 Gy (15, 16, 21, 24, 25). These results suggest that not all of the ATM is activated *in vivo* by radiation. Although it is not clear how ATM is activated in irradiated cells, it appears to be due to recognition of double strand breaks in DNA either by direct binding through a complex with other proteins such as the Brca1-associated genome surveillance complex (BASC) complex (51) or perhaps by responding to changes in the superhelicity of chromatin remote from the actual site of the break. The activation of only some of the ATM protein after irradiation is consistent with recent data demonstrating heterogeneous distribution of ATM within the nucleus (52). A subset of the ATM pool appears to rapidly associate with chromatin and the nuclear matrix at sites of double strand breaks (52). Most of the ATM is loosely tethered to the nucleus; however, both fractions contain at least some active ATM kinase.

It is evident from our data that activation of ATM from unirradiated cells is only observed at concentrations of ATP from 0.05 mM up to physiological concentrations. Under kinase reaction conditions, the concentration of ATP is 5–10 μM, which clearly distinguishes between immunoprecipitates from irradiated and unirradiated cells (15, 16). The difference between irradiated and unirradiated could be due to greater access of ATP to the kinase active site in the former case. Radiation damage to DNA could alter the conformation of ATM or its association with an interacting protein to provide unrestricted access to the active site. This phenomenon has been observed with TAK1, a member of the mitogen-activated kinase kinase kinase family, which is activated *in vivo* by different cytokines (53). When phosphatase-treated immunoprecipitates of TAK1 are subsequently incubated with cold ATP (1 mM) and reassayed, kinase activity is greatly increased (54). How do we explain activation by higher concentrations of ATP? Activation could simply be due to an excess of a charged molecule, which caused a switch from basal to active states. This seems unlikely since a variety of nucleotide analogs failed to appreciably raise activity, and poly ADP-ribose also failed to activate ATM (re-

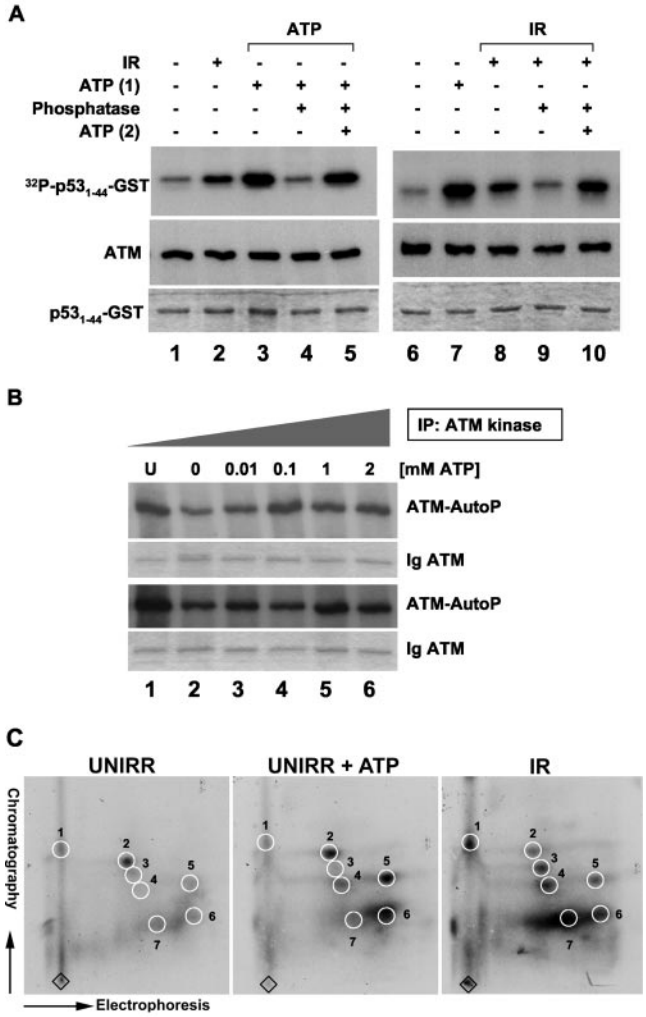

FIG. 6. **ATM is activated by autophosphorylation.** *A*, effect of successive phosphorylation and dephosphorylation on ATM kinase activity. Immunoprecipitated ATM was incubated for 30 min at 30 °C in kinase buffer or kinase buffer containing 1 mM ATP. After removal of ATP by extensive washing with kinase buffer, ATM was incubated in phosphatase buffer or phosphatase buffer containing 100 units of λ-phosphatase for 30 min at 30 °C. After subsequent removal of phosphatase by extensive washing with kinase buffer, ATM was again incubated in kinase buffer or kinase buffer containing 1 mM ATP for 30 min at 30 °C. After additional washing with kinase buffer, ATM was subjected to a normal kinase reaction using GST-p53$_{1-44}$ as substrate. *Left panel*, immunoprecipitates (*IP*) from unirradiated extracts (*lanes 3–5*). *Right panel*, immunoprecipitates from irradiated extracts (*lanes 8–10*). *IR*, 5 Gy of ionizing radiation. *ATP* (*1*), first round of incubation with ATP. *ATP* (*2*), second round of incubation with ATP. *^{32}P-p53$_{1-44}$-GST*, autoradiogram of radioactive phosphate incorporated into the substrate peptide. *ATM*, Western blot of the amount of ATM protein in the kinase reaction. *p53$_{1-44}$-GST*, Coomassie-stained gel indicating the amount of substrate in the reaction. *ATM-AutoP*, autoradiogram of radioactive phosphate incorporated into ATM. *B*, effect of pretreatment with ATP on ATM autophosphorylation. Immunoprecipitated ATM was incubated for 30 min at 30 °C in kinase buffer or kinase buffer containing ATP. After removal of ATP by extensive washing with kinase buffer, ATM was subjected to autophosphorylation reaction in the presence of 10 μCi of [γ-^{32}P]ATP. *Lane 1* depicts ATM autophosphorylation in immunoprecipitates from irradiated (*two upper lanes*) or unirradiated (*two lower lanes*) samples without ATP preincubation conditions. *Ig ATM*, immunoprecipitated and stained ATM. *C*, tryptic phosphopeptide mapping of ATM, autophosphorylated *in vitro* from unirradiated (*UNIRR*), irradiated, and ATP-activated ATM. Immunoprecipitates were subjected to ATM autophosphorylation conditions as described under "Experimental Procedures" followed by 5% SDS-PAGE. The labeled ATM protein band was enzymatically digested with trypsin. The resulting peptides were separated in the first and second dimension by electrophoresis and chromatography, respectively. The origins are marked by *diamonds*, and the positions of the phosphopeptides are indicated by *circles*.

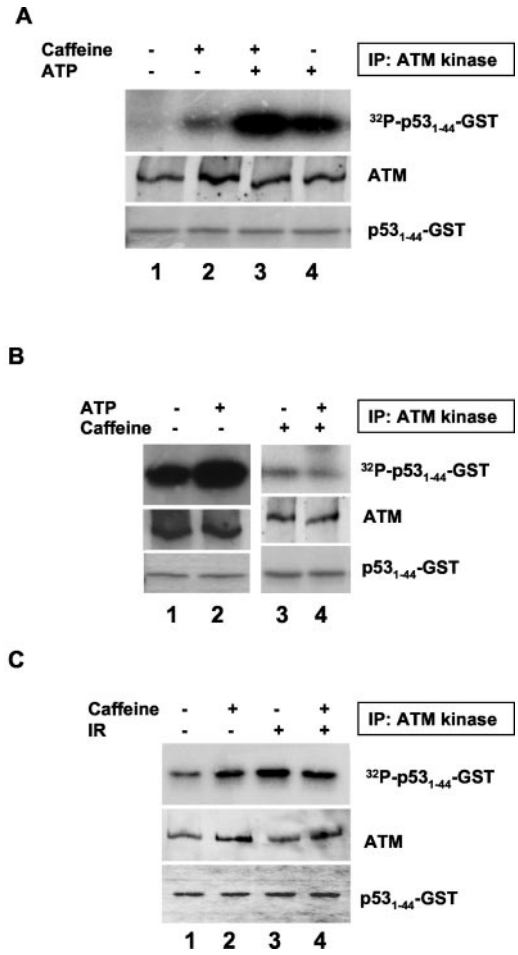

FIG. 7. **Effect of caffeine pretreatment on ATM kinase activity.** As shown in *A*, immunoprecipitated ATM was incubated for 30 min at 30 °C in kinase buffer or kinase buffer containing 1 mM caffeine and/or 1 mM ATP. After washing with kinase buffer, ATM was subjected to a standard kinase reaction using GST-p53$_{1-44}$ as substrate. *IP*, immunoprecipitate. *^{32}P-p53$_{1-44}$-GST*, autoradiogram of radioactive labeled substrate. *ATM*, Western blot of the amount of ATM protein the kinase reaction. *p53$_{1-44}$-GST*, Coomassie-stained substrate in the reaction. *B*, effect of caffeine in the kinase reaction. Immunoprecipitated ATM was incubated for 30 min at 30 °C in kinase buffer or kinase buffer containing 1 mM ATP. After washing with kinase buffer to remove the ATP, ATM was subjected to a kinase reaction in the presence of 1 mM caffeine, 10 μCi of [γ-^{32}P]ATP and GST-p53$_{1-44}$ as substrate. *C*, ATM kinase activity after treatment of cells with caffeine. Control (*C3ABR*) cells were treated with 3 mM caffeine for 1 h before irradiation. ATM was subsequently immunoprecipitated and subjected to a standard kinase assay using GST-p53$_{1-44}$ as substrate. *IR*, 5 Gy of ionizing radiation.

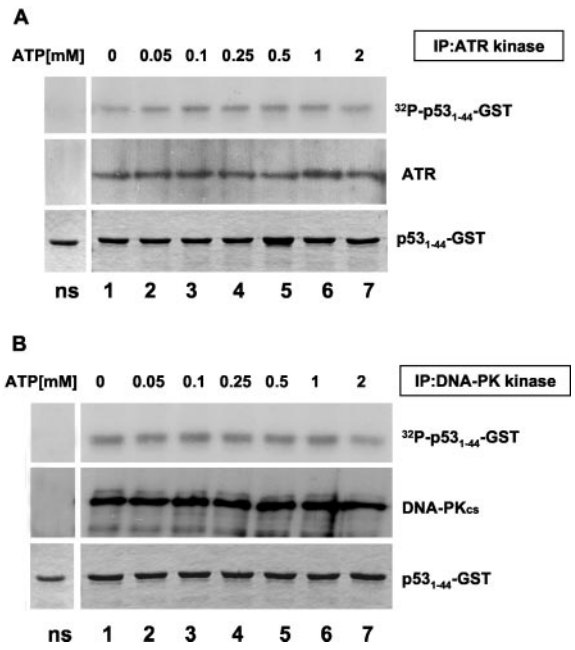

FIG. 8. **Effect of ATP on activation of other PI3-kinases.** Effect of ATP pretreatment on ATR and DNA-PKcs kinase activity. Immunoprecipitated ATR (*A*) and DNA-PKcs (*B*) were subjected to ATP pretreatment (0–2 mM as described above) before kinase activity was determined by standard kinase reaction in the presence of 10 μCi of [γ-^{32}P]ATP using GST-p53$_{1-44}$ as substrate. The amount of kinase in the reaction was determined by Western blot against ATR or DNA-PK, respectively. The specificity of the immunoprecipitation was verified by using nonspecific antiserum (*lane ns*). *IP*, immunoprecipitate. *^{32}P-p53$_{1-44}$-GST*, autoradiogram of radioactive labeled substrate. *DNA-PKcs*, Western blot of the amount of DNA-PK$_{cs}$ protein in the kinase reaction. *ATR*, Western blot of the amount of ATR protein in the kinase reaction. *p53$_{1-44}$-GST*, Coomassie-stained substrate in the reaction.

sults not shown). More likely, the K_m for ATP binding to the unirradiated ATM is considerably higher than that for irradiated, possibly as a consequence of the ATP/substrate-binding site being occluded by an inhibitory domain of ATM itself or due to interaction with another protein. This may be related to the behavior of ATM during purification where it binds tightly to γ-phosphate-linked ATP-Sepharose, but it is readily eluted from a metal-chelating Sepharose resin as a constitutively active phosphoprotein (45).

We have demonstrated here that autophosphorylation is a key event in the activation of ATM kinase *in vitro* and that the pattern of autophosphorylation induced by ATP bears considerable overlap with that seen in radiation-activated ATM. It appears that multiple autophosphorylation sites exist on ATM after both treatments. Treatment of ATP-activated ATM kinase with phosphatase abrogated activity, but this was fully restored upon subsequent incubation with ATP. Evidence for ATM autophosphorylation has been observed previously, but its importance was not highlighted (23, 25). In one of these reports, a radiation-induced increase in ATM phosphorylation in controls was not observed in an A-T cell line (25). [^{32}P]orthophosphate labeling *in vivo* provides evidence for ATM autophosphorylation in response to radiation damage supported by a failure to observe this effect in cells expressing kinase-dead ATM (23). The activation observed here was specific for ATM since it was absent in immunoprecipitates from A-T patients expressing mutant protein, was dependent on the presence of Mn^{2+}, and was inhibited by wortmannin. These data suggest that at least *in vitro*, no other kinase is required for the activation of ATM, and this may also be the case in irradiated cells. These observations appear to be contradictory to those of Sarkaria *et al.* (44), who reported that preincubation of cells with caffeine prior to radiation exposure failed to prevent the radiation-induced activation of ATM, as measured in immunoprecipitates, but prevented radiation-induced phosphorylation of p53 in the cell. They interpreted these results to mean that autophosphorylation was not part of the mechanism for ATM activation. We made the same observations as Sarkaria *et al.* (44) with pretreatment of cells with caffeine, but we described a differential effect of caffeine on autophosphorylation and its capacity to phosphorylate added substrate. Caffeine did not interfere with ATP-dependent activation of ATM kinase (*i.e.* autophosphorylation) at concentrations up to 4 mM, but it did prevent substrate phosphorylation when included in the kinase reaction. Thus the data are not contradictory but rather demonstrate that autophosphorylation of ATM, whether it be induced *in vivo* by radiation or *in vitro* by ATP, is not inhibited by caffeine, but the capacity of ATM to phosphorylate substrates such as p53 *in vivo* or *in vitro* is prevented by

caffeine. Although caffeine has been shown to interfere with cell cycle checkpoints and radiosensitize cells, the exact mechanism of action remains unknown. This compound inhibits both ATM and ATR kinase activities (44, 55), but it is not clear whether this purine analog competes with ATP or inhibits the enzymes by some other mechanism. The differential effect of caffeine on ATM autophosphorylation and substrate phosphorylation suggests that it is not simply competition with ATP for active site binding that causes inhibition. In this context, it is of considerable interest that several of the ATM substrates including p53, BRCA1, and BLM not only bind to the kinase domain *in vitro* but also bind to a region close to the N terminus of the protein (17, 25, 27). In addition, p53 and BRCA1 have been shown by co-immunoprecipitation to bind constitutively to ATM (17, 56). Thus, although two sites may be required for substrate phosphorylation, this would not be expected in the case of autophosphorylation and could explain differential inhibitory effects as observed here for caffeine. A greater understanding of the mechanism of ATM autophosphorylation will allow for the identification of more specific inhibitors than caffeine. Preincubation of cells with wortmannin, an inhibitor of ATM kinase, suggested that autophosphorylation is not required for the nuclear retention of ATM kinase (52). However, it should be pointed out that in that report, the bulk of ATM kinase was not tightly bound to chromatin but was nevertheless activated by radiation.

It is well established that serine/threonine protein kinases are activated due to conformational change in the molecule as a consequence of binding to a variety of ligands or by phosphorylation by upstream kinases (57). This alters the conformation of the protein, allowing increased access to ATP and substrate. In the present case, it is conceivable that a substrate such as p53 (*e.g.* as a tetramer), which is capable of binding to the two ends of the ATM molecule simultaneously, would effectively close off the active site by excluding access to ATP and maintaining the kinase in an inactive state. In the case of immunoprecipitates, removal of ATM and its associated proteins from their nuclear location may be sufficient to alter the conformation to an extent at which access to ATP occurs at higher concentrations. An alternative possibility for activation is that ATP binds at another site on ATM or on an associated protein and alters its conformation to allow more favorable access to the ATP-binding site within the active site. Previous data have demonstrated that ATP molecules bind co-operatively to the *Escherichia coli* chaperonin GroEL and cause long range conformational changes that determine the orientations of remote substrate-binding sites (58). ATP has also been shown to induce an increase in Ca^{2+}-dependent K^{+} channel (hIK1) activity, whereas several of its hydrolysable or non-hydrolysable analogs failed to do so (59). Another member of the P13-kinase family, mTOR, plays an important role in ribosome biogenesis and cell growth (36). Recent data demonstrate that the intracellular concentration of ATP alters the activity of mTOR independent of amino acid-induced changes (36). As ATP increases up to 1 mM, the activity of mTOR, detected as S6K1 activation, increased. These data were interpreted to mean that mTOR is a direct sensor of ATP in the cell. It is also of interest that mTOR bound to ATP agarose is efficiently eluted off the matrix with excess cold ATP (60). Phosphatidic acid also interacts with mTOR, which is positively correlated with the ability of mTOR to activate downstream substrates (61). Another small molecule inositol hexakisphosphate has been shown to stimulate the kinase activity of DNA-PK by interacting specifically with the Ku7u/80 heterodimer (35).

In summary, we have shown that ATP activates ATM kinase *in vitro* by a mechanism involving autophosphorylation. The process of autophosphorylation is resistant to caffeine inhibition, whereas substrate phosphorylation is sensitive to this agent. Activation is specific for ATM and is not observed to a significant extent with either ATR or DNA-PK. This is in agreement with previous results employing purified DNA-PK in which preincubation with unlabelled ATP in the absence of DNA failed to appreciably alter the activity (62). When DNA was included in the preincubation, autophosphorylation of DNA-PKcs reduced its capacity to phosphorylate exogenous substrates (38). It is intriguing that autophosphorylation has the opposite effect on the activities of these two P13-kinase family members that recognize double strand breaks in DNA. It is proposed that a similar mechanism for activation occurs in the cell, where DNA double strand breaks either indirectly alter the conformation of ATM or indirectly alter its association with an interacting protein, allowing unrestricted access to ATP for autophosphorylation and substrate phosphorylation. Clearly, it is important to determine how the observations made here impact upon *in vivo* activation of ATM kinase. Identification of the *in vivo* phosphorylation sites on ATM are critical to an overall understanding of the mechanism of activation.

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REFERENCES

1. Sedgwick, R. P., and Boder, E. (1991) in *Hereditary Neuropathies and Spinocerebellar Atrophies* (Vianney De Jong, J. M. B, and Alan R. Liss, eds) pp. 347–423, Elsevier Science Publishing Co., New York
2. Lavin, M. F., and Shiloh, Y. (1997) *Annu. Rev. Immunol.* **15,** 177–202
3. Gotoff, S. P., Amirmokri, E., and Liebner, E. J. (1967) *Am. J. Dis. Child.* **114,** 617–625
4. Taylor, A. M., Harnden, D. G., Arlett, C. F., Harcourt, S. A., Lehmann, A. R., Stevens, S., and Bridges, B. A. (1975) *Nature* **258,** 427–429
5. Chen, P. C., Lavin, M. F., Kidson, C., and Moss, D. (1978) *Nature* **274,** 484–486
6. Cornforth, M. N., and Bedford, J. S. (1985) *Science* **227,** 1589–1591
7. Foray, N., Priestley, A., Alsbeih, G., Badie, C., Capulas, E. P., Arlett, C. F., and Malaise, E. P. (1997) *Int. J. Radiat. Biol.* **72,** 271–283
8. Kim, S., Xu, B., and Kastan, M. B. (2002) *Genes Dev.* **16,** 560–570
9. Yazdi, P. T., Wang, Y., Zhao, S., Patel, N., Lee, E. Y.-H. P., and Qin, J. (2002) *Genes Dev.* **16,** 571–582
10. Beamish, H., and Lavin, M. F. (1994) *Int. J. Radiat. Biol.* **65,** 175–184
11. Morgan, S. E., and Kastan, M. B. (1997) *Adv. Cancer Res.* **71,** 1–25
12. Savitsky, K., Bar-Shira, A., Gilad, S., Rotman, G., Ziv, Y., Vanagaite, L., Tagle, D. A., Smith, S., Uziel, T, and Sfez, S., *et al.* (1995) *Science* **268,** 1749–1753
13. Abraham, R. T. (2001) *Genes Dev.* **15,** 2177–2196
14. Keating, K. E., Gueven, N., Watters, D., Rodemann, H. P., and Lavin, M. F. (2001) *Oncogene* **20,** 4281–4290
15. Banin, S., Moyal, L., Shieh, S., Taya, Y., Anderson, C. W., Chessa, L., Smorodinsky, N. I., Prives, C., Reiss, Y., Shiloh, Y., and Ziv, Y. (1998) *Science* **281,** 1674–1677
16. Canman, C. E., Lim, D. S., Cimprich, K. A., Taya, Y., Tamai, K., Sakaguchi, K., Appella, E., Kastan, M. B., and Siliciano, J. D. (1998) *Science* **281,** 1677–1679
17. Khanna, K. K., Keating, K. E., Kozlov, S., Scott, S., Gatei, M., Hobson, K., Taya, Y., Gabrielli, B., Chan, D., Lees-Miller, S. P., and Lavin, M. F. (1998) *Nat. Genet.* **20,** 398–400
18. Brown, K. D., Ziv, Y., Sadanandan, S. N., Chessa, L., Collins, F. S., Shiloh, Y., and Tagle, D. A. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 1840–1845
19. Shiloh, Y., and Kastan, M. B. (2001) *Adv. Cancer Res.* **83,** 209–254
20. Yang, D. O., and Kastan, M. B. (2000) *Nat. Cell Biol.* **2,** 893–898
21. Gatei, M., Young, D., Cerosaletti, K. M., Desai-Mehta, A., Spring, K., Kozlov, S., Lavin, M. F., Gatti, R. A., Concannon, P., and Khanna, K. (2000) *Nat. Genet.* **25,** 115–119
22. Zhao, S., Weng, Y. C., Yuan, S. S., Lin, Y. T., Hsu, H. C., Lin, S. C., Gerbino, E., Song, M. H., Zdzienicka, M. Z., Gatti, R. A., Shay, J. W., Ziv, Y., Shiloh, Y., and Lee, E. Y. (2000) *Nature* **405,** 473–477
23. Lim, D. S., Kim, S. T., Xu, B., Maser, R. S., Lin, J., Petrini, J. H., and Kastan, M. B. (2000) *Nature* **404,** 613–617
24. Cortez, D., Wang, Y., Qin, J., and Elledge, S. J. (1999) *Science* **286,** 1162–1166
25. Gatei, M., Scott, S. P., Filippovitch, I., Soronika, N., Lavin, M. F., Weber, B., and Khanna, K. K. (2000) *Cancer Res.* **60,** 3299–3304
26. Chen, G., Yuan, S. S., Liu, W., Xu, Y., Trujillo, K., Song, B., Cong, F., Goff, S. P., Wu, Y., Arlinghaus, R., Baltimore, D., Gasser, P. J., Park, M. S., Sung, P., and Lee, E. Y. (1999) *J. Biol. Chem.* **274,** 12748–12752
27. Beamish, H., Padmini, K., Kaneko, H., Chen, P., Fukao, T., Peng, C., Beresten, S., Gueven, N., Lees-Miller, S., Ellis, N., Kondo, N., and Lavin, M. F. (2002) *J. Biol. Chem.* **277** 30515–30523
28. Chehab, N. H., Malikzay, A., Stavridi, E. S., and Halazonetis, T. D. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 13777–13782
29. Maya, R, Balass, M., Kim, S. T., Shkedy, D., Leal, J. F., Shifman, O., Moas, M.,

Buschmann, T., Ronai, Z., Shiloh, Y., Kastan, M. B., Katzir, E., and Oren, M. (2001) *Genes Dev.* **15,** 1067–1077
30. Kastan, M. B., Zhan, O., el-Deiry, W. S., Carrier, F., Jacks, T., Walsh, W. V., Plunkett, B. S., Vogelstein, B., and Fornace, A. J., Jr. (1992) *Cell* **71,** 587–597
31. Khanna, K. K., Beamish, H., Yan, J., Hobson, K., Williams, R., Dunn, I., and Lavin, M. F. (1995) *Oncogene* **11,** 609–618
32. Falck, J., Petrini, J. H., Williams, B. R., Lucas, J., and Bartek, J. (2002) *Nat. Genet.* **30,** 290–294
33. Xu, B., Kim, S. T., and Kastan, M. B. (2001) *Mol. Cell. Biol.* **21,** 3445–3450
34. Lees-Miller, S. P. (1996) *Biochem. Cell Biol.* **74,** 503–512
35. Hanakahi, L. A., Bartlet-Jones, M., Chappell, C., Pappin, D., and West, S. C. (2000) *Cell* **102,** 721–729
36. Dennis, P. B., Jaeschke, A., Saitoh, M., Fowler, B., Kozma, S. C., and Thomas, G. (2001) *Science* **294,** 1102–1105
37. Keegan, K. S., Holtzman, D. A., Plug, A. W., Christenson, E. R., Brainerd, E. E., Flaggs, G., Bentley, N. J., Taylor, E. M., Meyn, M. S., Moss, S. B., Carr, A. M., Ashley, T., and Hoekstra, M. F. (1996) *Genes Dev.* **10,** 2423–2437
38. Chan, D. W., and Lees-Miller, S. P. (1996) *J. Biol. Chem.* **271,** 8936–8941
39. Douglas, P., Moorhead, G. B., Ye, R., and Lees-Miller, S. P. (2001) *J. Biol. Chem.* **276,** 18992–18998
40. Scott, S. P., Zhang, N., Khanna, K. K., Khromykh, A., Hobson, K., Watters, D., and Lavin, M. F. (1998) *Biochem. Biophys. Res. Commun.* **245,** 144–148
41. Chen, G., and Lee, E. Y. H. P. (1996) *J. Biol. Chem.* **271,** 33693–33697
42. O'Neill, T., Dwyer, A. J., Ziv, Y., Chan, D. W., Lees-Miller, S. P., Abraham, R. H., Lai, J. H., Hill, D., Shiloh, Y., Cantley, L. C., and Rathbun, G. A. (2000) *J. Biol. Chem.* **275,** 22719–22727
43. Frangioni, J. V., and Neel, B. G. (1993) *Anal. Biochem.* **210,** 179–187
44. Sarkaria, J. N., Busby, E. C., Tibbetts, R. S., Roos, P., Taya, Y., Karnitz, L. M., and Abraham, R. T. (1999) *Cancer Res.* **59,** 4375–4382
45. Chan, D. W., Son, S. C., Block, W., Ye, R., Khanna, K. K., Wold, M. S., Douglas, P., Goodarzi, A. A., Pelley, J., Taya, Y., Lavin, M. F., and Lees-Miller, S. P. (2000) *J. Biol. Chem.* **275,** 7803–7810
46. Ziv, Y., Banin, S., Lim, D. S., Canman, C. E., Kastan, M. B., and Shiloh, S. (2000) in *Methods in Molecular Biology: Stress Response: Methods and Protocols*, pp. 99–108, Humana Press Inc., Totowa, NJ
47. Boyle, W. J., van der Geer, P., and Hunter, T. (1991) in *Methods in Enzymology* (Wu, R., ed), Vol. 201, pp. 110–149, Academic Press Inc., San Diego
48. Katoh, T., and Fujisawa, H. (1991) *J. Biol. Chem.* **266,** 3039–3044
49. Szczepanowska, J., Zhang, X., Herring, C. J., Qin, J., Korn, E. D., and Brezeska, H. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 8503–8508
50. Deak, J. C., and Templeton, D. J. (1997) *Biochem. J.* **322,** 185–192
51. Wang, Y., Cortez, D., Yazdi, P., Neff, N., Elledge, S. J and Qin, J. (2000) *Genes Dev.* **14,** 927–939
52. Andegeko, Y., Moyal, L., Mittelman, L., Tsarfaty, I., Shiloh, Y., and Rotman, G. (2001) *J. Biol. Chem.* **276,** 38224–38230
53. Shirakabe, K., Yamaguchi, K., Shibuya, H., Irie, K., Matsuda, S., Moriguchi, T., Gotah, Y., Matsumoto, K., and Nishida, E. (1997) *J. Biol. Chem.* **272,** 8141–8144
54. Kishimoto, K., Matsumoto, K., and Ninomiya-Tsuji, J. (2000) *J. Biol. Chem.* **275,** 7359–7364
55. Blasina, A., Price, B. D., Turenne, G. A., and McGowan, C. H. (1999) *Curr. Biol.* **9,** 1135–1138
56. Watters, D., Kedar, P., Spring, K., Bjorkman, J., Chen, P., Gatei, M., Birrell, G., Garrone, B., Srinivasa, P., Crane, D. I., and Lavin, M. F. (1999) *J. Biol. Chem.* **274,** 34277–34282
57. Sowadski, J. M., Epstein, L. F., Lankiewicz, L., and Karlsson, R. (1999) *Pharmacol. Ther.* **82,** 157–164
58. Roseman, A. M., Ranson, N. A., Gowen, B., Fuller, S. D., and Saibil, H. R. (2001) *J. Struct. Biol.* **135,** 115–125
59. Gerlach, A. C., Syme, C. A., Giltinan, L., Adelman, J. P., and Devors, D. C. (2001) *J. Biol. Chem.* **276,** 10963–10970
60. Peterson, R. T., Beal, P. A., Comb, M. J., and Schreiber, S. L. (2000) *J. Biol. Chem.* **275,** 7416–7423
61. Fang, Y., Vilella-Bach, M., Bachmann, R., Flanigan, A., and Chen, J. (2001) *Science* **294,** 1942–1995
62. Lees-Miller, S. P., Chen, Y., and Anderson, C. W. (1990) *Mol. Cell. Biol.* **10,** 6472–6481

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