Involvement of novel autophosphorylation sites in ATM activation

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ATM kinase plays a central role in signaling DNA double-strand breaks to cell cycle checkpoints and to the DNA repair machinery. Although the exact mechanism of ATM activation remains unknown, efficient activation requires the Mre11 complex, autophosphorylation on S1981 and the involvement of protein phosphatases and acetylases. We report here the identification of several additional phosphorylation sites on ATM in response to DNA damage, including autophosphorylation on pS367 and pS1893. ATM autophosphorylates all these sites *in vitro* in response to DNA damage. Antibodies against phosphoserine 1893 revealed rapid and persistent phosphorylation at this site after *in vivo* activation of ATM kinase by ionizing radiation, paralleling that observed for S1981 phosphorylation. Phosphorylation was dependent on functional ATM and on the Mre11 complex. All three autophosphorylation sites are physiologically important parts of the DNA damage response, as phosphorylation site mutants (S367A, S1893A and S1981A) were each defective in ATM signaling *in vivo* and each failed to correct radiosensitivity, genome instability and cell cycle checkpoint defects in ataxia-telangiectasia cells. We conclude that there are at least three functionally important radiation-induced autophosphorylation events in ATM.

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Introduction

Ataxia-telangiectasia (A-T) is an autosomal recessive disorder characterized by neurodegeneration, immunodeficiency, hypogonadism and cancer susceptibility (Sedgwick and Boder, 1991). Cellular features of A-T include hypersensitivity to

agents that cause double-strand breaks (DSBs) in DNA and a decreased capacity to activate all cell cycle checkpoints (Lavin and Shiloh, 1997). The protein defective in A-T, ATM, is a member of the phosphatidylinositol-3-kinase like kinase family, which is activated in response to DNA DSBs and phosphorylates multiple substrates involved in cell cycle checkpoint control and DNA repair (Savitsky *et al*, 1995). This syndrome overlaps in its clinical and cellular phenotype with A-TLD, caused by mutations in Mre11, and Nijmegen breakage syndrome (NBS), mutated in Nbs1 (Carney *et al*, 1998; Stewart *et al*, 1999). All three syndromes show hypersensitivity to ionizing radiation, cell cycle anomalies, genome instability and cancer susceptibility for both NBS and A-T (Shiloh, 2003).

Exposure of cells to ionizing radiation and radiomimetic agents causes a rapid autophosphorylation of ATM kinase on S1981 (Bakkenist and Kastan, 2003). This phosphorylation leads to dissociation of an inactive ATM dimer to generate a catalytically active monomeric form. Under these conditions, a small number of DNA DSBs activated the entire nuclear pool of ATM, suggesting that the breaks *per se* were not activating ATM directly. The use of agents that disrupted chromatin structure without introducing breaks into DNA supported the hypothesis that gross alterations in chromatin structure were responsible for activation (Bakkenist and Kastan, 2003). The capacity of ATM to bind to DNA and associate with chromatin in response to radiation damage supports a role for ATM as a sensor of DNA damage (Andegeko *et al*, 2001). However, there is also evidence that Mre11 migrates rapidly to the sites of DNA DSBs and is retained on chromatin in a form of small granular foci (Mirzoeva and Petrini, 2003). Evidence supporting the Mre11 complex as the sensor of damage was demonstrated by inefficient activation of ATM by DNA DSBs in the absence of Nbs1 or Mre11 (Uziel *et al*, 2003; Cerosaletti and Concannon, 2004). In addition, a conserved C-terminus motif present in Nbs1, ATRIP and Ku80 is important in their interaction with ATM, ATR and DNAPK, respectively (Falck *et al*, 2005). Lee and Paull (2005) demonstrated *in vitro* that the Mre11 complex senses DNA DSBs and recruits ATM to broken DNA molecules. They showed that this complex was capable of activating ATM dimers to phosphorylate downstream cellular targets such as p53 and Chk2. Furthermore, depletion of Mre11 from *Xenopus* extracts abrogated DNA DSBs and ATM-dependent phosphorylation of H2AX, suggesting that the Mre11 complex acts upstream of ATM activation (Constanzo *et al*, 2004). Further support for this was provided recently by Dupre *et al* (2006), who showed that *Xenopus* ATM is activated by two independent steps both of which require the Mre11 complex.

ATM activation has also been demonstrated to be dependent on phosphatase activity (Ali *et al*, 2004; Goodarzi *et al*, 2004). These data suggest that PP2A has a negative regulatory role in controlling ATM activation, but PP5 interacts with ATM in a DNA damage-inducible fashion and depletion of PP5 was shown to attenuate DNA damage-induced

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activation of ATM. Protein modification by acetylation has also been shown to influence ATM activation. Sun *et al* (2005) revealed that suppression of TIP60 histone acetyltransferase (HAT) blocks ATM activation and prevents ATM-dependent phosphorylation of p53 and Chk2. ATM forms a stable complex with TIP60 through a conserved FATC domain at its C-terminus. A second HAT, hMOF, also interacts with ATM and influences its function (Gupta *et al*, 2005).

Although phosphopeptide mapping revealed a single major *de novo* phosphorylated peptide, from which S1981 was identified as the phosphorylation site, it was evident that other phosphopeptides were present in the ATM tryptic digest after exposure of cells to ionizing radiation (Bakkenist and Kastan, 2003). A separate study demonstrated the presence of at least seven phosphopeptides in ATM from irradiated cells using tryptic phosphopeptide mapping (Kozlov *et al*, 2003). Furthermore, a number of biological signaling processes that activate ATM protein kinase in cells do not require autophosphorylation on S1981 (Powers *et al*, 2004; Lee and Paull, 2005). These data suggest that the activation and cellular function of ATM is a complex process involving several phosphorylation events in addition to S1981. Accordingly, we employed mass spectrometry to identify additional phosphorylation sites in ATM from cells exposed to radiation damage and investigated their function.

Results

Identification of autophosphorylation sites on ATM

Multiple ATM phosphopeptides were isolated by a combination of Fe³⁺ immobilized metal affinity chromatography (IMAC) and reversed phase chromatography. Only a subset of all the phosphopeptides was detectable by mass spectrometry. In conjunction with phosphatase treatment, MALDI-TOF-MS confirmed the presence of at least three unique phosphopeptides (Figure 1A–C). Sequencing them by tandem MS/MS directly identified ATM_{363–375} and ATM_{1974–1992} and directly revealed two *in vivo* sites of phosphorylation, S367 and S1981. The S1981 site has been reported previously (Bakkenist and Kastan, 2003; Beausoleil *et al*, 2004). A third phosphopeptide sequence was ATM_{1883–1898}, but was ambiguous with respect to the exact site of phosphorylation. Despite this, the sequence unequivocally localized two new phosphorylation sites to two separate subdomains of the peptide: S1883 to T1885 and S1891 to S1893 (Figure 1D–F).

We initially determined whether the phosphorylation sites identified in ATM from irradiated cells could act as substrates for ATM kinase activity, that is, autophosphorylation. The results in Figure 2A demonstrate that all three GST-fusion proteins containing the above three peptides are phosphorylated *in vitro* by ATM in response to radiation. This was expected for ATM_{1974–1992} as it contains the pS1981 site (Bakkenist and Kastan, 2003) and for ATM_{363–375} as it contains the S367 site (ISQS), conforming to the general phosphorylation consensus sequence for ATM kinase (Kim *et al*, 1999; O'Neill *et al*, 2000). No ATM substrate consensus sequence was evident in ATM_{1883–1898}, but a sequence (S¹⁸⁹¹ES¹⁸⁹³E) was present in which glutamic acid residues were located next to serines (SE) instead of the consensus glutamine (SQ). This is not unexpected as *in vitro* and *in vivo* phosphorylation of Brca1 by ATM has been demonstrated at sites other than S/TQ (Cortez *et al*, 1999). As there are other S/T residues

in ATM_{1883–1898}, we prepared two GST-fusion fragments, GST-(ATM)-RSTTPANLD and GST-(ATM)-ANLDSESEHFFR, by dividing the second tryptic phosphopeptide (Figure 1E) into two overlapping pieces, each of which contains one of the subdomains that are phosphorylated *in vivo*, to determine whether ATM could phosphorylate these fragments *in vitro*. Phosphorylation by ATM from radiation-treated cells was only observed for the peptide containing the SESE sequence (data not shown). In order to determine which of the serines in the S¹⁸⁹¹ES¹⁸⁹³E sequence was being phosphorylated by ATM, we prepared GST-fusions with the two serines individually mutated to alanines. The results in Figure 2B show that both the wild-type and the S1891A mutant are phosphorylated by ATM in response to radiation but no phosphorylation is observed with the S1893A substitution (lanes 5), indicating that S1893 is the site phosphorylated by ATM. We confirmed that the S1893 was an autophosphorylation site *in vitro* by demonstrating inhibition of GST-S¹⁸⁹¹ES¹⁸⁹³E phosphorylation by wortmannin (Figure 2C). We provided evidence for ATM dependence in this reaction by showing that no radiation-induced phosphorylation occurred in an A-T cell line (Figure 2D).

ATM is autophosphorylated on S1893 in response to DNA damage

In order to determine whether S1893 is phosphorylated by ATM *in vivo* in response to radiation-induced damage to DNA, we generated rabbit polyclonal antibodies that recognize this phosphorylation site (anti-pS1893). The specificity of this antibody was demonstrated by detection of a peptide containing pS1893 and no reactivity against the corresponding unphosphorylated peptide or a phosphorylated peptide containing pS1981 (Supplementary Figure 1A and B). This antibody did not detect pS1893 in cell extracts from unirradiated cells but a signal was clearly detectable in irradiated cells at 15 and 60 min post-irradiation (Supplementary Figure 1C). For comparison, we also employed an anti-pS1981 antibody, which detected an increase in phosphorylation in response to radiation (Supplementary Figure 1C) as described previously (Bakkenist and Kastan, 2003). Time-course analysis revealed that phospho-S1893 was detected by 15 min post-irradiation and increased to a maximum by 1–2 h (Figure 3A). This paralleled that seen for phospho-S1981, but phosphorylation at S1981 reached a maximum more rapidly. In both cases, the response had begun to decrease by 24 h post-irradiation. Much the same was observed with increasing radiation dose, where an optimum response was observed for phospho-S1893 at 5 Gy whereas that for phospho-S1981 was optimal by 1 Gy (Figure 3B).

We confirmed radiation-induced phosphorylation on S1893 by measuring the response in ATIABR cells, an A-T cell line expressing a mutant but less stable form of ATM (7636del9) (Spring *et al*, 2001). No phosphorylation signal was detected in these cells post-irradiation or in another A-T cell line not expressing any ATM protein (Figure 3C). This was also the case for phospho-S1981 (Figure 3C). We demonstrated that radiation-induced phosphorylation on S1893 was autophosphorylation by showing that both wortmannin (Figure 3D) and Ku-55933 (Figure 3E), a specific ATM inhibitor (Hickson *et al*, 2004), reduced significantly pS1893 ATM. Under these conditions, pS1981 was also inhibited.

Recent data show that the Mre11 complex is required for efficient activation of ATM kinase after exposure to radiation (Cerosaletti and Concannon, 2004) or the radiomimetic agent neocarcinostatin (Uziel *et al*, 2003). Accordingly, we exam-

ined the capacity of cells from patients deficient in Nbs1, a member of the Mre11 complex, for S1893 autophosphorylation. There was minimal response to radiation compared to controls (Figure 4A) and S1981 autophosphorylation was also

reduced. In cells from an ATLD patient lacking Mre11, radiation-induced phosphorylation of S1893 was again minimal, whereas autophosphorylation of S1981 was reduced but was still detected (Figure 4B). Cells deficient in a third member of the Mre11 complex, Rad 50, also failed to display a radiation-induced response to phosphorylation at pS1893 but retained a partial response for pS1981 (Figure 4C). These results reveal clear differences between these two autophosphorylation sites. Radiation-induced phosphorylation of S1893 is completely dependent on the presence of all members of the MRE complex, whereas that of S1981 is only partly dependent.

Effect of autophosphorylation site mutations on radiation-induced ATM signaling

We next investigated the role of autophosphorylation on ATM activation and signaling, and the dependence of the different phosphorylation sites on each other. To address this, we introduced S367 to Ala (S367A), S1893 to Ala (S1893A) and S1981 to Ala (S1981A) mutations into full-length ATM cDNA, transfected these into A-T cells (ATIABR) and generated stable cell lines in which the mutant proteins were inducible with CdCl₂ (Zhang *et al*, 1997). The level of expression of all three proteins was comparable after induction (Figure 5A). We initially determined the interdependence of the different phosphorylation events on each other. When the ATM S1981A mutant was induced in ATIABR cells exposed to radiation, no S1981 phosphorylation was detected as expected (Figure 5A). However, under these conditions, phosphorylation occurred at the S1893 site. In cells transfected with the S1893A mutant, a similar pattern was observed; no S1893 phosphorylation was seen, but activity was evident at the S1981 site (Figure 5A). Again in the case of the S367A mutant, it was possible to detect both S1893 and S1981 phosphorylation in response to radiation in transfected cells (Figure 5A). No phosphospecific antibody was available to check the dependence of S367 for phosphorylation on the other two sites. These data suggest that regardless of which phosphorylation site is mutated, ATM still retains autophosphorylation activity for the other sites. This was further supported by immunoprecipitation of the three mutant ATMs from ATIABR cell extracts, where it is apparent that they

all possess *in vitro* kinase activity after irradiation (Supplementary Figure 2).

Bakkenist and Kastan (2003) have also shown that the ATM S1981A mutant has protein kinase activity *in vitro* and that mutation at this site impacts on downstream signaling in transient transfections. Induction of wild-type ATM (pMAT1) in A-T cells gave rise to p53 phosphorylation on S15 at 60 min post-irradiation, which was comparable to that observed in control untransfected cells (Figure 5B, right-hand panel). On the other hand, a reduced p53 phosphorylation response to radiation exposure was detected in A-T cells transfected with either the S367A, S1893A or S1981A mutant forms of ATM. At later times after irradiation, p53 S15 phosphorylation was evident but was delayed in all cases (data not shown), reminiscent of that seen in A-T cells (Khanna and Lavin, 1993; Khanna *et al*, 1998). The defective p53 response was also evident for p53 protein stabilization (Figure 5B). Although p53 stabilization by the inducing agent CdCl₂ was present in all cases, it did not conceal the radiation-induced increase in pMAT1-transfected cells. This increase was not observed for any of the three mutant ATMs. As Nbs1 is also phosphorylated by ATM in response to radiation, (Gatei *et al*, 2000), we determined its response in transfected cells. The results reveal S343 phosphorylation of Nbs1 and an Nbs1 mobility shift upwards for wild-type ATM (Figure 5B), indicative of phosphorylation, post-irradiation, whereas that phosphorylation and the shift were also defective in all of the mutant forms of ATM, indicating defective ATM signaling. Radiation-induced phosphorylation of Chk2 on T68 was also reduced in the mutants compared to pMAT1-transfected cells (Figure 5B). The defective radiation-induced signaling in the ATM mutant-transfected cells was also shown by reduced signal for pS957-SMCI (Figure 5B). We also generated phosphomimics for these ATM autophosphorylation sites, substituting glutamic acid for serine (S1893E, S1981E) to further investigate the effect on radiation-induced ATM activation. Using the CdCl₂ induction system, we demonstrated efficient expression of both glutamic acid-substituted proteins in ATIABR cells (Supplementary Figure 3A–C). There was no evidence for constitutive activation of these forms of ATM in *in vitro* kinase assays but both showed induction of kinase activity post-irradiation. Further-

Figure 1 Detection and identification of ATM phosphorylation sites by mass spectrometry. (A) A monoisotopic peptide signal was detected at m/z 1593.8 by MALDI-TOF-MS. This signal was sensitive to treatment with Antarctic phosphatase (PPase). It disappeared, and the signal at m/z 1513.8 (80 U smaller) was stronger after PPase treatment. The signal at m/z 1593.8 matched the theoretical m/z of phospho-ATM_{363–375} (m/z 1593.7). A meta-stable signal, characteristic of neutral loss of phosphoric acid (–98 U) from a phosphopeptide (shown with an asterisk) confirmed the presence of a phosphopeptide. (B) Signals at m/z 1917.8 and 80 U higher at 1998.0 disappeared after PPase treatment and a new signal at m/z 1837.8 appeared. This shows that the signals at m/z 1917.8 and 1998.0 are mono- and di-phosphorylated forms of the same peptide (80 and 160 U larger, respectively). The insets expand the region from m/z 1996 to 2004 to show that the signal at m/z 1998.0 was removed by PPase treatment. These signals matched the theoretical m/z of mono- and di-phospho-ATM_{1883–1898} (m/z 1917.8 and 1997.8). (C) A signal at m/z 2080.9 was no longer present after PPase treatment, indicating that it was a phosphopeptide. A signal at 80 U less, at m/z 2000.9, was stronger after PPase treatment. The signal at m/z 2080.9 matched the theoretical mass of phospho-ATM_{1974–1992} (m/z 2080.9). (D) The phosphopeptide in (A) was sequenced by QqTOF-MS/MS. A doubly charged parent ion at m/z 797.4 was selected for fragmentation. The spectrum shows most of the y-type and b-type fragment ions that matched the sequence of ATM_{363–375} where S367 is phosphorylated (pS). The transition from y_8 to y_9 demonstrates the presence of a phosphoserine residue. (E) The triply charged parent ion at m/z 639.9 from the phosphopeptide in (B) was selected for fragmentation. The signal intensity in the region from m/z 850 to 1300 has been multiplied by a factor of 8 to improve clarity. The spectrum matches the sequence of phospho-ATM_{1883–1898}. Two independent sequences with different phosphorylation sites were detected. There was sufficient information to confirm that these peptides can be phosphorylated in either of two subdomains: either near its N-terminus (S1883, T1884 or T1885, upper sequence and ions shown with an asterisk) or its C-terminus (S1891 or S1893, lower sequence). The overlapping sequences are demonstrated by the presence of particular fragment ions in both their phosphorylated and non-phosphorylated forms (y_{11} and y_{13}). (F) The triply charged parent ion of the phosphopeptide in (C) at m/z 694.3 was selected for fragmentation. The spectrum matches the sequence of phospho-ATM_{1974–1992} where S1981 (pS) is phosphorylated. Multiple y-type and b-type ions rule out the possibility of phosphorylation at any site other than S1981. Note: the parent ions in (D, E) have been truncated in height to improve the clarity of the fragmentation ions.

Figure 2 ATM phosphorylates S367, S1893 and S1981 *in vitro*. (A) Untreated or irradiated (10 Gy, 1 h) C3ABR lymphoblastoid cells were collected, lysed in the ATM kinase immunoprecipitation buffer (see Materials and methods) and immunoprecipitated with ATM antibody followed by *in vitro* kinase assays using recombinant GST-ATM 1, 2 and 3 as substrates, containing each of the three tryptic peptides from this figure and Figure 1. (B) Untreated or irradiated (10 Gy, 1 h) C3ABR cells were used in the ATM *in vitro* kinase assays as described in (A). The following GST substrates were derived from the second tryptic peptide in (B) and Figure 1B (ATM₁₈₈₃₋₁₈₉₈) and used in ATM *in vitro* kinase assays: GST-ATM 4-wild type: ANLDSESEHFFR; GST-ATM 5-S1893A: ANLDSEAEHFFR; and GST-ATM 6-S1891A: ANLDAESEHFFR. (C) Sensitivity of phosphorylation of GST-4 by ATM to wortmannin. Untreated and irradiated (10 Gy, 1 h) C3ABR cells were employed for ATM *in vitro* kinase activity in the presence or absence of 1 μ M wortmannin. (D) A-T (AT1ABR) cells are deficient in radiation-induced phosphorylation of GST-4. AT1ABR cells contain near-full-length ATM, homozygous for 7636del 9. This is a kinase dead mutant that expresses a less stable form of ATM. In this experiment, equal loading with control was achieved by immunoprecipitating four-fold more cell extracts. Protein kinase activity was determined under the same conditions as described above.

more, there was no evidence for constitutive activation when phosphorylation of p53, Nbs1, Chk2 and SMCI was used as an indicator of ATM kinase activity and normal radiation induction was observed in all cases (Supplementary Figure 3D).

ATM autophosphorylation site mutants fail to correct DNA repair, radiosensitivity and cell cycle checkpoint defects in A-T cells

Exposure of cells to radiation leads to the rapid accumulation of a variety of DNA damage recognition proteins to the sites of DNA damage in the form of small granular foci (Maser *et al*, 1997; Paull *et al*, 2000). Bakkenist and Kastan (2003) have previously shown that immunofluorescent staining of pS1981 ATM in fibroblasts is diffusely spread across the nucleus immediately after irradiation, some of which local-

Figure 3 ATM is autophosphorylated on S1893 in response to ionizing radiation. (A) Time course of ATM S1893 and S1981 autophosphorylation after 10 Gy of radiation. Control (C3ABR) lymphoblastoid cells were exposed to 10 Gy of radiation, extracts prepared at indicated time points post-irradiation and immunoprecipitated with anti-ATM antibody before immunoblotting with phosphospecific antibodies against ATM pS1893 and pS1981. A-T represents irradiated AT25ABR. (B) Effect of increasing radiation dose on ATM autophosphorylation on S1893 and S1981. Cells were irradiated and incubated for 60 min before preparation of extracts, immunoprecipitation and immunoblotting as described under panel A. A-T is AT25ABR. (C) Autophosphorylation is not detected in A-T cells with either mutant ATM (AT1ABR) or cells not expressing ATM protein (AT25ABR). These cells were irradiated with 10 Gy and incubated for 60 min before processing as described under panel A. C refers to C3ABR cells. Equal loading of ATM mutant was achieved by immunoprecipitating four-fold more protein. (D) Phosphorylation of ATM on S1893 is inhibited by wortmannin. Control (C3ABR) lymphoblastoid cells were pretreated with 20 μ M wortmannin for 0.5 h before mock irradiation or exposure to 10 Gy of radiation. Extracts were prepared 1 h post-irradiation and immunoprecipitated with anti-ATM antibody followed by immunoblotting with phosphospecific antibodies against ATM pS1893 and pS1981. (E) Phosphorylation of ATM on S1893 is inhibited by the ATM-specific inhibitor Ku-55933 (KuDOS Pharmaceuticals). C3ABR cells were pretreated with 10 μ M Ku-55933 for 1 h before irradiation (10 Gy). Cell extracts were prepared 1 h after irradiation and analyzed as described in panel D.

izes to discrete foci at later times. We detected the presence of pS1981 ATM foci immediately after irradiation in A-T lymphoblastoid cells transfected with full-length ATM (pMATI) cDNA (Figure 6A). These foci colocalized with those for γ H2AX. However, when A-T cells were transfected with either S367A or S1893A mutant forms of ATM, reduced numbers of pS1981 and γ H2AX foci were observed. In both

Figure 4 The Mre11 complex is required for radiation-induced autophosphorylation of ATM in S1893. (A) NBS lymphoblastoid (NBS03LA) and control (C3ABR) cells were exposed to 3 Gy of radiation and extracts prepared for immunoprecipitation and immunoblotting at 15 and 60 min post-irradiation. ATM was immunoprecipitated as in Figure 3A followed by immunoblotting with anti-ATM and anti-pS1981 and pS1893 antibodies. Part of the extract was separated directly in 7.5% SDS-PAGE and immunoblotted for Mre11, Rad50 and Nbs1. As expected, no Nbs1 was detected in NBS03LA cells. (B) A-TLD6 and CBABR lymphoblastoid cells were exposed to 3 Gy of radiation and extracts prepared at 15 and 60 min post-irradiation. ATM was immunoprecipitated followed by immunoblotting with anti-ATM and pS1981 and pS1893 antibodies. A portion of the extracts was directly blotted for Mre11, Rad50 and Nbs1 after separation on SDS-PAGE. ATL66 is a compound heterozygote with truncating (R571X) and missense (T481K) mutations. The weaker Mre11 band in ATL66 represents the missense form. Note that both Rad50 and Nbs1 are also expressed at a lower level in those cells, as observed previously (Delia *et al*, 2004). (C) Rad50-deficient (Ha239) and C3ABR lymphoblastoid cells were exposed to radiation (10 Gy) and incubated for 60 min before preparation of extracts. ATM immunoprecipitated and immunoblotting was carried out as described above. A portion of the extract was also immunoblotted for Mre11, Rad50 and Nbs1. The Ha239 cells contain low levels of Rad50. Again in these cells Mre11 and Nbs1, the other two members of the complex, show reduced levels. Some variability was observed in ATM labeling for both the phospho-S1893 and phospho-S1981 in untreated cells.

S1893A- and S367A-transfected cells, foci numbers were approximately 30% of those in pMAT1 cells at 0.5 h post-irradiation (Figure 6B). By 6 h, in cells transfected with pMAT1 foci, numbers decreased by approximately five-fold. In contrast, the basal levels observed in S367A- and S1893A-transfected cells did not change appreciably. Only minimal levels of γ H2AX foci were detectable in S1981A-transfected cells (Figure 6B). Overall numbers of DNA damage-induced foci are low in the ATM mutant cells and change little up to 6 h after damage, indicating that the response to DNA damage is minimal compared to wild-type transfected cells.

In order to establish further the functional significance of the three ATM autophosphorylation sites, we investigated the

ability of the phosphorylation site mutants to correct aspects of the A-T cellular phenotype (Zhang *et al*, 1997). Control and ATIABR cells can be readily differentiated on the basis of radiosensitivity, which is a well-established characteristic for distinguishing these cell types (Lavin and Shiloh, 1997). Transfection of ATIABR cells with full-length ATM cDNA (pMAT1) significantly corrected the radiosensitivity in these cells. Exposure of ATIABR cells, transfected with either S367A or S1893A mutant ATM, to radiation exhibited the same degree of radiosensitivity as the parental cell line (Figure 7A). It is of interest that there appeared to be a small but not significant correction of radiosensitivity in ATIABR cells transfected with S1981A ATM. A-T cells also show increased numbers of radiation-induced chromosome aberrations (ICA) compared to controls (Zhang *et al*, 1997), which represents another measure of capacity to maintain genome stability and survive DNA damage. The number of ICA/metaphase was approximately three-fold higher in ATIABR cells than in control (Figure 7B). Full-length ATM cDNA restored ICA to control levels in ATIABR cells but none of the three autophosphorylation site mutants did so (Figure 7B).

A-T cells are characterized by defects in all cell cycle checkpoints, post-irradiation (Lavin and Shiloh, 1997). We determined whether the autophosphorylation site mutants would correct the defective G2/M checkpoint in ATIABR cells. As a measure of G2 delay, we determined the number of cells entering mitosis with time after irradiation (Chen *et al*, 1999). A rapid inhibition of mitotic index, approximately 20-fold, followed by recovery over a 6 h period is apparent in control cells exposed to 1.5 Gy of radiation (Figure 7C). The extent of inhibition of entry of ATIABR cells into mitosis post-irradiation was 25-fold less. Introduction of full-length ATM cDNA into ATIABR cells imposed a normal pattern of G2/M delay in these cells (Figure 7C). None of the three autophosphorylation site mutants corrected the G2/M checkpoint defect in ATIABR cells.

Discussion

ATM orchestrates the cell cycle checkpoint and DNA repair responses to DNA DSBs by phosphorylating a variety of intermediate substrates in these signaling pathways (Shiloh, 2003). The complexity of this control is evident from separate substrate phosphorylations, direct and indirect, to regulate individual checkpoints (Lavin, 2005). Thus, it is not surprising that the activation of ATM is itself highly regulated. Previous data have described the importance of a single site of ATM autophosphorylation (S1981) in its activation (Bakkenist and Kastan, 2003). We have identified this same site by a different approach and also described two additional autophosphorylation sites, S367 and S1893, that are also essential for activation. The functional significance of yet another site located at S1883, T1884 or T1885 remains to be determined as well as the identity of the protein kinase involved. Indeed the set of phosphorylations described here may be an under-representation of the mechanism of complete activation, as we have also observed a number of other phosphopeptides in our tryptic digestions. Multiple sites of autophosphorylation are also a feature of DNA-PK activation (Chan *et al*, 2002; Ding *et al*, 2003). Once recruited by the Ku heterodimer to the ends of a DNA DSBs, DNA-PK catalytic subunit autophosphorylates at six well-conserved sites before

Figure 5 Effect of autophosphorylation site mutants on the activation of ATM. **(A)** Stable cell lines were established in AT1ABR cells after transfection with the autophosphorylation site mutants S367A, S1893A and S1981A. Mutant ATM protein expressed from an EBV-based construct (pMEP4) was induced for 18 h with CdCl₂ and cells were irradiated with 10 Gy and incubated for a further 10 min. Extracts were prepared and immunoprecipitated with anti-ATM antibody followed by immunoblotting with ATM, pS1893 and pS1981 antibodies. All three mutant proteins were induced to approximately the same amount. The band in the uninduced lanes is owing to endogenous mutant ATM in AT1ABR cells. **(B)** Transfected stable cell lines were induced with CdCl₂ before exposure to radiation (10 Gy) and 1 h incubation before preparation of extracts. Full-length ATM (pMAT1) and mutant constructs were employed. In this case, ATM was immunoprecipitated with anti-FLAG antibodies. The pMEP4 ATM constructs all contain a FLAG tag (Zhang *et al*, 1997). Control (C3ABR) and AT1ABR were included for comparison with the transfected cells (right panel). After immunoprecipitation with anti-ATM antibody, immunoblotting was carried out with ATM and downstream substrates p53, pS15-p53, pS343-Nbs1, Nbs1, pT68-Chk2, Chk2, pS957-SMCI and SMCI.

localizing with other DNA repair proteins at DNA damage foci (Chan *et al*, 2002). Mutation of individual sites leads to a block of DNA end-processing and increased sensitivity to radiation. In the present study, use of a phosphospecific antibody revealed that S1893 is rapidly autophosphorylated in response to radiation exposure and is detected at low radiation doses. As in the case of S1981 autophosphorylation, this satisfies the requirement for ATM activation and signaling (Bakkenist and Kastan, 2003). It is notable that the S1893 site, with an adjacent Glu residue, does not satisfy the predominant predicted recognition sequence, S/TQ, for ATM. This site is readily phosphorylated by ATM *in vitro* and *in vivo* in response to radiation. Non-S/TQ-containing sites have been described previously for *in vitro* and *in vivo* phosphorylation of BRCA1 by ATM (Cortez *et al*, 1999).

Radiation-induced autophosphorylation at S1893 is ATM kinase-dependent and occurs less efficiently in cells that are deficient in components of the Mre11 complex. This is in keeping with a role for the Mre11 complex as a sensor of DNA DSBs. Abrogation of this complex by mutations in Nbs1 or Mre11 have been shown to reduce the efficiency of ATM activation (Uziel *et al*, 2003; Cersalett and Concannon, 2004). Defective activation of ATM was also manifested as defective substrate phosphorylation of p53, Chk2, Nbs1 and SMCI in A-T cells transfected with ATM cDNA mutated in the S1893 site (S1893A). However, this mutant ATM was not devoid of protein kinase activity as it was still capable of S1981 autophosphorylation and phosphorylation of p53 substrate *in vitro*. This was also the case for the S1981A ATM mutant, which exhibited a defective ATM signaling response

Figure 6 ATM autophosphorylation site mutants are deficient in radiation-induced DNA repair. **(A)** ATM mutant proteins are defective in formation of radiation-induced phospho-S1981 and γ H2AX foci. Stable cell lines expressing full-length ATM (pMAT1) and the three autophosphorylation site mutants were induced with CdCl₂ for 18 h, irradiated (10 Gy) and collected after 0.5 and 6 h incubation on slides by cytocentrifugation. Cells were stained with antibodies to phospho-S1981 ATM and γ H2AX and analyzed for foci formation using immunofluorescence microscopy. **(B)** Quantitation of pS1981-ATM and γ H2AX foci. The number of both forms of foci per cell was determined in concordance with that observed in the overlays. Merged foci were quantitated and plotted for each cell line at 0.5 and 6 h post-irradiation.

in cells but was capable of S1893 autophosphorylation and substrate phosphorylation *in vitro*. Bakkenist and Kastan (2003) have previously shown that the ATM S1981A mutant possesses protein kinase activity *in vitro*. The S1893A mutant also failed to correct radiosensitivity, radiation-induced chromosome aberrations and the defective G2/M checkpoint in A-T cells, providing further supportive evidence for a functionally important role for S1893 autophosphorylation in the activation of ATM. The capacity of ATM to autophosphorylate any two of the sites when the third site was mutated, together

with the functional importance of all three phosphorylations suggests that all three sites are involved in ATM activation and downstream signaling. Substitution of S1893 or S1981 with glutamic acid, a phosphomimic, did not lead to constitutive activation of ATM *in vivo*. This is not entirely surprising, as there are many other examples where phosphomimics fail to create active protein kinases (Connell-Crowley *et al*, 1993; Huang and Erikson, 1994; Groban *et al*, 2006) and in this case multiple sites may be required for *in vivo* activation. However, unlike the S1893A and S1981A, the glutamic acid

Figure 7 Failure of ATM autophosphorylation site mutants to correct radiosensitivity, genomic instability and cell cycle defects in AT1ABR cells. **(A)** Survival of control (C3ABR), AT1ABR and AT1ABR cells transfected with pMAT1, S367A, S1893A and S1981A mutant forms of ATM. Cells were induced with CdCl₂, exposed to radiation over the range 0–4 Gy and incubated for 3 days, before determination of cell survival. Survival is expressed as a percentage of irradiated/unirradiated. Each point represents an average of triplicate experiments. Error bars represent s.e.m. **(B)** Radiation-induced (1 Gy) chromosome aberrations in AT1ABR cells transfected with the wild-type and mutant forms of ATM. Cells were induced as described above and aberrations determined as described in Materials and methods. **(C)** Effect of radiation (1.5 Gy) on the G2/M checkpoint, measured by number of mitotic figures appearing with time after irradiation. Error bars represent s.e.m.

substitutions retained normal radiation-inducible ATM-dependent downstream signaling capacity.

Previous data have shown that ATM is a highly phosphorylated protein (Chen and Lee, 1996). The results described here provide evidence for several radiation-induced, functionally significant autophosphorylations in ATM. Coupled to this are parallel dephosphorylations, also implicated in ATM activation (Ali *et al*, 2004; Goodarzi *et al*, 2004). It has been shown that dimerization of ATM is a mechanism to suppress its activation (Bakkenist and Kastan, 2003). Once autophosphorylated on S1981, monomerization occurred to generate active kinase. The additional autophosphorylations described here may also contribute to the monomerization. On the other hand, Lee and Paull (2005) demonstrated that autophosphorylation on ATM S1981 was not required for ATM monomerization in the presence of the Mre11 complex *in vitro*. These results suggest that additional factors or events (such as the additional phosphorylation observed here) are necessary for ATM activation *in vitro*. More recently, evidence has been provided that PP2A interaction with ATM in undamaged cells may also contribute to the suppression of activation

(Goodarzi *et al*, 2004). Exposure of cells to radiation causes a phosphorylation-dependent dissociation of PP2A from ATM and loss of its associated protein phosphatase activity. Thus, PP2A phosphatase activity may play a role in maintaining ATM in the basal state by dephosphorylating ATM at more than one site. On the other hand, DNA damage enhanced the interaction between ATM and another phosphatase, PP5 (Ali *et al*, 2004). Downregulation of PP5 suppressed the activation of ATM by radiation and led to a defect in the intra-S-phase checkpoint. Recent data also reveal that acetylation of ATM and chromatin proteins play a key role in ATM activation (Gupta *et al*, 2005; Sun *et al*, 2005). Our findings demonstrate that like DNA-PK, activation of ATM involves several sites of autophosphorylation with functional significance. Each of the three phosphorylation sites discussed here has been conserved throughout evolution and is therefore likely to be integral to the biological function of ATM. The mechanism of activation of ATM is complex and these results will assist in delineating how each phosphorylation site of ATM relates to DNA damage.

Materials and methods

Cell culture and irradiation

Lymphoblastoid cell lines (LCLs) were established from normal healthy individuals (C3ABR), A-T patients (AT25ABR and AT1ABR), an NBS patient (NBS03LA), an ATLD patient (ATLD 6) and a Rad-50-deficient patient (Ha239). The LCLs were cultured in RPMI 1640 medium with 10% fetal calf serum (15% fetal calf serum in case of ATLD and Rad 50-deficient cells), 100 U/ml penicillin and 100 U/ml streptomycin (Invitrogen). All irradiations were performed at room temperature using a Gammacell 40 Exactor research irradiator (1 Gy/min, MDS Nordion, Canada).

Antibodies

Affinity-purified ATM-specific sheep polyclonal antibodies were prepared as described earlier (Kozlov *et al*, 2003). ATM 2C1 monoclonal antibody (Abcam/GeneTex), phospho-S1981 ATM rabbit polyclonal (Rockland Immunochemicals), Mre11-2D7 monoclonal (GeneTex), Rad50-2C6 monoclonal (Upstate/Chemicon), Nbs1 polyclonal (GeneTex), phospho-S343 Nbs1 polyclonal (Novus Biologicals), gamma-H2AX (pS139) polyclonal (Novus Biologicals), phospho-S15 p53 polyclonal (Cell Signalling Technology), phospho-T68 Chk2 (Cell Signalling Technology), Chk2 polyclonal (Upstate/Chemicon), phospho-S957 SMC1 (Upstate/Chemicon) and SMC1 (Cell Signalling Technology) antibodies were used for immunoblotting.

Phosphospecific antibody production

All peptides were synthesized by Mimotopes (Australia). Phosphopeptides were conjugated to KLH and phospho-Ser-1981 ATM was prepared by immunizing sheep (University of Queensland Veterinary Farm) with a phospho-S1981 peptide conjugate (Bakkenist and Kastan, 2003) followed by affinity purification on affinity column prepared by coupling of the corresponding phosphorylated and non-phosphorylated S1981 ATM peptides to Sulfolink resin (Pierce). Phospho-S1893 ATM antibody was produced in rabbits (IMVS, Australia) by immunization with a conjugate of KLH-LDSEp-SEHFFRC (where pS is the phosphoserine) and was purified by consecutive affinity chromatography on non-phosphorylated and phosphorylated S1893 ATM peptide Sulfolink columns.

Cell extracts, immunoblotting and ATM immunoprecipitation

Cell extracts were prepared by lysing cells in ATM kinase lysis buffer or RIPA buffer containing phosphatase and protease inhibitors (Kozlov *et al*, 2003). For immunoblotting, 50 µg of cell lysate was electrophoresed and transferred to nitrocellulose membranes (Amersham). Membranes probed with antibodies were visualized by using the ECL kit (NEN/DuPont). ATM protein was immunoprecipitated using sheep polyclonal ATM antibodies and resolved on SDS gels (5% acrylamide) as described for the *in vitro*

ATM kinase assays. After transfer to nitrocellulose membrane, they were probed consecutively with phospho-S1893 ATM, phospho-S1981 ATM and ATM2C1 antibodies with stripping between each step. Scaled-up ATM immunoprecipitations were used to obtain ATM protein for mass spectrometry analysis. A portion of the ATM immunoprecipitations was used for *in vitro* autophosphorylation reactions with 20 μ Ci of [γ - 32 P]ATP to produce 32 P-labeled ATM to facilitate analysis by mass spectrometry.

Mass spectrometry

Immunoprecipitated ATM from irradiated cells was separated by SDS-PAGE and excised from gels. An estimated 8–16 μ g of ATM was available after combining multiple lanes from the gel. Each band was digested with trypsin in multiples of four, and the extracted phosphopeptides were pooled and enriched using Fe $^{3+}$ IMAC, as described previously (Larsen *et al*, 2004). The peptides eluting from IMAC were applied to a reverse phase HPLC column as described previously (Graham *et al*, 2004) except that the gradient was 100% phase A (0.1% trifluoroacetic acid aqueous solution) for 6 min, to 12% phase B (0.1% trifluoroacetic acid in acetonitrile) for 0.5 min, to 17.5% phase B for 13.5 min, to 35% phase B for 11 min and then to 100% phase B for 6 min. The radioactive samples were spread across multiple fractions and were vacuum dried and resuspended in 4 μ l of 10% acetonitrile aqueous solution. A portion (0.5 μ l) was spotted onto nitrocellulose membrane and the radiation detected by a phosphorimager (Storm 860, Amersham Biosciences, USA) to determine which fraction contained phosphopeptides. Another portion (0.5 μ l) of each radioactive fraction was treated with phosphatase as described previously (Graham *et al*, 2004) except that Antarctic phosphatase was used at 22°C. The phosphatase-treated sample was desalted using a microcolumn packed with Poros R3 (Applied Biosystems, Boston, MA, USA) and compared to an untreated portion (0.5 μ l) by mass spectrometry using a Voyager-DE Pro MALDI-TOF-MS (Applied Biosystems, USA) as described previously (Graham *et al*, 2004; Larsen *et al*, 2004). Three phosphopeptides were clearly observed (see Figure 1), whereas phosphopeptides from other radioactive fractions remained undetectable (data not shown).

Tandem mass spectrometry peptide sequencing of these phosphopeptides was accomplished using a QSTAR XL QqTOF mass spectrometer (MDS Sciex, Canada) as described previously (Larsen *et al*, 2004).

In vitro ATM kinase assays

ATM protein kinase assays using GST-p53 as substrate and ATM autophosphorylation assays were performed essentially as described previously (Kozlov *et al*, 2003). GST-ATM fusion proteins containing each tryptic phosphopeptide (containing S367, S1893 and S1981) of ATM were prepared according to standard techniques and were called GST-ATM 1, 2 or 3 respectively. GST-ATM 1 contains the peptide sequence ATM_{363–375} (S367), GST-ATM 2 contains the peptide sequence ATM_{1883–1898} and GST-ATM3 contains the peptide sequence ATM_{1974–1992} (S1981). The second peptide was further prepared in a series of truncated and mutated GST forms for the ATM *in vitro* kinase assays: GST-ATM 4-wild type: ANLDSESEHFRR; GST-ATM 5-S1893A: ANLDSEAEHFRR; and GST-ATM 6-S1891A: ANLDASEHFRR.

Expression vectors and transfection

Construction of full-length ATM cDNA expression vector pMAT1 was described previously (Zhang *et al*, 1997). ATM cDNA was placed under the control of heavy metal-inducible metallothionein promoter in pMEP4 vector (Invitrogen). Site-directed mutagenesis of the pMAT1 construct was performed using the Quick-Change mutagenesis kit essentially as described by the manufacturers (Stratagene) to generate both S→A and S→E substitutions for the autophosphorylation sites. Transfection of AT1ABR LCL was performed using Lipofectamine 2000 reagent as described by the manufacturer (GIBCO). A total of 2×10^6 exponentially growing AT1ABR cells were transfected with 5 μ g of pMAT1, S367A, S1893A,

S1981A, S1893E or S1981E ATM DNA. Selection for resistant cells was initiated 48 h post-transfection by addition of 0.2 mg/ml of hygromycin B (Boehringer Mannheim). Cells containing stable replicating episomal vector are usually obtained as survivors in suspension culture after 3–4 weeks of hygromycin selection. Expression of wild-type and mutant ATM proteins in AT1ABR was achieved by induction of stably transfected cells with 5 μ M CdCl₂ for 18 h.

Immunofluorescence microscopy

Transfected cells were collected onto glass slides by cytocentrifugation and stained by indirect immunofluorescence. The sheep phospho-S1981 ATM antibody was used at 1:100 dilution and gamma-H2AX antibody (Cell Signalling Technology) was used at 1:300 dilution. Anti-sheep and anti-rabbit secondary antibodies labeled with Alexa dyes (Molecular Probes) were used to visualize binding of primary antibodies. Coverslips were mounted with the Vectashield antifade solution containing DAPI (Molecular Probes) as DNA counterstain. Images were collected using a digital camera attached to a Zeiss Axioskop (Zeiss) fluorescence microscope. Initial digital image processing was performed using Zeiss software. Digital images were prepared for publication using Adobe Photoshop CS software.

Cell survival

Control, AT1ABR and AT1ABR LCLs transfected with wild-type ATM (pMAT1), S367A, S1893A or S1981A ATM were induced for ATM protein expression. The cells were collected by centrifugation and resuspended at 2×10^5 cells/ml in the cell culture medium. Cells were irradiated with 4 Gy. Cell viability was determined by adding 0.1 ml of 0.4% Trypan blue solution to a 0.5 ml of cell suspension as described previously (Zhang *et al*, 1997). The number of viable cells was counted up to 4 days after irradiation.

Mitotic index

The analysis of radiation-induced mitotic delay was performed as previously described (Chen *et al*, 1999). Briefly, non-transfected and transfected LCLs were irradiated with 1.5 Gy and samples were collected at 1 h intervals for the first 8 h. Both non-irradiated and irradiated cells were washed with 0.075 M KCl and fixed in methanol-acetic acid (3:1) before dropping on glass slides. Cells were stained with diluted Giemsa stain (BDH) for 5 min and nuclei from approximately 1000 cells were counted under a light microscope for each time point.

Induced chromosome aberrations

The analysis of ICA was performed essentially as described previously (Zhang *et al*, 1997). Control, non-transfected and transfected AT1ABR LCLs were irradiated with 1 Gy. For G2-phase cells, colcemid at a final concentration of 0.1 mg/ml was added immediately after irradiation, approximately 1–2 h before harvesting. The cells were treated with 0.075 M KCl, fixed and stained as described above. A total of 50 metaphases were analyzed for each sample.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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