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Telomeric Protein Pin2/TRF1 as an Important ATM Target in Response to Double Strand DNA Breaks*

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ATM mutations are responsible for the genetic disease ataxia-telangiectasia (A-T). **ATM** encodes a protein kinase that is activated by ionizing radiation-induced double strand DNA breaks. Cells derived from A-T patients show many abnormalities, including accelerated telomere loss and hypersensitivity to ionizing radiation; they enter into mitosis and apoptosis after DNA damage. Pin2 was originally identified as a protein involved in G₂/M regulation and is almost identical to TRF1, a telomeric protein that negatively regulates telomere elongation. Pin2 and TRF1, probably encoded by the same gene, *PIN2/TRF1*, are regulated during the cell cycle. Furthermore, up-regulation of Pin2 or TRF1 induces mitotic entry and apoptosis, a phenotype similar to that of A-T cells after DNA damage. These results suggest that ATM may regulate the function of Pin2/TRF1, but their exact relationship remains unknown. Here we show that Pin2/TRF1 coimmunoprecipitated with ATM, and its phosphorylation was increased in an ATM-dependent manner by ionizing DNA damage. Furthermore, activated ATM directly phosphorylated Pin2/TRF1 preferentially on the conserved Ser²¹⁹-Gln site *in vitro* and *in vivo*. The biological significance of this phosphorylation is substantiated by functional analyses of the phosphorylation site mutants. Although expression of Pin2 and its mutants has no detectable effect on telomere length in transient transfection, a Pin2 mutant refractory to ATM phosphorylation on Ser²¹⁹ potentially induces mitotic entry and apoptosis and increases radiation hypersensitivity of A-T cells. In contrast, Pin2 mutants mimicking ATM phosphorylation on Ser²¹⁹ completely fail to induce apoptosis and also reduce radiation hypersensitivity of A-T cells. Interestingly, the phenotype of the phosphorylation-mimicking mutants is the same as that which resulted from inhibition of endogenous Pin2/TRF1 in A-T cells by its dominant-negative mutants. These results demonstrate for the first time that ATM interacts with and phosphorylates Pin2/TRF1 and suggest that Pin2/TRF1 may be involved in the cellular response to double strand DNA breaks.

Mutations in the **ATM** gene are responsible for the rare autosomal human recessive disorder ataxia-telangiectasia (A-T),¹ characterized by progressive neurological degeneration, telangiectasia, growth retardation, premature aging, immunodeficiency, mitotic checkpoint defect, hypersensitivity to ionizing radiation, gonadal atrophy, genomic instability, and predisposition to cancer (1). **ATM** encodes a protein kinase that is activated by ionizing DNA damage and is critical for maintaining genome stability, telomere maintenance, and induction of cell cycle checkpoints by double strand DNA breaks (2–4). **ATM** has been shown to bind and/or phosphorylate many key regulators, including p53, β -adaplin, c-Abl, Chk1–2, Brca1, and Nijmegen breakage syndrome protein (5–15). Identification of these ATM target proteins opens new avenues for understanding the physiological function of ATM as well as for explaining the pleiotropic phenotypes associated with ATM mutations in A-T patients and cells.

Double strand DNA breaks induced by ionizing irradiation activate ATM and trigger multiple pathways to ensure that cells delay entry into mitosis following DNA damage to repair the damaged DNA before cell division. Many of these pathways ultimately lead to the inhibition of cyclin B/Cdc2, a major protein kinase that regulates entry into mitosis (7, 8, 16–22). However, in A-T cells, the ATM-dependent mitotic checkpoint is disrupted, and cyclin B/Cdc2 cannot be kept in an inactive state after DNA damage (22–24). Therefore, A-T cells are hypersensitive to ionizing radiation; they fail to delay entry into mitosis and instead are prone to enter into mitosis and then apoptosis after irradiation (25–28).

Radiation hypersensitivity of A-T cells has been shown to correlate with their telomere loss (25–28). There is compelling evidence supporting an important role for ATM in the regulation of telomere metabolism. Cells derived from humans and mice with a defective **ATM** gene show a prominent defect related to telomere dysfunction (1, 29–32). These cells have an accelerated rate of telomere loss and chromosome end-to-end associations and show premature senescence (25–28, 33, 34). Furthermore, ATM has recently been shown to regulate the interaction between telomeres and the nuclear matrix (35). In addition, the yeast ATM homologues TEL1 and MEC1 control telomere length and the G₂/M checkpoint; their mutations result in shortened telomeres, a G₂/M checkpoint defect, and genomic instability (36–38). Furthermore, TEL1 substitutes for ATM in rescuing telomere shortening, radiation hypersensitivity, and the G₂/M checkpoint defect in A-T cells (39). These results indicate that ATM plays a crucial role in regulating

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¹ The abbreviations used are: A-T, ataxia-telangiectasia; GST, glutathione S-transferase; HA, hemagglutinin; NCS, neocarzinostatin.

telomere length and the DNA damage mitotic checkpoint. However, it is not fully clear how ATM is involved in coordinating these two events.

Telomeres are composed of repetitive DNA sequences of TTAGGG arrays concealed by a complex of telomeric proteins that protect the ends from exonucleolytic attack, fusion, and incomplete replication (40–42). Compelling evidence suggests that telomere length is regulated by telomeric DNA-binding proteins in yeast and mammalian cells (43–47). In human cells, inhibition of one such protein, TRF1, by the dominant-negative mutant increases telomere length, whereas overexpression of TRF1 accelerates telomere loss (47, 48). These results indicate that TRF1 negatively regulates telomere maintenance.

In our genetic search for proteins that are able to suppress premature mitotic entry induced by the mitotic kinase NIMA (never-in-mitosis A), we identified three proteins, Pin1–3, which are all involved in mitotic regulation (49–51). Pin1 is a phosphorylation-specific prolyl isomerase that regulates the conformation of the phosphorylated Ser/Thr-Pro motifs present in a defined subset of phosphoproteins (52–56). Pin2 is identical to TRF1 apart from an internal deletion of 20 amino acids and forms a homodimer or heterodimer with TRF1 (50). In cells, Pin2 is 5–10-fold more abundant than TRF1 (50), and Pin2 and TRF1 are probably two alternatively spliced isoforms of the *PIN2/TRF1* gene, as suggested by Young *et al.* (57). For clarity, we will here use TRF1 for the 20-amino acid-containing isoform and Pin2 for the 20-amino acid deletion isoform, as they were originally identified (48, 50), but refer to endogenous proteins as Pin2/TRF1 because it is still difficult to physically and functionally separate these isoforms. Pin2 and TRF1 contain a structural motif similar to destruction boxes present in mitotic cyclins, and their levels are regulated during the cell cycle, with a striking increase in late G₂ and M (50), as is the case for mitotic cyclins (58). Furthermore, overexpression of Pin2 induces mitotic entry and then apoptosis in cells containing short telomeres (59). These results suggest that Pin2/TRF1 may play a role in the regulation of mitotic entry. However, little is known about the regulation and function of Pin2/TRF1 in cell cycle checkpoints.

Here we show that Pin2/TRF1 formed stable complexes with ATM in cells. Furthermore, following ionizing radiation, activated ATM phosphorylated Pin2/TRF1 preferentially on Ser²¹⁹ both *in vitro* and *in vivo*. Significantly, like Pin2, Pin2^{S219A} induced mitotic entry and apoptosis and increased radiation hypersensitivity of A-T cells. In contrast, Pin2^{S219D} or Pin2^{S219E} completely failed to induce apoptosis and reduced radiation hypersensitivity of A-T cells. Interestingly, these phenotypes of phospho-mimicking Pin2 mutants were the same as those that resulted from inhibition of endogenous Pin2/TRF1 in A-T cells by dominant-negative mutants. These results suggest that phosphorylation of Pin2 on Ser²¹⁹ by ATM probably inhibits its function after DNA damage.

EXPERIMENTAL PROCEDURES

Site-directed Mutagenesis and Recombinant Protein Production—The mutants of Pin2 were generated using polymerase chain reaction mutagenesis procedures and confirmed by sequencing. GST fusion proteins containing Pin2 and various mutants were purified as described (50, 54).

Transient Transfection, Immunoprecipitation, and Immunoblotting Analysis—cDNAs encoding Pin2, TRF1, and its various mutants with an NH₂-terminal HA epitope tag were subcloned into the pUHD expression vector and then transfected into tTA-1 HeLa cells or ATM-negative A-T221JE-T cells using the Superfect reagents (Qiagen) as described (50, 60, 61). In the case of A-T221JE-T cells, tTA expression vector was used for cotransfection as described (62). The expression of the transgenes was determined by immunoblotting analysis using anti-HA antibody (12CA5). For coimmunoprecipitation, cells were lysed in lysis

buffer I (50 mM Hepes, pH 7.5, 150 mM NaCl, 0.5% Triton X-100, 1 mM EDTA, 2.5 mM EGTA, 100 mM NaF, 1 mM Na₃VO₄, 2 μg/ml aprotinin, 5 μg/ml leupeptin, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride), followed by immunoprecipitation with anti-Pin2/TRF1 antibody (5804), preimmune sera, anti-ATM antibody (Ab-3/ATM-6), or anti-c-Abl antibody (Ab-3) as described (50). For the detection of the endogenous protein interaction with anti-ATM antibodies, nuclear extracts were used for immunoprecipitation as described (63). We used cell lysates that were five times more than those in inputs, and we only loaded half of the immunoprecipitates to the gel for Western blotting with the appropriate antibodies.

In Vitro Binding Assay for ATM and Pin2—To detect binding of ATM and ³⁵S-Pin2 *in vitro*, the cDNA encoding Pin2, Pin2^{1–316}, and Pin2^{317–419} were cloned into pcDNA3 (Invitrogen) and subjected to *in vitro* transcription and translation (Promega) in the presence of [³⁵S]Met. Translated proteins were incubated for 12 h at 4 °C in 500 μl of buffer II (50 mM Hepes, pH 7.5, 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 100 mM NaF, 1 mM Na₃VO₄, 2 μg/ml aprotinin, 5 μg/ml leupeptin, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride) with ATM that was immunoprecipitated from equal amounts of lysates prepared from HeLa or A-T221JE-T cells. After being washed three times with buffer II plus 0.25% Triton X-100, precipitated proteins were visualized by SDS-containing gels, followed by autoradiography and immunoblotting analysis with anti-ATM antibodies. To detect the interaction between GST-Pin2 proteins and ATM, ATM that was immunoprecipitated from equal amounts of HeLa lysates was mixed with GST-Pin2 and its mutants. After incubation for 12 h at 4 °C in 500 μl of buffer II with protein A beads, complexes of beads were washed three times with buffer II plus 0.25% Triton X-100, and precipitated proteins were visualized by SDS-containing gels, followed by immunoblotting analysis with anti-GST and anti-ATM antibodies, respectively.

In Vivo and In Vitro Phosphorylation of Pin2/TRF1 by ATM—For the detection of *in vivo* phosphorylation of Pin2/TRF1, HeLa cells were labeled overnight with [³²P]orthophosphate (54), and Pin2/TRF1 was immunoprecipitated with anti-Pin2/TRF1 antibodies, followed by SDS-polyacrylamide gel electrophoresis and phospho-amino acid analysis as described (50, 64). For examining Pin2/TRF1 phosphorylation after irradiation, cells were irradiated and labeled with ³²P for 1–4 h as described (65). For transient expression of ATM, pcDNA3 vectors containing wild-type FLAG-ATM or its kinase-dead mutant were transfected to 293T cells, and expressed proteins were immunoprecipitated using M2 antibody, followed by Pin2 phosphorylation as described (7, 8). Briefly, cells were lysed by freezing and thawing in buffer III (50 mM Hepes, pH 7.5, 150 mM NaCl, 0.2% dodecyl maltoside, 1 mM EDTA, 2.5 mM EGTA, 50 mM NaF, 1 mM NaVO₄, 2 μg/ml aprotinin, 5 μg/ml leupeptin, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride) ATM was then immunoprecipitated with anti-ATM polyclonal antibody (Ab-3). Immune complexes were washed twice with buffer III, once with a buffer containing 100 mM Tris, pH 7.5, and 0.5 M LiCl, and twice with a kinase buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 10 mM MnCl₂, 10 mM MgCl₂, 10% glycerol, 50 mM NaF, 100 μM NaVO₄, 2 μg/ml aprotinin, 5 μg/ml leupeptin, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride). 1 μg of GST-Pin2 or its mutant proteins and 5 μM [γ-³²P]ATP were added; the reaction mixtures were incubated at 30 °C for 30 min; and the reaction products were visualized by SDS-polyacrylamide gel electrophoresis and autoradiography.

Fluorescence in Situ Hybridization and Flow Cytometry—Combinatorial immunostaining and telomere fluorescence *in situ* hybridization was carried out as described (66). Cells grown on coverslips were washed once in Tris-buffered saline and incubated in 3.7% formaldehyde in Tris-buffered saline for 10 min at room temperature. The cells were then permeabilized and blocked with Tris-buffered saline containing 2% normal goat serum and 0.4% Triton X-100 for 30 min. Transfected HA-Pin2 and its mutants were first immunostained with the anti-HA (12CA5) antibody and with TRITC-conjugated mouse secondary antibodies. After immunostaining, the cells were fixed again under the same conditions as the first time. These prepared cells were then denatured in a hybridization mixture containing 70% deionized formamide, 20 mM Tris, pH 7.0, 1% bovine serum albumin, and 10 nM fluorescein isothiocyanate-labeled peptide nucleic acid telomere repeat probe (PerSeptive Biosystems, Framingham, MA) for 10 min at 80 °C. A hybridization was performed for 12 h at room temperature. Finally, DNA was counterstained with 0.5 μg/ml 4',6-diamidino-2-phenylindole, and preparations were mounted in antifade solution (Vectashield, Vector Laboratories). For the preparation of samples for flow cytometric analysis, cells were harvested by trypsinization, and suspended cells were fixed and stained as described above. Flow cytometric analysis was performed by FACScan™ (Becton Dickinson). Transfected cells

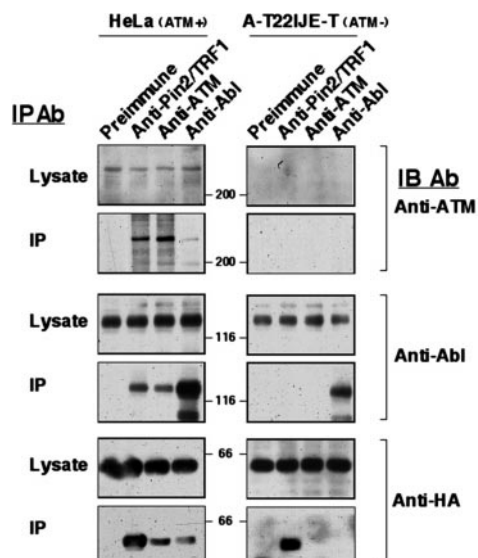


FIG. 1. Interaction between ATM and ectopically expressed HA-Pin2. Coimmunoprecipitation of expressed Pin2 with ATM and also with c-Abl via ATM. An HA-Pin2 expression construct was transfected into HeLa cells (ATM+) and A-T221JE-T cells (ATM-), followed by immunoprecipitation (IP) with the indicated antibodies. Immunoprecipitates were separated on SDS gels and subjected to immunoblotting analysis (IB) with the proper antibodies. An aliquot of each lysate taken prior to immunoprecipitation was also separated on the same gels to show similar amounts of each protein present in lysates.

were gated by FL2 channel, and the intensity of telomere fluorescence was determined by FL1 channel as described (67, 68).

RESULTS

Pin2/TRF1 Coimmunoprecipitates with ATM in Cells—To examine whether ATM is involved in the regulation of Pin2/TRF1, we first determined whether Pin2/TRF1 interacts with ATM. A construct expressing HA-Pin2 was transfected into HeLa cells and then subjected to coimmunoprecipitations as described (50). Consistent with earlier reports (5, 6), ATM coimmunoprecipitated with c-Abl (Fig. 1). Interestingly, HA-Pin2 was also detected both in anti-ATM and anti-c-Abl immunoprecipitates. Conversely, antibodies (anti-Pin2/TRF1) recognizing both Pin2 and TRF1 also immunoprecipitated ATM and c-Abl, although pre-immune sera did not (Fig. 1). These results indicate that expressed Pin2 coimmunoprecipitates with ATM and c-Abl.

To rule out the possibility that the observed coimmunoprecipitations were due to the cross-reactivity of the antibodies and to determine whether ATM was needed for Pin2 to associate with c-Abl, we performed the same experiments in an A-T cell line, A-T221JE-T (61). A-T221JE-T cells were originally derived from primary A-T fibroblasts (69), which harbor a homozygous frameshift mutation at codon 762 of the *ATM* gene and contain no ATM protein, because the truncated protein is not stable (61, 70, 71). Although Pin2 and c-Abl were expressed in A-T221JE-T cells at levels similar to those in HeLa cells, neither anti-ATM nor anti-Abl antibodies were able to immunoprecipitate Pin2 in A-T221JE-T cells. Furthermore, anti-Pin2/TRF1 antibodies did not immunoprecipitate c-Abl. A similar interaction between ATM and the TRF1 isoform was also observed (Fig. 2A). Two COOH-terminal truncation mutants were used to approximately map the domain in Pin2 that interacts with ATM. ATM coimmunoprecipitated with mutants that were truncated up to residue 316 (Fig. 2A), suggesting that the interacting domain may be located at the NH₂-terminal 316 amino acids of Pin2. To further confirm the binding domain, Pin2 was divided into the NH₂-terminal Pin2¹⁻³¹⁶ and the COOH-terminal Pin2³¹⁷⁻⁴¹⁹, and the mutant proteins were

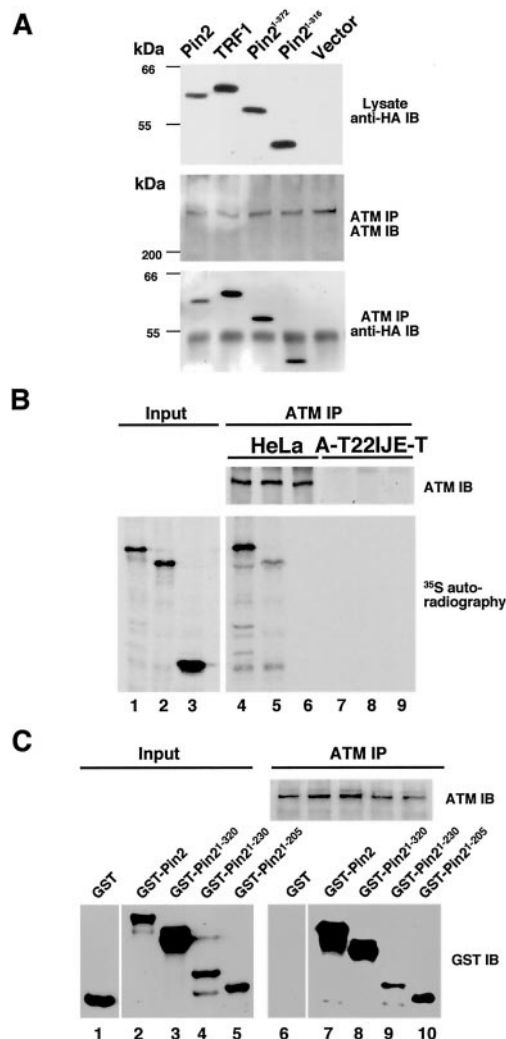


FIG. 2. Identification of the ATM-interacting domain in Pin2. A, interaction of ATM with TRF1 or Pin2 truncation mutants. HeLa cells were transfected with HA-TRF1 or HA-Pin2 truncation mutant constructs. Cell lysates were immunoprecipitated using anti-ATM antibodies, followed by immunoblotting analysis with anti-ATM antibodies or anti-HA antibody (12CA5). Note that slightly more TRF1 than Pin2 was coimmunoprecipitated by anti-ATM antibodies, which is probably due to the fact that more TRF1 protein was expressed in cells. B, interaction of ATM with the NH₂-terminal, but not the COOH-terminal, domain of Pin2. Pin2 and the Pin2 NH₂-terminal (Pin2¹⁻³¹⁶) and COOH-terminal (Pin2³¹⁷⁻⁴¹⁹) mutants were synthesized by *in vitro* transcription and translation in the presence of [³⁵S]Met. The labeled proteins were incubated with ATM that was immunoprecipitated from equal amounts of lysates prepared from HeLa or A-T221JE-T cells. After washing, the bound proteins were subjected to SDS-polyacrylamide gel electrophoresis, followed by autoradiography (bottom panel) or immunoblotting analysis with anti-ATM antibodies (top panel). Lanes 1, 4, and 7, Pin2; lanes 2, 5, and 8, Pin2¹⁻³¹⁶; lanes 3, 6, and 9, Pin2³¹⁷⁻⁴¹⁹. C, interaction of ATM with the NH₂-terminal half of Pin2. Pin2 and its COOH-terminal truncation mutants were expressed and purified as GST fusion proteins and then incubated with ATM that was immunoprecipitated from equal amounts of HeLa lysates. After washing, the bound proteins were subjected to SDS-polyacrylamide gel electrophoresis, followed by immunoblotting analysis with anti-ATM antibodies (top panel) or anti-GST antibodies (bottom panels). Lanes 1 and 6, GST; lanes 2 and 7, Pin2; lanes 3 and 8, Pin2¹⁻³²⁰; lanes 4 and 9, Pin2¹⁻²³⁰; lanes 5 and 10, Pin2¹⁻²⁰⁵. IB, immunoblotting analysis; IP, immunoprecipitation.

produced and labeled with ³⁵S by *in vitro* transcription/translation. When the labeled proteins were incubated with anti-ATM immunoprecipitates prepared from HeLa cells, both full-length Pin2 and its NH₂-terminal Pin2¹⁻³¹⁶, but not the COOH-terminal Pin2³¹⁷⁻⁴¹⁹, were precipitated by anti-ATM

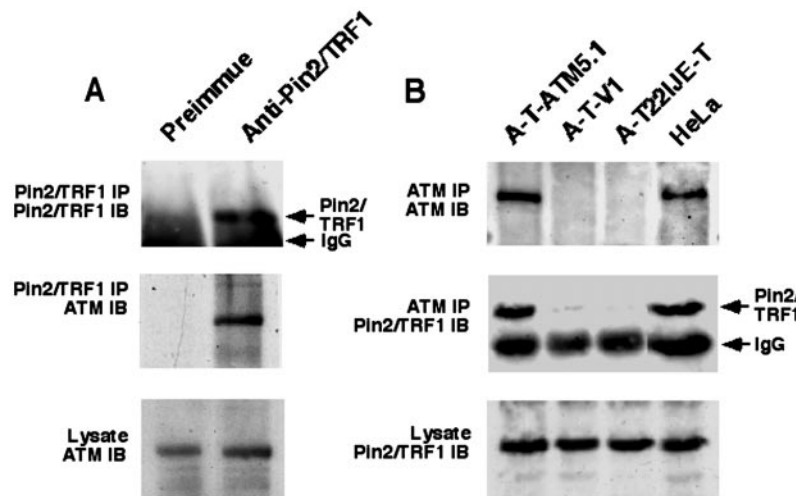


FIG. 3. Coimmunoprecipitation of ATM with endogenous Pin2/TRF1. A, coimmunoprecipitation of ATM with Pin2/TRF1. Cell lysates from HeLa cells were immunoprecipitated with anti-Pin2/TRF1 antibodies or preimmune sera, followed by immunoblotting analysis (IB) with anti-ATM antibodies or anti-Pin2/TRF1 antibodies. B, coimmunoprecipitation of Pin2/TRF1 with ATM. A construct expressing full-length ATM or an empty vector was stably transfected into A-T221JE-T cells. Cell lysates from these stable cells and HeLa cells were immunoprecipitated with anti-ATM antibodies, followed by immunoblotting analysis with anti-ATM (top panel) or anti-Pin2/TRF1 antibodies (middle panel). Pin2/TRF1 was present at similar levels in all cells, as shown in the bottom panel. HeLa, an ATM-positive cell line; A-T221JE-T, an ATM-negative parent cell line; A-T-ATM5.1, a representative A-T221JE-T-derived cell line that stably expressed ATM; A-T-V1, a representative A-T221JE-T-derived cell line that was stably transfected with the control vector.

antibodies (Fig. 2B). Furthermore, the binding was not observed with anti-ATM immunoprecipitates from ATM-negative A-T221JE-T cells (Fig. 2B), demonstrating that anti-ATM does not nonspecifically precipitate Pin2. Finally, we performed *in vitro* binding experiments using ATM immunoprecipitates and GST-Pin2 or its mutants. Pin2 and three different COOH-terminal truncation mutants, Pin2^{1–320}, Pin2^{1–230}, and Pin2^{1–205}, were generated and purified as GST fusion proteins, followed by incubation with ATM immunoprecipitated from HeLa cells. All these three mutants, but not the control GST, bound to ATM. Together, these results indicate that the NH₂-terminal half of the Pin2 molecule specifically interacts with ATM.

To examine whether ATM associated with endogenous Pin2/TRF1, we performed coimmunoprecipitation experiments in non-transfected ATM-positive cells (HeLa cells) and A-T cells (A-T221JE-T) using anti-ATM or -Pin2/TRF1 antibodies. As shown in Fig. 3A, anti-Pin2/TRF1 antibodies, but not their pre-immune sera, coimmunoprecipitated ATM. Conversely, anti-ATM antibodies, but not pre-immune sera, coimmunoprecipitated Pin2/TRF1 in HeLa cells, but not in A-T221JE-T cells (Fig. 3B and data not shown). These results indicate that Pin2/TRF1 and ATM coimmunoprecipitation occurs in ATM-positive cells but not in ATM-negative cells. To rule out the possibility that the failure of anti-ATM antibodies to immunoprecipitate Pin2/TRF1 in A-T221JE-T cells is due to genetic factors other than the lack of ATM in these cells, we transfected an ATM expression vector or its control vector into A-T221JE-T cells as described (61). After selection with G418, multiple cell lines that were stably transfected with ATM (A-T-ATMs) or with the control vector (A-T-Vs) were obtained, as confirmed by immunoblotting analysis with anti-ATM antibodies (Fig. 3B). ATM, but not the vector, restored the coimmunoprecipitation between Pin2/TRF1 and ATM in A-T cells (Fig. 3B). Because Pin2/TRF1 levels were similar in these ATM-negative cells, these results indicate that anti-ATM antibodies do not nonspecifically precipitate Pin2/TRF1. As compared with amounts of Pin2/TRF1 and ATM proteins present in total lysates, about 10–15% of ATM coimmunoprecipitated with Pin2/TRF1 (Fig. 3). These results demonstrate that a fraction of ATM interacts with Pin2/TRF1 in cells.

DNA Damage Increases Phosphorylation of Pin2/TRF1 in an

ATM-dependent Manner in Vivo—Because Pin2/TRF1 interacts with ATM, a kinase activated by ionizing radiation, Pin2/TRF1 may be a substrate for ATM. To test this possibility, we first asked whether Pin2/TRF1 is phosphorylated in the cell and, if so, whether its phosphorylation is increased upon ionizing radiation. When HeLa cells were subjected to irradiation and labeled with [³²P]orthophosphate, phosphorylation of Pin2/TRF1 was significantly increased (Fig. 4A), indicating that ionizing radiation increases Pin2/TRF1 phosphorylation *in vivo*. To determine whether ATM is required for irradiation-induced phosphorylation *in vivo*, we repeated the same experiments in A-T-ATM5.1 (ATM-positive) and A-T-V1 (ATM-negative) cells. Although Pin2/TRF1 was still phosphorylated at low levels in A-T-V1 cells, its phosphorylation was not increased after irradiation (Fig. 4B), suggesting that Pin2/TRF1 can be phosphorylated by other protein kinase(s) that are not activated by irradiation. Importantly, phosphorylation of Pin2/TRF1 was significantly increased after irradiation in A-T-ATM5.1 (Fig. 4B), as is the case in HeLa cells (Fig. 4A). These results demonstrate that ionizing radiation increases phosphorylation of Pin2/TRF1 in an ATM-dependent manner *in vivo*.

Activated ATM Directly Phosphorylates Pin2 Preferentially on Ser²¹⁹ in Vitro and in Vivo—We next asked whether ATM is directly responsible for the phosphorylation of Pin2/TRF1 following ionizing radiation. To address this question, we needed to determine whether ATM can directly phosphorylate Pin2/TRF1. ATM belongs to a family of phosphatidylinositol 3-kinase-related and wortmannin-sensitive protein kinases, which phosphorylate proteins preferentially on an SQ motif (Fig. 5A) (5–13). Consistent with this idea, endogenous Pin2/TRF1 was phosphorylated mainly on Ser residues *in vivo* (Fig. 5B). Although Pin2/TRF1 contains three SQ sequences (Ser²¹⁹-Gln, Ser²⁷⁴-Gln, and Ser³⁴⁶-Gln), only one SQ sequence (Ser²¹⁹-Gln) is conserved in all known vertebrate homologues, even in the mouse TRF1 that shares only 67% identity to the human counterpart (Fig. 5A) (48, 50, 72, 73). Significantly, this SQ is not conserved in human TRF2 (Fig. 5A), a closely related but functionally distinct telomeric protein (74). This analysis suggests that Ser²¹⁹-Gln might be a potential phosphorylation site of ATM. To test this possibility, fragments spanning the NH₂-terminal 230 or 320 residues of Pin2, containing one or two of

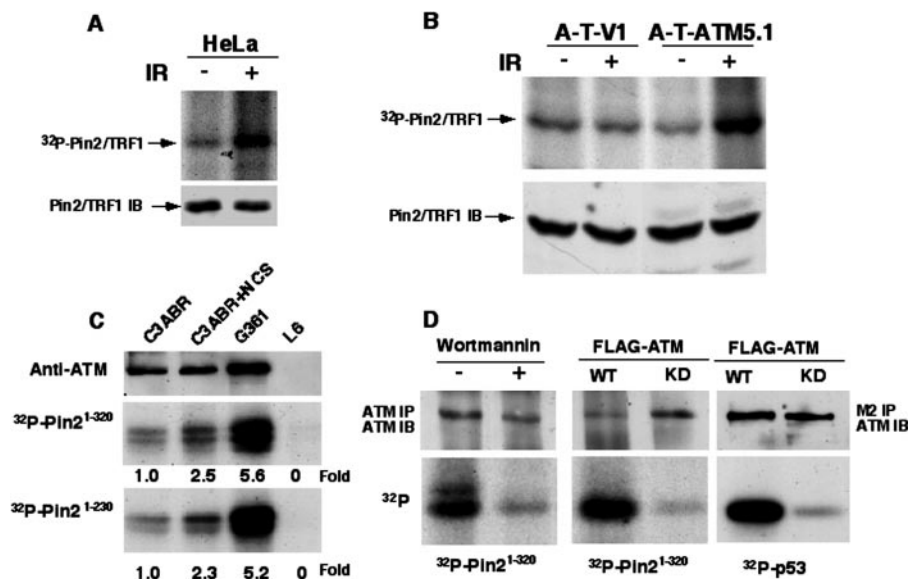


FIG. 4. Phosphorylation of Pin2/TRF1 by ATM *in vivo* and *in vitro*. *A* and *B*, induction of Pin2/TRF1 phosphorylation by ionizing radiation (IR) in an ATM-dependent manner. HeLa cells (*A*) or A-T-V1 and A-T-ATM5.1 cells (*B*) were irradiated with γ -rays (+) or mock-irradiated (–) and then labeled with [^{32} P]orthophosphate. Pin2/TRF1 was immunoprecipitated and separated on SDS-containing gels, followed by autoradiography or by immunoblot with anti-Pin2/TRF1 antibodies. *C*, phosphorylation of Pin2 by ATM *in vitro*. ATM was immunoprecipitated from equal amounts of total proteins in various cell lines and visualized by immunoblotting with anti-ATM antibodies (*top panel*). The ATM immunoprecipitates were used to phosphorylate GST-Pin2^{1–320} and GST-Pin2^{1–230}. Phosphorylation was quantified with a phosphorimaging device and normalized to phosphorylation of GST-Pin2/TRF1^{1–320} by ATM obtained from untreated C3ABR cells. C3ABR, a normal lymphoblastoid cell line; C3ABR+NCS, C3ABR cells treated with NCS; G361, a melanoma cell line overexpressing ATM; L6, an ATM-negative lymphoblastoid cell line. *D*, direct phosphorylation of Pin2/TRF1 and p53 by ATM. *Left panels*, ATM was immunoprecipitated from G361 cells using anti-ATM antibodies and subjected to immunoblotting analysis with the same antibodies (*top panel*) or to kinase assay using GST-Pin2^{1–300} as a substrate in the presence (+) or absence (–) of 0.5 μ M wortmannin (*bottom panel*). *Middle and right panels*, FLAG epitope tagged wild-type (WT) and kinase-dead (KD) ATM expression constructs were expressed in 293T cells and then immunoprecipitated using the M2 antibody specifically against the FLAG tag, followed by immunoblotting analysis using anti-ATM antibodies (*top panels*) or by kinase assays using GST-Pin2^{1–300} or p53 as a substrate (*bottom panels*). IB, immunoblotting analysis; IP, immunoprecipitation.

the SQ sequences, respectively, were produced as GST fusion proteins, GST-Pin2^{1–230} and GST-Pin2^{1–320}, and used as a substrate for ATM. Both proteins were readily phosphorylated to a similar extent (Fig. 4C). These results are consistent with the idea that Ser²¹⁹ is preferentially phosphorylated by ATM kinase, whereas Ser²⁷⁴ is probably not phosphorylated by ATM.

To confirm that phosphorylation of Pin2 by ATM immunoprecipitates is indeed due to ATM kinase, we used the following five different criteria, which had previously been used to validate phosphorylation of various proteins by ATM (7–9). First, Pin2 was phosphorylated *in vitro* by ATM immunoprecipitated from ATM-positive cells (C3ABR, G361, and HeLa) but not from ATM-negative cells (L6) (Fig. 4C) (7). Second, the extent of Pin2 phosphorylation *in vitro* correlated with the amounts of ATM present in the cells (7). The increased level of ATM protein isolated from an ATM-overexpressing melanoma cell line, G361, led to an increased level of Pin2 phosphorylation relative to that seen with ATM isolated from a low ATM-expressing normal lymphoblastoid cell line, C3ABR (Fig. 4C). Third, treatment of cells with a radiomimetic drug, neocarzinostatin (NCS), or ionizing radiation has been previously shown to activate ATM kinase activity 2–3-fold, although neither of the treatments affects levels of ATM protein (7, 8). Accordingly, when three different cell lines, C3ABR, G361, and HeLa, were treated with NCS (Figs. 4C and 5C) or irradiated (Fig. 5D), ATM kinase activity toward Pin2 was increased 2–3-fold. Fourth, Pin2 phosphorylation by ATM immunoprecipitates was strongly inhibited by wortmannin (Fig. 4D), a phosphatidylinositol 3-kinase inhibitor that has been shown to inhibit ATM kinase (7, 9). Finally, to rule out the possibility that Pin2 phosphorylation by ATM immunoprecipitates is due to contaminating or associated kinases, we used transfected cells with wild-type FLAG-ATM and its kinase-dead point mu-

tant, followed by isolating the expressed proteins using M2 monoclonal antibody as described previously (8). Although the expressed wild-type ATM was able to phosphorylate Pin2 or p53 (Fig. 4D), its kinase-dead mutant had little activity toward Pin2 or p53, even though the mutant protein was expressed and immunoprecipitated (Fig. 4D). This result indicates that, like p53 phosphorylation, Pin2 phosphorylation is intrinsic to the ATM protein. These results provide strong evidence that ATM specifically phosphorylates Pin2 and suggest that activation of ATM by DNA damage is critical for Pin2 phosphorylation.

After demonstrating that ATM directly phosphorylates Pin2 *in vitro*, we examined whether Ser²¹⁹ in Pin2 is indeed phosphorylated by ATM by replacing Ser²¹⁹ with Ala in Pin2^{1–320}. The mutant protein could still be weakly phosphorylated by ATM immunoprecipitates (Fig. 5, C–E), suggesting that ATM may phosphorylate other sites *in vitro*. However, the extent of phosphorylation in the mutant protein was reproducibly reduced slightly, as compared with that of the wild-type protein (Fig. 5, C and D). Furthermore, this difference became much more dramatic when DNA damage-activated ATM was used (Fig. 5, C–E). Although ionizing radiation or NCS treatment increased ATM kinase activity to Pin2^{1–320} about 2–3-fold, there was no increase in phosphorylation of the mutant Pin2^{S219A} protein (Fig. 5, C and D). To examine whether Ser²¹⁹ in Pin2 is also phosphorylated by ATM *in vivo*, wild-type and S219A mutant Pin2 were transfected into HeLa cells and labeled with ^{32} P, followed by ionizing radiation. As shown in Fig. 5E, irradiation increased phosphorylation of wild-type Pin2 but did not significantly affect phosphorylation of the mutant Pin2^{S219A}, although both proteins were expressed at similar levels. Taken together, the above results indicate that although Pin2 may be phosphorylated on other sites, Ser²¹⁹ is the pref-

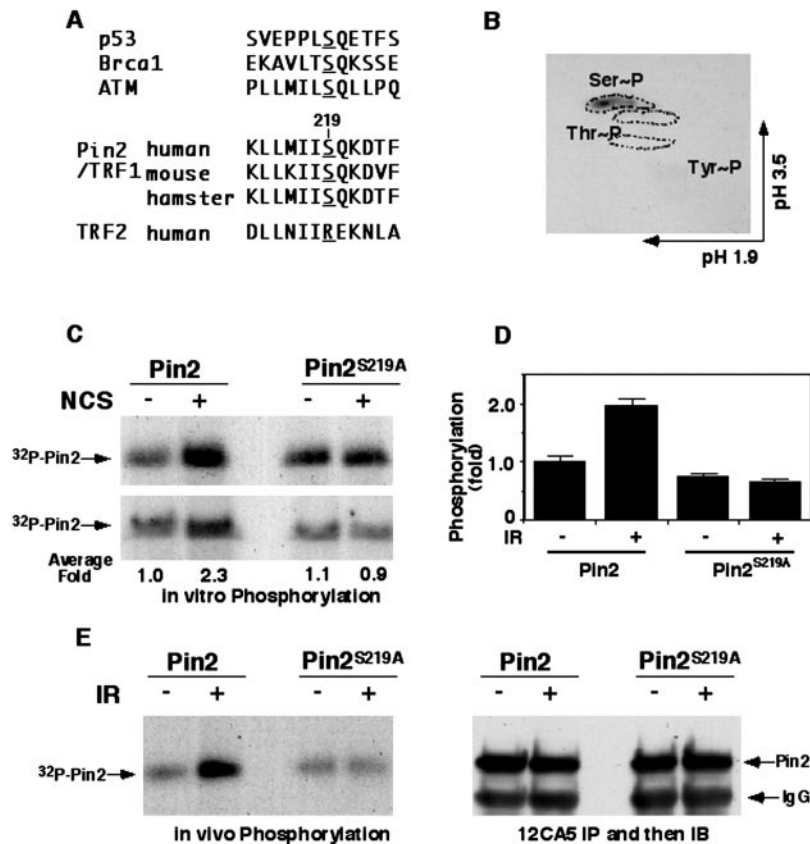


FIG. 5. Phosphorylation of Pin2 by activated ATM preferentially on Ser²¹⁹ *in vitro* and *in vivo*. *A*, alignment of selected known ATM phosphorylation sites and a potential ATM phosphorylation site in Pin2/TRF1 and its related proteins. Only one SQ in Pin2/TRF1, Ser²¹⁹-Gln, is conserved in all known Pin2/TRF1 homologues but not in TRF2, a distinct telomeric protein. *B*, Ser phosphorylation of Pin2/TRF1 *in vivo*. *In vivo* ³²P-labeled Pin2/TRF1 was immunoprecipitated and separated on SDS-containing gels, followed by phospho-amino acid analysis on TLC plates together with phospho-amino acid standards as indicated. *C*, phosphorylation of Pin2 preferentially on Ser²¹⁹ by NCS-activated ATM. After G361 cells were treated either with 50 ng/ml NCS or control buffer for 2 h, ATM protein was immunoprecipitated to phosphorylate GST-Pin2¹⁻³²⁰ (Pin2) or its S219A mutant (Pin2^{S219A}). Phosphorylation was quantified from two independent experiments and normalized to that from mock-treated cells. *D*, phosphorylation of Pin2 preferentially on Ser²¹⁹ by irradiation-activated ATM. After HeLa cells were irradiated with 5 Gray of γ -rays (+) or mock-irradiated (-), ATM protein was immunoprecipitated to phosphorylate wild-type or mutant GST-Pin2¹⁻³²⁰. Data are representative of four independent experiments. *E*, abolishing Pin2 phosphorylation by the S219A mutation *in vivo*. HeLa cells were transfected with an HA-Pin2 or HA-Pin2^{S219A} expression vector and irradiated with γ -rays (+) or mock-irradiated (-), followed by labeling with [³²P]orthophosphate. HA-Pin2 was immunoprecipitated with anti-HA antibody and separated on SDS-containing gels, followed either by autoradiography (*left panel*) or by immunoblot with the same antibody (*right panel*). IR, ionizing radiation.

erential phosphorylation site by ATM following ionizing DNA damage both *in vivo* and *in vitro*.

Pin2 Phosphorylation Site Mutants or Dominant-negative Mutants Partially Complement Radiation Hypersensitivity of A-T Cells—Given that ATM interacts with and phosphorylates Pin2 on Ser²¹⁹, we examined whether this phosphorylation has any biological significance, affecting the function of Pin2, the phenotype of A-T cells, or both. We have previously demonstrated that Pin2/TRF1 levels are significantly increased at the G₂/M transition and that overexpression of Pin2/TRF1 triggers mitotic entry and then apoptosis (53, 59). Interestingly, this Pin2-induced phenotype is similar to that of A-T cells after ionizing radiation (25–28, 59). These results suggest that phosphorylation of Pin2/TRF1 by ATM upon irradiation may be a mechanism to prevent Pin2 from inducing abnormal mitotic entry and apoptosis, thereby reducing the radiation sensitivity.

To test this possibility, we first examined whether Ser²¹⁹ phosphorylation affects the ability of Pin2 to induce mitotic entry and apoptosis by substituting Ser²¹⁹ either with the non-phosphorylatable residue Ala or with Glu or Asp, which might mimic phosphorylation on Ser²¹⁹. The mutant Pin2 proteins were expressed in cells at levels similar to that of the wild-type protein, interacted with ATM, formed dimers, and localized at telomeres in the cells (Fig. 7A and data not shown). Because long term expression of TRF1 has been shown to cause

telomere shortening (47), we first examined whether expression of Pin2 and its Ser²¹⁹ mutants affects telomere length in transient transfection before apoptosis induction. Because the experiments were performed in transient transfection, with the transfection efficiency being about 20–40%, this made it difficult to determine telomere length by genomic Southern blotting analysis. To overcome this difficulty, we used two different but complementary methods to detect telomere length in transiently transfected cells. One method is to measure telomere length by flow cytometry, and the other is to measure telomere length by fluorescence microscopy after fluorescence *in situ* hybridization of telomere repeats; both methods have been successfully used to estimate telomere length in cells (67, 68, 75). To optimize the conditions, we used HeLa cells and HeLa 1.2.11, which have the same genetic background but have telomere lengths of 1–3 and 15–40 kilobases, respectively, as determined by genomic Southern analysis (59, 76). As shown in Fig. 6A, HeLa 1.2.11 cells had almost 10 times longer telomeres than those in HeLa cells, as measured by the fluorescence intensity of telomeric fluorescence *in situ* hybridization, which validates the assays. Therefore, we doubly stained the cells that had been transiently transfected with HA-Pin2 and its mutants with the anti-HA antibody and the fluorescence telomeric probe and measured telomere length of HA-positive cells by flow cytometry and by fluorescence microscopy. As shown in

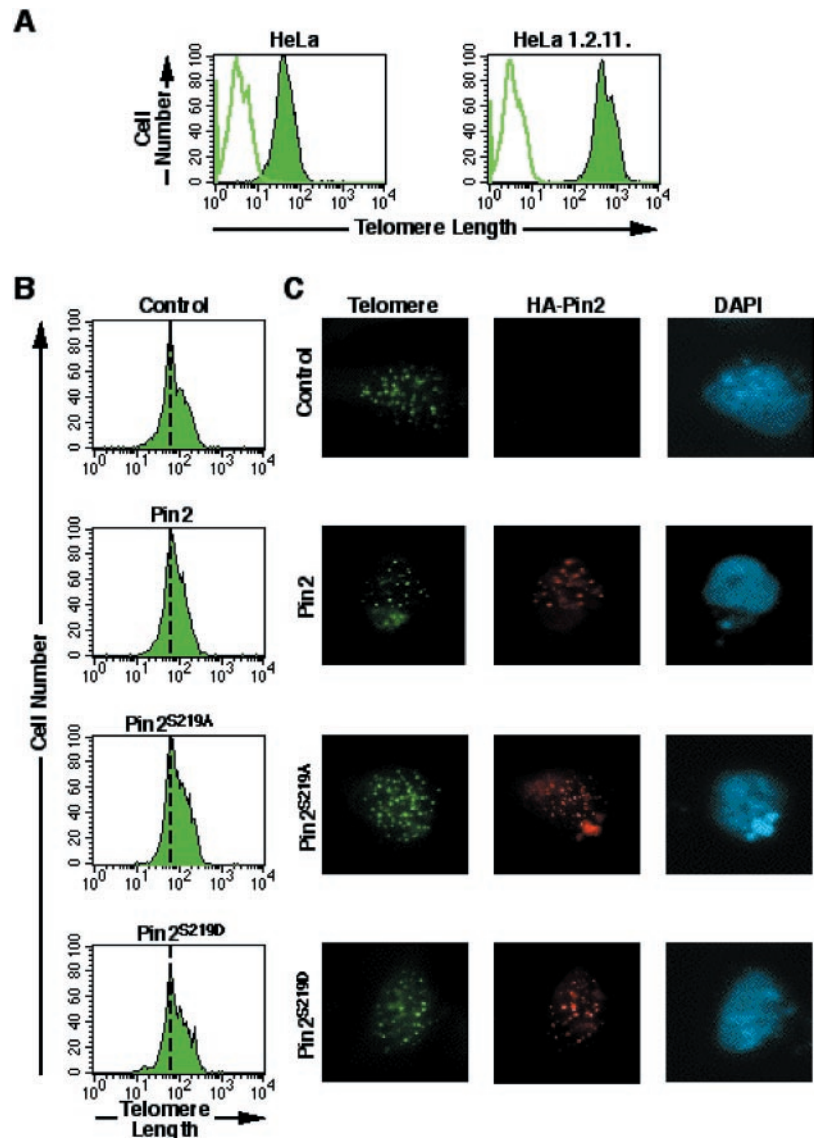


FIG. 6. Pin2 and Ser²¹⁹ phosphorylation site mutants have no effect on telomere length in transient transfection. *A*, telomere length in HeLa and HeLa1.2.11 cells. Cells were stained with fluorescence telomeric DNA probe and then subjected to flow cytometrical analysis. *B* and *C*, the effect of Pin2 and its mutants on telomere length. HeLa cells were transfected with the control vector or vector expressing HA-Pin2 or its Ser²¹⁹ mutants as indicated for 20–24 h. Cells were fixed and doubly stained with the anti-HA antibody and fluorescence telomeric DNA probe, followed by flow cytometrical analysis (*B*) and fluorescence microscopy.

Fig. 6, *B* and *C*, there was no detectable difference in nontransfected control cells and those expressing Pin2 or its mutants in both assays. These results indicate that Pin2 and Ser²¹⁹ mutants have little effect on telomere length during transient expression, which is consistent with the previous findings that it takes many population doublings to detect the effect of Pin2/TRF1 on telomere length (47).

However, we observed strikingly different phenotypes induced by Pin2 and its mutants. As shown earlier (59), Pin2 was more potent in inducing mitotic entry and apoptosis in A-T-V1 cells stably transfected with the control vector than in HeLa cells or A-T-ATM 5.1 cells stably expressing ATM (Fig. 7, *B–D*). Importantly, although Pin2^{S219A} potentially induced apoptosis, Pin2^{S219D} and Pin2^{S219E} were completely inactive both in ATM-positive cells and in ATM-negative cells (Fig. 7, *B–D*). These results indicate that the substitutions of Ser²¹⁹ with the phosphoserine-mimicking residues completely abolish the ability of Pin2 to induce mitotic entry and apoptosis.

We then examined whether Pin2 phosphorylation site mutants affect radiation hypersensitivity of A-T cells. A-T22IJE-T cells were transfected with various GFP-Pin2 mutants and then irradiated; we then examined the apoptotic phenotype of the transfected cells. As shown previously (59, 61, 77), the apoptosis rate was increased 5-fold from 80 to 42% after irradiation in vector-transfected cells (Fig. 8*A*). However, if

A-T22IJE-T cells were transfected with GFP-Pin2^{S219A}, the apoptosis rate was high in the absence of irradiation and was slightly higher after irradiation (Fig. 8*A*). In contrast, if the cells were transfected with GFP-Pin2^{S219D} or GFP-Pin2^{S219E}, the apoptosis rate decreased down to 20%. These results indicate that both Pin2^{S219D} and GFP-Pin2^{S219E} decrease radiation hypersensitivity of A-T cells. To further confirm this observation and to examine whether the protective effect of phosphorylation-mimicking mutants is due to the loss of ATM, we examined the effects of Ser²¹⁹ mutants on cell survival after irradiation using A-T-V1 and A-T-ATM 5.1 cells. To identify the transfected cells, different Pin2 constructs were cotransfected with a LacZ reporter construct. Cells were fixed at various times after irradiation and stained with LacZ, followed by counting surviving transfected cells as described (59). Consistent with other reports (61, 77), A-T-V1 cells showed the typical radiation hypersensitivity, and this phenotype was rescued by expression of ATM, as shown in A-T-ATM 5.1 cells (Fig. 8, *B* and *C*). Interestingly, whereas Pin2 and Pin2^{S219A} increased, Pin2^{S219D} decreased radiation hypersensitivity of A-T-V1 cells (Fig. 8*B*). Furthermore, when Pin2^{S219D} was transfected into A-T-ATM 5.1, there was no such protective effect on radiation sensitivity (Fig. 8*C*), indicating that the protective effect of Pin2^{S219D} on A-T cells is due to the loss of ATM. These results show that the Pin2 mutant refractory to ATM phosphorylation

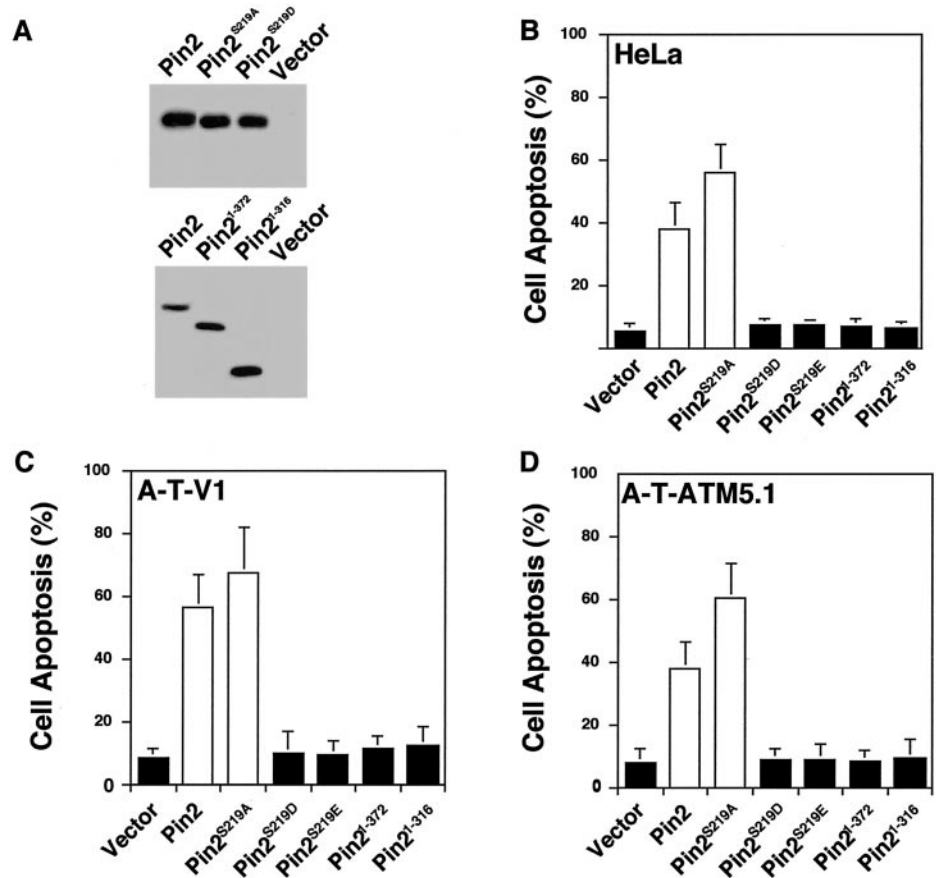


FIG. 7. Effects of Ser²¹⁹ phosphorylation site mutations on the ability of Pin2 to induce mitotic entry and apoptosis. A–D, the effect on the ability of Pin2 to induce apoptosis. HeLa (A and B), A-T-V1 (C), and A-T-ATM5.1 (D) cells were transfected with vectors expressing GFP-Pin2 or its mutants or with a GFP vector, fixed at 30 h after transfection, and stained with 4',6-diamidino-2-phenylindole; then the percentage of apoptotic cells was counted. Results represent averages of three experiments. A shows levels of expressed proteins, as determined by immunoblotting analysis.

on Ser²¹⁹ and the Pin2 mutants mimicking ATM phosphorylation have opposite effects, increasing and decreasing radiation hypersensitivity of A-T cells, respectively. We concluded that phosphorylation of Pin2 on Ser²¹⁹ may inhibit its function.

To confirm that inhibition of Pin2/TRF1 reduces radiation hypersensitivity of A-T cells, we used dominant-negative Pin2 mutants to inhibit the function of endogenous Pin2/TRF1. Because Pin2/TRF1 functions as a dimer via its NH₂-terminal dimerization domain, COOH-terminal truncation Pin2 mutants act as dominant-negative mutants by forming heterodimers with the endogenous protein and preventing endogenous Pin2/TRF1 from performing its normal functions (47, 50). When two different dominant-negative Pin2/TRF1 mutants, Pin2¹⁻³⁷² and Pin2¹⁻³¹⁶, were transfected into cells, neither of the mutants induced apoptosis in ATM-positive or -negative cells (Fig. 7, B–D), although mutant proteins were expressed at levels similar to those of wild-type protein (Fig. 7A). Furthermore, the dominant-negative Pin2 mutants reduced radiation-induced apoptosis and increased cell survival of A-T cells after irradiation (Fig. 8, A and B). Importantly, if A-T cells stably expressed ATM, Pin2¹⁻³¹⁶ had little effect on their radiation sensitivity, as shown in A-T-ATM 5.1 cells (Fig. 8C). Interestingly, all the phenotypes of the dominant-negative Pin2 mutants were the same as those induced by phosphorylation-mimicking Pin2 mutants (Figs. 7 and 8). These results support the notion that phosphorylation of Pin2/TRF1 by ATM following DNA damage may inhibit its function and contribute to the cellular response to DNA damage.

DISCUSSION

The following results support the conclusion that Pin2/TRF1 is an important downstream substrate for ATM following DNA damage. First, endogenous ATM specifically interacts with the NH₂-terminal half of Pin2/TRF1 and forms stable complexes

with both ectopically expressed and endogenous Pin2/TRF1 in cells (Figs. 1–3). Second, ionizing DNA damage induces phosphorylation of Pin2/TRF1 in an ATM-dependent manner (Fig. 4). Third, ATM activated by DNA damage directly phosphorylates Pin2/TRF1 preferentially on Ser²¹⁹ both *in vitro* and *in vivo* (Fig. 5). Fourth, the biological significance of this phosphorylation is substantiated by functional analyses of the phosphorylation site mutants. Although expression of Pin2 and Ser²¹⁹ mutants has no detectable effect on telomere length under the conditions used (Fig. 6), Pin2^{S219A}, a mutant refractory to ATM phosphorylation on Ser²¹⁹, potently induces mitotic entry and apoptosis and increases radiation hypersensitivity of A-T cells (Figs. 7 and 8). In contrast, Pin2^{S219D} or Pin2^{S219E}, mutants potentially mimicking ATM phosphorylation on Ser²¹⁹, completely fails to induce apoptosis and also reduces radiation hypersensitivity of A-T cells (Figs. 7 and 8). Fifth, the phenotype of Pin2^{S219D} is the same as that we see when endogenous Pin2/TRF1 in A-T cells is inhibited by dominant-negative Pin2/TRF1 mutants (Figs. 7 and 8), suggesting that phosphorylation of Pin2/TRF1 on Ser²¹⁹ by ATM probably inhibits its function after DNA damage. Finally, neither Pin2^{S219D} nor the dominant-negative Pin2 mutant has any protective effect on radiation sensitivity if ATM is reexpressed in A-T cells (Fig. 8), indicating that the protective effects are due to the loss of ATM. These results indicate that ATM binds and phosphorylates Pin2/TRF1 and probably negatively regulates its function in DNA damage response.

The notion that Pin2/TRF1 is involved in the ATM-dependent DNA damage response is consistent with the expression pattern and function of Pin2/TRF1 during the cell cycle, which is tightly regulated. Pin2/TRF1 contains a motif related to the destruction box that mediates degradation of many mitotic proteins, including cyclin B (50, 58). Furthermore, the levels of

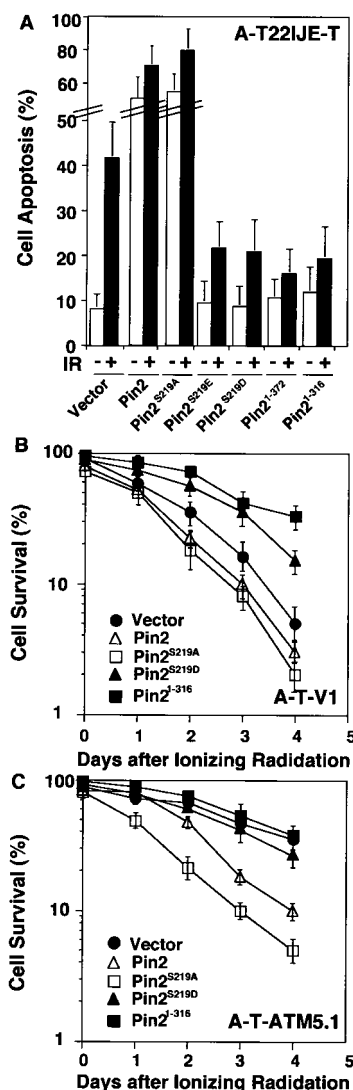


FIG. 8. Reversal of radiation hypersensitivity of A-T cells either by phosphorylation-mimicking Pin2 mutants or dominant-negative Pin2 mutants. A, effects on radiation-induced apoptosis. A-T221JE-T cells were transfected with control vector or various GFP-Pin2 mutant constructs for 16–18 h and irradiated (+) or mock-treated (–). Cells were harvested 12 h later and stained with 4',6-diamidino-2-phenylindole; then the percentage of cells with apoptotic phenotype in transfected cells was counted. B and C, effects on cell survival after irradiation. A-T221JE-T cells stably transfected with the control vector (B, A-T-V1) or the ATM construct (C, A-T-ATM5.1) were cotransfected with different Pin2 mutants or control vector and the reporter LacZ construct for 12 h and then irradiated. Cells were harvested at various times after irradiation and stained for LacZ expression; then the number of surviving blue cells was counted.

Pin2/TRF1 are increased at the G₂/M transition, followed by degradation as cells exit from mitosis. Our previous study indicates that overexpression of Pin2/TRF1 promotes mitotic entry and apoptosis (59). The current investigation has further shown that inhibition of Pin2/TRF1 either by the dominant-negative Pin2 mutant or the Pin2 mutants mimicking ATM phosphorylation significantly reduces radiation hypersensitivity of A-T cells, preventing A-T cells from entering mitosis and apoptosis after DNA damage. In contrast, the Pin2 mutant refractory to ATM phosphorylation on Ser²¹⁹ potentially induces mitotic entry and apoptosis and increases radiation hypersensitivity of A-T cells. These results indicate that the level and function of Pin2/TRF1 are tightly regulated during the cell cycle, reaching their maximum at the G₂/M transition.

Our findings that Pin2/TRF1 is an important downstream

substrate for ATM can help explain some phenotypes associated with ATM mutations. A-T cells have two closely correlated and prominent defects, radiation hypersensitivity and telomere loss (25–28). Because inhibition of Pin2/TRF1 reduces radiation hypersensitivity, the lack of ATM to suppress Pin2/TRF1 in A-T cells contributes to radiation hypersensitivity of A-T cells (1, 61, 77). Because up-regulation of Pin2/TRF1 accelerates telomere shortening (47), the lack of ATM to suppress the Pin2/TRF1 function in A-T cells may also contribute to accelerated telomere loss (25–28). If Pin2/TRF1 would indeed regulate both radiation response and telomere length, this would provide an explanation for why radiation hypersensitivity is correlated with telomere loss in A-T cells (25–28). In addition, because Pin2/TRF1 mediates the interaction between telomeres and the nuclear matrix (78), our results are also consistent with the findings that ATM is able to regulate the interactions between telomeres and the nuclear matrix (35).

In summary, we have demonstrated for the first time the interaction between ATM kinase and the telomeric protein Pin2/TRF1. ATM coimmunoprecipitated with Pin2/TRF1 in cells and, upon irradiation, phosphorylated Pin2/TRF1 preferentially on the only conserved Ser²¹⁹-Gln site. Significantly, like Pin2, Pin2^{S219A} potentially induced mitotic entry and apoptosis and increased radiation hypersensitivity of A-T cells. In contrast, Pin2^{S219D} or Pin2^{S219E} completely failed to induce apoptosis and also reduced radiation hypersensitivity of A-T cells. Because the phenotype of phosphorylation-mimicking Pin2 mutants is the same as that which resulted from inhibition of endogenous Pin2/TRF1 by dominant-negative mutants, phosphorylation of Pin2/TRF1 by ATM probably inhibits its function in the DNA damage response. Therefore, Pin2/TRF1 is an important ATM substrate in response to double strand DNA breaks. Further studies on how Pin2/TRF1 is involved in the DNA damage response will help understand the physiological and pathological functions of ATM and elucidate the molecular mechanisms of cellular responses to DNA damage.

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Telomeric Protein Pin2/TRF1 as an Important ATM Target in Response to Double Strand DNA Breaks

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