A Critical Role for Pin2/TRF1 in ATM-dependent Regulation

INHIBITION OF Pin2/TRF1 FUNCTION COMPLEMENTS TELOMERE SHORTENING, RADIOSENSITIVITY, AND THE G2/M CHECKPOINT DEFECT OF ATAXIA-TELANGIECTASIA CELLS*

Received for publication, November 28, 2001
Published, JBC Papers in Press, December 13, 2001, DOI10.1074/jbc.M111365200

Shuji Kishi‡ and Kun Ping Lu§

From the Cancer Biology Program, Division of Hematology/Oncology, Department of Medicine, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts 02215

Cells derived from patients with the human genetic disorder ataxia-telangiectasia (A-T) display many abnormalities, including telomere shortening, premature senescence, and defects in the activation of S phase and G2/M checkpoints in response to double-strand DNA breaks induced by ionizing radiation. We have previously demonstrated that one of the ATM substrates is Pin2/TRF1, a telomeric protein that binds the potent telomerase inhibitor PinX1, negatively regulates telomere elongation, and specifically affects mitotic progression. Following DNA damage, ATM phosphorylates Pin2/TRF1 and suppresses its ability to induce abortive mitosis and apoptosis (Kishi, S., Zhou, X. Z., Nakamura, N., Ziv, Y., Khoo, C., Hill, D. E., Shiloh, Y., and Lu, K. P. (2001) J. Biol. Chem. 276, 29282–29291). However, the functional importance of Pin2/TRF1 in mediating ATM-dependent regulation remains to be established. To address this question, we directly inhibited the function of endogenous Pin2/TRF1 in A-T cells by stable expression of two different dominant-negative Pin2/TRF1 mutants and then examined their effects on telomere length and DNA damage response. Both the Pin2/TRF1 mutants increased telomere length in A-T cells, as shown in other cells. Surprisingly, both the Pin2/TRF1 mutants reduced radiosensitivity and complemented the G2/M checkpoint defect without inhibiting Cdc2 activity in A-T cells. In contrast, neither of the Pin2/TRF1 mutants corrected the S phase checkpoint defect in the same cells. These results indicate that inhibition of Pin2/TRF1 in A-T cells is able to bypass the requirement for ATM in specifically restoring telomere shortening, the G2/M checkpoint defect, and radiosensitivity and demonstrate a critical role for Pin2/TRF1 in the ATM-dependent regulation of telomeres and DNA damage response.

Mutations in the ATM gene are responsible for the genetic disease ataxia-telangiectasia (A-T) (1). Cells derived from A-T patients display many abnormalities, including telomere shortening, premature senescence, and hypersensitivity to ionizing radiation (2, 3). Exposure of normal mammalian cells to ionizing radiation leads to a delay in progression of the cell from G1 to S phase, inhibition of DNA synthesis, and a delay in progression from G2 phase into mitosis (4, 5). Similar mechanisms are also presented in yeast cells (6, 7). These cell cycle checkpoints allow cells to repair damaged DNA and to maintain genomic stability (3, 8). However, A-T cells are defective in activating checkpoints at the G1/S, during S phase, and at the G2/M in response to ionizing radiation exposure (2). ATM is a protein kinase that is activated by ionizing DNA damage and is critical for genome stability, telomere maintenance, and induction of cell cycle checkpoints (2, 3). ATM has been shown to phosphorylate and regulate many key regulators, including p53, β-adaptin, c-Abl, Chk1–2, Brca1, and Nijmegen breakage syndrome protein (9–19). For example, ATM phosphorylates p53 and thereby increases transcription of the Cdk inhibitor p21 and the Cdc2 sequester 14-3-3r. Furthermore, ATM also phosphorylates Chks, which eventually leads to inhibition of Cdc2 activation. These multiple and redundant pathways have been shown to be involved in cell cycle checkpoint regulation (2, 3).

An increasing body of evidence supports an important role for ATM in regulating telomere metabolism. Cells derived from humans and mice with a defective ATM gene show prominent defects related to telomere dysfunction (1, 20–23). Both primary and transformed A-T cells have been found to have unusually short telomeres and chromosome end-to-end associations, and primary A-T cells show premature aging/senescence phenotype (24–29). Furthermore, expression of a dominant-negative ATM fragment in normal cells results in a decrease in average telomere repeat length (29, 30). Moreover, ATM has been implicated in regulation of chromosome end associations and telomere nuclear matrix interactions (31). In yeast, deletion of ATM homologous genes TEL1 and MEC1 also leads to accelerated telomere shortening, premature aging, and the G2/M checkpoint defect (32–35). Interestingly, yeast TEL1 partially substitutes for human ATM in suppressing ionizing radiation-induced apoptosis and telomere shortening in A-T cells (36), and overexpression of telomerase elongates telomeres and extends the life span of A-T cells (37). These results indicate that ATM plays a crucial role in regulation of telomere maintenance and the G2/M checkpoint. However, overexpression of telomerase does not rescue radiosensitivity, telomere fusion, or cell cycle checkpoint defects in A-T cells (37). In addition, deletion of both TEL1 and MEC1 in yeast does not affect telomerase activity and still allows telomerase to act when the

Dye:
- DAPI, 4',6-diamidino-2-phenylindole
- FITC, fluorescein isothiocyanate
- GFP, green fluorescent protein
- x-gal, 5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside
- FISH, fluorescent in situ hybridization

* This work was supported by National Institutes of Health Grants R01GM56230 and R01GM58556 (to K. P. L.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Present address: Dept. of Cancer Biology, Dana-Farber Cancer Inst., and Dept. of Pathology, Harvard Medical School, 1 Jimmy Fund Way, Boston, MA 02115.
§ A Pew Scholar and Lymphoma and Leukemia Society Scholar. To whom correspondence should be addressed: Beth Israel Deaconess Medical Center, HIM 1047, 330 Brookline Ave., Boston, MA 02215. Tel.: 617-667-4143; Fax: 617-667-6010; E-mail: kh@caregroup.harvard.edu.

1. The abbreviations used are: ATM, ataxia-telangiectasia-mutated; A-T, ataxia-telangiectasia cells; PBS, phosphate-buffered saline; BrdUrd, deoxyuridomuridine; SA-β-Gal, senescence-associated β-galactosidase; FITC, fluorescein isothiocyanate; GFP, green fluorescent protein; BrdU, bromodeoxyuridine; ICRF, 1-iodo-2-deoxyuridine; dUrd, deoxybromouridine; SA-GFP, sensor-linked GFP; SA-β-Gal, senescence-associated β-Galactosidase; FISH, fluorescent in situ hybridization; DAPI, 4',6-diamino-2-phenylenediamine.
A Critical Role for Pin2/TRF1 in ATM-dependent Regulation

The telomere structure is disrupted (38). These results indicate that the primary function of ATM in telomere maintenance is not to regulate telomerase activity but rather to act on telomeres or telomere proteins (38).

Our previous studies indicate that one of the ATM substrates in the regulation of telomeres and mitotic progression is the telomere protein Pin2/TRF1 (39). Pin2/TRF1 was originally identified by its ability to bind telomeric DNA repeats (TRF1) (40) or to bind the mitotic kinase NIMA and suppress its ability to induce mitotic catastrophe (Pin2) (41, 42). Pin2 is identical to TRF1 with the exception of a 20-amino acid internal deletion but is 5-10-fold more abundant than TRF1 in the cell (43); they are likely generated from the same gene PIN2/TRF1 (44). For clarity, we will use Pin2 for the 20-amino acid deletion isoform and TRF1 for the 20-amino acid-containing isoform, as they were originally identified (40, 41, 43), and use Pin2/TRF1 for endogenous proteins. Overexpression of Pin2/TRF1 accelerates telomere loss, whereas dominant-negative Pin2/TRF1 increases telomere length, indicating that Pin2/TRF1 is a negative regulator of telomere elongation (45). Furthermore, Pin2/TRF1 interacts with tankyrase and Tin2, which modulate telomere metabolism (46, 47). Although Pin2/TRF1, tankyrase, and Tin2 do not directly inhibit telomerase activity (45–47), we have recently demonstrated that Pin2/TRF1 binds a potent telomerase inhibitor, PinX1, which directly inhibits telomerase, shortens telomeres, and induces cells into crisis (48). These results indicate that Pin2/TRF1 plays a key role in the regulation of telomere maintenance.

In addition, we have shown that both the protein level and subcellular localization of Pin2/TRF1 are tightly regulated during the cell cycle. Pin2/TRF1 levels are increased in the G2/M phase (43). Furthermore, Pin2/TRF1 specifically localizes to the mitotic spindle during mitosis and affects microtubule assembly (49, 50). Moreover, overexpression of Pin2/TRF1 induces abortive mitosis and apoptosis in cells containing short telomeres but not in those containing long telomeres (51). These results indicate that Pin2/TRF1 also plays an important role in mitotic progression. This is consistent with other studies linking telomere regulation to mitotic progression. For example, elimination or mutation of telomeres causes a Rad9p-mediated cell cycle arrest in G2 in budding yeast (52), triggers abortive mitosis and apoptosis in Drosophila (53), or causes a severe delay or block in anaphase, displaying an anaphase bridge in Tetrahymena (54).

Interestingly, we have also demonstrated that Pin2/TRF1 binds with ATM in vitro and in vivo (39). Furthermore, ATM activated by DNA damage directly phosphorylates Pin2/TRF1 preferentially on serine 219 and also suppresses its ability to induce abortive mitosis and apoptosis (39). Moreover, point mutations in Pin2/TRF1 mimicking ATM phosphorylation completely abolished its ability to induce apoptosis, whereas replacing the ATM phosphorylation site with a nonphosphorylatable residue rendered Pin2 resistant to suppression by ATM (39). In addition, overexpression of Pin2/TRF1 results in phenotypes similar to those of ATM mutations, including accelerated telomere shortening (45–48), abortive mitosis, and apoptosis (51). These results suggest that ATM may inhibit the function of Pin2/TRF1 during DNA damage response. However, the physiological importance of Pin2/TRF1 in mediating ATM-dependent regulation remains to be determined.

To address this question, we here inhibited the function of endogenous Pin2/TRF1 in A-T cells by stable expression of two different dominant-negative Pin2/TRF1 mutants. Both the mutants increased telomere length in A-T cells. More importantly, both the mutants reduced radiosensitivity and restored the G2/M checkpoint defect without inhibiting Cdc2 activation in A-T cells. In contrast, neither of the mutants affected the S phase checkpoint defect in same cells. These results indicate that inhibition of Pin2/TRF1 can specifically suppress telomere shortening, the G2/M checkpoint defect, and radiosensitivity in A-T cells and demonstrate a critical role for Pin2/TRF1 in mediating some aspects of phenotypes associated with ATM mutations.

EXPERIMENTAL PROCEDURES

Stable Expression of ATM or Dominant-negative Pin2/TRF1 Mutants in A-T Cells—For stable expression of ATM, pEBSV vector encoding full-length ATM tagged with FLAG or the control vector were stably transfected into parental A-T22LE-T cells as described (39, 55). After selection with hygromycin B (200 μg/ml) and limited dilution, multiple clones were isolated and checked for ATM expression by immunoblotting analysis with anti-ATM antibody (Ab-3) and anti-FLAG antibody (M5). For stable expression of Pin2 mutants, cDNA encoding Pin2 (1–372) and Pin2 (1–316) were cloned into the pEGFP-C1 vector and stably transfected into A-T22LE-T cells. After selection with G418 (1 mg/ml), GFP-expressing cells were picked up under a fluorescence microscope. Multiple stable clones were obtained with similar properties. To detect expression of GFP fusion proteins in the cells, trypanized cells were resuspended in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, washed in phosphate-buffered saline (PBS), and then immediately analyzed by flow cytometry (BD PharMingen) for detection of the GFP fluorescence intensity of individual cells with nontransfected cells as a negative control or by immunoblotting analysis with anti-GFP antibodies.

Analysis of Telomere Restriction Fragment Length—Telomere restriction fragment length was determined as described previously (48). Briefly, genomic DNA was isolated and digested with restriction enzymes HinfI and RsnI (New England Biolabs), separated on 0.7% agarose gels (2 μg of DNA per lane). The gels were dried, but not completely, and then hybridized in gel with a 500-bp telomeric DNA fragment labeled with α[32P]dCTP by standard protocols, followed by autoradiography.

Telomere FISH Analysis—Telomere FISH was carried out as described previously (39). Briefly, cells grown on coverslips were washed once in Tris-buffered saline (TBS) and incubated in 3.7% formaldehyde in TBS for 10 min at room temperature. These prepared cells were then denatured in a hybridization mixture containing 70% deionized formamide, 5× sodium formate, 60 mM magnesium chloride, and 0.5 mg/ml of DNA per lane). The gels were dried, but not completely, and then hybridized in gel with a 500-bp telomeric DNA fragment labeled with α[32P]dCTP by standard protocols, followed by autoradiography.

Analysis of Telomere Restriction Fragment Length—Telomere restriction fragment length was determined as described previously (48). Briefly, genomic DNA was isolated and digested with restriction enzymes HinfI and RsnI (New England Biolabs), separated on 0.7% agarose gels (2 μg of DNA per lane). The gels were dried, but not completely, and then hybridized in gel with a 500-bp telomeric DNA fragment labeled with α[32P]dCTP by standard protocols, followed by autoradiography.

Senescence-associated β-Galactosidase Activity Assay—To stain for senescence-associated β-galactosidase (SA-β-Gal), cells grown in dishes or on coverslips were washed with PBS and fixed in 0.5% glutaraldehyde. The cells were then incubated with staining solution (1 mg/ml 5-bromo-4-choro-3-indolyl-galactoside, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, and 1 mM MgCl2 in PBS at pH 6.0) for 12 h at 37 °C, as reported previously (48, 56). Cells were rinsed in PBS, and the staining and cell morphology were determined under a microscope.

Cell Cycle Analysis—For cell cycle analysis, cells were harvested by trypsinization, resuspended in Dulbecco’s modified Eagle’s medium supplemented with 10% serum, washed in PBS, and then fixed in 70% ethanol. After washing cells once with PBS containing 1% bovine serum albumin, DNA was stained with propidium iodide (10 μg/ml) containing 250 μg/ml of ribonuclease A, followed by flow cytometry analysis (BD Pharmingen) as described (42, 51). DNA synthesis was assayed by incubation with 10 μM BrdUrd for 60 min, and incorporation of BrdUrd into cells was determined by staining cells with PE-conjugated anti-BrdUrd monoclonal antibody, followed by flow cytometry according to the manufacturer’s protocol (BD Pharmingen) as described (48). Tyr-15 phosphorylation status and histone H1 kinase activity of Cdc2 were assayed as described previously (42, 58).
RESULTS

Stable Expression of Two Different Dominant-negative Pin2/TRF1 Mutants, Pin2-(1–372) and Pin2-(1–316), or ATM in A-T Cells—Overexpression of Pin2/TRF1 induces telomere shortening, abortive mitosis, and apoptosis (45, 51). Furthermore, ATM phosphorylates Pin2/TRF1 and suppresses its ability to induce abortive mitosis and apoptosis (39). Interestingly, A-T cells contain shortened telomeres and enter abortive mitosis and apoptosis upon ionizing radiation (25–28, 30, 55, 59). These results suggest that one of the major functions of ATM activation following DNA damage may be to inhibit Pin2/TRF1 function. If this is the case, direct inhibition of endogenous Pin2/TRF1 function in A-T cells may bypass the requirement for ATM in restoring some phenotypes of A-T cells.

To test this hypothesis, we used two different dominant-negative mutants, Pin2-(1–372) and Pin2-(1–316), to inhibit the function of endogenous Pin2/TRF1. Pin2/TRF1 functions as a dimer and contains an N-terminal dimerization domain and a C-terminal telomeric DNA-binding domain (43, 45). Overexpression of the C-terminal truncation Pin2 mutants would act in a dominant-negative manner by forming heterodimers with the endogenous protein and preventing endogenous Pin2/TRF1 from performing its normal functions, as shown previously (43, 45). To facilitate the identification of transfected cells and to monitor expression of mutant proteins in stable cell lines, we inserted a GFP epitope tag, which does not affect Pin2/TRF1 function, as shown previously (39, 51). We transfected A-T22LJE-T (A-T cells) with the GFP-Pin2 mutants and control GFP and generated multiple independent cell lines stably expressing GFP-Pin2-(1–316) (A-T-GFP-Pin2-(1–316)), GFP-Pin2-(1–372) (A-T-GFP-Pin2-(1–372)), or GFP (A-T-GFP) (Fig. 1, A and B). As controls, ATM and the control vector were also stably transfected into the same A-T cells, producing A-T-ATM and A-T-V cells, respectively (Fig. 1C), as described previously (39, 55). A-T cells were originally derived from primary A-T fibroblasts and immortalized by SV40 (60), 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, as shown previously (39, 55, 61, 62). Protein levels of GFP-Pin2 mutants in stable cell lines were similar to that of control GFP, as determined by immunoblotting analysis with anti-GFP antibodies (Fig. 1A) and by measuring the intensity of the GFP fluorescence using flow cytometry (Fig. 1B). Furthermore, without DNA damage, there was no detectable difference either in the cell cycle profile or the cell morphology between the control GFP and GFP-Pin2/TRF1 mutant-expressing cells (Figs. 4B, 5B, and 7B). Similarly, re-expression of ATM in A-T cells did not affect the cell cycle profile (Figs. 4A, 5A, and 7A). Similar phenotypes as described below were observed in multiple independent cell clones isolated from each stable transfection (Fig. 5A, data not shown). These results indicate that stable expression of dominant-negative Pin2/TRF1 mutants does not have any general effect on cell cycle progression, similar to re-expression of ATM.

Dominant-negative Pin2/TRF1 Mutants Elongate Telomeres in A-T Cells—Primary A-T fibroblasts and lymphocytes, as well as transformed A-T fibroblasts and lymphoblasts, have been found to have unusually short telomeres (26–28). Furthermore, expression of a dominant-negative ATM fragment in normal cells results in a decrease in average telomere repeat length (29), whereas expression of the yeast ATM homologue TEL1 in A-T cells restores telomere shortening (36). These results indicate that telomere shortening is one of the characteristic features of A-T cells. Because expression of a dominant-negative Pin2/TRF1 mutant causes telomere elongation in other cells (45), we would be interested in examining whether stable expression of dominant-negative Pin2/TRF1 mutants affects telomere length in A-T cells.

To examine this possibility, we determined telomere length, after about 5 months of subculture from the establishment of stable cell lines, using two different methods. First, we used fluorescence in situ hybridization with fluorescent telomere repeats (Telomere FISH) to qualify the intensity of telomere signals in nuclei as described previously (39). As shown in Fig. 2A, the telomeric signal intensity in both of the mutant-expressing cells was stronger than that in control GFP-expressing cells. When the fluorescence intensity was measured in more than 300 cells using the NIH Image software, quantitative increases were readily found in both GFP-Pin2-(1–372) or GFP-Pin2-(1–316)-expressing A-T cells, as compared with the cells expressing control GFP cells (Fig. 2B). To confirm these results, we measured telomere restriction fragment length in stable cell lines using genomic Southern analysis as described previously (48). As shown in Fig. 2B, control A-T-GFP cells contained rather short and relatively uniform telomeres, which is similar to other A-T cell lines (26–28, 36). However, telomeres in both A-T-GFP-Pin2-(1–372) and A-T-GFP-Pin2-(1–316) cells were significantly lengthened and more heterogeneous (Fig. 2C). Furthermore, there was a good correlation in the telomere signals determined by telomere FISH and genomic Southern analysis (Fig. 2). Interestingly, expression of yeast TEL also results in a similar increase in telomere length in A-T cells (36). Therefore, these results indicate that both Pin2-(1–372) and Pin2-(1–316) mutants increase telomere length in A-T cells, confirming that they indeed act as dominant-negative mutants to inhibit endogenous Pin2/TRF1 function in A-T cells, as shown previously in other cells (45).

Dominant-negative Pin2/TRF1 Mutants Correct the Radiosensitivity of A-T Cells—A prominent and characteristic abnormality associated with ATM mutations is hypersensitivity to ionizing radiation, which can be observed in A-T patients exposed to therapeutic levels (63, 64) and in cultured cells from these patients (65, 66). We have previously demonstrated that following ionizing radiation, ATM phosphorylates Pin2/TRF1 (39). Furthermore, ATM-phosphorylation site mutants or dominant-negative mutants partially reduce the radiosensitivity of A-T cells in transient transfection (39). Together with the results described below in Fig. 5, these results suggest that dominant-negative Pin2/TRF1 mutants might be expected to enhance cell survival in A-T cells post-irradiation.

To examine the effects of the Pin2/TRF1 mutants on the radiosensitivity of A-T cells, various GFP stable cell lines were treated with ionizing radiation, followed by monitoring of cell morphology and growth at various times after the treatment. At 24 h post-irradiation, the majority of A-T-GFP and A-T-V cells were contracted, rounded up, and loosely attached to culture flasks (Fig. 3, data not shown). DAPI staining revealed that nuclei in the cells became condensed and fragmented, with some cells blocked at anaphase (Fig. 3). Surprisingly, both A-T-GFP-Pin2-(1–372) and A-T-GFP-Pin2-(1–316) were not contracted or rounded up, and nuclei were not condensed. Instead, most of these cells exhibited interphase cell morphologies that were similar to those of A-T-ATM cells (Fig. 3, data not shown). These results indicate that the dominant-negative Pin2/TRF1 mutants prevent A-T cells from entering apoptosis right after ionizing radiation.

After further culture for 3–4 days post-irradiation, a small fraction of A-T-GFP and A-T-V cells that did not round up eventually exhibited increased size and a flattened morphology (Fig. 4, A and B). Furthermore, they were also stained positively for SA–β-Gal (Fig. 4, A and B), a biomarker for identify-
ing senescent human cells in culture and in aging skin in vivo (56). These cells failed to proliferate, and almost no cell colonies were observed after 2–3 weeks of further culture (Fig. 4C). Because the parental A-T cell line AT22JE-T was immortalized by SV40 to escape premature senescence (60), these data suggest that DNA damage induces immortalized A-T cells to enter premature senescence. More importantly, under the same conditions, senescent phenotypes, including the morphological changes and positive SA-β-Gal staining, were rarely found in A-T-GFP-Pin2-(1–372) or A-T-ATM cell lines at 3–4 day post-irradiation (Fig. 4, A and B), indicating that these cells are refractory to premature senescence after DNA damage. Moreover, these cells continued to divide and eventually formed many cell colonies after 2–3 week of further culture (Fig. 4C). Similar results were also observed with A-T-GFP-Pin2-(1–316) (data not shown). These results on both short term and long term cell survival consistently indicate that expression of either of the dominant-negative Pin2/TRF1 mutants corrects the radiosensitivity of A-T cells, with potency similar to that of re-expression of ATM.

**Fig. 1. Stable expression of two different dominant-negative Pin2/TRF1 mutants or ATM in A-T cells.** A and B, expression of GFP-Pin2 mutants. AT22JE-T cells were transfected with control GFP vector (A-T-GFP) or GFP-Pin2 mutants (A-T-GFP-Pin2-(1–372)) or A-T-GFP-Pin2-(1–316), and multiple stable cell lines were established from each transfection. Expression of transgenes was detected by immunoblotting analysis with anti-GFP antibodies (A) or by measuring the fluorescence intensity of GFP using flow cytometry (B). C, re-expression of ATM in A-T cells. AT22JE-T cells were stably transfected with pEBS7 vector encoding full-length ATM tagged with FLAG (A-T-ATM) or the control vector (A-T-V), followed by detection of expression of ATM using immunoblotting analysis with anti-ATM antibodies (top panel) or with anti-FLAG antibodies (bottom panel). Note, ATM-positive HeLa cells were used as a control for ATM expression.
Dominant-negative Pin2/TRF1 Mutants Restore the G2/M Checkpoint in A-T Cells—It has been reported that radiosensitive fibroblasts from A-T patients exhibit less mitotic delay than cells from normal donors when exposed to irradiation (59). These findings have been confirmed using lymphoblastoid cells synchronized at G2/M, demonstrating that the G2 checkpoint is not activated in A-T cells shortly after irradiation (25). This failure to delay in G2 leads to premature entry into mitosis and then apoptosis (25), which is believed to be the major reason for the hypersensitivity of A-T cells to ionizing radiation. Indeed,
expression of yeast Chk1 or Tel1 reduces radiosensitivity as well as the G2/M checkpoint defect (36, 67). Given that the dominant-negative Pin2/TRF1 mutants correct the radiosensitivity of A-T cells, rendering them resistant to apoptosis after ionizing radiation, they may also restore the G2/M checkpoint defect in A-T cells.

To examine this possibility, we determined the cell cycle status of these cells using flow cytometry analysis. As shown previously (25, 36, 67), following ionizing radiation both A-T-V and A-T-GFP cells failed to delay entry into mitosis, as indicated by the lack of accumulation of cells with the 4N DNA content (Fig. 5). Instead, these cells entered abortive mitosis and apoptosis, as indicated by the appearance of cells containing the sub-G1 DNA content (Fig. 5), a characteristic feature of apoptosis. This is consistent with apoptotic cell morphology (Fig. 3). In contrast, A-T-ATM cells were delayed entry into mitosis because of accumulation of the 4N DNA content (Fig. 5A). These results indicate that GFP has no significant effect on the G2/M checkpoint and confirm that ATM restores the G2/M checkpoint defect in A-T cells, as shown previously (25, 36, 67). Importantly, under the same conditions, both A-T-GFP/Pin2-(1–372) and A-T-GFP/Pin2-(1–316) were accumulated with the 4N DNA content after ionizing radiation (Fig. 5B). Because these cells contained the interphase nuclear morphology (Fig. 4), they were not blocked in mitosis but rather in G2, a phenotype similar to that resulting from re-expression of ATM (Fig. 5A). These results indicate that, as with re-expression of ATM, inhibition of Pin2/TRF1 can specifically restore the G2/M checkpoint defect in A-T cells in response to DNA damage.

Dominant-negative Pin2/TRF1 Mutants Do Not Affect Cdc2 Activation after DNA Damage—After demonstrating that inhibition of Pin2/TRF1 function corrects the defective G2/M checkpoint in A-T cells, we were interested whether it could prevent activation of Cdc2. Following ionizing DNA damage, ATM regulates several Cdc2 upstream regulators to ensure that cyclin B/Cdc2 is not activated as would be indicated by an increase in the phosphorylation of Cdc2 on Tyr-15 and a decrease in the Cdc2 H1 kinase activity following DNA damage. However, in A-T cells, cyclin B/Cdc2 cannot be kept in an inactive state after DNA damage, as suggested by no increase in phosphorylation of Cdc2 on Tyr-15 and no decrease in the Cdc2 H1 kinase activity (68, 69).
To examine whether inhibition of Pin2/TRF1 function affects Cdc2 activation, we subjected stable A-T cell lines to ionizing radiation and then assayed Cdc2 Tyr phosphorylation and its histone H1 kinase activity. As shown previously (68, 69), following ionizing radiation both Cdc2 Tyr phosphorylation and its histone H1 kinase activity remained unchanged in A-T-V1 cells, whereas in A-T-ATM5.1 cells, Tyr phosphorylation of Cdc2 increased, and its kinase activity was inhibited (Fig. 6A). Under the same conditions, both Cdc2 Tyr phosphorylation and its histone H1 kinase activity remained unchanged in A-T cell lines stably expressing either GFP, GFP/Pin2-(1–372), or GFP/Pin2-(1–316) (Fig. 6B), although they were arrested in G2 after ionizing radiation (Figs. 3 and 5B), like A-T-ATM5.1 cells (Fig. 5A). These results indicate that inhibition of Pin2/TRF1 appears not to inhibit activation of Cdc2 in A-T cells even though they were arrested in G2 after ionizing radiation. This is consistent with the notion that ATM directly regulates the function of Pin2/TRF1 by binding and phosphorylating Pin2/TRF1, as proposed earlier (39).

Dominant-negative Pin2/TRF1 Mutants Do Not Restore the S Phase Checkpoint in A-T Cells—Given that inhibition of Pin2/TRF1 corrects the G2/M checkpoint in A-T cells, we were interested in the specificity of this rescuing effect. To address this question, we examined whether the Pin2/TRF1 mutant could also influence the S phase checkpoint. Exposure of mammalian cells to ionizing radiation causes inhibition of both initiation of DNA replication and chain elongation (70). This characteristic pattern of inhibition of DNA synthesis is very much reduced in A-T cells, and the phenomenon has been referred to as radioreistant DNA synthesis (70, 71). Expression of yeast Chk1 or Tel1 in A-T cells restores the G2/M but not the S phase checkpoint defect (36, 67), confirming that different pathways control these two checkpoints. Because endogenous Pin2/TRF1 protein is cell cycle regulated, with a low level during G1 and S (43), we suspected that dominant-negative Pin2/TRF1 mutants might not have significant effects on the S phase checkpoint in A-T cells.

To examine this possibility, we subjected the stable cell lines to ionizing radiation, pulsed them with BrdUrd for 60 min, and followed by measuring the incorporation of BrdUrd into DNA using immunostaining and then flow cytometry. As reported
previously in A-T cells (70, 71). A-T-V1 cells continued to synthesize their DNA (Fig. 7A). In contrast, A-T-ATM5.1 cells displayed a normal response to DNA damage, reducing the DNA synthesis approximately 3-fold after ionizing radiation (Fig. 7A), consistent with previous studies (36, 67, 70, 71).

Importantly, GFP-, GFP-Pin2-(1–372), or GFP-Pin2-(1–316)-expressing cells continued to synthesize DNA following ionizing radiation (Fig. 7B), with the percentage of BrdUrd-positive cells being similar to that of A-T-V1 cells (Fig. 7). These results indicate that the dominant-negative Pin2/TRF1 mutants do not restore the S phase checkpoint defect in A-T cells, demonstrating that the ability of Pin2/TRF1 inhibition to restore the G2/M checkpoint in A-T cells is highly specific.

**DISCUSSION**

To examine the importance of Pin2/TRF1 in mediating ATM-dependent regulation, we here stably expressed two different dominant-negative Pin2/TRF1 mutants to inhibit the function of endogenous Pin2/TRF1 in A-T cells. Both Pin2/TRF1 mutants increased telomere length in A-T cells. Surprisingly, both mutants reduced radiosensitivity and restored the G2/M checkpoint defect in A-T cells. These rescuing effects are highly specific because neither of them corrected the S phase checkpoint defect in the same cells. These results indicate that inhibition of Pin2/TRF1 in A-T cells is able to bypass the requirement for ATM in specifically suppressing telomere shortening, radiosensitivity, and the G2/M checkpoint defect. Given our previous findings that following DNA damage ATM phosphorylates Pin2/TRF1 and suppresses its ability to induce abortive mitosis and apoptosis, these results indicate that Pin2/TRF1 plays a critical role in ATM-dependent regulation of telomeres and mitotic regulation.

Whereas the length of telomeres decreases with each cell division in primary cells, the average telomere length of immortalized, telomerase-positive cells is generally stable during long term culture (72–74). Consistent with these observations, our A-T cells (A-T22IJE-T) contain active telomerase activity, and their mean telomere length is not obviously affected by continuous culturing or by expression of GFP (Fig. 2, data not shown). Furthermore, they are very similar to the telomere lengths reported for other A-T cell lines (26–28, 36). Therefore, telomere length in our A-T cells is stable at least over the period of our experiments. However, after 5 months of culturing following transfection, telomere length in A-T cells expressing dominant-negative Pin2/TRF1 mutant, either GFP-Pin2-(1–372) or GFP-Pin2-(1–316), but not control GFP, is significantly increased to almost double those of the parental A-T line (Fig. 3). Interestingly, a similar increase in telomere length is also reported by overexpression of yeast TEL1 in A-T cells (36). These results indicate that both Pin2/TRF1 mutants increase telomere length in A-T cells. Because Pin2/TRF1 contains only one single Myb-type telomeric DNA-binding motif at its C terminus, it needs to form dimers to bind telomeres. Given that both Pin2-(1–372) and Pin2-(1–316) contain the dimerization domain and form heterodimers with the full-length protein (43, 75), they likely act in a dominant-negative fashion to inhibit the ability of endogenous Pin2/TRF1 to bind telomeres, as shown previously (45). Although Pin2/TRF1 does not directly inhibit telomerase activity (45), it can directly bind the potent telomerase inhibitor PinX1 and target it to telomeres (48). Therefore, it is possible that Pin2/TRF1 inhibits telomere elongation by telomerase via PinX1. However, further experiments are needed to determine how Pin2/TRF1 inhibits telomere elongation.

The most significant finding of our study is that inhibition of Pin2/TRF1 can complement radiosensitivity and the G2/M checkpoint defect in A-T cells, which can be readily detected by both short term and long term cell survival assays. Most control vector-transfected or GFP-expressing A-T cells enter apoptosis 24 h after ionizing radiation (Fig. 3), and a small fraction of them that do not enter apoptosis at 24 h eventually enter senescence 3–4 days post-irradiation (Fig. 4). Therefore, almost no cell colonies are observed 2–3 weeks post-irradiation (Fig. 4). In sharp contrast, A-T cells stably expressing dominant-negative Pin2/TRF1 mutants do not enter apoptosis right after ionizing radiation (Fig. 3). Instead, these delay entry into mitosis and are accumulated in G2 (Figs. 3 and 5), a normal DNA damage response (4, 5, 25, 59). These Pin2/TRF1-inhibited A-T cells do not enter senescence but continue to divide, eventually leading to the formation of many cell colonies (Fig. 4).

Interestingly, these phenotypes of Pin2/TRF1-inhibited A-T cells are indistinguishable from those of A-T cells re-expressing ATM (Figs. 4 and 5). In contrast, inhibition of Pin2/TRF1 completely fails to correct the S phase checkpoint defect in the same cells, whereas ATM can restore both the S and G2/M checkpoint defect in A-T cells (Fig. 7). Similarly, expression of yeast CHK1 or TEL1 gene in A-T cells complements radiosensitivity and the G2/M checkpoint defect but not the S phase checkpoint defect (36, 67). These results indicate that inhibition of Pin2/TRF1 can specifically complement radiosensitivity and the G2/M checkpoint defect in A-T cells.

The finding that direct inhibition of endogenous Pin2/TRF1 function can bypass the requirement for ATM in ATM-negative cells by specifically rescuing telomere shortening, radiosensitivity, and the G2/M checkpoint provides convincing evidence for the functional importance of Pin2/TRF1 in mediating ATM-dependent regulation. These results strongly argue that the negative regulation of Pin2/TRF1 by ATM, presumably via phosphorylation, plays a critical role in maintaining telomeres and mitotic regulation. The fact that this negative regulatory mechanism is missing in ATM-negative cells may suggest why these cells contain shortened telomeres and are hypersensitive to ionizing radiation (1, 26–30, 55). Thus, Pin2/TRF1 is a critical ATM downstream target in the regulation of telomeres and in mitotic checkpoint regulation.

The notion that Pin2/TRF1 is involved in the ATM-dependent G2/M, but not S phase, checkpoint is consistent with other studies linking telomere regulation specifically to mitotic progression (52–54). More importantly, it is consistent with the tightly regulated expression pattern and function of Pin2/TRF1 during the cell cycle. Pin2/TRF1 contains a motif related to the destruction box that mediates degradation of many mitotic proteins, including cyclin B (43, 76). Furthermore, the levels of Pin2/TRF1 are strikingly increased at the G2/M transition, followed by degradation as cells exit from mitosis. This cell cycle-dependent regulation of Pin2/TRF1 is likely due to regulated protein degradation. Our previous study indicates that overexpression of Pin2/TRF1 promotes mitotic entry and apoptosis (51). Thus, these results indicate the function of Pin2/TRF1 is tightly regulated during the cell cycle, reaching its maximum at the G2/M transition when it exerts its function.

It remains to be determined how Pin2/TRF1 is involved in ATM-dependent G2/M checkpoint regulation. Upon double-strand DNA breaks, activation of ATM kinase in normal cells phosphorylates several downstream target proteins, including p53 and checkpoint kinases Chks (2, 3). Chks inhibit Cdc25C and activate Wee1, which are the protein phosphatase and kinase that activate and inhibit Cdc2, respectively. In addition, Chks and ATM also phosphorylate p53, resulting in an increase in transcription of the Cdk inhibitor p21 and the Cdc2 sequestrator 14–3-3r. These multiple and redundant pathways have been shown to ensure that Cdc2 is not activated, and cells will delay entry into mitosis following DNA damage. However, be-
cause these G2/M checkpoint cascades are disrupted and Cdc2 cannot be kept in an inactive state after DNA damage in ATM-negative cells, they fail to delay entry into mitosis and instead enter abortive mitosis and apoptosis. Surprisingly, it appears that inhibition of Pin2/TRF1 restores the G2/M checkpoint without inhibiting Cdc2 in A-T cells after ionizing radiation, at least as assayed by Cdc2 tyrosine phosphorylation and H1 kinase activity (Fig. 6). These results suggest that ATM may also regulate the G2/M checkpoint via controlling the function of Pin2/TRF1. Indeed, ATM regulates the mitotic function of Pin2/TRF1 via phosphorylation on Ser-219 (39). These results suggest that Cdc2 and Pin2/TRF1 may be collaboratively or sequentially involved in the cascade of the G2/M checkpoint control, although their exact relation remains to be determined.

In summary, we have demonstrated for the first time that inhibition of Pin2/TRF1 via two different dominant-negative mutants is sufficient to bypass the requirement for ATM in restoring telomere shortening, increasing radiosensitivity, and correcting the G2/M checkpoint defect in A-T cells. However, neither of the Pin2/TRF1 mutants restores the S phase checkpoint defect in the same cells, demonstrating the specificity of rescuing effects by inhibition of Pin2/TRF1. Together with the fact that ATM phosphorylates Pin2/TRF1 and inhibits its mitotic function, these results provide strong evidence that Pin2/TRF1 plays an critical role in the ATM-dependent regulation of telomeres and DNA damage response. Further studies on how Pin2/TRF1 is involved in telomere regulation and DNA damage response will help understand the physiological and pathological functions of ATM and elucidate the molecular mechanisms of telomere maintenance and DNA damage response.

Acknowledgments—We thank Y. Shiloh, R. Abraham, T. Hunter, B. Neel, and L. Cantley for constructive discussions.

REFERENCES

1. Savitsky, K., Bar, S. A., Glad, S., Rotman, G., Ziv, Y., Vanapaite, L., Tagle, D. A., Smith, S., Uziel, T., Sfaxi, S., Ashkenazi, M., Pecker, I., Frydman, M., Harnik, R., Patau, J., Bar-Sagi, D., Bass, S., Shapira, O., Lavin, M. F., and Lavin, A. J. (1996) Cell Mol. Genet. 25, 526–531.

2. Hartwell, L. H. (1995) Science 269, 629–634.

3. Matsuoka, S., Huang, M., and Elledge, S. J. (1998) Science 281, 1749–1753.

4. Leeper, D. B., Schneiderman, M. H., and Dewey, W. C. (1972) Proc. Natl. Acad. Sci. U. S. A. 69, 5848–5852.

5. Konig, K., and Baisch, H. (1980) Genet. Res. 35, 151–167.

6. Hartwell, L. H., and Kastan, M. B. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 5459–5463.

7. Dieder, D. B., Chang, E. W., and Dewey, W. C. (1973) Proc. Natl. Acad. Sci. U. S. A. 70, 2659–2663.

8. Hartwell, L. H., and Kastan, M. B. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 5464–5468.

9. Shafman, T., Khanna, K. K., Kedar, P., Spring, K., Kozlov, S., Yen, T., Hobson, J. M., Nes, M., Patel, F., and Sakaguchi, A. Y. (1997) Somatic Cell Mol. Genet. 23, 275–286.

10. van Steensel, B., and de Lange, T. (1995) Science 270, 655–657.

11. Banin, S., Moyal, L., Shieh, S., Taya, Y., Anderson, C. W., Chessa, L., and Kastan, M. B. (2000) Mol. Cell. Biol. 20, 1166–1176.

12. Canman, C. E., Lim, D. S., Cimprich, K. A., Taya, Y., Gabrielli, B., Chan, D., Lees-Miller, S. P., and Lavin, M. F. (1998) Science 281, 350–353.

13. Smidenov, L. B., Morgan, S. L., Melia, W., Sawant, S. G., Kastan, M. B., and Pandita, T. K. (1997) Oncogene 15, 2659–2665.

14. Morgan, S. E., Lovly, C., Pandita, T. K., Shiloh, Y., and Kastan, M. B. (1997) Mol. Cell. Biol. 17, 2020–2029.

15. Matsuoka, S., Bar-Sagi, D., and Pandita, T. K. (1999) Mol. Cell. Biol. 19, 6963–6971.

16. Richtie, K. B., Malory, J. C., and Petes, T. D. (1999) Mol. Cell. Biol. 19, 6055–6075.

17. Fritz, E., Friedell, A. A., Zewack, R. M., Eckardt-Schupp, F., and Meyn, M. S. (2000) Mol. Biol. Cell 11, 2665–2676.

18. Wood, L. D., Halvorsen, T. L., Dhar, S., Baur, J. A., Pandita, R. K., Wright, E. W., Hande, M. P., Calaf, G. H., Hsu, H. C., Fein, M. J., Wang, J. Y., and Pandita, T. K. (2001) Oncogene 20, 278–288.

19. Zhao, S., Weng, Y. C., Yuan, S. S., Lin, Y. T., Hsu, H. C., Lin, S. C., Gerbino, E., Song, M. H., Zdienicke, M. Z., Gatti, R. A., Shaya, J. W., Ziv, Y., Shiloh, Y., and Lee, E. Y. (2000) Nature 405, 473–477.

20. Barlow, C., Hirotsune, S., Paylor, R., Liyanage, M., Eckhaus, M., Collins, F., Shiloh, Y., Crawley, J. N., Ried, T., Tagle, D., and Wynshaw-Bass, A. (1996) Cell 86, 159–171.

21. Xu, Y., Ashley, T., Brunier, E. E., Bronson, R. T., Meyn, M. S., and Baltimore, D. (1996) Gene Dev. 10, 2411–2422.

22. Xu, Y., and Baltimore, D. (1996) Gene Dev. 10, 2410–2411.

23. Elson, A., Wang, Y., Daugherty, C. J., Morton, C. C., Zhou, F., Campos-Torres, L., and Leder, P. (1996) Mol. Cell. Biol. 16, 3084–3089.

24. Rudolph, N. S., and Latt, A. S. (1989) Mutat. Res. 211, 31–41.

25. Beamish, H., Kanna, K. K., and Lavin, M. F. (1994) Radiat. Res. 130–133.

26. Pandita, T. K., Pathak, S., and Geard, C. R. (1995) Cyto genetic. Cell Genet. 71, 257–261.

27. Xia, S. J., Shamas, M. A., and Shmoolker, R. J. (1996) Mutat. Res. 364, 1–11.

28. Mcetal, J. A., Parkhill, J., Campbell, L., Stacey, M., Biggs, P., Byrd, P. J., and Taylor, A. M. (1996) Nat. Genet. 15, 350–355.

29. Smidenov, L. B., Morgan, S. L., Melia, W., Sawant, S. G., Kastan, M. B., and Pandita, T. K. (1997) Oncogene 15, 2659–2665.

30. Morgan, S. E., Lovly, C., Pandita, T. K., Shiloh, Y., and Kastan, M. B. (1997) Mol. Cell. Biol. 17, 2020–2029.

31. Shafman, T., Khanna, K. K., and Lavin, M. F. (1999) Oncogene 18, 249–256.

32. Paules, R. S., Levealad, E. N., Wilson, S. J., Innes, C. L., Rhodes, N., Tisty, T. D., Galloway, D. A., Donchev, L. A., Tainyos, M. A., and Kausmann, F. (1996) Oncogene 11, 1519–1525.
A Critical Role for Pin2/TRF1 in ATM-dependent Regulation

W. K. (1995) Cancer Res. 55, 1763–1773
69. Beamish, H., Williams, R., Chen, P., and Lavin, M. F. (1996) J. Biol. Chem. 271, 20486–20493
70. Painter, R. B., and Young, B. R. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 7315–7317
71. Houldsworth, J., and Lavin, M. F. (1980) Nucleic Acids Res. 8, 3709–3720
72. Counter, C. M., Avilion, A. A., LeFeuvre, C. E., Stewart, N. G., Greider, C. W., Harley, C. B., and Bacchetti, S. (1992) EMBO J. 11, 1921–1929
73. Counter, C. M., Hirte, H. W., Bacchetti, S., and Harley, C. B. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 2900–2904
74. Klingelhutz, A. J., Barber, S. A., Smith, P. P., Dyer, K., and McDougall, J. K. (1994) Mol. Cell. Biol. 14, 961–969
75. Bianchi, A., Smith, S., Chong, L., Elias, P., and de Lange, T. (1997) EMBO J. 16, 1785–1794
76. King, R. W., Deshaies, R. J., Peters, J. M., and Kirschner, M. W. (1996) Science 274, 1652–1659
A Critical Role for Pin2/TRF1 in ATM-dependent Regulation: INHIBITION OF Pin2/TRF1 FUNCTION COMPLEMENTS TELOMERE SHORTENING, RADIOSENSITIVITY, AND THE G2/M CHECKPOINT DEFECT OF ATAXIA-TELANGIECTASIA CELLS
Shuji Kishi and Kun Ping Lu
J. Biol. Chem. 2002, 277:7420-7429.
doi: 10.1074/jbc.M111365200 originally published online December 13, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M111365200

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

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

This article cites 75 references, 34 of which can be accessed free at http://www.jbc.org/content/277/9/7420.full.html#ref-list-1