Role of Pin1 in the Regulation of p53 Stability and p21 Transactivation, and Cell Cycle Checkpoints in Response to DNA Damage*

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DNA damage leads to stabilization and accumulation of p53, which plays a pivotal role in transcriptional activation of p21 and cell cycle arrest. The increase in p53 stability depends critically on its phosphorylation on serine/threonine residues, including those preceding a proline (Ser(P)/Thr-Pro). The Ser(P)/Thr-Pro moiety exists in the two distinct cis and trans conformations and their conversion is catalyzed specifically by the prolyl isomerase Pin1. Pin1 regulates the conformation and function of certain phosphorylated proteins and plays an important role in cell cycle regulation, oncogenesis, and Alzheimer’s disease. However, nothing is known about the role of Pin1 in DNA damage. Here we found that DNA damage enhanced the interaction between Pin1 and p53, which depended on the WW domain in Pin1 and Ser33/46-Pro motifs in p53. Furthermore, Pin1 regulates the stability of p53 and its transcriptional activity toward the p21 promoter. As a result, p53 and p21 barely increased after DNA damage in Pin1 knock-out embryonic fibroblasts or in neoplastic cells depleted of Pin1. Moreover, Pin1 null cells displayed significant defects in cell cycle checkpoints induced by DNA damage. These results demonstrate a new role of Pin1 in regulating p53 function during DNA damage.

The tumor suppressor protein p53 regulates multiple cellular functions, including cell cycle checkpoint, and transcriptional activation (1). DNA damage induced by such as ionizing radiation leads to stabilization and accumulation of p53 (1). This p53 induction plays a pivotal role in transcriptional activation of the cell cycle inhibitor p21 and cell cycle arrest (2–4) and is at least partially due to its dissociation from the ubiquitin ligase MDM2 (5, 6). The increases in the p53 stability and/or transcriptional activity depend critically on its phosphorylation on multiple serine/threonine residues, including those preceding a proline (Ser(P)/Thr-Pro) (1, 7–9). However, it is not clear whether the stability and/or function of p53 are regulated after Pro-directed phosphorylation.

Ser(P)/Thr-Pro motifs in proteins exist in cis and trans conformations, whose conversion is normally inhibited by phosphorylation, but is specifically catalyzed by the prolyl isomerase Pin1 (10–13). Pin1 contains an N-terminal WW domain and C-terminal isomerase domain. The WW domain binds specific Ser(P)/Thr-Pro motifs and targets Pin1 to a subset of phosphoproteins where the PPIase domain induces conformational changes by isomerizing specific Ser(P)/Thr-Pro bonds. Such conformational changes have profound effects on catalytic activity, dephosphorylation, protein-protein interactions, subcellular location, and/or stability of Pin1 substrates (10–22). Functionally, Pin1 has been shown to regulate several phases of the cell cycle, including G1/S and G2/M as well as the DNA replication checkpoint (10, 11, 20–23). Furthermore, Pin1 is strikingly overexpressed in many human cancers and affects the stability of certain phosphoproteins (20–22). However, it is not known whether this mechanism is important in DNA damage response. Here we report that Pin1 binds phosphorylated p53 and is important for p53 stabilization, p21 transactivation, and cell cycle check points in response to DNA damage.

MATERIALS AND METHODS

Cell Culture, DNA Constructs, and Transfection—Primary embryonic fibroblasts (MEFs)† were prepared and transfected as described (22). Breast cancer cells MCF7 and T47D cells were transfected using FuGENE 6 (Roche Molecular Biochemicals), as described (20). Constructs expressing Pin1, its point mutants, or Pin1ΔWW have been described (11, 13). p53, its dominant-negative mutant (R248W), as well as the phosphorylation site mutants Ser20 and Ser20 expression constructs and the p21 promoter luciferase constructs were as described (2, 8).

GST Pull-down, Immunoprecipitation, and Immunoblotting Analysis—Cells were irradiated with 5 Gy of γ-ray or mock-irradiated, as described (24). The cells were harvested 4 h later and lysed in a lysis buffer containing 1% Triton X-100. The supernatants were subjected to GST-Pin1 pull-down assay, followed by immunoblotting analysis using monoclonal antibodies against p53 (Ab-6 for human p53 or Ab-3 for murine p53/Oncogene), as described (15, 17, 20). For co-immunoprecipitation, we used agarose-conjugated anti-p53 polyclonal antibodies (Santa Cruz), with unrelated antibodies as a control, as described (15, 17, 20).

Promoter Reporter Assays—Promoter reporter assays were performed with the dual-luciferase reporter assay system (Promega) at 24 h after transfection, as described (20).

Cell Cycle Analysis—Cells were harvested at 24 h after irradiation, followed by flow cytometric analysis in a FACScan (Becton Dickinson), as described (11, 24).

RESULTS

DNA Damage Enhances the Interaction between Pin1 and p53—DNA damage induces phosphorylation of p53 on several Ser/Thr sites, including Ser33, 38-Pro and Ser46-Pro motifs, and these phosphorylation sites are important for p53 function (1, 7–9). Moreover, Ser33 and Ser46 have multiple upstream hydrophobic residues (Leu, Val, and/or Met) and are preceded by

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† The abbreviations used are: MEF, murine embryonic fibroblast; GST, glutathione S-transferase; Ab, antibody; Gy, gray; WT, wild-type; PPIase, peptidyl-prolyl cis/trans isomerase.
single point mutants Ser 33 and Ala/Ser46, cell lysates were obtained 4 h after ionizing radiation with 5 Gy, at a time when Ser33 is shown to be significantly phosphorylated (7). A substantial amount of transfected wild-type p53 was pulled down by Pin1, but not by its mutant containing the Ser33→Glu mutation (Fig. 1F). Given that the Ser33→Glu mutation disrupts the ability of the Pin1 WW domain to bind phosphoproteins, these results confirm that Pin1 binds p53 via its WW domain. Furthermore, Pin1 binding to the Ser33→Ala or Ser46→Ala p53 single point mutant was considerably decreased, while no binding was detected between Pin1 and the Ser33→Ala/Ser46→Ala double mutant of p53 (Fig. 1F), indicating the requirement of both Ser residues for the binding. The above results indicate that DNA damage enhances the specific interaction between Pin1 and p53, and the binding depends on the WW domain in Pin1 as well as the Ser33 and Ser46 residues in p53.

**Effects of Pin1 on the Protein Stability of p53 upon DNA Damage**—We next asked whether Pin1 affects p53 accumulation and p53-dependent responses after DNA damage. To address the first question, we used primary embryonic fibroblasts (MEFs) derived from wild-type (WT) or Pin1 knock-out (Pin1−/−) mice. The DNA damage-induced p53 accumulation was time- and dose-dependent in WT MEFs (Fig. 2, A and B). However, both the usual time- and dose-dependent increase in p53 levels in response to ionizing radiation was abolished in Pin1−/− MEFs (Fig. 2, A and B). These results indicate that p53 protein fails to increase in Pin1−/− MEFs. To further support that Pin1 is important for p53 accumulation following DNA damage, we depleted Pin1 from WT MEFs via expression of the Pin1 antisense construct (Pin1AS). Indeed, depletion of Pin1 from WT MEFs drastically suppressed induction of p53 after DNA damage (Fig. 2C). These results indicate that Pin1 is required for the accumulation of p53 after DNA damage. Next we examined the effects of Pin1 on p53 stability after DNA damage using a cycloheximide chase, as described (27). Pin1−/− MEFs were co-transfected with p53 and control vector, Pin1, or the S16E Pin1 mutant that failed to bind p53 (Fig. 1F). After irradiation, cycloheximide was added to inhibit de novo p53 synthesis and the steady-state levels of p53 were determined using antibodies that detected only transfected human p53 (Fig. 2, D and E). In Pin1−/− MEFs transfected with the control vector, the half-life of p53 was about 60 min (Fig. 2, D and E). Importantly, the half-life of p53 was significantly increased to ~6 h by expression of Pin1. In contrast, the Pin1 mutant that failed to bind p53 did not have detectable effect on the p53 stability.

To further support that phosphorylation-dependent association is important for Pin1 to stabilize p53, we examined the stability of the mutant S33A/S46A p53 mutant that failed to bind Pin1 (Fig. 1F). Pin1 stabilized p53, but not its mutant S33A/S46A mutant after DNA damage (Fig. 2, E and F). The above results indicate that Pin1 and its phosphorylation-dependent association is required for stabilization of p53 and also reveal an important new role of phosphorylation of Ser33 and Ser46 in regulating the stability of p53.

**Effects of Pin1 on p53 Transactivation toward p21 after DNA Damage**—We assayed the effects of Pin1 on p53-dependent responses. We first examined whether Pin1 affects p53-dependent p21 transactivation without DNA damage given the detectable, although weak, interaction (Fig. 1, A–D). As shown in Fig. 3A, Pin1 alone had a modest stimulatory effect on the p21 promoter activity. This stimulatory effect was significantly enhanced when
FIG. 2. Loss or depletion of Pin1 decreases p53 stability and p21 induction after DNA damage. A and B, Pin1−/− MEFs lack the time- and dose-dependent increase of p53 and p21 after DNA damage. WT or Pin1−/− MEFs were irradiated with the indicated doses of γ-ray and then harvested 6 h after irradiation (A) or with γ-ray and harvested at the indicated time points (B). Lysates were subjected to immunoblotting with antibodies against p53, p21, or Pin1, with actin or tubulin as a control. C, lack of p53 and p21 induction after DNA damage in Pin1-inhibited MEFs. WT MEFs (first two panels) were transfected with either control vector or antisense Pin1 (Pin1AS) for 24 h. Six hours after mock (−) or irradiation (+), cells were subjected to immunoblot analysis. D and E, requirement of Pin1 for stabilization of p53. Pin1−/− MEFs were co-transfected with p53 and control vector, Pin1, or its S16E mutant for 24 h, followed by irradiation. 4 h after irradiation, cycloheximide was added, and lysates were prepared at the indicated time points, followed by immunoblotting with anti-human p53 (upper panel) or anti-actin (lower panel) antibodies (D). p53 levels were semiquantified using Imagequant and normalized using actin as an internal control. For comparing the p53 stability in the absence or presence of Pin1 or its mutant, p53 levels at 0 was defined as 100%. F and G, requirement of Ser33/46 for stabilization of p53. p53 or its mutant was co-transfected into Pin1−/− MEFs, and their stability was assayed as described in the legends to D and E.

Co-transfection with p53, but was inhibited by dominant-negative p53 (Fig. 3A). In contrast, depletion of Pin1 by Pin1AS blocked the ability of p53 to activate the p21 promoter (Fig. 3A). Moreover, a mutation in the p53-binding site in the p21 promoter completely abolished the effects of Pin1 on the p21 promoter (Fig. 3B). These results show that Pin1 can enhance the ability of p53 to activate the p21 promoter.

Since the Pin1 and p53 interaction is enhanced after DNA damage, we next examined the effects of irradiation on the ability of Pin1 to enhance the p21 promoter. As shown in Fig. 3B, irradiation increased p21 transactivation after transfection with Pin1 alone in comparison with vector control. However, p21 transactivation in response to irradiation was greatly enhanced when Pin1 and p53 were co-transfected (Fig. 3B). This stimulatory effect of Pin1 and p53 depended on DNA damage (Fig. 3B). Again, the mutation in the p53-binding site in the p21 promoter completely abolished the effects of Pin1 on the p21 promoter (Fig. 3B). Similarly, a dominant-negative p53 mutant suppressed the effects of Pin1 (Fig. 3B). Finally, the Pin1 point mutant that either could not bind p53 (Pin1S16E, Pin1W34A) or isomerize Ser(P)/Thr-Pro motifs (Pin1K63A) failed to increase p53 transactivation (Fig. 3B). These results indicate that Pin1 enhances the p53-dependent p21 transactivation upon DNA damage and that both the WW domain and the PPIase activity of Pin1 are required for this cooperation.

If Pin1 enhances the p53-dependent p21 transactivation upon DNA damage, it would be expected that inhibition of Pin1 might affect the accumulation of p21 after DNA damage. p21 levels are strongly increased in WT MEFs after ionizing radiation, and this induction was time-dependent as well as dose-dependent (Fig. 2, A–C). However, under the same conditions, p21 levels failed to increase in Pin1−/− MEFs or WT MEFs that were depleted of Pin1 by Pin1AS (Fig. 2, A–C). Furthermore, both the time- and dose-dependent increases in p21 levels were abolished in Pin1−/− MEFs (Fig. 2, A and B). These results demonstrate that loss of Pin1 abolishes the accumulation of p53 and p21 after DNA damage.

Effects of Pin1 on DNA Damage-induced Cell Cycle Checkpoints—To examine whether Pin1 affects cell cycle checkpoint control in response to DNA damage. To address this question, we subjected WT or Pin1−/− MEFs to 5 Gy of γ radiation. Before DNA damage, Pin1−/− cells had slightly more cells in G1, and fewer cells in S phase, as compared with WT cells (Fig. 4, A and B). This phenotype is consistent with the previous findings that Pin1 is an important regulator of the G1/S activator cyclin D1 (22).

Importantly, cell cycle profiles were observed after DNA damage. WT MEFs displayed interphase cell morphologies, whereas significantly more Pin1−/− MEFs were rounded up (data not shown). These differences were further confirmed by flow cytometrical analysis (Fig. 4). WT MEFs in S phase were dramatically reduced, and the majority of cells were accumulated with the 4n DNA content (Fig. 4). However, Pin1−/− MEFs in S phase were not changed. Furthermore, DNA dam-
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**FIG. 3.** Pin1 increases p53 transactivation toward the p21 promoter, which is enhanced by DNA damage. A, cooperation between Pin1 and p53 in activating the p21 promoter. Cells were co-transfected with expression constructs for Pin1, Pin1S16E, Pin1W34A, or WW domain Pin1 mutant (Pin1AS), and p53 or dominant-negative p53 (dnp53), together with a p21 luciferase reporter construct. The activity of the reporter luciferase was expressed relative to the activity in control vector transfected cells, which is defined as 1.0. B, DNA damage enhances cooperation between Pin1 and p53 in activating the p21 promoter. Cells were co-transfected with the indicated expression constructs and a p21 luciferase reporter construct. For the assays in the last two sets of experiments labeled as Mt p21 promoter, the same p21 luciferase construct, but containing a mutated p53-binding site, was used. 24 h after transfection cells were mock-treated (white bars) or irradiated (solid bars), followed by luciferase assay.

**FIG. 4.** Loss of Pin1 causes prominent defects in the cell cycle checkpoints in response to DNA damage. WT or Pin1−/− MEFs were subjected to mock- or 5 Gy of irradiation. 12 h later, the cells were harvested and stained with propidium iodide, followed by flow cytometry.

that the Pin1 binding or isomerase mutant fails to increase the stability and transactivation activity of p53. Furthermore, the S33A/S46A mutant that fails to bind Pin1 fails to be stabilized after DNA damage. These results strongly suggest for the first time that phosphorylation of Ser33 or Ser46 plays an important role in regulating the stabilization of p53. This is consistent with the fact that both residues lie in immediate vicinity of the Mdm2-binding site and affect transcriptional activity of p53 (5, 6, 28). Therefore, we propose that when p53 is phosphorylated following DNA damage, Pin1 binds and isomerizes p53 on phosphorylated Ser33/46. This conformational change may prevent binding of p53 to its ubiquitin ligase MDM2, affect phosphorylation of p53 on other sites, and/or affect transcriptional activity of p53 toward p21.

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**AGE-INDUCED G2 ARREST WAS SIGNIFICANTLY INHIBITED IN PIN1−/− MEFs (FIG. 4).** These results indicate that loss of Pin1 leads to multiple cell cycle checkpoint defects in response to DNA damage.

**DISCUSSION**

Here we report that Pin1 plays an important role in regulating p53 function and in modulating cellular response to DNA damage. Pin1 bound p53 and also increased its protein stability, which depended on the binding of Pin1 to phosphorylated Ser33/46 of p53. Furthermore, Pin1 potentiated the transcriptional activity of p53 toward the p21 promoter. Moreover, p53 and p21 barely increased after ionizing radiation in Pin1−/− MEFs or in neoplastic cells depleted of Pin1. Finally, Pin1−/− MEFs displayed significant defects in cell cycle checkpoints induced by DNA damage. These results demonstrate that Pin1 is a novel regulator of p53 in DNA damage response.

It remains to be determined how Pin1 regulates the ability of p53 and p21 to up-regulate during DNA damage. We have shown...