Protein phosphatase 1, but not protein phosphatase 2A, dephosphorylates DNA-damaging stress-induced phospho-serine 15 of p53

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Abstract Okadaic acid (OA) is a protein phosphatase (PP) inhibitor and induces hyperphosphorylation of p53. We investigated whether the inhibition of PP1 by OA promotes the phosphorylation of the serine 15 of p53. In vitro dephosphorylation assay showed that PP1 dephosphorylated ultraviolet C (UVC)-induced phospho-ser15 of p53, and that OA treatment inhibited it. One of the PP1 regulators, growth arrest and DNA damage 34 (GADD34), disturbed PP1 binding with p53, interfered with the dephosphorylation of p53 and increased the amount of phospho-p53 after UVC-treatment. This report provides the first evidence that PP1, but not PP2A, dephosphorylates phospho-serine 15 of p53.

Keywords: Okadaic acid; DNA-damaging stress; p53 Phosphorylation; Protein phosphatase 1; Protein phosphatase 2A; GADD34

1. Introduction

Tumor suppressor p53 is tightly regulated by phosphorylation. The phosphorylated p53 induces cell-growth arrest and/or apoptosis through p53 response gene transcription. DNA-damaging stress, such as γ-irradiation and ultraviolet (UV) irradiation, activates ATM/ATR kinases, which in turn phosphorylate the serine 15 residue of p53 protein [1]. The phosphorylated serine 15 promotes the interaction with p300 [2], and p300 acetylated p53 and histones, which enhances promoter activity of p53 response gene. It remains unclear whether phospho-serine 15 stabilizes p53 protein. On the other hand, the serine 20 residue of p53 is phosphorylated by CHK proteins [3–5], and is the important site of interaction to MDM2 protein. Phosphorylation of serine 20 dissociates from MDM2, which promotes p53 stability [6].

It has been shown that multiple protein phosphatases (PPs) can dephosphorylate p53 in vitro. These phosphatases include PP1, PP2A, PP5, Wip1 and Cdc14 [7–10]. Under normal condition, PP1/PP2A dephosphorylates the C-terminal of p53 protein; this site is phosphorylated by protein kinase C, which influences the DNA binding ability of p53 and transcriptional activity [11]. Meanwhile, the phosphorylation at the N-terminal of p53 is important for transcription and stabilization [12,13], but dephosphorylation of that site has not been well studied. Okadaic acid (OA), the inhibitor of PP1 and PP2A [14], accumulates hyperphosphorylated p53 [15,16] and the PP2A inhibitor, SV40 small t antigen, promotes the DNA binding ability of p53 and the transcriptional activity [17]. One of the PP1 regulators, growth arrest and DNA damage 34 (GADD34) (PPP1R15A, myd116), increases the amount of total and phosphorylated p53, and promotes p21 (waf1) mRNA expression [18]. These studies show that PPs regulate p53 through these phosphatase activities. Under stressed condition, however, the relations between PPs and p53 have been unclear.

In the present study, we show the relation between p53 and PP1 under DNA damaging conditions. We demonstrate that OA increases the amount of phosphorylation at the serine 15, which is related to the inactivation of PP1. Furthermore, by using GADD34 deficient MEFs, we show that the regulation of PP1 influenced p53 phosphorylation in vivo. These results suggest that PP1 is the key regulator of p53 and is the major phosphatase of phospho-serine 15 of p53.

2. Materials and methods

2.1. Cell lines and transfection

Human WI38 cells and HEK293 cells were maintained in Dulbecco's modified Eagle's medium (GIBCO) supplemented with 10% fetal bovine serum (GIBCO). Human p53 null cell lines SOAS-2 were maintained in modified McCoy's 5A medium (GIBCO) with 15% fetal bovine serum (FBS). All cells were grown at 37 °C in a humidified atmosphere of 5% CO_2. Transfections were performed with the effectene reagent (Qiagen). Methyl methanesulfonate (MMS) was purchased from Sigma–Aldrich.

2.2. Preparation of mouse embryonic fibroblasts

Mouse embryonic fibroblasts (MEFs) from GADD34-deficient mice and wild-type mice were prepared from 14.5-day-old embryo [19]. All cultures were maintained in Dulbecco's modified essential medium (Sigma) with 10% FBS. Cells were plated 2×10^6 cells/10-cm plates for subculture.

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Abbreviations: OA, okadaic acid; GADD, growth arrest and DNA damage; UVC, ultraviolet C; MMS, methyl methanesulfonate

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2.3. Plasmids

Human p53 expression vector was provided by Bai and Merchant [20]. PP1 cDNA was purchased from Invitrogen and cloned into the *Bam*HI/*Xba*I site of pcDNA3.1HisA vector with Xpress tag. Mouse GADD34 myc-tagged expression vector was described previously [18]. SV40 small t antigen cDNA was obtained by polymerase chain reaction (PCR) amplification using the cDNA of COS-1 cells as a template and employing 5′- and 3′-oligonucleotide primers. The oligonucleotides used in this experiment were as follows: small t antigen 5′-probe: 5′- CGGGATCCATGGATAAAGTTTTAAACAGAG -3′; small t antigen 3′-probe: 5′-CCGCTCGAGTTAGAGCTTTAAATCTCTG-TA -3′. The fragments were subcloned into the *Bam*HI/*Xba*I site of pcDNA3.1HisA vectors. Sequences were confirmed by dideoxy sequencing. When cDNA expression vectors were transfected, protein synthesis was inhibited by the phosphorylation of eukaryotic translation initiation factor 2 alpha (eIF2α). We employed pAdVantage vector (Promega) to preclude the termination of protein synthesis.

2.4. DNA-damaging and phosphatase-inhibitor treatments

WI38 cells and MEFs were grown to 50–80% confluence in 6-cm dishes in DMEM containing 10% FBS. DNA damage was achieved by exposing the cells to ultraviolet C (UVC) (50 J/m2) or MMS (80 μg/ml). Cells were harvested at indicated time points. In some experiments, they were treated with increasing concentrations of OA (from 1 to 100 nM) 6 h before DNA-damage treatment. Four hours after irradiation, cells were treated with 10 μg/ml of cyclohexamide to arrest new p53 protein synthesis and cells were then harvested at the indicated time points. OA and cyclohexamide were purchased from Sigma.

2.5. Immunoblotting

Proteins were analyzed by SDS–PAGE and detected by a chemiluminescent ECL kit (Amersham) with one of the following antibodies: anti-p53 antibody, anti-phospho-ser15 p53 antibody (Cell signaling), anti-GFP antibody (BD Biosciences), anti-GADD34 antibody (Santa Cruz), anti-eIF2α antibody (Santa Cruz), anti-phospho-ser 51 eIF2α antibody (BIOSOURCE international), anti-p21 (waf1) antibody (Santa Cruz), and anti-β-actin antibody (Amersham).

2.6. In vitro dephosphorylation assay

WI38 cells wereUVC-treated (100 J/m^2) and 1 h later the cells were lysed in low-stringency buffer (50 mM Tris–HCl, pH 7.5; 120 mM NaCl; 0.5 mM EDTA; and 0.5% NP-40) in the presence of PMSF and protease inhibitors. After pre-cleaning with protein G beads (Amersham), the extracts were immunoprecipitated with anti-p53 antibody in the presence of protein G beads for 4 h at 4 °C. The beads were then washed three times with low-stringency buffer, and twice with PP1 reaction buffer containing 1 mM MnCl2 and 5 mM caffeine. One unit of PP1 (New England Biolabs, Inc.) was added to the immune complexes for 1 h at 30 °C. For inhibition studies, the immune complexes and PP1 were incubated with 1 μM of OA in PP1 reaction buffer for 1 h at 30 °C. For positive control studies, Calf intestine alkaliphosphatase (CIAP) (Takara) was added to the immune complexes in CIAP buffer for 1 h at 37 °C. Dephosphorylation was analyzed by SDS–PAGE and was detected by immunoblotting with anti phospho-serine 15 of p53 antibody.

3. Results

3.1. Okadaic acid increases UVC-induced phosphorylation at serine 15 residue of p53

OA is a inhibitor of PP1 and PP2A. To evaluate the role of PP1/PP2A in regulating p53 phosphorylation after DNA-damaging stress, WI38 cells were exposed to UVC (50 J/m^2) with increasing concentrations of OA. As shown in Fig. 1, the high concentrations of OA (100 nM) induced an increase in phosphorylated serine 15 of p53. On the other hand, the low concentrations of OA (1 nM) slightly reduced the phosphorylation of p53 after UVC treatment. The same treatment did not affect the amount of β-actin. These results demonstrate

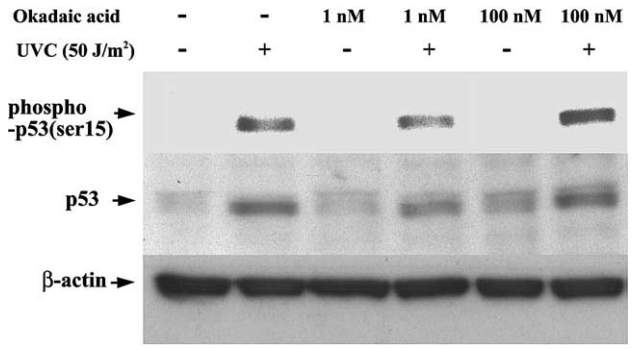

Fig. 1. OA increases p53 phosphorylation by UVC treatment. WI38 cells were treated by UVC (50 J/m^2) for 4 h with increasing concentrations of OA. Phosphorylated p53 (top panel) and total p53 (middle panel) were determined. As a control, β-actin levels were determined (bottom panel). These are the representative data of more than two experiments.

that PP1 and/or PP2A regulated the phosphorylation at the serine 15 of p53 through phosphatase activity.

3.2. Protein phosphatase 1, but not protein phosphatase 2A, is the key regulator of phospho-serine 15 of p53

High concentrations of OA (100 nM) promoted the phosphorylation of p53, but it was unclear whether PP1 or PP2A was more important for the regulation of phospho-serine 15 of p53. To rule out the possibility that PP2A dephosphorylates p53, the PP2A inhibitor, SV40 small t antigen, was co-transfected to the culture cells to block p53 dephosphorylation. After UVC treatment, small t antigen did not affect the amount of p53, but induced a decrease in phospho-serine 15 of p53 (Fig. 2). To confirm the possibility that PP1 dephosphorylates p53, the PP1 regulator protein, GADD34, was co-transfected to the cultured cells to block p53 dephosphorylation. After UVC treatment, mouse GADD34 increased the amount of p53 and induced phosphorylation at the serine 15 of p53 (Fig. 2). These results suggested that GADD34 interfered in the dephosphorylation of p53, causing an increase in phospho-serine 15.

3.3. Protein phosphatase 1 dephosphorylates the serine 15 residue of p53 in vitro

Because we found that PP1 bound p53 in vivo, we next investigated whether PP1 could actually dephosphorylate phospho-ser15 of p53. We employed in vitro dephosphorylation assay. As shown in Fig. 3, PP1 dephosphorylated UVC-induced phospho-ser15 of p53. 1 μM of OA treatment inhibited the phosphatase activity of PP1. CIAP also dephosphated p53.

3.4. The PP1 regulator GADD34 enhances DNA damage-induced p53 phosphorylation

To determine the relation between p53 phosphorylation and GADD34, we analyzed the p53 protein expression and p53 phosphorylation using GADD34 deficient MEFs. In wild-type MEFs, GADD34 was induced 8 h after MMS treatment (Fig. 4). And the protein levels of p53 and phospho-serine 18 of mouse p53 (corresponding to serine 15 of human p53) were highly expressed in a time-dependent manner at 8 h after MMS treatment. On the other hand, in GADD34-deficient MEFs, the levels of both p53 and phospho-serine 18 of p53

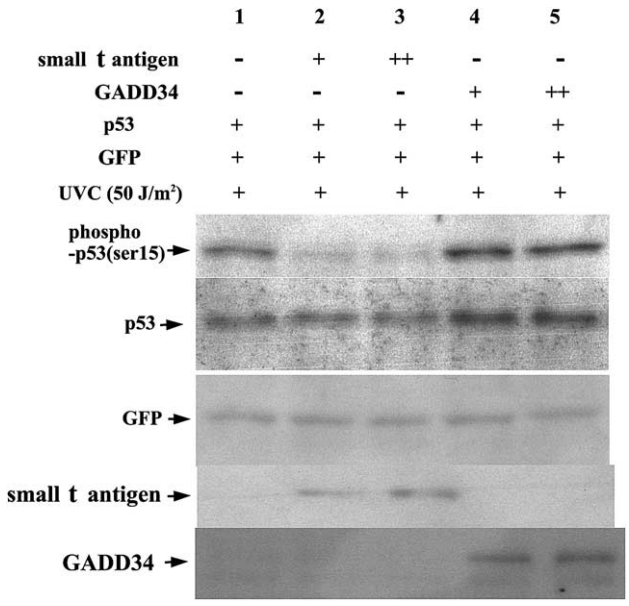

Fig. 2. Mouse GADD34 promotes phospho-ser 15 of p53 and interferes with the interaction between PP1 and p53. GADD34 promotes phosphorylation of p53 at serine 15, but SV40 small t antigen decreases phospho-serine 15. SOAS-2 cells were transfected with 0.1 μg of human p53 wild-type, 0.1 μg of GFP and 0.2 μg of Advantage vector (all lanes), and co-transfected with one or more of the following vectors: 0.2 μg of mock vector (lane 1), 0.1 μg of mock vector and 0.1 μg of small t antigen expression vector (lane 2), 0.2 μg of small t antigen expression vector (lane 3), 0.1 μg of mock vector and 0.1 μg of mouse GADD34 expression vector (lane 4), and 0.2 μg of GADD34 expression vector (lane 5). Phosph-ser 15 of p53 (top panel) and total p53 (middle panel) were determined. GFP was determined as the transfection internal control (bottom panel). These are the representative data of more than two experiments.

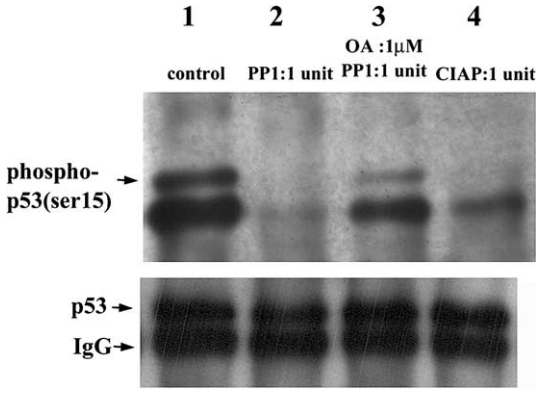

Fig. 3. PP1 dephosphorylates phospho-ser15 of p53 in vitro. WI38 cells were UVC-treated (100 J/m^2) and p53 protein was immunoprecipitated with anti-p53 antibody. As a control, the immune complexes were incubated without PP1 (lane1). One unit of PP1 was added to the complexes (lane 2). For inhibition studies, the complexes and PP1 were incubated with 1 μM of OA (lane 3). As a positive control, CIAP was added to the complexes (lane 4). The products were probed with anti phospho-serine 15 of p53 antibody (upper panel). Same membrane was re-probed with anti p53 antibody (lower panel). These are the representative data of more than two experiments.

expressions were lower than in wild-type MEFs. The fold induction of phosphorylated p53 per total p53 by MMS treatment was lower than that of wild-type MEFs. In GADD34-deficient MEFs, the level of p21 (waf1, cip1) expression was

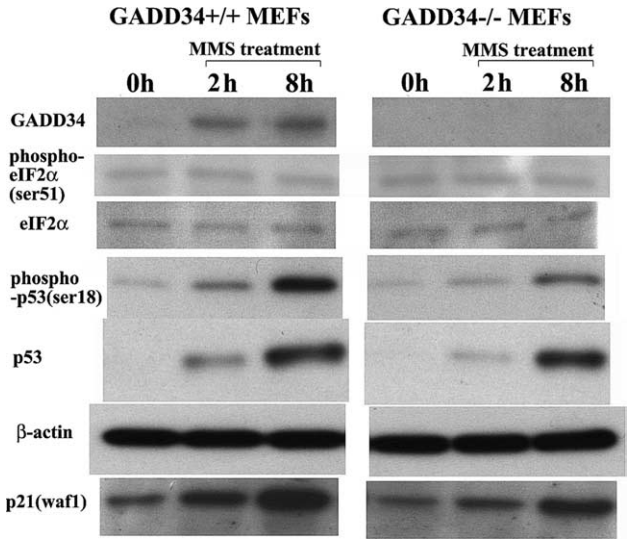

Fig. 4. Determination of p53 and phospho-p53 protein levels in wild-type and GADD34 deficient MEFs after MMS treatment. Cells were treated with 80 μg/ml MMS and cell lysates were prepared at the indicated time points. 50-μg lysate was run on 10% SDS–PAGE. To investigate the status of protein synthesis, we examined total eIF2α and phosphorylated eIF2α. Total p53, phosphorylated p53 and p21 (waf1) were determined by immunoblotting. As a control, β-actin levels were determined. These are the representative data of more than two experiments.

lower than that in wild-type MEFs. MMS treatment, unlike endoplasmic reticulum (ER) stress, did not induce the phosphorylation of eIF2α (Fig. 4). The same treatment did not affect the expression of β-actin, indicating that GADD34 protein did not have an overall positive effect on protein expression after MMS treatment.

4. Discussion

PP1 is the cellular protein serine/threonine phosphatase and the regulator of protein function through dephosphorylation. Here, we showed that OA treatment enhanced the phosphorylation of p53 at serine 15 after UVC treatment (Fig. 1). OA is the inhibitor of PP1 and PP2A, and this agent inhibits PP2A (50% inhibitory dose IC_{50}, 1–10 nM) more potently than PP1 (IC_{50}, 100 nM to 1 μM) [21]. Since 100 nM OA promoted the phosphorylation of p53, PP1 was thought to be more important in phospho-serine 15 of p53. To rule out the possibility that PP2A dephosphorylates p53, the PP2A inhibitor, SV40 small t antigen, was co-transfected with p53 expression vector to block p53 dephosphorylation. After UVC treatment, small t antigen did not affect the amount of p53, but induced a decrease in phospho-serine 15 of p53 (Fig. 2). The corresponding mechanisms remain unknown, but it is suggested that PP2A may act as a negative regulator of PP1 or activate the serine 15 kinase.

The PP1 regulator, GADD34 (PPP1R15A, myd116), is induced by ER stress [19,22,23], and GADD34 relocates PP1 to ER for dephosphorylation of eIF2α. We employed GADD34 to confirm the possibility that PP1 dephosphorylates p53. After UVC treatment, co-transfected mouse GADD34 expression increased the amounts of total and phospho-ser 15 of

p53 (Fig. 2). By using GADD34 deficient MEFs, we confirmed the relation between GADD34 and p53 phosphorylation (Fig. 4). In a previous study, we reported that PP1 regulator GADD34 induced phospho-serine 15 of p53 and promoted p21 (waf1) mRNA expression through p53 DNA binding sites [18].

The phosphorylation at the N-terminal site of p53 is important for transcription and stabilization [12,13], but the dephosphorylation of N-terminal site has not been well studied. Here, we revealed for the first time a relationship between N-terminal phospho-serine 15 residue of p53 and PP1, and that phospho-serine 15 temporarily promotes the stability of p53 protein. By in vitro dephosphorylation assay (Fig. 3), we showed that PP1 actually dephosphorylated phospho-serine 15 of p53. However, it is still unclear whether other phosphorylated sites of p53 are dephosphorylated by PP1.

In conclusion, we propose that in response to UVC treatment, p53 is phosphorylated by the serine 15 kinases and continuously dephosphorylated by PP1 but not by PP2A. This dephosphorylation requires the phosphatase ability of PP1. The PP1 regulator GADD34 interferes with the dephosphorylation of p53 and increases the phosphorylation at serine 15 of p53. These results support the speculation that GADD34 regulates p53 by modulating its phosphorylation levels.

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