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Protein phosphatase 1 inhibits p53 signaling by dephosphorylating and stabilizing Mdmx

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ABSTRACT

The activation and stabilization of the p53 protein play a major role in the DNA damage response. Protein levels of p53 are tightly controlled by transcriptional regulation and a number of positive and negative post-translational modifiers, including kinases, phosphatases, E3 ubiquitin ligases, deubiquitinases, acetylases and deacetylases. One of the primary p53 regulators is Mdmx. Despite its RING domain and structural similarity with Mdm2, Mdmx does not have an intrinsic ligase activity, but inhibits the transcriptional activity of p53. Previous studies reported that Mdmx is phosphorylated and destabilized in response to DNA damage stress. Three phosphorylation sites identified are Ser342, Ser367, and Ser403. In the present study, we identify protein phosphatase 1 (PP1) as a negative regulator in the p53 signaling pathway. PP1 directly interacts with Mdmx and specifically dephosphorylates Mdmx at Ser367. The dephosphorylation of Mdmx increases its stability and thereby inhibits p53 activity. Our results suggest that PP1 is a crucial component in the ATM-Chk2-p53 signaling pathway.

1. Introduction

Genomic DNA of eukaryotic cells is under the constant attack of chemicals, free radicals, and UV or ionizing radiation from environmental exposure, by-products of intracellular metabolism, or medical therapy. The DNA damage response in cells translate DNA damage signal to responses of cell cycle arrest, DNA repair or apoptosis. A central component of the DNA damage response is tumor suppressor p53 [1,2]. P53 is a transcription factor and serves as a pivotal tumor suppressor in animals. More than 50% of human tumors contain a mutation or deletion of the *TP53* gene [3]. Upon DNA damage, the p53 tumor suppressor is activated to direct a transcriptional program that prevents the proliferation of genetically unstable cells. Inappropriate regulation of p53 results in a severe consequence for cells. While the loss of p53 function predisposes cells to tumorigenesis, errant p53 activation can lead to premature senescence or apoptosis.

An exquisite control mechanism prevents errant activation of p53 in cells. Central to this mechanism is the negative regulation exerted by Mdm2 and Mdmx (or Mdm4) [4]. Mdm2 is a RING domain containing E3 ubiquitin ligase that facilitates the ubiquitination of p53. Once poly-ubiquitinated, p53 is subject to proteasome-dependent degradation. Interestingly, p53 not only transcriptionally regulates genes involved in cell cycle arrest or apoptosis, but also its own negative regulator, Mdm2. Thus, p53 and Mdm2 participate in an auto-regulatory feedback loop [2]. Mdmx was identified as a p53-binding protein that has structural similarity with Mdm2, but lacked ubiquitin-ligase function. Similar to Mdm2, Mdmx deficiency in mice causes early embryonic lethality rescued by p53 loss [5]. Thus, Mdmx and Mdm2 have non-redundant roles in the regulation of p53. Recent in vitro and in vivo studies suggested that Mdm2 mainly controls p53 stability, whereas Mdmx functions as an important p53 transcriptional inhibitor [6,7].

In stressed cells, p53 is activated through mitigating the inhibitory activity of Mdm2 and Mdmx. A major mechanism that leads to the activation of p53 was purported to be the post-transcriptional modifications of p53 such as phosphorylation and acetylation that prevent Mdm2 from binding to or ubiquitinating p53 [8]. Many phosphorylation sites are located in the N-terminus of p53 that is adjacent to or overlapping with its Mdm2 binding domain, which may interfere with p53-Mdm2 interaction [9]. However, data from knockin p53 mutant mouse models as well as the observation that p53 does not have to be phosphorylated to be activated in cells have challenged the biological effects of phosphorylation events for p53. Mice expressing endogenous p53 mutated at the murine equivalents of serine 15 or 20 have only mild effects in p53 activity and stability, which is contrary to the predictions from the in vitro studies suggesting that

Abbreviations: PPM, protein phosphatase, magnesium-dependent; MKK4, mitogen-activated protein kinase kinase 4; MKK6, mitogen-activated protein kinase kinase 6; JNK, c-Jun amino-terminal kinase.

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serine 15 and threonine 18 phosphorylation prevented the negative regulation of p53 by Mdm2 [10,11]. Whereas phosphorylation of p53 may fine-tune its function under various physiological contexts, an alternative view was brought up in which p53 regulation primarily depends on Mdm2 and Mdmx.

Mdm2 and Mdmx have also been phosphorylated in the DNA damage response. Oren et al. first reported that Mdm2 undergoes ATM-dependent phosphorylation at Ser395 in response to ionizing radiation and radiomimetic drugs [12]. We previously showed that Mdm2 has reduced stability and accelerated degradation in the presence of Ser395 phosphorylation [13]. Mdmx is also phosphorylated and destabilized after DNA damage. Three phosphorylation sites have been identified on Mdmx, which are Ser342, Ser367 and Ser403 [14–16]. While Ser403 is directly phosphorylated by ATM, the other two sites are phosphorylated by Chk1 and Chk2, two crucial kinases that are activated by ATM/ATR and in turn initiate cell cycle checkpoints [14–17]. ATM-mediated phosphorylation destabilizes Mdmx and promotes their auto-degradation, which facilitates rapid p53 induction.

Opposed to protein kinases, protein phosphatases may play active roles in modulating the p53 signaling. The Prives group reported that cyclin G recruited PP2A to dephosphorylate Mdm2. Disruption of cyclin G leads to the hyperphosphorylation of Mdm2 and a higher level of p53 [18]. The specific B regulatory subunit of PP2A B56γ was identified to be associated with p53 in vivo and responsible for Thr55 dephosphorylation [19]. We and other groups identified Wip1 as a master inhibitor in the ATM–p53 pathway [20]. Three of the Wip1 targets in the pathway are kinases that phosphorylate and activate p53 (Chk1, Chk2, and p38 MAP kinase) [21–23]. We have also shown that Wip1 dephosphorylates Mdm2 and Mdmx at their ATM phosphorylation sites (Ser395 on Mdm2 and Ser403 on Mdmx). Unphosphorylated forms of Mdm2 and Mdmx have increased stability and affinity for p53, facilitating p53 degradation and deactivation. In the current study, we identify PP1 as the phosphatase that specifically dephosphorylates Mdmx at Ser367. The PP1-mediated dephosphorylation increases the stability of Mdmx and extends its half-life. Our results suggest that PP1 may serve as a homeostatic regulator in the p53 signaling pathway.

2. Materials and methods

2.1. Cell lines and cell culture

U2OS (p53 wildtype) cell line is a human osteosarcoma line that was obtained from the American Type Culture Collection (ATCC). Primary *p53 Ala18/Ala* and wildtype (*p53 Ser18/Ser18*) mouse embryonic fibroblasts were provided by Dr. Stephen Jones at the University of Massachusetts Medical Center. They were harvested and cultured as previously described [10]. To obtain cells stably expressing Mdmx, expression vector expressing HA-tagged or FLAG-tagged wildtype or mutant human Mdmx was transfected into U2OS cells and stable cells were selected with 1000 μg/mL G-418. Positive colonies were isolated, amplified, and checked for expression of Mdmx protein individually.

2.2. Plasmid constructs

Human serine/threonine phosphatase clones were purchased from ATCC and Open Biosystem. Full-length cDNA sequence of each phosphatase was subcloned into pcDNA3.1 expression vector (Invitrogen). Human PP1γ expression vector was kindly provided by Dr. Sergei Nekhai (Howard University, Washington DC) and PPP5C was obtained from Dr. Xiaofan Wang (Duke University, NC). The p21-Luciferase was obtained from Dr. Gigi Lozano (The University of Texas MD Anderson Cancer Center, TX). P53 consensus RE-luciferase and Mdm2-Luciferase constructs were provided by Dr. Lawrence Donehower (Baylor College of Medicine, TX). The vectors expressing wildtype and mutant Mdmx were previously described [24]. To express wildtype and mutant Mdmx proteins, full-length or mutant (point mutation and deleted mutation) Mdmx cDNA fragments were amplified by high-fidelity PCR from the above wildtype or mutant Mdmx expression vectors, and cloned into pGEX-4 T-3 vector (GE Healthcare) as indicated in Fig. 4. GST-Mdmx fusion proteins were purified from crude Escherichia coli using Glutathione Sepharose 4B (GE Healthcare) column according to the manufacturer's protocol and detected by Western blotting. PP1 shRNA expression vectors were purchased from Openbiosystems and the ShRNA and ORFeome Core at the M.D. Anderson Cancer Center.

2.3. Cell transfection with plasmid DNA

Cells were transfected with plasmid DNA by Lipofectamine and Plus reagents (for transformed cell lines, Invitrogen, catalog # 18324–012 and #11514-015) or Lipofectamine 2000 reagent (for mouse embryonic fibroblasts, Invitrogen, catalog # 11668–019). Transfection experiments were performed following the instruction manuals provided with reagents.

2.4. Luciferase assays

Cells were cotransfected with phosphatase expression vector, p21-Luciferease vector (or p53RE-Luciferase and Wip1-Luciferase vectors), and Renilla Luciferase vector. Cells were harvested at various time points and lysed. Luciferase activity was measured using a Turner 20/20n luminometer and normalized to Renilla luciferase according to the instructions provided with the dual-luciferase assay kit (Promega). Data are presented as the mean ± standard deviation and are representative of three independent experiments.

2.5. Western blot analysis, antibodies, and purified proteins

Immunoprecipitations, Western blot analysis, and immunoprecipitation-Western blot analyses were performed by standard methods described previously [23]. Anti-actin (#1616), anti-ubiquitin (#8017), HRP-anti-goat IgG (#2020), HRP-anti-rabbit IgG (#2302), and HRP-anti-mouse IgG (#2302) were purchased from Santa Cruz; Anti-HA (A190-108A) and anti-Mdmx (#A300-287A) were purchased from Bethyl Laboratories; anti-Mdmx (pS403) and anti-Mdmx (pS367) were provided by Dr. Yosef Shiloh (Tel Aviv University, Isreal) and Dr. Jiandong Chen (Lee Moffitt Cancer Center, FL). They were generated and described previously [14,16]. FLAG-tagged PP1 proteins were immunopurified using anti-FLAG beads (Sigma-Aldrich). PP2A proteins (#14-111) and PP1α proteins (#14-595) used for in vitro binding assays were purchased from EMD Millipore.

2.6. Protein stability measurement

Cell stably expressing wildtype or mutant HA-Mdmx were transfected with PP1 or control expression vector DNA. Twenty-four hours after transfection, cells were treated with 500 ng/ml NCS and 50 μg/ml cycloheximide. Cells were harvested at the indicated time points after treatment, and cell lysates were immunoblotted with anti-HA antibody. Levels of Mdmx at each time point were quantitated by phosphorimager for Mdmx bands on immunoblots.

2.7. In vitro phosphatase assays

The in vitro phosphatase assays and control peptide sequences have been described previously [25]. Mdmx and control phosphopeptides were custom synthesized by New England Peptide. Their sequences are: Mdmx (pS342): Ac-LTHSLpSTSDIT-amide; Mdmx(pS367): Ac-CRRTIpSAPVVR-amide; Mdmx (pS403): Ac-AHSSEpSQETIS-amide; Chk1 (pS345): Ac-QGISFpSQPTCPD; negative control phospeptide: RHFSKpTNELLQ.