Biochemical and Biophysical Research Communications 434 (2013) 659–663

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Nucleolar protein, Myb-binding protein 1A, specifically binds to nonacetylated p53 and efficiently promotes transcriptional activation

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ARTICLE INFO

Article history:
Received 22 March 2013
Available online 11 April 2013

Keywords:
Nucleolar protein MYBBP1A
Nucleolar stress
p53 acetylation
p53 activation

ABSTRACT

Nucleolar dynamics are important for cellular stress response. We previously demonstrated that nucleolar stress induces nucleolar protein Myb-binding protein 1A (MYBBP1A) translocation from the nucleolus to the nucleoplasm and enhances p53 activity. However, the underlying molecular mechanism is understood to a lesser extent. Here we demonstrate that MYBBP1A interacts with lysine residues in the C-terminal regulatory domain region of p53. MYBBP1A specifically interacts with nonacetylated p53 and induces p53 acetylation. We propose that MYBBP1A dissociates from acetylated p53 because MYBBP1A did not interact with acetylated p53 and because MYBBP1A was not recruited to the p53 target promoter. Therefore, once p53 is acetylated, MYBBP1A dissociates from p53 and interacts with nonacetylated p53, which enables another cycle of p53 activation. Based on our observations, this MYBBP1A–p53 binding property can account for efficient p53-activation by MYBBP1A under nucleolar stress. Our results support the idea that MYBBP1A plays catalytic roles in p53 acetylation and activation.

1. Introduction

The tumor suppressor p53 is a critical mediator of cellular stress response, which maintains genomic integrity and prevents oncogenic transformation [1]. The protein p53 regulates many target genes that induces cell cycle arrest or apoptosis [2,3].

DNA damage is known to activate p53 as a transcription factor through post-translational modifications [4,5] such as phosphorylation, ubiquitination, and acetylation, which are critical in regulating p53 function [6–12]. The p300/CBP protein that possesses histone acetyltransferase activity and acetylates p53, acts as a coactivator of p53 and augments p53 transcriptional activity [13–15]. p53 acetylation occurs at multiple lysine residues in the C-terminal regulatory domain (CRD) of p53 (residues 370, 372, 373, 381, 382, and 386) in response to DNA-damaging agents [16–18]. p53 acetylation also correlates with its sequence-specific DNA binding [19], and augments recruitment of transcriptional activators to p53 [20]. Based on these observations, p53 acetylation is considered to play a vital role in p53 activation [21,22].

DNA damage induces repression of rRNA transcription by RNA polymerase I, resulting in disruption of nucleolar structure [23,24]. Low concentrations of actinomycin D (ActD) specifically inhibit RNA polymerase I-driven transcription, but do not affect RNA polymerase II-driven transcription [25,26]. Therefore, ActD treatment also induces nucleolar disruption [27].

We previously reported that nucleolar disruption induces acetylation and accumulation of p53 without phosphorylation. The nucleolar protein Myb-binding protein 1A (MYBBP1A) binds to p53 and facilitates p53 acetylation to enhance p53-mediated transcription by enhancing the p53–p300 interaction [28]. However, the mechanism by which MYBBP1A enhances p53 acetylation and induces p53 activity is still ambiguous.

Here we demonstrate that MYBBP1A specifically recognizes nonacetylated lysine residues of p53, promotes its acetylation, and dissociates from acetylated p53. The dissociated MYBBP1A may recognize nonacetylated p53 for another cycle of p53 activation. We predict that this MYBBP1A–p53 binding property may explain the effective p53-activating function of MYBBP1A.

2. Materials and methods

2.1. Cell culture and treatments

MCF-7 human breast cancer cells and H1299 p53-deficient human lung cancer cells were maintained in DMEM (Sigma). All

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http://dx.doi.org/10.1016/j.bbrc.2013.04.006

media were supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin solution (Nacalai Tesque). Cells were maintained at 37 °C in an atmosphere containing 5% CO_2 and 100% humidity. To induce nucleolar stress, cells were exposed to ActD (5 nM).

2.2. siRNA and plasmid DNA transfection

For siRNA transfection, cells at 30–50% confluency were transfected using Lipofectamine RNAi MAX (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. All siRNAs were purchased from Invitrogen. The siRNA duplexes MYBBP1A, 5′-UCUUUCAGUCAGGUCGGCUGGUGAA-3′, p300 5′-AUUAUAGG AGAGUUCACCGGGCAGG-3′. Stealth RNAi negative control Medium or High GC was used as a negative control. Protein and RNA were extracted at 48 h after transfection of siRNA. For transfection of plasmid DNA, cells at 70–80% confluency were transfected using Lipofectamine LTX (Invitrogen) according to the manufacturer's protocol. Protein was extracted 24 h after transfection of plasmid DNA.

2.3. Expression vectors, antibodies

cDNAs encoding full-length and indicated mutants of p53 and MYBBP1A were amplified by PCR and subcloned into the pcDNA3 plasmid (Invitrogen) containing sequences encoding FLAG, FLAG-HA, HA or myc sequences. β-Actin (Sigma) and anti-human-p53 (DO-1, Santa cruz) monoclonal antibodies and rabbit anti-p53-K382Ac (Cell Signaling Technology) polyclonal antibodies were used according to the manufacturers' instructions. Rabbit anti-human MYBBP1A antibody was raised against a synthetic peptide corresponding to 1265–1328 amino acids of human MYBBP1A.

2.4. GST pull-down assay

cDNAs encoding full-length human p53 or MYBBP1A and its deletion mutant derivatives were cloned into pGEX-4T-1 (Amersham Biosciences). GST-fusion proteins were expressed in BL-21 cells following induction with IPTG and purified with glutathione Sepharose 4B beads (Amersham). *In vitro* translated MYBBP1A was synthesized using an *in vitro* transcription/translation-coupled reticulocyte lysate system (Promega). Binding was performed in TNE buffer [150 mM NaCl, 0.5% Nonidet P-40, 50 mM Tris–HCl (pH 8.0), 5 mM EDTA] for 30 min under rotation at 4 °C, and the beads were washed 5 times with TNE buffer. Beads were boiled in SDS sample buffer for 5 min, and the supernatants were loaded onto SDS–polyacrylamide gels followed by immunoblotting.

2.5. Coimmunoprecipitation and immunoblotting

Cells were lysed in TNE buffer. Extracted proteins were immunoprecipitated with antibody-coated protein G Sepharose (Amersham) beads. Bound proteins were separated by SDS–PAGE, transferred to polyvinylidene difluoride membranes (Millipore), and detected with appropriate primary antibodies and horseradish peroxidase-conjugated secondary antibodies. Specific proteins were visualized using an enhanced chemiluminescence (ECL) immunoblot detection system (Amersham).

2.6. Chromatin immunoprecipitation (ChIP) and real-time PCR detection

ChIP assay was performed according to the published procedure [29]. The primers for real-time PCR were as follows:

p21-p53RE fw primer 5′-GTGGCTCTGATTGGCTTTCTG-3′
p21-p53RE rv primer 5′-CTGAAAACAGGCAGCCCAAG-3′

3. Results and discussion

3.1. Lysine residues in the CRD region of p53 play an important role in MYBBP1A–p53 interaction

We have previously demonstrated that cellular stresses induce nucleolar disruption, which leads to MYBBP1A translocation from the nucleolus to the nucleoplasm. Subsequently, MYBBP1A binds to p53 and promotes acetylation by enhancing p53–p300/CBP association, thereby activating the p53 function [28]. However, determination of the MYBBP1A–p53 binding properties remains. To investigate this, we attempted to dissect the mechanism of p53–MYBBP1A interaction. Co-immunoprecipitation experiments revealed the binding of endogenous MYBBP1A to p53 in MCF-7 cells under conditions of nucleolar stress (Fig. 1A), which is consistent with previous results.

Our previous results indicated that MYBBP1A binds to the CRD region in p53. Therefore, to further narrow the regions of p53 responsible for binding with MYBBP1A, we generated a series of truncation mutants of the p53 CRD region and tested the interaction with MYBBP1A by a GST pull-down assay (Fig. 1B). The GST pull-down assay determined that CRD-1 and CRD-3 interacted with MYBBP1A. In contrast, CRD-2 and CRD-4 did not bind to MYBBP1A. These data indicate that the 367–386 amino acid (aa) region of p53 is responsible for binding with MYBBP1A (Fig. 1C).

To identify the residues responsible for the binding, we subsequently performed alanine-scanning mutagenesis of the p53-CRD region construct and subjected it to GST pull-down assay (Fig. 1D). It was significant that substitution of lysine with alanine residues at K370, K372, K373, K381, K382, or K386, located in the 367–386 aa region specifically attenuated the MYBBP1A–p53 interaction (Fig. 1E). Moreover, MYBBP1A did not bind to the p53 6KA mutant, which bears simultaneous lysine substitutes 370, 372, 373, 381, 382, and 386 to alanines (Fig. 1F). These results (Fig. 1E and F) demonstrate that lysine residues in the CRD region of p53 play an important role in the p53–MYBBP1A interaction.

3.2. MYBBP1A binds specifically to nonacetylated p53

Because these lysine residues, responsible for the binding to MYBBP1A, are sites for p53 acetylation, we explored the effect of acetylation of these residues on binding to MYBBP1A. First, to test whether acetylation of lysine residues influences MYBBP1A–p53 binding, we generated a p53–6KQ mutant that mimics acetylated status (Fig. 2A). Our GST pull-down experiments demonstrated that the 6KQ mutant did not bind to MYBBP1A (Fig. 2B). Next we overexpressed p300 in H1299 cells to enhance p53 acetylation, and found that overexpression of p300 increased acetylation status of p53 and decreased MYBBP1A–p53 binding (Fig. 2C). Moreover, we generated a p53–6KR mutant (Fig. 2A), which acts as an acetylation-deficient missense mutant [21]. Co-immunoprecipitation experiments revealed that interaction between p53–6KR mutants and MYBBP1A is stronger than that between wild-type p53 and MYBBP1A (Fig. 2D, compare lanes 2, 3). We also examined the effect of decrease in p53 acetylation on p53–MYBBP1A interaction in MCF-7 cells by knockdown of p300. We found that p300 knockdown decreased endogenous p53 acetylation and increased the MYBBP1A–p53 interaction (Fig. 2E and F). Taken together, these data indicate that MYBBP1A specifically interacts with nonacetylated p53.