Archives of Biochemistry and Biophysics 655 (2018) 26–36

Contents lists available at ScienceDirect

Archives of Biochemistry and Biophysics

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

PIM2 survival kinase is upregulated in a p53-dependent manner in cells treated with camptothecin or co-treated with actinomycin D and nutlin-3a

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ARTICLE INFO

Keywords:
Apoptosis
p53
PIM2
BAD
β-catenin

ABSTRACT

The p53 protein is an inducer of apoptosis, acting as a transcriptional regulator of apoptotic genes. In a previous study, we found that actinomycin D and nutlin-3a (A + N) synergistically activate p53. To better understand the molecular consequences of this synergism, we incubated arrays of antibodies against apoptotic proteins with extracts of A549 cells in which p53 had been activated. We found that strong activation of p53, marked by serine 46 and 392 phosphorylation, was associated with inactivating phosphorylation of proapoptotic BAD protein on serine 136. Investigation of the source of this phosphorylation revealed that activation of p53 was associated with accumulation of PIM2, a survival kinase. The accumulation of PIM2 following treatment with A + N was suppressed in p53-knockdown cells. Others discovered that *PIM2* was activated by cooperatively acting p53 molecules. Our results are consistent with this finding. Moreover, we found that in A549 cells, the treatment with A + N stimulated in p53-dependent fashion the expression of other high cooperativity p53 target genes, *DRAXIN* and *H19*. Activation of antiapoptotic *H19* can mechanistically explain relatively low rate of apoptosis of A549 cells exposed to A + N. We conclude that *PIM2, DRAXIN and H19* are efficiently stimulated by strongly activated p53 molecules, probably acting cooperatively.

1. Introduction

The p53 protein positively regulates both the receptor-activated and intrinsic pathways of apoptosis. For instance, p53 promotes the expression of death receptor DR5 and the apoptosis-inducing FAS ligand/receptor system [1,2], upregulates proapoptotic genes of the BCL-2 family (e.g. *BAX*), and downregulates anti-apoptotic proteins such as survivin [3,4]. Moreover, p53 can induce apoptosis in a direct, transcription-independent fashion by associating with mitochondrial membrane proteins to promote the release of cytochrome *c* [5]. Apoptosis is also regulated by a complex system of post-translational modifications. For instance, the proapoptotic protein BAD is inactivated by phosphorylation catalyzed by so-called "survival kinases": AKT, which phosphorylates BAD on Ser136, and PIM1, which phosphorylates BAD on Ser112. Phosphorylated BAD is sequestered by 14-3-3 proteins and can no longer neutralize anti-apoptotic proteins such as BCL-X_L (reviewed by Danial [6]). Thus, apoptosis is the result of a shift in the balance between the levels and activation status of pro- and anti-apoptotic proteins. p53 modulates apoptosis not only by regulating the amounts of these proteins, but also by influencing their phosphorylation/activation status. For instance, p53 promotes the expression of PTEN phosphatase, which indirectly decreases the activity of AKT kinase [7].

p53 acts primarily as a transcription factor, binding to a consensus sequence consisting of two half-sites, RRRCWWGYYY (R - purine, Y pyrimidine, W - A or T), separated by a spacer of 0–13 bp [8]. In this manner, p53 regulates the expression of hundreds of genes. However, not all p53-regulated genes respond in the same fashion. Some genes are very sensitive to activated p53 (e.g., *MDM2*, a negative regulator of p53), whereas other (mostly proapoptotic) genes respond only when p53 is "hyperactivated". In general, the level of p53 activation depends on the degrees of phosphorylation on its N-terminus and acetylation on its C-terminus [9]. For instance, phosphorylation of Ser46 is considered to be a marker of strongly activated p53 [10].

Recently, we showed that treatment of cells with two substances that activate p53 by different mechanisms led to synergistic activation of p53, reflected by a synergistic increase in phosphorylation of p53 on Ser46 [11]. Actinomycin D induces nucleolar stress by blocking RNA

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https://doi.org/10.1016/j.abb.2018.08.005
Received 26 April 2018; Received in revised form 17 July 2018; Accepted 6 August 2018
Available online 07 August 2018
0003-9861/

polymerase I, whereas nutlin-3a directly inhibits MDM2, which normally binds to p53 and promotes its degradation [12,13]. Consistent with observations by others [14], we found that, in the A549 cell line, camptothecin, an inhibitor of topoisomerase I, promoted strong phosphorylation of p53 on Ser46; this amino acid was also modified by co-treatment with actinomycin D and nutlin-3a [11]. However, despite the strong phosphorylation of p53 on Ser46 in A549 cells treated with either camptothecin or actinomycin D/nutlin-3a, the rate of apoptosis was considerably higher following camptothecin treatment [11]. To obtain insight into the molecular mechanism underlying the different abilities of camptothecin and actinomycin D/nutlin-3a to promote apoptosis, we used arrays of antibodies against apoptosis-regulating factors to estimate the levels of relevant proteins and their post-translationally modified forms in control and treated A549 cells. This assay did not reveal the molecular mechanism responsible for the inability of actinomycin D and nutlin-3a to induce extensive apoptosis; however, we unexpectedly found that strong activation of p53 was associated with significant accumulation of the survival kinase PIM2. Moreover, this accumulation was p53-dependent. Thus, we obtained plausible evidence that *PIM2* is another gene positively regulated by p53.

2. Material and methods

2.1. Cell culture, reagents, imaging, treatment and PIM2 knockdown

A549 (lung adenocarcinoma, from American Type Culture Collection [ATCC]), NCI-H1299 (non-small-cell lung cancer, ATCC) and NCI-H28 (mesothelioma, ATCC) cells were grown in Dulbecco's modified Eagle's medium (low-glucose DMEM; Sigma-Aldrich) containing 1 g/L glucose. A375 (malignant melanoma, ATCC) cells were grown in DMEM containing 4.5 g/L glucose. Media were supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA) and 1 × penicillin/streptomycin solution (Sigma-Aldrich). All cells were grown at 37 °C in an atmosphere containing 5% CO_2.

The following stock solutions of chemicals were used: actinomycin D (10 μM in DMSO; Sigma-Aldrich), camptothecin (10 mM in DMSO; Calbiochem-Merck, Darmstadt, Germany), nutlin-3a (10 mM in DMSO; Selleck Chemicals LLC, Houston, TX, USA), cisplatinum (1 mg/ml, Accord Healthcare, Warsaw, Poland). Stock solutions were diluted in culture medium to the required final concentrations: 5 nM actinomycin D, 5 μM nutlin-3a, 5 μM camptothecin and indicated concentrations of cisplatinum. Control cells were mock-treated with medium containing DMSO.

Knockdown of p53 was performed as described previously [15]. A549 cells were treated with transduction-ready lentiviral particles (Santa Cruz Biotechnology, Dallas, TX, USA) containing three constructs encoding 19–25-nucleotide shRNA sequences targeting *TP53* mRNA. Control cells were transduced with lentiviral particles containing scrambled shRNA sequences that do not induce the specific degradation of any known mRNA. Positively transduced cells were selected using puromycin. Due to the high efficiency of transduction, selection of clones was not necessary. Efficiency of knockdown was monitored by Western blotting.

Cell-cycle profiles were obtained by flow cytometry (FACSCanto, Becton Dickinson, Franklin Lakes, NJ, USA) following trypsinization of attached cells, ethanol fixation, RNase treatment, and propidium iodide (PI) staining.

For microscopic observations, cells were seeded on chambered coverslips (Nunc-LabTech, Roskilde, Denmark) in low-glucose DMEM medium with 10% FBS and penicillin/streptomycin. Treatment was started on the next day. The cells were incubated with camptothecin or A + N for 48 h under standard conditions, and then the unfixed cells were photographed using the inverted microscope (Axio Observer. Z1, Carl Zeiss, Germany), equipped with EC-Plan Neofluar 40 × /1.30 Oil DIC M27 objective. Images were acquired using standard software (ZEN lite 2012, Carl Zeiss).

Pim2-shRNA (h) lentiviral particles were purchased from Santa Cruz Biotechnology (Dallas, TX, USA), and the transduction was performed according to the manufacturer's protocol. Control cells were transduced with lentiviral particles containing scrambled shRNA sequences. Positively transduced A549 cells were selected using puromycin. Clonal selection was not performed, because most cells were puromycin-resistant after transduction.

2.2. Analysis of protein expression with antibody arrays

A549 cells, seeded on 6 cm culture plates, were treated for 48 h as described above with actinomycin D, nutlin-3a, both substances, or camptothecin. Subsequently, the culture medium was removed, and the cells were washed with ice-cold PBS and lysed on plates on ice in cell lysis buffer supplemented with protease inhibitor cocktail provided by the manufacturer of the PathScan Stress and Apoptosis Antibody Array kit for chemiluminescent readout (Cell Signaling Technology, Danvers, MA, USA). Cell lysates from three independent experiments were prepared in advance and stored at −70 °C. Before the procedure, lysates were thawed on ice, and their protein concentrations were adjusted to 0.9 μg/μl with array diluent buffer. All lysates were incubated simultaneously on a single glass containing 16 arrays in separate wells, one for each lysate (one array was left empty). Images were captured using chemiluminescent film.

2.3. Semi-quantitative real-time PCR

Total RNA samples were prepared using the RNeasy mini kit (Qiagen, Hilden, Germany). cDNA was synthesized with MuLV reverse transcriptase and random hexamers (Applied Biosystems, Foster City, CA, USA). Measurements of mRNA levels were performed using Real-Time 2 × PCR Master Mix SYBR (A&A Biotechnology, Gdynia, Poland). The following oligonucleotides were used as primers: *PIM2*, ACT GAC TTT GAT GGG ACA AGG G and TTA GGG CAC AGC AGT CTG GG (BioTeZ, Berlin-Buch, Germany); *DRAXIN*, CGA AAC ATC ACC AGC CGA AG and CCT TCC ACA CAC ATG CAG TC; *H19*, AAT CGG CTC TGG AAG GTG AAG and GCT GCT GTT CCG ATG GTG TC, (Genomed, Warsaw, Poland); β-actin (internal reference), GCA AGC AGG AGT ATG ACG AG and CAA ATA AAG CCA TGC CAA TC (BioTeZ). Amplification was performed on a CFX96 Real-Time System (Bio-Rad, Hercules, CA, USA). In each PCR run, cDNA samples were amplified in triplicate. Relative quantitation of mRNA was performed using the $\Delta\Delta C_T$ method with β-actin as a reference. Means and standard deviations were calculated from three independent treatments.

2.4. Western blotting

Whole-cell lysates were prepared using IP buffer, supplemented with protease and phosphatase inhibitors as described previously [11]. Aliquots of lysates (35–50 μg) were separated by SDS-PAGE on 8% or 13% gels and electrotransferred onto PVDF membranes. Before incubation with primary antibody, the membranes were incubated for 1 h at room temperature in blocking solution (5% skim milk in PBS with 0.1% Tween-20). The following primary antibodies were obtained from Cell Signaling Technology: anti–phospho-Ser15 p53 (rabbit polyclonal antibody), anti–phospho-Ser46 p53, anti-phospho-Ser392, anti-cleaved caspase-3 (Asp175) (5A1E), anti-phospho-AKT (Ser473) (D9E), anti–PIM-1 (C93F2), anti–PIM-2 (D1D2), anti–PIM-3 (D17C9), anti-BAX (D2E11), anti–phospho-BAD (Ser112) (40A9), anti–phospho-BAD (Ser136) (D25H8), anti-BAD (D24A9). Anti-p53 (DO-1), anti-$p21^{WAF1}$ (F-5), and loading control anti-HSC70 (B-6) antibodies were obtained from Santa Cruz Biotechnology. Anti-14-3-3 sigma antibody (Ab14116) was obtained from Abcam (Cambridge, UK). All incubations with primary antibodies were performed overnight at 4 °C in blocking solution. HRP-conjugated secondary antibodies (Thermo Fisher Scientific, Waltham, MA, USA) were detected by chemiluminescence (SuperSignal