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Counteracting MDM2-induced HIPK2 downregulation restores HIPK2/p53 apoptotic signaling in cancer cells

Lavinia Nardinocchi [a,*], Rosa Puca [a,b], David Givol [c], Gabriella D'Orazi [a,b,**]

[a] Department of Experimental Oncology, Molecular Oncogenesis Laboratory, National Cancer Institute "Regina Elena", 00158 Rome, Italy
[b] Department of Oncology and Experimental Medicine, School of Medicine, University "G. d'Annunzio", 66100 Chieti, Italy
[c] Department of Molecular Cell Biology, The Weizmann Institute of Science, 76100 Rehovot, Israel

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ABSTRACT

Homeodomain-interacting protein kinase-2 (HIPK2) is a crucial regulator of p53 apoptotic function by phosphorylating serine 46 (Ser46) in response to DNA damage. In tumors with wild-type p53, its tumor suppressor function is often impaired by MDM2 overexpression that targets p53 for proteasomal degradation. Likewise, MDM2 targets HIPK2 for protein degradation impairing p53-apoptotic function. Here we report that zinc antagonised MDM2-induced HIPK2 degradation as well as p53 ubiquitination. The zinc inhibitory effect on MDM2 activity leads to HIPK2-induced p53Ser46 phosphorylation and p53 pro-apoptotic transcriptional activity. These results suggest that zinc derivatives are potential molecules to target the MDM2-induced HIPK2/p53 inhibition.

1. Introduction

P53 is a potent tumor suppressor gene and one of the key players in apoptosis signaling [1]. P53 activation is regulated through specific posttranslational modifications, leading to p53 transcriptional activation of target genes [2]. Because of its critical function, p53 is frequently targeted for inactivation and suffers disabling mutations or deletions in about 50% of all malignant tumors. The other half of human cancers express wild-type p53 protein that however, can be inactivated by deregulation of regulatory proteins [3]. An important mechanism that regulates p53-apoptotic function involves MDM2 oncogene by means of its E3 ubiquitin ligase activity that targets p53 for proteasomal degradation [4]. DNA-damage-induced phosphorylation of its N-terminus contributes to p53 stability by preventing MDM2 from degrading it [5]. Homeodomain-interacting protein kinase-2 (HIPK2) was identified as a p53-interacting protein [6,7] that specifically phosphorylates p53 at Ser46 for transcriptional activation of pro-apoptotic factors such as p53AIP1, PIG3, Bax, Noxa, Puma and KILLER/DR5 [7–9], as well as for the repression of the anti-apoptotic factor galectin-3 [10]. Moreover, HIPK2-induced p53Ser46 phosphorylation prevents the MDM2-mediated p53 ubiquitination recovering p53 apoptotic function [11]. Therefore, preserving HIPK2 function is crucial for p53 apoptotic activity.

MDM2 is not only a p53 inhibitor but also contributes to modulate the p53-mediated biological outcomes (i.e., cell cycle arrest versus apoptosis) by regulating HIPK2. In response to non-lethal DNA damage, p53-activated MDM2 inhibits the p53 apoptotic pathway by targeting HIPK2 at residue lysine 1182 for proteasomal degradation [12]. It is worth noting that the *mdm2* gene is amplified in a significant proportion of human tumor types, thereby contributing to tumor progression by efficiently reducing the availability of a functional p53 as well as most likely the availability of its apoptotic activator HIPK2. Several approaches have been tested, leading to the discovery of small molecules that can restore p53 function in tumor cells by targeting MDM2 through different mechanisms [13]. This prompted us to evaluate whether it would be possible to restore p53 apoptotic function by reactivating HIPK2 in the presence of MDM2.

2. Materials and methods

2.1. Cell culture and reagents

Human embryo kidney 293 and human colon carcinoma HCT116 cells were maintained in DMEM (Life Technology-Invitrogen), while

* Corresponding author.
** Corresponding author at: Department of Experimental Oncology, Molecular Oncogenesis Laboratory, National Cancer Institute "Regina Elena", 00158 Rome, Italy. Fax: +39 0652662505.
E-mail addresses: lavix@inwind.it (L. Nardinocchi), gdorazi@unich.it (G. D'Orazi).

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human colon carcinoma RKO cells, human epithelial cervical cancer cells HeLa, and human lung cancer H1299 (p53 null) cells were maintained in RPMI-1640 (Life Technology-Invitrogen), all supplemented with 10% heat-inactivated fetal bovine serum plus glutamine and antibiotics in humidified atmosphere with 5% CO_2 at 37 °C.

Treatments: cells were incubated with 100 μM $ZnCl_2$, 200 μM Cobalt Chloride ($CoCl_2$, hypoxia mimetic), 10 μmol/l Nutlin-3 (Cayman), and 5-10 μmol/l proteasome inhibitor MG132 (Biomol Research Laboratories) for the indicated time.

2.2. Transient transfection and vectors

Cells were plated in 60 mm Petri dishes and, the day after, transfected with the modified version of the calcium phosphate procedure or the cationic polymer LipofectaminePlus method (Invitrogen), according to the manufacturer's instructions.

Expression vectors used were: wild-type HIPK2-Flag [7] and mutant HIPK2-K1182R-Flag (MDM2-degradation resistant) [12], human HA-MDM2 (kindly provided by M. Oren, Weizmann Institute of Science, Rehovor, Israel), and pCAG3.1wtp53 (kindly provided by E. Appella, NIH, Bethesda, MD, USA).

2.3. Western immunoblotting and ubiquitination

For Western immunoblotting, total cell extracts were prepared by incubating at 4 °C for 30 min in lysis buffer as reported [11] and resolved by SDS–polyacrylamide gel electrophoresis. Proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore). Immunoblottings were performed with: mouse monoclonal anti-p53 (DO1), rabbit polyclonal anti-p53 (FL393), mouse monoclonal anti-MDM2 (Ab1), rabbit polyclonal anti-$p21^{Waf1}$ (all from Santa Cruz Biotechnology), rabbit polyclonal anti-phospho-Ser46 (Cell Signaling Technology), rabbit polyclonal anti-HIPK2 (kindly provided by M.L. Schmitz, Justus-Liebig-University, Giessen, Germany), mouse monoclonal anti-Flag (M2, Sigma, Bio-Sciences), rat polyclonal anti-HA (Roche Diagnostic), mouse monoclonal anti-tubulin (Immunological Sciences), and mouse monoclonal anti-Hsp70 (Stressgene).

P53 ubiquitination was performed essentially as described [11]. Briefly, H1299 cells were co-transfected with 1 μg pCAG3.1wtp53 and 4 μg HA-MDM2 expression vectors and 24 h later treated with $ZnCl_2$ and 10 μM MG132 for, respectively 16 and 8 h. Equal amounts of total cell extracts were immunoprecipitated with mouse monoclonal anti-p53 antibody (DO1) preadsorbed to protein G-agarose (Pierce). For endogenous HIPK2 ubiquitination assay, RKO cells were treated with $ZnCl_2$ for 24 h, $CoCl_2$ and 5 μM MG132 for 16 h. Equal amounts of total cell extracts were immunoprecipitated with rabbit polyclonal anti-HIPK2 antibody preadsorbed to protein G-agarose (Pierce). Immunocomplexes were collected by centrifugation, separated by SDS–PAGE, and blotted onto nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). Immunoreactivity was detected by enhanced chemiluminescence kit (ECL; Amersham).

2.4. Luciferase assay

Cells were co-transfected with expression vectors along with the luciferase reporter gene driven by the p53-dependent promoter Noxa-luc (kindly provided by T. Taniguchi, University of Tokyo, Japan) vector. Transfection efficiency was normalized with a co-transfected β-galactosidase plasmid. Luciferase activity was assayed on whole cell extract and the luciferase values were normalized to β-galactosidase activity and protein content.

2.5. Reverse transcription polymerase chain reaction (RT-PCR)

Cells were harvested in TRIzol reagent (Invitrogen) and total RNA was isolated following the manufacturer's instructions. The first strand cDNA and the semi-quantitative RT-PCRs were carried out essentially as described [14]. PCR products were run on a 2% agarose gel and visualized by ethidium bromide staining using UV light. The housekeeping glyceraldehydes-3-phosphate dehydrogenase (GAPDH) or aldolase-A (ald-A) mRNAs were used as internal control.

2.6. TUNEL assay and immunofluorescence

For TUNEL assay, 4×10^4 cells were spun on a slide by cytocentrifugation and subsequently fixed in 4% paraformaldehyde for 30 min at room temperature. The samples were permeabilized and then incubated in the TUNEL reaction mix for 1 h at 37 °C according to the manufacture's instructions (Roche, Germany). Cells were counter-stained with Hoechst 33 342 before analysis with a fluorescent microscope (Zeiss).

For indirect immunofluorescence, HeLa cells were transfected with HIPK2-Flag expression vector and 24 h after transfection treated with 100 μM $ZnCl_2$ and 200 μM $CoCl_2$ for 24 h and then fixed in methanol/acetone (1:1). Dried cells were blocked for 30 min at room temperature in phosphate-buffered saline containing 10% (v/v) goat serum, incubated with primary anti-Flag antibody at 4 °C for 16 h and for 45 min with an appropriate fluorochrome-conjugated secondary antibody. Chromosomal DNA was visualized by DAPI staining, stained cells were mounted on glass slides and examined using a confocal laser microscope (Nikon Eclipse).

2.7. Statistical analysis

All experiment unless indicated were performed at least three times. All experimental results were expressed as the arithmetic mean and standard deviation (S.D.) of measurements was shown. Student's *t*-test was used for statistical significance of the differences between treatment groups, determined as $P < 0.05$.

3. Results

3.1. MDM2-induced downregulation of HIPK2 and inhibition of p53Ser46 phosphorylation, are rescued by zinc treatment

In order to identify natural compounds that restore HIPK2 function, the effect of zinc was investigated. 293 cells were co-transfected with HIPK2-Flag or the K1182R-Flag mutant and MDM2-HA expression vectors and treated with zinc. As shown in Fig. 1, HIPK2 expression induced p53Ser46 phosphorylation (lane 2) while MDM2 co-transfection induced HIPK2 downregulation and abolishment of p53Ser46 phosphorylation (lane 3); interestingly, the MDM2-induced HIPK2 downregulation was completely rescued by zinc treatment with re-establishment of HIPK2-induced p53Ser46 phosphorylation and downregulation of MDM2 levels (lane 4). As expected, the MDM2 degradation-resistant HIPK2-K1182R mutant induced p53Ser46 phosphorylation (Fig. 1, lane 5) and was not downregulated by MDM2 (lane 6) nor its activity towards p53Ser46 phosphorylation was further affected by zinc treatment (lane 7).

3.2. Reactivation of HIPK2-induced p53 transcriptional and apoptotic activities

The clear effect of zinc on HIPK2 stability and p53Ser46 phosphorylation in the presence of MDM2 prompted us to evaluate its biological consequences. First, the p53 responsive reporter