Free Radical Biology & Medicine 48 (2010) 1338–1346

Contents lists available at ScienceDirect

Free Radical Biology & Medicine

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

Original Contribution

Nox1 is involved in p53 deacetylation and suppression of its transcriptional activity and apoptosis

Rosa Puca [a,b,1], Lavinia Nardinocchi [a,b,1], Giuseppe Starace [a,c], Gideon Rechavi [d], Ada Sacchi [a], David Givol [e], Gabriella D'Orazi [a,b,*]

[a] Department of Experimental Oncology, Molecular Oncogenesis Laboratory, National Cancer Institute Regina Elena, Rome, Italy
[b] Department of Oncology and Neurosciences, School of Medicine, University "G. d'Annunzio," 66013 Chieti, Italy
[c] Institute of Neurobiology and Experimental Medicine, CNR, Rome, Italy
[d] Cancer Research Center, Chaim Sheba Medical Center, Tel-Hashomer, and Sackler School of Medicine, Tel-Aviv University, Tel-Aviv, Israel
[e] Department of Molecular Cell Biology, Wiezmann Institute of Science, Rehovot, Israel

ARTICLE INFO

Article history:
Received 19 October 2009
Revised 10 February 2010
Accepted 11 February 2010
Available online 18 February 2010

Keywords:
p53 lysine 382 acetylation
HIPK2
SIRT1
p53 transcriptional activity
Reactive oxygen species
Free radicals

ABSTRACT

HIPK2 is a stress-induced kinase and a transcriptional corepressor that functionally cooperates with p53 to suppress cancer. Activation of the p53 proapoptotic function requires a cascade of phosphorylations and acetylations, and HIPK2 takes part in both modifications in that it phosphorylates p53 Ser46 and induces p53 Lys382 acetylation. Here, to further investigate the role of HIPK2 in p53 activation, we started with the finding that HIPK2 inhibition upregulated Nox1, a homolog of the catalytic subunit of the superoxide-generating NADPH oxidase, involved in tumor progression and ROS production. We found that Nox1 inhibited p53 Lys382 acetylation, which is a target of SIRT1 deacetylase, and impaired p53 proapoptotic transcriptional activity. By the use of either small interfering RNAs to target SIRT1 or the SIRT1 inhibitor nicotinamide we found that Nox1-dependent inhibition of p53 transcriptional activity was SIRT1-dependent. Thus, Nox1 was unable to inhibit p53 when coexpressed with a SIRT1 deacetylase-defective mutant (SIRT1HY), suggesting a link between Nox1 and SIRT1 activity. Finally, recovery of HIPK2 function downregulated Nox1 expression with rescue of p53 Lys382 acetylation and p53 activity. Together, our findings indicate that Nox1 upregulation may activate SIRT1 and inhibit p53 and that Lys382 is important for p53 proapoptotic function.

© 2010 Elsevier Inc. All rights reserved.

The oncosuppressor p53 plays a critical role in cell growth and apoptosis in response to various stress signals [1]. The activation of p53 as a sequence-specific transcription factor depends on a cascade of phosphorylations and acetylations that entail the correct function of kinases and acetyltransferases [2,3]. Homeodomain-interacting protein kinase-2 (HIPK2)[2] phosphorylates p53 at Ser46 for apoptotic commitment [4]. Moreover, HIPK2 affects p53 Lys382 acetylation [5], which, along with phosphorylation of Ser46, is necessary for p53 proapoptotic activity [6]. Lys382 of p53 is a substrate for the SIRT1 (NAD-dependent histone deacetylase)-mediated deacetylation, which antagonizes p53-dependent transcriptional activation and apoptosis in response to DNA damage and oxidative stress [7,8]. HIPK2 inhibition has been shown to strongly impair p53 activity [9] and promote tumor progression, angiogenesis, and chemoresistance [10], although the understanding of the exact role of HIPK2 in p53 regulation and in restraining tumor progression is far from being complete.

NADPH oxidase 1 (Nox1), a homolog of gp91*phox*, the catalytic subunit of the phagocyte superoxide-generating NADPH oxidase, constitutively produces both superoxide and H_2O_2 when overexpressed in fibroblasts [11]. Nox-generated ROS, at least in part, behave as second-messenger-like molecules at low concentrations and elicit cellular signals, whereas aberrant levels of Nox-derived ROS can perturb the balance of cellular homeostasis, resulting ultimately in pathological states [12]. Although the functional role of NADPH oxidases in nonphagocytic cells is not fully understood, their deregulated expression has been linked to various pathological conditions, including cancer [13–15]. It has been shown that Nox1 causes aggressive growth of tumors in vivo by triggering the angiogenic switch [13]; moreover, overexpression of Nox1 induces genome instability [16], a key factor in the induction or progression of human cancer [17]. In addition to the control of Nox1 activity by regulatory components, the transcriptional regulation of *Nox1* gene expression is critical for its activity. It has been shown that *Nox1* is

Abbreviations: HIPK2, homeodomain-interacting protein kinase-2; ADR, adriamycin; Dox, doxycycline; Nic, nicotinamide; Nox, NADPH oxidase; HEK-293, human embryo kidney; PARP, poly(ADP-ribose) polymerase; ROS, reactive oxygen species; RT-PCR, reverse transcriptase–polymerase chain reaction; TSA, trichostatin A.

* Corresponding author. Department of Experimental Oncology, Molecular Oncogenesis Laboratory, National Cancer Institute Regina Elena, Rome, Italy. Fax: +39 652662505.
E-mail address: gdorazi@unich.it (G. D'Orazi).

[1] These authors contributed equally to this work.

0891-5849/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.freeradbiomed.2010.02.015

regulated by a combination of GATA factors, hepatocyte nuclear factor-1α, and the *caudal*-related homeobox proteins Cdx1 and Cdx2 in human colon cancer cells [18,19].

SIRT1 protein is a NAD-dependent deacetylase that governs gene expression via deacetylation of substrate proteins that function as transcriptional activators or transcriptional repressors [20]. Several SIRT1 substrates have been characterized and few molecules have been identified as SIRT1 regulators [21]. SIRT1 is upregulated in several types of cancers, including prostate, skin, and colorectal [22], probably because of inactivation of hypermethylated in cancer 1 (HIC1), which binds directly to the SIRT1 promoter and represses gene transcription [23]. Furthermore, DBC1 (deleted in breast cancer 1) acts as an inhibitor of SIRT1 and promotes p53-mediated apoptosis [24]. In contrast, AROS (active regulator of SIRT1) enhances SIRT1-mediated p53 deacetylation both in vitro and in vivo and inhibits p53-mediated transcriptional activity [25]. Changes in the NAD^+/NADH ratio may also impact SIRT1 function. For instance, NAMPT (nicotinamide phosphoribosyltransferase), a rate-limiting enzyme in the NAD biosynthesis pathway, has been shown to enhance SIRT1 activity in mammalian cells by increasing NAD^+ levels [26]. Finally, the microRNA miR-34a, which is a target of p53, inhibits SIRT1 and thus increases p53 activity in a positive feedback loop [27].

We recently found that HIPK2 is involved in p53 Lys382 acetylation, which strongly affects the transcription of proapoptotic genes [6]. This led us to investigate the molecular mechanisms underlying HIPK2 regulation of the p53 acetylation/deacetylation balance. In this study, starting from the finding that HIPK2 depletion induced Nox1 upregulation, we investigated whether Nox1 was involved in SIRT1-induced p53 Lys382 deacetylation. In the experiments described below, we provide evidence that Nox1 induces p53 Lys382 deacetylation with inhibition of p53 proapoptotic transcriptional activity. Interestingly, we found that SIRT1 deacetylase activity was required by Nox1 for p53 inhibition. Finally, restoration of HIPK2 activity, in a model of chemical hypoxia, correlated with Nox1 downregulation, with rescue of p53 Lys382 acetylation and of p53 proapoptotic activity, suggesting a novel mechanism by which HIPK2 may regulate p53 function in tumors.

Material and methods

Cells and reagents

Human colon carcinoma RKO, human breast cancer MCF7, and human lung cancer H1299 (p53 null) cells were maintained in RPMI 1640 (Life Technology–Invitrogen), and human embryo kidney HEK-293 cells were maintained in DMEM (Life Technology Invitrogen), both supplemented with 10% heat-inactivated fetal bovine serum plus glutamine and antibiotics in a humidified atmosphere with 5% CO_2 at 37 °C. The doxycycline (Dox)-inducible MCF7 (MCF7-indsi/HIPK2) cell line expressing HIPK2 interference has been described [28]. For inducible HIPK2 knockdown Dox (1 μg/ml) was added to MCF7-indsi/HIPK2 cells every 3 days until HIPK2 knockdown was successfully reached (usually in about 5 days). After HIPK2 knockdown was reached, cells were cultured without Dox for an additional 5 days before being harvested and processed for the indicated experiments.

For treatments, Dox (1 μg/ml), adriamycin (ADR; 2 μg/ml), $ZnCl_2$ (150 μM), nicotinamide (Nic; 5 mM), and trichostatin A (TSA; 100 nM), all from Sigma (St. Louis, MO, USA), were used as indicated; chemical hypoxia was obtained by adding cobalt chloride ($CoCl_2$) into the culture medium to a final concentration of 200 μmol/L for the indicated period.

Transfection, plasmids, and transactivation assay

Transient or stable transfections were carried using the *N,N*-bis-(2-hydroxyethyl)-2-amino-ethanesulfonic acid-buffered saline version of the calcium phosphate procedure [29] (for HEK-293 and H1299 cells) or the cationic polymer Lipofectamine Plus reagent (Invitrogen, Carlsbad, CA, USA) (for RKO and MCF7 cells), according to the manufacturer's instructions. The amount of plasmid DNA in each sample was equalized by supplementing with empty vector.

For transactivation assay cells were cotransfected with the reported expression vectors along with the luciferase reporter gene driven by p53-target promoters or with the Nox1-luc reporter constructs [19]. Transfection efficiency was normalized with the use of a cotransfected β-galactosidase plasmid. Luciferase activity was assayed on whole-cell extract and the luciferase values were normalized to β-galactosidase activity and protein content. At least three independent experiments were performed in duplicate.

The following plasmids were used: HIPK2-Flag and kinase mutant HIPK2-K221R-Flag [4], pSuper and pSuper-HIPK2 (for RNA interference) [30], pCMV-wtp53, p53K382R [31], p300-Flag (kindly provided by M. Fanciulli, Regina Elena Cancer Institute, Rome, Italy), GFP-Nox-1 (kindly provided by M. Bignami, National Institute of Health–ISS, Rome, Italy), pCMV-SIRT1 and pCMV-SIRT1HY (deacetylase defective; kindly provided by M. Levrero, University Sapienza, School of Medicine, and Andrea Cesalpino Foundation, Rome, Italy), the luciferase reporter gene driven by the p53-dependent promoters Noxa-luc (kindly provided by T. Taniguchi, University of Tokyo, Japan) and Bax-luc, and the Nox1-luc reporter constructs encompassing the proximal 6.1-, 2.5-, 1.1-, and 0.44-kb promoter regions of the human Nox1 gene [19] (kindly provided by A.J. Valente, Department of Medicine, University of Texas Health Science Center, San Antonio, TX, USA).

Western immunoblotting

Total cell extracts were prepared by incubating at 4 °C for 30 min in lysis buffer (50 mmol/L Tris–HCl (pH 7.5), 50 mmol/L NaCl, 5 mmol/L EDTA, 150 mmol/L KCl, 1 mmol/L dithiothreitol, 1% NP-40) and a mix of protease inhibitors (Sigma) and resolved by SDS–polyacrylamide gel electrophoresis. Proteins were transferred to a polyvinylidene difluoride membrane (Millipore) and incubated with the primary antibodies followed by an anti-IgG–horseradish peroxidase antibody (Bio-Rad). Immunoblotting was performed with the following antibodies: mouse monoclonal anti-p53 (DO1; Santa Cruz Biotechnology), rabbit polyclonal anti-phospho-Ser46 (Cell Signaling Technology), polyclonal anti-acetyl-p53 (Lys382) (Cell Signaling), monoclonal anti-GFP (Roche Diagnostic), polyclonal anti-Bax (N20; Santa Cruz Biotechnology), monoclonal anti-poly(ADP-ribose) polymerase (PARP; BD Pharmingen), polyclonal anti-SIRT1 (Sigma), and monoclonal anti-Hsp70 (Stressgene). Immunoreactivity was detected by enhanced chemiluminescence kit (ECL; Amersham).

Viability assay

Exponentially proliferating cells were exposed to ADR (2 μg/ml) in the presence or absence of Nox1 or SIRT1 overexpression. Both floating and adherent cells were collected and counted in a hemocytometer after the addition of trypan blue. The percentage of dead cells (i.e., blue/total cells) was determined by scoring 100 cells per chamber three times. At least three independent experiments were performed and cell numbers were determined in duplicate.

RNA extraction and reverse transcription (RT)-PCR analysis

Cells were harvested in TRIzol reagent (Invitrogen) and total RNA was isolated following the manufacturer's instructions. The first-strand cDNA reaction and the semiquantitative RT-PCRs were carried out essentially as described [9] by using gene-specific oligonucleotides under conditions of linear amplification. PCR was performed in duplicate in two different sets of cDNA. PCR products were run on a 2%

Download English Version:

https://daneshyari.com/en/article/1909510

Download Persian Version:

https://daneshyari.com/article/1909510

Daneshyari.com