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Oligomerization conditions Mdm2-mediated efficient p53 polyubiquitylation but not its proteasomal degradation

Roland Hjerpe[a,1], Fabienne Aillet[a,1], Fernando Lopitz-Otsoa[a], Valerie Lang[a], Mónica Torres-Ramos[a], Rosa Farrás[c], Ronald T. Hay[d], Manuel S. Rodríguez[a,b,*]

[a] The Proteomics Unit, CIC bioGUNE, CIBERehd, 48160 Derio, Bizkaia, Spain
[b] Biochemistry Department, University of the Basque Country, 48940 Leioa, Bizkaia, Spain
[c] Centro de Investigación Príncipe Felipe, 46013 Valencia, Spain
[d] Centre for Interdisciplinary Research, School of Life Sciences, University of Dundee, Scotland DD1 5EH, United Kingdom

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ABSTRACT

In normal cells p53 is maintained at low level through the action of the ubiquitin–proteasome system. As a consequence of p53 transcriptional activity, various regulators of this tumor suppressor are produced, forming a negative feedback loop tightly controlling p53 stability. One of the most prominent is the ubiquitin-ligase Mdm2. Here, we have used a transfer of signals strategy to study the p53 degradation process promoted by Mdm2 in the absence of p53 transcriptional activity. Our results show that in a p53 null background, transcriptionally silent p53-fusions require multiple N- and C-terminal signals to be optimally targeted to proteasomal degradation. As for WT p53, p53-fusions able to form tetramers are polyubiquitylated and optimally degraded by the proteasome. However, p53 molecules unable to oligomerize, show Mdm2-mediated polyubiquitylation deficiency but are still targeted to proteasome degradation *in vitro* and *ex vivo*. In the presence of Mdm2, nuclear shuttling of p53 monomeric fusions favours proteasome-dependent degradability but not its polyubiquitylation. *In vitro*, 26S proteasome fails to drive degradation of OD mutants in the presence of Mdm2, suggesting the contribution of additional cellular factors in this process. All together, our results indicate that Mdm2-mediated proteasome-dependent degradation of polyubiquitylation deficient p53 monomers is mechanistically possible, taking alternative pathways to better achieve their proteolysis. These results support the existence of additional levels to regulate p53 stability and activity acting on individual subunits of the functional tetramer.

1. Introduction

The transcription factor p53 is a critical tumor suppressor protein, mutated in approximately half of all occurrences of human cancer (Levine, 1997). P53 plays a central role in the control of DNA-damage repair and cell proliferation (Sturzbecher et al., 1996). Under normal conditions, p53 levels are maintained low, whereas upon cell stress, such as DNA damage induced by ionizing radiation or genotoxic drugs, activated p53 accumulates in the nucleus and induces transcription of genes involved in cell cycle arrest and apoptosis.

Ubiquitylation is the process where a target protein is post-translationally modified with the 76 amino acid protein ubiquitin. This involves the sequential action of three types of enzymes, an E1, E2 and E3. The E1 or ubiquitin activating enzyme forms a thiolester bond between its active site and ubiquitin, and subsequently transfers ubiquitin to the E2, or ubiquitin conjugating enzyme. The E3, or ubiquitin ligase, largely determines substrate specificity, and facilitates the transfer of ubiquitin from the conjugating enzyme to the target protein (Hershko and Ciechanover, 1998). Mdm2 binds to a N-terminal p53 sequence element, occluding the p53 transactivation domain, thus silencing p53 transcriptional activity. In addition, Mdm2 has been shown to ubiquitylate p53 promoting its efficient proteasomal degradation (Honda et al., 1997). The Mdm2 gene itself is a transcriptional target of p53 (Barak et al., 1994), leading to the formation of a negative feedback loop. Several cellular and viral ubiquitin E3s have been proposed to regulate p53 activity (Brooks and Gu, 2006). The apparent redundancy of some

Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; DBD, DNA binding domain; DMEM, Dulbecco's modified eagles medium; EGFP, enhanced green fluorescent protein; KO, knock out; Mdm2, mouse double minute 2; NEDD8, neural precursor cell expressed developmentally down-regulated 8; SUMO, small ubiquitin-like modifier; NES, nuclear export signal; NLS, nuclear localization signal; OD, oligomerization domain.

* Corresponding author at: Proteomics Unit, CIC bioGUNE, Bizkaia Technology Park, Building 801A, 48160 Derio, Spain. Tel.: +34 946 061 323; fax: +34 946 061 324.
E-mail address: mrodriguez@cicbiogune.es (M.S. Rodríguez).
[1] Both authors contributed equally to this work.

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E3 ligases suggests the existence of a tight control of p53 activity during various physiological and pathological processes, through molecular mechanisms that remain to be fully characterized.

p53 can conceptually be seen as a modular protein, where functionally independent domains perform specific tasks. The central part of p53 contains the DNA binding domain, which is N-terminally flanked by a natively unfolded region (Dawson et al., 2003) containing the transactivation domain (TAD), the proline rich domain (PRD) and a proteolysis sensitive region (PSR) (Gu et al., 2000). The C-terminus of p53 contains the oligomerization domain (OD) and the cytoplasmic sequestration domain (CSD) (Liang and Clarke, 1999). The OD is required not only for the formation of the transcriptionally active tetramer but also for the efficient polyubiquitylation of p53 (Maki, 1999). Both N- and C-terminus contain regulatory sequence elements, such as nuclear export signals (NES), nuclear localization signals (NLS) and sequences involved in protein–protein interactions. In both ends of p53, several amino acids are targeted by a wide variety of posttranslational modifications including the ubiquitin-like molecules SUMO-1 (Rodriguez et al., 1999) and NEDD8 (Xirodimas et al., 2004). Previous *in vitro* experiments have underlined the importance of the six C-terminal lysines of p53 in Mdm2-mediated proteasomal degradation (Nakamura et al., 2000; Rodriguez et al., 2000). Conjugation of SUMO-1 to p53 is mainly having a positive effect on p53 transcriptional activity (Rodriguez et al., 1999), in contrast, NEDD8 ligation, act as an inhibitory modification (Xirodimas et al., 2004).

To analyze the role of sequence elements involved in p53 degradation avoiding its transcription-dependent auto-regulatory feedback loops (Rahman-Roblick et al., 2007; Horn and Vousden, 2007) we choose a strategy of transfer of signals to unrelated proteins. We show that delimited signals of p53 confer an optimal Mdm2-mediated proteasome-dependent degradability to heterologous proteins, in a p53 transcriptionally silent background. More importantly, this strategy allowed us to demonstrate that although p53 monomers present a defective Mdm2-mediated polyubiquitylation, these molecules can still be degraded via the proteasome.

2. Materials and methods

2.1. Reagents and plasmids DNA manipulations

Proteasome inhibitors MG132 and Lactacystin were obtained from Sigma and Biomol, and used at 10 μM. Plasmid encoding Mdm2 has been previously described (Midgley et al., 2000). p53-EGFP/β-galactosidase plasmids were generated by cloning standard PCR amplifications of specific regions of p53 in frame with either EGFP (from plasmid pEGFP-C1, with GenBank Accession No. U55763) or β-galactosidase (from plasmid pSVβ, with GenBank Accession No. U02435). p53 fragments were cloned into the vector pcDNA3-Sv5 using the restriction sites KpnI–KpnI (amino terminal p53 fragments) and BamHI–EcoRI (carboxy terminal p53 fragments). For constructs 7 and 8, the amino terminal fragment of p53 has been cloned using HindIII–KpnI. Subsequently, either EGFP or β-galactosidase was cloned in frame between the two p53 fragments, using the restriction sites BamH1–BamH1.

2.2. Cell culture and transfection

p53 null H1299 human lung cancer cells, p53 null Saos-2 human osteosarcoma cells and $p53^{-/-}$ $Mdm2^{-/-}$ mouse double knock out cells (2KO) were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS) and antibiotics. Cells were transfected with p53-EGFP or p53-β-galactosidase plasmid and Mdm2 using lipofectamine (Invitrogen). Transfection efficiency was controlled with vector expressing a V5-tagged version of EGFP or β-galactosidase, indicated in the figures as charge control (CC). For degradation and ubiquitylation experiments a 1:5 p53-fusion:Mdm2 ratio was used. After transfection cells were grown in six-well plates for 24 h, and harvested for analysis by Western blotting or immunofluorescence. For measurement of transcriptional activity, H1299 cells were co-transfected with plasmids expressing WT p53 or the p53-EGFP fusions and the pG13-Luc and pSV-β-galactosidase reporter plasmids. Luciferase and β-galactosidase activities were measured as previously described (Rodriguez et al., 1996).

2.3. Tetramerization assays

H1299 cells were transfected with indicated plasmids, and lysed in buffer containing 1% NP-40 (Igepal CA-630), 100 mM NaCl, 100 mM TRIS, pH 8. Samples were split in two and treated or not with crosslinking agent (glutaraldehyde), final concentration 0.1%. The reaction was quenched after 10 min with 100 mM PBS/glycine. Monomers or multimers were detected by Western blot with the indicated antibodies.

2.4. Preparation of cell extracts and Western blotting

Harvested cells were lysed in Laemmli buffer and boiled for 15 min. Proteins were then separated by gel electrophoresis on 10% (for EGFP constructs) or 8% (β-galactosidase constructs) polyacrylamide gel, to be subsequently transferred to polyvinylidene difluoride membranes (Millipore) by electroblotting. His6-tagged proteins were purified as reported (Feng et al., 2005). To be able to detect multiple mono-ubiquitylated forms when using His-Ubiquitin KO (all lysine residues involved in the formation of ubiquitin chains are mutated to arginine), washing conditions were less stringent (no detergent was included) resulting in higher background levels. A fraction (1/20) of the initial lysate was used as control input. Chimeric proteins were immunodetected by Western blot. Primary antibodies used were mouse anti-p53 DO1; mouse anti GFP (Roche); mouse anti-SV5 (MCA1360, Serotec); mouse anti-Mdm2 (Ab5 4B2C1.11, Calbiochem). Anti-β-actin (Sigma) was obtained from Santa-Cruz Biotechnology. Mdm2 co-immunoprecipitation experiments were performed using Protein A cross-linked with the SV5 antibody to immunoprecipitate p53-fusions.

2.5. In vitro degradation assays

Degradation assays were performed using 3 μg of purified human 26S proteasomes (Hjerpe et al., 2009; Coux and Goldberg, 1998) or 1 μg mouse 20S proteasomes (Boston Biochem) in a ubiquitylation buffer (see Supplementary material). Mdm2, p53 or OD mutants were *in vitro* transcription/translated using reticulocytes kit (Promega). Reactions included 2 μl of translated substrates and when indicated 0.5 μl of Mdm2. To analyze the implication of ATP, ATP-regenerating system was removed and 20 units of Apyrase (Sigma) added to the reaction. After incubation at 37 °C for 120 min the reaction products were stopped with SDS Laemmli buffer, and analyzed by Western blot.

3. Results

3.1. p53 degradation signals can be dissociated from activation of transcription

Using EGFP as carrier protein, a transfer of signal strategy allowed us to assess the contribution of p53 signals in the Mdm2-mediated proteasomal degradation (Fig 1A and data not shown).