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

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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 or ubiquitin activating enzyme, an E2 or ubiquitin conjugating enzyme, and an 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 activation 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...
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 Voussen, 2007) we choose a strategy of transfer of signals to unrelated proteins. We show that delimited signals of p53 confer an optimal effect on p53 transcriptional activity (Rodriguez et al., 1999), in contrast, NEDD8 ligation, act as an inhibitory modification (Xirodimas et al., 2004).

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 BamHI–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 crossinglinking 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 D01; 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 reticulocyte 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).
Chimeric proteins were co-transfected in H1299 p53<sup>−/−</sup> cells, either with Mdm2 or an empty vector. Both N-terminal (including the main Mdm2 binding domain) and C-terminal (containing the six lysine acceptors for ubiquitylation) extensions of p53 were required to promote efficient proteasomal degradation of EGFP-p53-fusions (Fig. 1B). Based on quantifications with the SV5 antibody and taking in consideration the charge control β-galactosidase, it was observed that the short fusion proteins 3, 4, 5 and 6 were degraded 21%, 77%, 48% and 41% with respect to the situation without Mdm2. However degradation was improved for some of the longer p53-fusions up to 59%, 78% and 93% for constructs 9, 10, 11, respectively. Due to its capacity to autoubiquitylate (Honda et al., 1997), the stability of Mdm2 was systematically compromised in conditions where p53-fusions showed high degradability.

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**Fig. 1.** Degradation signals of p53 do not activate p53-dependent transcription. (A) Schematic representation of p53-fusions. The exact number of amino acid sequences is indicated. TAD—trans activation domain; NES—nuclear export signal; PRD—proline rich domain; PSR—proteolysis sensitive region; DBD—DNA binding domain; NLS—nuclear localization signal; OD—oligomerization domain; CSD—cytoplasmic sequestration domain. p53-fusions contain a C-terminal SV5-tag (star) and (B) H1299 cells were transfected with plasmids encoding WT p53 or p53-EGFP fusion proteins, together with empty vector or Mdm2 vector as indicated. Whole cell extracts were analyzed by Western blot with the indicated antibodies. (C) Transcriptional activity of WT p53 and p53-EGFP fusions. A p53-dependent luciferase reporter was used. Activity was normalized with an RSV-β-gal reporter (n = 6). CC indicates charge control, co-transfected β-galactosidase-SV5, detected with anti-SV5 antibody.
Fig. 2. Tetramerization conditions polyubiquitylation but not multiple mono-ubiquitylation. (A) Mdm2-mediated p53-EGFP fusions degradation depends on the proteasome. H1299 cells were transfected as indicated in Fig. 1B and treated or not with 10 μM MG132 during 8 h. Whole cell extracts were prepared and analyzed by Western blot with the indicated antibodies. Co-transfected β-galactosidase-SV5, detected with anti-SV5 antibody was used as charge control (CC). (B and C) Ubiquitylation pattern of p53-fusions. H1299 cells were co-transfected with plasmids expressing His6-ubiquitin WT (B) or His6-ubiquitin KO (C) and indicated constructs. Cell extracts were prepared under denaturing conditions and ubiquitylated proteins were purified using nickel columns. Pull down and input samples were analyzed by Western blot with the indicated antibodies. Star indicates unmodified forms of fusion protein. N.S., nonspecific. (D) Oligomerization capacity of p53-fusions. H1299 cells were transfected with the indicated constructs. Lysates from transfected H1299 cells were treated or not with 0.1% glutaraldehyde (GA) and analyzed by Western blot using anti-p53 or SV5 antibodies as indicated.

(Fig. 1B) but recovered in the presence of proteasome inhibitors (Fig. 2A). Degradation of WT p53 was always better than any of the p53-fusions due to the cooperative action of p53-dependent genes, whose expression is induced by the WT molecule. Similar results were obtained in p53 null Saos-2 and mouse p53−/− Mdm2−/− cells (data not shown) and using p53-β-galactosidase fusions (data not shown). The absence of the p53 DBD in all these constructs results in the complete abrogation of p53 transcriptional activity (Fig. 1C), indicating that under these experimental conditions, Mdm2 is sufficient to drive this proteasome-mediated proteolytical process without other p53-dependent positive or negative regulators.

3.2. Oligomerization of p53-fusions conditions efficient polyubiquitylation but not proteasome-dependent degradation by Mdm2

Degradation of p53-EGFP chimeras mediated by Mdm2 is prevented by proteasome inhibitors MG132 (Fig. 2A) or Lactacystin.
suggesting its self-induced degradation (Fang et al., 2000). Interestingly, constructs 9–11 and WT p53 accumulate high molecular weight species in different proportions, after treatment of the cells with proteasome inhibitors (Fig. 2A), suggesting that these proteins are likely ubiquitylated. Ubiquitin conjugated forms of all constructs were purified using nickel beads and denaturing conditions (Rodriguez et al., 1999) from H1299 cells co-transfected with all p53-fusions and vectors expressing Mdm2 and His6-ubiquitin WT. Constructs 9–11 show the highest ubiquitylation levels, corresponding to polyubiquitylated forms comparable to WT p53, whereas constructs 2–6 and 8 accumulate ubiquitylated forms of lower molecular weight (Fig. 2B). The deficient pattern of ubiquitylation observed for constructs 2–6 and 8 is composed, in a large proportion, of multiple mono-ubiquitylation as it is similar to the one obtained with His6-Ubiquitin KO, which cannot form ubiquitin branches (Fig. 2C) (see Section 2). As previously reported (Hjerpe et al., 2009), when ubiquitin KO is expressed, the ubiquitylation pattern of WT p53 is then composed by lower molecular weight forms indicating a switch to multiple mono-ubiquitylation. These mono-ubiquitylated forms can be purified with His6-Ubiquitin KO for WTP53 and for constructs 9–11 (Fig. 2C).

Since polyubiquitylation is conditioned by the tetramerization of p53 (Maki, 1999), the capacity of the fusions to form tetramers was investigated. H1299 cells were transfected with vectors expressing EGFPP53-fusions and analyzed as previously described (Carter et al., 2007). Constructs 1–8 lacking OD domain do not form tetramers or dimers, whereas the tetramerization of constructs 9–11 was very efficient (Fig. 2D). In agreement with previously published information (Maki, 1999), our results highlight a perfect correlation between optimal Mdm2-mediated polyubiquitylation and proteasome-mediated degradation of the p53-fusions with their oligomerization potential. However, our data also show that some of the constructs unable to oligomerize (such as constructs 4–6 and 8), exhibit different levels of Mdm2-mediated proteasome-dependent degradability (Figs. 1B and 2A) without an efficient formation of polyubiquitin chains.

3.3. Role of C-terminal lysines of p53 in the degradation of monomeric and tetrameric p53-fusions mediated by Mdm2 and the proteasome

To investigate the contribution of C-terminal lysine ubiquitylation in the Mdm2-mediated proteasome-dependent degradation of tetramerization deficient p53-fusions, single K-to-R mutations were introduced in construct 4. As a control, we have also generated K-to-R in the tetramerizable construct 11. Both constructs were individually expressed in the absence or presence of Mdm2 in H1299 cells. Simultaneous mutation of the residues 370, 372, 373 (3MKR) or 381, 382 and 386 (3CKR) is not sufficient to block Mdm2-mediated proteasomal degradation of construct 4 (Fig. 3A and B). The same results were obtained with construct 11 (Fig. 3A and C). However, the combined K-to-R mutation of all these residues (6KR) results in a protective effect for construct 4 (Fig. 3B) but not for construct 11 (Fig. 3C). The complete protective effect for construct 11 is obvious when, in addition to the 6KR mutations, lysine residues 319, 320 and 321 (3NKR) are simultaneously mutated to generate mutant 9KR (Fig. 3C). Accordingly, when inhibiting Mdm2-mediated proteasomal degradation of K-to-R mutants by using Lactacystin, slowly migrating forms can be easily detected with WT construct 11 and mutants 3CKR, 3MKR, 3NKR (Fig. 3D). In contrast, a significant reduction of slowly migrating forms is observed with mutants 6KR and 9KR, suggesting a defective ubiquitylation potential for these mutants. These results indicate that the cluster of lysines 319–321 contribute to the proper polyubiquitylation and degradation of the p53-fusion 11. Because lysines 319–321 are also part of the main NLS of p53, it was decided to analyze the sub-cellular distribution of the 3NKR and 9KR mutants. The distribution of mutants 3NKR and 9KR shows a defective nuclear localization (Fig. 3E). Consequently it has an impact on both ubiquitylation and resistance to Mdm2-mediated proteasomal degradation. All together these results underline the importance of C-terminal lysine residues and the nuclear localization of p53 in its degradation.
Fig. 4. Mdm2 binding to p53-fusions contributes to their nuclear localization and proteasomal degradation. (A) Interaction of p53-EGFP fusions with Mdm2. Cells were co-transfected with the different constructs, and the catalytically inactive Mdm2 C464A. Constructs were immunoprecipitated with anti-SV5, and co-immunoprecipitated Mdm2 was detected with anti-Mdm2 by Western blot analysis. (B) Sub-cellular distribution of p53-fusions. H1299 cells were transfected with a WT p53 encoding vector or plasmids expressing the different p53-EGFP fusions. GFP fluorescence was analyzed. DNA was detected by DAPI. N/c: mainly nuclear distribution, n/c: equal distribution in nucleus and cytoplasm, N: dominant nuclear distribution, and C: dominant cytoplasmic distribution. (C) Mdm2 promotes nuclear localization of p53-EGFP fusions. Cells were co-transfected as described in A. GFP fluorescence was used to observe distribution of p53-EGFP fusions and for Mdm2 indirect immunofluorescence was performed as previously reported (Midgley et al., 2000). Indicated scale bars are 40 μm.

3.4. Nucleo-cytoplasmic shuttling enhances Mdm2-mediated proteasomal degradation but not polyubiquitylation of tetramerization deficient p53-fusions

While the C-terminus of p53 is required for the ubiquitylation of p53-fusions, the N-terminus provides the main binding site to Mdm2 (Fig. 4A). Importantly, Mdm2 binding to p53-fusions favours their nuclear localization (Fig. 4C compared to B). Considering these results and the importance of the nuclear shuttling signals in the Mdm2-mediated proteasomal degradation of p53 (O’Keefe et al., 2003), an artificial NLS, NES or both shuttling signals were incorporated in tetramerization deficient p53-fusions proteins presenting...
Fig. 5. Importance of nucleo-cytoplasmic shuttling for multiple mono-ubiquitin-mediated degradation p53-EGFP fusion. (A) Sub-cellular distribution of construct 4 and construct 8, fused to an NES, NLS or both. H1299 cells were transfected with the indicated constructs, and analyzed for GFP and DAPI fluorescence. (B and C) Mdm2 and proteasome-mediated degradation of nuclear shuttling mutants. H1299 cells co-transfected with plasmids expressing indicated constructs in the absence or presence of a vector expressing Mdm2. (C) 10 μM Lactacystin was used during 8h to block degradation of the indicated constructs. Cell lysates were analyzed by Western blot. (D) Mdm2-mediated ubiquitylation of p53-EGFP fusion 11 and fusions 4 and 8 containing artificial shuttling signals. H1299 cells were co-transfected with plasmids expressing His6-ubiquitin WT with indicated constructs. Cell extracts were prepared under denaturing conditions and ubiquitylated proteins were purified using nickel columns. Eluates were analyzed by Western blot with the indicated antibodies. Star indicates unmodified forms of fusion protein. CC indicates charge control, co-transfected β-galactosidase-SV5, detected with anti-SV5 antibody.
Poor (18% for construct 8) or more efficient (38% for construct 4) degradation (Fig. 5A). Including one or both nuclear shuttling signals promotes a better Mdm2-mediated proteasomal degradation of constructs 8 (up to 75%) (Fig. 5B). However, under these experimental conditions, the effect of the NES on the degradation of construct 4, can only be seen when both NES and NLS are present (up to 82%) (Fig. 5B), probably reflecting the passive diffusion of these molecules due to their size. Interestingly, degradation of construct 8NES/NLS is very similar to the one of construct 11. Accumulation of slowly migrating forms were investigated by Western blot in these constructs after proteasome inhibitors treatment (Fig. 5C). Ubiquitylated forms of constructs 4, 4NLS, 4NES, 4NESNLS, 8, 8NLS, 8NES, 8NESNLS and construct 11 WT were purified using His6-ubiquitin WT on nickel beads and denaturing conditions. Modified forms of monomeric p53-fusions were always of low molecular weight compared to the high molecular weight forms isolated from tetramerizable construct 11 (Fig. 5C). These results suggest that most of the slowly migrating forms purified

Fig. 6. Oligomerization deficient p53 mutants are targeted to proteasomal degradation by Mdm2 in the absence of polyubiquitylation. (A) Single point mutations were introduced in residues F328 or L330 of construct 11 to generate OD1 and OD2 mutants. (B) Oligomerization capacity of WTp53 and OD mutants was tested. (C) Using luciferase reporter under the control of p21 and Bax promoters the transcriptional activity of OD1 and OD2 mutants was investigated and compared to p53WT. Activity was normalized with an RSV-

\[ \text{H9252}/\text{H9252} \text{ /H9252} \text{+ /H9252}-\text{gal reporter (n = 6).}

(D) Sub-cellular localization of p53WT, OD1 and OD2 was investigated. (E) Mdm2-mediated proteasomal degradability and proteolysis blockade by proteasome inhibitor. Indicated scale bars are 40 μm. (F) Purification of ubiquitylated forms using His6-Ubiquitin WT and His-Ubiquitin KO was evaluated for p53WT, OD1 and OD2 in H1299 cells. Ubiquitin patterns were analyzed by Western blot analysis using the indicated antibodies. CC indicates charge control by anti-β-actin.
from monomeric p53-fusions correspond in a large proportion to multiple mono-ubiquitylations (Fig. 5D). From these experiments it can be concluded that the ubiquitylation defects of oligomerization defective p53-fusions cannot be compensated by their nuclear or cytoplasmic localization, nevertheless, nuclear shuttling has a positive effect on Mdm2-mediated proteasomal degradability.

3.5. Mdm2-mediated proteasomal degradation of p53 OD mutants does not require efficient formation of polyubiquitin chains

Nuclear shuttling, but not oligomerization appears to promote Mdm2-mediated degradation of tetramerization deficient EGFP-p53-fusions. To confirm these observations in the context of the construct 11 and full-length p53, mutations in the OD domain named OD1 (L330R) and OD2 (L330R/F328V) were generated (Fig. 6 and Supplementary Fig. 1). These previously reported point mutations (Fig. 6A) have negative effects on the transcriptional activity of p53 due to disruption of p53 tetramerization capacity (Kato et al., 2003), and have moreover been described to exist in human tumors (Kobayashi et al., 1996; Duddy et al., 2000; Kawaguchi et al., 2003), and have moreover been described to exist in human tumors. These amino acid changes affected the oligomerization capacity (Fig. 6B and Supplementary Fig. 1A), resulted in lack of transcriptional activity (Fig. 6C) but showed a nuclear distribution (Fig. 6D and Supplementary Fig. 1B). Mdm2-mediated proteasomal degradation of OD mutants was observed in H1299 cells (Fig. 6E and Supplementary Fig. 1C) but it was systematically less efficient than the one observed with p53 WT or construct 11 WT. Degradation of OD1 and OD2 mutants is blocked by proteasome inhibitors MG132 (Fig. 6E and Supplementary Fig. 1C) and Epoxomicin (data not shown) and slowly migrating forms of low molecular weight were observed in both OD mutants in conditions where WT construct 11 and WT p53 showed higher molecular weight forms detected by Western blot with anti-SV5 and DO1 antibodies, respectively (Fig. 6E and Supplementary Fig. 1C middle panels). These slowly migrating forms mainly correspond to multiple mono-ubiquitylated forms (for OD mutants) and polyubiquitylated forms (for WT construct 11 and WT p53) that can be purified with His6-Ubiquitin WT and His6-Ubiquitin KO (Fig. 6F and Supplementary Fig. 1D). The polyubiquitin chain preference of the Tandem Ubiquitin Binding Entities (TUBEs) allow the efficient capture of WTp53 but not the trapping of OD mutants which are mainly multi-ubiquitylated (Hjerpe et al., 2009). Ubiquitylation defects of the OD mutants cannot be overcome in vitro using recombinant hdm2 alone or in association with hdm4 or complemented with the HeLa fraction 2.5 (Supplementary Fig. 2A and B). Moreover, OD mutants always showed a similar modification pattern with both ubiquitin WT or KO under the same experimental conditions (Supplementary Fig. 2C). Thus, the incapacity of the OD mutants to be polyubiquitylated ex vivo is related to their oligomerization defects and not to secondary effects due to the over expression of the transdominant negative OD mutants or KO ubiquitin moieties.

3.6. Proteasomal degradation of p53 OD mutants in vitro

To investigate if the poorly ubiquitylated p53 OD mutants are degraded by the 26S and/or the 20S proteasomes, in vitro degradation assays were performed. Whereas WT p53 is degraded by both 26S and 20S proteasomes in the presence of Mdm2, OD mutants are better substrates for the 20S proteasome (Fig. 7A). Increasing the amount of Mdm2 used in these assays does not increase degradability of OD mutants by the 26S proteasomes. Tetrameric WT p53 is however not as efficiently degraded than OD mutants by the 20S proteasomes. Tetramerization WT p53 is however not as efficiently degraded than OD mutants by the 20S proteasomes in the presence or absence of Mdm2 (Fig. 7B). Under our experimental conditions Mdm2 and ATP contribute to optimise WTp53 degradation by the 26S proteasomes (Fig. 7C and data not shown). In contrast, degradation of WTp53 and OD mutants by the 20S does not require Mdm2 (Fig. 7B and C and data not shown). All together our in vitro results indicate that in the absence of Mdm2, 20S proteasomes drive an efficient degradation of p53 monomers, perhaps contributing to their basal turnover. This mechanism appears to be different that the one driving WTp53 degradation by the 26S proteasomes that requires Mdm2. In the

![Fig. 7. In vitro degradation of OD mutants by 26S and 20S proteasomes. (A) 20S or 26S proteasomes were used to drive in vitro degradation of WTp53, OD1 and OD2 mutants in presence of Mdm2. When indicated, proteasome inhibitors were used to block this process. Reactions were incubated at 37 °C during 2 h before Western blot analysis with anti-p53 (DO.1) antibody and anti-Beta3 subunit of the proteasome. (B) Degradation of WTp53 and OD mutants occurs in the absence of Mdm2. Similar conditions as in panel (A) were used to test the implication of Mdm2 in this reaction (− and +). Western blot analysis with anti-p53 (DO.1) was done. Rnx-Bfr.: reaction buffer, C) ATP conditions 26S but not 20S proteasome-mediated degradation of WT p53 and OD mutants. Similar conditions as in panel (A) were used to test the implication of ATP in this reaction (− and +). Western blot analysis with anti-p53 (DO.1) was done. ATP, ATP-regenerating system, Apyrase; −: no ATP, no ATP-regenerating system, no Apyrase, +: ATP, ATP-regenerating system, no Apyrase). Western blot analysis with anti-p53 (DO.1) was realized.](image-url)
case of OD mutants, 26S-dependent degradation might involve the participation of additional cellular factors.

4. Discussion

The strategy used in this study allows the assessment of p53 sequence elements in the process of its Mdm2-mediated proteasomal degradation in the absence of any p53-mediated transcription. This eliminates the possibility of interference of any of the p53-dependent negative or positive regulators reported in previous studies (Rahman-Roblick et al., 2007; Horn and Vousden, 2007). The most efficient degradation is observed with the longest p53-fusions, which have, as WT p53, intact nuclear shuttling signals, oligomerization capacity and optimal polyubiquitylation (Figs. 1 and 2). A major element conditioning polyubiquitylation of p53 is the tetramerization domain (Maki, 1999). Tetramerization is also known to be required for p53 transcriptional activity, leading to p53 ubiquitylating enzymes expression (Brooks and Gu, 2006). Here we demonstrate that constructs unable to tetramerize due to the absence of the oligomerization domain or single point mutations affecting this capacity, present a deficient Mdm2-mediated ubiquitylation pattern but are still degraded by the proteasome (Figs. 2 and 6). These results contradict the actual model of ubiquitin-mediated proteasomal degradation of p53 by Mdm2 (Maki, 1999) and suggest that alternative mechanisms of degradation should exist. The fact that in our ex vivo experiments Mdm2 efficiently drives p53 monomers to proteasomal degradation even in a p53 transcriptional silent background, underlines the importance of this ubiquitin E3 in the feedback mechanism controlling p53 stability and activity.

Degradation of OD mutants by the 26S proteasomes in our in vitro experiments is poor, probably reflecting the low level of ubiquitylation of these mutants and/or the requirement of other cellular factors to mediate this process. Interestingly, the 20S-mediated degradation of OD mutants is more efficient than the one observed for WT p53 indicating that the lack of tetramerization renders these mutants more susceptible to degradation by the so-called “default pathway” (Asher et al., 2006; Asher and Shaul, 2006). However, in vitro, 20S-mediated degradation of OD mutants is independent of the action of Mdm2 and ATP suggesting that both processes are distinct. Therefore, our in vitro degradation systems and in particular the one mediated by the 26S proteasomes, might lack a cofactor or enzymatic activity contributing to p53 OD mutants degradation. All together, our results suggest that both ubiquitin-dependent and independent mechanisms might co-exist to guarantee the efficient removal of nontetramerizable transdominant negative p53 mutants that are not efficiently polyubiquitylated. If WT p53 monomers can be regulated ex vivo and in vivo in a similar way remains an interesting open question (Asher and Shaul, 2006; Asher et al., 2005).

Although the mechanism of Mdm2-mediated proteasomal degradation of oligomerization/polyubiquitylation deficient p53 mutants needs to be fully characterized, ubiquitylation on the C-terminal lysine residues mediate their proteasomal degradation (Fig. 3 and data not shown). Furthermore, a catalytically inactive form of Mdm2 cannot drive proteolysis of p53 OD mutants, clearly indicating that this process requires the ubiquitin-ligase activity of Mdm2 (data not shown). A defective Mdm2-mediated ubiquitylation pattern has also been reported for DNA binding defective p53 mutants (Lukashchuk and Vousden, 2007). These mutants and the ones used in this study have in common that they are transcriptionally silent. DNA binding defective mutants require the ubiquitin E3 CHIP to compensate or mediate this ubiquitylation deficiency (Lukashchuk and Vousden, 2007). Although our investigations do not allow us to conclude that multiple mono-ubiquitylation is a signal directly recognized by the proteasome for degradation, this type of modification might well contribute to the proteolysis of p53 mutants with the collaboration of alternative enzymes such as CHIP (Lukashchuk and Vousden, 2007) or promote nuclear shuttling to favour degradation. Understanding how p53 dominant negative phenotypes are established will be crucial to develop new treatments.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.biocel.2010.01.010.

References


