A split-ubiquitin-based assay detects the influence of mutations on the conformational stability of the p53 DNA binding domain in vivo

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Abstract Many mutations in the human tumor suppressor p53 affect the function of the protein by destabilizing the structure of its DNA binding domain. To monitor the effects of those mutations in vivo the stability of the DNA binding domain of p53 and some of its mutants was investigated with the split-ubiquitin (split-Ub) method. The split-Ub-derived in vivo data on the relative stability of the mutants roughly correlate with the quantitative data from in vitro denaturation experiments as reported in the literature. A variation of this assay allows visualizing the difference in stability between the wild-type p53 core and the mutant $p53_{V143A}$ by a simple growth assay.

Key words: Protein stability; Protein folding; p53; Cancer; Split-ubiquitin

1. Introduction

The protein p53 is a transcriptional activator that controls tumor suppression and apoptosis in higher eukaryotes [1]. Its role in tumor suppression is highlighted by the fact that approximately 50% of all human cancers display a mutation in the protein p53. Most of those mutations fall into the central DNA binding domain and can be roughly divided into mutations that directly affect the DNA binding properties and mutations that destabilize or distort the structure of the protein and thereby affect the DNA binding properties of the protein more indirectly. Assays that can distinguish between those two classes are therefore important in developing and monitoring the action of new chemicals that aim to stabilize p53 and its mutants [2]. Measuring the stability of p53 involves either purifying the core domain and recording its urea-induced denaturation curve or the use of conformation-sensitive antibodies [3,4]. Whereas the former technique requires purified proteins and provides very accurate and quantitative data, the latter technique also works in extracts but does not allow a quantification of the measured effects. However, both techniques ignore the influence of cellular factors on the folding or the stability of the protein. Since p53 and some of its mutants were reported to associate with heat shock proteins to different extents both approaches might be of limited use for evaluating the structural state of p53 in living cells [5,6]. The same argument would also favor cell-based over pure in vitro assays to monitor the influence of candidate compounds on the stability of the p53 core domain. To explore alternative ways to measure the stability of p53 and its mutants in living cells I made use of a modification of the split-ubiquitin system (split-Ub).

The split-Ub method is based on the ability of N_{ub} and C_{ub}, the N- and C-terminal halves of Ub, to assemble into a quasinative Ub. Ub-specific proteases recognize the reconstituted Ub, though not its halves, and cleave the Ub moiety off a reporter protein which has been linked to the C-terminus of C_{ub}. The release of the reporter serves as readout indicating the reconstitution of Ub. The assay is designed in a way that prevents efficient association of N_{ub} and C_{ub} by themselves, but permits such association if the two Ub halves are separately linked to proteins that interact in vivo [7]. Attaching N_{ub} and C_{ub} to the N- and C-terminus of the same polypeptide makes possible the measurement of intramolecular N_{ub} and C_{ub} reassociation by quantifying the ratio of cleaved to uncleaved fusion protein possible. This ratio is defined both by the affinity of N_{ub} to C_{ub}, as well as by the nature of the polypeptide separating N_{ub} from C_{ub}. For example, if the protein adopts a folded conformation where the N- and the C-terminus are in close proximity, the intramolecular association of N_{ub} and C_{ub} and the subsequent cleavage is favored compared to an unfolded conformation. The assay was used to monitor the conformational change of the γ-subunit of the trimeric G-protein upon binding its β-subunit and to follow the effect of a mutation onto the conformation of the N-terminal domain of Sec62p [8,9]. Here I developed the technique into an assay that can distinguish between mutants of p53 that disturb the structure of the protein from those that directly interfere with its DNA binding activity.

2. Materials and methods

2.1. Construction of fusion proteins

Fragments encoding the open reading frame (ORF) of p53, p53Δ, V143A or the mutant [S135Y; S240I] were obtained by PCR using plasmids encoding the full-length ORFs as a template and an oligonucleotide primer complementary to the 5′- and 3′-ends of the core domain respectively. All 5′-primers contained an additional *Bam*HI site and all 3′-primers an additional *Sal*I site to allow for the in-frame fusion with the N_{ub} and C_{ub} moieties. The region connecting the sequence of N_{ub} to the sequence of the core domain of p53 reads: GGG ATC CCT GGG GAT ACC. The *Bam*HI site is underlined and the

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Abbreviations: Ub, ubiquitin; N_{ub} and C_{ub}, N- and C-terminal half of Ub; ORF, open reading frame; Dha, dihydrofolate reductase extended by the hemagglutinin epitope

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last codon of the N_{ub} and the first residue of $p53_C$ (T102) are in bold letters. The sequence connecting the end of $p53_C$ to the sequence of C_{ub} reads: **GGG** TCG AC**C GGT**. The *Sal*I site is underlined. The last codon of $p53_C$ (G 293) and the first codon of C_{ub} are in bold letters. The obtained PCR fragments were cut with *Bam*HI and *Sal*I and introduced into the correspondingly cut P_{CUP1}-N_{ub}-C_{ub}-Dha (dihydrofolate reductase extended by the hemagglutinin epitope) cassette on a pRS314 vector. All other mutations were introduced by site-directed mutagenesis using the Quick-change protocol from Stratagene (Amsterdam, The Netherlands).

The oligonucleotides used for the mutagenesis read: R175H forward: ACGGAGGTTGTGAGGCACTGCCCCCACCATGAG; R175H reverse: CTCATGGTGGGGGCAGTGCCTCACAACCTCCGT; C242S forward: CATGTGTAACAGTTCCTCCATGGGCGGCATGAACC; C242S reverse: GGTTCATGCCGCCCATGGAGGAACTGTTACACATG; R248Q forward: ATGGGCGGCATGAACCAGAGGCCCATCCTCACC; R248Q reverse: GGTGAGGATGGGCCTCTGGTTCATGCCGCCCAT; R273H forward: CGGAACAGCTTTGAGGTGCATGTTTGTGCCTGTCCTGGG; R273H reverse: CCCAGGACAGGCACAAACATGCACCTCAAAGCTGTTCCG; N268D forward: AATCTACTGGGACGGGACAGCTTTGAGGTGCGT; N268D reverse: ACGCACCTCAAAGCTGTCCCGTCCCAGTAGATT.

The corresponding C_{ub}-RURA3 constructs were obtained by inserting the *Eag*I–*Sal*I cut P_{CUP1}-N_{ub} ORF in front of the C_{ub}-RURA3 module on a pRS313 vector.

DNA sequences were determined by the MPIZ DNA facility on PE Biosystems ABI Prism 377 and 3700 sequencers using BigDye-terminator chemistry. Oligonucleotides were purchased from Metabion (Martinsried, Germany).

2.2. Immunoblotting

Saccharomyces cerevisiae cells expressing the different N_{ub}-$p53_C$-C_{ub}-Dha were grown at 30°C in 10 ml of SD medium to an optical density at 600 nm (OD_{600}) of ~0.8 and supplemented with 100 μM $CuSO_4$ and when indicated shifted to 37°C 1 h prior to cell extraction. Cell extraction for immunoblotting was performed essentially as previously described [10]. Proteins were fractionated by SDS–12.5% PAGE and electroblotted onto nitrocellulose membranes (Schleicher&Schuell, Dassel, Germany), using a semi-dry transfer system (Hoefer, Pharmacia Biotech Inc., San Francisco, CA, USA). Blots were incubated with a monoclonal anti-hemagglutinin (HA) antibody (Babco, Richmond, CA, USA). Bound antibody was visualized with horseradish peroxidase-coupled rabbit anti-mouse (Bio-Rad, Hercules, CA, USA), using the chemiluminescence detection system (Pierce, Rockford, IL, USA). The chemiluminescence was quantified with the aid of the lumi-imager system (Boehringer, Mannheim, Germany).

2.3. Yeast strains, growth and functionality assays

S. cerevisiae strains were JD53 (*MATα his3-Δ200 leu2-3,112 lys2-801 trp1-Δ63 ura3-52*), yeast rich (YPD) and synthetic minimal media with 2% dextrose (SD) or 2% galactose (SG) followed standard recipes.

Growth assays: *S. cerevisiae* cells were first grown at 30°C in liquid selective media containing uracil. Cells were diluted in water to an OD_{600} of 1 and 4 μl were spotted on agar plates selecting for the presence of the fusion constructs and containing 5-FOA at a concentration of 1 mg/ml. The plates were incubated at 30°C for 3.5 days.

3. Results

3.1. Monitoring the influence of mutations on the structure of p53 in vivo

The mean spatial distance between the N- and the C-terminus in a protein depends on their spatial arrangement and on the stability and rigidity of the folded structure. Mutations that change the stability or the conformation of the protein will therefore alter this distance [11]. The reassociation of N_{ub} and C_{ub} that are attached to the N- and C-terminus of the core domain of p53 ($p53_C$) should be a sensitive measure of monitoring such changes in p53 in living cells (Fig. 1). According to the extent of the N_{ub}–C_{ub} reassociation the assay should allow classifying the mutations occurring in p53 into two groups. Native p53 and those mutants that only affect the DNA binding should share very similar ratios of cleaved to uncleaved N_{ub}-$p53_C$-C_{ub} reporter whereas structurally destabilizing mutations should alter this ratio (Fig. 1).

To test this concept I first studied a mutation that most probably should unfold $p53_C$ under physiological conditions. A deletion of 28 residues between positions 115 and 143 of the full-length protein removes the L1 loop and the S2–S2′ hairpin β-sheet to yield p53Δ. Since the deleted regions contain central structural elements of $p53_C$, p53Δ should be completely unfolded [12].

The central DNA binding domains of native $p53_C$ and p53Δ (residues 102–293) were introduced between the N- and the C-terminal half of Ub (N_{vg} and C_{ub}). N_{vg} is a mutant of the wild-type N_{ub} that binds with a reduced affinity to C_{ub} [9].

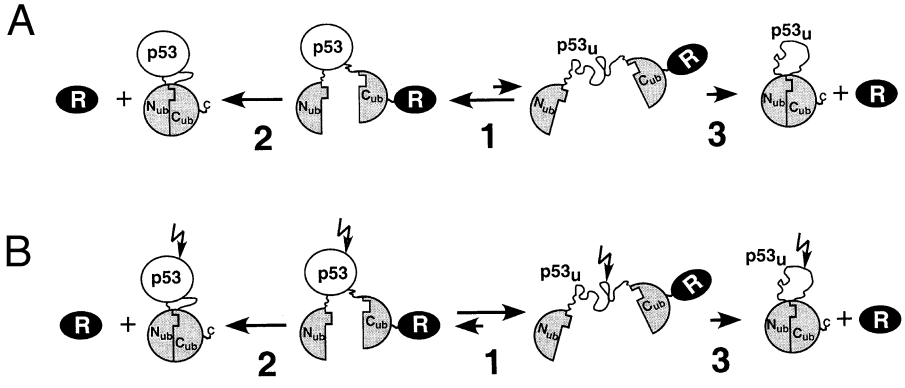

Fig. 1. Rational of the split-Ub assay. A: The majority of $p53_C$ is folded (closed circle) under physiological conditions as indicated by length of the arrows (1) and the efficiency of reassociation of the attached N_{ub} and C_{ub} is dictated by the arrangement of the N- and C-terminus in the folded structure (2). The reassociation of N_{ub} and C_{ub} is monitored by the cleavage and release of the reporter protein R. B: A mutation that destabilizes the structure of p53 will shift the equilibrium to a higher proportion of unfolded molecules as indicated by the lengths of the arrows (1). The reassociation of the attached N_{ub} and C_{ub} is now dominated by the mean distance of the N- and C-terminus in the denatured state (wavy line). Provided that the rates of N_{ub}–C_{ub} reassociation are different between the folded and the unfolded conformations as indicated by the length of the arrows (2) and (3), the influence of a mutation on the stability of $p53_C$ can be measured by the altered ratio of the cleaved to uncleaved fusion protein (here the ratio of cleaved to uncleaved p53 fusion protein is decreased by the mutation).

The C_{ub} moiety was extended with the mouse dihydrofolate reductase that contains a HA-tag at its C-terminus (Dha) to create N_{vg}-$p53_C$-C_{ub}-Dha and N_{vg}-p53Δ-C_{ub}-Dha respectively. The expression of the fusion proteins was induced in yeast cells for 1 h at 37°C or 30°C and the ratio of cleaved to uncleaved fusion protein was determined by Western blotting. The inspection of the anti-HA Western blot reveals a clear difference in cleavage between N_{vg}-$p53_C$-C_{ub}-Dha and its mutant (Fig. 2). Whereas 77% of N_{vg}-$p53_C$-C_{ub}-Dha are cleaved at 30°C, a majority of 85% of N_{vg}-p53Δ-C_{ub}-Dha remains uncleaved at this temperature. I conclude that upon unfolding of the core domain the spatial distance between the N- and the C-terminus of the polypeptide increases. To support this conclusion another mutant of p53 that should not adopt a folded structure was constructed. In this mutant a bulky tyrosine replaces the natural cysteine at position 135 (all numbers relate to the residue position in the full-length p53) and a hydrophobic leucine replaces the conserved serine at position 240 ([C135Y; S240I]). As observed for p53Δ the majority of the expressed N_{vg}-[C135Y; S240I]-C_{ub}-Dha remains uncleaved (Fig. 2A, lane 3). The very similar ratio of cleaved to uncleaved fusion protein suggests that both mutants of p53 are equally unfolded (Fig. 2A). In Fig. 2B the effect of a certain mutation onto the stability of the $p53_C$ is given as relative destabilization. This value is calculated for each mutant by subtracting the percentage of cleaved N_{vg}-$p53_{CMut}$-C_{ub}-Dha from the percentage of cleaved N_{vg}-$p53_C$-C_{ub}-Dha. By extrapolating the results of p53Δ and [C135Y; S240I] a positive value will reflect a reduced stability of the mutant p53. A value close to zero will indicate a similar stability as the wild-type p53 whereas a negative value will indicate a higher stability of the mutant. To validate this interpretation and to compare their conformational properties in vivo I measured the cleavage ratios of five further p53 mutants that are known to be associated with cancer. The urea denaturation curves of these five mutants were precisely determined and their degree of unfolding extrapolated to more physiological conditions at 37°C [13]. The mutant R273H harbors a histidine instead of an arginine in position 273. R273 protrudes into the solvent and is directly involved in DNA recognition [12]. The global stability of the mutant is only marginally decreased. As can be seen in Fig. 2A,B the cleavage of the N_{ub}-R273H-C_{ub}-Dha is nearly identical to that of N_{ub}-$p53_C$-C_{ub}-Dha at both temperatures measured. The assay therefore correctly confirms that the R273H exchange does not impair the stability of the protein.

The R248Q exchange is one of the more frequently observed mutations in p53-ssociated tumors. The arginine in positions 248 directly contacts the DNA and is fully exposed [12]. Urea denaturation experiments revealed a decrease in stability of 1.9 kcal/mol compared to the wild-type protein. This decrease translates into an estimated 14% of unfolded molecules at 37°C [13]. In accordance with this relatively small percentage of unfolded molecules I observe no significant difference between the wild-type protein and R248Q in the cleavage of the respective fusion proteins at 30°C. However, at 37°C the mutant yields in average a higher fraction of cleaved Dha indicating a slight stabilization relative to wild-type. This interpretation contradicts the reported in vitro stability measurements. Despite this discrepancy the assay confirms that the R248Q mutation does not exert its effect via a global destabilization of $p53_C$.

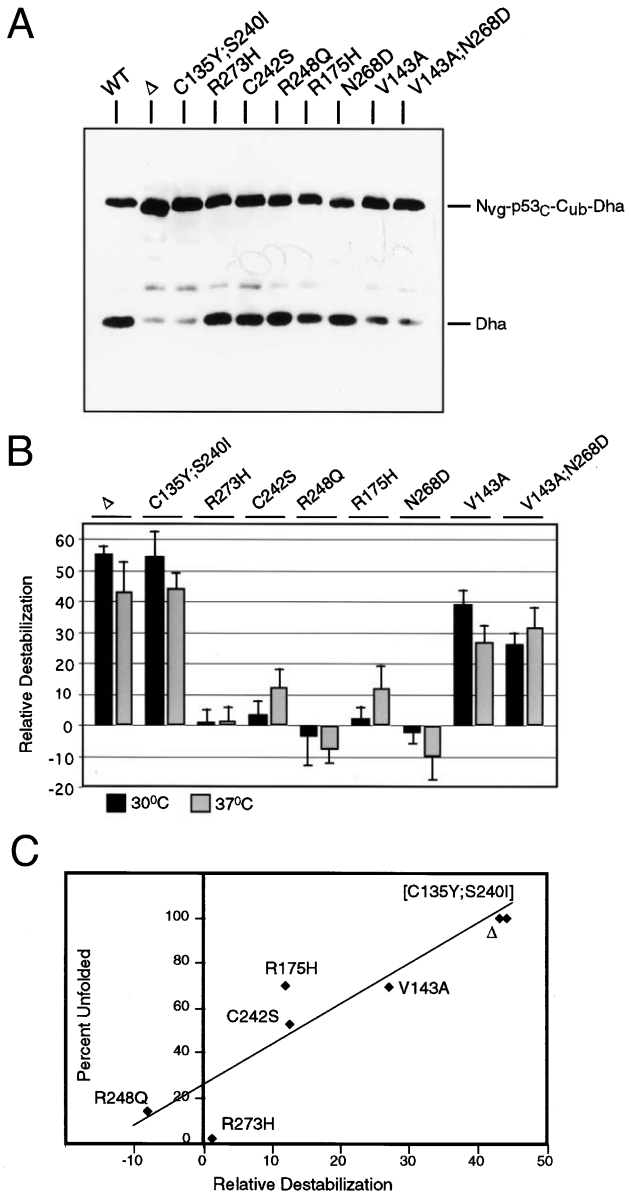

Fig. 2. Measuring the effects of cancer-causing mutations on the stability of $p53_C$ in vivo. A: N_{vg}-$p53_C$-C_{ub}-Dha and different $p53_C$ mutants were expressed in yeast at 37°C and the cleaved and uncleaved fractions of the fusion proteins were probed with the anti-HA antibody on a nitrocellulose blot after cell extraction and SDS–PAGE. The amount of cleaved and uncleaved fusion protein was measured by quantitative chemoluminescence. The amino acid exchanges of the different $p53_C$ mutants are indicated at the top of the blot. A stretch of 28 amino acids of $p53_C$ is missing in Δ. B: Summary of the effect of different mutations on the stability of $p53_C$. For each experiment the percentage of cleaved N_{vg}-$p53_C$-C_{ub}-Dha was calculated. This value was subtracted from the percentage of cleaved N_{vg}-$p53_C$-C_{ub}-Dha of the different mutants to give the value of relative destabilization. A positive value, indicating a higher fraction of uncleaved fusion protein, indicates destabilization of the mutant protein whereas a negative value indicating a higher fraction of cleaved Dha indicates stabilization of $p53_C$. Experiments were performed at 30°C (black bars) and 37°C (gray bars). Five independent experiments at each temperature are shown except for C242S (four experiments at 37°C) and R175H (six experiments at 37°C). C: Correlation between the split-Ub-derived values of relative destabilization and the calculated fraction of unfolded molecules at 37°C according to Bullock et al. [12].

Other known mutants primarily disturb the structure of p53 or reduce the stability of the protein. P53 binds zinc and this binding stabilizes the core. I tested two known cancer-causing mutants of $p53_C$ that both destroy the ability of p53 to bind zinc [12]. As a consequence both mutants are less stable than the wild-type protein by 3.5 (R175H) and 3.1 (C242S) kcal/mol respectively [13]. At 30°C the two mutants and the native $p53_C$ give nearly identical cleavage ratios whereas at 37°C R175H and C242S can be distinguished from the wild-type core by an increase in the amount of uncleaved fusion protein (Fig. 2). In accordance with the literature the values of a relative destabilization of +12 indicate that both mutants display a higher proportion of unfolded molecules at 37°C.

The V143A exchange affects the stability of the core by 3.5 kcal/mol [13]. This destabilization is clearly reflected in the split-Ub assay by the relatively small fraction of cleaved N_{vg}-V143A-C_{ub}-Dha at 30°C and at 37°C resulting in a value of relative destabilization of +42 and +27 respectively (Fig. 2). A second side mutation in position 268 (N268D) restores some of the functions of V143A at 30°C [14]. I constructed N_{vg}-[V143A; N268D]-C_{ub}-Dha to test if the in vivo assay reveals a stabilization of the conformation of V143A by the additional N268D exchange. At 30°C N_{vg}-[V143A; N268D]-C_{ub}-Dha has a higher fraction of cleaved Dha than the single mutant N_{vg}-V143A-C_{ub}-Dha. This decrease in relative destabilization clearly indicates a higher stability of the double mutant [V143A; N268D] compared to the single mutant V143A [15]. However, the difference between the two mutants disappears when the values of relative destabilization of N_{vg}-[V143A; N268D]-C_{ub}-Dha and N_{vg}-V143A-C_{ub}-Dha are compared at 37°C (Fig. 2).

To measure the influence of the single N268D exchange on the stability of the wild-type core of p53 I performed the split-Ub assay of N_{vg}-N268D-C_{ub}-Dha. At 30°C the cleavage rate of N_{vg}-N268D-C_{ub}-Dha is nearly identical to that of the wild-type core. At 37°C the value of relative destabilization of −10 indicates an increase in the conformational stability of N268D. One should note that the assay would detect an increase in stability of a mutant only if a significant fraction of the wild-type $p53_C$ already unfolds at 37°C in the N_{ub}-$p53_C$-C_{ub}-Dha fusion protein. This might explain why the effect of the N268D mutation is only seen at 37°C.

In Fig. 2C I plotted the values of relative destabilization for the different p53 mutations at 37°C as measured and calculated by the split-Ub technique against the proportion of unfolded molecules as calculated by Bullock et al. [13]. I assumed that a value of relative destabilization of +45 (p53Δ and [C135Y; S240I]) reflects the completely unfolded population of molecules. I further assumed that the split-Ub-derived value for destabilization linearly correlates with the proportion of unfolded molecules of the mutants as calculated by Bullock et al. on the basis of their equilibrium measurements of the same mutants [13]. In agreement with these two assumptions the two sets of data correlate (Fig. 2C). The graph intercepts the *y*-axis at a value of 20% of unfolded molecules as if $p53_C$ in the fusion protein is already partially unfolded under physiological conditions.

3.2. A cell-based growth assay to monitor the structure of the p53 core

Compounds that stabilize the conformation of p53 are discussed as possible drugs for the treatment of cancers that are associated with mutations in p53 [2,16]. It is therefore desirable to develop cell-based assays that allow testing for such compounds [17]. Towards this end I exchanged the Dha reporter against the RUra3p reporter to create N_{ub}-$p53_C$-C_{ub}-RUra3p. Since cleavage exposes an arginine at the N-terminus of RUra3p the freed reporter is targeted for rapid destruction via the N-end rule pathway [18,19]. Cells expressing fusion proteins that are completely cleaved do not grow on Sd-ura but will grow on medium containing the substance 5-FOA since Ura3p that otherwise would convert 5-FOA to the toxic 5-F-uracil is rapidly degraded. Mutations in p53 that substantially increase the amount of uncleaved fusion protein will increase the Ura3p activity and will cause the cells to die on medium containing 5-FOA. To test the feasibility of this approach I focused on the V143A mutant since this mutation exclusively affects the stability of p53 to an extent that can be easily measured by the split-Ub assay at 37°C as well as 30°C. To be able to detect an alteration in the growth of the cells requires that the majority of the RUra3p have to be cleaved off from the C_{ub} and degraded. Since N_{vg}-$p53_C$-C_{ub}-Dha leaves a significant fraction of the fusion protein uncleaved different N_{ub} mutants with a higher affinity to C_{ub} than N_{vg} were tested [8,9]. Cells expressing either wild-type $p53_C$ or V143A in different N_{ix}-$p53_C$-C_{ub}-RUra3 fusions were spotted on medium containing 5-FOA. As can be seen in Fig. 3C, the cells containing the N_{ia} and to a lesser extent the cells containing the N_{ig} distinguish between wild-type $p53_C$ and the mutant V143A. The cells containing the wild-type $p53_C$ grow whereas the cells containing the mutant V143A die. A similar experiment using the same N_{ub} mutants but the Dha as a reporter confirmed the growth assay by a cell extraction and Western blotting experiment. All constructs containing the wild-type protein gave a higher proportion of cleaved fusion protein than the V143A mutant independent of the N_{ub} that was used for the assay (Fig. 3). Importantly, the difference in growth between the cells containing the N_{ia}-$p53_C$-C_{ub}-RUra3p constructs is not due to a difference in the steady state expression of the fusion as the sums of cleaved and uncleaved N_{ia}-$p53_C$-C_{ub}-Dha fusions of the wild-type and the V143A mutant are very similar (Fig. 3A).

4. Discussion

To monitor the molecular defects of some of the cancer-causing mutants of p53 in a cellular environment, I used the split-Ub technique to measure the mutation-induced alteration in the stability of the DNA binding domain of p53. The comparison with the published thermodynamic data allowed evaluating the predictive and quantitative value of this technique. The application of the RUra3p reporter made it possible to monitor the reduced stability of the V143A mutant by a simple growth assay of the yeast expressing the respective fusion proteins.

The application of the split-Ub assay on the analysis of the mutations in p53 rested on two prerequisites. First, the unfolding of $p53_C$ had to alter the cleavage of the corresponding N_{ub}-$p53_C$-C_{ub} fusion protein. Secondly, the amount of unfolding must correspond to the change in the ratio of cleaved to uncleaved fusion protein. The mutants p53Δ and [C135Y; S240I] served as a reference for the unfolded state of $p53_C$. Both mutants displayed a significant decrease in the ratio of cleaved to uncleaved fusion protein. A correlation between the

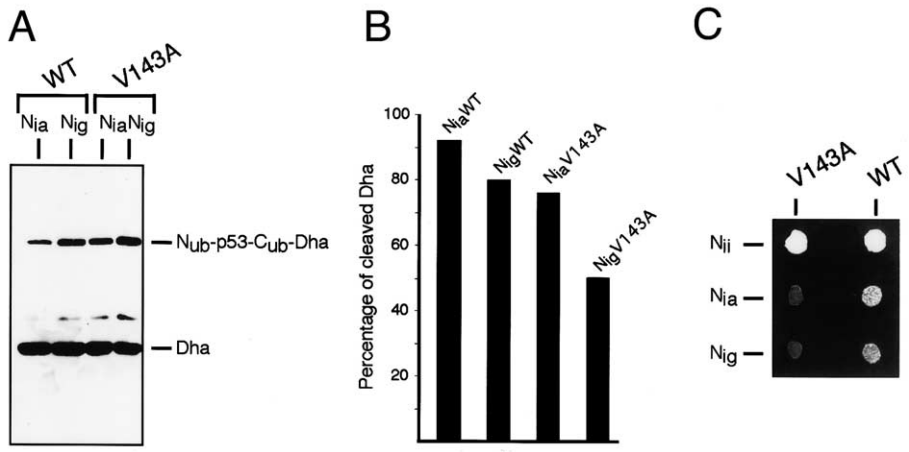

Fig. 3. Monitoring the conformational stability of $p53_C$ and the V143A mutant of $p53_C$ by a simple growth assay. A: Western blot of protein extracts of yeast cells expressing N_{ub}-$p53_C$-C_{ub}-Dha and N_{ub}-V143A-C_{ub}-Dha containing the N_{ub}-mutants N_{ia} and N_{ig}. The quantification of this experiment is shown in (B). Bars indicate the percentage of cleaved Dha. C: Growth assay of yeast cells expressing the corresponding N_{ii}-, N_{ia}-, N_{ig}-$p53_C$-C_{ub}-RUra3p fusion proteins on media containing 5-FOA at 30°C. The N_{ia} fusion protein allows most clearly to distinguish between the cells expressing the wild-type $p53_C$ (growth) and the cells expressing the mutant V143A (non-growth).

fraction of unfolded polypeptides and the reduction in cleavage (expressed in relative destabilization) of the corresponding N_{ub}-$p53_C$-C_{ub}-Dha of the other tested mutants was therefore postulated. Fig. 2D confirms this assumption by showing a correlation between the two data sets. However, two of the tested single mutants stand out in deviating from the expectation. R248Q is destabilized by 1.9 kcal/mol at 37°C yet has a smaller value of relative destabilization in the assay than the wild-type $p53_C$ (Fig. 2A,B). R175H leads to the predicted reduction in cleavage of the fusion protein. However, the amount of the relative destabilization is much smaller than the value for V134A, although both mutants display very similar denaturation curves in the in vitro experiment [13]. A realization of the inherent limitations of the split-Ub technique might help to understand these discrepancies. The assay relies on the change of a single parameter, which is the time-averaged distance between the N- and the C-terminus of the p53 core. This parameter might not only change upon global unfolding but might also respond to local alterations in the structure. R248Q shows only a moderate destabilization of 1.9 kcal/mol and a majority of 86% was calculated to be folded at 37°C. However, nuclear magnetic resonance spectroscopy revealed local structural changes in the fold of this mutant which might cause the observed increase in cleavage of the corresponding N_{vg}-R248Q-C_{ub}-Dha [20]. Furthermore, the assumption that the cleavage ratio of N_{vg}-p53Δ-C_{ub}-Dha will serve as the universal reference for the denatured state might not equally apply to all mutants. Recent studies showed that denatured proteins could preserve a specific structural signature [21–25]. Depending on the nature of the mutation the conformation(s) of the denatured states might differ between the p53 mutants leading to distinct cleavage ratios of the unfolded proteins in the corresponding split-Ub fusions.

Despite these limitations the technique allowed to correctly classify all the p53 mutations tested. R248Q and R273H are DNA contact mutations and were shown to decrease the stability of the core only very moderately. Both mutations displayed compared to wild-type p53 either no change or only a modest increase in cleavage of the corresponding N_{vg}-$p53_C$-C_{ub}-Dha (Fig. 2). The N268D mutation does not affect the functions of p53 but instead leads to an increase in stability by −1.22 kcal/mol [15]. This increase is reflected by the largest ratio of cleaved to uncleaved fusion protein in the split-Ub assay (Fig. 2). In contrast C242S, R175H and V143A are structural mutants leading to a compromised stability of $p53_C$. Accordingly all three mutants decrease the cleavage of N_{vg}-p53-C_{ub}-Dha at 37°C leading to the predicted positive value of relative destabilization (Fig. 2B).

A closer inspection of the relative destabilization values of p53Δ, [C135Y; S240I] and V143A reveals a seeming inconsistency. All three proteins appear more stable at 37°C than at 30°C (Fig. 2B). This interpretation is misleading since the ratio of cleaved to uncleaved N_{vg}-p53-C_{ub}-Dha decreases from 77% to 52% as the temperature rises from 30°C to 37°C whereas the ratio is naturally much less reduced for those mutants that are already significantly unfolded at 30°C. The decrease in relative destabilization of those three mutants therefore reflects the increase in the population of unfolded $p53_C$ at 37°C rather than a stabilization of the mutants.

Using the RUra3p reporter in the split-Ub assay I could translate the difference between wild-type core and V143A into a read out that is based on growth of the transformed cell on 5-FOA. Compounds that restore the stability of V143A and penetrate the cell wall and the membrane of the yeast should be able to restore the growth of the cells on this medium. Based on the appearance of a fluorescent signal the split-Ub compatible reporter RGFP might allow a similar assay that will make possible the screening for p53 stabilizing compounds in mammalian cells [26].

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