Biochemical characterization of different conformational states of the Sf9 cell-purified p53His175 mutant protein

Pascale A. Cohen[a,b,*], Ted R. Hupp[c], David P. Lane[a], Dion A. Daniels[a]

[a] *CRC Laboratories, Department of Biochemistry, Medical Sciences Institute, Dundee University, Dundee DD1 4HN, UK*
[b] *CNRS UMR 9921, UFR Pharmacie, 15 Av. Flahault, 34060 Montpellier Cedex 2, France*
[c] *Department of Molecular and Cellular Pathology, Ninewells Hospital and Medical School, Dundee University, Dundee DD1 9SY, UK*

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Abstract In this study, we expressed and purified the p53 mutant encoded by the His175 allele (p53His175) in a baculovirus expression system in order to study the folding and the DNA binding activity of the protein. A two-site ELISA revealed that purified p53His175 protein preferentially displayed a PAb1620 conformation, which appeared to be not sufficient to interact specifically with DNA. The cryptic DNA binding activity of this mutant was then investigated by electrophoretic mobility shift assay in the presence of anti-p53 antibodies, and shown to be refractory to significant activation by PAb421 (a potent allosteric activator of wild-type p53's DNA binding activity). Nevertheless, p53His175 DNA binding was regulated by antibodies targeting the N-terminal region of the protein. Furthermore, while the protein preferentially displayed a PAb1620 conformation, our data suggested the existence of an equilibrium between at least two folding states of the protein (PAb1620 and PAb240 conformations). A model rationalizing the conformation, antibody-interacting ability and DNA binding regulation potential of p53His175 is presented.

Key words: p53; Mutant; Conformation; DNA binding; His175 mutation

1. Introduction

In human tumors, most of the mutations identified in the tumor suppressor p53 protein are localized within the central DNA binding domain. Strikingly, six residues within this region have been found to be preferentially targeted for mutation (Arg175, Gly245, Arg248, Arg249, Arg273 and Arg282) and correspond to 40% of all p53 mutations [1]. Analysis of the co-crystal of the central DNA binding domain of p53 and its cognate binding sequence has given great insight into how mutations at these hotspots lead to abrogation of p53 sequence-specific DNA binding [2]. The existence of these mutational hotspots emphasizes the importance of the conformation and the sequence-specific DNA binding in the normal biological function of p53.

*Corresponding author. Fax: (33) 4-67 54 86 10.
E-mail: p.cohen@valdorel.fnclcc.fr

Abbreviations: p53His175, p53 mutant protein encoded by the His175 allele; aa, amino acid; scFv, single chain variable fragment; ELISA, enzyme linked immunosorbent assay; EMSA, electrophoretic mobility shift assay; mAb, monoclonal antibody; Mdm2, protein encoded by the murine double minute gene 2; wt p53, wild-type p53

Anti-p53 monoclonal antibodies (mAbs) which interact with the conformationally-flexible DNA binding domain of p53 have been used to elucidate the global affects of missense mutations on the structure of p53's core, and to permit the definitions of wild-type (wt) and mutant conformations of p53. PAb240 and DO-12 antibodies are defined as being able to detect the mutant conformation of p53 [3–6]. These two antibodies target respectively two different cryptic linear epitopes within the conformationally flexible central core of the molecule. These epitopes are only exposed upon denaturation of the wild-type p53 (wt p53) protein, and are accessible in a variable fraction of point-mutant p53 molecules [4–6]. PAb1620 displays a reciprocal behavior, as it interacts preferentially with native wt p53, fails to bind to denatured protein, and precipitates none or a fraction of mutant p53 expressed in different cell lines [4,5,7–9]. However, the border between these mutant and wild-type conformations is not clearly defined. Indeed, during formation of hetero-oligomers with mutant proteins [10], during cell division [11,12], or when bound to DNA [13], wt p53 can adopt the mutant conformation. In contrast, in cell lines expressing defined p53 point mutations, it appeared that PAb1620 was able to precipitate a fraction of p53 mutant proteins, suggesting that the total pool of the p53 mutant proteins encoded by the same allele can contain complementary fractions of both PAb1620-positive/PAb240-negative molecules and PAb1620-negative/PAb240-positive molecules. The proportion of these two forms was found to vary greatly from mutation to mutation, and from cell line to cell line [4–6,9]. These observations taken together suggested the ease with which p53 proteins seem to be able to adopt alternate conformations. The association of these alternate conformations of p53 with different biological activities led to the suggestion that such conformational changes may be important for the normal regulation of p53 function.

In order to decipher the meaning of p53 mutation for tumor suppression, a number of studies have characterized the DNA binding and transactivation activities of tumor-derived p53 mutants [14–16]. However, the majority of this work has focused on the analysis of p53 protein derived from crude cell lysates rather than biochemically pure entities. In this study, we expressed and purified the p53 mutant protein encoded by the His175 allele (p53His175) in a baculovirus expression system. The conformation and the DNA binding activity of this protein have been investigated by a two-site enzyme linked immunosorbent assay (ELISA) and electrophoretic mobility shift assay (EMSA), using a set of well-characterized anti-p53 antibodies. These findings provide an opportunity for us

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to understand the regulation of the conformation and the DNA binding properties of the most highly represented and oncogenic p53 mutants in human neoplasia.

2. Materials and methods

2.1. Antibodies

Anti-p53 mAbs DO-2, DO-1, PAb1801, PAb241, PAb243, PAb421, PAb122, PAb1620, PAb240, and DO-12 [3,4,17–21] were purified using protein A-Sepharose. CM1, a rabbit polyclonal antiserum, was raised against recombinant human p53 [22]. DO-2 interacts with an epitope on p53 comprising the amino acids (aa) 10–16 [23]; DO-1 recognizes aa 20–25 [23]; PAb1801 targets aa 46–55 [18]; 200.47 binds aa 81–95 [24]; PAb241 and PAb243 [4] both recognize an epitope corresponding to aa 296–305 on human p53 [22]; PAb421 [19] and PAb122 antibodies [20] bind to a similar region on human p53 comprising aa 371–380 [23]; PAb240 recognizes a linear epitope within the central core of p53, aa 212–217 [23]; DO-12 binds to another cryptic linear epitope within p53's core corresponding to aa 256–270 [21]. Production and characterization of the DO-1 single chain variable fragment (scFv) is described elsewhere [25].

2.2. ELISA of wt p53 and p53His175 proteins

Human wt p53 and p53His175 were purified from recombinant baculovirus infected *Spodoptera frugiperda* (Sf9) insect cells by previously described methods [26]. Mabs used for capture were first adsorbed onto a 96-well PVC assay plate (Falcon) overnight at 4°C (50 μl, 10 μg/ml in a phosphate-buffered saline solution (PBS)). After blocking in the presence of 5% (w/v) low fat milk powder and 0.1% (v/v) Tween 20 (PBSMT), purified p53His175 or wt p53 were incubated for 1 h at 4°C (3 μg/ml in a volume of 50 μl in PBSMT). Control wells contained only the capture monoclonals. After washing, the rabbit anti-p53 serum CM1 was incubated for 2 h at 4°C (50 μl, 1/2000 in PBSMT). Subsequent to a final wash, p53-antibody complexes were detected by a swine anti-rabbit horseradish peroxidase-conjugated serum (Dako, Glostrup, Denmark). A Dynatech plate reader was used to measure absorbance at 450 nm.

2.3. Transient transfection

The p53 null human lung carcinoma H1299 cells were maintained in Dulbecco's modified Eagle medium and supplemented with 10% fetal calf serum at 37°C. Cells were seeded 24 h before transfection to 70–80% confluence. Calcium phosphate mediated transfections were performed as previously described [27]. Two micrograms of a plasmid encoding the human p53His175 protein (pCMV-Neo-p53His175) was transfected into a sub-confluent 10 cm dish culture. Control transfections were performed with 2 μg of the pCMV-Neo-Bam vector [28]. Forty-eight hours after transfection, cell lysates were prepared [29] and tested in the two-site ELISA where PAb1620 or PAb240 were used as the capture antibodies and CM1 was used for detection of p53His175.

2.4. Electrophoretic mobility shift assay

Binding conditions using the specific p53 consensus sequence (PG) were as previously described [30]. Standard reactions were assembled at 4°C with 190 nM of Sf9-produced p53His175 mutant in presence of 330 nM of an anti-p53 mAb. The anti-Mdm2 (protein encoded by the murine double minute gene 2) mAb 4B2 [31] was used as negative control (330 nM). Characterization of the PAb1620 and PAb240 activities of p53His175 (190 nM) in EMSA experiments was performed by assembling reaction mixtures containing 460 nM of DO-1 with various amounts of either PAb1620 or PAb240, details are given in the figure legends. Incubations were performed for 40 min on ice before the DNA binding assay. Control experiments were carried out by substituting p53His175 mutant with wt human p53 (10 nM).

3. Results

3.1. Purified p53His175 reveals a 'wild-type conformation' in ELISA

A panel of mAbs that interact with an array of epitopes between the N- and C-terminus of p53 was used to decipher subtle differences in epitope availability between wt p53 and p53His175 in ELISA. The results (Fig. 1) revealed that each of the targeted epitopes were equally available for binding across both p53His175 and the wild-type protein. The data suggested that point mutation at this site did not alter the structure of the mutant p53 sufficiently to make a series of linear epitope determinants across the molecule differently accessible to wt p53.

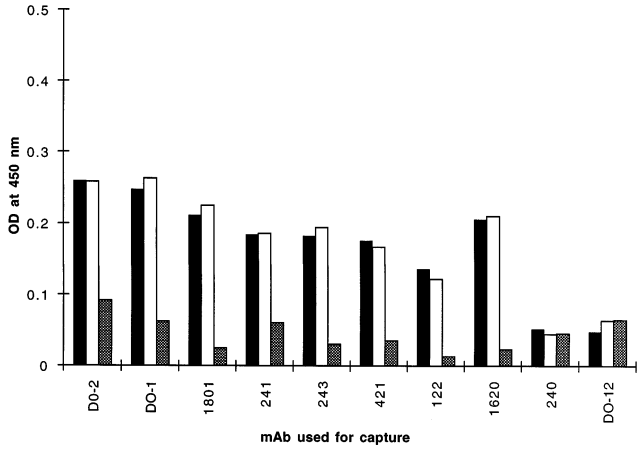

Fig. 1. Epitope analysis of Sf9-derived p53His175 and wt p53. The anti-p53 mAbs were independently immobilized in ELISA wells and then incubated with either p53His175 (50 μl, 3 μg/ml) black column, or, wt p53 (50 μl, 3 μg/ml) white column. Binding was compared with a buffer control (gray column). Detection of p53-antibody complexes was performed with the polyclonal serum CM1.

The p53His175 was predicted to be a denaturing or unfolding mutation [32], and previous analysis of p53His175 proteins in immunoprecipitation assays from tumor cell lysates revealed that the majority of the protein adopted a PAb1620-negative/PAb240-positive/DO-12-positive conformation [21,33]. When tested in ELISA, the majority of the p53His175 protein was strikingly seen to mainly adopt a PAb1620 rather than a PAb240 or a DO-12 conformation, similarly to the wt protein (Fig. 1). As the conformation of the p53His175 might be a consequence of a peculiarity of the Sf9 cell type, we analyzed the conformational status of p53His175 expressed in null human lung carcinoma H1299 cells. The results (Fig. 2) showed that H1299-derived p53His175 contained in equal proportion a pool of PAb1620-positive proteins as well as a pool of PAb240-positive proteins. In contrast, the majority of Sf9-derived p53His175 mainly adopted, as said above, a PAb1620 conformation. Taken together, these data suggested the point mutation Arg→His at the codon 175 does not always significantly unfold the structure as defined by classic PAb240-positive/PAb1620-negative reactivity, as the p53His175 protein was able to adopt the PAb1620 conformation in two independent cell lines.

3.2. N-terminal regulation of the cryptic DNA binding activity of the p53His175 mutant

The 'wild-type' PAb1620 conformation of p53 has been previously described as being a pre-requisite for sequence-specific DNA binding [34]. When an EMSA was performed on p53His175 purified fractions, the results demonstrated this point mutant possessed a very weak and unstable innate abil-

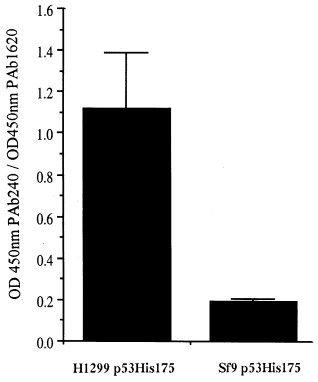

Fig. 2. Quantitative analysis of the PAb1620/PAb240 conformational status of p53His175 in Sf9 and H1299 cell lines. The Sf9- and H1299-expressed p53His175 proteins were tested in a two-site ELISA using either PAb1620 or PAb240 as capture mAbs. Results were expressed as the ratio of the PAb240-capture signal and the PAb1620-capture signal ($OD_{450\ nm}$PAb240/$OD_{450\ nm}$PAb1620 ratio). These data represent the mean of three independent experiments, the error bars represent the standard error of the mean.

ity to interact with DNA, as observed by Friedlander and colleagues [35] (Fig. 3, lane 1). These findings suggest that, at least for this particular mutant, the PAb1620 conformation cannot be considered as a sufficient condition for DNA binding.

In order to explore the regulation of the cryptic DNA binding function of p53His175, we tested a panel of anti-p53 mAbs in EMSA experiments. The C-terminal specific mAbs, PAb421 and PAb122, have previously been shown to greatly activate the latent DNA binding activity of wt p53 [13,30] and the point mutants p53Lys285 and p53His273 [36]. As illustrated in Fig. 3, neither PAb421 (lane 8), nor PAb122 (lane 9) demonstrated any ability to activate the DNA binding activity of p53His175. Similarly, no visible effect was observed in the presence of Pab241 (lane 6), Pab243 (lane 7), PAb1620 (lane 11), PAb240 (lane 12), DO-12 (lane 13), 200.47 (lane 14), or an irrelevant anti-Mdm2 antibody (lane 10). On the contrary, the N-terminal specific mAbs, DO-2, DO-1, and PAb1801, were demonstrated to greatly enhance p53His175's DNA binding activity (lanes 2, 3 and 5, respectively). As the p53 tetrameric state is essential for the DNA binding activity of wt p53 [26], we sought to determine what effect bivalence and size played upon the activating potential of N-terminal specific mAb interaction with p53His175 by performing the assay in the presence of the DO-1 scFv [25]. As illustrated in Fig. 3, lane 4, the DO-1 scFv was also able to enhance the DNA binding activity of p53His175.

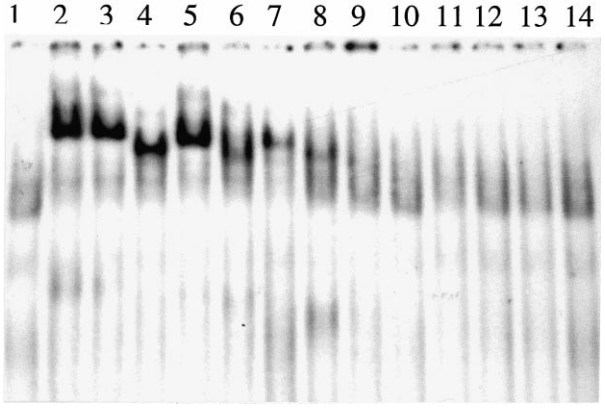

Fig. 3. DNA binding activity of the p53His175 protein. EMSA demonstrating the interaction of latent p53His175 (190 nM) with DNA after incubation (30 min at 4°C) alone (lane 1), or in presence of a panel of anti-p53 mAbs (300 nM): DO-2 (lane 2), DO-1 (lane 3), PAb1801 (lane 5), PAb241 (lane 6), PAb243 (lane 7), PAb421 (lane 8), PAb122 (lane 9), PAb1620 (lane 11), PAb240 (lane 12), DO-12 (lane 13) and 200.47 (lane 14). The anti-Mdm2 mAb 4B2 was used as a negative control (lane 10). DO-1 scFv (500 nM) was tested on lane 4.

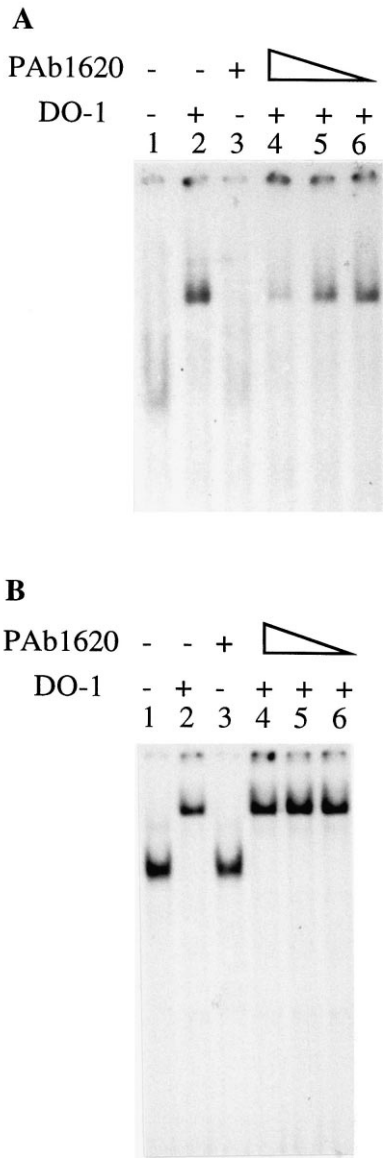

Fig. 4. The DNA binding activity of DO-1-p53His175 is inhibited by PAb1620 interaction. EMSA demonstrating inhibition of DNA binding of DO-1-activated p53His175 by PAb1620. p53His175 (190 nM) (A), or wt p53 (10 nM) (B), were assembled alone (lane 1), with 460 nM of DO-1 (lane 2), with 530 nM of PAb1620 (lane 3), or in presence of a DO-1 reaction (460 nM) containing decreasing amounts of PAb1620 (530 nM, 180 nM, and 60 nM) (lanes 4–6).

Taken together, these results suggested that the C-terminal region of this mutant does not negatively regulate the DNA binding activity of the protein as it does for wt p53 or other mutant p53 proteins [13,30,36]; in contrast, the binding of even a small molecule to the N-terminus of the p53His175

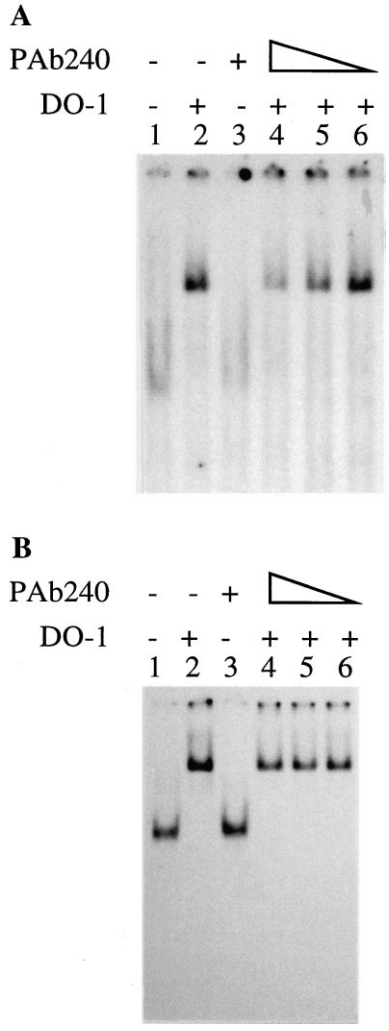

Fig. 5. The DNA binding activity of DO-1-p53His175 is destabilized by PAb240 interaction. EMSA demonstrating inhibition of DNA binding of DO-1-activated p53His175 by PAb240. p53His175 (190 nM) (A), or wt p53 (10 nM) (B), were assembled alone (lane 1), with 460 nM of DO-1 (lane 2), with 530 nM of PAb240 (lane 3), or in presence of a DO-1 reaction (460 nM) containing decreasing amounts of PAb240 (460 nM, 150 nM, and 50 nM) (lanes 4–6).

mutant induces a conformational change in the protein, thereby allowing sequence-specific DNA binding, and defining a new allosteric regulatory domain for this particular mutant.

3.3. Probing the PAb1620/PAb240 conformation of the p53His175 mutant by destabilization of DO-1-p53His175-DNA complexes

Given the fact that p53His175 appeared to have a distinct method for allosteric activation to wt p53, we wondered whether the accessibility of the conformationally sensitive antibody epitopes targeted by PAb1620 and PAb240 in the DNA binding conformation would be different to the wt protein. As illustrated in Fig. 4A, DNA binding assays performed in the presence of DO-1-activated p53His175 and an increasing amount of PAb1620 showed a dramatic reduction in the amount of p53His175-DNA complex formation (lanes 4–6 versus lane 2). In contrast, PAb1620 did not interfere with the formation of wt p53-DNA complexes (Fig. 4B). Due to the difference in specific DNA binding activity, wt p53 was used at a 20 times less concentration than p53His175 in these assays. Similar experiments carried out with PAb240 revealed that this antibody behaved surprisingly similarly to PAb1620. The increasing amounts of PAb240 which were incubated in the presence of DO-1 activated p53His175 led to reduced formation of p53His175-DNA complexes (Fig. 5A, lanes 4–6 versus lane 2). In contrast, the extent of wt p53-DNA complex formation was unaffected by PAb240 (Fig. 5B). The effect of PAb1620 and PAb240 on p53His175's DNA binding activity was specific, as the assay was performed in the presence of excess of non-specific protein (bovine serum albumin, 1 mg/ml), and as this phenomenon was not observed when another antibody such as PAb421 was tested (data not shown). These observations suggested that PAb1620 and PAb240 interaction with p53His175 locks the conformation of the latter into one that is inactive for DNA binding and resistant to N-terminal regulation of the molecule by N-terminal specific antibodies. The effect of PAb240 in these EMSA experiments was unexpected, as the p53His175 proteins were found by ELISA mostly in a PAb1620 conformation (Figs. 1 and 2). However, these results were not contradictory, as the EMSA is a more sensitive assay than the ELISA to detect weak proteinic interactions. Taken together, these data suggested the existence of an equilibrium between the PAb240 and the PAb1620 conformations of p53His175, while the latter was the preferred one. These observations also suggested that the DNA binding activation of p53His175 by N-terminal antibodies is a weak process (easily destabilized by PAb1620 and PAb240 interaction). This was reinforced by the comparison of the intensity of the DNA binding signals obtained with wt p53 and the DO-1-activated p53His175. Indeed, even when the p53His175 protein tested was 20 times more concentrated than wt p53 in the EMSA experiments (190 nM versus 10 nM), the signal obtained for the DNA binding of DO-1-activated mutant was still weaker than for wt p53 (compare lanes 2 in Fig. 4A,B, or in Fig. 5A,B).

4. Discussion

This report has allowed a more detailed understanding of the conformational states in which the p53His175 protein can reside. Our data suggested that this mutation does not spontaneously unfold the protein, as defined by PAb1620 immunoreactivity, when the protein was expressed in two different cell lines. Previous predictions have suggested that refolding of mutant p53's into the PAb1620-conformation may restore

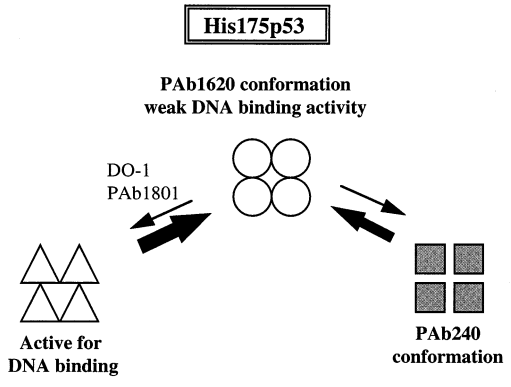

Fig. 6. Conformational flexibility of the p53His175 mutant protein.

function to the protein. However, our data indicated that acquisition of the 'folded' conformation to p53His175 does not restore sequence-specific DNA binding and reveals a yet undefined structural defect within the core domain. Investigation of the cryptic DNA binding activity of the p53His175 mutant suggested that the C-terminal region of the protein does not appear to be an allosteric regulatory domain as it does for wt p53 [13,30,36].

The amino terminus of p53 harbors the transactivation domain that drives p53-dependent transcription [37], and appeared also to allosterically regulate core DNA binding activity. The first clear evidence for this came from the observation that Mdm2-p53 complexes synthesized in vivo are inherently inactive for DNA binding, suggesting Mdm2 protein can distort the core DNA binding domain through conformational alterations [38]. The ability of the N-terminal antibody DO-1 to stimulate CK2-modified or HSP70-modified mutant p53 proteins [36], also suggest that the N-terminus contains regulatory domains that can effect core DNA binding activity. Furthermore, modification of the N-terminus of p53 protein by mAbs can protect the DNA binding activity of wt and mutant forms of p53 protein from thermal denaturation [35,39].

Our results demonstrated that the Arg→His point mutation of p53 did not lock the protein in a state that is refractory to DNA binding. Rather (Fig. 6), the protein exists in an equilibrium between different DNA binding conformations, with the inactive state being the preferred one. Factors (like mAbs) interacting with a N-terminal regulatory domain of the protein can stimulate the weak DNA binding function of this mutant, and reverse, at least for a population of the p53His175 proteins, the conformation of molecule into one active for DNA binding. The process of DNA binding activation of the p53His175 protein by N-terminus specific molecules was shown to be weak, as easily destabilized by PAb1620 and PAb240 antibodies. These results demonstrated that the His175 mutation revealed a new equilibrium between the PAb240 and the PAb1620 conformations. The PAb1620 conformation was the exacerbated one detected in the Sf9-produced p53His175 proteins, while when expressed in an other cell line (H1299), an equal proportion of PAb1620 and PAb240 fractions was detected (Fig. 2). As temperature was shown in vitro to affect the PAb1620 conformation of wt p53 [39], the difference in the proportion of PAb1620 and PAb240 populations of the p53His175 mutant expressed in the Sf9 and H1299 cell lines may reflect the difference of temperature at which the cells were grown (22°C and 37°C, respectively), as well as possible interactions of the p53His175 mutant with specific cellular factors influencing the folding state of the protein.

Taken together, our data suggested the flexibility of the p53His175 mutant to adopt different conformational states. This was in agreement with the work from Bullock and colleagues [40] who investigated thermodynamic stability of wild-type and mutant p53 core domains. The results of this study demonstrated that different p53 mutant core domains (including the p53His175 one), while less stable than the wild-type, are not locked in an unfolded state, but do retain cooperative conformational states. Moreover, it has recently been shown that the DNA binding activity of wt p53 can be modulated via the conformational equilibrium between the wt and the mutant states of the protein [41]. Current work is geared into developing probes that can dissect in vivo the sites of structural change within the p53His175 protein, in order to understand the regulation of the conformation and the DNA binding properties of the most highly represented and oncogenic p53 mutants in human neoplasia.

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