p53 DNA binding can be modulated by factors that alter the conformational equilibrium

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The p53 tumor suppressor protein is a dimer of dimers that binds its consensus DNA sequence (containing two half-sites) as a pair of clamps. We show here that after one wild-type dimer of a tetramer binds to a half-site on the DNA, the other (unbound) dimer can be in either the wild-type or the mutant conformation. An equilibrium state between these two conformations exists and can be modulated by two types of regulators. One type modifies p53 biochemically and determines the intrinsic balance of the equilibrium. The other type of regulator binds directly to one or both dimers in a p53 tetramer, trapping each dimer in one or the other conformation. In the wild-type conformation, the second dimer can bind to the second DNA half-site, resulting in drastically enhanced stability of the p53–DNA complex. Importantly, a genotypically mutant p53 can also be in equilibrium with the wild-type conformation, and when trapped in this conformation can bind DNA.

Keywords: conformational states/DNA binding/p53/pAb240/p53–DNA complex

Introduction

The p53 tumor suppressor gene is the most frequently mutated cancer-associated gene identified to date, with >50% of all tumors containing mutant p53 (Hollstein *et al.*, 1996). Tumorigenesis selects for mutations in p53 that either disrupt p53–DNA interactions directly or alter the overall conformation of the central DNA-binding domain (Cho *et al.*, 1994; Friend, 1994). The latter class of p53 mutants fails to bind DNA and displays various epitopes spanning the central domain (Gannon *et al.*, 1990; Legros *et al.*, 1994; Vojtesek *et al.*, 1995). These mutants, called class II or structural p53 mutants, are reactive with the monoclonal antibody (mAb) PAb240, which defines the mutant conformation (Gannon *et al.*, 1990; Cho *et al.*, 1994; Friend, 1994).

The conformation of wild-type p53 has been defined by its reactivity with the mAb PAb246 (murine-specific) or PAb1620 (murine- and human-reactive) (reviewed by Milner, 1995). However, the conformation of wild-type p53 is by no means static with little or no flexibility. For

example, in murine fibroblasts that contain genotypically wild-type p53, a change from the wild-type to the mutant phenotype is observed upon serum starvation followed by the addition of fresh medium (Milner and Watson, 1990). Wild-type p53 can also be converted to the mutant conformation when it is subjected to oxidation with physiological concentrations of Cu(II) (Hainaut and Milner, 1993; Hainaut *et al.*, 1995). The observation that p53 can be activated *in vivo* by the redox-regulatory protein Ref-1 also suggests that p53 is under redox control (Jayaraman *et al.*, 1997). Another event that converts wild-type p53 to a mutant conformation is denaturation (Gannon *et al.*, 1990). The latter observation suggests that the PAb240 epitope is linear, but is largely inaccessible in the conformationally wild-type protein due to the localization of the PAb240 epitope in the hydrophobic core of a β -sandwich (Cho *et al.*, 1994).

In vitro, the batch of rabbit reticulocyte lysate (RRL) used to translate p53 RNA determines what phenotype of p53 is produced (Cook and Milner, 1990; McLure and Lee, 1996). When two different batches are mixed, the type of RRL that produces the mutant conformation of p53 is dominant even when mixed 1:7 with wild-type producing RRL (Cook and Milner, 1990). This indicates that a factor, possibly a protein, is present in the mutant p53-producing RRL, and that this factor either prevents formation of the wild-type form or converts wild-type p53 to the mutant conformation. Intriguingly, it has been found that heat shock protein 90 (hsp90) is required in order for p53 to attain the mutant conformation (Blagosklonny *et al.*, 1996).

Although p53 can be converted between the mutant and wild-type conformations, it is not understood how they are related at a molecular level. Of particular interest is that some p53 has been found to display both the wild-type and mutant conformations simultaneously (Gannon *et al.*, 1990; McLure and Lee, 1996). Moreover, p53 tetramers can bind DNA in this 'dual positive' (PAb246⁺–PAb240⁺) conformation, whereas DNA-bound dimers were constrained to be conformationally wild-type (PAb246⁺–PAb240[–]) (McLure and Lee, 1996). Since tetramers bind DNA as a dimer of dimers, and one dimer within the tetramer is sufficient for DNA binding (McLure and Lee, 1998), we investigate here the hypothesis that if one dimer in a tetramer is bound to DNA, the other dimer may be able to assume the mutant conformation. We demonstrate that the conformation of unbound dimers in a tetramer is in a state of flux (between wild-type and mutant conformation). This conformational equilibrium can be modulated by two types of p53 modifiers. One type modifies the p53 biochemically and the other type binds p53 directly. Stable p53–DNA binding occurs only when both dimers in a tetramer are in the wild-type conformation.

Results

DNA-bound p53 tetramers can display both wild-type and mutant conformations, whereas DNA-bound p53 dimers display only the wild-type conformation

To correlate p53–DNA binding with p53 conformation, a series of mutants of the p53 consensus sequence were synthesized (Figure 1). All have been described previously

Fig. 1. Various DNA sequences. The p53 consensus sequence (CON) is shown (actual sequence in Materials and methods), along with positions that are altered in variations of the consensus sequence. Quarter-sites are depicted by arrows to visualize the arrangement of quarter-sites in the various sequences. Mutated quarter-sites are depicted as dashed lines. The imperfect mutation in quarter-sites 2 and 4 of M24v is indicated by an open arrow.

with the exception of the M24v mutant (McLure and Lee, 1998). The CON contains two consecutive half-sites each of which interacts with a p53 dimer. The non-binding (NB) mutant has all four quarter-sites mutated, with substitutions introduced at the invariant C (or base-paired G) in each quarter-site. The M34 mutant has one consensus half-site followed by mutated third and fourth quarter-sites, thus allowing only one p53 dimer to bind. The M24 mutant has mutations in the second and fourth quarter-sites and does not bind p53. The M24v mutant is a variant of the M24 mutant with the indicated four pyrimidines substituted with purines. This mutant is bound by p53 tetramers, but not p53 dimers (see below). The H1 mutant contains a single half-site (two quarter-sites).

We have demonstrated previously that wild-type tetrameric p53 bound to CON can display both the wild-type (PAb246⁺) and the mutant (PAb240⁺) conformation (McLure and Lee, 1996). In Figure 2A, wild-type p53 translated *in vitro* in RRL was allowed to bind to ³²P-labeled CON and analyzed in an electrophoretic mobility shift assay (EMSA). The mAb PAb421 was included in some DNA-binding reactions because it binds to and inactivates a C-terminal region of p53 that would otherwise block DNA binding (Hupp *et al.*, 1992, 1995). The inclusion of PAb240 in the reaction caused some of the CON-bound p53 to be supershifted (Figure 2A, compare lane 2 with lane 3), whereas all the CON-bound p53 reacted with PAb246 (Figure 2A, compare lane 2 with lane 4). This suggests that all CON-bound p53 tetramers display the wild-type p53 conformation and a subset of this population displays the mutant conformation.

Similar analysis was carried out using the mutant p53LZ332 which has the C-terminal 58 amino acids of p53 containing the dimer/tetramerization domains replaced by a heterologous dimeric leucine zipper from the yeast

Fig. 2. Conformations of p53 tetramers and dimers bound to CON. (A) Wild-type murine p53 tetramers or dimeric p53LZ332 were translated *in vitro* in rabbit reticulocyte lysate (RRL) and then assayed for binding to ³²P-CON in the presence of antibodies as indicated. The migrations are indicated as follows: p53 tetramer, solid arrow; PAb240-supershifted p53, solid tailed arrow; PAb246-supershifted p53, open arrow; single p53LZ332 dimers, dot; pairs of p53LZ332 dimers, solid triangle; PAb246-supershifted p53LZ332, open triangle. (B) Wild-type human p53 tetramers or A344 dimers were translated *in vitro* and then assayed for binding to ³²P-CON in the presence or absence of PAb240. Ctrl indicates control lanes that had no p53 translated. PAb421 was present in all the reactions. The position of single dimers is indicated by a dot, tetramers or pairs of dimers by an arrow, and PAb240-supershifted tetramers by a tailed arrow.

transcription factor GCN4 (Halazonetis and Kandil, 1993). This mutant is dimeric and, since it lacks the C-terminal domain, does not require PAb421 for binding to CON (Figure 2A, lane 5). Two distinct CON-bound species have been identified (lane 5, dot and solid triangle), with the slower migrating species corresponding to two dimers bound side by side to the two half-sites of CON, and the faster migrating species corresponding to a single dimer bound to a half-site on CON (data not shown). However, unlike wild-type tetrameric p53, neither p53LZ322 species was reactive with PAb240 (Figure 2A, lane 6), but both were reactive with PAb246 (Figure 2A, lane 7, open triangle). The lack of a quantitative shift was due to partial dissociation of the complex by PAb246 (McLure and Lee, 1996).

The inability of p53 dimers to display the mutant conformation was confirmed using another dimeric construct, p53A344, which is wild-type human p53 with a point mutation (leucine to alanine) at residue 344 (Waterman *et al.*, 1995). This point mutation resides at the dimer-dimer interface and results in p53 dimers that are unable to form tetramers, but pairs of A344 dimers can bind to the two half-sites in CON (McLure and Lee, 1998). As is the case with p53LZ322, two CON-bound p53A344 species (single and double dimers) were detected, but neither was reactive with PAb240 (Figure 2B, compare lane 5 with lane 6).

The above experiments demonstrate that DNA-bound p53 tetramers can display both wild-type and mutant conformations, whereas DNA-bound p53 dimers display only the wild-type conformation.

DNA-bound p53 tetramers display the mutant conformation only when one of the dimers is not in contact with DNA

The major difference between two p53A344 dimers bound to CON and a tetramer bound to CON is that tetramers could possibly bind to one of the two half-sites in CON via only one dimer of a tetramer. In such a tetramer, the other dimer in the tetramer would not have to contact DNA directly, and could instead possibly display the mutant conformation and interact with PAb240. In line with this reasoning, some p53 tetramers that were bound to a single half-site, M34 (Figure 1), were again supershifted by PAb240 (Figure 3, compare lane 3 with lane 4). Thus, when tetramers were bound to one half-site in M34 via one dimer of each tetramer, a subset of this tetramer population could react with PAb240. In contrast, p53A344 could only bind to M34 as a single dimer and was unreactive with PAb240 (Figure 3, compare lane 5 with lane 6).

To test the concept that the mutant conformation of tetramers is displayed only when one of the dimers is unbound and that tetramers with both dimers contacting DNA always display the wild-type conformation, we made use of a new DNA construct, M24v (Figure 1). Unlike M24, which does not bind to p53, the M24v mutant has less stringent mutations in the second and fourth quarter-sites and forms stable complexes only with p53 tetramers, but not dimers (Figure 4A, compare lane 6 with lane 11). In such complexes, high enough stability for detection by

Fig. 3. Conformations of p53 tetramers and dimers bound to a half-site (M34). Human p53 tetramers or A344 dimers were translated *in vitro* and then assayed for binding to [³²P]M34 in the presence or absence of PAb240. Ctrl indicates control lanes that had no p53 translated. PAb421 was present in all the binding reactions. The position of single dimers is indicated by a dot, tetramers by an arrow and PAb240-supershifted tetramers by a tailed arrow.

EMSA only occurs when both dimers of a tetramer contact DNA. Dimer-dimer interaction is possibly responsible for the enhanced stabilization of this binding (McLure and Lee, 1998). To determine the conformational status of p53 bound to M24v, EMSA was carried out in the presence of PAb240 or PAb246. The results (Figure 4B) show that unlike CON-bound p53, M24v-bound p53 was unreactive with PAb240 (compare lane 2 with lane 5), but was reactive with PAb246 (lane 6) although this interaction led to the dissociation of the complex. Thus, p53 tetramers display only the wild-type, but not the mutant conformation, when both dimers are in contact with DNA.

Unbound dimers in DNA-bound p53 tetramers are in conformational equilibrium between the wild-type and mutant states

We then proceeded to determine whether the M24v-bound p53, which displayed only the wild-type (PAb246-reactive) conformation, contained the subset of CON- or M34-bound p53 population that displayed the mutant conformation. To this end, unlabeled M24v was allowed to compete with [³²P]M34 for p53 binding. The results (Figure 5A) show that p53 tetramers that could bind [³²P]M34 were competed by excess, unlabeled M24v (lane 5). In contrast, M24 did not compete any better than did NB for p53 binding (Figure 5A, lanes 4 and 3, respectively). Therefore, the population of p53 tetramers that bound M34 and displayed the mutant conformation could also bind M24v and display only the wild-type conformation. This was confirmed by the inclusion of PAb240 in the competition experiments (Figure 5B); both the PAb240⁻ and PAb240⁺ forms of [³²P]M34-bound p53 were competed out by M24v. Furthermore, immunodepletion by PAb240 removed all the p53 that was able to bind M24v (Figure 5C, lanes 3

Fig. 4. Conformations of p53 tetramers bound to M24v. (A) Human p53 tetramers or A344 dimers were translated *in vitro* and then assayed for binding to [32 P]DNA sequences (described in Figure 1) as indicated. PAb421 was present in all the reactions. RRL with no exogenous p53 translated was run in lane 1. The migration of a single DNA-bound dimer is indicated by a dot, tetramers by an arrow. Lanes 1–6 were exposed for longer than lanes 7–11 to compensate for higher levels of A344 protein in lanes 7–11. (B) Murine p53 was translated *in vitro*, then bound to [32 P]CON (lanes 1–3) or [32 P]M24v (lanes 4–6). The mutant conformation-specific PAb240 or wild-type conformation-specific PAb246 were added as indicated. PAb421 was present in all the reactions. p53 migration is marked by a solid arrow, the PAb240 $^{+}$ p53 by a solid tailed arrow and the PAb246 $^{+}$ p53 by an open arrow. Lanes 4–6 were exposed for longer than lanes 1–3 to compensate for weaker binding of p53 to M24v.

Fig. 5. Toggling of conformations of p53 dimers within a tetramer. (A) Wild-type p53 tetramers were translated, then bound to the 32 P-labeled non-binding (NB) sequence (lane 1) or to a half-site, M34 (lanes 2–7). A 500-fold excess of various unlabeled competitor DNA sequences (as indicated) was added at the same time as [32 P]M34. DNA-bound tetramer migration is indicated by an arrow. (B) p53 was allowed to bind [32 P]M34 (lane 1). Either PAb240 (lane 2) or PAb240 and 1000-fold excess of unlabeled M24v were added at the same time as [32 P]M34 (lane 3). (C) p53 was translated, then cleared by immunoprecipitation with control antibody (lanes 1 and 2) or PAb240 (lanes 3 and 4). The supernatants were then assayed for binding to [32 P]M24v in the presence (lanes 2 and 4) or absence (lanes 1 and 3) of PAb240.

and 4). Again, after binding to M24v, the same initial population of p53 was not reactive with PAb240 (Figure 5C, lane 2).

The above experiments demonstrate that unbound dimers (in a tetramer) which display the mutant conformation can assume the wild-type conformation and bind DNA. This suggests that the conformational states of the

two dimers in a tetramer are independent of each other and that these conformational states are not static in nature. It further suggests that p53 is in conformational equilibrium which is influenced by environmental factors. This concept is consistent with the observation that the relative amounts of 'dual positive' p53 generated *in vitro* vary with different batches of RRL.

Fig. 6. Dissociation of PAb240⁺ p53 from CON. Wild-type p53 synthesized *in vitro* was allowed to bind to [³²P]CON and PAb240 for 60 min, then 1000-fold excess unlabeled CON was added (time zero) to 'trap' any p53 that dissociated from the [³²P]CON. At various times indicated (in min), aliquots were taken and loaded on a running gel. The migration of p53 is marked by an arrow and PAb240-supershifted p53 by a tailed arrow.

DNA-bound p53 is destabilized when unbound dimer is 'trapped' in the mutant conformation

The consequence of both dimers in a tetramer binding to the two half-sites in a consensus sequence is drastic stabilization of DNA binding (McLure and Lee, 1998). Therefore, if only one dimer in a tetramer binds to one half-site in DNA, and the unbound dimer is trapped in the mutant conformation (and thus prevented from interacting with the other half-site) by a factor such as PAb240, then the stability of such a p53–DNA complex should be reduced significantly.

To test this idea, p53 was first allowed to bind to [³²P]CON in the presence of PAb240. Again, a subset of CON-bound p53 was supershifted by PAb240 (Figure 6, lane 1). The half-lives of the two p53 populations were then compared by the subsequent challenge of the reactions with excess, unlabeled CON to 'trap' any p53 that dissociated from DNA (Figure 6, lanes 2–8). After 30 s, most of the PAb240⁺ p53 had dissociated from [³²P]CON (compare lane 1 with lane 2, tailed arrow). However, the p53 population that was not supershifted by PAb240 had a much longer half-life (15–20 min) (lanes 1–8). These results demonstrate that 'dual positive' (PAb246⁺–PAb240⁺) p53 is bound to DNA via only one dimer, and that prevention of the other dimer from interacting with DNA drastically decreases the half-life of the p53–DNA complex.

Genotypic mutant p53 can be in conformational equilibrium and can be stabilized in the wild-type conformation

We used a murine temperature-sensitive mutant, A135V, to determine whether a conformational p53 mutant is in conformational equilibrium like wild-type p53. This mutant is conformationally wild-type at 32°C, but is rapidly converted to the mutant conformation at 37°C (Milner and Medcalfe, 1990; Hainaut and Milner, 1992). Even in the wild-type conformation (at 30°C), A135V

Fig. 7. Effect of wild-type-specific antibody on mutant p53–DNA binding. The murine temperature-sensitive conformational mutant A135V (Mut) was translated at 30°C, then incubated for 15 min at either 30 or 38°C (lanes 3–4 and 9–10, respectively). Negative and positive controls were no p53 (Ctrl, lanes 1–2 and 7–8) or wild-type murine p53 (WT, lanes 5–6 and 11–12). DNA-binding mix was preincubated to the same temperatures, then added to the *in vitro* translated p53 with prewarmed wild-type-specific PAb246 as indicated. All reactions contained PAb421 to inactivate the C-terminal negative regulatory domain. The migration of p53 is marked by a solid arrow and PAb246-supershifted p53 by an open arrow. Lanes 5, 6, 11 and 12 were all exposed to film for the same duration, but for a shorter time than the other lanes to compensate for the lower DNA-binding activity of A135V at 38°C.

bound DNA better when PAb246 was added (Figure 7, compare lane 3 with lane 4). This is in sharp contrast to wild-type p53 which binds DNA less well in the presence of PAb246 (Figure 7, compare lane 7 with lane 8; also see McLure and Lee, 1996).

At 38°C, A135V was in the mutant conformation and as expected, did not bind to DNA (Figure 7, lane 9). However, in the presence of PAb246 some A135V was able to bind DNA (lane 10, open arrow). Again this suggested that the A135V conformational p53 mutant was in conformational equilibrium even at 38°C and was able to bind DNA when it was stabilized in the wild-type conformation by PAb246.

Discussion

Tetramers of p53 can consist of one wild-type dimer that contacts DNA and one mutant dimer that does not contact DNA

It has been proposed that p53 can be regulated via its conformational flexibility; the wild-type conformation suppresses cell cycling, whereas the mutant conformation promotes cell cycling (Milner, 1995). This 'conformation hypothesis' was modified to include PAb246⁺–PAb240⁺ dual positive p53 (McLure and Lee, 1996). We have demonstrated that some DNA-bound p53 tetramers can be simultaneously in the wild-type and mutant dual positive (PAb246⁺–PAb240⁺) conformation, whereas p53 dimers display only the wild-type conformation (PAb246⁺–PAb240[−]) when bound to DNA. Tetramerization is required

because the dual positive subset of DNA-bound p53 is bound to DNA via one wild-type dimer, whereas the other (unbound) dimer is conformationally mutant. Conclusive evidence that the mutant dimer does not contact DNA is the short half-life of the PAb240-supershifted, DNA-bound p53. Whereas PAb240⁻ p53 has a half-life of almost 20 min, most of the PAb240⁺ p53 dissociates from CON within 30 s. This mirrors the difference between p53 tetramers binding via one dimer to the one half-site in M34, or via two dimers to the two half-sites in CON (<30 s and 25 min, respectively; McLure and Lee, 1998). This strongly suggests that p53 supershifted by PAb240 is bound to one half-site in CON via only one dimer in the tetramer. Therefore, when one wild-type dimer of dual positive tetramers is bound to one DNA half-site, the other mutant dimer is not bound to DNA.

Dimers of p53 tetramers are in equilibrium between the wild-type and mutant conformations

Our results show that some genotypically wild-type p53 tetramers can bind CON via two wild-type dimers, whereas another subset of tetramers has one unbound, mutant dimer tethered to DNA via one wild-type dimer. However, the unbound dimer does not always display the mutant conformation. This was clearly demonstrated using the M34 DNA construct that contains only a single half-site. All p53 tetramers bound to this construct should have unbound dimers. However, ~50% of M34-bound tetramers are unreactive with PAb240 (Figure 3). Based on these observations, two possible scenarios can be envisaged. The first scenario is that the wild-type and the mutant conformations displayed by the unbound dimers represent static, alternatively folded states. The second scenario is that the two conformations are in equilibrium. The use of the construct M24v has allowed us to demonstrate that all dual positive p53 tetramers lose the mutant phenotype (and become wild-type) when both dimers bind DNA. This has provided compelling evidence that the mutant and wild-type conformations represent alternate structural (and functional) states of the same dimer in the tetramer, and that each dimer is conformationally independent.

One consequence of the binding mechanism is that the concept of p53 binding to DNA in a tight (T state) or relaxed (R state) conformation needs clarification (Halazonetis and Kandil, 1993; Waterman *et al.*, 1995). Each dimer has been depicted as following the concerted model of conformational change (Monod *et al.*, 1965; Halazonetis and Kandil, 1993; Waterman *et al.*, 1995). Because each dimer can be only wild-type or only mutant, DNA binding by subunits of a dimer can indeed be modeled as concerted. However, because the two dimers of a tetramer are in independent conformational equilibrium even in DNA-bound p53, the binding of each dimer in a p53 tetramer to DNA must be modeled by the sequential model of substrate binding (Koshland *et al.*, 1966).

p53-DNA-binding activity can be modulated via the conformational equilibrium

The sequential binding of each dimer of a tetramer to DNA has important implications for understanding how p53-DNA binding could be regulated. One dimer of the tetramer probably binds DNA first and the second dimer

in this DNA-bound tetramer can be in equilibrium between the wild-type and mutant conformation. In the mutant conformation, the second dimer can not bind to the second half-site. Consequently, the p53-DNA complex is unstable with a half-life of <30 s. If the second dimer is in the wild-type conformation, it can bind to the second half-site in a consensus sequence. This would result in dimer-dimer cooperativity, thereby extending the half-life of the p53-DNA complex to 25 min (McLure and Lee, 1998). Therefore, the DNA-binding activity of p53 can theoretically be modulated via the conformational equilibrium (Figure 8).

One way to control p53 is to alter the conformational equilibrium itself by promoting the formation of either the mutant or the wild-type conformation. Support for this notion has come from our observation (unpublished) that different batches of reticulocyte lysate produce p53 proteins with different levels of conformational flexibility and stability of the p53-DNA complex. For any given batch of reticulocyte lysate, a good correlation exists between conformational flexibility and the half-life of the complex: *in vitro*-synthesized wild-type p53 with a low degree of conformational flexibility tends to have a longer half-life and vice versa. Although the identity of the factors that modulate this conformational equilibrium have yet to be determined, potential candidates may well include protein modifiers such as kinases, phosphatases, acetylases and/or proteins that interact directly with p53. A good candidate for the last of these is the chaperone Hsp90, which is required for p53 to acquire the mutant conformation (Blagosklonny *et al.*, 1996). These factors might influence the conformational equilibrium and hence the fraction of p53 stably bound to DNA.

Another way to control p53-DNA binding is for a factor to bind specifically, and tightly, to either the wild-type or the mutant conformation of p53 (Figure 8). The result of this binding could be the prevention of the bound p53 from reverting to the other conformation. Thus, the binding of PAb240 to the mutant form of p53 could lock one or both dimers in a mutant conformation, thereby effectively destabilizing any DNA-bound p53 in the former case, or precluding DNA binding altogether in the latter case. The identification of two cellular factors that specifically interact with the mutant, but not the wild-type, conformation of p53 (Chen *et al.*, 1994) provide credence to this possibility. Over-expression of such factors might even be oncogenic if they could sequester genotypically wild-type p53 in a mutant conformation.

Conversely, the wild-type conformation could also be specifically recognized by protein factors. In theory, such factors might increase p53 activity by increasing the fraction of p53 in the wild-type conformation. The wild-type conformation is recognized by at least two cellular proteins, 53BP1 and 53BP2 (Iwabuchi *et al.*, 1994). Although 53BP2 would stabilize the wild-type conformation, DNA binding would be precluded because 53BP2 binds to the DNA-binding surface of p53 (Gorina and Pavletich, 1996). Two wild-type conformation-specific antibodies are PAb246 and PAb1620 (Milner and Cook, 1986; Milner *et al.*, 1987). These antibodies stabilize p53 in the wild-type conformation, but the result of them binding to p53 is that some p53 dissociates from, or can not bind to, DNA (Halazonetis *et al.*, 1993; Hall and

Fig. 8. Regulation of p53 conformational equilibrium. (A) Each dimer of a tetramer is in equilibrium between the wild-type (elliptical) and mutant (square) conformations. Each dimer is conformationally independent of the other dimer in the tetramer. (B) A tetramer initially binds to one half of the consensus DNA-binding site via one wild-type dimer. The unbound dimer can be in equilibrium between the wild-type and mutant conformations. The balance of this equilibrium is modulated by the primary sequence of p53 (class II mutations favor the mutant conformation) and by cellular factors. Molecules that bind tightly and specifically to the mutant or wild-type conformations can trap one or both dimers and prevent them from flipping to the other conformation.

Milner, 1995; Wolkowicz *et al.*, 1995; McLure and Lee, 1996). Nonetheless, some p53 reacts with wild-type-specific antibody and remains bound to DNA (Hall and Milner, 1995; McLure and Lee, 1996).

Significantly, a conformational mutant of p53 (A135V) was only able to detectably bind DNA after the addition of a wild-type conformation-specific antibody. The most probable explanation is that the p53 mutant was in equilibrium and was trapped in the wild-type, DNA-binding conformation. Consistent with this, two conformational mutants (V143A and R175H) have been reported to bind to the consensus sequence (Zhang *et al.*, 1993). The mutant we used is temperature sensitive, so it would be more likely to be in equilibrium than would a constitutive mutant. Nonetheless, a subset of human class II conformational mutants are also temperature sensitive, and can therefore assume the wild-type conformation and bind DNA at reduced temperature (Rolley *et al.*, 1995).

Presumably, the reason that wild-type antibody can destabilize wild-type p53–DNA binding would still apply to mutant p53. However, it might be possible for some molecule to react with and stabilize the wild-type conformation without inducing dissociation from or blocking binding to consensus DNA. Such a molecule would be particularly useful for stabilizing genotypic conformational mutants that are in equilibrium with the wild-type conformation, thereby restoring p53 tumor suppressor function.

Materials and methods

p53 constructs

The plasmids used were pSp65p53-H8 (wild-type human p53), pSp65p53-Ala (wild-type murine p53) and pSp65p53-Val (temperature-sensitive mutant murine p53; A135V) (Milner and Medcalf, 1990; Hainaut and Milner, 1992). The p53 dimer constructs used were p53A344,

which encodes wild-type human p53 containing a point mutation from leucine to alanine at amino acid 344 (Waterman *et al.*, 1995), and p53LZ332, which has the murine p53 C-terminal oligomerization domain replaced with the dimerization domain of GCN4 (Halazonetis and Kandil, 1993).

DNA-binding assay

Linearized plasmid DNA was transcribed using the Megascript kit (Ambion, TX). For translation of p53, aliquots of p53 RNA were added to micrococcal nuclease-treated RRL (Promega, WI). Translation was carried out according to the manufacturer's instructions, except that 20 non-radiolabeled amino acids were added and translation was allowed to proceed at 37°C for 30–90 min (except translation of A135V, which was carried out at 30°C).

Each DNA-binding reaction contained 2.5 µl of RRL (Promega) containing *in vitro* translated p53, 0.25 µl dithiothreitol (0.1 M, Sigma, MO), 1.0 µl ³²P-radiolabeled DNA (1 ng/µl), 1.0 µl salmon testes DNA as nonspecific competitor (0.1 µg/µl, Sigma), 1.2 µl glycerol, 0.25–0.5 µl of each mAb (1–5 mg/ml purified antibody or ascites fluid), and 5% bovine serum albumin (BSA) in Tris-buffered saline (TBS: 25 mM Tris pH 7.5, 130 mM NaCl, 3 mM KCl) to 10 µl final volume. Reactions were incubated at 23°C for 30–60 min, then briefly cooled to 4°C and electrophoresed in a high ionic strength, non-denaturing, EMSA (McLure and Lee, 1998). The A135V 38°C reactions were incubated at 38°C, then loaded immediately into the gel running buffer at 4°C.

The DNA-binding reactions were run in a 4% polyacrylamide gel at 150 V for between 1.5 and 3 h at 4°C. The gel was dried on Whatman 3-mm filter paper and exposed to Reflection autoradiography film with a Reflection intensifying screen (Dupont, Canada) for between 30 min and overnight at –70°C.

Immunodepletion

Aliquots of p53 were incubated with mAb PAb240, or fetal bovine serum as a control, for 30 min at 23°C. The ratio of reticulocyte lysate containing p53 to ascites antibody solution (v/v) was 4:1. Staphylococcus A (IgSorb, The Enzyme Center, MA) was then added to the mixtures and incubation was continued for an additional 20 min at 23°C. Bound p53 was pelleted by microcentrifugation, and supernatants were assayed for DNA binding as above.

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