How p53 binds DNA as a tetramer

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The p53 tumor suppressor protein is a tetramer that binds sequence-specifically to a DNA consensus sequence consisting of two consecutive half-sites, with each half-site being formed by two head-to-head quarter-sites (→← →←). Each p53 subunit binds to one quarter-site, resulting in all four DNA quarter-sites being occupied by one p53 tetramer. The tetramerization domain forms a symmetric dimer of dimers, and two contrasting models have the two DNA-binding domains of each dimer bound to either consecutive or alternating quarter-sites. We show here that the two monomers within a dimer bind to a half-site (two consecutive quarter-sites), but not to separated (alternating) quarter-sites. Tetramers bind similarly, with the two dimers within each tetramer binding to pairs of half-sites. Although one dimer within the tetramer is sufficient for binding to one half-site in DNA, concurrent interaction of the second dimer with a second half-site in DNA drastically enhances binding affinity (at least 50-fold). This cooperative dimer–dimer interaction occurs independently of tetramerization and is a primary mechanism responsible for the stabilization of p53 DNA binding. Based on these findings, we present a model of p53 binding to the consensus sequence, with the tetramer binding DNA as a pair of clamps.

Keywords: dimer–dimer interaction/p53 tumor suppressor protein–DNA binding/tetramer

Introduction

The p53 tumor suppressor gene is the most frequently mutated cancer-associated gene yet identified, with p53 mutations occurring in over half of all human tumors (Hollstein *et al.*, 1996). Most mutations occur in the DNA-binding domain, where point mutations may either disrupt protein–DNA interactions directly or alter the overall conformation of the DNA-binding domain (Cho *et al.*, 1994; Friend, 1994). Mutations in the p53 DNA-binding domain probably contribute to tumorigenesis due to the requirement for DNA binding of p53 tumor suppressor activity (reviewed by Ko and Prives, 1996; Soussi and May, 1996; Levine, 1997).

Wild-type, but not tumor-derived mutant, p53 binds to a double-stranded DNA consensus binding site containing two or more copies (consecutive or separated by one or

two helical turns) of the 10 bp half-site 5'-PuPuPuC(A/T)(T/A)GPyPyPy-3', where Pu and Py represent purines and pyrimidines, respectively (Kern *et al.*, 1991; El-Deiry *et al.*, 1992; Funk *et al.*, 1992; Cho *et al.*, 1994; Wang *et al.*, 1995; Waterman *et al.*, 1995). Thus, the consensus site comprises four inverted 5 bp quarter-sites, where the first quarter-site is underlined. X-ray crystallography has revealed how one core domain monomer (the central half of p53, residues 102–292) binds to one such quarter-site (Cho *et al.*, 1994), and a model was constructed in which four core domains can occupy the four quarter-sites in a full consensus sequence without steric clashes. This physical model of four p53 subunits bound to the consensus site is consistent with solution studies, where four p53 core domains can bind cooperatively to a consensus DNA sequence (Balagurumoorthy *et al.*, 1995; Wang *et al.*, 1995).

Tetramerization of p53 is a function of the C-terminal domain. This was deduced from the demonstration that C-terminal fragments of p53, spanning residues 311–367, form tetramers in solution (Pavletich *et al.*, 1993; Wang *et al.*, 1993). These tetramers are symmetric dimers of dimers in which all four subunits are geometrically equivalent (Clore *et al.*, 1994, 1995a,b; Lee *et al.*, 1994; Jeffrey *et al.*, 1995). However, a lower order symmetry must be exhibited by DNA-bound p53 if all four subunits in the tetramer contact a consensus DNA-binding site (Waterman *et al.*, 1995).

An important missing element in the understanding of p53 DNA binding is the global orientation of the oligomerization domains relative to the DNA-binding domains. This is because the structures of the DNA-binding and tetramerization domains, which consist of amino acids 102–292 and 324–355, respectively, and are joined by a flexible linker, have been determined separately (reviewed by Arrowsmith and Morin, 1996). Since the linker is relatively long, the relative orientations of the tetramerization and DNA-binding domains cannot be deduced from available biophysical data. As a result, two different models have arisen that conceptually connect the DNA-binding domains to the oligomerization domains in an intact p53 tetramer. In one model, one dimer of a tetramer contacts the first and second quarter-sites and the other dimer binds to the third and fourth quarter-sites in the consensus sequence (Halazonetis and Kandil, 1993; Clore *et al.*, 1994; Waterman *et al.*, 1995). In the other model, one dimer contacts the first and third quarter-sites and the other dimer binds to the second and fourth quarter-sites (Cho *et al.*, 1994; Lee *et al.*, 1994; Jeffrey *et al.*, 1995; Arrowsmith and Morin, 1996; Pennisi, 1996).

In order to differentiate between the two models of how tetrameric p53 binds to DNA, we analyzed the ability of *in vitro* synthesized tetrameric or dimeric p53 to bind to consensus DNA sequences that had various quarter-

Fig. 1. Various DNA sequences. The p53 CON is shown, 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.

sites mutated. Our results show that dimers bind to intact half-sites containing two consecutive quarter-sites but not to alternating quarter-sites. Furthermore, dimer-dimer interactions distinct from those involved in tetramerization drastically enhance the DNA-binding affinity of tetrameric p53.

Results

Wild-type 53 binds to a half-site (two consecutive quarter-sites), but not to alternating quarter-sites

To determine the nature of p53 DNA binding, a series of mutants of the p53 consensus sequence were synthesized (Figure 1). The consensus sequence (CON) contained two consecutive half-sites (four quarter-sites). The non-binding (NB) mutant had all four quarter-sites mutated, with substitutions introduced at the invariant C (or base-paired G) in each quarter-site. The M34 mutant had one consensus half-site followed by mutated third and fourth quarter-sites. The M24 mutant had mutations in the second and fourth quarter-sites. The H1 mutant contained a half-site (two quarter-sites). The m2 mutant was similar to H1 except that the second quarter-site was mutated.

Wild-type human p53 was translated *in vitro*, then assayed for binding to the various sequences. The results show that there were no novel bands with the control NB sequence (Figure 2A). There was a non-specific complex, present to varying extents with all [³²P]DNA sequences, which migrated approximately one-third to half of the way down the gel. This was endogenously present in rabbit reticulocyte lysate (RRL), varied with the batch of RRL and was not supershifted by p53-specific antibodies (data not shown). Two novel bands appeared with CON and M34 (Figure 2A, open arrow and a closed arrow). In contrast, the sequence in which the second and fourth quarter-sites were mutated (M24) was not bound by p53. The bands specific for CON and M34 required the presence of polyclonal antibody pAb421 to inactivate the negative regulatory domain located downstream of the oligomerization domain (i.e. close to the very end of the C-terminus),

suggesting that the bound p53 was full-length (Figure 5, compare lanes 1 and 2).

Interestingly, the migration rates of both p53-CON and p53-M34 complexes were the same, implying that the same molecular mass and therefore oligomeric form of p53 was present in each complex. As will be shown below (Figure 4), the slower migrating band (Figure 4C, closed arrow) corresponded to tetrameric p53, and the faster migrating band (Figure 4C, open arrow) corresponded to dimeric p53. Our results (Figure 2A) suggest that the same species of full-length p53 could bind either to the one-half-site in M34 or to one or both of the two half-sites in CON, but not to alternating quarter-sites in M24. Identical results were seen with murine and human p53 (data not shown).

A competition experiment was then carried out in which *in vitro* translated p53 was added to a [³²P]-labeled CON sequence in the presence of excess unlabeled DNA (Figure 2B). As expected, neither NB nor M24 competed with [³²P]CON for binding to a constant amount of p53 (Figure 2B, lanes 29–31 and 14–18, respectively). CON competed the best for p53 binding, followed by M34 (Figure 2B, lanes 2–6 and 8–12, respectively). Therefore, wild-type p53 preferentially bound to CON, but also had some affinity for M34 (but not M24). Identical results were seen with murine p53 (data not shown). This confirms that the same species of p53 that could bind to CON alternatively could bind to M34.

Due to the nature of the selective mutations of two out of 10 bp in the second M34 half-site (Figure 1), it was possible that the intact p53 oligomer was making other stabilizing contacts in the two mutated quarter-sites of M34. To test this possibility, p53 binding to [³²P]CON was challenged with a single half-site (H1) not followed by a mutated half-site. There was virtually no difference between excess M34 and H1 (Figure 2B, compare lanes 8–12 with lanes 20–24). Therefore, p53 tetramers preferentially bind CON, and can bind single half-sites (when present in excess) but not single quarter-sites (Figure 2B, m2, lanes 26–28).

Cellular p53 binds DNA similarly to p53 translated *in vitro*

It was important to compare the DNA binding of *in vitro* translated p53 with that of cellular p53. To this end, subconfluent Balb/c 3T3 cells, which contain wild-type p53, were first subjected to DNA damage (actinomycin D treatment) in order to activate and stabilize p53. Extracts were then prepared from untreated (control) or DNA-damaged cells, and assayed for DNA-binding activity. In the absence of DNA damage, a very low amount of endogenous cellular p53 was activated for DNA binding by pAb421 (Figure 3, lane 14), but DNA damage-induced stabilization of p53 yielded a much greater amount of pAb421-activated DNA-binding complex (Figure 3, lane 6). The most slowly migrating form (Figure 3, lane 6, closed arrow) was the major DNA-bound species, and probably consisted of p53 tetramer-DNA complexes. The lower band (Figure 3, lane 6, open arrow) probably represented a lower concentration of p53 dimer-DNA complexes, as was observed with *in vitro* translated p53. These two complexes were also detected when [³²P]M34 was used as the probe, although they were present at a

Fig. 2. Binding of *in vitro* translated p53 to various sequences. (A) Direct DNA binding analysis by electrophoretic mobility shift assay. p53 or irrelevant RNA (luciferase) was translated in RRL, and aliquots were then mixed with equal counts of [32 P]DNA (NB, CON, M34 or M24) as described in Materials and methods. The migration rates of two forms of p53 are indicated by arrows. (B) DNA binding competition studies. Binding of p53 to [32 P]CON was carried out in increasing amounts of unlabeled competitor DNA as indicated. Ten ng of competitor was added to lanes 2, 8, 14, 20, 26 and 29; 33 ng of competitor was added to lanes 3, 9, 15 and 21; 100 ng of competitor was added to lanes 4, 10, 16, 22, 27 and 30; 333 ng of competitor was added to lanes 5, 11, 17 and 23; and 1000 ng of competitor was added to lanes 6, 12, 18, 24, 28 and 31.

significantly reduced level (Figure 3, lane 7). Interestingly, there was a low level of DNA-binding activity, unique to CON, which was activated by DNA damage and detected without pAb421 (Figure 3, lane 2). This probably represented p53 DNA binding that was not inactivated by the C-terminal negative regulatory domain.

The overall pattern of cellular p53 binding was similar to that of *in vitro* translated p53, as CON was bound the best, with some M34 being bound, but no M24 or NB (Figure 3, lanes 6, 7, 8 and 5, respectively). Binding competition experiments were also carried out using unlabeled competitor DNA, and the results (data not shown) concurred with the direct binding studies. It is noteworthy that p53 translated *in vitro* (Figure 2A, lane 7) bound M34 much better than did p53 from the cell

extract (Figure 3, lane 7). The reason for this is presently unknown. Another similarity to *in vitro* translated p53 was the non-specific band that migrated approximately one-third of the way down the gel (Figure 3, lanes 1–16). Overall, the pattern of DNA binding exhibited by cellular p53 was similar to that exhibited by *in vitro* translated p53.

Free dimers bind to half-sites or pairs of half-sites, but not to alternate quarter-sites

Binding of p53 tetramers to M34 (or H1) but not to M24 indicated that the binding was stabilized when two subunits of the tetramer interacted with the two quarter-sites within a half-site. However, these results do not differentiate between whether the dimers in a tetramer each bind to a half-site, or whether each half-site is bound by one subunit

from each dimer that makes up a tetramer. To differentiate between these two possibilities, the ability of free dimers to bind to the variations of CON was compared.

The p53 A344 mutant is a full-length wild-type human p53 clone containing a point mutation at residue 344 (from leucine to alanine) that disrupts the dimer-dimer interface and results in the formation of dimeric, rather than tetrameric p53 (Waterman *et al.*, 1995). Like the tetrameric form, dimeric A344 bound neither to NB nor to M24 (Figure 4A, lanes 1 and 5); however, it formed a single complex with M34 (Figure 4A, lane 4, open arrow). Interaction of A344 with CON resulted in the formation

of two complexes, one migrating identically to the A344-M34 complex, and one migrating more slowly (Figure 4A, lane 3, open and closed arrows, respectively). As was the case with tetrameric p53, pAb421 was required to activate (or stabilize) the binding of the A344 dimer to DNA (Figure 4A, compare lanes 2 and 3).

Based on the migration rates of the A344-M34 and A344-CON complexes, and in view of the dimeric nature of A344, it seemed logical to suggest that the lower band (Figure 4A, open arrow) that was common to both CON and M34 represented one dimer, and the upper band (Figure 4A, closed arrow) found only on CON represented two dimers (a tetramer). This interpretation was assessed further by varying the protein-DNA ratio in the reaction mixture (Figure 4B). Decreasing the concentration of A344 while keeping the concentration of [32 P]CON constant resulted in a clear bias against the formation of the upper band compared with the lower band. This was precisely what one would expect if the upper band and the lower band corresponded to double and single A344 dimers, respectively. Decreasing the concentration of dimer relative to DNA would give an unbound dimer a greater chance of binding one of two half-sites on an unoccupied CON molecule compared with one half-site on CON that already had one A344 dimer bound. Accordingly, the faster migrating band of A344-CON (Figure 4B, open arrow) most likely represented one A344 dimer bound to one half-site in CON, whereas the more slowly migrating A344-CON band (Figure 4B, closed arrow) represented two dimers of A344 bound to the two half-sites in CON.

Does wild-type p53 bind DNA similar to A344 dimers? The less abundant, faster migrating species of p53-CON and p53-M34 migrated at a position identical to that of A344-M34, probably representing one dimer bound to DNA (Figure 4C, lanes 2 and 3 compared with 7, open arrow). Likewise, the slower migrating species (Figure

Fig. 3. Binding of cellular p53 to various sequences. Balb/c 3T3 total cell extract was prepared from cells with or without prior DNA damage by actinomycin D. Cell extract was added to [32 P]DNA (NB, CON, M34 or M24), either with or without pAb421, as indicated.

Fig. 4. Binding of A344 dimers to DNA. (A) *In vitro* translated A344 was mixed with [32 P]DNA (with pAb421 except for lane 2) as indicated. The migration rates of two forms of A344 are indicated by arrows. (B) Binding of A344 to CON and M34 at various A344 concentrations. Decreasing amounts of *in vitro* translated A344 were added to [32 P]CON (the reactions in lanes 3 and 4 contained two-thirds and one-third the amount of A344, respectively, compared with the reaction in lane 2) or [32 P]M34 (lanes 6–8). RRL alone served as a control (lanes 5 and 9). Lane 1 is an increased autoradiography film exposure time of lane 4. The migration of a single A344 dimer is indicated by an open arrow, pairs of A344 dimers by a closed arrow. (C) Comparison of migration patterns of wild-type p53-DNA and A344-DNA complexes. *In vitro* translated wild-type p53 or A344 was mixed with [32 P]DNA as indicated and analyzed by EMSA.

Fig. 5. Stability of p53 bound to CON and M34. *In vitro* translated wild-type p53 was allowed to bind to [32 P]CON or [32 P]M34 for 45 min at 22°C. A 100-fold excess of unlabeled CON (100 ng per reaction) was then added (time 0) to 'trap' any p53 that dissociated from [32 P]CON or [32 P]M34 at various times thereafter. Aliquots were taken at the times indicated and loaded on a running gel. For comparison, some reactions (lanes 2, 8 and 9) did not have any unlabeled CON added (cold 'trap', - lanes). All reactions contained pAb421 except for lane 1.

4C, closed arrow) which migrated at the same position as the upper band of A344-CON probably contained one p53 tetramer. The observation that the predominant species bound to one half-site (M34) was tetrameric p53 suggested that most wild-type p53 had already formed tetramers before binding to DNA.

Tetramers bind CON (two half-sites), but not a single half-site, with high affinity

We noted that p53 tetramers (but not free dimers) invariably bound better to CON than to M34 (Figure 2A, compare lanes 6 and 7). To assess whether this reflected a difference in affinity, p53-[32 P]DNA complexes were first allowed to form, and excess unlabeled CON was then added to 'trap' any p53 that dissociated from the 32 P-labeled sequence over time. The results showed that the binding of p53 to CON was very stable, with a calculated half-life ($t_{1/2}$) of 25 min (Figure 5, lanes 2–8; Table I). In contrast, most p53-M34 had a $t_{1/2}$ much shorter than 5 min, estimated at ~30 s (Figure 5, lanes 9–14). The dissociation of the majority of p53 from DNA probably follows first order kinetics and, therefore, the overall dissociation constant of p53-CON is 4.6×10^{-4} /s (Table I). It is interesting to note that although most p53 on M34 dissociated within 5 min (Figure 5, compare lanes 9 and 10), the p53 that remained bound after 5 min persisted with an estimated $t_{1/2}$ of ~5 min (Figure 5, lanes 10–14). This could represent a minor population of p53 that was bound to M34 more stably than the majority of p53.

High-affinity DNA binding results primarily from dimer-dimer interaction

While it seemed probable that the greater stability of the p53-CON complex compared with the p53-M34 complex was due to interaction of both dimers of a tetramer with

Table I. Half-lives ($t_{1/2}$) and dissociation constants (K_D) of p53-DNA complexes

Protein-DNA complex	$t_{1/2}$ (min)	K_D (s^{-1})
p53-CON	25	4.6×10^{-4}
p53-M34	0.5	1.4
(A344) $_2$ -CON	15	7.7×10^{-4}
A344 $_1$ -CON	~1 s	~0.012

The half-lives of various p53-DNA complexes were calculated based on time courses similar to those depicted in Figures 5 and 6. The assay was not sensitive enough to determine accurately the very short half-life of a single dimer bound to CON (A344 $_1$ -CON), so an estimate was made (based on a very faint A344 $_1$ -CON band evident at the 0.5 min time point when gels similar to the one depicted in Figure 6B were overexposed).

the two half-sites in CON, it was important to determine whether dimer-dimer interaction played any role in stabilizing this binding. To this end, the above finding (Figure 4) that two A344 dimers could bind side-by-side to CON was exploited to determine whether dimers stabilized one another by interacting after binding to DNA.

Two complexes of A344-CON were again observed: a faster migrating A344 dimer bound to one of the half-sites in CON and a slower migrating pair of A344 dimers bound to the two half-sites in CON (Figure 6A, lanes 1 and 7, open and closed arrows, respectively). When excess unlabeled CON was added to pre-formed A344-[32 P]CON complexes, the two A344 dimers bound side-by-side on CON had a $t_{1/2}$ of ~15 min (Figure 6A, lanes 1–7, closed arrow; Table I). This $t_{1/2}$ corresponds to a dissociation constant of 7.7×10^{-4} /s, assuming first order kinetics of dissociation (Table I). Strikingly, in contrast to the pair of A344 dimers, the single A344 dimer on DNA had a $t_{1/2}$ much less than 5 min (Figure 6A, compare lanes 1 and 2, open arrow). As expected, the single A344 dimer on M34 also had a short $t_{1/2}$ of <5 min (lanes 8–13). Shorter time courses revealed that the single A344 dimer on CON had a $t_{1/2}$ much less than 30 s (Figure 6B). Consequently, the $t_{1/2}$ of two dimers bound to CON (15 min) was much greater than double the $t_{1/2}$ of a single dimer bound to one half-site (estimated to be ~1 s; Table I footnotes).

The above experiment demonstrated that one A344 dimer cooperatively stabilized the binding of the second dimer, which would also presumably occur in dimers of a wild-type p53 tetramer bound to CON. Based on the $t_{1/2}$, p53-CON appeared to be more stable than (A344) $_2$ -CON, suggesting that the C-terminal tetramerization domain may contribute further to tetramer DNA binding.

Discussion

p53 binds DNA with each dimer of the tetramer contacting its own half-site

Although the individual structures of the major functional domains of p53 have been determined (reviewed by Pennisi, 1996), a major unresolved issue has been how the four DNA-binding domains are related to the oligomerization domains in a DNA-bound p53 tetramer (reviewed by Arrowsmith and Morin, 1996). Based primarily on NMR and crystallographic analysis of the C-terminal tetramerization domain of p53, it is now

Fig. 6. Stability of A344 bound to CON and M34. (A) *In vitro* translated A344 was allowed to bind to [32 P]CON or [32 P]M34 for 45 min. A 100-fold excess of unlabeled CON (100 ng per reaction) was then added (time 0) to 'trap' any A344 that dissociated from [32 P]CON or [32 P]M34 at various times thereafter. Aliquots were taken at the times indicated and loaded on a running gel. For comparison, some reactions (lanes 1, 7 and 8) did not have any unlabeled CON added (cold 'trap', -). The migration of one A344 dimer is marked by an open arrow; pairs of A344 dimers are marked by a closed arrow. All reactions contained pAb421. (B) Same as (A), except that aliquots were taken at shorter times after addition of unlabeled 'trap'.

generally accepted that p53 is a dimer of dimers (Clore *et al.*, 1994, 1995a,b; Lee *et al.*, 1994; Jeffrey *et al.*, 1995). Since each monomeric subunit of a dimer binds to a quarter-site, a dimer theoretically could bind to two contiguous quarter-sites that comprise a half-site, or to alternating quarter-sites within a full consensus sequence. Additionally, considering the head-to-head nature of the two quarter-sites within a half-site, binding of the two monomeric subunits within a dimer to contiguous quarter-sites would suggest that the dimer is rotationally symmetric (i.e. head-to-head). Conversely, binding of these monomers to alternating quarter-sites would suggest that the dimer is translationally symmetric (i.e. head-to-tail). These considerations are important since they have major implica-

tions for how the two dimers within a tetramer cooperatively interact upon DNA binding.

We demonstrate here for the first time how the DNA-binding domains are connected to the oligomerization domains in DNA-bound p53 tetramers. Tetramers could bind to a DNA sequence that contained quarter-sites one and two (M34), but not to a sequence that contained only quarter-sites one and three (M24). This suggests that stable DNA binding occurs only when the two adjacent quarter-sites in one half-site are occupied. However, this finding did not discriminate between which subunits of a tetramer were interacting with a half-site. The subsequent use of dimeric p53 (A344) resolved this issue. Like tetrameric p53, dimeric p53 bound only M34 but not M24, indicating that the two subunits of one dimer could bind to two adjacent quarter-sites within a half-site. It is easy to envisage how this binding would be more stable than one involving a single subunit and a single quarter-site. Furthermore, there may well be cooperative interaction between the two subunits that would enhance the overall stability of the complex further. In the case of the tetramer, binding to M34 is probably achieved via the interaction of a dimer in the tetramer with the one half-site in M34, leaving the other dimer either 'hanging off' the DNA or perhaps interacting non-specifically with DNA. Binding of a tetramer to CON would involve the binding of the two dimers to the two respective half-sites in CON.

Based on our present findings and other previous suggestions for various aspects of p53 DNA binding (Halazonetis and Kandil, 1993; Cho *et al.*, 1994; Clore *et al.*, 1994; Lee *et al.*, 1994; Waterman *et al.*, 1995), a model is depicted schematically in Figure 7A. In this model, p53 can be visualized as a pair of clamps, with each dimer being one clamp. The two arms of each clamp represent the two core domains that interact with the two adjacent quarter-sites within a half-site. As initially suggested by Cho *et al.* (1994), the bulk of the four core domains would lie on one face of the DNA helix. Flexible linkers extend to the other face of the helix and may have no fixed structure, thereby allowing the tetrahedrally symmetric tetramerization domain to retain its symmetry while the attached core domains are bound to DNA with a lower order of 2-fold cyclic symmetry (Waterman *et al.*, 1995). The oligomerization domain is a tetrahedrally symmetric dimer of dimers, consisting of two pairs of interlocking subunits (Clore *et al.*, 1994, 1995a,b; Lee *et al.*, 1994; Jeffrey *et al.*, 1995). Each dimer is formed by two monomers that dimerize via an antiparallel β -sheet followed by a turn and an α -helix, the latter of which forms the dimer-dimer interface (Clore *et al.*, 1994, 1995a,b; Lee *et al.*, 1994; Jeffrey *et al.*, 1995). The tetramerization domain probably resides on the opposite side of the helix relative to the core domains (Lee *et al.*, 1994; Jeffrey *et al.*, 1995).

A model that has the DNA-binding domains of one dimer in a tetramer binding to one half-site of a consensus DNA sequence has been postulated previously, but not demonstrated. One group demonstrated that free dimers could bind to half-sites, but it was not ascertained whether dimers in a tetramer could bind to quarter-sites one and two or one and three in the consensus DNA (Halazonetis and Kandil, 1993; Waterman *et al.*, 1995). Another group showed that tetramers (lacking the C-terminal negative

A **B**

Fig. 7. Model of a p53 tetramer binding to DNA. **(A)** Overall structure of the p53–CON complex. One dimer (green and yellow) binds to one half-site on the DNA. The second dimer (blue and red) of the tetramer binds to the adjacent half-site in a consensus DNA sequence. After binding, the dimers interact independently of the tetramerization domain to stabilize binding. **(B)** In search of CON by p53 in an environment of excess, random DNA. Initially, the p53 tetramer interacts transiently with non-specific DNA or with a single quarter-site (left; $f_{1/4 \text{ site}} = 64^{-1}$). Detectable, but unstable, binding occurs when a less frequently occurring half-site is encountered (middle; $f_{1/2 \text{ site}} = 4096^{-1}$). The tetramer becomes stably bound to DNA only when it encounters an infrequent full consensus sequence (right; $f_{\text{full-site}} = \sim 1.7 \times 10^{-7}$).

regulatory domain) could bind to half-sites much better than to DNA sequences that had only the first and third quarter-sites (Wang *et al.*, 1995). However, there was no discrimination between whether each dimer in the tetramer bound to one half-site or whether one subunit from each dimer bound to a half-site. These previous studies have nonetheless provided major clues and, together with our current findings, have led to the consolidation of the ‘double clamp’ model for p53 DNA binding.

Cooperative inter-dimer interactions stabilize tetramer binding to DNA

Tetramers could bind via one dimer interacting with one half-site, but binding was stabilized (extension of the $t_{1/2}$ from <30 s to ~ 25 min) if the second dimer of a tetramer simultaneously bound DNA beside the first dimer. This is consistent with previous observations that cooperative interactions occur between p53 monomers that bind to consensus half-sites (Pavletich *et al.*, 1993; Balagurumorthy *et al.*, 1995; Wang *et al.*, 1995). It is of note that p53 dimer–dimer DNA binding cooperativity has not been reported previously, since these studies were limited to the binding of monomeric subunits to consensus DNA sites.

One can entertain three possible explanations for the need for p53 to be tetrameric rather than dimeric, thereby interacting with two half-sites rather than one half-site. The first possibility is that pairing of the two dimers via the C-terminal tetramerization domain generates a conformational change in the DNA-binding domains of the component dimers, leading to enhanced DNA-binding activity. This would be consistent with a previous proposal that a conformational shift propagates through the oligomerization domain upon p53 tetramerization (Halazonetis and Kandil, 1993). This mechanism of enhanced binding is therefore distinct from other stabilizing factors such as cooperative binding that could occur only when both dimers interact with DNA (see below). It also predicts that the binding to a half-site by a tetramer (via a dimer) would be more stable than that by a single dimer. Results from our ‘cold trap’ experiments concur with this prediction.

The second possible explanation is based on simple arithmetical logistics. Assuming that no dimer–dimer interaction occurs outside of the oligomerization domain, the binding of the second dimer to the second half-site would double the ‘holding power’ of the tetramer, resulting in enhanced affinity of a tetramer. This was indeed observed

using a sequence with the two half-sites spatially separated by 10 bp, which effectively eliminates dimer-dimer interaction between the core domains while allowing the two subunits to bind to the two half-sites (data not shown). However, this enhanced affinity only contributes partially to the overall stability of the p53-CON complex (data not shown).

The third possibility is that cooperative dimer-dimer interaction contributes to tetramers binding better to a consensus sequence than to one half-site. This possibility is strongly supported by the finding that pairs of A344 dimers bound to CON with a much longer $t_{1/2}$ (15 min; Table I) than did single A344 dimers (much less than 30 s; Table I). It is possible that the dimer-dimer stabilization of DNA binding was mediated by the interaction that occurs between monomers in the crystal structure, namely Ser96, Ser99 and Thr170 of one subunit interacting with Thr140, Glu198, Gly199 and Glu224 of another subunit (Cho *et al.*, 1994). This interaction has been interpreted as the primary intra-dimer, inter-monomer interaction in the model of tetramers binding DNA as one dimer binding to quarter-sites one and three, the other dimer binding to sites two and four (Cho *et al.*, 1994; Lee *et al.*, 1994; Jeffrey *et al.*, 1995; Arrowsmith and Morin, 1996; Pennisi, 1996). The same interaction can now be reinterpreted as possibly occurring at the dimer-dimer interface, and may well be the most significant factor in stabilizing a DNA-bound tetramer.

Based on the relative capacity of p53 tetramers to bind various DNA sequences, the fate of a p53 tetramer when it encounters DNA can be envisaged (Figure 7B). Weak affinity of the dimers within the tetramer for non-specific DNA would first position the p53 on DNA. This possibly is mediated by the non-specific DNA-binding activity resident in the C-terminus (Pavletich *et al.*, 1993; Wang *et al.*, 1993). In a random sequence of DNA, single quarter-sites frequently would be encountered (one in sixty-four 5 bp sequences is a quarter-site), but relatively stable binding would only occur if a half-site was encountered. Since tetramers bound to a half-site would still have a very short half-life, they would be able to dissociate rapidly from the half-site and sample many more sequences of DNA. When a rare consensus site finally was encountered, both dimers within the p53 tetramer would bind, thereby enhancing the affinity of tetramer binding. Therefore, one function of the tetramerization domain may be to position the dimers of a tetramer such that once one dimer bound to a half-site, the probability of the second dimer binding to an adjacent half-site in a consensus sequence would be considerably elevated. The overall stability of the complex would be reinforced further by dimer-dimer interaction within the tetramer. Transcription of p53-activated genes, or other transcription-independent activities of DNA-bound p53, could then ensue.

Materials and methods

Plasmid p53 constructs

The plasmids used were pSp65p53-H8, which encodes wild-type human p53, and p53A344, which encodes wild-type human p53 with a mutation from leucine to alanine at amino acid 344. These were provided by Drs Jo Milner and Thanos Halazonetis, respectively.

The ability of p53 to bind DNA sequence specifically was determined using various double-stranded sequences. Two unphosphorylated strands

of each sequence were synthesized (University of Calgary Core DNA Services) and dissolved in TES buffer (10 mM Tris pH 8.0, 1 mM EDTA, 25 mM NaCl) to a concentration of ~2 mg/ml. The absorption at 260 nm was determined spectrophotometrically (1 OD = 33 µg/ml ssDNA), and equal amounts of each strand were mixed and incubated at 90°C for 10 min. The strands were allowed to hybridize by slowly cooling (60 min total) to 50, 37, 23 then 4°C.

The double-stranded sequences had 5' overhangs that were used for labeling with ³²P by the Klenow fragment of DNA polymerase. Labeled DNA sequences were then purified away from unincorporated nucleotide using the QIAquick Nucleotide Removal kit (Qiagen) and isolated in 100 µl of TE buffer (1 ng of original DNA per 1 µl final volume). For comparison of binding to different ³²P sequences, 2 µl of the labeled DNA was added to 3 ml of scintillation fluid (CytoScint, ICN) and counted in the ³²P channel of a scintillation counter (Beckman LS6500). The labeled DNA samples were then diluted to give equal counts per min.

The top and bottom strands of CON are 5'-AGCTTAGACATGCCTA-GACATGCCTA-3' and 5'-AGCTTAGGCATGTCTAGGCATGTCTA-3' (Figure 1). Various combinations of the underlined nucleotides were altered to construct other sequences. NB had the four underlined nucleotides in the top strand substituted with G, C, G and C, respectively. M34 had the third and fourth underlined nucleotides altered to G and C (top strand), while M24 had the second and fourth underlined nucleotides altered to C and C (top strand). The bottom strands were altered accordingly to yield complementary strands. The top and bottom strands of H1 are 5'-AGCTTAGACATGCCTA-3' and 5'-AGCTTAGGCATGTCTA-3'. The sequence of m2 is identical to that of H1 except that the underlined G and C are substituted with C and G, respectively.

In vitro transcription and translation

Purified DNA plasmids were linearized and then transcribed with Sp6 polymerase using the Megascript kit (Ambion). For translation of p53, aliquots of p53 RNA were added to micrococcal nuclease-treated RRL (Promega). 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.

Cell culture and preparation of cell extract

NIH 3T3 cells were grown in Dulbecco's modified minimal essential medium (DMEM) supplemented with 10% fetal bovine serum (Gibco-BRL). The cells were grown in 5% CO₂ at 37°C, and ~5×10⁶ cells were harvested at 80% confluency. Some cells were harvested after DNA damage was incurred by prior incubation for 16 h in DMEM containing 300 ng/ml actinomycin D (Calbiochem). The medium was aspirated, and the cells were rinsed with phosphate-buffered saline (PBS), then scraped off the surface of the tissue culture flask in 1 ml of ice-cold PBS using a rubber policeman. Cells were then centrifuged at 1000 g for 3 min, and washed in ice-cold PBS. The cell pellet was then resuspended and lysed in 100 µl of ice-cold hypotonic buffer [10 mM HEPES pH 7.6, 10 mM KCl, 1 mM MgAc, 1 mM dithiothreitol (DTT), 10% glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF)] for 10 min on ice, then passed through a 20 gauge needle. After centrifugation at 12 000 g for 5 min at 4°C, the supernatant was saved on ice and the pellet was extracted with continuous agitation in 40 µl of hypertonic buffer (hypotonic buffer supplemented to 0.5 M KCl) for 30 min on ice. The nuclei and cell debris were pelleted by centrifugation at 12 000 g for 5 min at 4°C, and the supernatant was added to the cytoplasmic extract. The final concentration of KCl was ~150 mM.

DNA binding analysis

Each DNA-binding reaction contained 2.5 µl of RRL containing *in vitro* translated p53 (or cell lysate), 0.25 µl of DTT (0.1 M; Sigma), 1.0 µl of ³²P-radiolabeled DNA (1 ng/µl), 1.0 µl of salmon testes DNA as non-specific competitor (0.1 µg/µl, Sigma), 1.2 µl of glycerol, 0.5 µl of pAb421 (1–2.5 mg/ml purified antibody or ascites fluid), and 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 cooled to 4°C and electrophoresed in a high ionic strength, non-denaturing electrophoretic mobility shift assay (EMSA) essentially as described (Chodosh, 1991).

When unlabeled double-stranded DNA sequences were used for competition, 1 µl of an appropriate dilution was added per reaction. The concentrations were determined by absorption at 260 nm (1 OD = 50 µg/ml dsDNA), then adjusted to give equal concentrations of quarter-sites (except NB, where the concentration was equal to the concentration of M34).

Densitometry

Autoradiography films were exposed to the ^{32}P gels such that the bands were readily visible. The film was scanned using a Hewlett Packard flatbed color scanner (ScanJet 5p), but it was essential to convert the scanned image from color to a grayscale image. No other computer adjustments were made to the image, which was then analyzed using SigmaGel (Jandel Scientific). Bands were quantified, and the only variable that was changed was the selection of a suitable threshold value that essentially represented background correction. When half-lives were measured, the threshold value was selected such that two separate pairs of time points in the same time course yielded a nearly identical reduction in band intensity over a constant time interval. This was found to be most reliable when relatively faint bands were analyzed.

Determination of $t_{1/2}$ and K_D

The change in band intensity between two time points, obtained from densitometry of the scanned image, was converted to relative values. Data were only used if the percentage reduction in band intensity was nearly identical between two different pairs of equally spaced time points in the same time course. An average percentage dissociation was then taken from the two pairs of time points and used to determine the half-life as follows (adapted from Larson and Hostetler, 1989):

$$t_{1/2} = t(\ln 2)/\ln(N_o/N_t)$$

where $t_{1/2}$ = half-life; N_o = band intensity at time point 1; N_t = band intensity at time point 2; t = time elapsed between time points 1 and 2

For 10 min time intervals, the $t_{1/2}$ in minutes is therefore:

$$t_{1/2} = -6.93/[\ln(10 \times N_o/N_t) - \ln(10)]$$

If the dissociation follows first-order kinetics, then the overall dissociation constant, K_D , can be calculated using the value of the $t_{1/2}$ (in s) as follows:

$$K_D = -\ln(0.5)/t_{1/2}$$

As above, $t_{1/2}$ is the half-life of the p53–DNA complex.

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