

Analysis of p53 “Latency” and “Activation” by Fluorescence Correlation Spectroscopy

EVIDENCE FOR DIFFERENT MODES OF HIGH AFFINITY DNA BINDING*

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The concept that the tumor suppressor p53 is a latent DNA-binding protein that must become activated for sequence-specific DNA binding recently has been challenged, although the “activation” phenomenon has been well established in *in vitro* DNA binding assays. Using electrophoretic mobility shift assays and fluorescence correlation spectroscopy, we analyzed the binding of “latent” and “activated” p53 to double-stranded DNA oligonucleotides containing or not containing a p53 consensus binding site (DNA_{spec} or DNA_{unspec}, respectively). In the absence of competitor DNA, latent p53 bound DNA_{spec} and DNA_{unspec} with high affinity in a sequence-independent manner. Activation of p53 by the addition of the C-terminal antibody PAb421 significantly decreased the binding affinity for DNA_{unspec} and concomitantly increased the binding affinity for DNA_{spec}. The net result of this dual effect is a significant difference in the affinity of activated p53 for DNA_{spec} and DNA_{unspec}, which explains the activation of p53. High affinity nonspecific DNA binding of latent p53 required both the p53 core domain and the p53 C terminus, whereas high affinity sequence-specific DNA binding of activated p53 was mediated by the p53 core domain alone. The data suggest that high affinity nonspecific DNA binding of latent and high affinity sequence-specific binding of activated p53 to double-stranded DNA differ in their requirement for the C terminus and involve different structural features of the core domain. Because high affinity nonspecific DNA binding of latent p53 is restricted to wild type p53, we propose that it relates to its tumor suppressor functions.

The tumor suppressor p53 is a DNA-binding protein with several DNA binding activities. Of those, sequence-specific DNA binding is the most important one because it mediates the transcriptional activity of p53 (1, 2). Sequence specificity of DNA binding is determined by the recognition of sequences that share homology to the consensus sequence 5'-(RRRC(A/

T)(T/A)GYYY)_n-3', where R is a purine nucleotide, and Y is a pyrimidine nucleotide (3). Although sequence-specific DNA binding of p53 has been analyzed in great detail since its discovery in 1991 (4), the detailed molecular interaction of p53 with its target sequences in promoter elements is still a matter of debate. In particular, the question of whether sequence-specific DNA binding of p53 requires an activation step is discussed controversially (Ref. 5; for review, see Ref. 6). Earlier observations had indicated that unmodified p53 seems to be inactive for sequence-specific DNA binding, whereas various posttranslational modifications in the p53 C-terminal domain, binding of the monoclonal antibody PAb421 that recognizes an epitope within the p53 C terminus, or deletion of the 30 C-terminal amino acids strongly enhance sequence-specific DNA binding under certain *in vitro* conditions (7). The data imply that the C terminus negatively regulates sequence-specific DNA binding of p53 (8). The inhibiting effects of the C terminus were explained by the “conformation” model, which postulates that the p53 protein exists in two distinct conformations termed “latent” (for DNA binding inactive p53) and “activated” (for DNA binding active p53). According to this model, the C-terminal domain in “latent” p53 directly interacts with the core domain, thereby inhibiting sequence-specific DNA binding of p53 (8). A conformational switch that converts latent p53 into an “activated” form relieves the allosteric inhibition. However, recent evidence strongly argues against the conformation model, because the overall conformation of p53 in a DNA binding active or inactive form seems to be quite similar (9). Although the latency concept could also be explained by other models (like *e.g.* the “competition” or “interference” model (10), the most critical argument against p53 latency is that activation of p53 does not seem to be required *in vivo* (11) or in different *in vitro* settings (11–13). Activation thus seems to be restricted to certain *in vitro* assays.

Several features have been delineated that might account for the apparent discrepancies, like type of binding assay (12, 14) and structure of the target DNA (12, 15, 16). In particular, the need for activation of p53 for sequence-specific DNA binding seems to be restricted to the interaction of p53 with short linear DNA and the application of electrophoretic mobility shift assay (EMSA)¹ for the analysis of sequence-specific DNA binding. Therefore, the concern has been raised that the “activation phenomenon” may be an artifact (11, 14). However, numerous

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¹ The abbreviations used are: EMSA, electrophoretic mobility shift assay; FCS, fluorescence correlation spectroscopy; TAMRA, 6-carboxytetramethylrhodamine; CRD, C-terminal regulatory domain; DBD, DNA binding domain.

laboratories have reproducibly observed the activation phenomenon in various assays. Therefore, its disqualification as an "artifact" could be shortsighted, as it might reflect an important and biologically relevant feature of p53-DNA interactions that, for some reasons, is only revealed under certain experimental conditions.

To further address the issue of p53 latency and activation, we employed EMSA and fluorescence correlation spectroscopy (FCS) to the analysis of the binding of p53 to double-stranded DNA oligonucleotides. FCS is based on the measurement of translational diffusion of fluorescent-labeled molecules through a confocal detection volume (10^{-15} liter). The method focuses on the detection of single particles rather than on averages over large numbers of particles as conventional macroscopic fluorescence detection methods. The fluorescence emission from the small detection volume is recorded in a time-resolved manner. Thus, the fluorescence quanta that belong to one fluorescing species can easily be identified by autocorrelating the time-resolved signals. Autocorrelation is a function of the diffusion times and the fractions of the different fluorescing species (17, 18). For the setup used in FCS readers, the optimal concentration range of the fluorescing species is between 1 and 10 nM, guaranteeing a good fluctuation of the fluorescence signal. At higher concentrations the spontaneous fluorescence fluctuation decreases and, thus, the measured signal as well. Differences in size and/or shape of free (unbound) or protein-bound fluorescent-labeled DNA molecules result in different translational diffusion times, thereby allowing quantitative analysis of protein-DNA interactions (*e.g.* K_D and K_I measurements) in solution. Specifically, the DNA-bound p53 protein (~192 kDa for the p53 tetramer) can be quantitatively discriminated from unbound oligonucleotide DNA (~20 kDa) in solution because of a 10-fold difference in molecular mass. Importantly, FCS enables a quantitative analysis of p53-DNA interactions in the absence or presence of various modifiers of p53 DNA binding without the need to "quench" nonspecific DNA interactions using competitor DNA.

Here we provide evidence that latent and activated p53 are able to bind to short double-stranded DNA oligonucleotides with high affinity. However, high affinity DNA binding of latent p53 is not sequence-specific. Sequence-specific recognition of the target DNA requires activation of p53 by PAb421. Activation is accompanied by a moderate increase in binding affinity for specific DNA and a significant drop in the affinity for nonspecific DNA. We propose that high affinity sequence-specific and nonspecific interactions of p53 with DNA require different modes of DNA recognition by the p53 core domain that are regulated by the p53 C terminus.

EXPERIMENTAL PROCEDURES

Protein Purification—Recombinant p53 proteins (human and mouse) expressed in insect cells were isolated as described in Bessard *et al.* (19) and purified by ion-exchange chromatography as described previously (20).

EMSA—DNA binding experiments were performed using 50 ng of recombinant p53 proteins in a reaction mixture containing 5 ng of poly(dI-dC) (Amersham Biosciences) and 2 μ g of bovine serum albumin in 50 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 1 mM dithiothreitol, 20% glycerol, and 50 mM NaCl. After a 20-min preincubation at room temperature 20,000 cpm of the labeled DNA probe was added, and the incubation was continued for an additional 25 min. Samples were loaded onto a 4% native polyacrylamide gel and separated by electrophoresis in 10 mM Tris-HCl (pH 7.8), 0.2 mM EDTA, 1.25 mM sodium acetate, and 8 mM acetic acid at 200 V for 2.5 h at room temperature. After electrophoresis gels were dried and analyzed by autoradiography.

DNA Binding Assay Using FCS—A confocal microscope (ConfoCor; EVOTEC BioSystems and Carl Zeiss, Germany) was used for FCS studies. An attenuated (to about 800 microwatts) beam from an argon ion laser, wavelength 543 nm, was focused to a spot of ~0.25- μ m radius, resulting in a diffusion time of ~60 μ s for 6-carboxytetramethylrh-

damine (TAMRA). The excitation intensity had generally been kept lower than or equal to a level characterized by about 15% amplitude of the triplet term of the autocorrelation function. Fluorescence emission was detected through a pinhole on the focal plane of the microscope using an avalanche photodiode detector SPCM-AQ 131 (EG&G) at 590 nm (bandwidth 35 nm).

For the binding and the competition experiments human or mouse p53 was incubated with the TAMRA-labeled DNA for 15 min at 20 °C. In the case of competition titrations, the competitor was added together with the labeled DNA. In the case of activation of the sequence-specific DNA binding of p53 the protein was preincubated with PAb421 for 15 min at 20 °C before the addition of the DNA. All experiments were performed in 15- μ l binding buffer (phosphate-buffered saline (pH 6.9), 0.05% Tween 20). Virtually identical results were obtained with human and mouse wild type p53. Binding data were fitted according to the standard hyperbolic binding model or according to the Hill equation (where mentioned), applying the fitting program Origin 6.0.

RESULTS

High Affinity Nonspecific DNA Binding Is a Specific Feature of Unmodified Wild Type p53 Protein—Sequence-specific DNA binding of p53 is commonly assessed by EMSA in the presence of competitor DNA to inhibit nonspecific DNA interactions. Depending on the kind of competitor DNA, its effects on sequence-specific DNA binding may greatly vary (10). Nonspecific DNA binding of p53 thus may represent an important parameter influencing sequence-specific DNA binding. We first analyzed by EMSA sequence-specific DNA binding and nonspecific DNA binding of p53 in the absence or presence of the antibody PAb421 ("unmodified" and "PAb421-modified" p53, respectively). PAb421 recognizes an epitope within the p53 C terminus (21), and its binding is thought to mimic the binding of cellular proteins or posttranslational modifications that activate p53 for sequence-specific DNA binding. Unmodified wild type p53 bound with similar apparent affinities to both specific and unspecific DNA (DNA_{spec} and DNA_{unspec} , respectively) in the absence of competitor DNA (Fig. 1, A and B, lanes 2, complex A and A', respectively). The addition of PAb421 strongly enhanced binding to DNA_{spec} (Fig. 1A, lane 3, complex B'), in accordance with the proposed ability of PAb421 to activate sequence-specific DNA binding (7). With DNA_{unspec} , PAb421 only supershifted the complex p53-DNA_{unspec} (Fig. 1B, lane 3, complex B) but did not enhance its formation.

To estimate the impact of unspecific competitor DNA on sequence-specific DNA binding, we analyzed the effects of poly(dI-dC) on DNA binding of p53. Depending on whether or not p53 was modified by PAb421, poly(dI-dC) competed with specific and unspecific DNA with a different dose dependence. Although already the presence of 10 ng poly(dI-dC) almost completely abolished binding of PAb421-modified p53 to DNA_{unspec} (Fig. 1B, lane 7, complex B), complete inhibition of binding of unmodified p53 to DNA_{unspec} required much higher amounts (100 ng) of poly(dI-dC) (Fig. 1B, lane 11, complex A). The data suggest that PAb421-modified p53 binds weaker to unspecific DNA than unmodified p53. A reversed pattern was observed with DNA_{spec} , as poly(dI-dC) much more efficiently competed out binding of unmodified p53 compared with binding of PAb421-modified p53 to DNA_{spec} (Fig. 1A, lanes 2–17, complex A' or B', respectively).

The results indicate that unmodified and PAb421-modified p53 differ in their binding to DNA_{spec} and to DNA_{unspec} . Whereas unmodified p53 binds DNA_{unspec} and DNA_{spec} equally well, the binding affinity of PAb421-modified p53 is shifted toward DNA_{spec} . The weaker binding of PAb421-modified p53 to DNA_{unspec} compared with unmodified p53 suggests that the p53 C terminus is involved in the high affinity interaction of unmodified p53 with DNA_{unspec} . The conclusion is supported by the finding that nonspecific DNA binding of p53 was completely abolished by deletion of the C-terminal regulatory domain

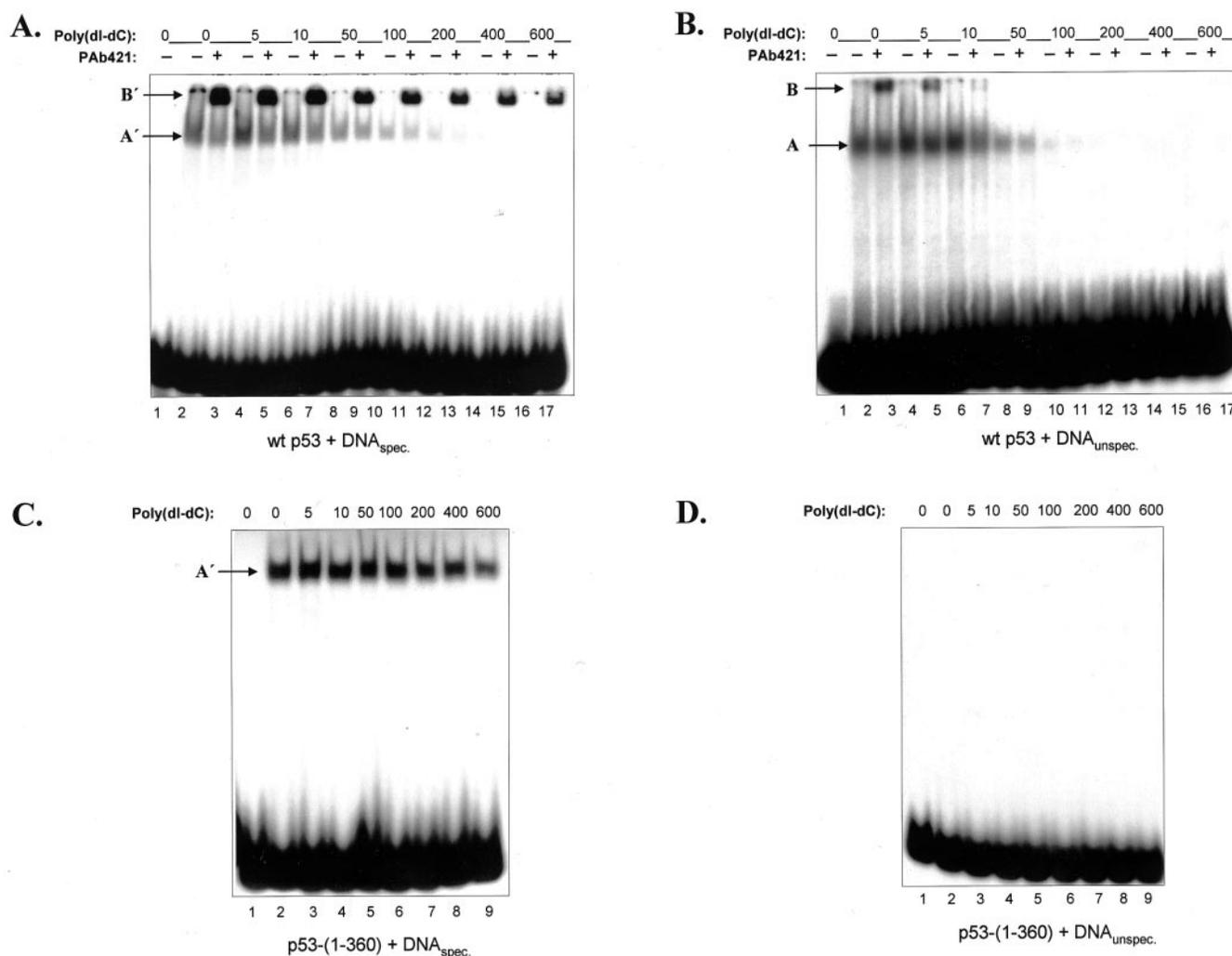


FIG. 1. Analysis of DNA binding by PAB421-modified or unmodified p53 by EMSA. 50 ng of recombinant wild type p53 protein were incubated with DNA_{spec} (A) or DNA_{unspec} (B). Binding was performed either in the presence or in the absence of PAB421. Amounts of poly(dI-dC) present in binding mixtures are indicated. C and D, binding of the p53-(1-360) deletion mutant to DNA_{spec} or DNA_{unspec}, respectively, analyzed under the same conditions as in A and B. Complexes A, A', B, and B' are described in "High Affinity Nonspecific DNA Binding Is a Specific Feature of Unmodified Wild Type p53 Protein" under "Results." wt, wild type.

(CRD), as the deletion mutant p53-(1-360) lacking the CRD strongly bound to DNA_{spec} (Fig. 1C, lanes 2-9) but completely failed to bind DNA_{unspec} (Fig. 1D, lanes 2-9) even in the absence of competitor DNA (Fig. 1D, lane 2). Thus, high affinity nonspecific DNA binding of unmodified p53 requires the CRD.

Importantly, mutant p53 proteins R248P and G245S not only were unable to bind DNA_{spec} but also did not bind DNA_{unspec} regardless of whether poly(dI-dC) was present or not (EMSA data not shown). Thus, the ability to bind unspecific double-stranded DNA with high affinity is an intrinsic biochemical property of wild type p53 that correlates with its potential to bind DNA sequence-specifically.

Unmodified Wild Type p53 Does Not Discriminate between Specific and Unspecific DNA—To quantitatively analyze p53-DNA interactions in solution, we next examined by FCS the effects of PAB421 on DNA binding of wild type p53. Two types of TAMRA-labeled double-stranded DNA oligonucleotides were used, DNA_{spec}, containing a p53 specific binding site, or DNA_{unspec}, lacking such a sequence (see Table I for oligonucleotides used in FCS). The same DNA_{spec} oligonucleotide had been used in the initial study describing the phenomenon of p53 latency and activation (8). We first analyzed DNA binding of unmodified purified wild type p53 in the absence of poly(dI-dC). Binding titrations were performed at a constant concentration of TAMRA-DNA and increasing p53 concentration.

Very similar dissociation constants (K_D) of 17.7 ± 2.1 and 18.5 ± 2.2 nM were determined for the binding of tetrameric unmodified wild type p53 to TAMRA-DNA_{spec} and to TAMRA-DNA_{unspec}, respectively (Fig. 2A and Table II).

The minimal size of p53-specific binding sites corresponds to 20 base pairs and accommodates a single p53 tetramer (1, 4). Although the oligonucleotides used here were only slightly larger (26 base pairs; see Table I), we still considered the possibility that more than one p53 tetramer could bind to a single oligonucleotide molecule. Such a binding behavior would influence the interpretation of the binding data shown above. To analyze the binding mode of p53 to DNA with respect to stoichiometry and cooperativity, we alternatively fitted the binding data applying the Hill equation $y = n \times [p53]^n / (K_D + [p53]^n)$, where y is the portion of DNA bound to p53, and n is the number of p53 binding sites on the DNA (see Table II). The fitting results clearly show that for both, specific and unspecific DNA, the binding stoichiometry is 1:1 for the interaction partners and that no cooperativity is observed. In accordance with our EMSA experiments, mutant p53 R248P did not bind to either DNA, indicating that high affinity unspecific binding to linear DNA is a specific property of wild type p53 (Fig. 2A).

The binding of unmodified wild type p53 to both DNA_{spec} and to DNA_{unspec} with similar high affinity suggested that such p53 was not able to discriminate between specific and unspecific

TABLE I
Oligonucleotides (ODNs) used for binding analysis of p53

The complementary, unlabeled strands are not shown. Double-stranded ODNs obtained by annealing of complementary single-stranded ODNs all have blunt DNA ends. p53 recognition sequences are underlined.

ODN	Sequence (from 5' to 3')
26-mer TAMRA-DNA _{spec}	TAMRA-AGCTTAGACATGCCTAGACATGCCTA
DNA _{spec}	AGCTTAGACATGCCTAGACATGCCTA
35-mer DNA _{unspec}	TATGTCTAAGGGACCTGCGGTTGGCATTGATCTTG
26-mer TAMRA-DNA _{unspec}	TAMRA-TATGTCTAAGGGACCTGCGGTTGGCA

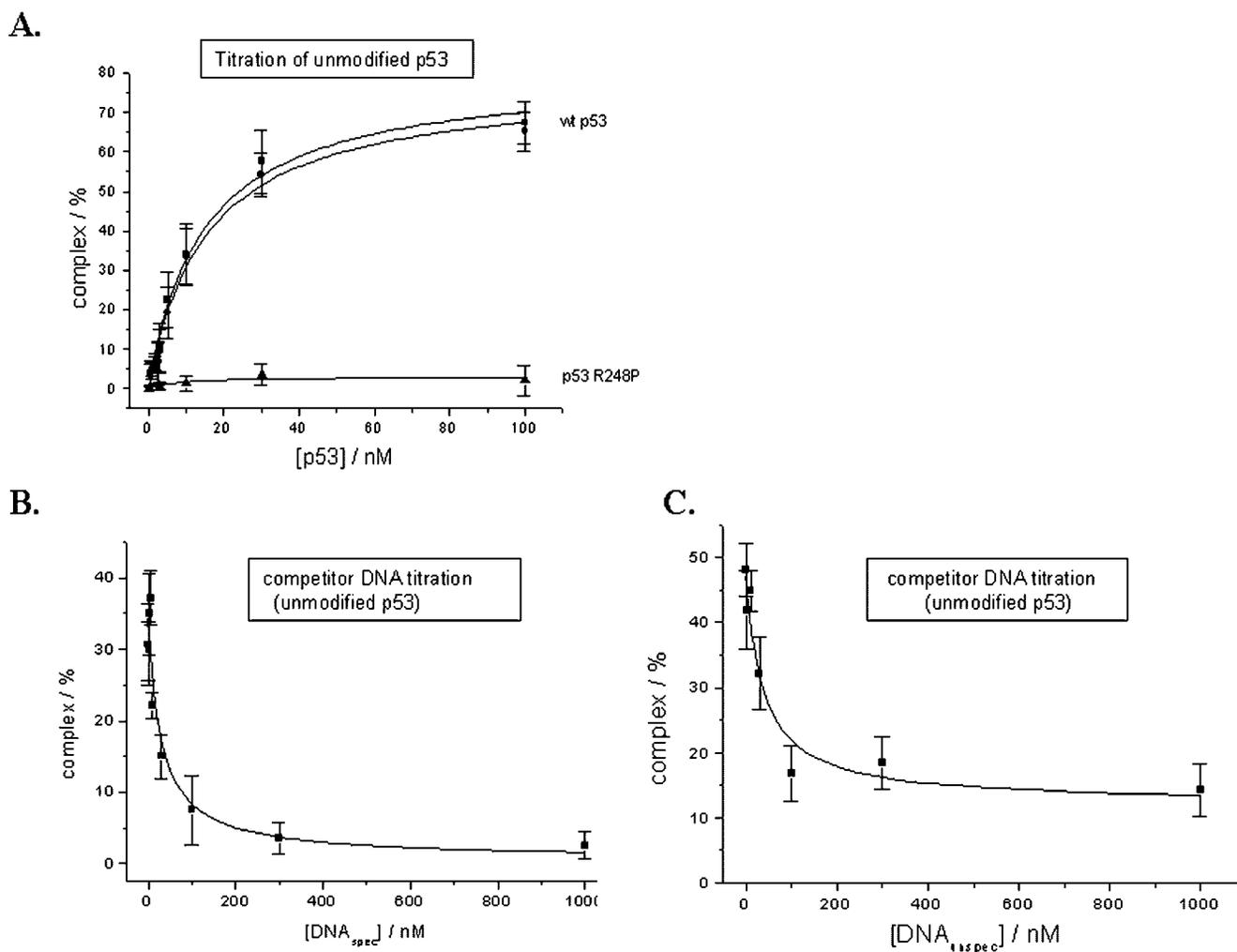


FIG. 2. **Characterization of DNA binding specificity of unmodified wild type p53 by FCS.** A, protein titration experiments were performed to determine the binding affinity of wild type (*wt*) p53 and of mutant p53 R248P to DNA_{spec} and DNA_{unspec} using FCS. Titrations were performed at a constant concentration of TAMRA-labeled DNA (1 nM). The concentration of tetrameric p53 was varied. ■, p53 wild type + TAMRA-DNA_{spec}; ●, p53 wild type + 26-mer TAMRA-DNA_{unspec}; ▲, p53 R248P + TAMRA-DNA_{spec}. B, displacement of TAMRA-DNA_{spec} bound to unmodified wild type p53 with unlabeled DNA_{spec}. The concentrations of tetrameric p53 (20 nM) and TAMRA-DNA_{spec} (1 nM) were constant. The concentration of unlabeled DNA_{spec} was varied. C, displacement of TAMRA-DNA_{spec} bound to non-modified wild type p53 with unlabeled DNA_{unspec}. The concentrations of tetrameric p53 (20 nM) and of TAMRA-DNA_{spec} (1 nM) were constant. The concentration of unlabeled DNA_{unspec} was varied.

DNA. The conclusion was corroborated by competition experiments which showed that TAMRA-DNA_{spec} bound by unmodified p53 was displaced from the p53-DNA complex by unlabeled DNA_{spec} and DNA_{unspec} with comparable efficiencies ($K_I = 19.2 \pm 6.0$ and 21.1 ± 4.5 nM, respectively, Fig. 2B and C, Table II). The results of our FCS experiments are in accordance with our EMSA data and show that unmodified wild type p53 does not discriminate between sequence-specific and unspecific DNA and binds with high affinity to double-stranded linear DNA independent of the presence of a cognate binding motif.

PAb421 Increases the Binding Affinity of Wild Type p53 for DNA_{spec} While Concomitantly Decreasing the Binding Affinity for DNA_{unspec}.—We next analyzed the effects of PAb421 on the

binding of p53 to DNA_{spec} and to DNA_{unspec}. Fig. 3A and Table II show that the affinity of PAb421-modified p53 for TAMRA-DNA_{spec} increased only moderately, *i.e.* by a factor of ~ 15 ($K_D = 1.1 \pm 0.2$ nM). In accordance, the K_I for DNA_{spec} also dropped to 0.7 ± 0.4 nM (Fig. 3B, Table II). In contrast to the increase in affinity for TAMRA-DNA_{spec} by PAb421, the affinity of PAb421-modified p53 for TAMRA-DNA_{unspec} was greatly reduced ($K_D = 169 \pm 17$ nM, Fig. 3A and Table II). The net outcome of these adverse effects of PAb421 on the respective p53-DNA interactions is a drastic (150-fold) difference in the affinity of PAb421-modified p53 for DNA_{spec} compared with DNA_{unspec}. Competition experiments using the 35-mer DNA_{unspec} used in the initial study describing the latency

TABLE II
 K_D and K_I values of wild type p53 interactions with DNA

DNA	Binding affinity (standard hyperbolic fitting)	Binding affinity and Hill coefficient (Hill equation ^a)	Location of results	poly (dI-dC)	PAb421
TAMRA-DNA _{spec}	$K_D = 17.7 \pm 2.1$ <i>nM</i>	$K_D = 27.9 \pm 9.1$ nM $n = 1.3 \pm 0.1$	Fig. 2A	-	-
TAMRA-DNA _{spec}	Not detectable		Fig. 5	+	-
26-mer TAMRA-DNA _{unspec}	$K_D = 18.5 \pm 2.2$	$K_D = 30.2 \pm 8.7$ nM $n = 1.3 \pm 0.1$	Fig. 2A	-	-
DNA _{spec}	$K_I = 19.2 \pm 6.0$		Fig. 2B	-	-
35-mer DNA _{unspec}	$K_I = 21.1 \pm 4.5$		Fig. 2C	-	-
TAMRA-DNA _{spec}	$K_D = 1.1 \pm 0.2$	$K_D = 1.0 \pm 0.2$ nM $n = 1.4 \pm 0.1$	Fig. 3A	-	+
TAMRA-DNA _{spec}	$K_D = 2.9 \pm 0.5$		Fig. 5	+	+
26-mer TAMRA-DNA _{unspec}	$K_D = 169 \pm 17$	$K_D = 627 \pm 170$ nM $n = 1.3 \pm 0.1$	Fig. 3A	-	+
DNA _{spec}	$K_I = 0.7 \pm 0.4$		Fig. 3B	-	+
35-mer DNA _{unspec}	$K_I = 2700 \pm 1300$		Fig. 3C	-	+

^a Hill equation: $y = n \times [p53]^n / (K_D + [p53]^n)$ where y is the degree of DNA bound to p53, and n is the number of binding sites of p53 on DNA.

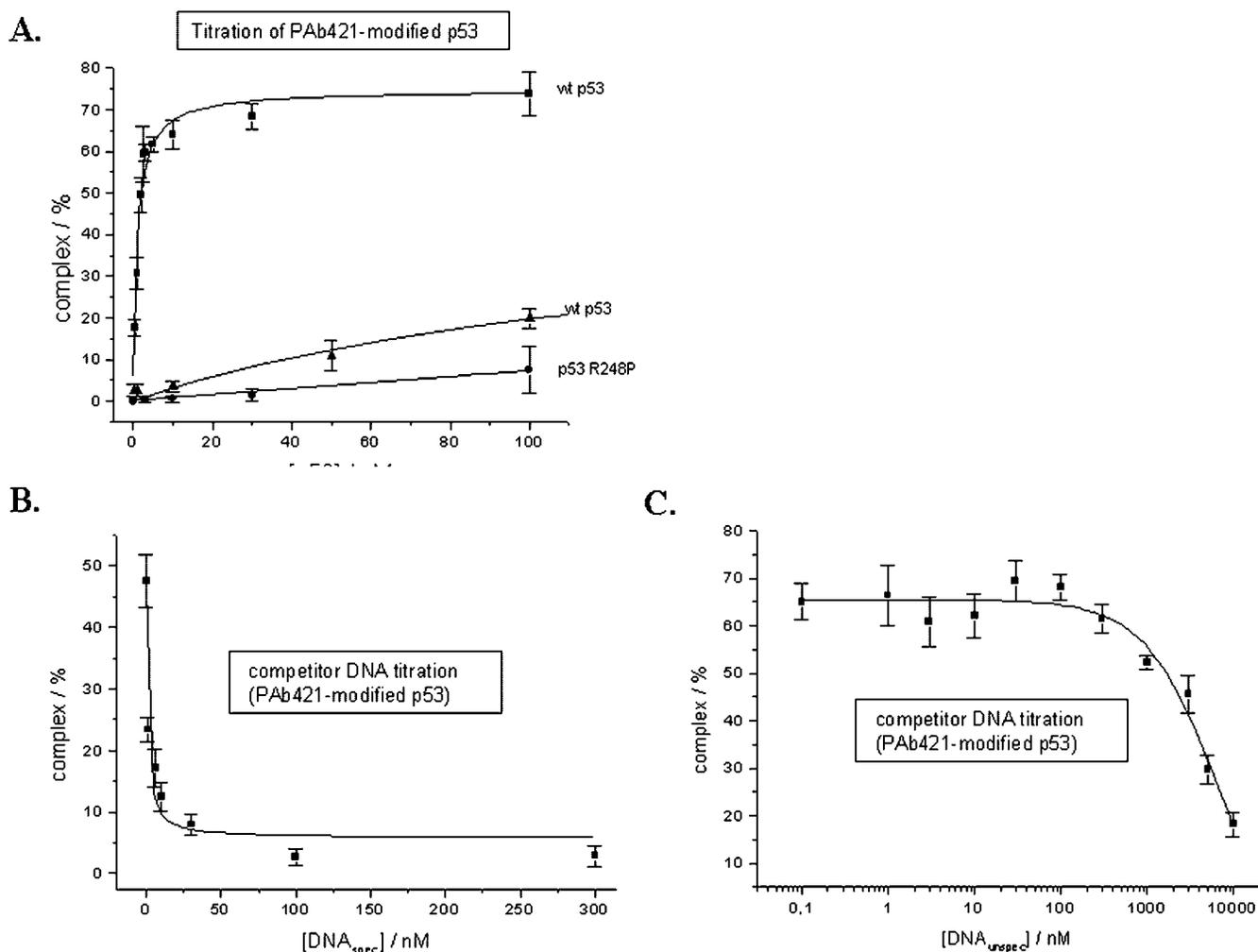


FIG. 3. DNA binding specificity of PAb421-modified wild type p53 analyzed by FCS. A, protein titration experiments were performed to determine the binding affinity of wild type (*wt*) p53 to DNA_{spec} and DNA_{unspec} after the addition of PAb421 (250 nM). Titrations were performed at a constant concentration of TAMRA-labeled DNA (1 nM). The concentration of tetrameric p53 was varied. ■, wild type p53 + TAMRA-DNA_{spec}; ▲, wild type p53 + 26-mer TAMRA-DNA_{unspec}; ●, mutant p53 R248P + TAMRA-DNA_{spec}. B, displacement of TAMRA-DNA_{spec} bound to PAb421-modified wild type p53 with DNA_{spec}. The concentrations of tetrameric p53 (2 nM), of TAMRA-DNA_{spec} (1 nM), and PAb421 (250 nM) were constant. The concentration of DNA_{spec} was varied. C, displacement of TAMRA-DNA_{spec} bound to PAb421-modified wild type p53 with DNA_{unspec}. The concentrations of tetrameric p53 (2 nM), TAMRA-DNA_{spec} (1 nM), and PAb421 (250 nM) were constant. The concentration of DNA_{unspec} was varied.

phenomenon (8), showed an even larger (~3,000-fold) difference in the affinity of PAb421-modified p53 to unspecific DNA. The 35-mer DNA_{unspec} was only able to effectively displace TAMRA-DNA_{spec} from the complex with PAb421-modified p53

with a K_I of 2700.0 ± 1300 nM (Fig. 3C, Table II). The results imply that modification of p53 by PAb421 leads to a significant reduction of its binding affinity for unspecific DNA, with the degree of reduction possibly varying with DNA length. Further

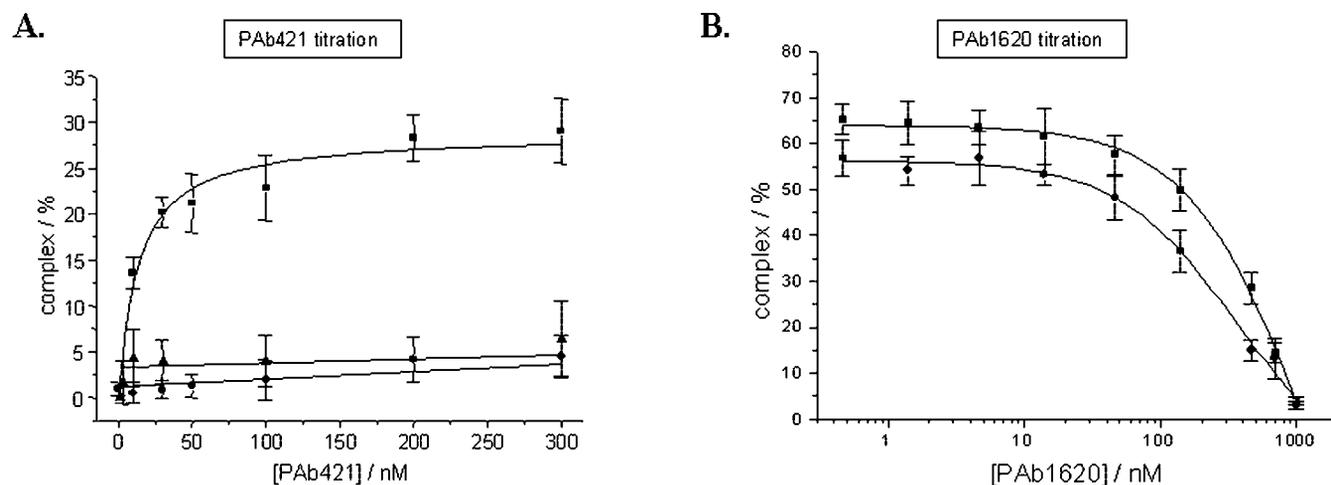


FIG. 4. Influence of p53 specific antibodies on p53 binding to DNA_{spec}. *A*, enhancement of p53 DNA_{spec} binding by monoclonal antibody PAb421 at a low p53:DNA ratio. The concentrations of tetrameric p53 (2 nM) and of TAMRA-DNA_{spec} (1 nM) were constant. The concentration of PAb421 varied. ■, p53 + TAMRA-DNA_{spec} + PAb421; ▲, no p53 + TAMRA-DNA_{spec} + PAb421. ●, N-terminal PAb1801 was added to p53-DNA_{spec} complexes in the same amounts as PAb421. *B*, dissociation of mouse wild type p53-DNA complexes by the conformation-dependent monoclonal antibody PAb1620. Displacement of TAMRA-DNA_{spec} bound to PAb421-modified, mouse wild type p53 (3 nM) (■) and to unmodified mouse wild type p53 (30 nM) (●) with PAb1620 using FCS. The concentrations of tetrameric p53, PAb421 (250 nM), and DNA_{spec} (1 nM) were constant. The concentration of PAb1620 was varied. Because of the difference in binding affinities of unmodified and PAb421-modified p53 to DNA_{spec} (see Table II), a 10-fold higher concentration of unmodified p53 compared with PAb421-modified p53 was used to achieve comparable p53-DNA complex formation.

analysis of the binding data using the Hill equation showed that PAb421 did not influence stoichiometry or cooperativity of binding (Table II). As expected, mutant p53 R248P did not bind to DNA_{spec} even in the presence of PAb421 (Fig. 3A).

Again, the results of the FCS analyses are concordant with our EMSA data (Fig. 1). The data suggest that PAb421 modulates the specificity of p53 DNA binding rather than increasing its binding affinity for DNA_{spec}. We propose that PAb421 has a dual effect on p53, leading to sequence-specific recognition of DNA_{spec} accompanied by a significant decrease in the affinity of p53 for DNA_{unspec}.

Fig. 4A demonstrates another important aspect of the activation of p53 DNA binding by PAb421. At low molar ratios of p53:DNA_{spec} (2:1 nM in Fig. 4A), DNA binding by unmodified p53 was hardly detectable because the concentrations of the binding partners were much below the K_D of 17.7 ± 2.1 nM (see Fig. 2A). Under such conditions, the addition of PAb421 strongly enhanced the fraction of DNA_{spec} bound by p53. The enhancement, however, can be solely explained by the moderately higher affinity of PAb421-modified p53 to DNA_{spec} ($K_D = 1.1 \pm 0.2$ nM) compared with that of unmodified p53 ($K_D = 17.7 \pm 2.1$ nM). Fig. 4A thus exemplifies that under certain conditions even a moderate difference in the binding affinity of p53 to a given DNA substrate can be relevant. In addition, Fig. 4A, as an important control, shows that PAb421 as such does not bind DNA. Furthermore, activation of p53 is specific for PAb421, because the addition of PAb1801, binding to an epitope in the p53 N terminus (22), did not have an effect on p53 binding to DNA_{spec}.

The results of our FCS and EMSA experiments led to the following conclusions. First, high affinity nonspecific DNA binding is an intrinsic property of unmodified wild type p53. High affinity nonspecific DNA binding usually is not seen in EMSA due to the routine use of unspecific competitor DNA like poly(dI-dC). Second, FCS as well as EMSA experiments showed that mutant p53 proteins containing a hot-spot mutation were not able to bind linear double-stranded DNA with high affinity in the absence or presence of either poly(dI-dC) or PAb421 (Figs. 2A and 3A). Therefore, not only high affinity sequence-specific DNA binding by PAb421 modified p53 but also high

affinity nonspecific DNA binding by unmodified p53 is restricted to wild type p53. Third, modification of the p53 protein by PAb421 mainly modulates the specificity of p53 DNA binding by enabling sequence-specific recognition of DNA_{spec} rather than by enhancing the affinity of p53 for DNA_{spec}. The latter conclusion is also supported by the FCS experiments shown in Fig. 5; in the presence of excess poly(dI-dC), binding of unmodified p53 to DNA_{spec} was not detectable due to high affinity nonspecific DNA binding of p53 to competitor poly(dI-dC) DNA. However, the addition of PAb421 changed the specificity of p53 toward a preferred binding of DNA_{spec}. PAb421 reduced the affinity of p53 for nonspecific DNA binding, thereby mediating high affinity DNA_{spec} binding in the presence of excess unspecific competitor DNA. Very similar results were obtained with human and mouse wild type p53 analyzed in parallel (data not shown), further supporting the notion that human and mouse wild type p53 do not differ significantly in their DNA binding properties.

High Affinity Binding of Unmodified p53 to Double-stranded DNA Requires Both the p53 Core Domain and the p53 C-terminal Domain—The results so far indicate that high affinity nonspecific DNA binding by unmodified p53 differs strikingly from high affinity sequence-specific DNA binding by PAb421-modified p53. As demonstrated previously (7) and shown in Fig. 1C, deletion of the C-terminal regulatory domain has similar effects on the DNA binding properties of p53 as on the addition of PAb421. We therefore analyzed by FCS the binding of DNA_{spec} and of DNA_{unspec} to p53-(1–360) in the absence of competitor poly(dI-dC) DNA. Although strongly binding to DNA_{spec} (Fig. 6A) p53-(1–360) did not bind to DNA_{unspec}. Therefore, binding of DNA_{spec} and DNA_{unspec} to p53-(1–360) qualitatively resembles binding to PAb421-modified p53. Quantitative evaluation demonstrated that p53-(1–360) bound DNA_{spec} with a K_D of 16.1 ± 2.1 nM, *i.e.* with an about 15-fold lower affinity as PAb421-modified p53 ($K_D \sim 1.1 \pm 0.2$ nM, Table III). Competition experiments (Fig. 6B) verified that p53-(1–360) bound DNA_{spec} with a much higher affinity than DNA_{unspec}, as DNA_{spec} was able to compete for TAMRA-DNA_{spec} with a K_I of 3.9 ± 0.5 nM, whereas the K_I for DNA_{unspec} was 1101 ± 226 nM (Table III).

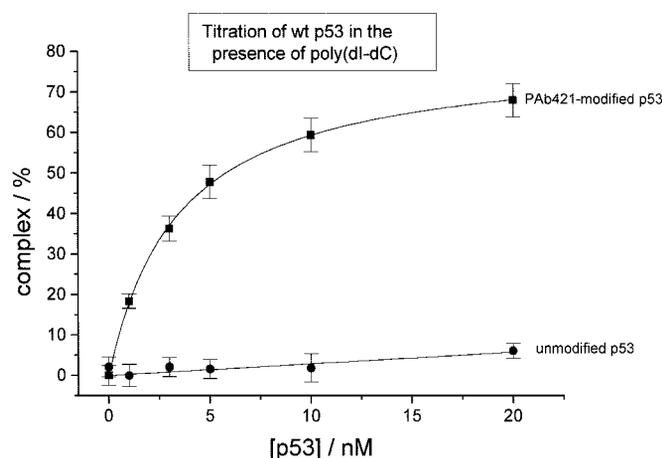


FIG. 5. Influence of poly(dI-dC) on the binding of PAb421-modified wild type p53 to DNA_{spec}. Binding titrations using FCS were performed to determine the binding affinity of wild type (*wt*) p53 to TAMRA-DNA_{spec} in the absence and presence of activating PAb421 (250 nM) and in the presence of poly(dI-dC) (1 ng/ μ l). Titrations were performed at a constant concentration of TAMRA-labeled DNA (1 nM). The concentration of tetrameric p53 was varied. ■, p53 + TAMRA-DNA_{spec} + PAb421; ●, p53 + TAMRA-DNA_{spec}.

The results are in accordance with results of EMSA experiments showing that p53-(1–360) bound strongly to DNA_{spec}, whereas it failed to bind to DNA_{unspec} even in the absence of poly(dI-dC) (Fig. 1D). The findings imply that high affinity nonspecific DNA binding of wild type p53 requires the p53 C-terminal domain. Modification of this domain by PAb421 or its deletion strongly reduces the affinity of p53 for sequence unspecific DNA binding but unravels its sequence-specific DNA binding properties.

Unspecific DNA binding of p53 can be mediated by the isolated C-terminal domain and the central DNA binding domain (DBD) of p53 (23, 24). Our FCS and EMSA results demonstrate that C-terminally-truncated p53 is unable to bind DNA nonspecifically, although it contained an intact DBD, arguing for a significant role of the DBD in high affinity nonspecific DNA binding. To test the contribution of the p53 DBD to high affinity nonspecific DNA binding, we first analyzed by FCS the binding to p53 Δ DBD, *i.e.* to p53 with a deleted DBD (amino acids 110–280). No binding of p53 Δ DBD was observed with either DNA_{spec} or DNA_{unspec} (data not shown), indicating that the C terminus cannot mediate DNA binding in the absence of DBD. It thus appears that both the C-terminal domain and the DBD mediate nonspecific DNA binding in an inter-dependent fashion.

To further assess the impact of the DBD in nonspecific and sequence-specific DNA binding, we examined the effects of the monoclonal antibody Pab1620 directed against a conformational epitope on the DBD of wild type p53 (25). Pab1620 recognizes p53 in a wild-type conformation, and its epitope is lost when the DBD becomes mutated or denatured (26). Pab1620 previously has been shown to displace mouse (but not human) wild type p53 from the complex with specific DNA formed by the core domain (27). We analyzed the effects of Pab1620 on the binding of either unmodified (exerting nonspecific DNA binding) or Pab421-modified (exerting sequence-specific DNA binding) mouse p53 to TAMRA-DNA_{spec}. Because unmodified p53 does not discriminate between DNA_{unspec} and DNA_{spec}, binding of unmodified p53 to DNA_{spec} reflects high affinity nonspecific DNA binding. The results show that Pab1620 released TAMRA-DNA_{spec} from the complex with unmodified mouse p53 with comparable efficiency as from Pab421-modified mouse p53 (Fig. 4B). The findings indicate

that the p53 DBD is involved in high affinity nonspecific DNA binding by unmodified p53 as well as in sequence-specific DNA binding of PAb421-modified p53. Altogether our results show that two binding domains on p53, the DNA binding domain and the C terminus, engage in the high affinity nonspecific interaction of p53 with DNA in an inter-dependent manner.

DISCUSSION

In this study we further investigated the phenomenon of p53 latency and activation, applying fluorescence correlation spectroscopy and EMSA. The concept of p53 as a latent DNA-binding protein that needs further activation by posttranslational modification or binding of activator proteins recently has been challenged by several independent studies, and it even has been suggested that activation might be an artifact of certain *in vitro* DNA binding assays (11). However, the present study demonstrates that the phenomenon of a Pab421-dependent enhancement of sequence-specific DNA binding is not an experimental artifact but, rather, reflects an intrinsic feature of the interaction of wild type p53 with DNA. Therefore, clarification of its molecular basis could provide new insights into the complex interactions of p53 with DNA that are relevant for understanding p53 function *in vivo*.

Our studies using FCS and EMSA provide evidence for two completely different modes of high affinity interactions of p53 with double-stranded DNA; unmodified p53 bound to such DNA in a sequence-independent manner, whereas PAb421-modified p53 was only able to bind with high affinity to DNA_{spec}, but its affinity for DNA_{unspec} was greatly reduced. Our data show that high affinity nonspecific DNA binding involves two binding domains of p53, DBD and the CRD. Inactivation of either binding domain abolished high affinity nonspecific DNA binding. The finding demonstrates that the contribution of both binding domains to high affinity nonspecific DNA binding is additive. In contrast, high affinity sequence-specific DNA binding of PAb421-modified p53 is mediated mainly by the DBD and does not require the p53 CRD. We conclude that the DBD of PAb421-modified p53 binds to DNA_{spec} with higher affinity than the DBD of unmodified p53 to DNA_{unspec}. The conclusion is supported by the high affinity binding of p53-(1–360) to DNA_{spec} but not to DNA_{unspec}. In line with this interpretation is the finding that high affinity nonspecific DNA binding of unmodified p53 requires the CRD, whereas high affinity binding of DNA_{spec} by p53-(1–360) does not.

We recently reported that unmodified p53 strongly binds in a sequence-dependent manner to DNA presented in a non-B DNA conformation (12), already demonstrating that unmodified p53 is not a latent, but an active DNA-binding protein. However, the binding of unmodified or PAb421-modified p53 to double-stranded DNA oligonucleotides described in this study differs strikingly from the interactions of p53 with non-B DNA. Whereas Pab421 strongly inhibits sequence-specific binding of p53 to non-B DNA (12), the same modification enhances sequence-specific recognition of double-stranded DNA_{spec} (this study). Thus the terms latent and activated p53 still apply for sequence-specific interactions of p53 with double-stranded linear DNA.

Activation of sequence-specific binding to double-stranded DNA by Pab421 consists of two different but probably interconnected processes, inhibition of nonspecific DNA binding and activation of sequence specific recognition of the target DNA by the p53 DBD. Because the DBD is involved in both types of interactions, one must assume that it can mediate various types of p53 interactions with nucleic acids. Indeed, the DBD of wild type p53 mediates versatile DNA interactions, including high affinity binding to Holliday junctions (28), to DNA-mim-

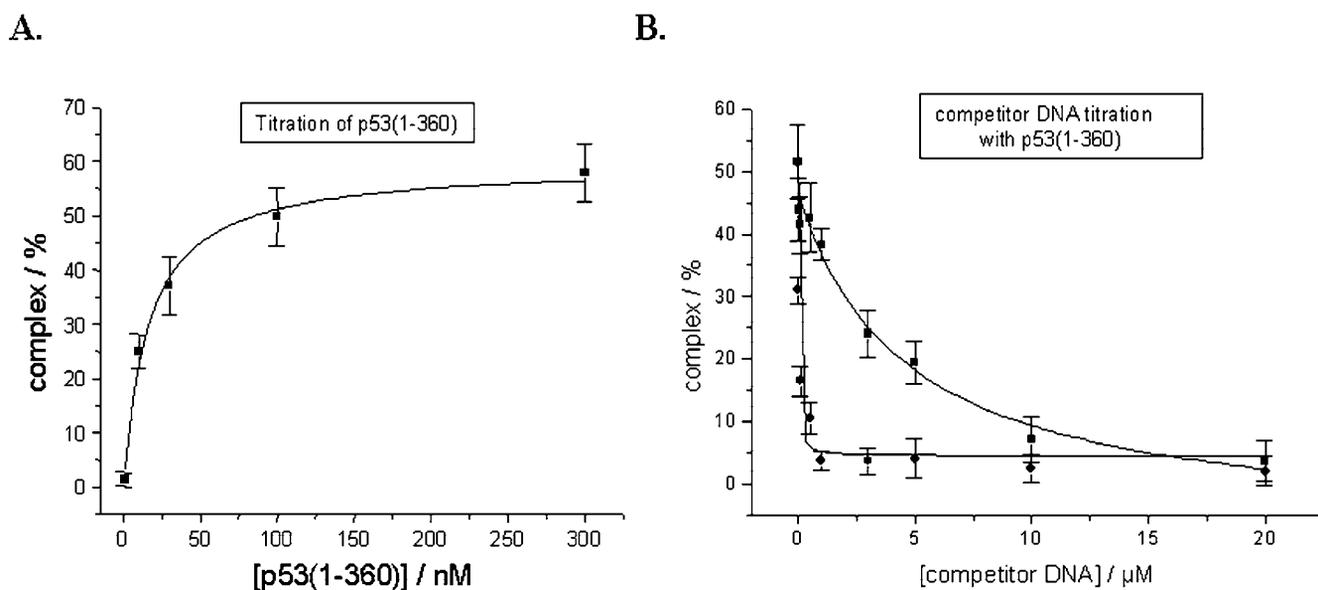


FIG. 6. FCS analysis of DNA binding specificity of the p53(1-360) protein. A, protein titrations were performed at a constant concentration of TAMRA-DNA_{spec} (1 nM) to determine the binding affinity of p53(1-360) to TAMRA-DNA_{spec} by FCS. The concentration of p53(1-360) was varied. B, displacement of TAMRA-DNA_{spec} bound to p53(1-360) with DNA_{spec} (■) and DNA_{unspec} (●) using FCS. The concentrations of tetrameric p53 (50 nM) and of TAMRA-DNA_{spec} (1 nM) were constant. The concentration of the competitor DNA was varied.

TABLE III
 K_D and K_I values of p53 deletion mutant p53(1-360) interactions with DNA

DNA	Binding affinity	Location of results
<i>nM</i>		
TAMRA-DNA _{spec}	$K_D = 16.1 \pm 2.1$	Fig. 6A
DNA _{spec}	$K_I = 3.9 \pm 0.5$	Fig. 6B
35mer DNA _{unspec}	$K_I = 1101 \pm 226$	Fig. 6B

icking recombination intermediates (29) and to RNA stem-loop structures (30). Finally, the p53 DBD also mediates the p53 exonuclease activity (31). The instrumental role of the CRD in regulating the binding of the DBD to various types of DNA probably involves conformational changes in the DBD. The assumption is supported by the recent analysis of the crystal structure of the mouse p53 DBD, which suggested that this domain can undergo structural rearrangements (32). As an important conclusion of our data we postulate that structural features of the DBD favoring high affinity nonspecific DNA binding in unmodified wild type p53 are different from those required for sequence-specific DNA binding by the DBD in PAb421-modified or C-terminally-truncated forms of p53. Our observation that point mutations in the DBD abolish nonspecific DNA binding of mutant p53 proteins supports the hypothesis and demonstrates the specificity of high affinity nonspecific DNA binding for wild type p53.

The physiological significance of the activation phenomenon observed with double-stranded DNA remains unclear. At least three principally different possibilities regarding the role of an activated sequence-specific binding to double-stranded DNA can be envisioned. (i) Activation may be important for the transcriptional activation of a subset of p53-regulated promoters that contains p53 response elements in a conformation resembling linear DNA. In such a scenario, selective activation of p53 binding to either B or to non-B target sites would provide an effective means for the selective transcriptional activation of p53 target genes (for review, see Ref. 6). (ii) High affinity nonspecific interactions of p53 with DNA might be important for the initiation of transcriptional activation of p53-regulated promoters. High affinity nonspecific interactions of p53 with DNA could keep p53 tightly associated with genomic DNA,

thereby ensuring that binding of p53 to specific target sites occurs promptly when the p53 transcriptional response must be quickly initiated upon genotoxic stress. In this context the tight association of p53 with multiple nonspecific sites in genomic DNA might as well be important for modulating the level of the p53 protein. It has been demonstrated that the binding of p53 to DNA protects it from ubiquitin-dependent proteolytic degradation (33). In this regard, it is important to note that different amounts of the p53 protein are required for various p53 responses, such as cell cycle arrest or apoptosis (34). (iii), high affinity nonspecific DNA binding of latent p53 and sequence-specific DNA binding of activated p53 may reflect DNA interactions that are not relevant for transcription-dependent functions of p53, but rather, to functions of p53 in chromatin remodeling. Considering the ability of p53 to interact with proteins influencing DNA topology such as topoisomerases (35, 36), helicases (37), and with chromatin-remodeling complex SWI/SNF (38), one could envision that switching of p53 from nonspecific to sequence-specific binding in genomic DNA would lead to the redistribution of p53-associated chromatin-remodeling factors and, thereby, to changes in chromatin structure. In such a scenario the effects of p53 on chromatin topology would be considerable, as a large number of p53 consensus-like sequences are predicted to exist in the human genome (39, 40). Recently it has been reported that wild type p53 can act as a chromatin accessibility factor (41), a function that is likely to require high affinity nonspecific DNA binding by p53. This property also could relate to the recent report that latent wild type, but not mutant p53 protein, can prevent aggregation of DNA *in vitro* (42), an activity that is abolished by modification of PAb421. On the other hand, recent evidence indicates that some genomic sequences that conform to the p53 consensus and to which PAb421-modified p53 binds *in vitro* cannot mediate transcriptional activation (43, 44). Also in these cases p53-induced changes in chromatin structure might be important for the biological outcome. For example, binding of p53 to the α -fetoprotein promoter, which is repressed by p53, causes structural changes in the chromatin distally from the binding site (45). It could be envisioned that a switch from the nonspecific to the sequence-specific mode of DNA binding will lead to changes in specific structural profiles in chromatin.

Although the implications of high affinity nonspecific DNA binding of p53 for its function as a tumor suppressor are not yet known, we propose that nonspecific high affinity DNA binding of latent wild type p53 is an important feature that needs to be further characterized. Our finding that this type of interaction is lost in mutant p53 proteins suggests that it will be relevant for the tumor suppressor functions of p53.

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