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Investigations of the supercoil-selective DNA binding of wild type p53 suggest a novel mechanism for controlling p53 function

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The tumor suppressor protein, p53, selectively binds to supercoiled (sc) DNA lacking the specific p53 consensus binding sequence (p53CON). Using p53 deletion mutants, we have previously shown that the p53 C-terminal DNA-binding site (CTDBS) is critical for this binding. Here we studied supercoil-selective binding of bacterially expressed full-length p53 using modulation of activity of the p53 DNA-binding domains by oxidation of cysteine residues (to preclude binding within the p53 core domain) and/or by antibodies mapping to epitopes at the protein C-terminus (to block binding within the CTDBS). In the absence of antibody, reduced p53 preferentially bound scDNA lacking p53CON in the presence of 3 kb linear plasmid DNAs or 20 mer oligonucleotides, both containing and lacking the p53CON. Blocking the CTDBS with antibody caused reduced p53 to bind equally to sc and linear or relaxed circular DNA lacking p53CON, but with a high preference for the p53CON. The same immune complex of oxidized p53 failed to bind DNA, while oxidized p53 in the absence of antibody restored selective scDNA binding. Antibodies mapping outside the CTDBS did not prevent p53 supercoil-selective (SCS) binding. These data indicate that the CTDBS is primarily responsible for p53 SCS binding. In the absence of the SCS binding, p53 binds sc or linear (relaxed) DNA via the p53 core domain and exhibits strong sequence-specific binding. Our results support a hypothesis that alterations to DNA topology may be a component of the complex cellular regulatory mechanisms that control the switch between latent and active p53 following cellular stress.

Keywords: monoclonal antibodies; p53 latency; redox state; supercoil-selective DNA binding; tumor suppressor protein p53.

The tumor suppressor protein p53 has been called 'the guardian of the genome' due to its functions in maintaining genetic integrity of cells (reviewed in [1–4]). Mutations of the *p53* gene are frequently connected with malignant transformation. Under stress conditions, wild type p53 acts as transcriptional activator for genes including *p21*, *gadd45*, *bax* and *mdm2* [5]. In addition, it has been proposed that in normal cells p53 participates in DNA replication, recombination and repair in a transcription-independent manner [6,7].

The biological activities of p53 are closely connected with its ability to interact with DNA. The protein is organized into several functional domains [8]. The N-terminal domain contains a transactivation region [amino acids (aa) 1–42] and a proline-rich region (aa 61–94), and mediates interactions with other transcription factors or the mdm2 protein. The evolutionary conserved core domain (CD; aa ≈ 100–300) is involved in sequence-specific binding to p53 response elements (consensus sequences; p53CONs) that occur within promoters of p53 downstream genes [9]. This domain also exhibits sequence nonspecific binding to internal regions of single-stranded and double-stranded DNA [10], conformation-specific interaction with DNA motifs mimicking early recombination intermediates [11,12], hairpin DNA structures [13] and insertion/deletion mismatches [14]. The C-terminal part of the protein contains a tetramerization domain (aa 325–356) and the basic DNA-binding site (CTDBS; aa 363–382). The CTDBS binds DNA sequence nonspecifically but exhibits a remarkable selectivity for certain DNA structures, including single-stranded DNA ends [10], single-stranded gaps within double-stranded DNA molecules [15], γ-irradiated [16] or *cis*-platinated DNA [17,18], and supercoiled DNA [19–24].

Regulation of the p53 DNA-binding activities is achieved through post-translational modifications of the protein molecule [4,25–27]. In the unmodified protein, a segment of the C-terminus (aa 369–383), overlapping with

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Abbreviations: aa, amino acids; CD, core domain; CON, 20 mer ODN spanning the p53CON; CTDBS, C-terminal DNA-binding site; fl, full length; linDNA, linearized DNA; ocDNA, open circular DNA; ODN, oligodeoxyribonucleotide; NODN, nonspecific 20 mer ODN; $p53^{red}$, reduced p53; $p53^{ox}$, oxidized p53; p53CON, p53 consensus DNA binding sequence; relDNA, relaxed DNA; scDNA, supercoiled DNA; SCS, supercoil-selective.

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the p53 CTDBS [28], acts as a negative regulator of sequence-specific binding and is connected with the apparent p53 'latency' typically observed in unstressed cells. Latency of p53 has been explained either by allosteric control of the CD via intramolecular protein–protein interactions [25,29] or by strong nonspecific p53–DNA binding mediated by the unmodified CTDBS, preventing the p53 core from interacting efficiently with the consensus sequences [30–32]. The negative regulatory effect of the C-terminus is abolished by phosphorylation [25,33], acetylation [34,35], deletion [25] or blocking by noncovalent effectors including antibodies [26,36,37], cellular proteins such as Ref1 [26], or short nucleic acid molecules [10,28,30]. Another mechanism that may be involved in regulating DNA binding activity of the CD is connected with changes of the protein redox state [9,21,26,38,39]. The p53 core contains 10 cysteine residues; these residues are absent in other parts of the p53 molecule [40]. In general, the reduced state of cysteines is critical for the proper DNA binding function of the p53 core. It has been shown that extensive oxidation of the p53 thiol groups results in a loss of DNA binding within the p53 core while controlled oxidation of Cys277 leads to altered sequence-specificity of the p53–DNA interaction [9]. DNA binding activities within the p53 C-terminus are not prevented by protein oxidation [21,22,39].

Full length (fl) p53 and the protein deletion variants possessing the CTDBS exhibit a distinct interaction with supercoiled (sc) DNA (independent of the presence of the p53CON), forming stable p53–DNA complexes that can be observed as band ladders in agarose gels [19–21,23]. Electron microscopy revealed formation of nucleoprotein filaments at higher protein/DNA ratios [23]. In competition experiments with linear (lin) DNA, fl p53 binds scDNA with a high preference (supercoil-selective; SCS binding) [20,23]. Recent observations suggest that DNA topology and DNA conformation transitions related to DNA supercoiling also markedly affect p53 binding to the p53CONs, resulting in either stimulation [41] or inhibition [42] of sequence-specific interactions. By means of protein deletion studies [23], the ability of p53 to recognize scDNA was attributed primarily to the protein C-terminus. Truncated p53 forms lacking the CTDBS were unable to selectively bind scDNA, while isolated p53 C-terminal domains exhibited SCS binding. It has been proposed that SCS binding involves cooperative interactions of the oligomeric p53 C-terminal domain with two segments of the DNA double helix within the plectonemic DNA superhelix, and stabilization of the complexes (filaments) by further protein–DNA and protein–protein interactions [23].

In this paper we employed redox modulation of the p53 CD and antibody manipulations at the protein C-terminus to study binding of bacterially expressed full length p53 protein to various topological forms of DNA either containing or lacking the p53CON. We demonstrate that the p53 CTDBS is critical for p53 SCS binding while the p53 CD is responsible for the supercoil nonselective DNA binding of p53 immune complexes in which the CTDBS is blocked by an antibody. Possible roles of p53 SCS DNA binding in the regulation of its biological activities are discussed.

Materials and methods

DNA samples

Supercoiled (sc) DNA of plasmid pBSK(–) (not containing p53CON) was isolated form *Escherichia coli* DH5α cells and purified by CsCl/ethidium bromide gradient ultracentrifugation. Superhelix density of the scDNA estimated from chloroquine agarose gels [43] was about −0.06. Linear DNAs were prepared by *Sma*I (Takara) cleavage of the pBSK(–) or pPGM1 (containing a p53CON identical to the CON oligonucleotide below) plasmids. Relaxed covalently closed circular (rel) DNA was prepared using wheat germ topoisomerase I (Promega). To generate open circular (oc) DNA, the scDNA sample was irradiated with γ-rays from a Chisostat ^{60}Co source (Chirana, Brno, Czech Republic) in 10 mM Tris/EDTA buffer; the dose (about 40 Gy) was adjusted empirically to achieve 50% relaxation of the scDNA. Synthetic 20 mer oligonucleotides (ODNs), the specific 5′-AGACATGCCTAGACATGCCT-3′ (CON) and nonspecific 5′-GCATCATAGCGCATCATAGC-3′ (NODN), including their complementary strands, were purchased from VBC Genomics (Vienna, Austria).

Monoclonal antibodies

The following anti-p53 mouse monoclonal antibodies (mAbs) were generated against full length p53 protein expressed in bacteria (DO-1, Bp53-10.1, Bp53-6.1, Bp53-30.1) and against synthetic peptide (ICA9). The method of development of antibodies was described in [44]. DO-1 binds to an epitope (aa 21–25) in the N-terminal region of the protein (Fig. 1) [44,45]. The other mAbs bind to the C-terminal region of p53, including ICA9 (aa 388–393) [46], PAb421 (aa 371–380) [45,47], Bp53-10.1 and Bp53-30.1 (aa 375–379), and Bp53-6.1 (aa 381–390) (Fig. 1) [48,49].

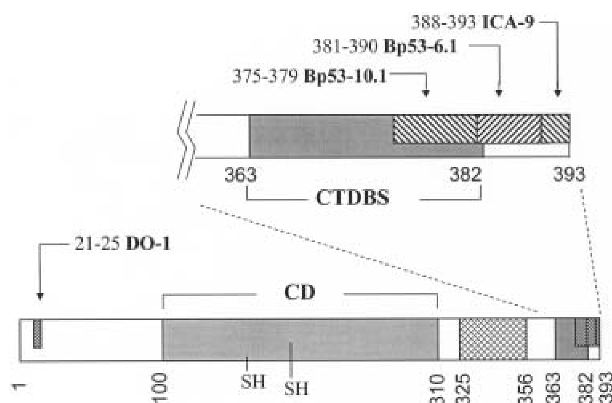

Fig. 1. Scheme of the domain structure of a subunit of full length p53 protein. The two p53 DNA binding sites, the core domain (CD; aa 80–310) and the basic C-terminal DNA binding site (CTDBS; aa 363–382), are indicated by grey boxes. The cross-hatched region (aa 325–356) is the p53 tetramerization domain. Diagonally hatched boxes indicate epitopes of mAbs DO-1 (aa 21–25), Bp53-10.1 (aa 375–379), Bp53-6.1 (aa 381–390) and ICA-9 (aa 388–393). The p53 CD contains cysteine residues (indicated by 'SH') which are the objects for redox modulation of the protein. The C-terminal region is expanded for better resolution of the CTDBS and the mAb epitopes.

The mAbs were purified from cell culture supernatants or ascites by means of affinity chromatography using either protein G-Sepharose (Pharmacia) or protein L-Sepharose (Pierce).

Purification of p53

Human full length p53 protein (wild type) was expressed in bacterial *E. coli* BL21/DE3 cells containing plasmid pT77Hup53. The cells were grown at 20 °C using a two-step induction with isopropyl thio-β-D-galactoside to limit protein aggregation. The protein was purified according to a modified protocol reported by Hupp *et al.* [36]. Protein was eluted from a Heparin-Sepharose Hi-Trap column (Pharmacia) by a 40 mL linear gradient of KCl at 0.5 M, followed by gel filtration through Superdex HR 10/10 (Pharmacia). Purity of the p53 preparation was checked by SDS/PAGE. The protein concentration was determined densitometrically from Coomassie Blue R-250 stained gels, using bovine serum albumin as a standard.

Modification of the p53 protein

Oxidation of cysteine residues was achieved through incubation of the protein with 1 mM diamide (Sigma) in 50 mM KCl, 5 mM Tris, 0.5 mM EDTA, 0.01% (v/v) Triton X-100 (pH 7.6) at 0 °C for 20 min. Reduced p53 was prepared by incubation with 2 mM dithiothreitol under the same conditions. Immune complexes of reduced or oxidized p53 were prepared by addition of the relevant mAb to the reaction mixture, followed by a 20 min incubation (more details in Results and Figure legends).

DNA binding assay

scDNA (400 ng) of pBSK(–) plasmid were mixed with the pretreated p53 samples (see above) at p53 tetramer/DNA molar ratios between 2.5 and 10, and incubated for 30 min on ice to reach equilibrium. In competition experiments 400 ng of the plasmid competitor DNAs or 50 ng of ^{32}P end-labeled ODNs, were added to the samples at the same time as the scDNA. After binding, the samples were loaded on 1 or 1.3% agarose gel containing 0.33× Tris/Borate/EDTA buffer, pH 8.0. Agarose gel electrophoresis was performed for 3 or 10 h at 120 V and 4 °C. (The higher agarose concentration and longer separation times were used in the sc/linear (lin) competition assays to achieve better separation of the nucleoprotein complexes. It should be noted that under the given conditions, linDNA migrates faster than scDNA of the same length.) Gels were stained with ethidium bromide and photographed. Radioactively labeled competitor ODNs were detected by autoradiography of the gel.

Immunoblotting analysis of p53–DNA complexes

Agarose gels were blotted onto nitrocellulose membrane BioTrace NT (Pall Life Sciences, Ann Arbor, MI, USA) in 3 M NaCl, 0.3 M sodium citrate (pH 7.0) on a vacuum blotting system (Bio-Rad) under 80 mPa. The membrane was then blocked with 5% low-fat powdered milk in NaCl/P_i solution and p53 was detected with primary rabbit polyclonal antibody CM1 (diluted 1 : 5000), followed by a horseradish peroxidase conjugated anti-rabbit IgG (Sigma) diluted 1 : 5000. Bands were visualized with the ECL detection system (Amersham). Band intensities were quantified by IMAGE-QUANT software.

Results

Effects of monoclonal antibodies on binding of reduced and oxidized p53 to scDNA

Full length p53 was preincubated with either 2 mM dithiothreitol (p53^{red}) or 1 mM diamide (p53^{ox}) followed by addition of a 5-fold molar excess (relative to the p53 tetramer) of one of the following antibodies: DO-1 (mapping to aa 21–25), ICA-9 (aa 388–393), Bp53-6.1 (aa 381–390) or Bp53-10.1 (aa 375–379) (Fig. 1). Then, scDNA of plasmid pBSK(–) (lacking the p53CON) was added (ratio of p53/scDNA = 5) and the effects of the antibodies on formation of p53–scDNA complexes were followed using mobility shift assay in agarose gel. Reduced p53 alone (Fig. 2A, lane 2) as well as all of its immune complexes (lanes 3–6) bound to scDNA, yielding band ladders in the ethidium stained gel (Fig. 2A). Based on the electrophoretic mobility shift and immunoblotting data and on parallel electron microscopic observations, we have concluded previously that individual retarded bands in the ladders differ in number of the p53 tetramers bound per scDNA molecule [20,21,23,50]. In the presence of the mAbs, the retarded bands were supershifted providing evidence for the formation of ternary mAb–p53^{red}–scDNA complexes [37]. The antibodies alone (in absence of the p53 protein) had no effect on the mobility of scDNA (Fig. 2B, lanes 11 and 12, for Bp53-10.1 and ICA-9, respectively).

Oxidized p53 alone (Fig. 2A, lane 7) as well as its immune complex with the p53 N-terminus mapping mAb DO-1 (lane 8) bound to scDNA, showing similar binding when compared to p53^{red} (lane 2) and p53^{red}–DO-1 (lane 3), respectively. Strikingly, effects of the mAbs mapping to the protein C-terminus on formation of p53^{ox}–scDNA complexes differed markedly, depending on the positions of their epitopes relative to the CTDBS (Fig. 1). ICA-9 (Fig. 2A, lane 9), mapping to the extreme C-terminus of p53, caused about 40% inhibition of p53^{ox}–scDNA binding (data obtained from densitometric tracing of the free scDNA band). The complex of p53^{ox} with Bp53-6.1 (mapping to epitope aa 381–390 just next to the CTDBS, Fig. 1) exhibited only 20–25% of scDNA binding (Fig. 2A, lane 10), compared to p53^{ox} in the absence of mAb. The stronger inhibition caused by Bp53-6.1 was in qualitative agreement with previous observations made with the p53(320–393) fragment [23]. With both ICA-9 and Bp53-6.1, highly visible, distinct bands exhibiting characteristic supershifts could be identified (Fig. 2A, lanes 9 and 10). On the contrary, the mAb Bp53-10.1 (within the CTDBS) fully inhibited binding of p53^{ox} to scDNA under the same conditions (Fig. 2A, lane 11). Strong inhibition of the p53^{ox}–scDNA binding was also exhibited by the mAbs PAb421 and Bp53-30.1 (not shown), whose epitopes overlap with that of Bp53-10.1 [37,49].

Effects of the antibody concentration on binding of p53^{ox} to scDNA were examined for the mAbs Bp53-10.1 and ICA-9 (Fig. 2B,C). Increasing the Bp53-10.1/p53 ratio

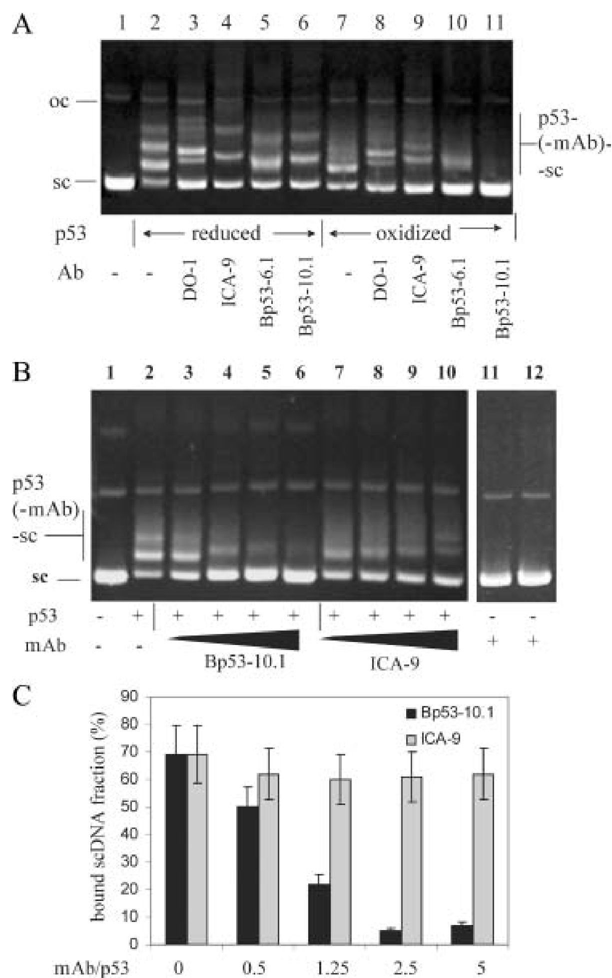

Fig. 2. Influence of monoclonal antibodies on binding of reduced and oxidized p53 to scDNA. (A) Effects of monoclonal antibodies recognizing different epitopes in p53 on binding of reduced or oxidized p53 to pBSK(–) scDNA. Protein was preincubated with 2 mM dithiothreitol ($p53^{red}$) or 1 mM diamide ($p53^{ox}$) in 10 μL of 50 mM KCl, 5 mM Tris/HCl, 0.5 mM EDTA, 0.01% (v/v) Triton X-100 (pH 7.8) on ice for 20 min. Then, the mAbs were added (200 ng per sample) and the mixtures were incubated on ice for 20 min, followed by subsequent addition of 400 ng of scDNA (molar ratio p53 tetramer/DNA = 5) and incubation of the samples on ice for 30 min. After electrophoretic separation in 1% agarose, DNA was stained with ethidium bromide and the gel was photographed. Lanes 2–6, reduced p53; lanes 7–11, oxidized p53; lane 1, scDNA only; lanes 2 and 7, no mAb; lanes 3 and 8, DO-1; lanes 4 and 9, ICA-9; lanes 5 and 10, Bp53-6.1; lanes 6 and 11, Bp53-10.1. Bands denoted as 'sc' and 'oc' correspond to free monomeric scDNA and open circular DNA, respectively. Species migrating between sc and ocDNA are p53–scDNA or mAb–p53–scDNA complexes. (B,C) Effects of increasing amounts of the mAbs Bp53-10.1 and ICA-9 on binding of oxidized p53 to scDNA. (B) Ethidium stained agarose gel: lane 1, scDNA only; lane 2, no mAb; lanes 3–6, Bp53-10.1; lanes 7–10, ICA-9. mAb/p53 tetramer molar ratios: lanes 3 and 7, 0.5; lanes 4 and 8, 1.25; lanes 5 and 9, 2.5; lanes 6 and 10, 5. Control samples loaded on lanes 11 and 12 contained scDNA and 200 $ng \cdot mL^{-1}$ of Bp53-10.1 or ICA-9, respectively, but no p53. (C) Graph showing the effects of Bp53-10.1 or ICA-9 concentration on relative bound fraction of scDNA [data calculated from densitometric tracing of the free scDNA bands in (B)]. For other details, see Fig. 2A.

(Fig. 2B, lanes 3–6) resulted in gradual decrease of the $p53^{ox}$–scDNA binding. Although supershifting of the band of p53–scDNA complex was observed at the Bp53-10.1/p53 tetramer ratio ≥ 1.25, further additions of the antibody resulted in diminishing of the band intensity (Fig. 2B, lanes 4–6). Intensity of the band of free scDNA simultaneously increased, at Bp53-10.1/p53 = 2.5 (lane 6) reaching 93% of the intensity of the scDNA band in the absence of p53 (lane 1). On the contrary, ICA-9 did not inhibit binding of the $p53^{ox}$ to scDNA in the same antibody concentration range (Fig. 2B, lanes 7–10). Instead, continuous retardation of the p53–scDNA complexes was observed with increasing the ICA-9/p53 ratio up to 5. Intensity of the scDNA band only slightly increased with the ICA-9 concentration (Fig. 2B,C). The results suggest that amino acid residues 375–379 (the Bp53-10.1 epitope) within the p53 CTDBS are of critical importance for binding of the oxidized p53 to scDNA. Blocking of this segment with Bp53-10.1 resulted in a strong inhibition of the $p53^{ox}$–scDNA complex formation. On the other hand, antibody binding outside the CTDBS did not prevent formation of the $p53^{ox}$–scDNA complexes.

Influence of mAbs on the supercoil-selective DNA binding of reduced p53

Although $p53^{red}$ was capable of binding to scDNA in the presence of either of the mAbs tested, the results shown in Fig. 2 did not reveal whether this binding was supercoil-selective. We therefore performed competition experiments involving sc and linear pBSK(–) DNA (sc/lin competition assay) to evaluate the influence of individual mAbs on the preference of $p53^{red}$ for scDNA. Figure 3 shows that at the p53/scDNA ratio = 2, $p53^{red}$ alone bound scDNA selectively, yielding a detectable complex only with scDNA (Fig. 3A, lane 3). This SCS binding was also retained in the presence of antibodies DO-1, Bp53-6.1 and ICA-9 (Fig. 3A, lanes 4, 6 and 7), i.e. the mAbs mapping to epitopes outside the CTDBS. In all cases, supershifted complexes were observed with scDNA but not with linDNA. On the contrary, immune complexes of $p53^{red}$ and Bp53-10.1 bound both sc and linDNA, yielding retarded bands of about the same intensities (Fig. 3A, lane 5). The behavior of antibody-free $p53^{red}$ and of $p53^{red}$–Bp53-10.1 immune complexes was also compared in competition experiments with circular relaxed DNAs (at p53/scDNA = 5). The pBSK(–) DNA was either treated with topoisomerase I [generating relaxed covalently closed circular DNA (relDNA); Fig. 3B], or irradiated by γ-rays inducing single strand breaks [resulting in formation of open circular DNA (ocDNA); Fig. 3C]. Similarly to the sc/lin competition assay (Fig. 3A), the mAb-free $p53^{red}$ bound scDNA with a high preference, producing no detectable bands of the relDNA–p53 or ocDNA–p53 complexes. On the other hand, $p53^{red}$–Bp53-10.1 bound both relDNA and ocDNA in the presence of scDNA (Fig. 3B,C). Analogous results were observed with the mAbs PAb421 and Bp53-30.1 that also bind within the CTDBS (not shown). Therefore, blocking the p53 CTDBS by mAbs resulted in loss of the protein preference for the scDNA, although these $p53^{red}$ immune complexes were still capable of binding to both scDNA and the relaxed DNA forms (Figs 2 and 3).

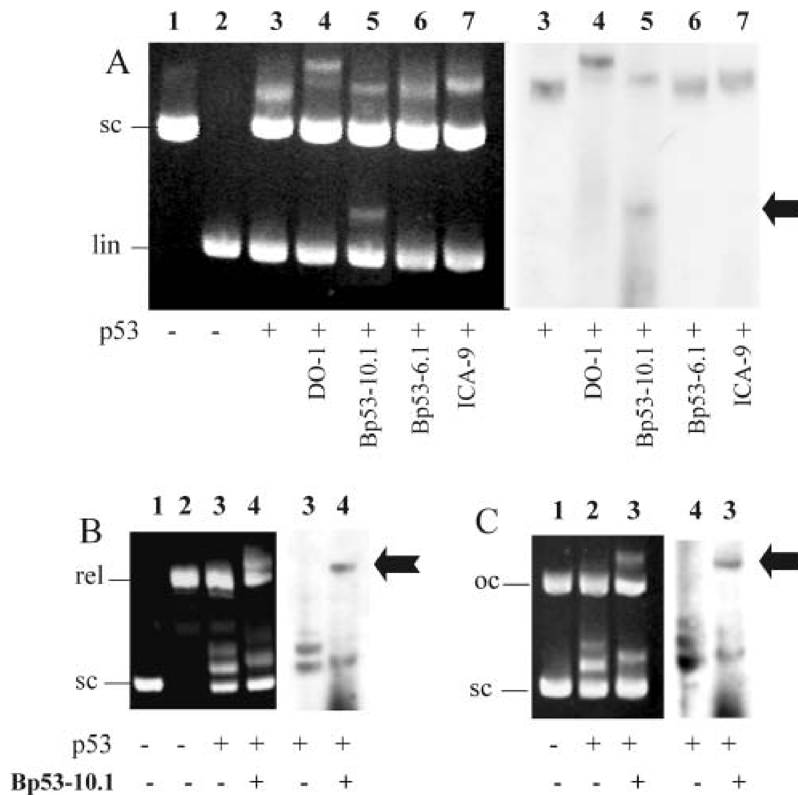

Fig. 3. Influence of monoclonal antibodies on the supercoil-selective DNA binding of reduced p53. (A) Effects of different mAbs on preferential binding of reduced p53 to scDNA in competition with linDNA. Both forms of the pBSK(–) DNA (400 ng of each) were added to the protein and subsequently preincubated with dithiothreitol and/or with the given antibody at the same time. After photographing of the ethidium stained DNA (as in Fig. 2), the gel was blotted onto nitrocellulose membrane. Visualization of the p53 with anti-p53 rabbit polyclonal antibody CM1 and secondary anti-rabbit IgG peroxidase conjugate using the ECL technique. Left, ethidium stained gel; right panel, immunoblot: lane 1, scDNA only; lane 2, linDNA only; lanes 3–7, sc/lin competition for p53 in the presence of: lane 3, no mAb; lane 4, DO-1; lane 5, Bp53-10.1; lane 6, Bp53-6.1; lane 7, ICA-9. The samples were run on 1.3% agarose gel and the p53/scDNA ratio was 2 : 1. For other details see Fig. 2. (B,C) Analogous competition assays performed with topoisomerase relaxed (rel) or open circular (oc) pBSK(–) DNA instead of the lin DNA. (B) Lane 1, scDNA only; lane 2, relDNA only lanes 3–4, sc/rel competition for p53: lane 3, no antibody; lane 4, Bp53-10.1. (C) Lane 1, 1 : 1 mixture of sc and ocDNA, no p53; lanes 2 and 3, sc/oc competition for p53: lane 2, no antibody; lane 3, Bp53-10.1. The smear below the p53–Bp53-10.1–scDNA complexes on the immunoblots corresponds to the DNA–unbound p53 immune complex. The samples were run on 1% gel with p53/scDNA = 5; other details as in (A). Arrows indicate positions of p53–mAb complexes with the competitor DNAs.

To examine the effects of Bp53-10.1 or ICA-9 on the $p53^{red}$ preference for scDNA at different antibody concentrations, the sc/lin competition experiments were performed at a ratio of p53/scDNA = 5 (Fig. 4A,B). Under these conditions, $p53^{red}$ alone again bound scDNA with a high preference, apparently producing no linDNA complex (Fig. 4A,B, lane 3). At Bp53-10.1/p53 = 0.5, a faint band of the p53–linDNA complex appeared on the blot (lane 4). Starting from a Bp53-10.1/p53 ratio of 1.25 (lane 5), the protein preference for scDNA was lost. Immune complexes of p53 with ICA-9 exhibited a different behavior (Fig. 4A,B, lanes 8–11). A weak band of p53–linDNA appeared on the blot at ICA-9/p53 = 1.25 (lane 9) and the intensity of this band steadily increased with the ICA-9 concentration; the p53 preference for scDNA simultaneously decreased slightly (Fig. 4). Nevertheless, at the highest ICA-9/p53 ratio (= 5, lane 11) p53 still exhibited distinctly preferential scDNA binding, providing 10–15-times higher intensity of the p53–scDNA bands as compared to p53–linDNA (Fig. 4C; data obtained from densitometric tracing of the blot on Fig. 4B). Taken together, these results revealed an essential role of the CTDBS for p53 SCS binding and moreover indicated that blocking of only a part of the Bp53-10.1 epitopes within the p53 tetramer (at the mAb/p53 tetramer molar ratio of 1.25) was sufficient for a loss of the $p53^{red}$ preference for scDNA.

Effects of p53 oxidation on SCS binding

We further examined the effect of p53 oxidation on its preference for scDNA (in the absence of mAbs, at p53/scDNA ratios of 3 and 6, Fig. 5). Oxidized p53 exhibited similar binding to scDNA and linDNA in the absence of the other DNA form (Fig. 5, lanes 8–11). Nevertheless, in the sc/lin competition assay, $p53^{ox}$ bound scDNA with a strong preference, yielding a faint band of $p53^{ox}$–linDNA complex only at p53/DNA = 6 (Fig. 5B, lane 13). Distinctly supercoil-selective DNA binding was thus retained by $p53^{ox}$, although the overall p53 DNA interactions were partially decreased due to oxidation of the protein cysteine residues.

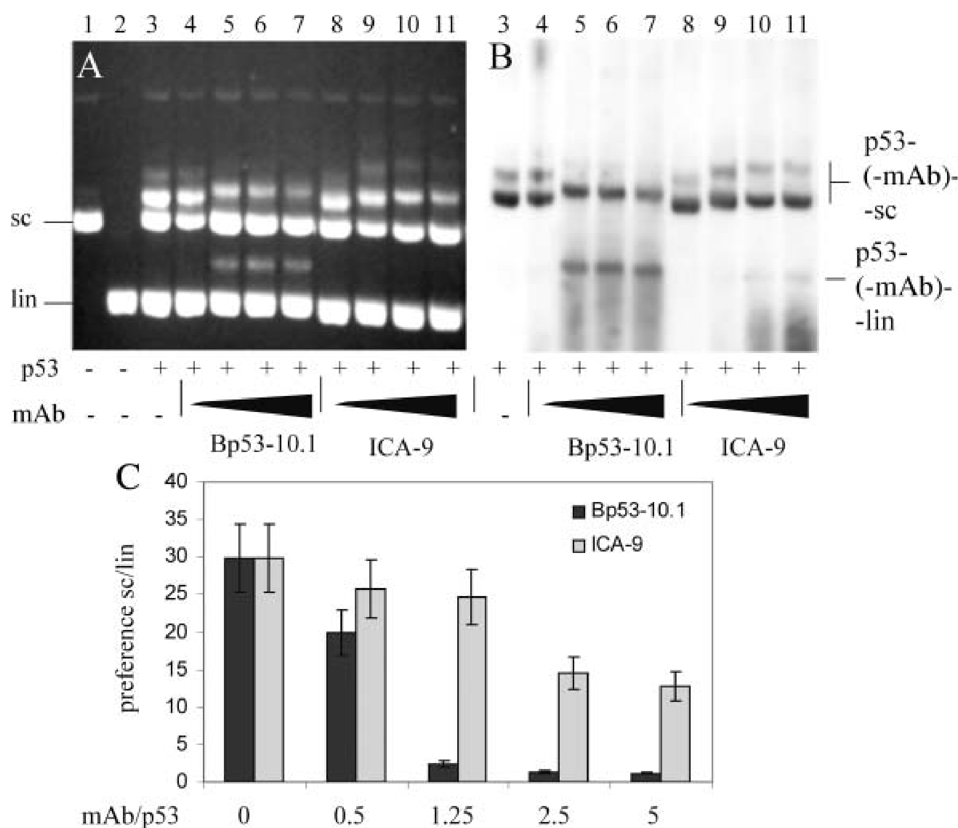

Fig. 4. Effects of the concentration of the mAbs Bp53-10.1 and ICA-9 on preferential binding of reduced p53 to scDNA in competition with linDNA. (A) Ethidium stained agarose gel. (B) Immunoblot: lane 1, scDNA only; lane 2, linDNA only; line 3, no mAb; lanes 4–7, Bp53-10.1; lanes 8–11, ICA-9; mAb/p53 tetramer ratios: lanes 4 and 8, 0.5; lanes 5 and 9, 1.25; lanes 6 and 10, 2.5; lanes 7 and 11, 5. (C) Graph showing the effects of mAbs concentration on p53 preference for scDNA [expressed as ratios of the intensities of bands of p53–scDNA/p53–linDNA complexes on the blot (B)]. For other details, see Fig. 2.

Competition between supercoiled DNA and p53CON for p53

We performed further competition assays to compare binding affinities of p53^{red} (in the absence of antibodies) and of its Bp53-10.1 immune complex to pBSK(–) scDNA (not containing the p53CON) with the sequence-specific binding of the two p53 forms. We used competitor DNAs containing the p53CON either within a linear ≈ 3 kb plasmid pPGM1 (Fig. 6A,B), or in a 20 mer double-stranded oligonucleotide (Fig. 6C,D).

The lin pPGM1 DNA was used in a molar ratio of 1 : 1 to the sc pBSK(–) DNA. In the absence of mAb, p53^{red} bound pBSK(–) scDNA with a remarkable preference (Fig. 6A,B, lanes 3–5). On the other hand, p53^{red}–Bp53-10.1 complex exhibited a strong bias towards the lin pPGM1 DNA (Fig. 6A,B, lanes 6–8) under the same conditions, indicating that this p53 immune complex bound to the p53CON within the linDNA with a higher affinity than to the scDNA lacking the consensus sequence. Competition experiments involving the 20 mer p53CON oligonucleotide (CON) provided qualitatively the same results. The presence of a 20-fold molar excess of the ^{32}P-labeled ODNs (regardless of their sequences) was apparently without effect on p53^{red}–scDNA binding (Fig. 6C, lanes 3 and 5). Autoradiogram of the agarose gel revealed the formation of p53–ODN complexes only in the absence of scDNA (Fig. 6D, lanes 4 and 6). A strikingly different behavior was exhibited by p53–Bp53-10.1. In the presence of the CON, binding of the p53 immune complex to scDNA was fully abolished (Fig. 6C, lane 8). Supershifted spots corresponding to the CON–p53–Bp53-10.1 complexes appeared on the autoradiogram both in the presence and absence of scDNA (Fig. 6D, lanes 8 and 9). On the other hand, NODN did not inhibit formation of the scDNA–p53–Bp53-10.1 complexes (Fig. 6C, lane 10), and spots corresponding to the NODN–p53–Bp53-10.1 complexes were detected only in the absence of scDNA (Fig. 6D, lane 11). It should be emphasized that no radioactive signal matched the ethidium-stained band ladders corresponding to the p53–scDNA complexes (Fig. 6D, lanes 3, 5, 7 and 10).

Results shown in Fig. 6 suggest that p53^{red} with an unmodified C-terminus (i.e. in the absence of Bp53-10.1) bound more strongly to scDNA not containing p53CON than to the specific sequence in both long plasmid linDNA molecule and the 20 mer ODN. On the other hand, the immune complex p53–Bp53-10.1 bound preferentially to the p53CON. This agrees well with inactivation of the p53 CTDBS (responsible for the p53 SCS binding) and activation of the protein sequence-specific

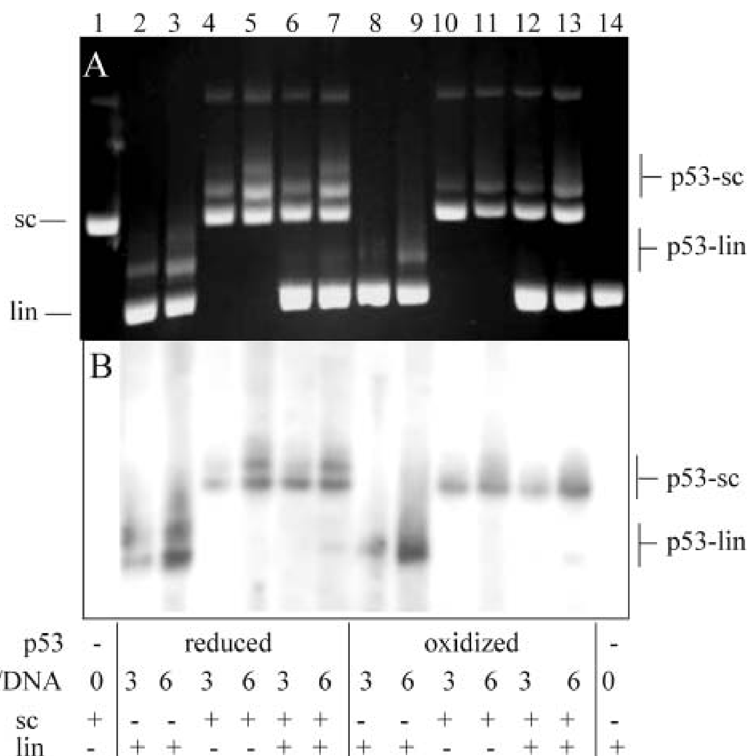

Fig. 5. Effect of the p53 redox state on preferential binding to scDNA. (A) Ethidium stained agarose gel. (B) Immunoblot: lane 1, scDNA only; lane 14, linDNA only; lanes 2–7, reduced p53; lanes 8–13, oxidized p53; lanes 2–3 and 8–9, p53 binding to linDNA alone; lanes 4–5 and 10–11, p53 binding to scDNA alone; lanes 6–7 and 12–13, competition between sc and linDNA; lanes 2, 4, 6, 8, 10 and 12, p53/DNA = 3 : 1; lanes 3, 5, 7, 9, 11 and 13, p53/DNA = 6 : 1. For other details, see Fig. 2.

DNA binding by Bp53-10.1 [37]. Experiments with the CON oligonucleotide did not provide any sign of formation of tentative ternary CON–p53–scDNA complexes, in which p53 CD would bind the oligonucleotide while CTDBS bound scDNA.

Discussion

Redox and antibody modulation of DNA binding of p53 core and C-terminal domains

In our recent papers [20,23], we used p53 deletion mutants to identify the roles of p53 domains in supercoil-selective DNA binding. It was shown that the isolated C-terminal domain of p53 (amino acids 320–393) binds scDNA with a high preference, while p53 lacking the CTDBS did not, suggesting that it is the p53 CTDBS which is crucial for strong SCS binding. The protein deletion studies are useful for characterization of the isolated p53 DNA binding sites but cannot directly confirm their roles in SCS DNA binding of full length p53 that represents a more complex entity. We have therefore combined the deletion experiments with a parallel study of contributions of the p53 CD and the CTDBS to the SCS binding of fl p53. The DNA binding activities of the two domains in fl p53 were separately modulated by a thiol-oxidizing agent diamide and by monoclonal antibodies mapping to epitopes within the protein C-terminus. It has been established previously that the redox state of p53 is essential for its sequence-specific DNA binding [21,26,38–40]. Oxidation of cysteine residues in the p53 CD results in substantial changes in the protein structure, including release of zinc ion from the CD [21,22,38,50,51] and adopting a mutant-like conformation unable to bind the p53CON [38]. The p53 structural changes due to thiol oxidation are reversible under certain conditions [9,21,26] and redox modulation has been taken into consideration as one of the possible mechanisms involved in the complexities of p53 control *in vivo* [9,21,26,40]. Our preliminary results (M. Fojta, M. Brazdova & H. Pivonkova, unpublished data) showed that sequence-nonspecific binding of the isolated p53 CD [p53(94–312)] or C-terminally truncated p53(1–363) to both linDNA and scDNA lacking the p53CON are also strongly inhibited by p53 oxidation. Because the C-terminal domain does not contain cysteine residues, fl $p53^{ox}$ retained the ability to bind DNA via this site [21,22,38,39]. On the other hand, the CTDBS can be 'switched off' by antibodies mapping to epitopes within it (such as antibodies PAb421 and Bp53-10.1 [37,49,52,53]).

The p53 CTDBS is critical for preferential binding of full length p53 to scDNA

Our oxidation and antibody-interference experiments suggest that fl $p53^{ox}$ can bind scDNA unless a segment of its CTDBS is blocked by mAbs Bp53-10.1 (Fig. 2), PAb421 or Bp53-30.1. In the presence of these mAbs, binding of fl $p53^{ox}$ to scDNA or linDNA (regardless of the presence or absence of the p53CON) was abolished, suggesting a crucial role of the CTDBS in $p53^{ox}$ DNA binding. Immune complexes of $p53^{red}$ with mAbs mapping to the protein C-terminus bound scDNA in the absence of linDNA efficiently, regardless of the epitope position (Fig. 2). However, the sc/lin competition assay (Figs 3 and 4) revealed striking differences in DNA binding of $p53^{red}$–Bp53-10.1 and $p53^{red}$ complexes with

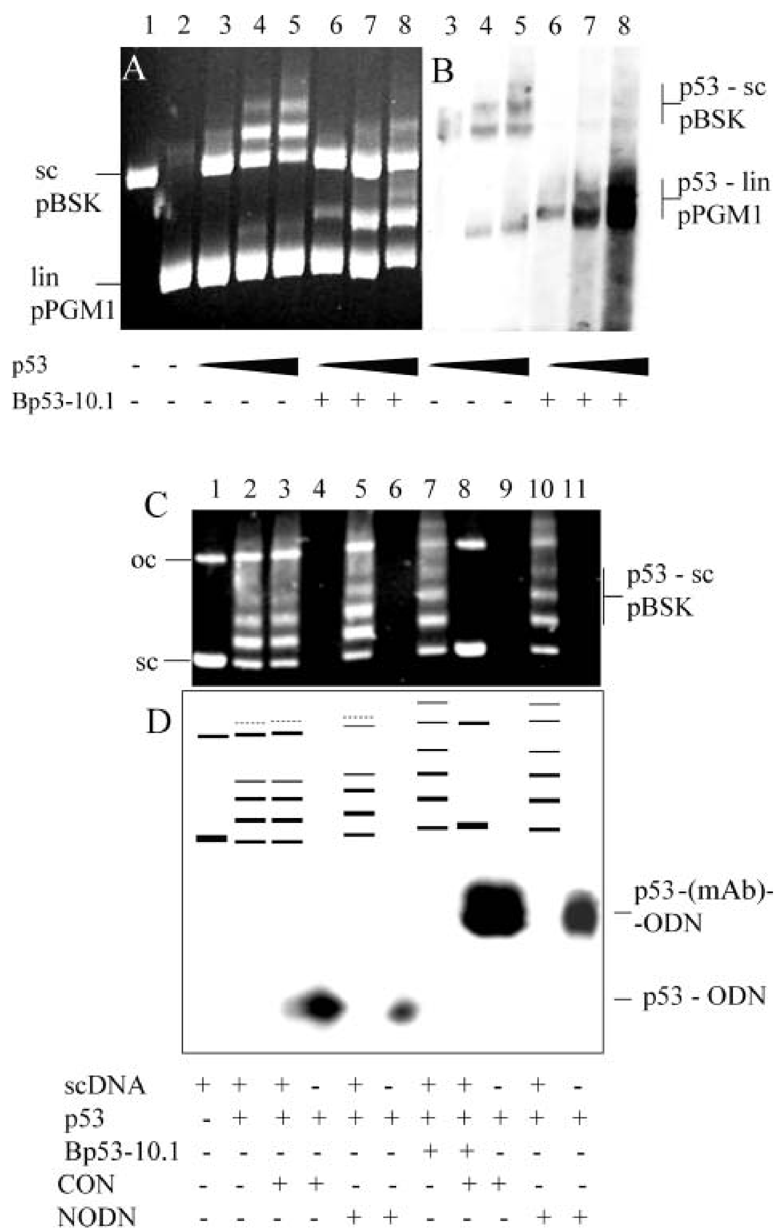

Fig. 6. Competition between scDNA (lacking p53CON) and p53CON for reduced p53 or its Bp53–10.1 immune complex. (A,B) Binding of p53 to sc pBSK(–) in the presence of linear pPGM1 DNA (containing the p53CON): lane 1, scDNA alone; lane 2, lin pPGM1 alone; lanes 3–8, both DNAs (molar ratio 1 : 1); lanes 3–5, no antibody; lanes 6–8, Bp53-10.1; lanes 3 and 6, p53/scDNA = 2.5; lanes 4 and 7, p53/scDNA = 5; lanes 5 and 8, p53/scDNA = 10. (A) Ethidium stained gel. (B) Immunoblot. (C,D) Binding of p53 to sc pBSK(–) in the presence of p53CON in 20 mer oligonucleotides: lane 1, scDNA alone; lanes 2–6, no antibody; lanes 7–11, Bp53-10.1; lanes 1, 2 and 7, no ODN; lanes 3, 4, 8 and 9, CON; lanes 5, 6, 10 and 11, nonspecific ODN (NODN). The ODNs were radioactively end-labeled and applied in about eightfold molar excess (20 ng per sample), as compared to scDNA. In the autoradiogram (D), horizontal bars represent superimposition of the ethidium stained bands in (C); p53/scDNA = 5. For other details, see Fig. 2.

mAbs binding outside the CTDBS. The former $p53^{red}$ immune complex bound both sc and linDNA, both lacking the p53CON, and also oc or relDNA (Fig. 3B,C), without significant preference for DNA form. Titration experiments showed that blocking of only two of the CTDBS copies in the p53 tetramer (by one molecule of the divalent antibody) was sufficient for a strong decrease of SCS binding (Fig. 4). This was in qualitative accordance with our previous suggestions that efficient p53 SCS binding requires cooperative protein–DNA interactions in the multivalent form of the protein CTDBS, conferred by the oligomeric state of the deletion protein constructs [23]. It should nevertheless be noted that the tetrameric fl p53 with two copies of CTDBS blocked by the mAb behaved differently than dimeric constructs of p53 C-terminal domain used previously [23]. The latter were able to bind scDNA with a high preference in the sc/lin competition assay. A comparison of results shown in this paper in Figs 2B and 4 suggests that the presumably semisaturated Bp53-10.1 immune complex of $p53^{ox}$ could bind scDNA, yielding a supershifted band of the nucleoprotein complex (Fig. 2B, lane 4); on the other hand, binding of $p53^{red}$ at the same p53/mAb ratio was not supercoil-selective (Fig. 4, lane 5). It can be speculated that in the semisaturated immune complex of fl p53, the remaining two CTDBS copies were not capable of efficient cooperative binding to scDNA for steric reasons. Steric interference of bulky antibody molecules bound to the protein C-terminus can also be the source of a partial inhibition of scDNA binding in the p53–ICA-9 immune complex (Figs 2B,C and 4).

We reported previously [21,22] that oxidized insect cell-expressed p53 was able to bind scDNA in the absence of linDNA, albeit with a partially decreased affinity. In this

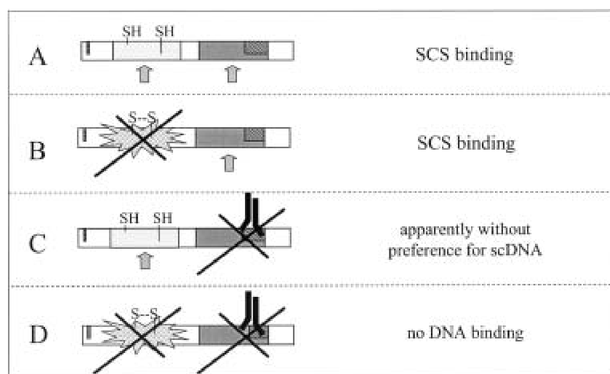

Fig. 7. Schematic summarization of the effects of cysteine oxidation within the p53 CD and mAb binding within the p53 CTDBS on the SCS DNA binding of bacterially expressed fl p53. Arrows indicate available DNA binding sites. (A) $p53^{red}$ in the absence of mAbs exhibits a highly selective scDNA binding; (B) $p53^{ox}$ in the absence of the mAbs also displays the SCS DNA binding; (C) $p53^{red}$ preference for scDNA is lost due to blocking of a part of the CTDBS by a mAb; (D) $p53^{ox}$ does not efficiently bind DNA when its CTDBS is blocked by the mAb.

paper we demonstrate that the same behavior is exhibited by post-translationally unmodified bacterially expressed p53. Moreover, we show for the first time that $p53^{ox}$ retains its preferential binding to scDNA in the sc/lin competition assay (Fig. 5). Therefore, 'switching off' the DNA binding activity of the p53 CD by thiol oxidation does not result in abolishment of the p53 SCS DNA binding, as long as the protein CTDBS is available (Fig. 7B). Conversely, blocking the CTDBS of $p53^{red}$ by mAbs causes loss of SCS binding (Fig. 7C). Taken together, these observations demonstrate that the tetrameric p53 CTDBS is critical for SCS binding of full length p53.

Supercoil-nonselective DNA binding of the full length p53 complex with Bp53-10.1 is located within the protein core domain

The behavior of the reduced form of the immune complex p53–Bp53-10.1 was analogous to that earlier established for p53 deletion constructs lacking the CTDBS but possessing the CD, such as p53(94–312) [20], p53(1–363) or p53(45–349) [23]. These constructs bound scDNA with apparently no or only weak preference in competition experiments with lin or relaxed circular DNAs (in the absence of the p53CON) [20,23]. Competition experiments involving the p53CON (Fig. 6) support the idea that the p53 CD is primarily responsible for the supercoil-nonselective DNA binding of p53–Bp53-10.1). Both the linear plasmid pPGM1 DNA and the CON oligonucleotide were strong inhibitors of binding of this p53 immune complex to pBSK(–) scDNA, but not of p53–scDNA binding in the absence of mAb, indicating that the sequence-specific DNA-binding site was essential for the interaction of p53–Bp53-10.1) with scDNA lacking the p53CON. When this site was occupied by the CON ODN, the p53 immune complex completely lost its ability to bind to scDNA. Similar results were obtained with the p53(1–363) deletion mutant (not shown).

It has been established that post-translational modifications (phosphorylation [4,27,29] or acetylation [4,34]) of the negative-regulating region within the p53 C-terminal domain, overlapping with the CTDBS [28], result in activation of the sequence-specific binding activity of the CD [4,26,29,31,32,37]. Blocking of this site by antibodies such as PAb421 [29] or Bp53-10.1 [37,49], or its deletion [30,36], have a similar effect. The differences between the post-translationally unmodified fl p53 and its Bp53-10.1 immune complex (or the C-terminally truncated p53 constructs [23]) can thus also be discussed in terms of p53 activation. In the presence of the activating mAb Bp53-10.1 [37], the affinity for p53CON increased and the immune complex (possessing only the CD available for DNA binding) bound preferentially to the pPGM1 linDNA (Fig. 6A,B) or to the CON ODN (Fig. 6C,D) in the presence of pBSK(–) scDNA. Activated p53 might also exhibit increased binding to degenerative p53CON-like sequences that are present in the pBSK(–) plasmid (including three p53CON half-sites containing a single base mismatch [17]). Such 'semispecific' p53–DNA interactions may partially contribute to the nonpreferential p53–Bp53-10.1 binding to both sc and lin pBSK(–) DNA (Figs 3 and 4).

Is the core domain involved in the SCS DNA binding of unmodified full length p53?

Results presented in this paper together with those published previously [20–23] lead us to conclude that the p53 CTDBS is primarily responsible for the p53 SCS DNA binding. On the other hand, the ability of the p53 CD to recognize some DNA conformational motifs, including those characteristic for negatively scDNA, was discussed ([20,23] and refs therein). The isolated p53 CD (aa 94–312) exhibited a certain degree of preference for scDNA in the sc/lin competition assay [20]. The p53 CD may therefore take part in the SCS DNA binding of fl p53.

One of the earlier proposed models of p53 latency, the 'steric' hypothesis [30], was based on mutual exclusivity of the p53 CD and CTDBS in DNA binding, implying that strong interactions of the unmodified CTDBS with nonspecific DNA prevent the CD from binding p53CON. However, there are growing amounts of data inconsistent with such a concept. Recent observations suggest that one p53 tetramer can interact with one DNA molecule by both DNA binding sites at the same time. In a double-stranded ODN possessing single-stranded overhangs, a p53 tetramer bound the overhangs via its C-terminal domains, simultaneously interacting with the central part of the ODN via its core domains [15]. Using fluorescence correlation spectroscopy it has been shown that the CD of a C-terminally unmodified p53 can interact with long double-stranded DNA molecules sequence nonspecifically [31]. These data recently resulted in the formulation of a 'two-site' model of p53 latency [31,32], involving simultaneous interaction of both core and C-terminal domains of 'latent' p53 with nonspecific DNA sequences. In addition, other observations suggest that the p53 C-terminus may stimulate sequence-specific binding to some p53 response elements within topologically constrained DNA molecules [41,54]. Structure-selective interactions of the CTDBS with DNA

facilitated p53 binding to a p53 response element within small DNA circles [54] or to p53CONs adopting non-B structures in ODNs [13,55,56] as well as within large plasmid scDNA molecules [41]. The absence of ternary complexes of CON–p53^{red}–scDNA demonstrated in this paper (Fig. 6) suggests that upon p53^{red} binding to scDNA via its CTDBS, the CD could not behave as an independent DNA binding site. There is thus no analogy between binding of the p53 C-terminus to the known noncovalent p53 activators of sequence-specific DNA binding and the interaction of p53 with scDNA. An explanation for this observation may be that the p53 sequence-specific DNA binding site was occupied, taking part in the fl p53^{red} interaction with the scDNA molecule.

Possible consequences of the p53 SCS DNA binding in the regulation of p53 biological activities

It has been proposed [6,7] that p53 may play a dual role in cells, acting either as a transcription factor under stress conditions (in its 'induced', 'activated' state, being able to bind DNA mainly sequence-specifically) or taking part directly in control of DNA replication, recombination [11,12] or repair (in a transcription-independent pathway) in unstressed cells. The latter function has been attributed to the 'noninduced' ('latent') forms of p53, exhibiting primarily conformation-selective DNA binding [6,7,55,56]. Interactions of some p53 mutants with non-B DNA structures specific for certain elements of chromatin architecture may be related to the p53 'gain of function' effect [57,58]. The possible involvement of p53 nucleoprotein filament formation (that correlates with the p53 SCS binding) in DNA recombination was discussed [23].

The relationships between DNA supercoiling and biological functions are well established [59–62]. In the nuclei of eukaryotic cells, rearrangements of chromatin structure occur during fundamental processes such as DNA replication, recombination, DNA repair or transcription and are connected with dynamic changes in DNA supercoiling [60–62]. Interactions of p53 with DNA will therefore be likely to change in synchrony with local alterations in DNA topology. In particular, our observations that post-translationally unmodified p53 binds strongly to scDNA suggest an additional mechanism for the induction of p53 as a sequence-specific transcription factor. The recently proposed 'two-site' [31,32] as well as the 'steric' [30] models of p53 latency involve strong interactions of p53 CTDBS with genomic DNA molecules, which prevents the p53 core domain from binding to p53 response elements. As the affinity of post-translationally unmodified p53 (regardless of its redox state) to scDNA is much higher than to relaxed (lin) DNA molecules, it is likely that DNA supercoiling in the nuclei contributes to p53 'latency' by sequestering p53 to scDNA. Sequestering of p53 will be overcome by relaxation of the local superhelical stress (due to chromatin rearrangement processes or, for instance, as a result of exposure to genotoxic agents that induce single- or double-strand DNA breaks), allowing post-translational modifications and subsequent activation of sequence-specific binding to p53 response elements. Strong binding of p53 to supercoiled DNA domains in the nucleus might also result in escape of p53 from the protein modification machinery to maintain the latent state, in keeping with the observations that p53-activation *in vivo* is highly variable between different tissues and cell populations within tissues [63,64]. This 'sequestration' model for determining the balance between 'latent' and active p53 is not incompatible with the two previously proposed models, and each may act in combination or separately in different cells under different conditions of growth and/or stress to regulate the overall p53 response pathway. Changes of DNA superhelicity and their impact on p53–DNA interactions, including the p53 SCS binding outside the p53 response elements [19–23,37,50] and DNA topology-dependent conformation transitions of some p53-inducible promoters [41,42], together with post-translational modifications of the p53 protein, might thus represent a complex p53 regulatory network.

Acknowledgements

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