DNA topology influences p53 sequence-specific DNA binding through structural transitions within the target sites

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The tumour suppressor protein p53 is one of the most important factors regulating cell proliferation, differentiation and programmed cell death in response to a variety of cellular stress signals. p53 is a nuclear phosphoprotein and its biochemical function is closely associated with its ability to bind DNA in a sequence-specific manner and operate as a transcription factor. Using a competition assay, we investigated the effect of DNA topology on the DNA binding of human wild-type p53 protein. We prepared sets of topoisomers of plasmid DNA with and without p53 target sequences, differing in their internal symmetry. Binding of p53 to DNA increased with increasing negative superhelix density (−σ). At −σ ≤ 0.03, the relative effect of DNA supercoiling on protein–DNA binding was similar for DNA containing both symmetrical and non-symmetrical target sites. On the other hand, at higher −σ, target sites with a perfect inverted repeat sequence exhibited a more significant enhancement of p53 binding as a result of increasing levels of negative DNA supercoiling. For −σ = 0.07, an approx. 3-fold additional increase in binding was observed for a symmetrical target site compared with a non-symmetrical target site. The p53 target sequences possessing the inverted repeat symmetry were shown to form a cruciform structure in sufficiently negative supercoiled DNA. We show that formation of cruciforms in DNA topoisomers at −σ ≥ 0.05 correlates with the extra enhancement of p53–DNA binding.

Key words: cruciform, DNA binding, protein–DNA complex, p53, supercoil, topoisomer.

INTRODUCTION

The p53 tumour suppressor protein is an important transcription factor that induces cell-cycle arrest or apoptosis in response to oncogenic transformation and DNA damage. Point mutations and deletions in the p53 gene are the most frequently observed alterations in human cancers [1]. The central role of p53 as a tumour suppressor protein has led several studies to explore the possibility of restoring p53 function for therapeutic use [2–4]. Its interaction with DNA in a sequence-specific manner is crucial for its function. The consensus sequence for the p53 binding sites consists of two copies of the motif 5′-RRRC(A/T)(T/A)GYYY-3′, separated by 0–13 bp [5]. Surprisingly, some sequences perfectly matching the p53 consensus sequence were found not to bind p53. A comprehensive list of known p53 targets can be found in the literature [6,7]. While the basic features of p53–DNA binding are known, it is still poorly understood which parameters determine the selectivity of these interactions. Wt (wild-type) p53 possesses the ability to diffuse linearly along DNA to find the target sequence [8,9]. It was proposed that non-sequence-specific binding of the p53 protein plays an important role in the activation of sequence-specific binding. A family of specific structural elements recognized by p53 has been established, including base mismatches [10], Holliday junctions and cruciform structures [11], bent DNA [12,13] and structurally flexible MAR (matrix attachment region)/SAR (scaffold attachment region) DNA [14]. The p53 protein binds preferentially to scDNA (supercoiled DNA) even in the absence of the target sequence in the scDNA molecule [15–17]. Many p53 target sequences display an internal symmetry and so are capable of assuming a non-B DNA conformation [18]. These structures are known to be stabilized by negative DNA supercoiling [19]. Contemporary analysis of the symmetry of natural p53 target sequences showed a strong palindromic coupling between the p53 half-sites [20]. The target sites can be very often described as two inverted repeats, and the two half-sites are highly repetitive. The symmetric nature of the p53 target sequence corresponds to the symmetric binding of the p53 tetramer [21]. Previous work provides strong evidence that the DNA binding activity of p53 is remarkably dependent on structural features within the target site. In addition to DNA bending [13], formation of stem–loop or cruciform structures can enhance p53–DNA binding [22,23]. Importantly, p53 has a dramatically greater affinity for a DNA microcircle containing a target site than for the same, but linear, DNA [12]. The p53 protein has also been shown to co-operate in DNA binding with other proteins which recognize and/or modulate the DNA structure, such as the cruciform binding protein HMGB1 (high-mobility group box 1), topoisomerases and various proteins of chromatin remodelling complexes [24,25]. HMGB1 selectively recognizes cruciform DNA [26] and is capable of interacting with DNA in a non-sequence-specific manner, causing a significant bending of the DNA double helix and acting as an activator of p53 sequence-specific DNA binding [24]. The role of DNA superhelical-induced cruciform extrusion as an event stimulating p53 sequence-specific DNA binding has been proposed [27], but this has not been clearly demonstrated at the level of structural transitions of a particular target sequence on changes of the DNA topology.

In the present paper, we have examined p53 binding to topologically constrained DNAs containing internally symmetrical or non-symmetrical target sites. Using a competition assay, we demonstrate a considerable correlation between the change of negative superhelix density, cruciform extrusion within the p53...
target sites and a remarkable extra enhancement of p53–DNA binding. The p53 targets having adopted cruciform structures always exhibited stronger p53 binding than p53 target sites not forming the cruciform or having a cruciform formed within a non-target sequence for p53.

MATERIALS AND METHODS

DNA

Duplex oligodeoxynucleotides containing sequences for CFNO (CFNO (cruciform structure no consensus sequence) 5′-CATGATGTGATCACATCATG-3′, PGM2 5′-AGCATGCTA-GGATGTGAT-3′, PGM3 5′-AGGCATGCCTAGGCTG-3′, PGM4 5′-AACATGTATGACATGTTT-3′) and PEV (5′-AGGCTAGTCTGAGCATGTTC-3′) with HindIII restriction sites were digested with HindIII and inserted into the HindIII site of SK (pBluescriptSK(−)). The ligated DNA was then used to transform electrocompetent Top10 Escherichia coli (Invitrogen) following the manufacturer’s instructions. DNA from single colonies selected for ampicillin resistance were used to transform electrocompetent Top10 Escherichia coli site of SK (pBluescriptSK). Clones with a PvuII fragment longer than 448 bp were subjected to single colony selected for ampicillin resistance were used to transform electrocompetent Top10 Escherichia coli site of SK (pBluescriptSK). Clones with a PvuII fragment longer than 448 bp were subjected to digestion with PvuII and electrophoresed. For competition experiments, 0.6 µg of the plasmid DNA was digested with PvuII and electrophoresed in 1% agarose gel. Because two other restriction sites which were located in the vicinity of the consensus sequence could give false results, we mutated 728A to G and 735G to A in the SK plasmid using the QuikChange® site-directed mutagenesis kit (Stratagene) following the manufacturer’s instructions. For the modification reaction, 2 µg of the plasmid DNA was incubated with 2 µM osmium tetroxide 2,2′-bipyridine complex (Fluka) in DNA binding buffer at 37°C for 30 min in a final volume of 50 µl. After modification, the samples were precipitated by ethanol, dissolved in the binding buffer and digested with XspI for 90 min at 37°C before being separated on a 1% agarose gel.

Proof of the cruciform structure by two-dimensional electrophoresis

For cruciform analysis by means of two-dimensional electrophoresis [28], prepared topoisomers [29] were mixed, loaded on to a 1.2% (w/v) TAE (Tris/acetate/EDTA)/agarose gel and run in the first dimension for 20 h at 40 V. After electrophoresis, the gel was soaked in TAE buffer containing 2.5 µg/ml chloroquine for 8 h. For the second dimension, the gel was run at 90°C and electrophoresis was performed at right angles to the first dimension in the TAE/chloroquine buffer for 20 h at 40 V. During electrophoresis, the buffer was circulated between the anode and cathode spaces.

Preparation of plasmid DNA topoisomers

Topoisomeric samples were prepared as described previously [29]. Briefly, 40 µg of DNA per ml was incubated in a solution containing 50 mM Tris/HCl (pH 7.5), 50 mM NaCl, 0.1 mM EDTA, 1 mM DTT (dithiothreitol) and 20% (v/v) glycerol (Promega). Appropriate concentrations of ethidium bromide (−σ = 0.01, 1.69 µM; −σ = 0.03, 3.8 µM; −σ = 0.05, 6.13 µM and −σ = 0.07, 9.1 µM) and 10 units of DNA topoisomerase I (Promega) was added and the samples were incubated at 37°C for 3 h. After complete relaxation, ethidium bromide and topoisomerase were removed by two phenol extractions, three chloroform extractions and ethanol precipitation. The relaxed (covalently closed circular) DNA was prepared in the same way without the addition of ethidium bromide.

Determination of DNA superhelix density

Superhelix densities (−σ) of the plasmid DNA samples were determined by electrophoresis on 1% agarose gels containing different concentrations of chloroquine (0, 1, 5 or 10 µg/ml) [29,30]. The electrophoresis was performed for 6 h at 100 V. The gels were stained with ethidium bromide, photographed, scanned and evaluated digitally. The mean superhelix densities of the topoisomers were determined from the strongest band in the distribution.
RESULTS

As shown previously, the p53 protein preferentially binds to scDNA regardless of the presence or absence of the target sequence [15], and DNA supercoiling can enhance p53 binding to certain target sites [27]. The positive effect of negative DNA supercoiling on sequence-specific DNA binding was highly pronounced, especially targets exhibiting internal symmetry being able to adopt cruciform structure under negative superhelical stress (such as pPGM1), but not those incapable of forming such structures as a result of a lack of inverted repeat symmetry [such as pRGC (plasmid RGC)]. Here, we focused on the effects connected with changes in the DNA negative superhelix density (the driving force of structural transition giving rise to the cruciform structures in inverted repeat DNA segments [19,29]) on p53 binding to various plasmid DNAs.

We prepared the following plasmid DNA constructs: (i) synthetic p53 target sequences with a perfect inverted repeat symmetry (pPGM2–4); (ii) perfect inverted repeat but a non-target sequence for p53 (pPGEV); (iii) asymmetrical p53 target sequence (pPEV); and (iv) SK vector as a control. In agreement with previous results, all of these scDNAs bound p53 protein, forming ladders of bands in agarose gels as a result of p53–scDNA complexes migrating more slowly than DNA alone (Figure 1A). No significant differences between DNA containing (Figure 1A, lanes 3–7) or not containing p53 target sequences (Figure 1A, lanes 1 and 2), or between DNA containing internally symmetrical (Figure 1A, lanes 1, 4–7) or non-symmetrical (Figure 1A, lanes 2 and 3) target sites were observed when using this simple, non-competitive gel-shift assay, showing efficient p53 binding to any scDNA. After PvuII digestion of the same DNA plasmids we observed specific p53 binding to the shorter (474 bp) fragments, provided that these fragments contained a p53 target site (Figure 1B, lanes 1–4). No retardation of any analogous fragments from SK and CFNO was detected, in agreement with the absence of the p53 target site.

To probe differences in relative p53 binding affinity to various scDNAs, we used a competition assay proposed previously [27].

Briefly, the 474 bp fragment resulting from PvuII cleavage of pPGM1 (containing a p53 target site) was used as an indicator substrate in the competition experiments. Binding of the protein to this fragment yielded a well resolved R band (retarded band) (Figure 2, lane 2). The intensity of this band was affected by the addition of the tested scDNAs. The scDNAs represented the competitors (Figure 2, lanes 3–9). Decrease of the R band intensity relative to the intensity detected in the absence of the competitors reflected the relative affinity of p53 for a given competitor. Complete absence of the R band was considered to indicate 100% affinity.

The intensity of the indicator R band decreased by approx. 70–85% on addition of scDNA (with native $\sigma$) with p53 target sequences possessing inverted repeat symmetry (pPGM1–4), but the intensity decreased only by approx. 50% on addition of scDNA involving a non-target sequence for p53 but possessing an inverted repeat (pCFNO). There was also a clear difference between DNA lacking the inverted repeats. The intensity of the indicator R band was decreased by approx. 62% (pPEV, containing a non-symmetrical target site) and approx. 40% (SK containing neither target site nor a perfect inverted repeat) respectively. Taken together, the p53 target sequences with the inverted repeat symmetry were the best competitors, followed by a non-symmetrical p53 target and lastly by DNA without any p53 target site.

Effect of DNA superhelix density variations on p53–DNA binding

To study the effects of the DNA topological state on p53 binding to various DNA substrates, we prepared sets of topoisomers of SK, pCFNO, pPEV and pPGM2 and employed them in the competitor assay (Figure 3). All DNAs exhibited increasing competitiveness towards the indicator pPGM1 fragment, but the dependence of p53 affinity on the individual plasmid DNA differed remarkably as a result of $\sigma$. Relaxed ($\sigma = 0$) circular duplex DNA (SK or pCFNO, both not containing a p53 target site) bound p53 with similar affinities, causing an approx. 30% decrease in the indicator band intensity (Figure 3). Apparent affinities of p53 for relaxed DNA containing a target site (both pPGM2 and pPEV)
The ability of all plasmids in their linearized forms to compete substantially worse competitor than pPEV even at

were higher that those observed for the non-target plasmids, but also similar to one another (50–55 % competition). Binding of p53 to SK increased slightly with increasing −σ, following an almost linear dependence and reaching 40 % competition for −σ = 0.07. pPEV also displayed a linear increasing dependence of p53 binding on −σ, showing 60–65 % competition at −σ = 0.07. On the other hand, the behaviour of pPGM2 DNA was different. At −σ = 0–0.03, the affinity of p53 for this plasmid followed practically the same trend as observed for pPEV: at a more negative superhelix density, a considerably steeper increase of binding with −σ was detected. At −σ = 0.05, pPGM2 DNA caused almost 70 % competition (60 % for pPEV) and at −σ = 0.07 85 % competition was observed (60–65 % for pPEV). Interestingly, a break on the apparent binding affinity dependence on −σ was also observed for pCFNO, which became a better substrate for p53 starting from −σ = 0.05 (but remained a substantially worse competitor than pPEV even at −σ = 0.07).

The ability of all plasmids in their linearized forms to compete for p53 (results not shown) was similar to that observed for the respective DNA in their relaxed (covalently closed circular) forms (Figure 3A).

**Cruciform extrusion in plasmids involving inverted repeat inserts**

The above results showed that differences in p53 binding (competition ability) within the groups of plasmid DNA substrates either containing (pPGM2 or pPEV) or lacking (SK or pCFNO) the p53 target sites were not statistically significant (Figure 3A) whether the DNA was relaxed or slightly negatively supercoiled (−σ ≤ 0.03). However, after the negative superhelix density reached a certain critical value (−σ = 0.05), pPGM2 (with the internally symmetrical target site) became a significantly better competitor than pPEV (without a non-symmetrical target site) for p53. Such behaviour suggests that formation of the cruciform structure within the symmetrical p53 target site of pPGM2 DNA at sufficient −σ may be responsible for the observed enhanced p53 binding. To test this assumption, we performed an analysis of structural transitions in pPGM2 based on S1 nuclease cleavage (Figure 4A), two-dimensional agarose-gel electrophoresis (Figure 4B) [19] and modification by osmium tetroxide 2,2'-bipyridine.

The S1 nuclease cleaves selectively single-stranded DNA and base-unpaired sites in double-stranded DNA (such as loop parts of hairpins and cruciforms). Cleavage of supercoiled pPGM2 (at native −σ) by S1 nuclease followed by Scal digestion produced two specific DNA fragments (1720 bp and 1267 bp), indicating the presence of the cruciform structure within the p53 target site of this scDNA (see Figure 4C). On the same treatment of relaxed pPGM2, we obtained only full-length linear DNA (2987 bp), suggesting the absence of the cruciform structure representing the specific cleavage site for the S1 nuclease (Figure 4A, lane 1).

The same experiments were performed with pPGM2 topoisomers with different −σ levels. We did not observe any indication of the specific S1 cleavage at −σ = 0.01 or 0.03, but we detected the cruciform in supercoiled pPGM2 at superhelical densities of −σ = −0.07, −σ = 0.05 or −σ = 0.07 (Figure 4A, lanes 4 and 5). A comparison of the band intensities resulting from S1 nuclease cleavage suggested that the cruciform content in pPGM2 increased strongly from −σ = 0.05 to −σ = 0.07. The SK vector which was used for construction of all plasmid substrates in this study contains a few imperfect inverted repeats [31,32] that may undergo local structural transitions in the scDNA. The weaker bands in lanes 4–6 and 8–11 of Figure 4(A), corresponding to approx. 2211 and 776 bp DNA fragments, may have arisen from S1 cleavage close to the origin of replication, where the major intrinsic inverted repeat is located. We obtained the same results using a single-strand selective chemical probe, osmium tetroxide 2,2'-bipyridine [19,33], combined with a restriction cleavage inhibition assay (results not shown).

We also detected extrusion of the cruciform structure in pPGM2 by two-dimensional gel electrophoresis. Presence of the supercoil-stabilized cruciform was indicated by the shift of the DNA band curve [28] at approx. −σ = 0.05 (Figure 4B, arrow), thus confirming the results from the S1 nuclease cleavage experiments. The superhelix density level, which was critical for cruciform appearance (−σ = 0.05), correlated well with the −σ value at which pPGM2 (and also pCFNO) displayed the break on superhelix density relative to p53 binding (Figure 3A).

Similarly, we detected the negative superhelical-dependent structural transitions in scDNA involving the p53 non-target inverted repeat (pCFNO, Figure 4A, lane 10) as well as in plasmids containing other symmetrical p53 target sites (pPGM3–4, results not shown). S1 nuclease cleavage occurred in all cases at sites of anticipated cruciform extrusion (i.e. within the inserted inverted repeats). On the other hand, no indication of a distinct
supercoil-dependent structural transition was detected under the same conditions with pPEV and SK (Figure 4, lanes 9 and 11) plasmids not containing the sequence motifs prerequisite for such transitions (Figure 3B).

**DISCUSSION**

The presence of cruciform structure in p53 target sequences is an important attribute in p53–DNA binding

Previous work has provided evidence that DNA binding of p53 is strongly dependent on the structural features of the target DNA [13,17,21,34]. It has been shown that full-length p53 was able to bind sequence specifically to target oligonucleotides adopting stem–loop structures in the absence of ‘activating’ antibodies such as PAb421 [18], McKinney and Prives [12] showed that a p53 target site within a topologically constrained DNA minicircle was bound by full-length p53 with a higher affinity than the same target site in non-constrained linear DNA. Our previous observations revealed enhanced binding of the full-length p53 protein to certain target sites within large (∼3 kb) DNA plasmids compared with the same target sites in 20-mer oligonucleotides or in the same linearized DNA plasmids. Strikingly, the effects of negative DNA superhelicity were especially well pronounced for symmetrical target sites able to adopt cruciform structures under topological constraints in the negatively scDNA. Hence it has been tentatively concluded that cruciform extrusion was the cause of the enhanced sequence-specific p53–DNA binding [27].

In this present paper we demonstrate, for the first time, that the relative affinity of the p53 protein for the same (internally symmetrical) p53 target site is remarkably changed on extrusion of a cruciform structure within the inverted repeat featuring the target site. Thus the structural transition in the binding site on topological stress represents a switch between two apparently distinct states which differ in their affinity for p53–DNA recognition (Figure 3B). The change from the low-affinity to the high-affinity binding state correlated well with the jump of the topoisomer spots in the two-dimensional agarose-gel electrophoresis and the cruciform extrusion starting at $\sigma = 0.05$ (Figure 4). On the other hand, relative binding of p53 to pPEV scDNA (in which the p53 target site was non-symmetrical and thus unable to form the cruciform structure, Figure 3B) had a monophasic, approximately linear dependence on $\sigma$ (Figure 3A). This trend reflected the known ability of full-length p53 to selectively bind scDNA with native superhelicity density regardless of the presence or absence of a target site. The SK vector, containing neither a p53 target site nor a perfect palindromic DNA motif, exhibited a dependence that was parallel to that observed for pPEV, but was shifted to lower relative binding (Figure 3). Hence in pPEV the overall p53 binding affinity appears to be a simple sum of the sequence-specific binding to the target site (not undergoing a distinct structural transition on changes of $\sigma$), and the supercoil-selective (but sequence non-specific) p53–DNA binding. Interestingly, pCFNO displayed the same behaviour as SK for $\sigma \leq 0.03$, but exhibited a certain extra enhancement of p53 binding at superhelicity densities sufficient for cruciform extrusion. This suggests that the cruciform structure itself (lacking features of the sequence-specific p53 binding site) can be bound by the protein with a certain selectivity, in agreement with results obtained previously by scanning force microscopy [11]. The difference in competition abilities of pCFNO and SK at $\sigma = 0.07$ was approx. 10%, in contrast to at least a 20% difference between pPGM2 (or pPGM3 and 4) and pPEV. The best substrate for p53 protein binding is therefore a p53 target sequence with the extruded cruciform structure. Natural p53 responsive elements often possess more or less perfect inverted repeat symmetry [18], and our preliminary experiments with some of them [such as Mdm2 (murine double minute 2 human analogue) and gadd45 (growth arrest and DNA damage 45)] revealed similar effects of negative DNA superhelicity as reported here for the synthetic p53 targets (results not shown). A combination of the structural and sequence features of the DNA substrate can thus be important for fine tuning the protein–DNA interaction.

The binding of p53 to its target DNA presents an apparent steric problem: p53 target sites may (but need not) involve insertions of variable DNA segments between the half-site. The question arises as to how p53 is able to bind such a broad spectrum of targets differing in both nucleotide sequence and conformation. It is unclear just how p53 can recognize specific DNA sequences and at the same time how it can recognize different geometries with varying sequences and conformations. Cruciform extrusion itself might solve the possible problem, with the intervening sequences (being displaced into the single-stranded loops in the cruciform structure, whereas the target half-sites are located in the hairpin stems). It simultaneously provides the binding site with considerable flexibility at the four-way junction. For target sites without intervening stretches, central parts of the binding sequence are inherently located within the cruciform apical loops, which may cause another problem for p53–DNA recognition. Nevertheless, Kim et al. [18] have demonstrated sequence-specific p53 binding to various hairpin oligonucleotide substrates, including those mimicking the p53 target (half) sites, which have adopted the cruciform (stem–loop) structure.
In more complex DNA, such as scDNA or DNA in the chromatin environment, specific structural profiles may be influenced by DNA supercoiling and/or by chromatin remodelling activities maintaining the structural fluidity of the chromatin [35]. It seems that flexibility of the p53 target sequences in long DNA targets can be one of the crucial factors in p53–DNA binding. Determination of the p53 protein bound to DNA by X-ray crystallography showed that the p53 protein bends the DNA by about 20° [13,36]. Our results are in good agreement with the new view of p53 performing regulatory tasks in the context of chromatin assembly in eukaryotic cells. In this view, the p53 protein will not bind simultaneously to all p53 target sequences, but only to those p53 targets that exhibit a structural architecture that is compatible with p53 preferred DNA structure. Cruciform target sites stabilized by DNA supercoiling are one of the favoured structures for effective p53–DNA binding in vivo. The correlation between p53 binding affinity and the presence of cruciform structures in topologically constricted DNA reflects the complexity of protein–DNA interactions. Transient supercoils occurring in the eukaryotic genome during DNA replication and transcription (processes that involve a local separation of DNA strands [37,38]), as well as a result of protein binding [39,40], may facilitate formation of secondary structures in DNA. Moreover, active chromatin remodelling is a typical feature for many promotors as well as a result of protein binding [39,40], may facilitate formation of secondary structures in DNA. The correlation between p53 sequence-specific binding in vitro predicts that the ability of p53 to bind its target sites will be strongly influenced by factors capable of modifying chromatin structure in a living cell. Changes in DNA supercoiling and stabilization of the non-B DNA structures may thus up- and down-regulate p53–DNA binding abilities.

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