Biochemical and Biophysical Research Communications 441 (2013) 83–88

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Preferential binding of p53 tumor suppressor to p21 promoter sites that contain inverted repeats capable of forming cruciform structure

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ARTICLE INFO

Article history:
Received 2 October 2013
Available online 14 October 2013

Keywords:
p53
Protein–DNA binding
Cruciform

ABSTRACT

p53 Is one of the most critical proteins involved in protecting organisms from malignancies and its gene is frequently mutated in these diseases. p53 Functions as a transcription factor and its role in the cell is mediated by sequence-specific DNA binding. Although the genome contains many p53-binding sequences, the p53 protein binds only a subset of these sequences with high affinity. One likely mechanism of how p53 binds DNA effectively underlies its ability to recognize selective local DNA structure. We analyzed the possibility of cruciform structure formation within different regions of the p21 gene promoter. p53 protein remarkably activates the transcription of p21 gene after genotoxic treatment. *In silico* analysis showed that p21 gene promoter contains numerous p53 target sequences, some of which have inverted repeats capable of forming cruciform structures. Using chromatin immunoprecipitation, we demonstrated that p53 protein binds preferentially to sequences that not only contain inverted repeats but also have the ability to create local cruciform structures. Gel retardation assay also revealed strong preference of the p53 protein for response element in superhelical state, with cruciform structure in the DNA sequence. Taken together, our results suggest that p53 response element's potential for cruciform structure formation could be an additional determinant in p53 DNA-binding machinery.

1. Introduction

The p53 tumor suppressor plays an essential role in response to genotoxic stress [1] by regulating the transcription of genes involved in cell cycle arrest, apoptosis and senescence [2,3]. As a key inhibitor of cell growth, p53 gene is often mutated or functionally inactivated in most human tumors [4,5]. A fundamental prerequisite for p53-modulated gene expression is the proximity of p53 protein to the basal transcriptional machinery of its target gene promoter, mediated via sequence-specific binding to p53 response element (RE) [6].

p53 protein and its related family members, p63 and p73, recognize and bind DNA not only through conserved amino acid residues, but also with similar affinity and transactivation profile [7]. The p53-family RE consists of two decameric half-sites: RRRCWWGYYY separated by 0–13 nucleotides [8]. This p53-binding consensus has been confirmed via large scale chromatin immunoprecipitation (ChIP) analyses [9]. Nevertheless, the range of functional p53-binding sites includes many REs with one or more base pairs (bp) that do not match such consensus [10]. In addition, non-canonical p53-binding motifs such as the pig3 (TGYCC)n microsatellite RE [11], the mdr1 "head-to-tail" promoter orientation [12], as well as p53 half-site and/or quarter-site [13,14] further broaden the spectrum of genetic configuration that p53 could modulate. A comprehensive list of verified p53 REs has been provided by Riley et al. [15]. A key question in p53 RE recognition is how p53 protein searches its target that is buried in a sea of other potential p53-binding sequences. It was demonstrated that p53 owns the ability to linearly diffuse along the DNA [16,17]. Within the nucleus, the DNA is strictly and economically organized into high order structures such that linear diffusion of p53 in nucleus would be limited to short transcriptionally active sites. Another important characteristic of p53 RE is its internal symmetry which is capable of assuming non-B DNA conformation [18]. Notably, p53 REs are not only direct repeats of half-sites, but rather two half-sites that can be coupled to form a higher order 20-bp palindrome [19]. The most frequently observed 3-bp insertion in human p53 REs is associated with enhancing the half-site palindrome formation. Moreover, the statistical frequencies of the coupling between the half-sites in the human genome are well-correlated with grouped experimental p53 affinities with p53 REs [19].

It was revealed that p53 protein binds preferentially to RE presented in cruciform structure [20,21]. In fact, protein binding to DNA cruciform structure is a common feature of an extensive array of protein families involved in junction-resolving, DNA replication and repair, transcription and chromatin-association [22]. The DNA-binding affinity of several proteins is also strongly

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http://dx.doi.org/10.1016/j.bbrc.2013.10.015

augmented by cruciform extrusion from inverted DNA repeats [23–25]. It has also been shown that synthetic p53 RE with an inverted repeat symmetry could form cruciform structure in a negative supercoiled state, which is correlated with further enhancement of p53 DNA-binding [21].

One of the key genes regulated by p53 protein in response to DNA damage is p21. Similar to p53, p21 is involved in cell cycle control and apoptosis, as well as in directing anti-apoptotic responses following DNA damage [26]. Regulation of p21 by p53 has been shown to occur via binding of p53 protein to three p21 promoter regions at positions −1400, −2300 [27] and −4500 [28]. p21 promoter is also activated coordinately by other transcriptional factors including E1AF and VDR [28,29]. Under conditions such as senescence, serum deprivation, contact inhibition, differentiation and exposure to genotoxic stresses, expression of p21 has been demonstrated to be up-regulated [30].

In this study, we analyzed in detail the regulation of p21 promoter by p53 protein. We demonstrated using ChIP that p53 protein binds preferentially to p21 promoter sequences that have the ability to form cruciform structures. These results were confirmed further via gel retardation assay. Our data taken together suggest that p53 RE's potential for cruciform structure formation may be another important factor in modulating effective p53 DNA-binding.

2. Materials and methods

2.1. Cell culture

Human breast cancer cell line MCF7 was grown in DMEM medium supplemented with 10% fetal bovine serum (PAA). MCF7 cells were kindly provided by Dr. B. Vojtěšek (Masaryk Memorial Cancer Institute, Brno).

2.2. Western blot analysis

MCF-7 lysates were analyzed by Western blot analysis as described [31]. Primary antibodies: p21 (1:250, Cell Signaling Technology), DO-1 (1:10,000) or actin (1:250, Abcam).

2.3. Immunofluorescence

Cells were fixed, permeabilized and incubated as described [24]. Primary antibodies: p53 polyclonal antibody CM-1 and p21 monoclonal antibody (Cell Signaling Technology). Each antibody was diluted 1:500 in 1% BSA dissolved in PBS. Secondary antibodies: FITC-goat anti-mouse IgG-whole molecule (Sigma), Dylight 594 anti-rabbit IgG (Abcam). Images were acquired using a Leica SP5 confocal system. Sequential scanning was used to eliminate emission spectral overlap. All emission spectra were measured independently. Controls were incubated while omitting the primary antibodies.

2.4. Chromatin immunoprecipitation

Cells were treated for 24 h with 50 μM 5-fluorouracil (5FU), 20 μM doxorubicin (DOX), 20 μM roscovitine (ROS) or 3 μM cis-platine (Cis-Pt) (Sigma). Cell density was $10^5/cm^2$. For immunoprecipitation, Chip Kit (Abcam) was used. For p53 precipitation, DO-1 mouse monoclonal antibody (kindly provided by Dr. Bořivoj Vojtěšek) was used, with antibody binding to histone H3 (Abcam) as a control.

2.5. Polymerase chain reaction

One of the three primer pairs corresponding to p21 promoter region was used in the PCR reaction: Seq1 −1335 to −1688 (5′-GAAATGCCTGAAAGCAGAGG-3′ and 5′-GCTCAGAGTCTGGAAATCTC-3′); Seq2 −2029 to −2478 (5′-CACCACTGAGCCTTCCTCAC-3′ and 5′-CTGACTCCCAGCACACACTC-3′); Seq3 −4149 to −4525 (5′-CGCGGTGCTTGGTCTCTATG-3′ and 5′-CCTTTCCCAACAAACAAGGGG-3′) (VBC-Biotech). Total volume of the PCR reaction was 30 μl which also contained Taq polymerase and polymerase buffer (New England Biolabs), nucleotides and 10 μl DNA from ChIP as the template.

2.6. Detection of PCR products

PCR reaction (10 μl) was loaded on 1× TAE 1% agarose gel. 100-bp DNA ladder (New England Biolabs) was used as comparison. Electrophoresis was performed (1 h, 100 V, room temperature). The gels were stained with ethidium bromide and snapped on UV transilluminator by digital imaging system (Herolab) and processed digitally.

2.7. Competition gel retardation assay on agarose gel

Supercoiled plasmid DNAs of pBluescript II SK (−) and pP21 [20] were isolated from bacterial strain DH5α as described in the QIAGEN protocol. *ScaI* restriction enzyme (New England Biolabs) was used for linearization of plasmids. Short DNA fragments Seq1, Seq2 and Seq3 from p21 gene promoter were obtained by PCR with specific primers. DNA (60 ng of PCR fragment with same molar amount of plasmid DNA) and p53 protein were mixed at molar ratio 4:1 in 10 μl DNA binding buffer (5 mM Tris–HCl, pH 7.0, 1 mM EDTA, 50 mM KCl and 0.01% Triton X-100). Samples were incubated for 10 min at 4 °C and loaded onto a 1% agarose gel containing 0.33× Tris–borate–EDTA buffer. Agarose electrophoresis was performed (2 h, 90 V, 4 °C) and processed.

3. Results

3.1. In silico analysis of p53 RE in p21 promoter for presence of inverted repeats

p21 promoter region contains three p53 REs localized at positions −1400, −2300 and −4500 [28]. We investigated inverted repeats in these sequences that are capable of creating palindrome using EMBOSS software suite [32]. We analyzed inverted repeats in DNA fragments bound by primers used for ChIP of p53 target sites corresponding to: −1335 to −1688 (Seq1), −2029 to −2478 (Seq2) and −4149 to −4525 (Seq3) of p21 gene promoter. Since there were a large number of palindromes with inverted repeats being 6-bp long, we focus here only on 7–10-bp inverted repeats. Only Seq2 contains two 7-bp inverted repeats without any mismatches. On the other hand, Seq1 and Seq3 contain four and two inverted repeats of this length, respectively, but with one mismatch in each. Analyses of longer inverted repeats show that none of these three sequences contain inverted repeats without mismatches. Seq1 and Seq3 contain two 8-bp palindromes with one mismatch. Sequences with 10-bp in length were detected with two (Seq3) and three (Seq1 and Seq2) mismatches.

3.2. Activation of p21 protein expression by chemical treatment

To study the occupancy of p53 REs within p21 promoter region, we analyzed p21 and p53 protein levels by Western blot analysis before and after various chemical treatments (50 μM 5FU, 20 μM

DOX, 20 μM ROS or 3 μM Cis-Pt) for 24 h. We used MCF-7 cell line expressing wild-type p53 protein. Western blot analysis of untreated cells showed very low base levels of p21 and p53 proteins (Fig. 1, lane 1). We detected strong elevation of p21 protein level in cells treated with 5FU (Fig. 1, lane 2) and ROS (Fig. 1, lane 4). Similarly, we observed intense elevation of p53 protein level after chemical treatment, especially by 5FU (Fig. 1, lane 2), DOX (Fig. 1, lane 3) and ROS (Fig 1, lane 4). We also detected decent accumulation of p53 protein level post Cis-Pt treatment (Fig. 1, lane 5). However, we did not notice elevation of p21 protein level in cells treated with DOX (Fig. 1, lane 3) or Cis-Pt (Fig. 1, lane 5). We analyzed also localization of these proteins in cells via confocal microscopy (Fig. 2). In control cells without any treatment, we observed only low level of p21 protein (Fig. 2A-b) and no detectable signal for p53 protein (Fig. 2A-c). After 5FU treatment, we observed strong elevated levels of p21 and p53 proteins (Fig. 2B-b and B-c, respectively). While p21 protein localized mainly to the cytoplasm, p53 protein was located strictly in cell nucleus as evidenced by the co-localization of p53 and DAPI signals (Fig. 2B-d).

3.3. Comparison of p53 binding to p21 promoter sequences in situ and in vitro

Using ChIP, we analyzed p53 protein binding to three known p53 REs (p21 promoter region at positions −1400, −2300 and −4500) in cells before and after chemical treatment. We did not precipitate any DNA from p21 promoter region from cells without chemical treatment. In addition, cells treated with ROS also did not indicate any immunoprecipitation (not shown). We observed strong p21 promoter immunoprecipitation following 5FU treatment (Fig. 3). We precipitated similar amount of all the tested sequences using H3 control antibody (Fig. 3A, lanes 2, 6, 11). p53 protein bound with the highest affinity to target Seq2 (Fig. 3A, lane 5 and Fig. 3B column 2) followed by Seq1 and Seq3, with Seq3 (Fig. 3A, lane 10 and Fig. 3B column 3) showing the weakest affinity for p53 binding. In fact, the affinity of p53 for Seq2 was shown to be 6 time stronger compared to Seq3 (Fig. 3B). This result is in direct correlation with presence of perfect inverted repeats localized within Seq2.

After obtaining results from ChIP, these were verified by competitive *in vitro* Electrophoretic Mobility Shift Assay (EMSA). Wild-type p53 full length protein was incubated with Seq1–3. We observed formation of distinct retarded band after incubation of these fragments with purified p53 protein (Fig. 4A and B, lanes 2, 6, 10, arrow). As a competitor we used plasmid pBluescript without p53 target sequence in superhelical (Fig. 4A, lanes 3, 7, 11) or linear (Fig. 4A, lanes 4, 8, 12) state. Superhelical DNA of pBluescript competed remarkably for p53 binding to PCR fragments from p21 promoter sequences, in contrast to the same DNA but in a linear state (compare retarded band of Fig. 4A in lanes 3–4; 7–8; 11–12, arrow). To elucidate the influence of p21 target sequence more exactly, we also used plasmid pP21, which is derived from pBluescript by insertion of the 20-bp p53 RE. This sequence corresponds to p53 RE in Seq2 PCR fragment. As a competitor, both supercoiled and linearized forms of plasmid pP21 decreased the intensity of the retarded band (Fig. 4B, arrow). Moreover, we did not detect any retarded band in the competition assay of superhelical pP21 with PCR fragments Seq1 and Seq3 (Fig. 4B, lanes 3, 11). A very weak signal of retarded band was detectable with competition involving linearized pP21 plasmid (Fig. 4B, lanes 4, 12). All p53 protein appeared to bind preferentially to supercoiled pP21 which contains sequence capable of creating cruciform structure. Only a small proportion of p53 protein remains bound to Seq2 PCR fragments.

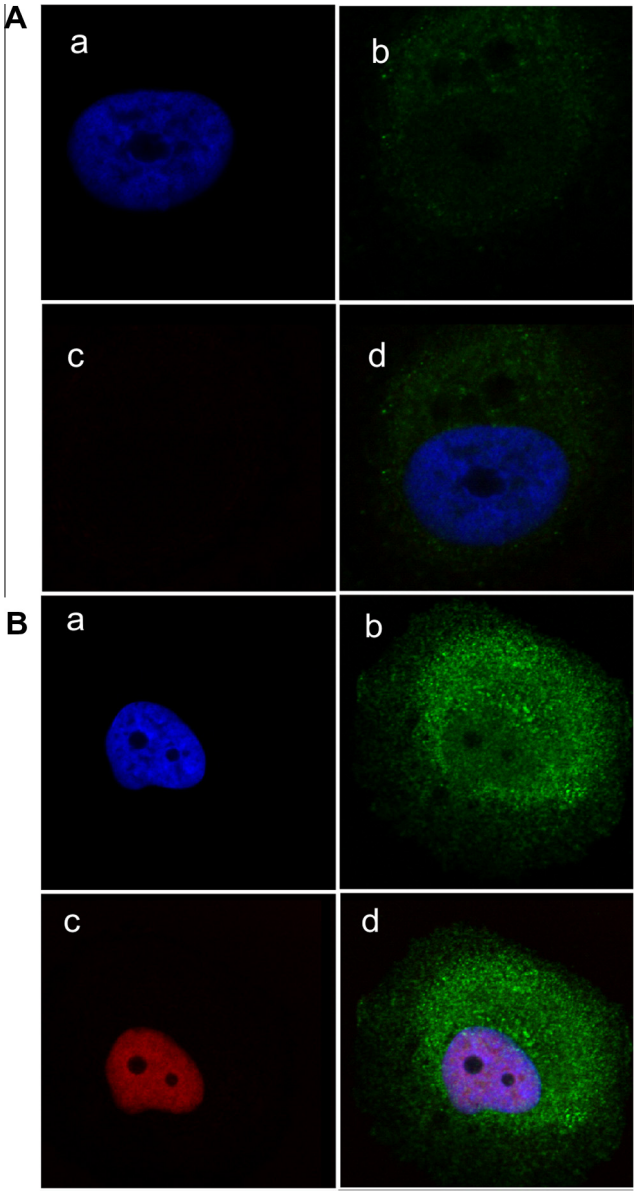

Fig. 2. Immunodetection of p53 and p21 proteins in MCF-7 cell line. Cells were grown on round coverslips coated with polylysine, treated with damaging agent 5FU (B) and compared with control sample (A). Cells were stained with specific antibodies against p53 and p21. Secondary antibodies were conjugated with fluorophores (p53 – red, p21 – green). After staining, samples were mounted with medium containing DAPI stain and visualized using confocal microscope Leica SP5. Picture a represents cell nucleus stained with DAPI; picture b represents p21 protein stained with its specific antibody; picture c represents p53 protein stained with its specific antibody; picture d is an overlay of pictures a, b and c. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

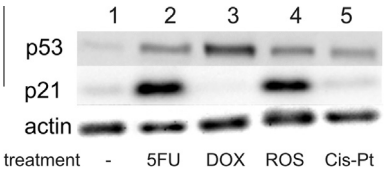

Fig. 1. Western blot analyses of p21 protein expression in MCF-7 cell line with and without genotoxic treatment. MCF-7 cells were treated with 5-fluorouracil (5FU), doxorubicin (DOX), roscovitine (ROS) and cis-platine (Cis-Pt) and compared with untreated control sample (−). Proteins were stained by specific antibody against p21 protein, actin and p53. Secondary antibodies were conjugated with peroxidase and signals were visualized using Las3000 system.

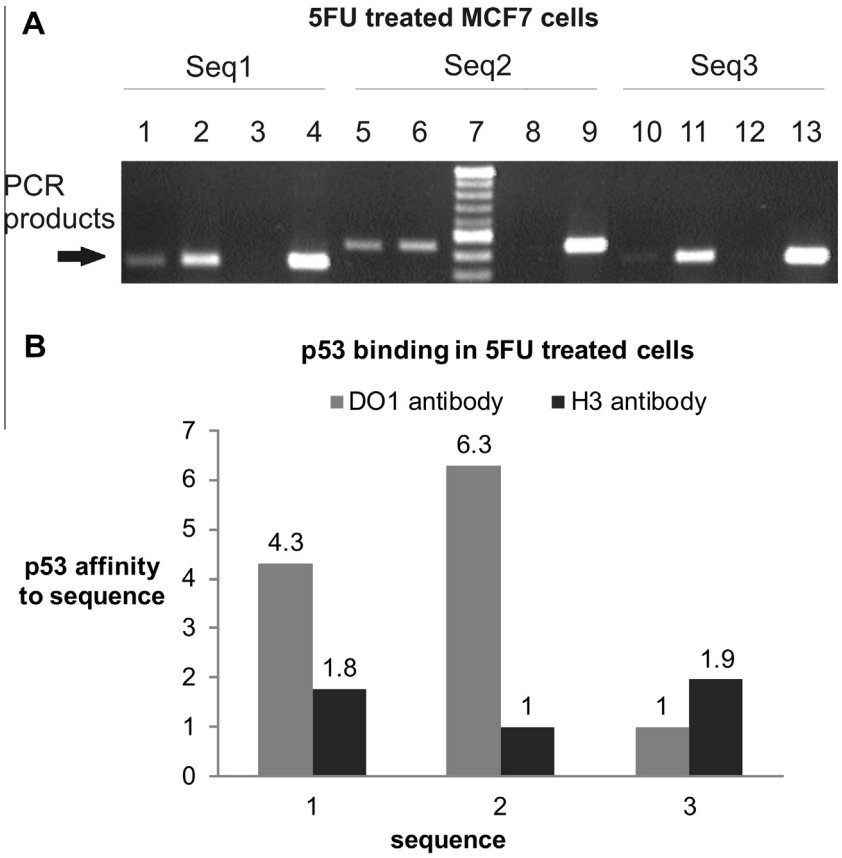

Fig. 3. Chromatin immunoprecipitation. (A) MCF-7 cells were treated with 5FU and compared with control sample without treatment. PCR was performed with 3 specific primer pairs covering the three p53-binding sites in p21 gene promoter. Lanes 1–4 were amplified with primer pair Seq1, lanes 5, 6, 8 and 9 were amplified with primer pair Seq2, lanes 10–13 were amplified with primer pair Seq3. Lane 7 contains only DNA ladder for identification of PCR products. DNA precipitated with DO-1 antibody was used as a template in lanes 1, 5 and 10. DNA precipitated with H3 antibody (verification of ChIP process) was used as a template in lanes 2, 6 and 11. DNA precipitated with beads only was used as a negative control in lanes 3, 8 and 12. DNA isolated from MCF-7 cells was used as a template in lanes 4, 9 and 13 (verification of PCR process). (B) Densitometrical analysis of PCR products by ImageJ software. Signals from one sample and one antibody were compared relatively against the weakest signal.

On the other hand, linearized plasmid without p53 response sequence was not as a sufficient competitor and the intensity of retarded bands with PCR fragments waned only slightly (compare retarded bands – Fig. 4A, lanes 3 and 4; 11 and 12).

4. Discussion

The p21 promoter region has been demonstrated to possess three p53 REs at positions around −1400, −2300 [27] and −4500 [28]. Several lines of evidence suggested the significance of DNA secondary structure formation for effective p53 binding to its REs [20,21]. Importantly, it was revealed that p53 target sequence is often associated with inverted repeats that are capable of forming cruciform structure [10,18]. *In situ* analyses also revealed a possible role of higher order 20-bp palindromes within p53-responsive sequences [19]. We therefore analyzed the association of palindromic sequences within p53 REs of p21 promoter and accessibility of these elements for p53 protein via ChIP. Surprisingly, all the tested sequences contain inverted repeats demonstrating the potential for non-B DNA structure formation in the p21 promoter sequence. Notably, only Seq2 contains the ideal inverted repeats without any mismatches, suggesting a higher probability for cruciform structure formation and sequence stability. Although we did not observe any interaction of p53 with p21 promoter sequences via ChIP after ROS treatment (not shown), it is likely that p21 under these conditions was expressed in a p53-independent manner as previously described [33]. Using confocal microscopy, we showed that treatment of MCF-7 cells by 5FU induced p53 and p21 protein expression significantly. In addition, p53 protein localized intensively to the nucleus after treatment. Although ChIP indicated that all the tested sequences were precipitated by p53 DO-1 monoclonal antibody, there was a strong preference of p53 for Seq2 as it is the only palindromic sequence without any mismatch within p21 promoter region. To validate the affinity of p53 for its responsive sequences, we performed EMSA experiments using PCR fragments of tested sequences and demonstrated that all three sequences were capable of binding p53 protein. However, under competition conditions, there was a strong preference for p53 binding to superhelical DNA, especially superhelical DNA containing p53 RE from Seq2 of p21 promoter.

Although p53 RE is believed to be bound via the core domain of p53 [34], the C-terminal domain of p53 remains to play an important role in DNA binding and recognition. In fact, the C-terminal domain of p53 can positively modulate p53 binding to several DNA structures including hemicatenated DNA, minicircular DNA, as well as supercoiled DNA [10,35–38]. Moreover, carboxy-terminally truncated p53 is impaired in binding not only p53 target promoters *in vivo* but also DNA templates in chromatin structure [16,39]. This indicates that the C-terminal domain of p53 is explicitly required for promoter binding in the context of chromatin. Our results with full length wild-type p53 protein revealed similar preferences in both EMSA and ChIP assays. It is therefore likely that even in the context of chromatin, there is a preserved preference

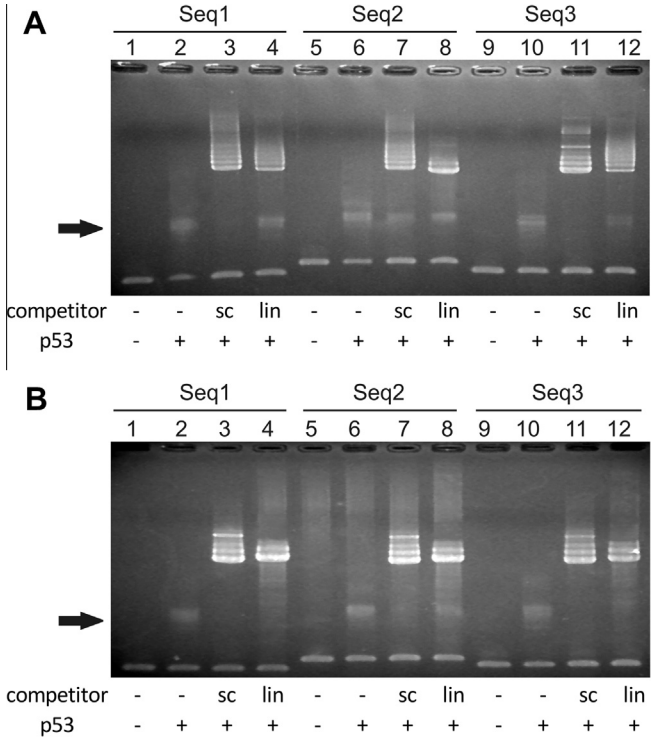

Fig. 4. p53 binding to PCR products *in vitro*. (A) Competition using DNA lacking p53 target sequence (pBluescript). (B) Competition using DNA containing p53 target sequence (pP21). p53 protein was incubated with PCR products (4:1 ratio) for 10 min on ice in binding buffer without competitor DNA (lanes 2, 6 and 10), and with DNA competitor in superhelical form (lanes 3, 7 and 11, sc) or in linear form (lanes 4, 8 and 12, lin). Lanes 1, 5 and 9 contain only PCR products. After incubation, samples were loaded on 1% agarose 0.33× TBE gel, run for 120 min, stained by ethidium bromide, snapped on UV transilluminator by digital imaging system (Herolab) and processed digitally.

for palindromic sequences in p53 REs. Although p53 REs may involve insertions of variable DNA segments between the half-sites, it was shown that p53 is also capable of binding half and/or quarter-sites [13,14]. The formation of non-B DNA structure could subsequently act as a catalyst to bring p53 REs that are separated by a considerable distance close together. Cruciform structures provide another possible mechanism for easier accessibility of distant p53-responsive half and/or quarter sites. Interestingly, it was demonstrated that superhelical density of native plasmid is sufficient for cruciform formation in p53 REs from p21 promoter [20]. Hence, the presence and potential of non-B DNA structure formation within p53 REs may play an important role in p53 DNA-binding affinity and regulation.

Acknowledgments

This work was supported by the Grant Agency of the Czech Republic (P301/11/2076, P206/12/G151).

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