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Specific Modulation of p53 Binding to Consensus Sequence within Supercoiled DNA by Monoclonal Antibodies

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Monoclonal antibodies (MAbs) were used to investigate the binding of insect cell-expressed, wild-type human p53 protein to the consensus sequence (p53CON) in a 474-bp DNA fragment and to supercoiled (sc) DNAs with and without p53CON. Supershifting of p53–DNA complexes by MAbs in agarose gels was applied to studies of activation of p53 for sequence-specific binding within scDNA. C-terminal specific antibody Bp53-10.1 activated the sequence-specific binding of p53 to p53CON within pPGM1 scDNA but did not influence binding of p53 to pBluescript scDNA (not containing p53CON). Incubation of p53 with DO-1 prior to addition of Bp53-10.1 prevented activation of p53 and induced dissociation of a portion of pPGM1 scDNA from the sequence-specific immune complex; no such dissociation was observed if pPGM1 scDNA was replaced by the 474-bp p53CON-containing DNA fragment.

The p53 protein was first described in 1979, but its ability to function as a tumor suppressor was not recognized until the 1980s [reviewed in (1)]. p53 contains 393 amino acids and is organized into several functional domains. The N-terminal domain contains a transactivation region (1–42) and a proline-rich region with five copies of the sequence PXXP (61-94 in human p53). The C-terminal part of the protein contains a flexible linker (~300–~325), a tetramerization domain (~325–~356) and the extreme C-terminus which is highly basic [reviewed in (2, 3)]. The latter domain contains a well-characterized negative regulatory domain which is able to contribute to the activation of latent p53 for sequence-specific DNA-binding. The sequence-specific DNA-binding domain is contained in the evolutionary conserved central region (~100–~300) which is involved not only in binding to the DNA consensus sequence [p53CON, consisting of two copies of PuPuPuC(A/T) (T/A)GPyPyPy separated by 0–13 bp (4)], but also in non-specific binding to internal regions of single-stranded DNA (5). Point mutations and deletions in the p53 gene are the most frequently observed alterations in human cancers (6, 7). Such mutations can inactivate sequence-specific DNA-binding through alterations in the folding of the core domain (8).

Monoclonal antibodies (MAbs) are indispensable tools in current p53 research. They can be used for detection, localization and quantification of p53 as well as in p53 structural and functional studies. p53 epitopes have been divided into two broad categories primarily on the basis of whether antibody reactivity is dependent on, or independent of a native p53 structure (9, 10). Conformational or discontinuous epitopes involve residues widely spaced in the primary sequence but brought into spatial proximity by p53 folding. In contrast, sequential or continuous epitopes are formed entirely by residues adjacent in the primary sequence. Methods for characterizing and locating the sequential epitopes are more abundant and rapid compared to those necessary for the conformational epitopes. One of the methods applicable to both types of epitopes is gel retardation.

We have previously shown that wild-type human p53 protein binds preferentially to supercoiled DNA regardless of the presence or absence of p53CON (11). As a result of this binding, a ladder of DNA bands can be observed on ethidium-stained agarose gels in which each retarded band contains a DNA–p53 complex (12, 13). Binding of p53 to scDNA is inhibited by oxidation of the protein (13) and by some metal ions (12). It has been suggested that both the core domain and the C-terminal domain may be involved in p53 binding to scDNA (11). Strong preference of p53 for scDNA (as compared to linear and relaxed DNA molecules) may

Abbreviations used: MAb, monoclonal antibody; p53CON, p53 consensus DNA binding sequence; sc, supercoiled.

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reflect the affinity of the protein for specific local secondary and/or tertiary structures stabilized by DNA supercoiling.

Here we demonstrate that p53–DNA complexes can be supershifted by MAbs using agarose gels. We also show that both the N-terminal and C-terminal specific antibodies supershift p53–scDNA complexes and modulate the p53 sequence-specific binding within scDNA.

MATERIALS AND METHODS

Plasmids. Supercoiled plasmid DNA of pBluescript SK II and/or pPGM1 was isolated from bacterial strain DH5α as described in the QIAGEN protocol (QIAGEN GmbH, Germany). pPGM1 plasmid contains an insertion of 22-mer oligonucleotide encoding the p53 consensus sequence (tAGACATGCCTAGACATGCCTa) in the *Hin*dIII restriction site of pBluescript SK II (14). The pPGM1 DNA was digested with the *Pvu*II restriction endonuclease (MBI Fermentas) resulting in a 2513-bp long fragment without consensus sequence and a short 474-bp fragment containing the consensus sequence. *Sma*I restriction enzyme (MBI Fermentas) was used for linearization of both pBluescript SK II and pPGM1.

Isolation and purification of p53 protein. Human wild-type p53 protein was expressed in Sf9 insect cells infected with a recombinant baculovirus and isolated as described (11). We used a p53 fraction eluted at 0.40 M KCl (storage buffer: 20% glycerol, 25 mM Hepes–NaOH, pH 7.6, 0.1% Triton X-100, 5 mM DTT and 1 mM benzamidine). Isolation and purification of the anti-p53 monoclonal antibodies was performed as described (15).

In vitro binding assays. Binding of wild-type p53 protein to DNA was performed as follows: 300 ng of DNA (supercoiled or linear DNA fragments) and 100 ng of wild-type p53 protein (molar ratio of p53 tetramer/DNA 3/1) was incubated in binding buffer (5 mM Tris, 0.5 mM EDTA, 50 mM KCl, 0.01% Triton X-100, pH 7.8) for 30 min on ice. p53–DNA complexes were incubated with monoclonal antibody (molar ratio of MAb/p53 tetramer 3/1) at 20°C for another 15 min.

Excess of competitor DNA (ratio of *Pvu*II fragment/competitor 1/3) was used in competition experiments. pBluescript scDNA and/or linear DNA or pPGM1 linear DNA (linearized with *Sma*I) was added (prior to addition of monoclonal antibody) and incubated for 30 min on ice.

Alternatively, wild-type p53 protein was preincubated with the first monoclonal antibody (molar ratio of MAb/p53 tetramer was 3/1) at 20°C for 10 min. Then scDNA (p53 tetramer/DNA 3/1) was added and incubation continued on ice for 15 min. After the formation of the MAb–p53–DNA ternary complex, the second monoclonal antibody was added (MAb/p53 tetramer 3/1) and the mixture was incubated for 15 min at 20°C. Loading buffer (40% sucrose, 0.25% bromphenol blue, 0.25% xylen cyanol FF) was then added and samples were run in 1% agarose gel as described (11).

RESULTS

Monoclonal antibodies supershift sequence-specific complexes of p53 with linear DNA fragments in agarose gels. We have previously shown (11) that binding of p53 to the consensus sequence (p53CON) in relatively long linear DNA fragments (several hundred bp) is manifested by a retarded band R in agarose gel. Here we used this band to study the effect of MAbs on the sequence-specific DNA-binding activity of insect cell-expressed p53. pPGM1 DNA was cleaved by *Pvu*II and the DNA fragments (2513 and 474 bp) were incubated with wild-type human p53 at molar ratio of p53 tetramer/DNA 3/1. After incubation, the monoclonal antibody DO-1 (which binds to the N-terminus at amino acids 20–25; (16)) was added at MAb/p53 tetramer 3/1. Formation of immune complexes resulted in a supershifted band R_s (Fig. 1A, lane 4) with no sign of activation of p53 for DNA binding. With the pBluescript *Pvu*II 448-bp fragment (not containing p53CON), no retarded bands appeared even at p53 tetramer/DNA 10/1 (not shown).

Using polyacrylamide gels, it was shown that PAb421, specific for the C-terminal part of p53 (aa 371–380), activated sequence-specific binding of p53 to radioactively-labeled short oligonucleotides (10). Using the same MAb with *Pvu*II DNA fragments and agarose gels, we obtained similar results (not shown) as Hupp *et al.* (10). In addition to PAb421, we used a similar MAb, Bp53-10.1 (15, 17) (binding to aa 371–380), which supershifted and activated p53 for DNA binding (Fig. 1A, lane 3) as indicated by stronger band R_s and by a large decrease in intensity of the unretarded band of the 474-bp (p53CON-containing) fragment compared to this band in lanes with non-supershifted (Fig. 1A, lane 2) and DO-1 supershifted (Fig. 1A, lane 4) p53–DNA complexes. Band R_s of the (DO-1)–p53–DNA complex was more retarded than band R_s of (Bp53-10.1)–p53–DNA (Fig. 1A, lanes 3 and 4). Similar difference in mobilities was observed with free DO-1 and Bp53-10.1 (not shown) suggesting that different mobilities of the immune complexes (Fig. 1A, lanes 3 and 4) may be related to mobilities of the free MAbs in the agarose gel. Further details documenting the ability of Bp53-10.1 to activate p53 for sequence-specific DNA binding will be published elsewhere (15).

Binding of p53 to scDNA. Sharp band R (or R_s) (Fig. 1A) can be conveniently used to study the effect of various agents on sequence-specific p53 binding to p53CON. Here we used these bands to examine the influence of addition of scDNA or linDNA on the stability of sequence-specific DNA-p53 and DNA–p53–(DO-1) complexes (Fig. 1B). Using densitometric tracing and a ratio of *Pvu*II fragment/competitor = 1/3, we found that supercoiled and linear pBluescript DNA had decreased the intensity of band R by 69 and 26%, respectively (Fig. 1B, lanes 3 and 4). With the DNA–p53–(DO-1) complex, band R_s decreased due to addition of sc and linDNAs by 65% and 24%, respectively (Fig. 1B, lanes 7 and 8). These results suggest that scDNA can be efficiently dissociated from the sequence-specific p53–DNA complex, in agreement with our previous findings (11). Binding of DO-1 to the N-terminal region of p53 does not substantially influence this process.

We attempted to study the nature of the earlier described ladder of bands (11–13, 18) produced by p53–scDNA complexes by supershifting them with MAbs.

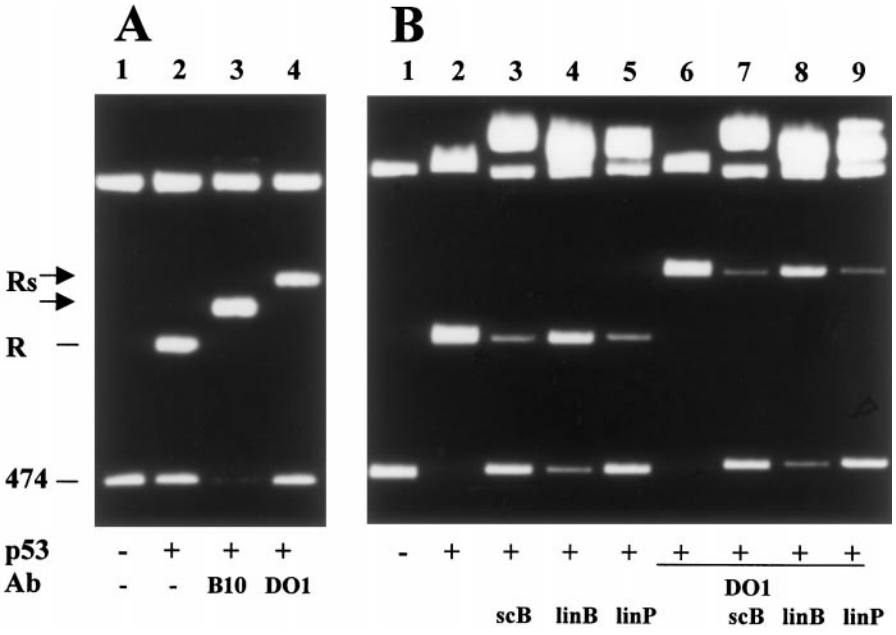

FIG. 1. Supershifting of p53–DNA complexes with monoclonal antibodies. *Pvu*II fragments of pPGM1 DNA were incubated with wild-type p53 protein; the molar ratio of p53 tetramer/DNA was 3/1 (A) or 7/1 (B). (A) Monoclonal antibodies Bp53-10.1 (B10; lane 3) and/or DO-1 (DO1; lane 4) were added at molar ratios MAb/p53 tetramer 3/1. (B) pBluescript scDNA (scB) and/or linear DNA (linB) or pPGM1 linear DNA (linP) were used to dissociate the sequence-specific p53–DNA complex (ratio of *Pvu*II fragment/competitor 1/3). Monoclonal antibody DO-1 (lanes 6–9) was added at molar ratio MAb/p53 tetramer 3/1. Samples were loaded on 1% agarose gel in 0.33× TBE. After electrophoresis, the gel was stained with ethidium bromide for 30 min. The free *Pvu*II restriction fragment containing the p53CON sequence (474 bp), the band R containing p53–DNA complex and the R_s bands composed of MAb–p53–DNA complexes are marked (the arrows represent positions of p53–DNA complexes bound to monoclonal antibodies).

Incubation of p53 with pBluescript scDNA (not containing p53CON) at molar ratios p53 tetramer/DNA 3/1 resulted in two retarded DNA bands 1 and 2 on the gel (Fig. 2, lane 2). As a result of incubation of the p53–scDNA complexes with excess of each MAb [DO-1, Bp53-10.1 or Bp53-30.1 which binds to aa 371–380 (15)], these bands were supershifted, while the mobility of the unretarded band 0 remained unchanged (Fig. 2, lanes 3–5). A titration of DO-1 antibody gave rise to an intermediate form of the MAb–p53–DNA complex, probably corresponding to one DO-1 molecule bound per tetrameric p53 (Fig. 3, lanes 4 and 5). Two DO-1 molecules completely supershifted the p53–DNA complex (Fig. 3, lane 6). Previous observations obtained with DNA oligomers suggested that two DO-1 epitopes are accessible on the tetrameric form of p53 (14, 19, 20). Similarly, molar ratio of Bp53-10.1/p53 tetramer = 2/1 was necessary for complete supershifting of p53–DNA complex (not shown).

Bp53-10.1 activates p53 binding to p53CON in supercoiled DNA. We examined activation of p53 by Bp53-10.1 for binding to p53CON within pPGM1 scDNA. As in the previous experiments with pBluescript (Figs. 2 and 3), supershifting of bands 1 and 2 was observed by Bp53-10.1 (Fig. 4, lanes 3 and 7) as well as by DO-1 (Fig. 4, lanes 4 and 8). In addition, p53 was activated for binding to pPGM1 scDNA as detected by a decrease in intensity of band 0 (Fig. 4, lane 7), while in scDNA pBluescript band 0 was unchanged (Fig. 4, lane 3). DO-1 did not induce activation of p53 for binding to these DNAs (Fig. 4, lanes 4 and 8; Fig.

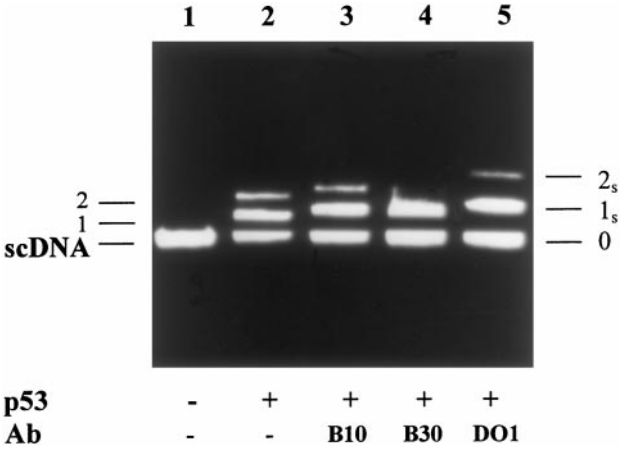

FIG. 2. Supeshifting of pBluescript scDNA–p53 complexes with monoclonal antibodies. pBluescript scDNA plasmid was incubated with wild-type p53 protein (lanes 2–5); the molar ratio of p53 tetramer/DNA was 3/1. Then, monoclonal antibodies Bp53-10.1 (B10; lane 3), Bp53-30.1 (B30; lane 4) and/or DO-1 (DO1; lane 5) were added (MAb/p53 tetramer 3/1). Samples were run on a 1% agarose gel in 0.33× TBE as described under Materials and Methods. Free scDNA corresponds to unretarded band 0. Bands 1 and 2 composed of p53–scDNA complexes and bands 1_s and 2_s composed of MAb–p53–scDNA immune complexes are marked.

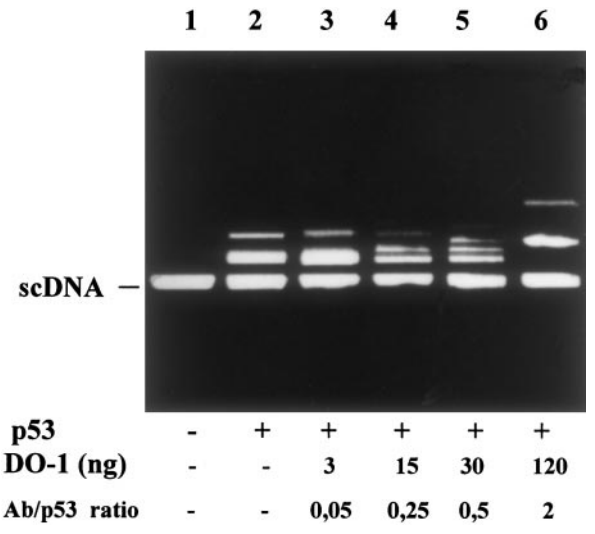

FIG. 3. The dependence of p53–scDNA complex mobility on DO-1 antibody concentration. pBluescript scDNA plasmid was incubated with wild-type p53 protein (lanes 2–6); the molar ratio of p53 tetramer/DNA 3/1. Then, different amounts of DO-1 monoclonal antibody (lanes 3–6) were added to the p53–scDNA complex (the amount of DO-1 antibody and its molar ratio to p53 tetramer is indicated below the figure). (DO-1)–p53–scDNA immune complexes were separated on a 1% agarose gel as described under Materials and Methods.

5B, lane 3). Activation of p53 binding to pPGM1 scDNA (but not to pBluescript scDNA) was observed at different p53/DNA and MAb/p53 ratios (not shown). We conclude that Bp53-10.1 activates the p53 protein for binding to p53CON within scDNA in a manner similar to p53CON binding in linear DNA fragments (Fig. 1A, lane 3).

The order of addition of antibodies determines modulations of p53 DNA-binding affinity to p53CON in scDNA. It was shown by Hupp *et al.* (14) that DO-1 supershifts the activated PAb421–p53–DNA complex (where DNA was a 26-mer containing p53CON). If the order of addition of antibodies was changed so that DO-1 was allowed to form the complex with p53 prior to the addition of PAb421, the activation of p53 was abolished. We also observed the loss of p53 activation in a similar experiment using scDNA pPGM1 and Bp53-10.1 instead of PAb421 (Fig. 5A, lane 8). In addition, when Bp53-10.1 was added to the (DO-1)–p53–DNA immune complex, the intensity of band 0 of pPGM1 scDNA strikingly increased (Fig. 5A, lane 8), indicating a dissociation of DNA from the p53 immune complex. The intensity of band 0 increased with the increasing level of Bp53-10.1 (Fig. 5B, lanes 4–6). No DNA dissociation was detected when scDNA was replaced by the 474-bp (p53CON-containing) pPGM1 DNA fragment (not shown). Incubation of the (DO-1)–p53 immune complex with Bp53-10.1 prior to addition of pPGM1 scDNA resulted in strong inhibition of p53 binding to scDNA (not shown). With scDNA pBluescript and high p53/DNA ratios (conditions where binding of p53 to sites other than p53CON in scDNA pPGM1 prevail), no dissociation of DNA was detected (not shown) indicating that the dissociation of pPGM1 DNA is related to the sequence-specific binding in scDNA.

DISCUSSION

Supershifting of p53–DNA complexes in agarose gels. Supershifting of p53 complexes with oligonucleotides and shorter DNA fragments by MAbs has been performed earlier in polyacrylamide gels (14, 21). Our results on agarose gels represent an extension of the existing technique for longer DNAs; with little reduction of sensitivity and with no DNA labeling, well defined bands can be obtained suitable for densitometric tracing. Using the *Pvu*II digest of pPGM1, no addition of competitor DNA is necessary because the presence of the 2513-bp fragment is sufficient for this purpose. On the other hand, with the isolated (p53CON-containing) 474 bp fragment, addition of competitor DNA was necessary to decrease the nonspecific binding of p53 to the 474-bp fragment (13). Supershifting of p53–DNA complexes by MAbs is a powerful technique and the possibility of using this technique with scDNA on agarose gels, opens the door for more detailed studies of the interactions of p53 with scDNA, as well as of the non-specific binding of this protein to long linear ds and ss DNAs.

Modulation of p53 binding to scDNA by MAbs. Preferential binding of p53 to scDNA was first described in 1997 (11). Recently, Mazur *et al.* (18) confirmed this new type of p53 binding and showed that p53 binds not only to negatively but also to positively supercoiled DNA. These authors also used MAbs (PAb421, DO-1, PAb1801 and PAb240) to supershift

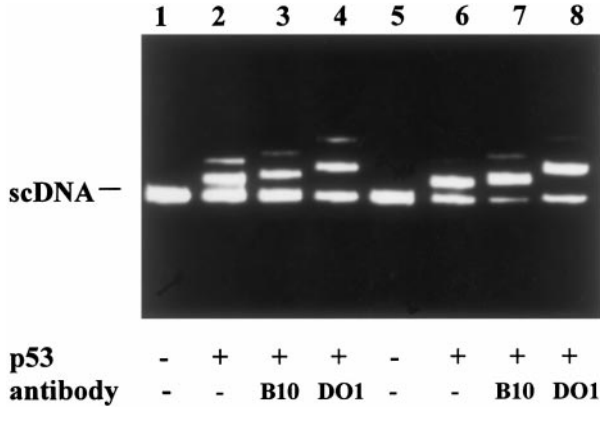

FIG. 4. Activation of p53 binding to p53CON in supercoiled DNA. pBluescript (lanes 1–4) and/or pPGM1 (lanes 5–8) scDNA, respectively, was incubated with wild-type p53 protein (lanes 2–4 and 6–8); the molar ratio of p53 tetramer/DNA 3/1. Then monoclonal antibody Bp53-10.1 (B10; lanes 3 and 7) and/or DO-1 (DO1; lanes 4 and 8) was added (MAb/p53 tetramer 3/1). Samples were run on a 1% agarose gel in 0.33× TBE as described under Materials and Methods.

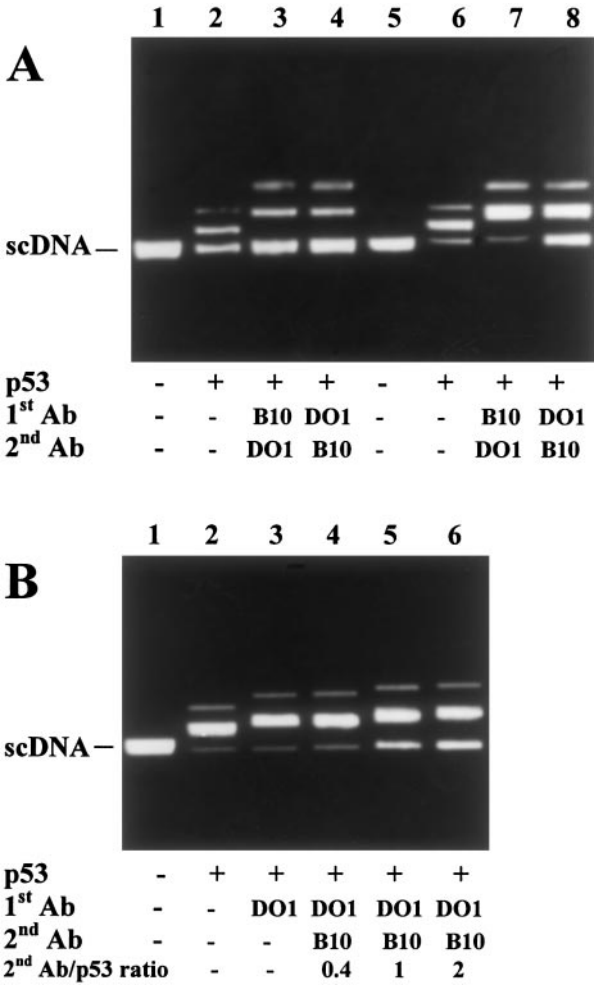

FIG. 5. Modulation of p53 binding to p53CON in supercoiled pPGM1 DNA by preincubation of the p53 protein with DO-1 antibody. (A) Immune complexes (1st Ab) of p53-(Bp53-10.1) (lanes 3 and 7) and/or p53-(DO-1) (lanes 4 and 8), respectively, were prepared prior to binding of p53 to scDNA (see Materials and Methods). Then, pBluescript (lanes 1–4) and/or pPGM1 (lanes 5–8) scDNA was added to p53 protein (lanes 2 and 6) and/or to p53 immune complexes (lanes 3, 4, 7, and 8), respectively. Finally, a second monoclonal antibody (2nd Ab) was added (addition of p53, 1st Ab and 2nd Ab is marked below the figure). (B) The dependence of the dissociation of (DO-1)–p53 complex from pPGM1 scDNA on an increasing level of Bp53-10.1. (DO-1)–p53–scDNA pPGM1 complexes (lanes 3–6) were prepared as described above. Then, increasing amounts of Bp53-10.1 antibody (2nd Ab) were added (denoted as 2nd Ab/p53 tetramer ratio below the figure). Samples were run on a 1% agarose gel in 0.33× TBE. Free scDNA corresponds to unretarded band 0.

the p53–scDNA complexes but for technical reasons they were unable to clearly resolve band 0 from other bands. Our results show that both N-terminal and C-terminal specific MAbs supershift the bands of scDNA in essentially the same way: the mobility of band 0 is unchanged while all other bands are supershifted, suggesting that band 0 is free of p53 while other bands contain DNA–p53 complexes (Figs. 2 and 3), in agreement with immunoblot results (12, 13). There is, however, a striking difference between N-terminal and C-terminal specific MAbs in their effect on the intensity of band 0 of pPGM1 DNA. While the N-terminal specific DO-1 is without effect (Fig. 4, lane 8), the C-terminal specific Bp53-10.1 decreases the intensity of this band, suggesting that the amount of p53-unbound DNA is decreased as a result of p53 activation for DNA binding by the MAb (Fig. 4, lane 7). In contrast to pPGM1, in pBluescript scDNA no changes in the intensity of band 0 induced by Bp53-10.1 were observed (Fig. 4, lane 3). This finding suggests that Bp53-10.1 activates the p53 protein for binding to p53CON in scDNA (pPGM1) but exerts no effect on binding of p53 to other sites in scDNA. Activation of p53 by C-terminal specific PAb421 for binding to p53CON in short linear DNAs was described earlier (14). Our results show that p53 can be activated by the C-terminal specific MAb for binding to p53CON within pPGM1 scDNA (Fig. 4, lane 7).

This activation of p53 was not prevented by addition of DO-1 to the (Bp53-10.1)–p53–scDNA pPGM1 complex (Fig. 5A, lane 7). However, when p53 was reacted with DO-1 prior to addition of Bp53-10.1, no activation was detected (Fig. 5A, lane 8) and the amount of scDNA free of p53 increased (as indicated by increasing intensity of band 0; Fig. 5A, lane 8; Fig. 5B, lanes 5 and 6), suggesting that binding of DO-1 to p53 protein induced dissociation of scDNA from the immune complex.

When bound to the N-terminus of p53, some proteins (such as TFIID) stimulate or stabilize binding of p53 to DNA (22). To explain the ability of DO-1 to stimulate the DNA-binding of CK2-modified or DnaK-modified mutant p53, it was suggested that DO-1 binding may regulate the core domain DNA-binding activity (23). DO-1 antibody has been reported to prevent the C-terminal specific MAb from inducing the conformational change in latent p53 necessary for activation of p53 binding to p53CON (23). Some MAbs that react with epitopes within the N-terminus can stabilize the temperature sensitive DNA-binding of wild-type (24) and mutant p53 (8), but to our knowledge no DNA dissociation from the immune complex due to binding of antibody to the p53 N-terminus has been reported. Our results suggest that this DNA dissociation is not due only to binding of DO-1 to p53 but results from binding of the C-terminal antibody to the (DO-1)–p53–DNA complex (Fig. 5A, lane 8) while binding of DO-1 to the (Bp53-10.1)–p53–DNA complex is without effect (Fig. 5A, lane 7). We may speculate that DO-1 binding to p53 either (i) generates some cryptic conformational change in p53 which is manifested when Bp53-10.1 is bound to the (DO-1)–p53–DNA complex or (ii) prevents p53 from adopting the conformation resulting normally from binding of the C-terminal MAb.

No dissociation of DNA from the immune complex involving pBluescript scDNA was observed (Fig. 5A, lane 4). Thus, the DO-1 induced dissociation of scDNA

is limited to p53 bound to p53CON in scDNA (Fig. 5A, lane 8; Fig. 5B, lanes 5 and 6). It is interesting that the DNA dissociation from the immune complex containing p53CON (in pPGM1 DNA) was observed only with scDNA but not with linear DNA fragments [V. Brazda, unpublished data; (10)]. This may indicate that the sequence-specific binding of p53 to p53CON in scDNA is not exactly the same as binding to the same site in a linear DNA molecule. Involvement of the C-terminal domain (in addition to the core domain) in sequence-specific binding of p53 to scDNA cannot be excluded. It was shown that PAb421 antibody strongly inhibited the binding of p53 to p53CON when this site was in a stem-loop structure, in contrast to the enhancing effect of this MAb on p53 binding to the same sequence in a regular duplex structure (25). It is possible that different conformations of consensus sequence in linear and scDNAs may contribute to the observed differences in modulation of p53 binding by MAbs (Fig. 5A, lane 8; Fig. 5B, lanes 5 and 6).

The N-terminal region is *in vivo* accessible for interaction with various proteins, including MDM2, whose binding site (aa 17–27) overlaps the DO-1 epitope (aa 20–25). The possibility that the p53 sequence-specific DNA binding can be influenced by binding of some protein to this region and by conformation of p53CON or of its flanking sequences (influenced by DNA supercoiling) may be of biological significance. Preferential binding of p53 to scDNA and its modulation by MAbs is a new phenomenon which deserves further attention because of its potential biological role such as stabilization of p53 and its release from scDNA due to changes in the DNA supercoiling.

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