

## Identification of an additional negative regulatory region for p53 sequence-specific DNA binding

BEATE F. MÜLLER-TIEMANN\*<sup>†‡</sup>, THANOS D. HALAZONETIS<sup>§</sup>, AND JAMES J. ELTING\*

\*Institute for Bone and Joint Disorders and Cancer, Bayer Corporation, Pharmaceutical Division, 400 Morgan Lane, West Haven, CT 06516; and <sup>§</sup>Department for Molecular Oncology, The Wistar Institute, 3601 Spruce Street, Philadelphia, PA 19104-4268

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**ABSTRACT** The DNA binding activity of p53 is crucial for its tumor suppressor function and is subject to tight regulation. Previous studies revealed that the inhibitory function of the p53 C terminus is implicated in the latent, low affinity sequence-specific DNA binding activity of p53 in the uninduced state. Sequence-specific DNA binding of p53 has been shown to be activated by several posttranslational modifications and interacting proteins that target predominantly the C terminus. Moreover, several authors have shown that synthetic peptides corresponding to p53 C-terminal sequences activate p53 sequence-specific DNA binding. In an effort to identify the interaction site of p53 with these activating peptides we assessed complex formation between p53 deletion constructs and C-terminal activating peptides by peptide affinity precipitation. This study revealed that two distal regions of the p53 molecule contribute synergistically to the interaction with activating C-terminal peptides: amino acids 80–93 and 364–393. The C-terminal residues 364–393 are already well characterized as having negative regulatory function. DNA binding analyses with these deletion constructs reveal a comparable negative regulatory activity for residues 80–93, defining this region as a previously unidentified negative regulatory domain of p53. Furthermore, synthetic peptides spanning this newly identified proline-rich negative regulatory region (residues 80–93) are able to activate p53 sequence-specific DNA binding *in vitro*. We suggest that both negative regulatory regions, residues 80–93 and 364–393, contribute cooperatively to the maintenance of the latent, low-affinity DNA binding conformation of p53.

The tumor suppressor p53 plays a central role in the regulation of cellular growth. Most critically, p53 regulates the response to genomic lesions by initiating cell cycle arrest and DNA repair or apoptosis (reviewed in refs. 1–3). Two functional characteristics of this nuclear phosphoprotein are prerequisite in these cellular responses: (i) its sequence-specific transactivation potential and (ii) its capacity to interact with a variety of cellular proteins.

p53 represents a transcription factor with its N-terminal transactivation domain (4–6) and the central sequence-specific DNA binding domain (7–10). Genes up-regulated by p53 are critically involved in the downstream propagation of growth control signals. Examples are the cyclin-dependent kinase inhibitor p21<sup>waf1/cip1</sup>, which induces cell cycle arrest (11–13); gadd45, which is implicated in DNA repair (14, 15); and the bcl-2 binding protein, bax, which induces apoptosis (16, 17). Protein factors directly interacting with the p53 protein can inhibit or support transduction of such growth control signals. The mdm2 oncogene product (18–20), the transcriptional complex TFIID (5, 6) and a number of protein kinases

(reviewed in ref. 21 and references therein; refs. 22 and 23) are all examples of proteins directly interacting with p53.

The transactivation function of p53 integrates a multiplicity of cellular signals and consequently is subject to tight control. A major regulatory mechanism controlling this transactivation activity is the modulation of the affinity of p53 for its target DNA sequences. *In vitro* analyses have led to the identification of several potential upstream regulators of p53. These factors activate p53 DNA binding activity by increasing the affinity of p53 to its target promoters. Serine/threonine-kinases like casein kinase II (24), protein kinase C (25, 26), as well as S and G2/M cyclin-dependent kinases (27) were shown to activate p53 by phosphorylation. p300 acetyltransferase has recently been shown to activate p53 DNA binding by C-terminal acetylation (28). DNA strand breaks have also been described as activators of p53 (29, 30). Other protein factors like the bacterial heat shock protein DnaK (24) and most potently the p53-specific mAb PAb421 (24, 25, 31) activate p53 by protein-protein interaction.

The majority of activators target the C terminus of p53. Deletion of the C-terminal 30 amino acids results in a constitutively active molecule, which unequivocally defines this region as a negative regulatory domain (8, 24). The underlying mechanism of p53 latency and activation still remains to be elucidated. However, it has been proposed by several authors that p53 is subject to allosteric regulation (8, 25, 28, 31, 32). The molecule can adopt a latent, low-affinity DNA binding state or an activated high-affinity DNA binding state. These conformations can be termed tense (T) state or relaxed (R) state, respectively, according to Monod *et al.*'s (33) nomenclature for allosteric enzymes. Current models propose that the negative regulatory C terminus interacts with another region on the p53 molecule. Such intramolecular (or intermolecular between the subunits of one p53 tetramer) interaction locks the DNA binding domain in a T state conformation with low affinity for its target sequences. Disruption of this interaction by C-terminal phosphorylation, protein binding or deletion leads to a conformational change. This active conformation, or R state, exhibits increased affinity for the target DNA.

The activation potential of PAb421, whose epitope is residues 371–380 of human p53 (34), led to the identification of peptides that activate p53. The sequences of these peptides overlap with the PAb421 epitope (29, 35–37). These C-terminal activating peptides are thought to interact with the p53 protein, however, an interaction site has not yet been identified. It can be hypothesized that the binding site for these synthetic C-terminal peptides corresponds to the intra- or intermolecular site to which the C terminus binds.

Abbreviations: EMSA: electrophoretic mobility shift assay; T, tense; R, relaxed.

<sup>†</sup>To whom reprint requests should be addressed. e-mail: beate.muellertiemann@schering.de.

<sup>‡</sup>Present address: Institute for Cellular and Molecular Biology, Department of Protein Chemistry, Schering AG, 13342, Berlin, Germany.

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We sought to test this hypothesis by mapping the interface of p53 and the C-terminal activating peptides. We demonstrate that two distal regions of the p53 molecule, amino acids 80–93 and 364–393, contribute synergistically to the interaction with activating C-terminal peptides. This discovery implicates a conformational motif rather than primary amino acid sequence alone in the allosteric regulation of p53 sequence-specific DNA binding. Remarkably, DNA binding analyses reveal that not only the last 30 amino acids of the p53 molecule, but also residues 80–93 have negative regulatory activity, defining the latter region as a previously unidentified negative regulatory domain of p53.

## EXPERIMENTAL PROCEDURES

**Preparation of Bacterial Lysates.** Recombinant p53 cDNAs were cloned into the bacterial expression vector pT5T (38) encoding an additional N-terminal epitope-tag. Recombinant proteins were expressed in *Escherichia coli* BL21. Vector construction and further characterization of these recombinant proteins will be described elsewhere (E. Stavridi and T.D.H., unpublished work). Bacterial cultures were grown at 30°C and expression was induced with 1 mM isopropyl- $\beta$ -D-thiogalactopyranoside at OD<sub>600</sub> of 0.8. Bacteria were harvested by centrifugation 4 hr after induction. The bacterial pellet was resuspended in glycerol with 0.7% Triton X-100 and 0.4% 2-mercaptoethanol. Bacteria were lysed in extraction buffer [10 mM Tris-HCl, pH 8/500 mM NaCl/5 mM EDTA/1 mM DTT/0.1 mM ZnOAc/6 mg/ml lysozyme/Complete protease inhibitors (Boehringer Mannheim)]. Bacterial DNA was degraded by addition of 50  $\mu$ g/ml of DNase I. The suspension was cleared by centrifugation at 120,000  $\times g$  at 4°C. Supernatant was stored at –80°C. p53 concentrations were determined by Western blot analysis by using the Amersham enhanced chemiluminescence system. The primary p53 mAb PAb240 (Oncogene Science) was used at a concentration of 0.1 ng/ $\mu$ l. The secondary horseradish peroxidase-linked anti-mouse Ig (Amersham) were used at a 1  $\times 10^{-4}$  dilution.

**Electrophoretic Mobility Shift Assays (EMSAs).** p53 DNA binding reactions were performed as described by Hupp *et al.* (35). Each reaction was performed with bacterial lysate containing 15 ng of p53 protein. The p21<sup>waf1/cip1</sup> promoter oligonucleotide Ep21 (39) was end-labeled with [ $\gamma$ <sup>32</sup>P]ATP and used at a final concentration of 25 nM in the DNA binding reaction. The nonspecific competitor oligonucleotide (5'-ACCACGTGGTACCACGTGGTACCACGTGGT-3') was added to the reaction at a concentration of 400 nM. KCl concentrations of 150 mM were used where indicated. Antibodies and peptides were added to the reaction at the concentrations given in the respective figure legend. Reaction products were analyzed by native gel electrophoresis in 2–15% gels in TGE buffer (25 mM Tris/192 mM glycine/1 mM EDTA, pH 8.5) at 90V for 2.5 hr. mAb PAb421 was obtained from Oncogene Science. Peptides were prepared by 9-fluorenylmethyloxy-carbonyl solid phase synthesis and were purified to 99% homogeneity by using reverse-phase HPLC. Proof of structure was obtained by mass spectrometry and amino acid analysis (data not shown). The sequences of the peptides used in these studies are as follows: (i) peptide 363–373, CRAHSSHLKSKK; (ii) peptide 369–383, (C)LKSKKGQSTRHKKL; and (iii) control peptide, CKLEGNFPEENNDKKSIV. Sequences of peptides 76–94 and 58–94 are displayed in Fig. 4A.

**Affinity Precipitations.** The peptide affinity matrices were prepared using SulfoLink Coupling Gel (Pierce) and synthetic peptides each containing an N-terminal cysteine residue. Coupling was performed as directed by manufacturer. Coupling efficiencies ranged from 85% to 97% as determined for each affinity matrix by using the BCA protein assay (Pierce). The cysteine-blocked control matrix was prepared by directly

applying the 50 mM L-cysteine solution to the SulfoLink gel. For each affinity precipitation reaction 30 nmol of immobilized peptide was incubated with bacterial lysate containing the indicated amount of p53 protein (see figure legends). Binding reactions were performed in p53 activation buffer (35) with 300 mM KCl for 3 hr at 4°C under agitation. For antibody preincubations, the indicated amounts of antibody were added to the binding mixture and incubated at 4°C under agitation for 1 hr before addition of the affinity matrix. Subsequently the affinity matrix was added and incubated for 3 hr as described above. Beads were washed twice with 1 ml TBS (50 mM Tris-HCl, pH 7.6/150 mM NaCl) at 4°C. Protein bound to the affinity matrix was eluted in sample buffer (125 mM Tris-HCl, pH 6.8/1% SDS/5% 2-mercaptoethanol/15% glycerol with bromophenol blue tracking dye) at 95°C. Aliquots of 20% of each eluted sample were separated by SDS/PAGE in a 4–20% gel (see Fig. 1) or a 10% gel (Fig. 5). As reference an aliquot of the respective p53 containing lysate was diluted in sample buffer and also subjected to SDS/PAGE. p53 was detected by enhanced chemiluminescence Western blot analysis as described above.

## RESULTS

### Peptide Affinity Precipitation Allows Analysis of Complex Formation Between p53 and Activating C-Terminal Peptides.

Synthetic peptides corresponding to the basic C terminus of the p53 protein activate p53 DNA binding (29, 35–37). In an effort to determine the interaction site of p53 with these peptides, we established a peptide affinity precipitation assay with which to assess complex formation between p53 and these C-terminal peptides. C-terminal peptides and an unrelated control peptide were crosslinked to an agarose matrix. The C-terminal peptides used were peptide 369–383, which had been demonstrated by Hupp *et al.* (35) to activate p53 DNA binding, and peptide 363–373, which also activates p53, but is 10-fold less potent compared with peptide 369–383 (data not shown). The control peptide contains three positively charged lysine side chains but does not exhibit strong net charge. Equimolar amounts of each coupled peptide were used for affinity precipitations. Bacterial lysate containing native, recombinant, human p53 was added to each matrix and the precipitation was performed under stringent salt conditions (300 mM KCl). Precipitates were washed and bound p53 was eluted in SDS sample buffer. p53 in the eluates was detected by Western blot analysis with antibody PAb240. As a reference for normalization, 0.5% of the input amount of p53 containing lysate was included in the Western blot analysis (Fig. 1A, lane 1). Matrix alone, which had been saturated with L-cysteine, does not bind to p53 (lane 2), neither does the control peptide (lane 3). Both C-terminal peptides, however, interact with full-length p53 (lanes 4 and 5). Under these experimental conditions, the C-terminal peptide 363–373 binds equivalent amounts of p53 as peptide 369–383. This interaction between p53 and the C-terminal peptides can be efficiently competed with a 100-fold excess of free peptides (data not shown). One-hundred-fold excess of control peptide did not show significant competition of the interaction between p53 and C-terminal peptide. These analyses validate the affinity precipitation assay for mapping the interaction site for C-terminal activating peptides on the p53 molecule.

### p53 Residues 80–93 and 364–393 Both Contribute to the Specific Interaction of p53 with C-Terminal Peptides.

To identify a potential interaction site on the p53 molecule for these C-terminal peptides, deletion mutants of p53 were generated (Fig. 2). Deletions are placed N-terminally and C-terminally of residues 94–363, which are required for efficient sequence-specific binding of the four pentanucleotide repeats in the p53 DNA consensus sequence (32, 39). This preserved region is comprised of the core domain (residues

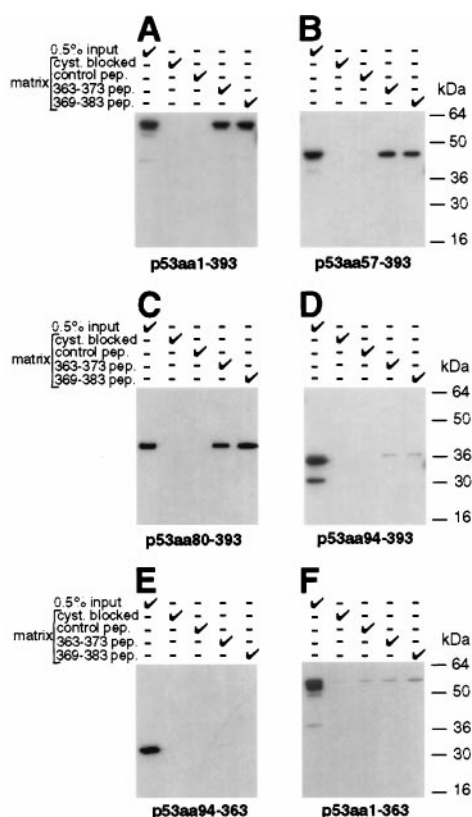


Fig. 1. Affinity precipitation of recombinant p53 deletion constructs with peptides, analyzed by Western blot. Bacterial lysates containing 1.5  $\mu$ g of the indicated p53 constructs were precipitated with different peptide affinity matrices. p53 in eluates was determined by Western blot analysis with PAb240. Lanes 1 show lysate samples containing 7.5 ng of the respective input p53 construct. The cysteine-blocked matrix was used for the control analyses in lanes 2. The indicated peptide-matrices were used for precipitation in lanes 3–5. Position of molecular mass standards is indicated.

90–290) with the sequence-specific DNA binding function (7–10, 40) and the tetramerization domain (residues 324–355) connected by the flexible linker region (10, 32, 41, 42). N-terminal deletions target the transactivation domain, within residues 1–57 (4–6), and the proline-rich domain (residues 61–94), characterized as a potential SH3 domain binding region (43). The respective p53 variants in each lysate were determined by Western blot analysis with PAb240 whose epitope, residues 213–217 (44), is common to all constructs.

To determine which p53 regions contribute critically to the specific physical interaction between p53 and the C-terminal peptides, the p53 deletion mutants were subjected to affinity precipitation studies with the matrix-coupled C-terminal peptides. None of the p53 constructs analyzed in this assay exhibit nonspecific interaction with the agarose matrix itself, nor with

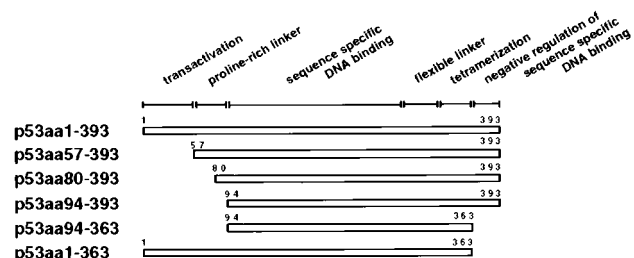


Fig. 2. Recombinant p53 constructs. Schematic overview of the recombinant p53 constructs that were generated and bacterially expressed.

the control peptide (Fig. 1 A–F, lanes 2 and 3). The two N-terminal deletion constructs p53aa57–393 (Fig. 1B) and p53aa80–393 (Fig. 1C) show readily detectable binding to both C-terminal peptide matrices, 363–373 (lanes 4) and 369–383 (lanes 5). Both deletion constructs exhibit similar peptide binding capacity as full-length p53 (Fig. 1 A–C). Additional N-terminal deletion of 14 amino acids in construct p53aa94–393 results in drastically decreased peptide binding (Fig. 1D, lanes 4 and 5). This suggests that these amino acids 80–93, harboring PXXP motifs termed “D” and “E” (43), are necessary for the interaction of p53 with the C-terminal peptides. However, the region encompassing residues 80–93 does not seem to be by itself sufficient for the specific interaction with the peptides. Construct p53aa1–363 (Fig. 1F), which contains region 80–93, but lacks the C terminus, shows an equally reduced affinity for the peptides as does construct p53aa94–393 (Fig. 1D), which lacks region 80–93, but contains the C terminus. Construct p53aa94–363 (Fig. 1E) lacks both regions and does not exhibit detectable binding.

These data indicate that the N terminus of p53 from amino acids 1–79 is dispensable for the specific interaction of p53 with the C-terminal activating peptides. Two distal regions of the p53 molecule, 80–93 and 363–393, however, both seem to contribute synergistically to the specific peptide binding. The presence of both regions confers specific and efficient binding of C-terminal activating peptides on p53 (Fig. 1 A–C, lanes 4 and 5). Absence of either of the two amino acid stretches, 80–93 or 363–393, results in significantly reduced peptide binding (Fig. 1 D and F, lanes 4 and 5).

**Residues 80–93 Have Negative Regulatory Activity on p53 Sequence-Specific DNA Binding.**

We have demonstrated that two separate regions on the p53 molecule are involved in the direct physical interaction with the C-terminal peptides. One of them, comprising the 30 C-terminal amino acids, has known negative regulatory function for p53 sequence-specific DNA binding (8, 24). Therefore we addressed the role of residues 80–93 for p53 DNA binding and whether the presence of both regions is required for peptide-induced p53 activation. Deletion constructs were subjected to EMSAs with the p21<sup>waf1/cip1</sup> promoter oligonucleotide (Fig. 3). DNA binding activities of each construct were analyzed in the absence of any activator, representing the basal binding activity (Fig. 3, lanes 1), in the presence of control peptide at 3 mM (Fig. 3, lanes 2), in the presence of C-terminal activating peptides 369–383 at 300  $\mu$ M (Fig. 3, lanes 3) and 363–373 at 3 mM (Fig. 3, lanes 4), as well as PAb421 (10 nM) for reference (Fig. 3, lanes 5).

Construct p53aa80–393, which retains region 80–93, exhibits the same low-affinity sequence-specific DNA binding activity as full-length p53aa1–393 in the absence of activators (Fig. 3 A and B, lanes 1 and 2). In addition, both proteins are efficiently activated by peptide 369–383 and by peptide 363–373 (Fig. 3 A and B, lanes 3 and 4), but not by the control peptide (lanes 2). PAb421 activates and supershifts both constructs (Fig. 3 A and B, lanes 5). In contrast, deletion of the region in question (residues 80–93), in construct p53aa94–393, gives rise to a constitutively activated p53 molecule (Fig. 3C, lane 1) despite retaining the C-terminal negative regulatory domain. This protein exhibits high-affinity DNA binding that is not further stimulated by either C-terminal peptide (Fig. 3C, lanes 3 and 4). While PAb421 does not further activate this constitutively active molecule, it still supershifts this construct (Fig. 3C, lane 5). The increased affinity of this construct for the target DNA in the absence of exogenous activators suggests that residues 80–93 have negative regulatory function, analogous to the C-terminal 30 amino acids. Deletion of this negative regulatory region can overcome the inhibitory effect of the C-terminal regulatory domain characterized by Hupp *et al.* (24). Construct p53aa1–363 shows constitutive DNA binding activity (Fig. 3E, lanes 1 and 2), due to deletion of the C-terminal negative regulatory domain, as described in many

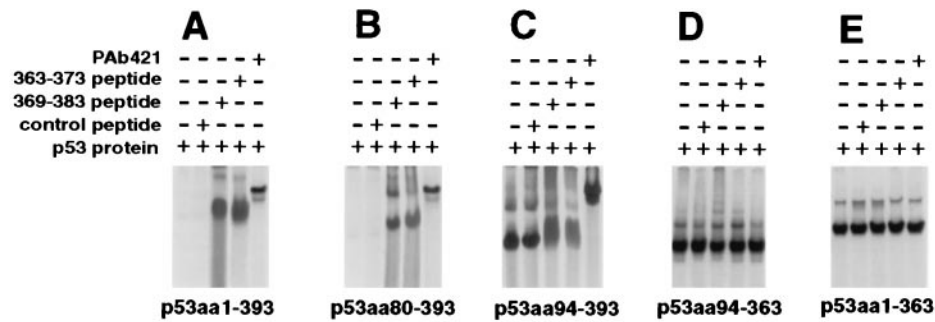


FIG. 3. Effects of C-terminal peptide and PAb421 on DNA binding of recombinant p53 deletion constructs. EMSAs of indicated p53 constructs with radiolabeled Ep21 oligonucleotide. Lanes 1 represent basal uninduced DNA binding activity of the respective proteins. In lanes 2–5 the indicated agents were added to the DNA binding reactions at following concentrations: 3 mM control peptide (lanes 2); 300  $\mu$ M peptide 369–383 (lanes 3); 3 mM peptide 363–373 (lanes 4); and 10 nM PAb421 (lanes 5).

reports (8, 24, 25, 32, 45). The high-affinity DNA binding activity of this construct can neither be stimulated further by C-terminal peptides (Fig. 3E, lanes 3 and 4) nor by PAb421. Due to deletion of the epitope it is not supershifted (Fig. 3E, lane 5). Construct p53aa94–363 (Fig. 3D), which carries neither of the two negative regulatory regions, exhibits the same constitutive DNA binding activity as p53aa1–363.

The constitutive activities of constructs p53aa1–363 and p53aa94–393 demonstrate that deletion of either one of the regulatory regions is sufficient to overcome the inhibitory activity of the other. This suggests a possible cooperative effect of these two negative regulatory regions, 80–93 and 364–393. The presence of both regulatory regions is essential for the activation of p53 by C-terminal peptides. Also, binding of p53 to the peptides depends on the presence of both structural determinants. Neither region alone is sufficient for peptide binding or negative regulation.

**Synthetic Peptides Spanning Residues 80–93 Activate p53 Sequence-Specific DNA Binding.** EMSA and affinity precipitation analyses with p53 deletion constructs reveal a comparable function of regions 80–93 and 364–393. Because C-terminal peptides are potent p53 activators we examined whether synthetic peptides spanning the newly identified negative regulatory region 80–93 could also activate p53 sequence specific DNA binding *in vitro*. We synthesized two proline-rich peptides, 76–94 and 58–94 (Fig. 4A). These peptides were tested in EMSAs for their ability to activate p53 as described above. Both peptides activate p53 DNA binding *in vitro* at concentrations of  $>0.25$  mM for the longer peptide 58–94 (Fig. 4B) and above 0.5 mM for the shorter one (Fig. 4C). The longer peptide 58–94 activates p53 with 10-fold less potency compared with the C-terminal peptide 369–383. These activation data present further evidence that indeed the two regions 80–93 and 364–393 of the p53 molecule have comparable function for the regulation of p53 sequence-specific DNA binding.

**Complex Formation Between p53 and C-Terminal Peptides Correlates with the Latent or T State of p53.** Deletion analysis indicates that both of the regions, 80–93 and 363–393, are required for binding of the C-terminal activating peptides. Furthermore, deletion of either region results in constitutive activation of sequence-specific DNA binding, a property previously attributed only to the negative regulatory region 363–393. This observation is consistent with the hypothesis that in the latent or T state conformation, p53 is capable of interacting with activating peptides, while the activated or R state conformation it is not. This implies that the ability to interact with these activating peptides is a property of the conformational state of p53 rather than of the p53 primary sequence alone.

To address this hypothesis, we attempted to determine whether conversion of T state p53 to R state p53 results in the loss of C-terminal peptide binding. We used PAb421, an

antibody, which interacts with the C terminus of p53 and activates sequence-specific DNA binding, to determine the conformational dependence of peptide binding. Increasing amounts of PAb421 were incubated with equal amounts of p53aa1–393. This mixture was subsequently tested for binding to peptide 363–373 matrix (Fig. 5A). Antibody 119.10 was used as a control for nonspecific IgG effects. This antibody binds to the N-terminally added epitope on the full-length p53 protein. This antibody was used at the same concentrations as PAb421 (Fig. 5B).

PAb421 reduces the affinity of p53 for the C-terminal activating peptide in a dose-dependent manner (Fig. 5A, lanes 2–4), while the control antibody at equivalent concentrations does not alter peptide binding (Fig. 5B, lanes 2–4). PAb421 binds to the C terminus of p53 between amino acids 371–380 (34). We determined, however, that PAb421 does not interact directly with the peptide 363–373 matrix (data not shown) and that the results cannot therefore be attributed to simple blocking of the matrix by antibody. The results are consistent with the hypothesis that interaction of p53 with C-terminal activating peptides is a property of the T state conformation and that the switch to the R state results in the loss of this binding function. However, we cannot completely rule out the possibility of a direct contact between the activating peptides and more C-terminal amino acids. Although this kind of

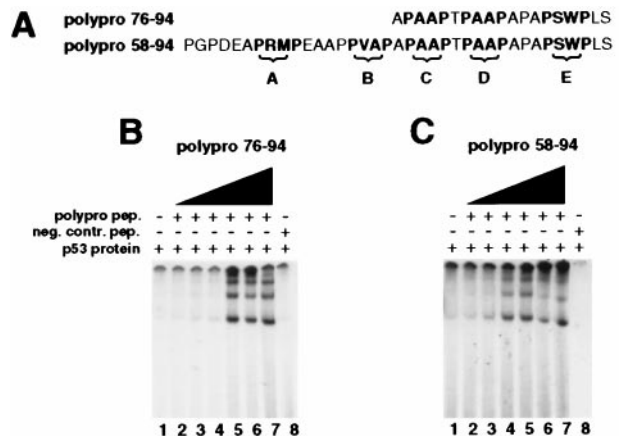


FIG. 4. Activation of p53 by synthetic polyproline peptides spanning residues 80–93. Sequences of the two peptides in single letter code. PXXP motifs, termed “A–E,” are depicted in boldface letters (A). EMSAs of full-length p53 with radiolabeled Ep21 oligonucleotide (B and C). Lanes 1 represent basal uninduced p53 DNA binding activity. In lanes 2–7 increasing concentrations of the indicated polyproline peptide had been added to the DNA binding reactions: 0.1 mM (lanes 2); 0.25 mM (lanes 3); 0.5 mM (lanes 4); 1 mM (lanes 5); 2.5 mM (lanes 6); 7.5 mM (lanes 7). Lane 8: 0.75 mM of control peptide had been added to the reaction mixture.

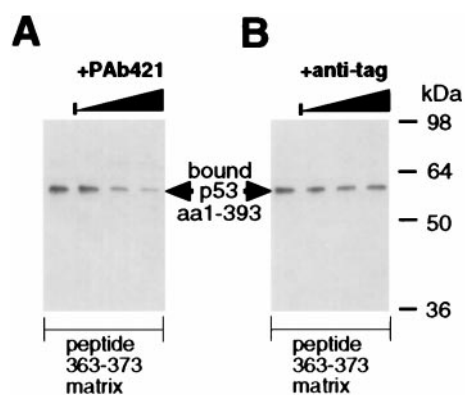


FIG. 5. Affinity precipitation of R vs. T state full-length p53 with C-terminal peptide analyzed by Western blot. p53aa1-393 (lysate containing 750 ng) was titrated into R state conformation with increasing amounts of PAb421. p53 was subsequently analyzed for peptide binding by affinity precipitation with peptide 363-373. p53 in eluates was analyzed by Western blotting with PAb240 (A). In parallel, same epitope-tagged p53aa1-393 lysate was preincubated with an anti-peptide-tag mAb as control (B). Lanes 1 in both A and B represent p53aa1-393 bound to the peptide-matrix without addition of any antibody. The following increasing amounts of PAb421 (A) and anti-peptide-tag antibody (B) were used for preincubation with p53: 1  $\mu$ g (lanes 2), 5  $\mu$ g (lanes 3), and 10  $\mu$ g (lanes 4). Position of molecular mass standards is indicated.

contact would contribute to a complex formation between p53 and the peptide, it would still not be sufficient for maintaining a stable complex. In such a case PAb421 binding could interfere sterically with the availability of potentially critical residues of the p53 C terminus for interaction with the peptide. A nonspecific steric interference of the bound antibody seems unlikely because p53aa1-363, which is in the R state, also does not bind to the peptide matrix. In this case, loss of peptide binding function cannot be attributed to the presence of blocking antibody.

### DISCUSSION

This study, initiated to address the mechanism by which C-terminal peptides activate the latent sequence-specific DNA binding activity of p53, revealed a previously unidentified negative regulatory region of p53 with similar activity as the well described C-terminal regulatory domain (8, 24, 25, 32, 45). We showed that p53 activation by C-terminal peptides encompasses a direct physical contact between p53 and the peptide. Deletion constructs were generated to determine the actual interaction site. Peptide binding studies and DNA binding activation analyses revealed that two distal regulatory regions of the p53 molecule, 80-93 and 364-393, are critical. Both of these p53 regions are required for (i) direct interaction with the C-terminal peptides and (ii) activation of DNA binding by these peptides. Unexpectedly, the DNA binding data with the deletion constructs indicated that not only the C-terminal 30 amino acids, but also region 80-93 has an inhibitory effect on p53 sequence-specific DNA binding, defining residues 80-93 as a negative regulatory domain of the tumor suppressor. Synthetic peptides spanning this newly identified negative regulatory region 80-93 activate p53 sequence-specific DNA binding *in vitro*. We propose that both regions cooperatively maintain p53 in the latent or T state with low-affinity for its target sequences.

The positive cooperativity of the two distal regulatory regions of the p53 molecule, residues 80-93 and 364-393, in binding C-terminal peptides strongly suggests a conformational requirement for p53 peptide interaction. Interaction of p53 with peptides correlates with the T state conformation of p53 (Fig. 6). A shift of p53 to the R state conformation,

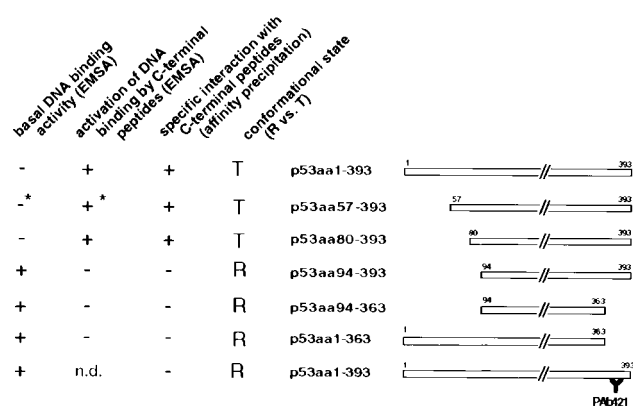


FIG. 6. Correlation of peptide binding with the T state conformation of p53. Columns 1 and 2 summarize DNA binding data. Column 1 scores basal DNA binding activity of the p53 deletion constructs as determined in EMSAs. -, Low-affinity DNA binding activity of p53; +, high-affinity DNA binding activity of p53. Column 2 scores the ability of the respective p53 deletion constructs to be activated by the C-terminal peptides. +, Significant activation of p53 DNA binding activity; -, nonsusceptibility of the respective protein to further activation by the peptides. \*, Data not shown. Column 3 scores the results of the peptide affinity precipitation analyses of the various deletion constructs and therefore summarizes the data presented in Fig. 1. +, Specific interaction of the respective protein with the C-terminal peptides; -, significantly reduced affinity of the protein for the peptides.

whether due to deletion of negative regulatory regions or PAb421 activation, reduces binding of peptides to p53 in affinity precipitation experiments. We propose that only T state p53 is capable of directly interacting with the C-terminal peptides via a T state specific structural motif. This interaction can be efficiently analyzed by affinity precipitation, although peptides induce transition of p53 from the T to the R state. This could be due to (i) the high avidity between peptides immobilized on agarose beads, because they represent a multivalent complex for the p53 tetramers, which are also polyvalent binding partners; (ii) the requirement of DNA to stabilize R state p53; (iii) peptide remaining bound in a specific binding pocket of full-length p53 even after conformational switch. In light of a conformational peptide binding model, the two negative regulatory regions, 80-93 and 364-393, would exert an indirect effect on peptide binding as they maintain this specific T state conformation.

This newly identified regulatory domain, 80-93, could represent an attractive candidate for the hypothetical intramolecular interaction site (or intermolecular between the subunits of one p53 tetramer) for the C terminus in light of current models for p53 latency. Using C-terminal peptides as a tool to study this interaction, our experimental data seem to refute this admittedly attractive hypothesis. Region 80-93 is necessary, but not sufficient for peptide binding. Yet the complex intramolecular interface could still involve the last 10 C-terminal amino acids (384-393) downstream of the PAb421 epitope. In light of this model, the result of the PAb421 titration analysis (Fig. 5) can be explained by specific steric interference of the large antibody with the entire C terminus. A very detailed deletion analysis of the p53 C terminus could address the validity of such hypothesis.

The newly identified negative regulatory region 80-93 comprises parts of the proline-rich region (residues 62-91) of p53. Residue 82 within this region is mutated in a Li-Fraumeni syndrome family (46). The region has recently been shown to play a central role in the transmission of p53-dependent antiproliferative signals uncoupled from transcriptional activation (43, 47). A p53 deletion mutant lacking PXXP motifs A-E (p53 $\Delta$ proAE) was analyzed for its transactivation potential in chloramphenicol acetyltransferase assays by Walker and

Levine (43). Quantitation of the transactivation data of this construct revealed a transactivation potential that is increased by  $\approx 50\%$  as compared with wild-type p53. This observation could be explained by our data as being due to the constitutive activation of DNA binding as a result of the deletion of the negative regulatory region 80–93. Interestingly, this transactivation competent p53 $\Delta$ proAE mutant was nevertheless unable to induce growth suppression and apoptosis. The authors suggest that interaction of an SH3 domain protein factor with this proline-rich region is necessary for mediating growth suppression and/or apoptosis, independent of the transcriptional activity of the construct. We propose that such an interacting protein could have a dual effect as it could additionally activate p53 DNA binding activity.

In conclusion, we have identified a novel negative regulatory domain on the p53 protein, a finding that contributes to the understanding of the complex allosteric regulation of the sequence-specific DNA binding of the tumor suppressor. Although this regulatory region is involved in the interaction with C-terminal peptides, it does not by itself seem to represent target site for the inhibitory C terminus, which is hypothesized to exert its negative regulatory effect by an intra- or intermolecular interaction locking the DNA binding domain in an inactive conformation. Rather our peptide binding analyses suggest that conformation is the critical determinant for binding of C-terminal peptides to p53.

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