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Dissociation of DNA Binding and *in Vitro* Transcriptional Activities Dependent on the C Terminus of p53 Proteins

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Wild type p53 protein requires posttranslational modification within a carboxy-terminal negative regulatory domain to activate DNA binding and transcription. Binding of monoclonal antibody PAb421 to the carboxy-terminal domain reproduces this activation. In the absence of PAb421, we found that wild type p53 bound actively to a template containing two copies of the *p21*^{WAF1} p53 binding site. However, in an *in vitro* transcription assay with partially purified basal transcription factors, p53 only partially activated transcription from the same binding site and required PAb421 for full activation. Oncogenic missense mutant p53 proteins (N239 to S239, G245 to S245, R273 to H273) bound the WAF1 doublet significantly and were activated further by PAb421. However, these mutants were inactive in the transcription assay, even with PAb421. These results indicate that sequence-specific binding and transcriptional activities of p53 can be dissociated through C-terminal interactions and suggest that conformational changes induced by the mutations alter p53 interactions with basal transcription factors. © 2001 Academic Press

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The process of transformation of normal cells into metastatic tumors involves the accumulation of genetic alterations through mutation and clonal selection. The genes targeted by these mutational events participate in the control of cellular proliferation, apoptosis, angiogenesis, and extracellular matrix invasiveness. One of the most commonly mutated genes in human cancers is *p53*. On average, nearly 40% of all cancer types present a missense point mutation of the *p53* gene, particularly in skin, pancreatic, colon, and lung cancers (1). The human *p53* gene encodes a homotetrameric 393 amino acid transcription factor, which activates transcription of target genes containing the responsive element 5′-PuPuPuC(A/T) (T/A)GPyPyPy. . .0–13 bp. . .PuPuPuC-(A/T) (T/A)GPyPyPy-3 in their promoters (2). The p53 protein is present in cells at very low levels, but after genotoxic stimuli (UV light, ionizing radiation, or genotoxic drugs) the levels of the protein are elevated through posttranscriptional mechanisms (3–5). Activation of wild type p53 induces several downstream genes with antiproliferative and apoptotic effects, including *p21*^{WAF1}, a gene coding for a cyclin-dependent kinase inhibitor that induces G1 arrest (6, 7). The relative contributions of p53 action to cell cycle arrest and apoptosis depend on the cell type and physiological conditions. The majority of mutations of the *p53* gene found in human cancers are missense point mutations in one allele, with loss of the other allele by rearrangement or deletion. Most of the point mutations map to three frequently mutated regions within the DNA binding domain, abolishing the DNA binding activity and increasing the protein half-life (8–10). Because of their increased stability, the mutant p53 proteins typically accumulate to high levels, in contrast to the wild type protein.

The levels and transcriptional activity of p53 protein need to be tightly regulated due to its growth inhibitory effects and predominant posttranslational induction. The last 30 amino acids of the carboxy terminus display negative regulatory activity; when these residues are deleted or modified by phosphorylation or acetylation, increased DNA binding activity of p53 is detected by electrophoretic mobility shift assays (EMSA) (11–13). These modifications can be mimicked

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by addition of monoclonal antibody PAb421, which binds with high affinity to its epitope within the last 30 amino acids of p53. Addition of peptides spanning the last 30 amino acids also activates the DNA binding function of p53 (14). These facts have lead to a model in which the carboxy-terminal residues interact with the main core of the protein in the p53 tetramers, keeping them locked in a conformation inactive for DNA binding. Removal of the interaction by posttranslational modifications such as phosphorylation, deletion, or PAb421 binding allows the tetramer to adopt a conformation suitable for DNA binding (14). The DNA binding activity of some p53 missense point mutants found in human cancers can be recovered by PAb421 or by incubation with peptides spanning the last 30 amino acids of the C terminus (15–18). Since mutant p53 protein is often overexpressed in cancer cells, reactivation of mutant p53 function by peptides or agents that mimic the action of PAb421 is a plausible strategy for cancer therapeutics (19–21).

The present study compared the DNA binding of recombinant purified wild type and oncogenic missense mutant p53 proteins and *in vitro* transcriptional activities under conditions of strong and weak binding to DNA templates. The results indicated that the requirement for activation of DNA binding by PAb421 was template-dependent and that DNA binding and transcriptional activities could be dissociated in both wild type and mutant p53 proteins. These results imply that the regulatory effect of the last 30 amino acids of p53 is not restricted to the DNA binding affinity of the protein but may extend to the interaction of p53 proteins with members of the basal transcriptional machinery.

MATERIALS AND METHODS

Construction of vectors for expression of recombinant wild type and mutant human p53 proteins in insect cells. cDNA sequences coding for the wild type and mutant human cDNA proteins were cloned into the baculovirus expression vector pFastBac (Gibco-BRL) and expressed as amino-terminal 6xhistidine-tagged proteins for purification through nickel-agarose columns. The sequences were generated by PCR amplification from plasmid pC53-SN3 (22) containing the human wild type *p53* cDNA sequence. The wild type cDNA was amplified using oligonucleotides 5′-AATTCGGATCCATGGAGGAGCCGCAGTCA-3′ (5′-end primer) and 5′-ATACAGAATTCTCAGTCTGAGTCAGGCCC-3′ (3′-end primer). The PCR product was digested with *Bam*HI and *Eco*RI and cloned into the *Bam*HI/*Eco*RI sites of pFastBac. This plasmid was used to create point mutants at amino acid positions 239 (N to S), 245 (G to S), and 273 (R to H), by means of *in situ*-directed mutagenesis using oligonucleotides 5′-CTACATGTGTA**G**CAGTTCCTGCATGG-3′, 5′-CCTGCATGGGC**A**GCATGAACCGG-3′, and 5′-GCTTTGAGGTGC-**A**TGTTTGTGCCTGTC-3′, respectively (with mutated bases in bold). To prepare the carboxy-terminally truncated wild type p53 form, PCR amplification was done using oligonucleotides 5′-AATTCGGATCCATGGAGGAGCCGCAGTCA-3′ (5′-end primer) and 5′-ATACAGAATTCTCACCTGCTCCCCCCTGGCTCCTT-3′ (3′-end primer). The PCR product was digested with *Bam*HI and *Eco*RI and cloned into pFastBac. The plasmids generated were confirmed by nucleotide sequencing.

Purification of recombinant baculovirus expressed human p53 proteins. Recombinant p53-expressing baculoviruses were obtained by transfection of the pFastBac-based vectors into DH10Bac *E. coli* competent cells (Gibco-BRL) carrying the baculovirus genome as an extrachromosomal element. The recombinant baculovirus genome was purified from bacteria and transfected into Sf-9 insect cells. Cells were lysed in 50 mM Tris–HCl (pH 8.0), 150 mM KCl, 10% glycerol, 1% NP-40, 10 mM β-mercaptoethanol, and 1 mM PMSF. The cell lysates were absorbed onto nickel-agarose resin. After sequential washing with 20 mM imidazole and 1 M KCl, the recombinant proteins were eluted with 100 mM imidazole and dialyzed against buffer containing 20 mM Hepes (pH 7.8), 100 mM KCl, 15 mM $MgCl_2$, 0.2 mM EDTA, 0.2 mM EGTA, and 10% glycerol. Purified proteins were stored at −80°C.

Determination of DNA-bound proteins by electrophoretic mobility shift assays (EMSA). The double-stranded oligonucleotide sequences used in EMSA were: RGC (5′-ACGTTTGCCTTGCCTGGACTTG-CCTGGCCTTGCCTT-3′), from the ribosomal gene cluster (23); hCON (5′-AGGCATGCCTAGGCATGCCT-3′), a consensus sequence defined from human genomic p53 binding sites (2); and WAF1 (5′-GAA-CATGTCCCAACATGTTGGGCGTCGGCTGTCGGAGGAACATGTC-CCAACATGTTGT-3′), containing two copies of the human $p21^{WAF1}$ gene p53 binding site (7). These probes were phosphorylated with γ-[^{32}P]-ATP and T4 nucleotide kinase and used in a 20 μl reaction mix containing 20 mM Hepes (pH 7.9), 25 mM KCl, 0.1 mM EDTA, 2 mM $MgCl_2$, 0.5 mM DTT, 0.25% NP-40, 2 mM spermidine, 50 nM of purified recombinant p53 protein, and 30,000 cpm of radiolabeled double-stranded oligonucleotide. Where indicated, 500 ng of antibody was included in reactions. The mixtures were incubated for 30 min at the indicated temperatures and protein complexes were separated by electrophoresis through 4% polyacrylamide under non-denaturing conditions. After electrophoresis, gels were dried and radioactivity was detected by autoradiography.

Construction of templates for in vitro transcription. The templates for p53-dependent transcription were constructed from plasmid pG5-MLT, a gift of Dr. Danny Reinberg (University of Medicine and Dentistry of New Jersey). This plasmid contains residues −50 to +1 of the adenovirus minimal late promoter (24). Transcripts initiated from this promoter contain a T1-RNase-resistant 380 nucleotide G-less cassette. The five GAL4 binding sites upstream of the promoter were replaced with p53 binding sites of RGC and WAF1 to produce pRGC-MLT and pWAF1-MLT, respectively. Plasmid pΔ50-MLT (also provided by Dr. Reinberg), containing the same minimal late promoter and a 210 nucleotide G-less cassette but lacking transcription factor binding sites, was used as a control for basal transcription.

In vitro transcriptional assays. Basal factors for *in vitro* transcription were obtained from HeLa cell nuclear extracts by salt extraction and partially purified by fractionation on P11 phosphocellulose as described by Dignam *et al.* (25, 26). Equal volumes of the flowthrough and the 500 and 1000 mM KCl fractions from the phosphocellulose column were used, each containing the basal transcription factors necessary for RNA polymerase II dependent transcription (26). Transcription reactions were carried out in a final volume of 20 μl, containing 250 ng of supercoiled plasmid pRGC-MLT or pWAF1-MLT, 250 ng of supercoiled plasmid pΔ50-MLT, 15 μg of the combined HeLa cell nuclear extract fractions, 10 mM Hepes (pH 7.8), 50 mM KCl, 7.5 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM EGTA, 10% glycerol, 100 μM 3′-*o*-methyl-GTP, 625 μM ATP, 625 μM CTP, 20 μCi [^{32}P]-α-UTP (800 Ci/mmol), 10 units RNasin inhibitor (Promega), 10 units T1-RNase (Gibco-BRL), and the indicated concentrations of purified recombinant human p53 proteins. PAb421 was dialyzed against this buffer (without nucleotides, RNasin, and T1Rnase) since phosphate buffer inhibited the transcription reaction. First, the transcription template, nuclear extract, and p53 proteins were pre-incubated at 25°C for 30 min, followed by addition of the remaining components and incubation for 30 min at 25°C. The

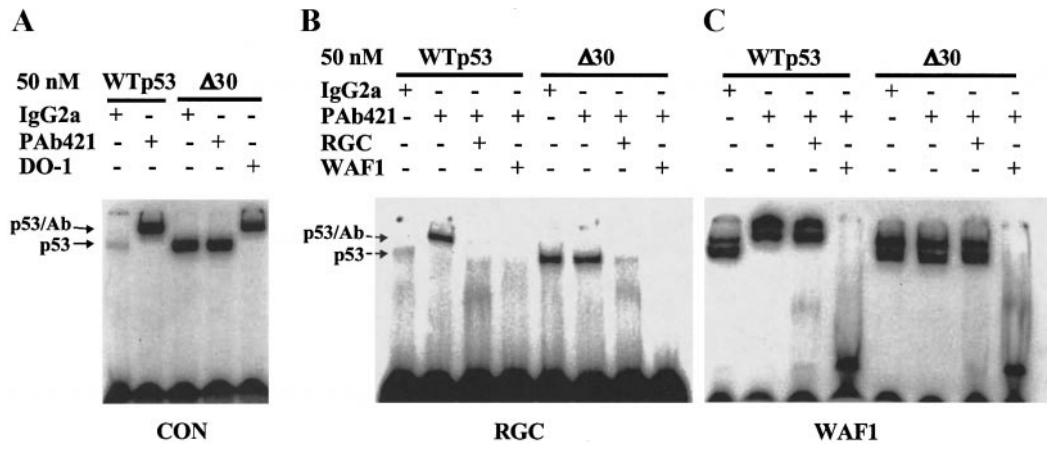

FIG. 1. DNA binding activities of wild type and carboxy-terminally deleted human p53 protein. A 50 nM aliquot of recombinant wild type p53 (WTp53) or p53 deletion mutant p53Δ30 (Δ30) was incubated at 25°C with 30,000 dpm of an end-terminally ^{32}P-phosphorylated oligonucleotide containing one of the following: (A) the human p53 binding consensus sequence (CON), (B) the ribosomal gene cluster p53 binding site (RGC), or (C) a doublet of the *p21*^{WAF1} gene p53 binding site. The reactions also included 500 ng of antibody where indicated: DO-1 (reactive against the amino terminus of p53), PAb421 (reactive with an epitope located in the last 30 amino acids of the carboxy terminus), or purified mouse serum IgG2a as control. 50-fold molar excess of unlabeled RGC or WAF1 doublet oligonucleotide competitor was included where noted at right in B and C. After 30 min incubation, the samples were separated by nondenaturing 4% PAGE and the retardation products were detected by autoradiography.

reaction was terminated by addition of SDS and proteinase K and incubation for 30 min at 50°C. RNA transcripts were extracted with phenol/chloroform and precipitated with ethanol/sodium acetate, separated by electrophoresis on urea/polyacrylamide gels and detected by autoradiography.

RESULTS

DNA binding activities of recombinant human p53 proteins. The histidine-tagged recombinant human wild type p53 (WTp53) and C-terminally truncated p53Δ30 proteins were compared for DNA binding (EMSA) to two commonly used p53 binding sequences: a consensus sequence (CON), deduced from multiple p53-binding sequences in the human genome (2), and the ribosomal gene cluster (RGC) (23). As expected, WTp53 bound weakly to these sequences in the absence of PAb421 (far left lane, Figs. 1A and 1B). Also as expected, binding was supershifted and increased by addition of PAb421, reaching levels equivalent to the constitutively active p53 protein p53Δ30. The p53Δ30 bound strongly under the same conditions in the absence of antibody and was unaffected by PAb421 due to loss of the epitope (Figs. 1A and 1B) but was supershifted by monoclonal antibody DO-1, recognizing the amino terminus of human p53 protein (Fig. 1A). Identical results were seen with the p53 binding site WAF1 found in the human *p21*^{WAF1} gene (not shown). In striking contrast, WTp53 bound as strongly as p53Δ30 to a probe containing two identical WAF1 binding sites, and addition of PAb421 supershifted but did not increase the overall p53 binding (Fig. 1C). In the absence of antibody, WTp53 exhibited slightly more of a faster migrating form (representing one of the two WAF1 binding sites bound) relative to the slowly migrating form (two sites bound), compared to the activated p53 forms WTp53 + PAb421 or p53Δ30. An excess of cold RGC or WAF1 probe competed completely with WTp53 and p53Δ30 binding to radiolabeled RGC probe (Fig. 1B). However, only the WAF1 probe itself, and not the unlabeled RGC probe, could compete with binding of either protein to the radiolabeled WAF1 doublet probe (Fig. 1C). This indicates that the binding activity of the p53 proteins for the WAF1 doublet sequence is greater than the binding for the CON or RGC sequences. Further, while the last 30 carboxy-terminal amino acids affect the DNA binding activity of p53 protein, the requirement for activation by PAb421 is DNA sequence and context-dependent.

Most of the mutations of the *p53* gene present in human cancers are single amino acid substitutions that compromise DNA binding. However, addition of PAb421 antibody to certain p53 missense point mutants can restore their *in vitro* DNA binding capacity. In light of these observations and our finding that wild type p53 appears fully active for binding to the doublet WAF1 sequence, we examined three of the most commonly occurring mutations in human cancers for DNA binding to the WAF1 doublet probe compared to the CON probe: 239^{Asn} to 239^{Ser} (S239), 245^{Gly} to 245^{Ser} (S245), and 273^{Arg} to 273^{His} (H273). As with WTp53, mutant p53 proteins required PAb421 antibody for binding to the CON probe (Fig. 2, lanes 2, 6, 8, and 10 compared to lanes 1, 5, 7, and 9). Binding intensity of the mutant S245 was indistinguishable from WTp53, while binding of H273 and S239 was diminished. However, mutant p53 proteins bound to the WAF1 doublet

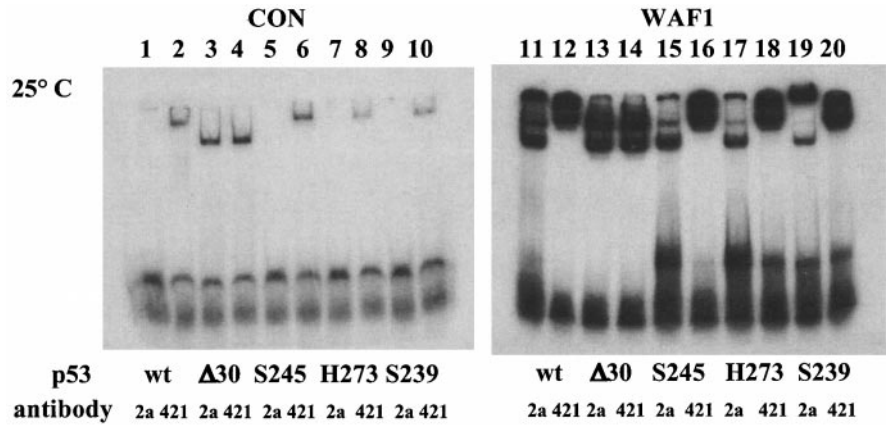

FIG. 2. Binding of wild type and mutant p53 proteins to p53 responsive elements CON and WAF1. A 50 nM aliquot of the recombinant p53 proteins (indicated at bottom) was incubated with 30,000 dpm oligonucleotide probe CON or WAF1 doublet at 25°C, in the presence of 500 ng mouse IgG2a (2a) control or PAb421 (421), followed by nondenaturing PAGE and autoradiography. S245, H273, and S239 correspond to human p53 point mutants 245Gly to 245Ser, 273Arg to 273His, and 239Asp to 239Ser, respectively.

probe in the absence of PAb421 (Fig. 2, lanes 15, 17, and 19), although with slightly less intensity than wild type protein. In the presence of PAb421, mutant p53 proteins were fully activated for DNA binding, exhibiting similar intensity to wild type p53 protein and the constitutively active p53Δ30.

In vitro transcriptional activities of wild type and p53Δ30 proteins. We examined the ability of the p53 proteins to activate transcription under the same conditions of temperature and template that enabled strong DNA binding by EMSA. In this assay, the transcription reaction is catalyzed by human basal transcription factors partially purified from HeLa cell nuclear extracts through P11 phosphocellulose chromatography. The transcription templates contain a p53 response element upstream of an adenovirus minimal late promoter (24) linked to a 380 nucleotide G-less cassette. As an internal control, a transcription template with the same promoter linked to a 210 nucleotide G-less cassette but without a p53 responsive element was included in the reaction. Transcriptional activation is measured by the ratio of the 380 to the 218 nucleotide transcription product (R), normalized to 1 for the lane without p53 protein addition. The constitutively active form p53Δ30 stimulated transcription via the RGC binding site, as demonstrated by accumulation of the p53-dependent 380 nucleotide product relative to the 210 nucleotide control transcript (Fig. 3A, lanes 3 and 4, compared to lane 1 without p53 protein). The wild type p53 protein did not stimulate transcription via RGC, even at the highest concentration tested (250 nM). These results correspond with those of the DNA binding experiments, in which no WTp53 protein binding to the RGC probe was detected

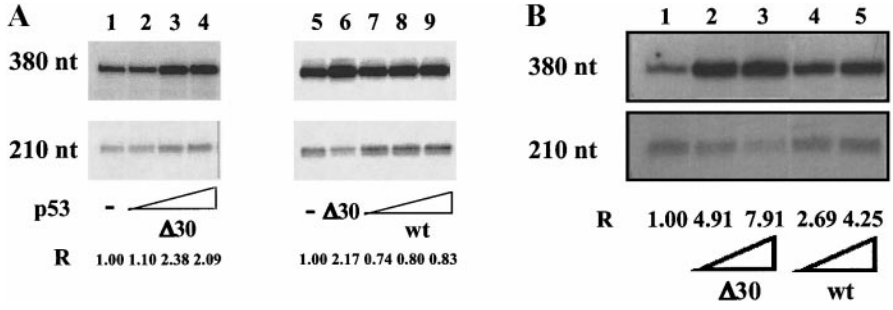

FIG. 3. p53-dependent *in vitro* transcription. Transcription reactions contained basal transcription factors from HeLa nuclear extracts and 500 ng of the supercoiled plasmid transcription templates containing (A) the RGC p53 binding site or (B) the WAF1 p53 binding site, which generate a p53-dependent 380 nucleotide transcript, and the control template without p53 binding sites, which generates a 210 nucleotide transcript. In A, lanes 2–4 and 7–9 contain 50, 150, and 250 nM of Δ30 or WTp53 protein, respectively, and lane 6 contains 150 nM Δ30. In panel B, lanes 2–3 and lanes 4–5 contain 150 and 250 nM of the p53 proteins, respectively. Plasmid templates, fractionated nuclear extracts, and the indicated p53 proteins were preincubated for 30 min at 25°C, followed by addition of ribonucleotides (including [^{32}P]-α-UTP) and T1-RNase for an additional 30 min at 25°C. Transcription products were analyzed by urea/polyacrylamide gel electrophoresis and detected by autoradiography. The ratio or fold difference between the 380 and 210 nucleotide transcripts is indicated (R).

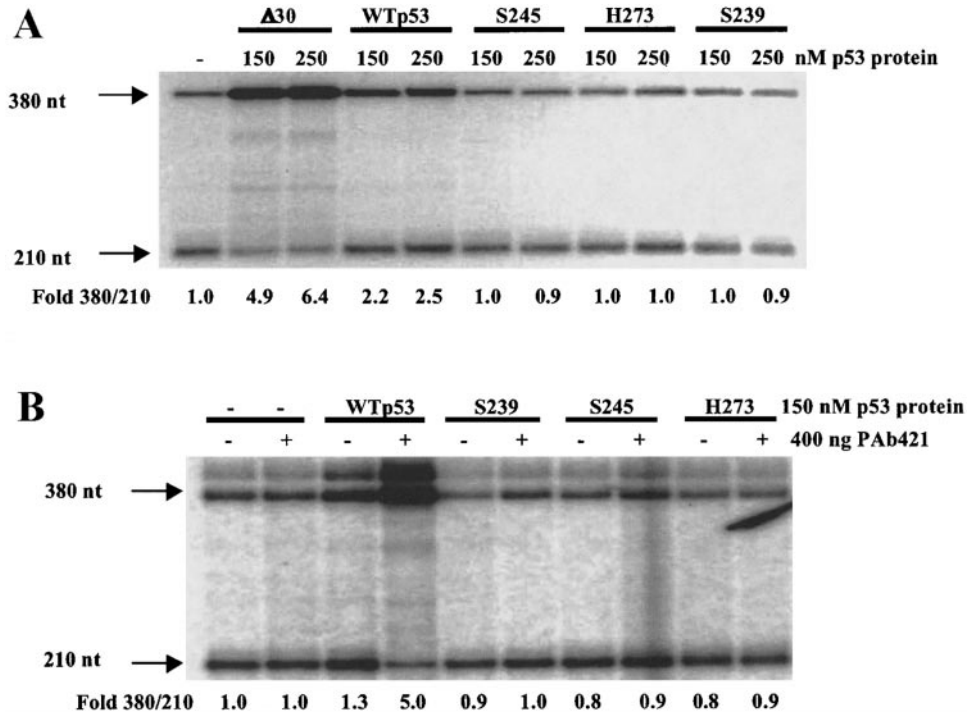

FIG. 4. Transcriptional activity of mutant p53 proteins. (A) The indicated amounts of Δ30, WTp53, and mutant S245, H273, and S239 proteins were incubated with fractionated HeLa cell nuclear extracts, WAF1 plasmid transcription templates, and ribonucleotides and analyzed under the same conditions as in Fig. 3. (B) The indicated p53 proteins were incubated in the presence or absence of 400 ng of mouse IgG2a or PAb421 under identical conditions.

in the absence of PAb421. Consistent with EMSA results, WTp53 stimulated transcription through the WAF1 doublet binding sites (Fig. 3B, lanes 4 and 5, relative to lane 1). However, WTp53 activated transcription at only half the levels of p53Δ30 (Fig. 3B, lanes 2 and 3) despite the fact that both WTp53 and p53Δ30 proteins bound equally as strong to the WAF1 doublet probe (Fig. 1C).

Transcriptional activity of mutant p53 proteins: Lack of activation by PAb421. Microinjection of PAb421 into cells containing a mutant p53 protein induces transcription from a β-galactosidase reporter gene controlled by a p53-dependent promoter (19). Therefore, the transcriptional activities of human p53 mutant proteins S239, S245, and H273 were tested using the WAF1 doublet transcription template under the conditions in which strong DNA binding was observed even in the absence of PAb421. As shown in Fig. 4A, the three mutant proteins were completely inactive, while control WTp53 increased transcription weakly compared to the active form p53Δ30. Addition of PAb421 antibody failed to restore the transcriptional activity of the three mutants (Fig. 4B) despite its ability to activate their DNA binding activities (Fig. 2). In contrast, PAb421 induced a dramatic increase in transcription by the wild type protein (Fig. 4B) despite producing no further increase in WTp53 binding to these same p53 binding sites (Fig. 2, lanes 11 and 12). PAb421 had no effect on basal transcription in the absence of p53 (Fig. 4B, first two lanes at left). In the presence of the antibody, wild type p53 transcriptional activity was increased, restricting transcription from the basal control template (210 nucleotide transcript) due to limiting transcription factors from the HeLa cell nuclear extract. This effect is also seen when the p53Δ30 protein is added alone at 150 to 250 nM, suggesting that wild type p53 activated by the PAb421 antibody was equivalent in transcriptional activity to the active form p53Δ30.

DISCUSSION

A model for allosteric regulation of p53 activity by the last 30 carboxy-terminal amino acids postulates that these residues interact with an internal domain, stabilizing the protein in a conformation that is not suitable for DNA binding (14). Thus, activation of the *in vitro* DNA binding function of p53 is expected when the domain is deleted (as in p53Δ30) or compromised by binding to a C-terminal reactive antibody (such as monoclonal PAb421). Weak binding of wild type p53 and activation of p53 protein by PAb421 or terminal deletion was observed by EMSA for the commonly used DNA binding sequences CON and RGC, as expected. A

single WAF1 sequence similarly requires p53 protein activation (7). However, wild type p53 with an intact carboxy terminus was fully active in binding to a doublet of the WAF1 sequence, without the need for activation by PAb421 or C-terminal truncation. These results indicate that the specific DNA binding activity of p53 protein is context-dependent. The binding of wild type p53 protein to the WAF1 doublet is independent of the extreme C terminus since it was neither increased nor interfered with by PAb421 binding, suggesting that it relied on other p53 protein domains. This is consistent with the report by Stenger *et al.* of interactions between central domains of p53 tetramers bound to templates containing more distant p53 DNA binding sites (27).

While the three binding sequences included in this study are widely used to test p53 *in vitro* DNA binding activity, only the *p21*^{WAF1} gene sequence has been shown to mediate p53 transcriptional activation in cells (7). The ribosomal gene cluster sequence was the first p53 binding sequence to be described, detected by screening a collection of cloned human genomic DNA sequences by immunoprecipitation of p53/DNA binding complexes with PAb421 (23); but to date, binding of p53 to this sequence has not been shown to be involved in p53-mediated transcriptional activation. The consensus sequence was derived from a collection of genomic p53 binding sequences isolated in a similar fashion, by immunoprecipitation of p53/DNA binding complexes in the presence of PAb421 antibody (2). The concept of *in vitro* p53-DNA binding activation by interaction with the carboxy terminus of the protein was initially suggested by experiments using a consensus DNA binding sequence (11). Mundt *et al.* (28) reported a requirement for PAb421 both for binding of wild type p53 recombinant protein to a p53 consensus sequence and for *in vitro* transcriptional activity. The EMSA probe used in their study contained one consensus site while the transcription template contained three consensus sites separated by 15 base pairs. Our results showing full activation for binding to the template containing two Waf1 sites separated by 17 base pairs predict that p53 protein might also be active for binding to the template containing the three consensus sites. The results of Mundt *et al.* (28) showing that transcriptional templates with even three consensus sites required p53 protein activation either by PAb421 or phosphorylation at the p53 C terminus make it unlikely that *in vitro* transcriptional activation would occur via a single consensus site in the absence of PAb421, since p53 binding to DNA is expected to be necessary for transcriptional activation. This is supported by our results showing a requirement for PAb421 for DNA binding and transcriptional activity from the RGC p53 binding site.

The DNA binding activities of wild type p53 and p53Δ30 toward the *p21*^{WAF1} gene sequence predicted equal *in vitro* transcriptional activities when using a template with the same responsive element. However, even though the wild type p53 protein bound fully to this WAF1 doublet template in the absence of PAb421, it was not fully active for *in vitro* transcription unless PAb421 was present. The dissociation of the *in vitro* binding and transcriptional activities of p53 protein may indicate that the PAb421 interaction is needed to alter the interaction of p53 with the basal transcriptional machinery, even when it is not necessary for activating DNA binding. The p53 protein is known to interact with both the TATA-binding protein and the TFIID components $TAF_{II}40$ and $TAF_{II}60$ (29–31). Interaction of p53 and the TATA-binding protein represses transcription and is mediated by short stretches in the amino- and carboxy-terminal domains (30, 32, 33). Overexpression of the carboxy-terminal domain of p53, transfected into the p53 deficient cell line Saos-2, can repress transcription mediated by cotransfected SP1, VP16, and p53 itself, and the last 30 amino acids are necessary for this effect (34). These reports and the current findings imply that binding of PAb421 to the carboxy terminus of p53 increases its *in vitro* transcriptional activity by disabling or modifying a putative inhibitory interaction with basal transcription factors (like TBP) or possibly by stabilizing an activating interaction (with TAFs) rather than simply by increasing p53 DNA binding activity. Taken together with our results, this suggests an alternative interpretation of the data of Mundt *et al.* (28) indicating that phosphorylation at the carboxy terminus can substitute for activation by PAb421. Our results suggest that phosphorylation at the carboxy terminus might transcriptionally activate p53 protein not only by increasing sequence-specific binding but also by altering interactions with basal transcription factors. Several residues in the carboxy terminus of p53 are subjected to both phosphorylation and acetylation, and these events are thought to participate in the activation of p53 after genotoxic stress (35). To test these possibilities, transcriptional activity for reporters with p53 DNA binding sites or for endogenous target genes should be compared among p53 mutants at residues known to be phosphorylated or acetylated *in vivo*, expressed in cells from weak promoters to reflect physiological p53 protein levels.

In vitro transcriptional activity was completely absent for the three mutant proteins S239, S245, and H273, despite the fact that the *in vitro* transcriptional assays were done in the presence of PAb421 and under conditions in which the mutant proteins displayed avid binding to the template containing the two high affinity *p21*^{WAF1} gene binding sites. Failure of the mutant proteins to transactivate in the presence of PAb421 suggests that the conformational changes induced by the mutation may not only affect the central DNA binding domain, reducing the bind-

ing affinity of the mutant protein, but also affect other domains of p53, reducing their interaction with basal transcription factors. That such a mechanism is possible is exemplified by the interaction of p53 with XPD (ERCC2) and XPB (ERCC3), components of TFIIH, a basal transcription factor involved both in transcriptional initiation and nucleotide excision repair (36). This interaction is mediated by the carboxy terminus of the p53 protein only, but it is abolished in p53 proteins mutated in the central DNA binding domain, including the H273 mutant used in this study (37, 38). Conformational changes induced by PAb421 may be sufficient to increase the binding affinity of the protein but not enough to produce further changes in the amino- or carboxy-terminal domains necessary for activation in *in vitro* transcriptional assays. However, activation of the transcriptional activity of mutant proteins (including H273) in whole cells, using microinjection of PAb421 or carboxy-terminal peptides, has been reported in three instances (19–21). The explanation for the dissociation of DNA binding and transcriptional events may lie among several possibilities. Further conformational change of the mutant protein may be needed for its transcriptional reactivation. These changes may require specific posttranslational modifications, translation of the mutant protein in the presence of activating factors such as PAb421 or the carboxy-terminal peptide, or putative cellular p53 regulatory factors. Other nuclear factors, not supplied in the fractionated nuclear extracts used in the current study, may be required to fully activate wild type p53 or to restore transcriptional activities of the mutant p53 proteins *in vitro.* Finally, the context of chromatin and of the nuclear architecture itself may be required for p53 protein to exhibit the full range of transcriptional regulation of its downstream target genes. The interplay of p53 protein in this context may be required in addition to the current *in vitro* transcriptional approaches for unraveling p53 protein transcriptional regulatory activities.

In summary, the current study indicates that activation of wild type and mutant p53 proteins for DNA binding can be dissociated from its role in transcription. In particular, the activation of p53 proteins for *in vitro* transcription is not due solely to increased efficiency of DNA binding and may be due to altered interactions of activated p53 protein with other proteins present in the *in vitro* transcription reactions, i.e., basal transcription factors. The implications are that not only altered interactions with DNA, but altered interactions with basal transcription factors, dependent upon the p53 C terminus, may underlie defects in p53 wild type and mutant proteins and their role in cancer.

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