Regulation of Mutant p53 Temperature-sensitive DNA Binding*

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We have examined in detail the DNA binding properties of several immunopurified tumor-derived mutant p53 proteins (Val-143 → Ala, Arg-175 → His, Arg-248 → Trp, Arg-249 → Ser, and Arg-273 → His). While all mutants were defective for binding to DNA at 37 °C, each bound specifically to several cognate p53 binding sites at sub-physiological temperatures (25–33 °C), and several mutants activated transcription from a p53-responsive promoter at 26 °C in transfected H1299 cells. Heating mutant p53 proteins at 37 °C irreversibly destroyed their ability to subsequently bind at 25 °C. However, several different monoclonal antibodies that each share the ability to recognize an epitope encompassing amino acids 46–55 markedly stabilized binding by mutant p53 proteins at 37 °C. Both intact antibody and Fab fragments allowed mutant p53 to bind to DNA. By contrast, antibodies that recognize epitopes located elsewhere within p53 stabilized mutant p53 binding significantly less effectively. Our data show that the major hot-spot mutations identified to date are located within the central DNA binding domain, especially within the C terminus, regulate the function of the DNA binding domain of wild-type p53 (Hupp et al., 1992; Halazonetis and Kandil, 1993; Wang and Prives, 1995).
well to DNA at 37 °C. The possibility of a general approach to stabilizing wild-type function in mutant p53 has therapeutic applications.

**MATERIALS AND METHODS**

**Purification of p53 Proteins**—Recombinant baculoviruses expressing wild-type and mutant p53 have been described (Friedman et al., 1990; Bargonetti et al., 1992). Extracts of infected sf21 insect cells were prepared, and p53 was purified from lysates by immunoaffinity procedures (Wang et al., 1989). Purified p53 protein was prepared with protein A-Sepharose columns cross-linked with the p53-specific monoclonal antibody PAB 421 (Harlow et al., 1981). The proteins were eluted either with a molar excess of PAB 421 epitope containing peptide (KKQGSTSRHKK-OH) (Wade-Evans and Jenkins, 1985) or with 50% ethylene glycol. Results were similar when proteins were eluted by either method. The protein was dialyzed into Dialysis Buffer containing 10 mM HEPES (pH 7.9), 0.1 mM NaCl, 1 mM dithiothreitol, and 50% glycerol. Our initial experiments were conducted with both wild-type and mutant p53(Ser-249) protein, which was not yet available as a recombinant baculovirus. More recently, after successfully constructing this baculovirus, we repeated many of the experiments with the p53(Ser-249) protein and, where possible, have included them as well.

**Papain Cleavage of Monoclonal Antibodies**—To a solution of PAB 190 (5 mg/ml) in 100 mM sodium acetate (pH 5.3) was added a 1/20 volume of cysteine from a 1 mM stock and a 1/20 volume of EDTA from a 20 mM stock. After addition of 10 μg of papain per mg of reaction, reaction mixtures were incubated for 12 h at 37 °C. Iodoacetamide was then added to a final concentration of 75 mM, and mixtures were incubated for 1 h at room temperature prior to dialysis in phosphate-buffered saline (PBS).1

**Gel Mobility Shift Assays**—EMSA was carried out as described (Peterson et al., 1990). The synthetic double-stranded oligonucleotides used in the study include the following: RGC, 5′-TTCGAGTTGGCTGGACCTGCGCTTTTTTCC-3′; mutant RGC, 5′-TTCGAGTTGGCTGGACCTGCGCTTTTTTCC-3′; SCS, 5′-TTCGACCGTCGATCGACGCTCAATGCTC-3′; GADD45, 5′-AATCTCGAGGCAGCAGCGATAGCGTCAGG-3′; and mutant GADD45, 5′-AATCTCGAGCGAGCAATCTGATGTTCGAGTC-3′. The probes were 32P-labeled using the Klenow fragment of Escherichia coli DNA polymerase. Binding reaction mixtures contained 20 mM HEPES (pH 7.9), 25 mM KCl, 0.1 mM EDTA, 2 mM MgCl2, 0.5 mM dithiothreitol, 0.25% Nonidet P-40, 2 mM spermidine, 10% glycerol, and 0.1 mg of bovine serum albumin, double-stranded poly(dI-dC) (60 ng), and 32P-labeled oligonucleotide (8 ng). p53 protein concentrations are indicated in figure legends, and in all cases volumes were equalized with Dialysis Buffer. Mixtures were incubated at the indicated temperatures for 30 min unless stated otherwise. In experiments with added antibodies the final reaction volumes remained at 20 μl. In time course of heating experiments, mixtures were incubated without the DNA probe for 0–10 min at 37 °C; the probe was added, and reactions were incubated for 30 min at 25 °C. In competition experiments unlabeled oligonucleotides were added at indicated concentrations directly to the reaction mixtures, and the binding reaction was continued for 30 min at 25 °C. In all cases mixtures were then loaded onto a native 4% polyacrylamide gel containing 0.5% Tris borate-EDTA (TBE) buffer, 1 mM EDTA, and 0.05% Nonidet P-40 and electrophoresed in 0.5× TBE at 4 °C at 180–200 V (not exceeding 40 mA current) for 2 h.

**DNA Filter Binding**—Reaction mixtures (20 μl) containing 20 mM HEPES (pH 7.9), 25 mM KCl, 0.1 mM EDTA, 10% glycerol, 2 mM MgCl2, 1 μl of 40 mM spermidine, 1 μl of 10 mM dithiothreitol, 1 μl of 0.5% Nonidet P-40, 1 μl of 60 μg/ml double-stranded poly(dI-dC), 1 μl of 2 mg/ml bovine serum albumin, 4 ng of 32P-labeled wild-type or mutant GADD45, and p53 protein were incubated for 30 min at 25 °C. Reaction mixtures were filtered through 0.45-μm nitrocellulose filters presoaked in 25 mM HEPES (pH 7.9), containing 10 ng/ml double-stranded poly(dI-dC), washed three times with 25 mM HEPES (pH 7.9), dried, and counted by liquid scintillation.

**Infection Assays**—H1299 cells at a density of 6×105 cells/6-cm dish were transfected by the calcium phosphate method with the p53 expressing pCMVneoBam or parental pCMVneoBam vectors (2 μg) containing wild-type or mutant p53a (Kern et al., 1992) along with the mdm2 promoter reporter construct, pGL2-NA(md2)-2-luc (Juven et al., 1993), which contains the Nsi-I-Apal fragment of the murine mdm2 gene cloned into the pG512-Basic vector (Promega). Six hours after incubation with the precipitate at 37 °C, cells were glycerol-shocked for 1 min (10% glycerol in DMEM) and then washed twice in DMEM before adding RPMI medium containing 10% fetal bovine serum. After incubation for an additional hour at 37 °C, cells were transferred to an incubator maintained at 26 °C and kept there for 42 h prior to extraction and determination of luciferase activity.

**RESULTS**

**DNA Binding by p53 Proteins Is Temperature-sensitive**—Our studies were performed with wild-type p53 and five tumor-derived mutant p53 proteins that were immunopurified from recombinant baculovirus-infected insect cells using a column containing the p53 monoclonal antibody PAB 421 (Wang et al., 1989). We estimate that the proteins produced by this protocol are approximately 80% pure as judged by silver-stained protein gels (Fig. 1a). The slight differences in electrophoretic mobility of the various p53 proteins may be due to altered structure resulting from the mutation and/or possible polymorphism(ies) elsewhere in p53, such as that at codon 72, shown to result in altered migration of p53 polypeptide (Matlashewski et al., 1987).

Using the electrophoretic mobility shift assay (EMSA), we examined DNA binding by p53 proteins at 25 or 37 °C to labeled oligonucleotides containing versions of the following p53 response elements: GADD45, RGC, and SCS (Fig. 1, b and c). GADD45 (Kastan et al., 1992) and RGC (Kern et al., 1991b) sites are sites that have been identified in human genomic while SCS contains an optimized p53 consensus sequence derived from Halazonetis et al. (1993). At 25 °C all mutants bind to the RGC and GADD45 oligonucleotides, albeit with varying efficiencies, while at 37 °C binding to these sites was either undetectable or extraordinarily weak. Wild-type p53 binding was also decreased at the higher temperature, consistent with recent findings of Hainaut et al. (1995) who reported that DNA binding at 37 °C by wild-type p53 synthesized in reticulocyte lysates is 30–40% of that observed at 25 °C. While binding at 25 °C by p53(Ala-143), p53(Trp-248), and p53(His-273) was robust, as quantitated by phosphorimaging, wild-type p53 bound at least 2-fold better than any mutant. DNA binding by either p53(His-175) or p53(Ser-249) mutant p53 proteins at 25 °C was significantly less than the above mentioned three, displaying only 5 and 10% of wild-type p53 activity, respectively. We frequently observed that fresh preparations of p53(His-175) protein bound very well to DNA but then rapidly lost activity, indicating its conformational instability. By contrast, binding by p53(Ser-249), while initially weak, reproducibly showed no deterioration. Therefore, in many cases further studies were carried out with all mutant proteins except p53(His-175).

To exclude the possibility that the loss of mutant p53 DNA binding at 37 °C was due to the activation of a contaminating protease, Western blotting using three different p53 monoclonal antibodies was performed on DNA binding reaction mixtures that had been incubated at 25 or 37 °C. Equivalent amounts of full-length, immunoreactive wild-type or mutant p53 polypeptides were detected after incubation at the two temperatures, showing that the loss of mutant p53 DNA binding at 37 °C did not result from selective degradation of the mutant p53 proteins (data not shown).

Although each mutant bound well to one or more sites at 25 °C there were some notable differences. In particular, we were surprised to observe that p53(Trp-248) showed no detectable binding to the idealized SCS site at 25 °C, even though p53(His-273) and p53(Ala-143) bound comparably well to this site, and wild-type p53 binds better to SCS than any other
Fig. 1. Mutant p53 proteins bind DNA at 25 °C. a, p53 proteins. Immunopurified wild-type (wtH) and p53(Ala-143), p53(His-175), p53(Trp-248), p53(Ser-249), and p53(His-273) proteins (quantities ranged between approximately 200 and 400 ng) were analyzed by SDS-polyacrylamide gel electrophoresis (a) and DNA binding assays at 25 °C (b) and 37 °C (c).
version of the consensus sequence (Halazonetis et al., 1993). P53(His-175) did not bind to SCS either; however, its generally weaker binding to the other sites makes this observation less significant.

There are two critical points to be made from these experiments. 1) All mutants tested have the potential to bind to DNA, and 2) the difference between wild-type and mutant forms of p53 at lower and higher temperatures is such that at physiological temperature wild-type p53 retains significant binding, whereas mutant p53 binding is virtually abolished.

**Mutant p53 Proteins Bind DNA in a Sequence-specific Manner**—Although the mutant proteins bind well to several DNA sites at lower temperatures, there were concerns that this binding might reflect interactions with DNA that were essentially nonspecific. A nonspecific DNA binding function has been mapped to the C-terminal portion of p53 (Wang et al., 1993), and in the absence of a functional central DNA binding domain, this region alone might be able to bind well to DNA in a sequence-independent manner. Moreover, we previously observed that while the proteolytically excised central “core” domain of wild-type p53 is capable of binding to DNA, the comparable domain released from p53(His-273) protein showed no detectable binding to DNA (Bargonnetti et al., 1993). To determine the specificity of DNA binding by mutant p53 proteins, we employed competition EMSA and filter binding assays. Using EMSA it was clear that different unlabeled oligonucleotides containing versions of the wild-type p53 binding site competed for binding to a labeled specific site oligonucleotide far better than did unlabeled oligonucleotides containing mutated binding sites (Fig. 2a). Although the amount of unlabeled binding site-containing DNA required for competition varied with the source of p53 and with the competitor, in all cases, at the highest level of competitor tested there were marked differences between the specific and nonspecific sources of DNA (Fig. 2a). Strong additional evidence for specificity of mutant p53 interactions with DNA was derived from filter binding experiments (Fig. 2, 6 and c) in which efficient binding to wild-type but not mutated p53 binding site oligonucleotides was determined.

**Mutant p53 Can Transactivate a p53-responsive Promoter at 26 °C in Vivo**—Since the mutant p53 proteins were temperature-sensitive for DNA binding it was of interest to determine whether they have any transcriptional activation capability in vivo at lower temperature. Numerous studies have shown that many mutant forms of p53 cannot activate transcription from biologically relevant p53-responsive genes in cells maintained at physiological temperatures (Vogelstein and Kinzler, 1992; Ko and Prives, 1996). We tested whether mutant constructs are able to activate a reporter containing a segment of the murine mdm2 promoter, pGL2-NA(mdml2)-luc (Juven et al., 1993), following transfection into p53-null H1299 cells at a temperature at which mutant p53 proteins bind in vitro. This construct was chosen because we wished to test a biologically relevant p53-responsive promoter. As expected we confirmed that, in contrast to wild-type p53, these mutants were incapable of activating transcription from this promoter at 37 °C (data not shown). In cells transfected at 32 °C we observed that only Ala-143 had transactivation capability, while all other mutants tested were inert (data not shown). That Ala-143 is transcriptionally active at 32 °C is consistent with experiments published by Zhang et al. (1994). When the wild-type and mutant p53-expressing constructs were transfected into H1299 cells and then cells were shifted to 26 °C, significant transactivation by three mutant p53 proteins, Ala-143, Trp-248 and His-273, was observed (Fig. 3). Ser-249 and His-175 mutant p53 constructs, however, did not detectably activate transcription over background levels seen with the empty vector. The failure of these latter two mutants to transactivate in vivo was not due to reduced expression since Western blotting of transfected cell extracts showed that all p53 constructs tested expressed detectable and roughly comparable levels of p53 protein (data not shown). These transient transfections were performed using a range of plasmid concentrations, and the values shown are the maximal plateau levels. Our data show that some mutant forms of p53 have the intrinsic ability to activate transcription from a physiologically relevant promoter. Those that cannot displayed greater impairment in DNA binding in vitro, suggesting a correlation between DNA binding and transactivation.

**Mutant p53 Proteins Are Less Thermostable Than Wild-type p53**—Since wild-type p53 DNA binding was also somewhat temperature-sensitive, we conducted time course of heating experiments in order to examine the relative thermostability of the p53 proteins (Fig. 4). Wild-type or mutant p53 proteins were incubated in DNA binding buffer at 37 °C for increasing periods; the RGC oligonucleotide was then added, and the reactions were incubated at 25 °C for a further 30 min. While preheating for up to 10 min caused only a 15% loss in DNA binding by wild-type p53, by 7 min of preheating DNA binding by all mutants was reduced to approximately 5% of that seen at 25 °C (Fig. 4). Note that the loss of binding by both the wild-type and mutant proteins was irreversible, as binding was not restored upon the shift to 25 °C.

There were also interesting differences among the mutants in their thermal inactivation properties; p53(Ala-143) and p53(Ser-249) proteins displayed the greatest temperature sensitivity with approximately 80–90% of their binding lost following 2 min of preheating (Fig. 4). By contrast, 2 min of preheating reproducibly actually caused a 2-fold increase in the DNA binding ability of both p53(His-273) and p53(Trp-248) proteins (Fig. 4). This transient stimulation was observed with a wild-type RGC oligonucleotide but not with mtRGC, suggesting that the increased binding is specific (data not shown).

To further understand the thermal sensitivity of p53 proteins, DNA binding over a range of temperatures (25–37 °C) was examined (data not shown). As expected, with increasing temperatures, all p53 proteins showed decreased ability to bind to an oligonucleotide containing the RGC site, although there was a relatively far sharper decline with the mutants than with wild-type form of p53 with the most drastic relative decrease in mutant p53 binding occurring between 33 and 37 °C.

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2 P. Friedlander, Y. Legros, T. Soussi, and C. Prives, unpublished data.
FIG. 2. Sequence-specific DNA binding by mutant p53 proteins. a, competition EMSA. Wild-type or mutant p53 proteins (200 ng) were bound to 32P-labeled oligonucleotides in the presence of unlabeled oligonucleotides as indicated. Mixtures with wild-type p53, p53(Ala-143), and

**Fold Excess Competitor**

b

![Graph showing fold, excess, and competitor](image)

![Graph showing percentage bound vs. p53 concentration](image)

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The Monoclonal Antibody PAb 1801 Stabilizes DNA Binding by Wild-type and Mutant p53 at 37 °C—Several studies have documented the regulation of p53 sequence-specific DNA binding by sequences and sites within its C terminus. In particular, the monoclonal antibody PAb 421 (Harlow et al., 1981) that interacts with an epitope (amino acids 373–381) within the C terminus of p53 (Wade-Evans and Jenkins, 1985) can enhance the DNA binding function of wild-type (Hupp et al., 1992; Halazonetis et al., 1993) and even certain mutant forms (Hupp et al., 1993; Halazonetis et al., 1993) of p53. Thus, it was of interest to compare the effect of PAb 421 on mutant p53 binding at lower and physiological temperatures. p53 proteins were incubated with oligonucleotides containing p53 binding sites at 25 or 37 °C in the presence or absence of PAb 421. As a control, we used an antibody that recognizes an epitope (amino acids 46–55) within the N terminus of p53, PAb 1801 (Banks et al., 1986; Legros et al., 1994). PAb 421 increased DNA binding by mutant p53 proteins at 25 °C (Fig. 5). However, binding in the presence of PAb 421 was significantly reduced at 37 °C. Additionally, DNA binding by wild-type p53 synthesized in vitro in reticulocyte extracts was stimulated by PAb 421 at 25 °C but rather poorly at 37 °C. These experiments therefore show that while PAb 421 increases the DNA binding function of wild-type and mutant p53 proteins at 25 °C, this stimulation is reduced at 37 °C and is thus temperature-sensitive. It should be noted, however, that at 37 °C there was detectable binding in the...
FIG. 6. PAB 1801 stabilizes but does not restore DNA binding by mutant p53 protein at 37 °C. a and b, gel mobility shift assays were performed to determine binding by p53(Trp-248) to 32P-labeled RGC (8 ng) (a) or by p53(His-175) to 32P-labeled GADD45 oligonucleotide (8 ng) (b) at 25 °C (lanes b and c) and 37 °C (lanes d and e). Binding was examined in the absence (lanes a, b, and d) and presence (lanes c and e) of purified monoclonal antibody PAb 1801 (500 ng). Lane a contains no p53 protein. 200 ng of mutant p53 proteins were used. c, EMSA was performed to determine binding by p53(Ser-249) (200 ng, lanes a, c, e, and g; or 400 ng, lanes d, f, and h) to 32P-labeled GADD45 oligonucleotide (8 ng) at 25 or 37 °C as indicated. Binding was examined in the absence (lanes a, b, e, and f) or presence (lanes c, d, g, and h) of PAB 1801 (600 ng). d, mixtures containing p53(Ala-143) (200 ng) were incubated either at 25 °C for 30 min (lanes b and c), 37 °C for 30 min (lanes d and e), or for 30 min at 37 °C followed by 30 min at 25 °C (lanes f, g, and h). PAB 1801 was present in mixtures run in lanes c, e, g, and h. In lane g the antibody was added following the 30 min at 37 °C, while in lane h the antibody was present prior to the 30 min at 37 °C.

We observed DNA binding at 37 °C by analyzing DNA bound by mutant p53 incubated with both PAB 1801 and PAB 421. We observed DNA binding at 37 °C comparable or slightly greater in amount to that seen when PAB 1801 alone was added (Fig. 5b, lanes n–p, and data not shown). The fact that the DNA protein complex migrated more slowly when both antibodies were used suggests that the PAB 1801-stabilized p53-DNA complexes still express the PAB 421 epitope and also that PAB 421 retains an affinity for p53 at 37 °C (Fig. 5b, lanes h, i, and p). Thus, the reduced ability of PAB 421 to rescue binding by p53 at 37 °C is not simply due to a lessened ability by the antibody to recognize the p53 protein at the higher temperature.

Stabilization in the presence of PAB 1801 was achieved for each mutant with each of the three different binding sites tested (Fig. 6 and data not shown). Indeed, even binding by p53(His-175), which, as expected, bound to GADD45 with markedly lower affinity than the other mutant proteins tested at 25 °C, was stabilized in the presence of PAB 1801 (Fig. 6). Additionally, PAB 1801, but not PAB 421, secured DNA binding of reticulocyte lysate expressed wild-type p53 at 37 °C (data not shown). Furthermore, the addition of SP21 cell extract to gel shift reaction mixtures containing purified mutant p53 protein did not alter 1) the temperature sensitivity of the p53 mutants, 2) the ability of PAB 1801 to thermostabilize DNA binding, and 3) the ability of PAB 421 to stimulate DNA binding in a temperature-sensitive manner. In summary, all of the data we have obtained with PAB 1801 suggest that this antibody prevents loss of binding by each mutant at physiological temperature but does not qualitatively change its intrinsic binding ability. For example, PAB 1801 did not allow p53(Trp-248) to bind SCS (data not shown). Moreover, this antibody did not stabilize binding by mutant p53 proteins to a mutated RGC sequence (data not shown).

To determine if DNA binding that was secured by PAB 1801 is dependent upon the presence of DNA, we incubated p53 at 37 °C in DNA binding buffer (lacking DNA) with PAB 1801. Then the RGC oligonucleotide was added and the reactions incubated at 25 °C for 30 min. Comparable binding to RGC by p53(Ala-143), p53(His-273), and p53(Trp-248) was seen in the reaction mixtures containing PAB 1801 to what was detected when DNA was present from the start of the reaction as in the normal protocol (data not shown). This demonstrates that the ability of PAB 1801 to stabilize p53 proteins in a DNA binding positive conformation does not depend upon the presence of a p53 DNA binding site at 37 °C.

PAB 1801 might affect binding at 37 °C either by maintaining the p53 protein in a DNA binding positive conformation or by restoring such a conformation to DNA binding negative protein. To differentiate between these possibilities, we incubated mutant p53 with RGC or SCS at 37 °C in the presence or absence of PAB 1801 (Fig. 6d). Then PAB 1801 was added to those samples lacking PAB 1801, and the incubation was continued at 25 °C. p53 proteins bound to DNA only when PAB 1801 was present throughout the 37 °C incubation period. A typical example is shown for p53(Ala-143) binding to SCS (Fig. 6d compare lane h to lanes f and g), and this result held for the other mutants binding to SCS as well as to RGC (data not shown). Thus, PAB 1801 allows the mutant proteins to remain in a conformation capable of binding DNA, as opposed to restoring such conformation to thermally inactivated p53 protein. The difference in the relative abilities of PAB 1801 and PAB 421 to prevent loss of mutant p53 binding at 37 °C led us to examine other antibodies recognizing either the same or different epitopes within p53. The immunogenicity of p53 has been studied in some detail previously. Both the N- and C-terminal regions are markedly immunodominant, and within these regions are a number of epitopes that have repeatedly elicited antibodies both in experimental models (Legros et al., 1994) and in human patients (e.g. Schlichtholz et al., 1992; Schlichtholz et al., 1994). We evaluated the ability of a number of
Fig. 7. Survey of antibodies affecting mutant p53 DNA binding at 37 °C. *a*, gel mobility shift assays were performed to determine the relative ability of various antibodies to stabilize binding by p53(Ala-143) to 32P-labeled GADD45 oligonucleotide (8 ng) at 37 °C. 200 ng of p53 protein was used. Binding was determined over a range of antibody concentrations (up to 750 ng), and the maximal protein-DNA complexes obtained were quantitatively using a phosphorimager. We used the values obtained for plateau levels of binding. Maximal binding in the presence of PAb 1801 was set at 100%, and the ability of the various antibodies to stabilize DNA binding were plotted relative to that value. The antibodies included L134 (epitope: amino acids 11–20) as ascitic fluid from mouse diluted 10-fold in PBS; B17, C36, and H461 (epitope: amino acids 16–30); PAb 1801, H279, and H447 (epitope: amino acids 46–55); HP64 (epitope: amino acids 171–185); PAb 421 (epitope: amino acids 370–378); and HR 231 (epitope: amino acids 371–380) all in PBS; and X77 (epitope: amino acids 16–25) in DMEM + 10% fetal calf serum. *b*, tabular representation of the relative ability of antibodies discussed in *a* to stabilize DNA binding by p53(Ala-143), p53(His-273), p53(Trp-248), and p53(Ser-249) at 37 °C. Stabilization by PAb 1801 was set at 100%.

purified monoclonal antibodies recognizing epitopes within amino acids 11–20, 16–25, 16–30, 46–55, 171–185, or 371–380 to stabilize the DNA binding function of p53. Fig. 7 shows our results expressed as maximal affect on DNA binding at 37 °C by a given antibody (previously determined after testing a range of concentrations of each antibody) normalized to the maximal binding in the presence of PAb 1801. We first determined that all the antibodies supershifted and did not inhibit the formation of p53-DNA complexes at 25 °C. Importantly, each of the mutants studied revealed essentially similar patterns of stabilization by the different antibodies. Two antibodies, H279 and H447, each recognizing a similar epitope to PAb 1801, were clearly the most effective, although neither was quite as impressive as PAb 1801, and the two varied in their relative ability to affect binding of different mutant proteins. Antibodies recognizing amino acids 16–30, particularly C36, also contributed significantly to p53 binding at 37 °C (12–45%), although to lesser extents than did the 46–55 series. An antibody to the epitope within amino acids 11–20 allowed for even less stabilization (5–20%). The antibody PAb HP64 that recognizes a cryptic epitope in the central portion of the p53 protein had no effect whatsoever. Finally, both PAb 421 and another monoclonal antibody, HR231, which each recognize an epitope within amino acids 371–380, provided only a small amount of stabilization of DNA binding at 37 °C (1–18%) even though both antibodies greatly stimulated DNA binding by 10–25-fold at 25 °C. Thus, antibodies recognizing the region spanning amino acids 46–55 are the most effective in stabilizing DNA binding by mutant p53.

**Fig. 8. Stabilization of DNA binding by monovalent FAb 1801 fragments.** *a*, p53(Trp-248) protein (350 ng) was bound to 32P-labeled GADD45 oligonucleotide (8 ng) at 25 °C (lanes a–j in *a*) or 37 °C (lanes k–t in *a*). PAb 1801 was added to the mixtures run in lane l (500 ng) and lanes c and m (800 ng). FAb 1801 was added to the mixtures run in lanes d and n (66 ng), lanes e and o (200 ng), lanes f and p (350 ng), lanes g and q (470 ng), lanes h and r (660 ng), lanes i and s (900 ng), or lanes j and t (1.1 µg). Mixtures run in lanes b and k had no antibody, and mixture run in lane a had no p53. *c*, graphical representation of stabilization of the DNA binding by mutant p53 proteins at 37 °C. Stabilization of FAb 1801 fragments is relative to that obtained when PAb 1801 was used as determined through phosphorimaging.

**Fab Fragments of PAb 1801 Stabilize Mutant p53 DNA Binding**—The effect on DNA binding by N-terminal antibodies was conceivably the result of cross-linking of the p53 tetramer. To address this possibility, we generated Fab fragments of PAb 1801 (Fab1801) and determined their ability to stabilize DNA binding by p53 (Fig. 8, *a* and *b*). Monovalent Fab 1801 fragments clearly allowed binding by all mutants tested at 37 °C. The slightly lower degree of stabilization by Fab 1801 versus PAb 1801 might result from a lower affinity for p53 or from cross-linking acting as an additional but not essential component of the effect of PAb 1801. Interestingly, we observed that the presence of three or four Fab fragments per p53 tetramer allowed significantly greater binding than did one or two Fab fragments per tetramer. This suggests that maximal stabiliza-
tion of mutant p53 binding requires interaction with all four of the monomers in a p53 tetramer but that the bivalent form of an antibody (i.e. one capable of cross-linking monomers) is not necessary for rescue of mutant p53 DNA binding from thermal inactivation.

**DISCUSSION**

DNA binding is very likely to be an important function of wild-type p53. The rather extraordinary clustering of tumor-derived mutations within the DNA binding domain of p53 strongly suggests that this region is critical for the normal role of p53 in tumor suppression. Reciprocally, the fact that mutations are focused in this region leads to the assumption that alteration of residues in this region would destroy or alter DNA binding. We have demonstrated, however, that hot-spot mutant p53 proteins are inherently capable of binding specifically to DNA. Binding varies somewhat with the mutation and the version of the consensus site used and occurs only at sub-physiological temperatures. Additionally, we have identified a means by which temperature-sensitive binding by mutant p53 can be stabilized such that significant levels of binding can be obtained at physiological temperatures.

The co-crystal structure of the central p53 DNA binding domain bound to DNA (Cho et al., 1994) has been most informative. Aside from providing the three-dimensional positions of the different amino acids that make up the domain and their relationship to a cognate p53 DNA site, the structure has provided insight into the role of the amino acids that are most frequently mutated in human cancer in binding to DNA. It is possible to divide p53 tumor-derived hot-spot mutations into two classes, those that affect directly the interaction between protein side chains and DNA and those that affect the stable conformation of the domain (reviewed by Cho et al., 1994). Two that contact the DNA directly are Arg-248, in the minor groove, and Arg-273 at a backbone phosphate. However, these are two out of several amino acids that make direct contact with DNA. It is interesting that the other contact residues are not mutated with unusual frequency. Rather, the other four hot spots are involved in maintaining the structure of the DNA binding motifs. The two classes of hot-spot mutants, contact and conformational, can be differentiated by other criteria as well; these include binding to heat shock cognate protein Hsc70 (Hinds et al., 1990), recognition by the conformation-specific antibody PAb 240 (Gannon et al., 1990), ability to function as a transcriptional activator when fused to a GAL4 DNA binding domain (Raycroft et al., 1991; Unger et al., 1992), and protease sensitivity (Bargonetti et al., 1993). The two classes of mutants might be expected to display significant variation in their interactions with DNA, and certainly differences among the mutants exist both in efficiency and specificity of binding. Nevertheless, given these differences it is extraordinary that all mutants tested show DNA binding capability and that all are temperature-sensitive. Possibly, under less stringent conditions, the very size and complexity of the p53 DNA binding domain allows for some degree of interaction with DNA by p53 gene products that have sustained a single mutation within this region.

One might view our results as follows: each missense mutation uniquely affects specific DNA or amino acid contacts or alters the over-all conformation of the DNA binding domain. This results in the varying degrees to which different mutants are impaired for binding to DNA, as well as some of the relative differences seen with different DNA binding sites. Any p53 protein, however, whether wild-type or mutant is sensitive to thermal stress at 37 °C. The combination of lower affinity DNA binding and thermal lability would result in no observable binding by mutant proteins at 37 °C. The temperature-sensitive phenotype of certain p53 mutants in cells has been observed previously. Both murine p53(Val-135) (Michalovich et al., 1990; Martinez et al., 1991) and human p53(Ala-143) (Zhang et al., 1994) display a temperature-conditional nature in cells. Chimeric polypeptides containing the GAL4 DNA binding domain fused with mutant p53 have also displayed temperature sensitivity for transcriptional activation (Unger et al., 1992). Indeed, a number of studies have documented DNA binding by mutant p53 in cell extracts, suggesting that even at physiological temperatures some mutants might have partial wild-type function (Chen et al., 1993; Chumakov et al., 1993; Miller et al., 1993; Zhang et al., 1993; Park et al., 1994; Niewold et al., 1995). However, it is clear from comparing these reports that the cell environment can affect whether and how various p53 mutants display DNA binding and transcriptional activation. Unfortunately, it is also frequently the case that mutants are “functional” in cells only when idealized consensus sites are used for DNA binding or as transcriptional response elements in reporter constructs. Our data show that purified mutant p53 proteins can bind to a variety of sites including ones from physiologically relevant genes. The ultimate goal will be to restore full wild-type p53 function to mutant forms of the protein in tumor cells.

The complexity and size of the DNA binding domain and the fact that it alone does not contain known regulatory sequences has led to the suggestion that it may be difficult to identify small molecules that interact directly with the DNA binding domain to restore function to defective mutants (Friend, 1994). However, there are several lines of evidence that sequences and signals outside of the DNA binding region can be propagated to affect the functioning of the DNA binding domain. Most of these are within the C-terminal portion of p53. Within the last 30 amino acids is a highly basic region that when bound by antibody or bacterial heat shock protein, dnaK, leads to marked activation of specific DNA binding (Hupp et al., 1992). Additionally, phosphorylation of p53 by casein kinase II (Hupp et al., 1992), protein kinase C (Delphin and Baudier, 1994), and G/S cyclin-dependent kinases (Wang and Prives, 1994) stimulates and alters (Wang and Prives, 1995) p53 sequence-specific DNA binding. Moreover, short DNA single strands stimulate DNA binding by the p53 central domain in a manner that is dependent on the C terminus (Jayaraman and Prives, 1995). Importantly, p53 from which the C-terminal 30 amino acids is deleted binds much better to DNA than full-length p53 (Hupp et al., 1992; Hallazomet et al., 1993). These data taken together suggest that p53 exists in DNA binding negative and positive conformations. The relationship between these two conformations holds for some mutant forms of p53 as well. Indeed, the observation of Bargonetti et al. (1993), who showed that a 27-kDa protease-resistant fragment spanning the central DNA binding domain from wild-type but not from p53(His-273) mutant p53 is capable of binding specifically to DNA at 25 °C, supports the likelihood that sequences outside of the DNA binding domain play critical roles in mutant p53 sequence-specific DNA binding. It was therefore disappointing that the powerful stimulation of both wild-type and mutant p53 DNA binding by the C-terminal specific monoclonal antibody, PAb 121, is temperature-sensitive. Our data showing that both PAb 421 and 1801 were able to bind together to mutant p53 at 37 °C are encouraging and provide the possibility that molecules reacting with both N and C termini of p53 might be developed to cooperate in restoring DNA binding to mutant p53.

Recent reports have provided evidence that p53 transcriptional activity is increased in cells microinjected with the monoclonal antibody, PAb 421 (Abarzua et al., 1995; Hupp et al.,
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