

# Allosteric Regulation of the Thermostability and DNA Binding Activity of Human p53 by Specific Interacting Proteins\*

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Conformational stability is a prerequisite for the physiological activity of the tumor suppressor protein p53. p53 protein can be allosterically activated for DNA binding by phosphorylation or through noncovalent interaction with proteins such as DnaK, the *Escherichia coli* homologue of the heat shock protein Hsp70. We present *in vitro* evidence for a rapid temperature-dependent change in the conformation and tetrameric nature of wild-type p53 upon incubation at 37 °C, which correlates with a permanent loss in DNA binding activity. We show that p53 is allosterically regulated for stabilization of the wild-type conformation and DNA binding activity at 37 °C by binding of two classes of ligands to regulatory sites on the N and C terminus of the molecule through which an intrinsic instability of p53 is neutralized. Deletion of the domain conferring instability at the C terminus is sufficient to confer enhanced stability to the total protein. DnaK binding to the C terminus can profoundly protect p53 at 37 °C from a temperature-dependent loss of the DNA binding activity but does not renature or activate denatured p53. In contrast, another activator of the DNA binding activity of latent p53, the monoclonal antibody PAb421, which also interacts with the C terminus of the protein, is not able to protect p53 from thermal denaturation. Two monoclonal antibodies to the N terminus of p53, PAb1801 and DO-1, do not activate the latent DNA binding function of p53 but can protect the p53 wild-type conformation at 37 °C. Thus, activation of the DNA binding function of p53 is not synonymous with protection from thermal denaturation, and therefore, both of these pathways may be used in cells to control the physiological activity of p53. The protection of p53 conformation from heat denaturation by interacting proteins suggests a novel mechanism by which p53 function could be regulated *in vivo*.

Wild-type p53 is a nuclear phosphoprotein of 393 amino acids (for reviews see Prives (1994) and Lane (1994)) with well characterized domains contributing to its function and regulation. The N-terminal domain functions as a transcriptional activator (Fields and Jang, 1990; Raycroft *et al.*, 1990; Farmer *et al.*, 1992; Unger *et al.*, 1992), and the C terminus has been shown to contain a tetramerization domain (Shaulian *et al.*, 1992;

Clore *et al.*, 1994; Waterman *et al.*, 1995) and to regulate the specific DNA binding function of p53 (Hupp *et al.*, 1992, 1993, 1995). The core region (residues 90–290) consists of the sequence-specific DNA binding domain (Bargonetti *et al.*, 1993; Pavletich *et al.*, 1993; Wang *et al.*, 1993) and contains four of the five regions that are evolutionarily conserved among vertebrates (Soussi *et al.*, 1990). The specific p53 DNA binding element is found to be a repeat of 10 base pairs (RRRC(A/T)(T/A)GYYY)<sub>2</sub> where each monomer of tetrameric p53 can bind to five base pairs (Kern *et al.*, 1991; El-Deiry *et al.*, 1992; Hazonetis and Kandil, 1993). Co-crystallization of the core domain with a DNA duplex containing a half consensus site revealed the critical amino acids for DNA binding (Cho *et al.*, 1994). Binding of p53 to the consensus element can drive the transcription of reporter genes (Funk *et al.*, 1992), and the element has been shown to be present in promoter or intron regions of several genes including p21<sup>waf1/cip1</sup> (El-Deiry *et al.*, 1993; Harper *et al.*, 1993), *gadd45* (Kastan *et al.*, 1992), the muscle creatine kinase gene (Weintraub *et al.*, 1991; Zambetti *et al.*, 1992), and the *mdm2* gene (Barak *et al.*, 1993).

The importance of this core region for the function of p53 is underlined by the fact that of more than 2000 described mutations of the p53 gene, the majority are point mutations clustered in the DNA binding domain (Pavletich *et al.*, 1993; Cho *et al.*, 1994). Many of these point mutations can be correlated with a loss of the wild-type conformation as detected by the loss of reactivity to the monoclonal antibodies PAb1620, specific for murine and human p53 (Milner *et al.*, 1987), and the mouse-specific PAb246 (Yewdell *et al.*, 1986) or the gain of reactivity to the mouse-specific monoclonal antibody PAb240 (Gannon *et al.*, 1990; Stephen and Lane, 1992).

Understanding the regulation of p53 conformation and biochemical activity is essential for understanding how cells use the p53 pathway to regulate growth control. To date, p53 protein is known to be activated for sequence-specific DNA binding by covalent modification (*i.e.* phosphorylation of its negative regulatory domain) or by noncovalent modification (binding by the monoclonal antibody PAb421 or bacterial Hsp70) (Delphin and Baudier, 1994; Hupp and Lane, 1994a, 1994b). The mechanism whereby an antibody activates p53 has been studied most extensively; activation occurs through a concerted mechanism in which the tetrameric nature of p53 is maintained (Hupp and Lane, 1994a). In addition, activation by casein kinase II or protein kinase C is reversed by monoclonal antibodies or phosphatases, respectively, indicating that activation of p53 is a reversible process possibly subject to refined metabolic control (Hupp and Lane, 1994a; Takenaka *et al.*, 1995).

Here, we demonstrate that the conformation of wild-type p53 is very temperature-sensitive. The loss of the PAb1620-reactive epitope correlates with a loss of the tetrameric nature of p53 and with a loss of the p53 DNA binding activity. We find that the *Escherichia coli* homologue of Hsp70, DnaK, and two hu-

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man Hsp70 proteins can protect p53 DNA binding at 37 °C. Specific monoclonal antibodies to the N terminus of p53 can also stabilize the PAb1620-positive conformation and DNA binding activity of p53 at 37 °C.

#### MATERIALS AND METHODS

**Antibodies**—The monoclonal antibodies PAb1620, PAb246, DO-1, PAb421, and PAb1801 have been described previously (Ball *et al.*, 1984; Milner *et al.*, 1987; Yewdell *et al.*, 1986; Vojtesek *et al.*, 1992; Harlow *et al.*, 1981; Banks *et al.*, 1986) and were purified using protein A-Sepharose. CM-1 is a rabbit polyclonal antiserum raised against recombinant human p53 (Midgley *et al.*, 1992).

**Purification of p53 Expressed in Bacteria**—Wild-type p53 was expressed in *E. coli* BL21 cells at room temperature using the T7 expression system encoded by the plasmid pT7hp53 (Midgley *et al.*, 1992). Expression and purification were described in detail elsewhere (Hupp *et al.*, 1992).

**Purification of Human and Murine p53 from Sf9 Cells**—*Spodoptera frugiperda* cells (Sf9 cells) were cultured at 27 °C in EX-CELL<sup>®</sup> 400 medium (JRH Biosciences) supplemented with 5% (v/v) fetal calf serum in 500-ml glass culture flasks stirred at 80 rpm. Cells growing logarithmically at  $1-3 \times 10^6$  cells/ml were subcultured to monolayers of  $3.3 \times 10^7$  cells, left to adhere onto 150-cm<sup>2</sup> plates for 2 h, and infected with the recombinant baculovirus pVL1393hp53 containing a human wild-type cDNA at a multiplicity of infection of 10. 90 min after infection the medium was changed, and cells were scrape-harvested in phosphate-buffered saline after 4 days, collected by centrifugation at 1500 rpm, and then stored in liquid nitrogen. Cell lysis was carried out at 4 °C for 20 min in lysis buffer (0.15 M NaCl, 1% Nonidet P-40, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 50 mM Tris-HCl, pH 7.5, 5 mM dithiothreitol (DTT),<sup>1</sup> 10 mM NaF, 1 mM benzamide). After centrifugation of the lysate, the supernatant was diluted 3-fold in 15% (v/v) glycerol, 25 mM HEPES, 0.1% Triton X-100, 5 mM DTT, 1 mM phenylmethylsulfonyl fluoride, and p53 was purified from the lysate on Hi-Trap heparin-Sepharose (Pharmacia Biotech Inc.) in a linear KCl gradient from 0.1 to 0.85 M KCl. Murine p53 was purified in the same way as human p53.

**Purification of C-terminal Deletion Mutant ( $\Delta 30$ ) from Sf9 Cells**—The deletion mutant has been described previously (Hupp *et al.*, 1992). Expression and purification were as described above.

**Purification of DnaK Expressed in Bacteria**—DnaK was purified from an overproducing *E. coli* strain. Cells were grown in LB media at 30 °C to an  $A_{600\text{ nm}}$  of 0.6. Subsequently cells were diluted two fold in prewarmed (55 °C) LB media and grown for 2 h at 42 °C. Cells were pelleted by centrifugation and resuspended in 10% sucrose/50 mM HEPES, pH 7.6. The cell suspension was lysed for 20 min on ice by adding KCl to 0.5 M, DTT to 1 mM, and lysozyme to 0.5 mg/ml. After centrifugation for 10 min, the crude lysate was applied to a DEAE-Sepharose column at a protein (mg):resin (ml) ratio of 10:1. Bound protein was eluted, and fractions containing DnaK were dialyzed against 10 mM imidazole, pH 7.0, 10% sucrose, 10 mM MgCl<sub>2</sub>, 0.1 mM EDTA, 20 mM KCl, and 1 mM DTT (buffer A). DnaK was applied to an ATP-agarose column (Sigma A-2682) in buffer A at a protein:resin ratio of 5:1. After washing in buffer A containing 1 M KCl and equilibration in buffer A, DnaK was eluted in buffer A containing 5 mM ATP.

Recombinant human Hsp70 (p72) and Hsc70 (p73), purified from *E. coli*, were obtained from Stress Gen, Biotechnologies Corp.

**ELISA**—Purified monoclonal antibodies were coated onto 96-well flat bottomed plates at 2  $\mu\text{g/ml}$  in 0.1 M CO<sub>3</sub>/HCO<sub>3</sub>, pH 9.1, at 4 °C overnight. The wells were blocked in BTP buffer (6% bovine serum albumin, 0.1% Tween 20 in 1  $\times$  phosphate-buffered saline) at 4 °C overnight. The subsequent steps were carried out at room temperature on one day; titrations of protein were added to the plates for 2 h. p53 was detected with the polyclonal rabbit antiserum CM-1, 1:1000 (1 h) and swine anti-rabbit IgG conjugated to horseradish peroxidase, 1:500, visualized with TMB substrate (Sigma T-2885) as described by Harlow and Lane (1988).

**Gel Mobility Shift Assay**—Binding conditions are described in detail elsewhere (Hupp *et al.*, 1992). The DNA binding buffer contained 20% (v/v) glycerol, 50 mM KCl, 40 mM HEPES, pH 8, 0.05 mM EDTA, 5 mM DTT, 0.1% Triton X-100, 10 mM MgCl<sub>2</sub>, 1.0 mg/ml bovine serum albumin. The specific p53 consensus site (PG) (El-Deiry *et al.*, 1992) was end-labeled with [ $\gamma$ -<sup>32</sup>P]ATP and used with a 20-fold excess of supercoiled competitor DNA (pBluescript II SK<sup>+</sup>, Stratagene). A Phosphor-

Imager (Molecular Dynamics) was used for the quantification of radioactive signals.

**Sucrose Gradient Sedimentation**—5–40% sucrose gradients were prepared in buffer containing 20 mM HEPES, pH 8.0, 0.1 M NaCl, 5 mM KCl, 0.1% Nonidet P-40, 1 mM DTT. The sedimentation was performed at 4 °C for 20 h at 35,000 rpm in a SW40 rotor.

**Protein Analysis**—Protein analysis by Western blot was performed as described by Harlow and Lane (1988).

#### RESULTS

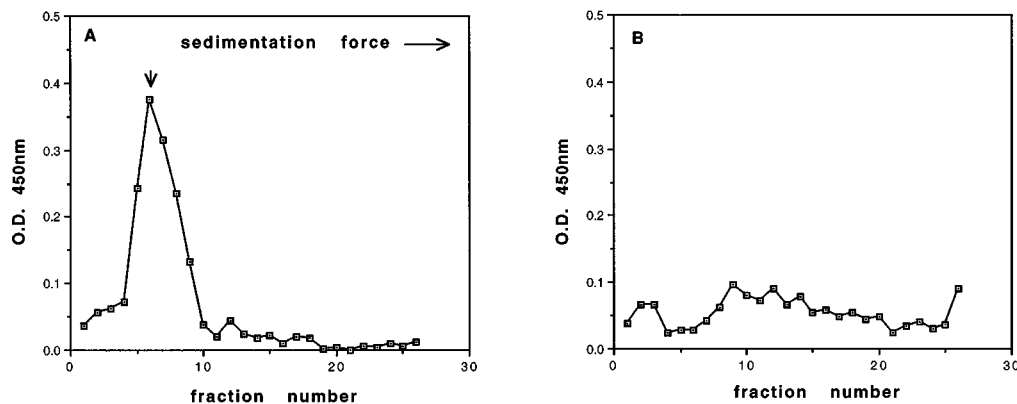
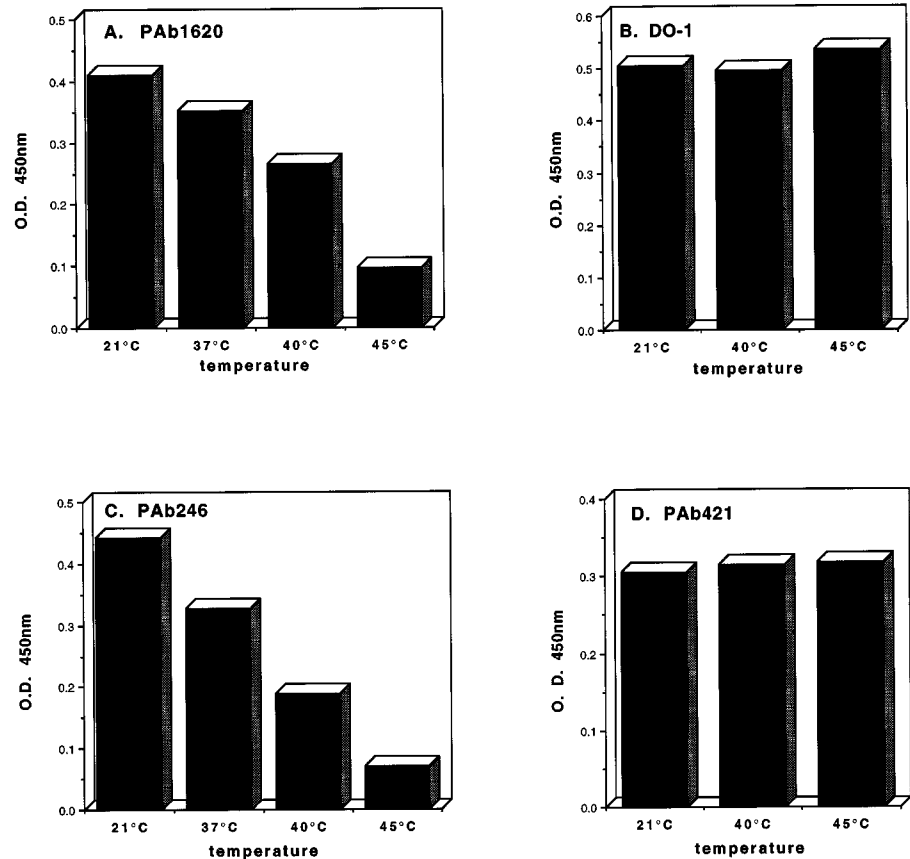
**Thermostability of Wild-type p53 Conformation**—p53 has been shown to be a conformationally dynamic protein *in vivo* and *in vitro* (Gannon *et al.*, 1990; Milner and Medcalf, 1990; Milner and Watson, 1990). Conformation-specific monoclonal antibodies can be used to analyze the structure of p53, and a number of antibodies recognizing a specific region of the protein associated with functional properties of p53 have been raised. By binding to the C terminus (amino acids 372–381), the antibody PAb421 can allosterically activate latent p53 for DNA binding (Wade-Evans and Jenkins, 1985; Hupp *et al.*, 1992, 1994). DO-1 binds to the highly antigenic N terminus (amino acids 20–25) of p53 (Stephen *et al.*, 1995). PAb1620 recognizes a conformationally sensitive epitope in the DNA binding domain of mouse and human p53 (Milner *et al.*, 1987; Ball *et al.*, 1984), which is absent from human p53 when bound to DNA (Hupp *et al.*, 1992). The mouse-specific PAb246, also conformationally sensitive (Yewdell *et al.*, 1986), recognizes a discontinuous epitope overlapping with the epitope of PAb1620. Here, these antibodies have been used to address whether the conformation of p53 is influenced by exposure to temperatures in the range of 21–45 °C. Recombinant human p53 was expressed in *E. coli*, purified as described under “Materials and Methods,” and incubated for 10 min at 21, 37, 40, or 45 °C. Protein conformation was analyzed in a two-site ELISA; p53 was captured with various monoclonal antibodies and detected with a polyclonal anti-p53 antiserum (CM-1, Midgley *et al.*, 1992) (Fig. 1, A–D). The increase in temperature resulted in a progressive loss of the PAb1620-reactive epitope (Fig. 1A). The epitopes recognized by DO-1 and PAb421, however, were not affected by temperature (Fig. 1, B and D). Murine p53 expressed in insect cells from a baculovirus expression vector and biochemically purified as described under “Materials and Methods,” displayed the same temperature-dependent loss of conformation recognized by the antibody and PAb246 (Fig. 1C). Since the loss of the PAb1620-recognized epitope was also observed for human p53 expressed in insect cells (data not shown), the thermostability seems to be a general phenomenon of p53 independent of the expression system and the species.

**Gradient Sedimentation Analysis of p53 Incubated at 37 °C**—In order to obtain more information on the effect of heat treatment on p53 oligomerization we compared untreated p53 with p53 incubated at 37 °C in the sedimentation profile of a sucrose gradient as measured by immunoreactivity of p53 with DO-1 (Fig. 2). Wild-type p53 focused at the top of the gradient as a sharp 6 S peak, representing the tetrameric structure of the molecule (Fig. 2A, compare Hupp and Lane (1994a)). 60-min incubation at 37 °C led to a complete loss of the tetramer and to the formation of higher molecular weight aggregates (Fig. 2B). Although there was a clear loss of structure of p53, the overall level of the epitope recognized by DO-1 (or PAb421) was not reduced after incubation at 37 °C for 60 min (data not shown), indicating that the 37 °C incubation affects not only the PAb1620-positive conformation in the core of individual molecules but also p53 oligomerization.

**Effects of Temperature on the Specific DNA Binding Activity of p53**—Having demonstrated a temperature-dependent loss of p53 conformation, we tested if the effects of temperature were

<sup>1</sup> The abbreviations used are: DTT, dithiothreitol; ELISA, enzyme-linked immunosorbent assay.

**FIG. 1. Thermal instability of wild-type p53 conformation determined by two-site ELISA.** Varying amounts of wild-type p53 incubated for 10 min at the indicated temperatures were added to microtiter well plates precoated with the indicated conformationally specific monoclonal antibody. p53 bound to the monoclonal antibody was detected with the rabbit polyclonal antiserum CM-1 and swine anti-rabbit IgG conjugated to horseradish peroxidase. The assay was carried out at 21 °C. Data is shown for 50 ng of recombinant human p53 expressed in *E. coli* (A, B, and D) and recombinant murine p53 expressed in Sf9 insect cells (C).



**FIG. 2. Effect of temperature on oligomeric structure of p53.** The differences in sedimentation of 1  $\mu$ g human p53 expressed in Sf9 insect cells, incubated for 1 h at 4 or 37 °C respectively, was determined by a 5–40% sucrose gradient at 4 °C and subsequent analysis of the collected fractions by a two-site ELISA using the monoclonal antibody DO-1 to capture p53 on the plate and the polyclonal antiserum CM-1 for detection. The position of tetrameric p53 (6 S) is indicated by the arrow.

also reflected in the DNA binding activity of p53. Bacterially expressed p53 is latent for DNA binding but can be activated by binding of the monoclonal antibody PAb421 (Hupp *et al.*, 1992). We tested p53 for its ability to bind to PG, its specific DNA consensus site, after 5 min of incubation at 35, 37, 40, 42, or 45 °C. Fig. 3A shows the results for human p53 produced in *E. coli*. p53 was preincubated at the stated temperature, followed by incubation of PAb421 and PG at 4 °C and a further incubation of 30 min before nondenaturing gel electrophoresis at 4 °C. The DNA binding activity was reduced following preincubation at 40 °C and abolished after preincubation at 45 °C. This inactivation appears to be permanent, since p53 did not refold during the 30-min incubation time on ice prior to the gel electrophoresis. To ensure that p53 was not degraded during the incubation time, the integrity of the protein was checked by

Western blot (Fig. 3B). The level of epitopes recognized by DO-1 and PAb421 was consistent at all temperatures (Fig. 1, B and D), suggesting that the abolition of DNA binding is due to conformational changes and not protein degradation.

**Regulation of DNA Binding at 37 °C**—Since biochemically purified p53 is a highly temperature-sensitive protein but exists and functions at 37 °C *in vivo*, we investigated the effect of incubation at this temperature on the DNA binding function of p53 purified from different expression systems. We started with a time course analysis of latent p53 produced in bacteria; incubations were performed at 37 °C in the range of 5–60 min prior to carrying out the DNA binding assay at 4 °C (Fig. 4). p53 was incubated at 37 °C and subsequently activated for DNA binding with the monoclonal antibody PAb421. The protein lost its DNA binding activity within 10 min (Fig. 4A), and

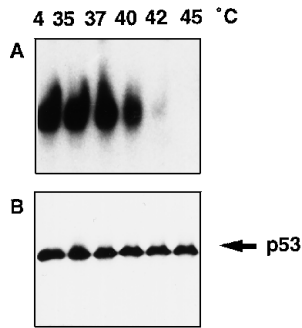


FIG. 3. **Effect of temperature on DNA binding activity of p53.** 150 ng of human p53 expressed in *E. coli* were incubated for 5 min at the indicated temperatures and subsequently subjected to a DNA binding assay including the monoclonal antibody PAb421 performed at 4 °C (A) or a Western blot detecting p53 with the monoclonal antibodies PAb421, DO-1 and PAb240 by ECL (Amersham Corp.) (B).

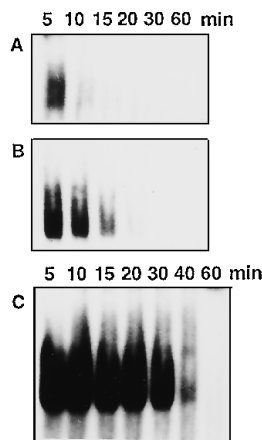


FIG. 4. **DNA binding of different forms of p53 incubated at 37 °C.** DNA binding assays were performed on 150 ng of p53 preincubated at 37 °C for 5–60 min with p53 produced in *E. coli*, which was activated with 100 ng PAb421 after the incubation at 37 °C (A), or p53 expressed in Sf9 insect cells (B). C, the C-terminal  $\Delta 30$  mutant produced in insect cells was subjected to the same incubation at 37 °C before analysis by DNA binding assay.

PAb421 was not able to activate denatured p53 for DNA binding (Fig. 4A, time points later than 10 min). The temperature sensitivity of p53 for DNA binding was also examined for recombinant human p53 purified from baculovirus-infected insect cells. The majority of this protein is multiply phosphorylated by kinases present in the eukaryotic expression system and, as a result, active for DNA binding without the addition of PAb421 (Hupp and Lane, 1994a). Therefore, it could display a different sensitivity to temperature. To examine the effect of these post-translational modifications on heat stability, insect cell-produced p53 was incubated at 37 °C from 5 to 60 min and assayed for DNA binding (Fig. 4B). The protein was slightly more stable than bacterially produced p53 alone (compare Fig. 4, A and B, thermostable for 15 min *versus* 5 min). Interestingly, deletion of the last 30 amino acids resulted in a dramatically enhanced stability at 37 °C for up to 40 min (Fig. 4C), suggesting that the C terminus might contribute an element of instability to p53.

**Effects of Heat Shock Proteins on the Stability of p53 at 37 °C**—The 66-kDa protein DnaK, an *E. coli* homologue of human Hsp70, has recently been shown to bind to the C terminus of p53 (amino acids 381–387) (Fourie *et al.* (1994) and data not shown) and to activate p53 for DNA binding (Hupp *et al.*, 1992, Hupp and Lane, 1994a). To analyze whether this interaction contributes to the stability of p53 DNA binding at 37 °C, we co-incubated DnaK with p53 at 37 °C. This resulted

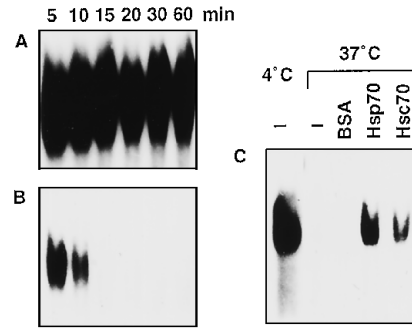
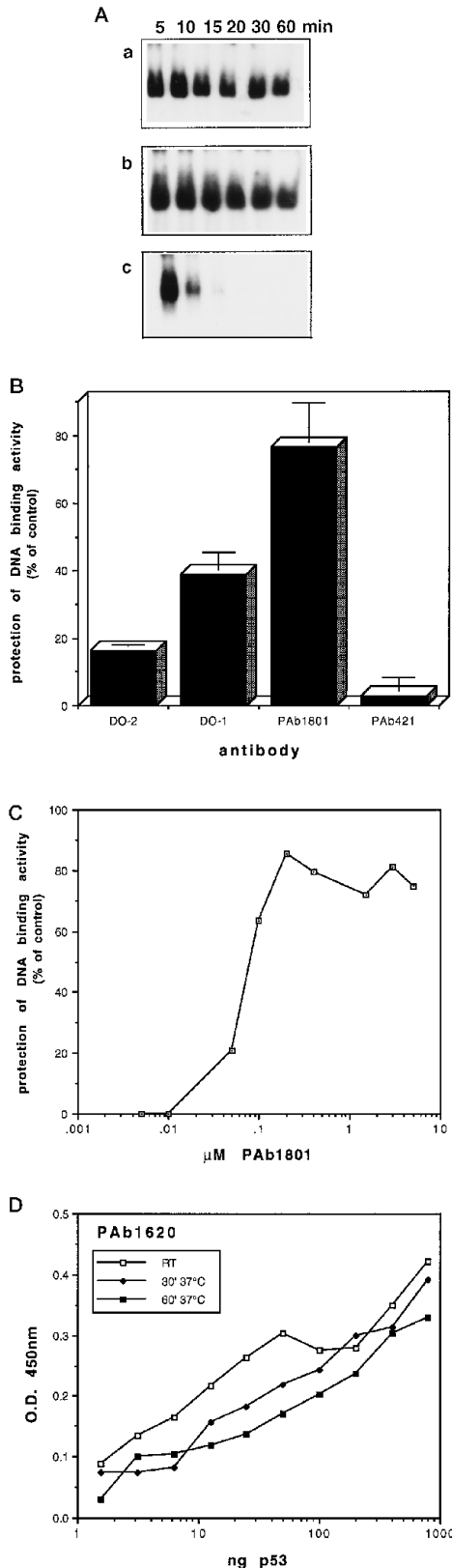


FIG. 5. **Effect of heat shock proteins on the p53 DNA binding activity at 37 °C.** A, human p53 expressed in bacteria (1  $\mu$ M) and DnaK (15  $\mu$ M) were incubated at 37 °C as indicated and subsequently analyzed for DNA binding activity. B, p53 was incubated alone at 37 °C for the indicated times and subsequently incubated with DnaK at 30 °C for 30 min. C, 1  $\mu$ M insect cell-produced p53 was analyzed for DNA binding after incubation at 4 °C and 60 min at 37 °C including bovine serum albumin (3.7  $\mu$ M), human Hsp70 (p72) (3.4  $\mu$ M), and human Hsc70 (p73) (3.4  $\mu$ M), respectively.

in a complete protection of the DNA binding activity even after 60 min of incubation (Fig. 5A). This is perhaps not surprising, since Hsp70 proteins are molecular chaperones that can protect or repair their specific cellular targets by refolding aggregated or incorrectly folded proteins or preventing heat-induced protein aggregation or disaggregation (for reviews see Morimoto *et al.* (1994) and Rassow *et al.* (1995)). To further characterize the interaction with DnaK, p53 was incubated at 37 °C over a range of 5–60 min and then incubated with DnaK for another 30 min at 30 °C (Fig. 5B). DnaK was only able to activate p53 for DNA binding after the first 5–10-min preincubation at 37 °C. This corresponds to the time span during which the DNA binding activity and conformation of p53 is still largely intact (compare with Fig. 4A). DnaK was not able to activate p53 for DNA binding when incubated at 4 °C, and it did not protect the DNA binding activity of p53 at 45 °C (data not shown). Thus the protection of the DNA binding activity of p53 by DnaK seems to be limited to 37 °C. In this system, DnaK protects p53 from denaturation but is not able to reactivate denatured protein. Since DnaK is 50% homologous to the human Hsp70 family, we tested human Hsp70 (p72) and Hsc70 (p73) for their ability to stabilize the DNA binding activity of p53 at 37 °C. Since neither of these proteins was able to activate latent p53 for DNA binding, activated p53 produced in insect cells was used for this assay (Fig. 5C). Both proteins (p72 and p73) conferred a weak protection to p53 DNA binding, indicating a possible role for human heat shock proteins in stabilizing p53.

**Stabilization of p53 at 37 °C by Monoclonal Antibodies**—Since activation of p53 by phosphorylation did not enhance the stability, but deletion of 30 C-terminal amino acids or incubation with DnaK contributed to temperature stability, we analyzed the involvement of the C terminus in the stabilization of DNA binding at 37 °C with the help of monoclonal antibodies. Co-incubation of the activating antibody PAb421 produced only a weak protection of the DNA binding activity of bacterially produced latent p53 at 37 °C for up to 15 min (Fig. 6A, part c). Thus, the activation for DNA binding itself did not contribute to the stabilization at 37 °C. Out of a number of other antibodies tested, we found that monoclonal antibodies binding to the N terminus of p53, DO-1 (amino acids 20–25) and PAb1801 (amino acids 46–55) (Legros *et al.*, 1994), displayed stabilizing properties over the time observed (Fig. 6A, parts a and b, respectively). DO-1 and PAb1801 exerted the same effect on bacterially produced p53 and on p53 produced in insect cells. Neither antibody could stabilize p53 at 45 °C, regardless of the source of protein; nor did addition of PAb1801 after incubation



**FIG. 6. Stabilization of p53 at 37 °C by monoclonal antibodies.** A, after co-incubation at 37 °C for 5–60 min with the indicated monoclonal antibodies, p53 (1  $\mu$ M) was analyzed in a DNA binding assay; insect cell-produced p53 was incubated with 0.6  $\mu$ M DO-1 (a) and 0.6  $\mu$ M PAb1801 (b). p53 produced in bacteria was incubated with 0.6  $\mu$ M PAb421 (c). B, quantitative analysis of maximal stabilization by DO-2, DO-1, PAb1801, and PAb421 of the DNA binding activity of p53 at 37 °C in relation to activity at 4 °C. The error bars represent the standard

error of the mean. C, stabilization of DNA binding of p53 produced in insect cells (1  $\mu$ M) at 37 °C by increasing concentrations of PAb1801 quantified by PhosphorImager. D, increasing amounts of bacterially produced p53 were analyzed by a two-site ELISA captured by PAb1620 and detected with CM-1 as in "Materials and Methods." p53 and PAb1801 (0.6  $\mu$ M) were incubated at 37 °C for 30 and 60 min compared with 21 °C.

at 37 °C renature p53 for DNA binding (data not shown). Thus the regulation of stabilization seems to occur only at 37 °C.

We determined the maximal protection achieved by the respective antibodies under optimized conditions after 60 min of incubation at 37 °C expressed as the relative percentage of DNA binding compared with binding at 4 °C (Fig. 6B). PAb1801 was the most potent stabilizing agent by protecting up to 77% of the DNA binding activity of p53. Interestingly, although DO-1 seemed to protect p53 consistently over the 60 min observed (Fig. 6A, part a), the effect was limited to only 39% of the total protein activity. Another antibody to the N terminus, DO-2, binding to amino acids 6–15 (Legros *et al.*, 1994), was also found to have some stabilizing properties (16%), whereas the effect of PAb421 was comparatively minor (3%) after 60 min at 37 °C. We characterized the kinetics of stabilization by titrating increasing amounts of PAb1801 into a constant amount of p53 and then incubated the mix for 60 min at 37 °C (Fig. 6C). Lower concentrations of 0.005–0.05  $\mu$ M PAb1801 did not stabilize the DNA binding activity significantly, but higher concentrations led to a dramatically increased stabilizing effect resulting in a sigmoidal binding curve, which is characteristic for an allosterically regulated reaction. Evidence for the notion that stabilization requires binding of PAb1801 to all four epitopes on tetrameric p53 comes from gel shift assays with a titration of PAb1801 at 4 °C. At PAb1801 concentrations at which only one antibody molecule is bound per tetramer, there is no protection of the DNA binding of p53 at 37 °C (data not shown).

In light of the stabilizing properties displayed by the N-terminal binding antibodies in a DNA binding assay, we investigated whether antibodies of this sort could also stabilize the native conformation as assayed by PAb1620 reactivity. The conformational changes of p53 were measured by ELISA as described under "Materials and Methods." p53 was incubated at 37 °C for 30 and 60 min before being captured with PAb1620 and detected by CM-1. When p53 was co-incubated with the monoclonal antibody PAb1801 at 37 °C, PAb1801 was able to protect the PAb1620-positive conformation (Fig. 6D).

#### DISCUSSION

Incubations at physiologically relevant temperatures affect p53 at the structural and functional level. The results reported here demonstrate that wild-type p53 is a highly temperature-sensitive protein in two respects. On a structural level, the protein undergoes irreversible conformational changes after short incubations (10 min) at temperatures in a range from 37 to 45 °C by losing its wild-type (PAb1620-positive) conformation. Because the assay for detecting the epitope changes of p53 is carried out at room temperature, the temperature-dependent differences are due to the initial heat treatment of 10 min, since the protein was theoretically allowed to refold during the progress of the ELISA. For this reason we believe that the monitored changes are irreversible and that there is no spontaneous refolding. Incubation at 37 °C abolishes the tetrameric nature of p53 and leads to formation of higher molecular weight aggregates. At a functional level, p53 is permanently inactivated for DNA binding by an incubation of 5 min at 45 °C, which confirms the PAb1620-positive conformation as a prerequisite for DNA binding (Halazonetis *et al.*, 1993). Longer incubations

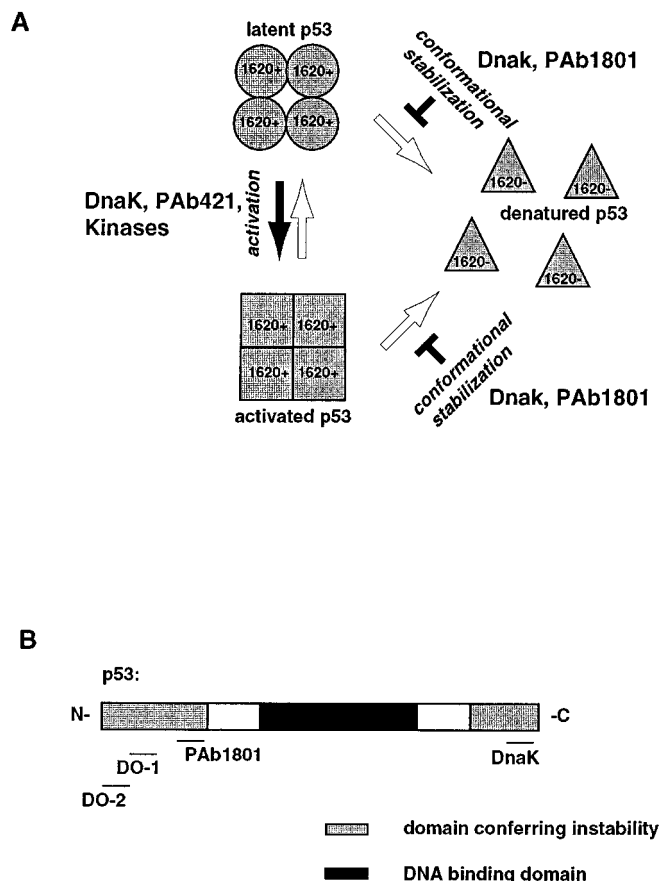


FIG. 7. *A*, activation for DNA binding and thermostability of p53 are two separate pathways. The activation for DNA binding of latent (1620-positive) p53 by kinases (protein kinase C and casein kinase II) can be mimicked by the monoclonal antibody PAb421 but not by PAb1801. Protein phosphatases (PP1 and PP2A) and monoclonal antibodies (ICA-9) can reverse the activation. Both forms of p53, activated and latent, are prone to thermal denaturation as monitored by a loss of the 1620-positive conformation, but activation does not confer significant stability to the protein. Although denatured p53 is drawn as monomers, it corresponds to aggregates of higher molecular weight (see Fig. 2). Binding of monoclonal antibodies to the N terminus (PAb1801) confers conformational stability to both forms of the protein, which is not achieved by PAb421. The interaction of the bacterial Hsp70 homologue DnaK with p53 is of a different nature, since it can act on both pathways: activation and protection from denaturation. *B*, model for the allosteric regulation of the thermostability of p53 DNA binding and conformation. p53 loses its wild-type conformation in the central core of the molecule in a temperature-dependent manner, with the N and C termini contributing to its instability. Deletion of a domain conferring instability within the last 30 amino acids of the C terminus or binding of DnaK within that region thermally stabilizes the core of the protein. In the same way, binding of monoclonal antibodies to specific domains at the N terminus also leads to different degrees of thermostability of p53.

at 37 °C also result in a loss of DNA binding activity. This thermoinstability is seen for both human and murine wild-type p53 expressed in eukaryotic and prokaryotic systems.

p53 has been shown to be a conformationally dynamic protein *in vivo* and *in vitro* (Gannon *et al.*, 1990; Milner and Medcalf, 1990). These results suggest that the conformation of the central core domain of p53 is essential for its biological activity. A loss of the PAb1620-reactive epitope has been reported for quiescent cells upon addition of fresh medium and reentry into the cell cycle (Milner and Watson, 1990). Interestingly, we show that PAb1620-negative p53 cannot be activated by PAb421 or DnaK for DNA binding. Thus it is no longer subject to the activation pathway (Fig. 7A), which is believed to contribute to the p53-mediated cell cycle arrest via a transcrip-

tional activation of target genes. Thus one can speculate that a possible physiological role of p53<sup>1620-</sup> is to allow cell proliferation due to loss of p53's physiological activity.

Investigations of the conformational stability of globular proteins (such as ribonuclease T1 and barnase) have shown that several factors account for stability: temperature, disulfide bonds, amino acid sequence, salt concentrations, and pH. Higher as well as lower temperatures can decrease protein stability and lead to unfolding (for review see Pace (1990)). Proteins show only a small free energy of stabilization as compared with the total molecular energy. As a result, molecular adaptations to extreme physical conditions like temperature require only marginal alterations of the intermolecular interactions and packing density. Enhanced stability can be achieved by mutations at the protein level or binding of extrinsic factors such as ions, co-factors or specific ligands *in vivo* and *in vitro*. We demonstrate here that the binding of specific ligands as well as the deletion of destabilizing domains can confer stability to the wild-type conformation of p53.

Phosphorylation of p53 at the C terminus allosterically activates the latent DNA binding activity of p53, possibly by removing the C-terminal domain from a site of interaction in the central part of the molecule (Hupp *et al.*, 1995). Similarly, deletion of the C-terminal 30 amino acids ( $\Delta 30$ ) leads to constitutive activation (Hupp *et al.*, 1992). Since this allosteric activation could thereby confer some stability to the molecule, we compared the thermostability of phosphorylated p53 produced in insect cells with unphosphorylated p53 expressed in bacteria and found no significant stabilization due to the phosphorylation. Surprisingly,  $\Delta 30$  protein shows a dramatically enhanced intrinsic stability to temperature, suggesting that a region at the C terminus confers some instability to p53.

*Specific Proteins Can Protect p53 from Temperature-dependent Instability*—We also tested the ability of another allosteric activator to contribute to the stabilization of p53 at 37 °C; the *E. coli* heat shock protein DnaK has been shown to bind to the C terminus of p53 (Fourie *et al.* (1994) and data not shown) in close proximity to the putative protein kinase C phosphorylation site and to activate p53 for DNA binding (Hupp *et al.*, 1992). Here, we demonstrate that DnaK has a second, separable function from p53 activation (Fig. 7A); it can protect PAb1620-positive p53 from a temperature-dependent loss of DNA binding activity but cannot renature and activate denatured p53. It has been shown before that DnaK protein can protect the *E. coli* RNA polymerase from heat inactivation (Skowyra *et al.*, 1990). Indeed, DnaK shows a very unusual behavior in recognizing and activating only native, *i.e.* 1620-positive p53 for DNA binding (Fig. 4B). In most cases, chaperones exert a selective activity for non-native proteins as is reported for Hsp70 cognate protein (Hsc); mutant forms of the p53 protein recognized by the monoclonal antibody PAb240 are found to bind to Hsc70, which correlates to their increased half-life (Hinds *et al.*, 1987; Finlay *et al.*, 1988). Hsc70 requires the C-terminal 28 amino acids of p53 for binding to p53 (Hainaut and Milner, 1992). It may involve a mammalian homologue of the bacterial protein DnaJ, Hsp40, in complex formation with p53 (Sugito *et al.*, 1995). Here, we have shown that incubation with Hsp70 and Hsc70, respectively, leads to a protection of some DNA binding activity of p53 at 37 °C. Hence we have developed an *in vitro* assay for human heat shock protein function. The fact that human heat shock proteins cannot substitute for DnaK in activating p53 for DNA binding may be due to the fact that a different substrate specificity has been shown for the three Hsp70 chaperones: DnaK, Hsc70, and BiP (Fourie *et al.*, 1994). It is possible that one of the Hsp70 family members in humans, alone or in combination with another chaper-



one, shares the activity of DnaK and protects p53 function *in vivo*.

To determine the role of allosteric activation in the stabilization of p53 at 37 °C, we used the monoclonal antibody PAb421, which activates p53 for DNA binding (Hupp *et al.*, 1992). PAb421 is not able to activate denatured p53 (Fig. 4A), nor is it able to protect p53 from denaturation (Fig. 6A, after a 15-min incubation at 37 °C), clearly showing that activation and conformational stabilization are two different pathways (Fig. 7A). We have identified a region at the N terminus (amino acids 46–55) of p53 that stabilizes p53 dramatically (60–70%) when bound by the antibody PAb1801. Indeed, the ability of antibodies to confer stability to p53 increases with antibodies binding in closer proximity to the PAb1801 epitope (Fig. 7B). One interpretation of these results is that the epitope recognized by PAb1801 confers some instability to p53, which can be neutralized by the binding of the antibody. A deletion of this region should therefore result in a gain of stability, as it is seen for  $\Delta 30$ . Indeed, a deletion at the N terminus of 102 N-terminal amino acids including the PAb1801 epitope renders p53 more stable to temperature (data not shown). We demonstrate that the ability of PAb1801 to stabilize p53 DNA binding at 37 °C increases dramatically with increasing concentrations and rapidly reaches a plateau, resulting in a sigmoidal binding curve (Fig. 6C). From a titration of PAb1801 at 4 °C it was evident that at nonprotective concentrations of PAb1801 only one antibody was bound per tetramer, since p53 was only partially supershifted to an intermediate form (data not shown). Thus, efficient stabilization only takes place when all subunits of the tetrameric p53 are bound by two bivalent antibody molecules. Fab fragment analysis showed that the bivalency of the antibody was not necessary for the stabilization, although the effect was somewhat weaker, but significant stabilization was achieved only with all four Fab fragments bound to p53 (data not shown). The stabilization effect of PAb1801 seems to be mediated by a protection of the wild-type conformation (Fig. 6D). Since p53 denatures in a rapid manner at 37 °C, the antibody concentration seems to be critical to protect p53. Over the time observed (up to 60 min), the population active for DNA binding remains unchanged (Fig. 6A, parts a and b), although it might only represent a small percentage of the total DNA binding activity (e.g. 39% stabilization by DO-1). A deletion of the C terminus of p53, while substantially delaying the inactivation of p53 at 37 °C, is less effective in protecting the activity of p53 than the binding of antibody or DnaK (Fig. 4C).

Taken together, our data indicate that p53 can be allosterically regulated for stabilization of the wild-type conformation and DNA binding activity at 37 °C by binding of two classes of ligands (e.g. PAb1801 and DnaK) to regulatory sites on both ends of the molecule, outside of the DNA binding domain, by which an intrinsic instability of p53 is neutralized (Fig. 7B). The deletion of motifs conferring instability either at the C or N terminus is enough to confer enhanced stability to the total protein as well as the binding of only one specific ligand. Hence we provide an assay to identify cellular factors that regulate p53 stability *in vivo*. We can clearly show that activation for DNA binding and stabilization are two separate pathways (Fig. 7A). In this model, misfolded protein (PAb1620-negative) would be targeted for rapid degradation by cellular proteolytic pathways. Monoclonal antibodies can often substitute for essential ligands or regulatory factors; binding of PAb421 to p53 can substitute for activation by protein kinase C and casein kinase II (Hupp and Lane, 1994). PAb1801 (and DO-1) could possibly mimic a class of cellular proteins that regulate p53 stability by interacting with the N terminus. It is of great interest that the DO-1 antibody interacts with critical amino acids in the N

terminus of the protein required for binding the *mdm2* oncogene and the transcriptional co-activators TAFII40 and TAFII60 (Picksley *et al.*, 1994; Thut *et al.*, 1995). Interestingly, the WT1 gene product, which binds to the 70 N-terminal amino acids of p53, has recently been shown to stabilize p53, resulting in increased steady-state levels of p53, and it partially protects against papillomavirus E6-mediated degradation of p53, which correlates to the presence of high levels of p53 protein in primary Wilms' tumors (Maheswaran *et al.*, 1995). Regulation of the thermostability of p53 may represent a new mechanism to control the biological activity of this important tumor suppressor gene product.

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