Molecular Evolution of the Thermosensitive PAb1620 Epitope of Human p53 by DNA Shuffling*

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Abstract

Conformational stability of the p53 protein is an absolute necessity for its physiological function as a tumor suppressor. Recent in vitro studies have shown that wild-type p53 is a highly temperature-sensitive protein at the structural and functional levels. Upon heat treatment at 37 °C, p53 loses its wild-type (PAb1620+) conformation and its ability to bind DNA, but can be stabilized by different classes of ligands. To further investigate the thermal instability of p53, we isolated p53 mutants resistant to heat denaturation. For this purpose, we applied a recently developed random mutagenesis technique called DNA shuffling and screened for p53 variants that could retain reactivity to the native conformation-specific anti-p53 antibody PAb1620 upon thermal treatment. After three rounds of mutagenesis and screening, mutants were isolated with the desired phenotype. The isolated mutants were translated in vitro in either Escherichia coli or rabbit reticulocyte lysate and characterized biochemically. Mutational analysis identified 20 amino acid residues in the core domain of p53 (amino acids 101–120) responsible for the thermostable phenotype. Furthermore, the thermostable mutants could partially protect the PAb1620+ conformation of tumor-derived p53 mutants from thermal unfolding, providing a novel approach for restoration of wild-type structure and possibly function to a subset of p53 mutants in tumor cells.

Activation of the p53 tumor suppressor protein appears to be an integrating mechanism in response to cellular stresses such as DNA damage and oxidative stress (1–3). This leads to activation of growth arrest or apoptotic pathways (4, 5), conferring maintenance of genomic stability (6, 7). Three separate functional domains at p53 have been identified. In the N terminus (amino acids 1–43) lies the transcriptional transactivation activity of the protein (8–11), whereas amino acids 100–290 form a protease-resistant hydrophobic core that is responsible for the sequence-specific DNA-binding activity (12, 13). The N terminus is composed of an oligomerization domain (amino acids 319–360) and a region that negatively regulates the sequence-specific DNA-binding activity of p53 (14–16).

Point mutations in the p53 gene have been identified in 50% of human tumors, indicating that p53 inactivation is an important step in tumor progression (17–19). The majority of these mutations (>90%) cluster in the central core domain of the protein and are responsible for the loss of the biological activity of p53 (20, 21). More specifically, these mutations involve either residues that make direct contact with DNA ("contact mutants") or residues that provide structural stability and proper positioning of the DNA contact residues ("structural mutants"). Examples of contact mutants include Arg-248 and Arg-273, and those of structural mutants include Arg-175, Gly-245, Arg-249, and Arg-282 (22).

Interestingly, many of the point mutations identified in the core domain of p53 produce a change in the global conformation of the protein, which can be monitored by a set of anti-p53 monoclonal antibodies. More specifically, PAb1620 (human and mouse p53-specific) (23) and PAb246 (mouse p53-specific) (24) recognize the wild-type native conformation of p53, but fail to react with most mutants found in tumors. These mutants react with PAb240 (25), which recognizes denatured, but not wild-type, p53. The definition of distinct p53 conformers is simplistic, however, since it appears that p53 is a dynamic protein, adopting different conformations in vitro and in vivo. In support of this statement, Milner and Medcalf (26) demonstrated that formation of hetero-oligomers of wild-type and mutant p53 proteins could drive the conformation of the wild-type protein into a mutant state. Furthermore, upon binding to DNA, p53 appears to switch from a PAb1620/PAb240 to a PAb1620/PAb240* conformation (27). Since these two conformations of p53 dictate the biological status of the protein, one could suggest that the balance between these two alternate states might determine the biological activity of p53 in cells. Identification of the mechanisms that govern this equilibrium could provide new insights into p53 structure/function.

In vitro studies on purified wild-type p53 have shown that the native conformation of the protein is very temperature-sensitive. Incubation of p53 at physiological temperatures (37 °C) causes an irreversible transition from PAb1620* to PAb1620 conformation and loss of the ability of p53 to act as a sequence-specific DNA-binding protein. However, p53-interacting proteins such as human HSF70, Escherichia coli DnaK, or N-terminal antibodies (DO-1, PAb1801) can protect p53 from temperature-induced denaturation, stabilizing to a certain degree the PAb1620* conformation of the protein (28). Similar studies have been performed on tumor-derived p53 mutants, which appear to be even less thermostable than wild-type p53, but again, N-terminal anti-p53 antibodies can partially protect these mutants from temperature-induced unfolding (29). Recently, a more quantitative approach regarding the stability of p53 has been performed using differential scanning calorimetry, where the core domains of wild-type p53 and several tumor-derived p53 mutants were subjected to urea-mediated denaturation (30). The results of this study demonstrated that the core domain of p53 is of moderate thermodynamic stability, with all the tested mutant core domains being less stable than the wild type. All these observations provide evidence that p53...
is a thermosensitive flexible protein switching between alternate conformations that can be modulated by different classes of ligands. A subset of the p53 mutations found in tumors destabilize the folded state of the protein, affecting the normal biological function of p53.

To further investigate the implications of the thermosensitive phenotype of p53 regarding its biological function, we created p53 mutants able to resist temperature-dependent unfolding. For this purpose, we applied a random polymerase chain reaction (PCR)-based mutagenesis technique called DNA shuffling (31, 32) and selected for p53 mutants that could resist temperature-dependent loss of PAb1620 reactivity. DNA shuffling involves random fragmentation of PCR-amplified related genes, followed by reassembly in a primerless PCR. Therefore, beneficial mutations existing in different genes can be united by homologous recombination in the same gene, mimicking the process by which proteins evolve in nature. Selected genes can then be pooled and used as a template for a new round of recombination and selection. DNA shuffling has been successfully applied for the molecular evolution of single gene products with enhanced activity (33), improved protein folding (34), or altered substrate specificity (35).

After three rounds of DNA shuffling, we isolated p53 mutants that retain PAb1620 reactivity at temperatures where wild-type p53 is completely unreactive. Sequencing and mutational analysis of the selected mutant identified a region of 20 amino acid residues in the core domain of p53 (amino acids 101–120) that is responsible for the observed thermostable phenotype. Furthermore, the thermostable mutants can partially protect the PAb1620 conformation of tumor-derived p53 mutants from thermal unfolding, providing a novel approach to reactivate or enhance wild-type p53 activity in tumor cells.

**EXPERIMENTAL PROCEDURES**

**Antibodies—**Anti-human p53 monoclonal antibodies were used in two-site ELISA or immunoprecipitations. DO-1 interacts with amino acids 20–25 (36); PAb1801 interacts with amino acids 46–55 (37); PAb240 recognizes an epitope in the core of p53 (amino acids 213–217) (38); and PAb421 reacts within the C terminus of p53 (amino acids 371–380) (39).

**DNA Shuffling—**The cDNA encoding wild-type human p53 (1.2 kilobases) was obtained by PCR from human p53 (P7-39) with SfiI (GGCCCCAGCGGGCCATGGATGAGGGCAGCTCAGA) and NotI (GCCGCCGCCTCGAGTACGCTAGCCCTCTCTG) primers and purified from a 1% low-melting-point agarose gel using the Wizard PCR purification system (Promega). 2–3 μg of the purified PCR product was digested with 0.15 units of DNase I (Sigma) for 10 min at room temperature in 100 μl of 50 mM Tris-HCl (pH 7.4) and 1 mM MgCl2. The reaction was stopped by addition of 1 mM EDTA and 0.1% SDS and incubation at 65 °C for 10 min. Fragments of 100–300 base pairs were purified from a 2% low-melting-point agarose gel using the QIAEX II gel extraction kit (Qiagen Inc.). The purified fragments were resuspended in a primerless 25-μl PCR (0.2 mM each dNTP, 2.2 mM MgCl2, 50 mM KCl, 10 mM Tris-HCl (pH 8.8), 0.1% Triton X-100, and 2.5 units of AmpliTaq (Perkin-Elmer) at 20–30 μg/ml. A PCR program of 94 °C for 2 min and 40 cycles of 94 °C for 40 s, 53 °C for 40 s, and 72 °C for 40 s was followed in a Perkin-Elmer DNA thermal cycler. The product of this reaction was diluted 40-fold in a 50-μl PCR (0.2 mM each dNTP, 2.2 mM MgCl2, 50 mM KCl, 10 mM Tris-HCl (pH 8.8), 0.1% Triton X-100, and 5 units of AmpliTaq) with the inclusion of SfiI/NotI primers at 0.2 pmol/μl, followed by a PCR program of 94 °C for 3 min and 25 cycles of 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 2 min. The correctly sized band (1.2 kilobases) was purified and digested with SfiI/NotI enzymes before being cloned into the pCantab5E phagemid vector. The in vitro recombined p53 library was transformed into HB2151 cells, plated on LB plates containing 100 μg/ml ampicillin and 2% glucose, and grown overnight at 30 °C.

**Screening Procedure—**For each round of DNA shuffling, 10,000 colonies transformed with the mutated p53 library were blotted on nitrocellulose filters, and protein expression was induced overnight at room temperature on LB plates containing 100 μg/ml ampicillin and 1 mM isopropyl-β-D-thiogalactopyranoside. At this stage, the master plates were kept at 4 °C until further use. Bacterial lysis was performed by incubating the colony blot filters in PBS, 1 mM EDTA, and 0.1% Triton X-100 for 15 min at room temperature. Under these lysis conditions, the PAb1620 conformation of expressed p53 was detectable. The filters were then washed twice for 15 min with PBS containing 0.1% Tween-20 (PBST) to remove the cell debris. Subsequently, the filters were subjected to heat treatment: incubation for 15 min at 37 °C in the first round of DNA shuffling and selection and at 42 °C in the second and third rounds.

**Immunological Selection—**After heat treatment, the filters were blocked in PBST containing 5% (w/v) Marvel (PBSTM) for 2 h and probed with PAb1620 at 2.5 μg/ml in PBSTM for 1 h before incubation with alkaline phosphatase-conjugated rabbit anti-mouse IgG (Dako D314) diluted 1:1000 in PBSTM for 1 h. Color development was performed by placing the filters in 10 ml of alkaline phosphatase buffer containing 44 μl of nitro blue tetrazolium chloride (75 mg/ml in 70% dimethylformamide) and 33 μl of 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt (50 mg/ml in dimethylformamide) and incubating for 7 min. All steps were carried out at room temperature, and the filters were washed twice for 15 min with PBST before each incubation step. For the first and second rounds, the 30 colonies from the master plates corresponding to the strongest signals were selected and used as a template for a PCR of a new cycle of recombination and selection. After the third round, the best mutant was further characterized.

**Bacterial Expression—**The mutant cDNA after the third round of selection was subcloned into the pT7-7 prokaryotic expression vector and transformed into BL21(DE3) bacteria. Protein expression and purification on a heparin-Sepharose column (Amersham Pharmacia Biotech) were performed as described (14), and samples were used in thermostability experiments. Equal amounts of protein were incubated at different temperatures for various periods of time. After heat treatment, the samples were transferred on ice, and serial dilutions were prepared for two-site ELISA as described below.

**In Vitro Transcription/Translation and Immunoprecipitations—**Wild-type human p53 and p53 mutants were translated in vitro using the TnT®-coupled reticulocyte lysate system (Promega). 1 μg of p53 p7T-7 plasmids was used in 50-μl reactions together with 350 μCi of [35S]methionine, and translation was performed at 30 °C for 90 min according to the manufacturer’s instructions. Further protein synthesis was blocked by adding cycloheximide at 100 μg/ml, and aliquots were used in thermostability experiments as described above. After heat treatment, the samples were transferred on ice and immunoprecipitated with 1 μg of PAb1620 in 100 μl of immunoprecipitation buffer (50 mM Tris-HCl (pH 8), 150 mM NaCl, 5 mM EDTA (pH 8), 1% Nonidet P-40, 2 mM dithiothreitol, and 2 mM phenylmethylsulfonyl fluoride) for 2 h on ice. The samples were then incubated with 20 μl of protein G beads for 45 min at 4 °C and washed three times with 200 μl of immunoprecipitation buffer. The [35S]-labeled immunoprecipitates were separated by 12% SDS-PAGE, and the gel was dried and exposed to x-ray film.

**Two-site ELISA—**96-well flat-bottomed plates (Falcon 3912) were coated with 50 μl/well of purified anti-p53 monoclonal antibodies at 5 μg/ml in 0.1 M CO3/HCO3 (pH 9) at 4 °C overnight. The wells were blocked for 2 h with 200 μl of PBST before adding 50 μl/well of p53 sample, diluted in a 1:1 ratio with PBST for 2 h. Detection of p53 was performed by adding 50 μl/well of anti-p53 rabbit polyclonal serum CM-1 (1:1000 in PBSTM for 1 h), followed by a 1-h incubation with 50 μl/well of horseradish peroxidase-conjugated swine anti-rabbit IgG (Dako P217; 1:1000 in PBSTM), and visualizing with 50 μl/well of TMB/H2O2 substrate (3,3',5,5'-tetramethylbenzidine; Sigma T 2885). All steps were carried out at room temperature, and the plates were washed four times with 200 μl of PBST between each incubation step. For each protein concentration, duplicates were performed, and each experiment was repeated three times. S.D. values were derived from arithmetic means.

**Mutagenesis—**Deletion mutants were created using the mutagenesis method described by Imai et al. (40). The sequences of the mutants were confirmed by automated sequencing performed in an ABI Prism 377 DNA sequencer.

**RESULTS**

**Acquisition of Mutants That Can Resist Temperature Denaturation—**DNA shuffling was used to randomly mutagenize the
human p53 cDNA and to select for mutants that retain the wild-type native conformation at temperatures where wild-type p53 becomes unfolded. For this purpose, we used, as a screening probe, the anti-p53 monoclonal antibody PAb1620, which is specific for the wild-type folded form of p53. The mutated p53 library was transformed into bacteria, and protein expression was induced on nitrocellulose filters. After cell lysis, the filters were incubated for 15 min at 37 °C in the first round of mutagenesis and screening and at 42 °C in the second and third rounds, before being probed with PAb1620 for the identification of clones expressing thermostable p53. Fig. 1 shows filters from all three rounds of shuffling and screening. Wild-type human p53 was used as a control in each round, and it failed to be recognized by PAb1620 after only a 15-min incubation of the filters at 37 °C, whereas there was a clear reactivity in untreated filters.

After the third cycle, we isolated single clones expressing p53 detected by PAb1620 after heat treatment. The strongest positive mutant protein (TR p53) was expressed initially in bacteria and purified through a 5-ml heparin-Sepharose column as described by Hupp et al. (14). Purified p53 mutant protein was then analyzed by two-site ELISA for epitope availability. A panel of anti-p53 monoclonal antibodies was used to capture p53, and antibody-associated p53 protein was then detected by the anti-p53 rabbit polyclonal serum CM-1 (Fig. 2, A and B). The DO-1 and PAb421 monoclonal antibodies recognize linear epitopes at the N and C termini, respectively, whereas PAb240 recognizes a linear epitope in the core domain of p53 that is cryptic in the wild-type folded core of the protein, but exposed only in point-mutated or denatured p53 (see “Experimental Procedures” and Fig. 2A). It appears that the isolated mutant protein displays decreased basal reactivity toward PAb1620 compared with wild-type p53, whereas it displayed very similar reactivity to all the other anti-p53 antibodies used (Fig. 2C).

We then addressed the question of the effect of temperature on epitope availability. Equal amounts of purified wild-type and TR p53 proteins were incubated at different temperatures for varying periods of time as described under “Experimental Procedures” before being serially diluted and added to ELISA wells precoated with anti-p53 monoclonal antibodies. As shown in Fig. 3, whereas there was no effect of temperature on the epitopes recognized by DO-1 and PAb421 (Fig. 3, A and B) for either wild-type p53 or the TR mutant, we observed profound differences in the reactivity toward the conformation-dependent antibodies, PAb1620 and PAb240 (Fig. 3, C and D). Heat treatment resulted in the gradual loss of PAb1620 recognition of wild-type p53, consistent with previous reports demonstrating that p53 is a thermosensitive protein (28). The TR mutant, however, displayed a different phenotype; temperature stress resulted in a reproducible 30–40% increase in PAb1620 reactivity, with a concurrent 30% decrease in the PAb240 epitope availability. Maximum resistance to temperature-dependent unfolding was achieved at 42 °C since further increase in tem-
FIG. 3. Effect of temperature on epitope availability for the TR mutant and wild-type p53. A two-site ELISA was used to monitor the effect of temperature on linear (A and B) and conformational (C and D) epitopes of the TR mutant and wild-type human p53. Equal amounts of protein (200 ng) were incubated at different temperatures for varying periods of time. The samples were cooled on ice, and serial dilutions were prepared and added to ELISA wells precoated with anti-p53 monoclonal antibodies. As before, detection of p53-antibody interaction was performed with the anti-p53 polyclonal antibody CM-1. Shown are the means ± S.D. of three independent experiments (two wells/condition). [ ], 4 °C; ○, 37 °C for 20 min; △, 37 °C for 45 min; ▽, 42 °C for 45 min.
human p53 proteins were expressed using the in vitro transcription/translation reticulocyte lysate system. Further translation was inhibited by cycloheximide, and equal amounts of protein were subjected to heat treatment before immunoprecipitation with 1 μg of PAb1620. The 35S-labeled immunoprecipitates were then analyzed by 12% SDS-PAGE. Lanes 1–5 represent the effect of temperature on PAb1620 reactivity for wild-type p53, and lanes 6–10 for the TR mutant. B, temperature treatment does not affect the total protein levels. After heat stress, 5 μl of each sample was separated by 12% SDS-PAGE. Lanes 1–4 demonstrate the protein levels of 35S-labeled wild-type p53, and lanes 5–8 the levels of TR mutant.

**Reticulocyte Transcription/Translation**—We then used the in vitro transcription/translation reticulocyte lysate system to investigate the thermostability of the isolated mutant in an alternative eukaryotic expression system. Wild-type human p53 and the TR mutant were expressed as described under “Experimental Procedures” at 30 °C for 90 min, and further transcription was inhibited by addition of cycloheximide. Equal amounts of protein were subjected to thermal treatment as described above and then immunoprecipitated with PAb1620. Fig. 4A shows that the amount of wild-type p53 recognized by PAb1620 progressively decreased after heat treatment, whereas an increasing amount of TR mutant p53 was immunoprecipitated by PAb1620, consistent with the thermostable phenotype observed using bacterially expressed protein in the ELISA.

Reticulocyte lysates have recently been shown to contain Mdm2-like proteins, present in p53/DNA complexes (41). Mdm2-p53 interaction appears to be crucial for the stability of p53 in cells since Mdm2 can target p53 for rapid degradation (42–44). We were interested to see whether there was p53 proteolysis in our heat treatment experiments, which could lead to decreased protein levels. Fig. 4B shows that heat treatment did not affect the overall protein levels of either wild-type or TR mutant p53. Therefore, the temperature-induced decrease in PAb1620 reactivity of wild-type p53 must be due to conformational changes and loss of native structure, which were resisted by TR mutant p53. The biochemical behavior of the thermostable mutant was consistent in both expression systems, i.e. decreased PAb1620 reactivity that is resistant to heat treatment. An interesting observation that arose from these experiments is that the isolated thermostable mutant has a much faster rate of migration compared with wild-type p53 on SDS-PAGE (Fig. 4, A and B). The explanation for this phenomenon lies in the sequence of the TR mutant described below.

**Sequencing and Mutational Analysis Identify a Region of 20 Amino Acid Residues Responsible for the Observed Phenotype**— Sequencing of the isolated TR mutant p53 gene after the third round revealed 14 point mutations and three nucleotide deletions. Different mutants were derived from the original by introducing various regions of the mutant gene onto the wild-type background (mutants VB7HP, 12C, and 6AB/3). In-frame 20-amino acid deletions (mutants 2b, 4b, 6b, and 8b) identified residues 101–120 in the core domain of p53 responsible for the thermostable phenotype. All mutants were classified either as + (TR thermostable phenotype) or − (wild-type thermosensitive phenotype) by determining their reactivity toward PAb1620 after heat treatment in two-site ELISA.

**Conformation-stabilizing Factors of p53 Have No Effect on the Thermostable Mutant**—It has been previously shown that N-terminal anti-p53 monoclonal antibodies can protect wild-type p53 from temperature-dependent unfolding (28, 29). We were interested to see whether such factors could further sta-
bibilize the thermostable mutant. Equal amounts of heparin-Sepharose-purified wild-type p53 and mutant 6b (Fig. 5) were incubated for 20 min on ice with the N-terminal anti-p53 antibodies DO-1 and PAb1801. As a control, we also used the C terminus-recognizing antibody PAb421, which was shown to have DNA-binding activating properties, but no stabilizing effect on p53. The samples were then subjected to thermal treatment and analyzed in a two-site ELISA for PAb1620 recognition as described above. As shown in Fig. 6, none of the antibodies used had any further stabilizing effect on the thermostable mutant, whereas wild-type p53 could be protected by N-terminal, but not C-terminal, antibodies as previously reported (28).

The Thermostable Mutant Confers Conformational Stability to the p53 His-175 Mutant—Structural point mutations in p53 appear to destabilize the global conformation of the protein, which results in abrogation of the sequence-specific DNA-binding activity of p53. These mutants exist predominantly in the TR p53 conformations with different biological activities. PAb1620 and PAb246 interact with the wild-type native structure of p53, but fail to recognize a subset of p53 mutants found in tumors. These mutants have lost sequence-specific DNA-binding activity and interact with PAb240, which recognizes denatured, but not wild-type, p53.

Previous studies have demonstrated that p53 is a temperature-sensitive protein at the structural and functional levels. Incubation of p53 at physiological temperatures results in an irreversible loss of the wild-type PAb1620 conformation and sequence-specific DNA-binding activity (28). In this study, our goal was to create p53 variants that could retain the wild-type native conformation at high temperatures. After three rounds of DNA shuffling and screening, we isolated mutants that could resist temperature-dependent loss of PAb1620 reactivity. Thermostability experiments on the strongest positive mutant protein (TR p53) expressed in two different systems confirmed the thermostable phenotype. Incubation of the TR p53 mutant at a range of temperatures from 37 to 42 °C for varying periods of time did not reduce its recognition by PAb1620. Instead, we observed a gradual increase in the fraction of protein reactive to PAb1620, which was accompanied by a reproducible decrease in PAb240 reactivity.

DISCUSSION

Conformation-dependent anti-p53 monoclonal antibodies have been extensively used for the discrimination of alternate p53 conformations with different biological activities. PAb1620 and PAb246 interact with the wild-type native structure of p53, but fail to recognize a subset of p53 mutants found in tumors. These mutants have lost sequence-specific DNA-binding activity and interact with PAb240, which recognizes denatured, but not wild-type, p53.

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The 35S-labeled immunoprecipitates were separated by 12% SDS-PAGE. p53 His-175 mutant protein was either expressed alone (0.5 μg of His-175 pT7-7 plasmid + 0.5 μg of vector; lanes 6–10) or coexpressed with the thermostable mutant 6AB/3 (0.5 μg of each plasmid; lanes 1–5). As a control, mutant 6AB/3 was translated alone (0.5 μg of 6AB/3 pT7-7 plasmid + 0.5 μg of vector; lanes 11–15). Equal amounts of protein were subjected to heat treatment before immunoprecipitation with 1 μg of PAb1620. The 35S-labeled immunoprecipitates were separated by 12% SDS-PAGE.

FIG. 7. The thermostable mutant confers conformational stability on the p53 His-175 mutant. p53 His-175 mutant protein was expressed either alone or coexpressed with the thermostable mutant 6AB/3. The temperature-sensitive mutant 6AB/3 was translated alone or coexpressed with the thermostable mutant 6AB/3. Equal amounts of protein were subjected to heat treatment before immunoprecipitation with 1 μg of PAb1620. The 35S-labeled immunoprecipitates were separated by 12% SDS-PAGE.

FIG. 8. Effect of temperature on the conformation of the thermostable (temperature-resistant) and temperature-sensitive mutants.

Molecular Evolution of the PAb1620 Epitope

several tumor-derived p53 mutants. Furthermore, anti-p53 monoclonal antibodies (DO-1, PAb1801) that can protect p53 from temperature-dependent unfolding (28, 29) had no further stabilizing effect on our thermostable mutants.

The thermostability profile of wild-type p53 in these experiments was consistent with a previous report, i.e., gradual temperature-dependent decrease in Ab1620 reactivity (28). The same study has demonstrated that heat treatment of wild-type p53 leads to protein aggregates that have lost reactivity both to Ab1620 and PAb240 (PAb1620/PAb240). Therefore, it is possible that heat treatment causes unfolding of wild-type p53 (loss of Ab1620 reactivity), which will finally lead to aggregates passing through the unfolded state (PAb240) as an intermediate step. This could explain why the PAb240 reactivity of wild-type p53 remains overall unchanged after heat treatment. Interaction with linear epitope-recognizing anti-p53 antibodies such as DO-1 and PAb421 was not affected in our thermostability experiments.

Temperature-sensitive p53 mutants that have been identified in many human tumors display a very different phenotype. The conformation of these mutants, for example, human Ala-143 (45) and murine Val-135 (46), appear to be flexible and very temperature-sensitive. At the permissive temperature (32.5 °C), these mutants adopt a wild-type conformation (PAb1620/PAb240) and are able to mediate transcription transactivation. At the restrictive temperature (37.5 °C), the conformation is switched to a mutant state (PAb1620/PAb240), and transcriptional activation function is greatly reduced or abolished. Therefore, the thermostable mutant isolated in this study displays a “temperature-resistant” phenotype, where temperature stress drives the balance toward the wild-type PAb1620+ conformation (Fig. 8).

Sequencing of the isolated clone revealed 14 point mutations that produced 12 amino acid substitutions. We also identified three nucleotide deletions that effectively replaced the p53 amino acid sequence 60–120 with a non-p53 peptide. Such mutational mechanisms have already been observed due to DNA shuffling (47) and, in our case, created a mutant protein that was one amino acid residue shorter than wild-type p53. Despite their almost identical sizes, the isolated mutant has a much faster rate of migration on SDS-PAGE than wild-type p53. Anomalous migration of p53 deletion mutants has already been reported (48), and it is believed that the proline-rich region in p53 may retain some structure on SDS-containing gels affecting migration. Even more strikingly, a sequence polymorphism found in the human p53 gene that results in either a proline or arginine at residue 72 (within the proline-rich region) also affects migration of the protein on SDS-PAGE (49) and may affect protein degradation (50). Mutational analysis of the isolated p53 mutant revealed the region responsible for the thermostable phenotype. Deletion of 20 amino acid residues in the core domain of p53 (residues 101–120) was necessary and sufficient to create the original mutant phenotype.

Recently, an initial characterization of the epitope for PAb1620 has been achieved by the use of phage-displayed random amino acid peptides by Ravera et al. (51). A sequence comparison of the selected PAb1620-interacting peptides and p53 revealed that the PAb1620 epitope is composed of two regions, residues 106–114 and 146–156. However, the authors did not exclude the possibility that other p53 residues could interact with PAb1620, but were not selected by the particular panning effort. One of the regions in p53 identified as part of the PAb1620 epitope (amino acids 106–114) is deleted in our derived thermostable mutants (mutant 6b, amino acids 101–120) (Fig. 5), which may explain the decreased basal reactivity of the mutants toward PAb1620 as compared with wild-type p53.

During the biochemical characterization of the thermostable mutant, we explored the ability of p53 to oligomerize in tetramers and asked whether the conformation of tumor-derived structural p53 mutants could be protected from temperature unfolding upon formation of hetero-oligomers with the thermostable mutant. Indeed, the wild-type (PAb1620+) conformation of p53 His-175, one of the most common structural p53 mutants found in tumors, is protected from temperature denaturation upon coexpression with our thermostable mutant.

Restoration of wild-type p53 activity in tumor cells is a desirable goal in anticancer therapy (52–54). One approach is to reintroduce wild-type p53, perhaps by gene therapy (55); alternatively, one could restore the wild-type function to endogenous mutant p53. In the second case, antibodies or peptides that interfere with the C-terminal regulatory domain of p53 may succeed in relieving the negative regulation of p53 DNA-binding activity (14, 56–58). Another approach toward this goal has been recently suggested, whereby, using a yeast genetic approach, second site mutations have been identified that suppress the effects of common p53 cancer mutations (59). Similarly, the increased thermostability displayed by a subset of tumor-derived mutants may also be a target for the restoration of wild-type function to mutant p53 molecules. Hence, small molecules or evolution of thermostable p53 variants that could increase the stability of the folded state of these mutants could provide a route for rescuing the native conformation and possibly the wild-type function of certain p53 mutants in tumor cells.

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