Isolation of Temperature-sensitive p53 Mutations from a Comprehensive Missense Mutation Library

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Temperature-sensitive (ts) mutations have been used as a genetic and molecular tool to study the functions of many gene products. Each ts mutant protein may contain a temperature-dependent intramolecular mechanism such as ts conformational change. To identify key ts structural elements controlling the protein function, we screened ts p53 mutants from a comprehensive mutation library consisting of 2,314 p53 missense mutations for their sequence-specific transacti

Missense mutations have not yet been examined. Recently, we constructed 2,314 missense mutations that covered almost all the tumor derived missense mutations, as well as a number of downstream genes through p53-binding sequences in Saccharomyces cerevisiae. We isolated 142 ts p53 mutants, including 131 unreported ts mutants. These mutants clustered in β-strands in the DNA-binding domain, particularly in one of the two β-sheets of the protein, and 15 residues (Thr183, Arg186, Met189, Ala191, Val192, His193, Ser195, Pro223, Thr231, Thr235, Ile236, Thr237, Ser238, Pro243, and Glu255) were ts hot spots. Among the 142 mutants, 54 were examined further in human osteosarcoma Saos-2 cells, and it was confirmed that 89% of the mutants were also ts in mammalian cells. The ts mutants represented distinct ts transactivities for the p53 binding sequences and a distinct epitope expression pattern for conformation-specific anti-p53 antibodies. These results indicated that the intramolecular β-sheet in the core DNA-binding domain of p53 was a key structural element controlling the protein function and provided a clue for finding a molecular mechanism that enables the rescue of the mutant p53 function.
of previously unreported missense mutations, and examined their ability to transactivate marker genes through distinct p53 binding sites when the mutants were expressed in yeast. We determined the functional effect of each mutant p53 and found that the p53 function correlated well with the structure and mutations (18).

Temperature-sensitive (ts) p53 mutations have been reported and used as tools for conditional p53 expression in mammalian cells. We identified previously four distinct ts p53 mutations in eight of the 91 human tumor cell lines using a yeast-based transactivation assay and predicted that 5–10% of the tumor-derived missense mutations should be ts mutations (19). To date, 61 ts p53 mutations have been isolated by using several different methods, including a yeast-based functional assay (Table I). Among these, the V272M ts mutant was reactivated by a small molecule, aminothiol WR1065 (20), at a non-permissive temperature, suggesting that ts mutants may be functionally rescued by small molecules.

The purpose of this study was the screening and isolation of a large number of ts mutations from a comprehensive missense mutation library, mapping them to the p53 structure, and considering the function-structure relationship through the ts mutants. To isolate a number of ts p53 mutations, we screened the p53 library containing 2,314 p53 missense mutations using a yeast-based p53 functional assay and found 142 ts p53 mutants, including previously unreported 131 mutants. We confirmed that most were also ts in p53-less mammalian cells. The ts mutants were preferentially mapped on one of the β-sheets, and there were hot spot sites for ts mutations. Because a fairly significant fraction of the p53 mutants in the TP53 database were ts mutants, these ts mutant proteins may be novel molecular targets through the ts mechanism and structure-dependent restoration of p53 function.

EXPERIMENTAL PROCEDURES

p53 Missense Mutation Library—2,314 p53 missense mutations were constructed recently through a 96-well, formatted, site-directed mutagenesis and stably expressed in a haploid yeast strain harboring a p53-responsive p21^WAF1 reporter plasmid (p5A500G) (21) or in diploid yeast strains harboring p53-responsive reporter plasmids with a MDM2 promoter or p53 binding sites derived from RAX (pKSS17R), 14-3-3ε (pKSS909), p53AI (pKSS11R), GADD45 (pKSS13R), Nco1 (pKSS15R), and p53R2 (pKSS17R) as described previously (18).

Screening ts p53 Mutants Using a Yeast Assay—The 2,314 yeast clones expressing the mutant p53 were grown on 25 96-well formatted plates containing synthetic complete (SC) media lacking leucine and tryptophane (SC – Leu – Trp) in the case of the haploid strains, or SC media lacking leucine, tryptophane and histidine (SC – Leu – Trp – His) in the case of the diploid strains.

Fluorescent Intensity—To evaluate the transactivty of each mutant p53 quantitatively, the yeast clones (haploid cells) were replicated on SC – Leu – Trp solid media using a 96-pin replicator and grown at 37 or 32 °C for 2 days. The plates were then directly processed in a 96-well formatted fluorometer (Fluosoraskn Ascent FL, Labsystems) to measure the fluorescent intensity (excitation, 485 nm; emission, 538 nm) of p53-dependent enhanced green fluorescence protein expression through a human p21^WAF1-derived p53 binding sequence. The diploid cells, selected by mating reaction, were incubated on SC – Leu – Trp – His plates at 37 or 30 °C for 2 days, and the fluorescent intensity of Ds-Red was measured using the same fluorometer (excitation, 544 nm; emission, 590 nm) to evaluate the p53-dependent Ds-Red expression through other p53-binding sequences. At least two independent experiments were performed for each reporter, and the fluorescence intensities were averaged. The averaged values were standardized in each p53 binding site and clustered, and visualized using the CLUSTER and TREEVIEW programs. The standardized data were also plotted on a two-dimensional graph for 30 and 37 °C. We defined the following criteria to select ts mutants from the p53 missense library, namely M_{30}/M_{37} ≥ 0.7, M_{30}/M_{37} ≤ 0.5, and M_{30}/M_{37} ≥ 2, where M_{30} and M_{37} indicate the fluorescent intensities of the p53 mutants at 30 and 37 °C, respectively, and M_{37} indicates the fluorescent intensities of the wild-type p53 at 30 and 37 °C, respectively.

Cell Culture and Transfection—A TP53-deficient human osteosarcoma cell line, Saos-2, was cultured in RPMI 1640 medium supplemented with 10% heat-inactivated (56 °C for 30 min) fetal calf serum (JRH Bioscience) in the presence of 5% CO₂. For luciferase assays, the cells were grown to 60–90% confluence in 96-well tissue culture plates at 37 °C and then cultured at 32 or 37 °C for another 24 h. For immunoprecipitation, the cells were grown in 90 × 20-mm tissue culture plates at 37 °C in the presence of 5% CO₂ and further incubated at 32 or 37 °C for another 18 h. Transient transfections were performed using the Effectene (Qiagen) transfection reagent. For luciferase assays, the cells were co-transfected with 12.5–50 ng of the expression vector (pCR259-p53WT, pCR259-p53MT, or a p53-less control pCR259 vector) (18) and 50–87.5 ng of the p53-responsive luciferase plasmid (p21P-luc, pMDMPs-luc, pB-AXPs-luc, pSIMGAP-luc, p53R2P-luc, or p53GADD45P-luc) (18, 21) and incubated for a further 24 h. For immunoprecipitation, the cells were transfected with 2 μg of the expression vector (pCR259-p53WT, pCR259-p53MT, or a control pCR259 vector) and further incubated for 36 h.

Luciferase Assay—After 24 h of transfection, luciferin (Steady-Glo luciferase assay system, Promega), a substrate of luciferase, was added to the culture media and further incubated for 60–120 min according to the manufacturer's instructions. The fluorescent intensity was measured using the Fluoroskan Ascent FL (Labsystems) with 485 nm excitation and 538 nm emission. The relative fluorescent intensity to the wild-type control was calculated from three sets of independent experimental data at 32 and 37 °C. The value differences at the two temperatures were statistically evaluated by t test. The ts mutants were defined when the p value was <0.001.

Immunoprecipitation and Immunoblotting of p53—Saos-2 cell lysates were prepared in 100 μl of NET buffer (150 mM NaCl, 50 mM Tris-HCl (pH 8.0), 5 mM EDTA, and 1% Nonidet P-40) containing 0.1 μg/ml phenylmethylsulfonfluoride. Fifty microliters of the cell lysates were immunoprecipitated with 10 μl of the PAb1620 (Ab-5; Oncogene) or the PAb240 (Ab-3; Oncogene) monoclonal antibody against human p53. The lysates, with 8 μl of the crude yeast lysate, were fractionated by SDS-polyacrylamide gel electrophoresis and transferred electrophoretically to an Optitran BA-S38 membranes (Schleicher & Schuell), and the expressed p53 mutants were detected using a HRP-conjugate anti-p53 antibody (p53FL395)HRP, Santa Cruz Biotechnology). The proteins were visualized and quantitatively analyzed using an ECL Western blotting detection system (Amersham Biosciences), a lumino-image analyzer (LAS1000, Fuji Film) and ID image analysis software (Kodak Digital Science).

Drawing p53 Peptide Structures—To map the ts p53 mutants on the p53 core domain, the NCBI structure file, 1TUP, was customized for our purpose and visualized using Cn3D 4.0 software (22).

RESULTS

Clustering of 2,314 Mutations on Transactivities at Two Distinct Temperatures—An unsupervised, hierarchical one-dimen- sional cluster analysis allowed us to cluster the 2,314 p53 mutants on the basis of similar measured transactivities for eight distinct p53 binding sequences (p53 binding sites) at 30 and 37 °C (Fig. 1A). The mutants are divided into two major clusters. In one of these clusters the mutants retain transactivities; in the other they lose activity, and these clusters are mostly temperature-independent. Notably, there is one temperature-dependent sub-cluster within the latter cluster (Fig. 1B). The cluster consists of 64 p53 mutants, and the transactivities of the mutants are inactive on almost all p53 binding sites at 37 °C but active on some p53 binding sites at 30 °C, indicating that a large number of mutants are ts for transactivation in yeast cells.

Isolation of ts p53 Mutants in Yeast—Although the cluster analysis found the typical ts mutants that represent temperature sensitivity for most p53 binding sites, there are mutants that show temperature sensitivity on limited types of p53 binding sites and, therefore, are not clustered. To also isolate such clones, the transactivities of the 2,314 mutant clones at 30 and 37 °C were standardized and overviewed by a scatter plot for each p53 binding site (Fig. 2). Among the 18,512 data points (8 × 2,314 clones), the majority had similar transcriptional
activity (either active or inactive) at both 30 and 37 °C, indicating that they were not ts. Obviously, there were significant numbers of p53 mutant clones that represented higher trans-activity at 30 °C than at 37 °C, showing ts mutants for the transactivation function (circled spots in Fig. 2). On the other hand, only a limited number of clones represented higher transactivity at 37 °C than at 30 °C, showing cold-sensitive mutants. As there is no clear boundary between ts and non-ts mutants, we defined the borders for convenience as described under “Experimental Procedures.” According to the definition, 142 p53 mutants were selected as ts for yeast transactivation assay (Fig. 3A), indicating that 6.1% (142 of 2,314) of the p53 mutants were ts for at least one of the p53 binding sites. The 142 mutants, including 131 previously unreported ts mutants, are summarized in Table I.

**Temperature-sensitive TP53 Mutation**

### Table I

| p53 mutant a | Experimental system b | p53BS or promoter c | Reference |
|--------------|-----------------------|---------------------|-----------|
| S99F***     | 1                     | BAX, CDKN1A, PIG3   | 27        |
| A119V        | 1                     | BAX, CDKN1A, PIG3   | 27        |
| Y126S        | 1                     | BAX, CDKN1A, PIG3   | 27        |
| Y126D        | 1                     | BAX, CDKN1A, PIG3   | 27        |
| K132N        | 3                     | CON                 | 28        |
| K132R        | 3                     | CON                 | 28        |
| M135T        | 1                     | RGC                 | 29        |
| V135* (mouse p53) | 2                  | CDKN1A   | 30, 31    |
| A138V***     | 2                     | BAX, BCL2, CDKN1A, MDM2 | 32        |
| T140Y        | 3                     | CON                 | 28        |
| V143A        | 1, 2                  | BAX, GADD45A, CCNG1, CDKN1A, CON, IGFBP3, MDM2, RGC | 33–35     |
| P152L***     | 1                     | BAX, CDKN1A, PIG3   | 27        |
| P152T**      | 1                     | BAX, CDKN1A, PIG3   | 27        |
| G154V**      | 2                     | GAL4                | 36        |
| T155I**      | 1                     | BAX, CDKN1A, PIG3   | 27        |
| M160A161T     | 1                     | BAX, CCNG1, CDKN1A, CON, GADD45A, IGFBP3, MDM2, RGC | 35        |
| I162F        | 1                     | BAX, CDKN1A, PIG3   | 27        |
| T170R        | 1                     | BAX, CDKN1A, PIG3   | 27        |
| V172F***     | 1                     | RGC                 | 29        |
| R175K*       | 2                     | BAX, FOS, IGFBP3    | 37        |
| R175*        | 2                     | BAX, FOS, IGFBP3    | 37        |
| R175P***     | 2                     | BAX, FOS, IGFBP3    | 37        |
| R175Q*       | 2                     | BAX, FOS, IGFBP3    | 37        |
| R175S***     | 2                     | BAX, FOS, IGFBP3    | 37        |
| R175M*       | 2                     | BAX, FOS, IGFBP3    | 37        |
| H179Q        | 2                     | GAL4                | 36        |
| E180K        | 1                     | BAX, CDKN1A, PIG3, RGC | 27, 38    |
| R181G        | 1                     | BAX, CDKN1A, PIG3   | 27        |
| R181H        | 1                     | BAX, CDKN1A, PIG3   | 27        |
| H193R***     | 1                     | BAX, CCNG1, CDKN1A, CON, GADD45A, IGFBP3, MDM2, RGC | 35        |
| V197L***     | 1, 2                  | BAX, CDKN1A, PIG3, RGC | 36–40     |
| Y205N***     | 1                     | BAX, CDKN1A, PIG3   | 27        |
| H214R***     | 1                     | RGC                 | 19        |
| P219I*       | 1                     | BAX, CDKN1A, PIG3, RGC | 27, 38    |
| Y220C        | 1                     | BAX, CCNG1, CDKN1A, CON, GADD45A, IGFBP3, MDM2, RGC | 35        |
| Y220H        | 1                     | BAX, CDKN1A, PIG3   | 27        |
| E224K        | 1                     | BAX, CDKN1A, PIG3   | 27        |
| D228V        | 1                     | BAX, CDKN1A, PIG3, RGC | 27, 38    |
| Y234C**      | 1                     | RGC                 | 19        |
| Y234H***     | 1                     | BAX, CDKN1A, PIG3   | 27        |
| M237R**      | 2, 3                  | CON, FOS, RGC       | 28        |
| N239S        | 3                     | CON                 | 28        |
| M246V**      | 3                     | CON                 | 28        |
| N247T***     | 2, 3                  | CON, GAL4           | 28, 36    |
| R248W        | 3                     | CON                 | 28        |
| P250L**      | 1                     | BAX, CDKN1A, PIG3, RGC | 27, 38    |
| L252F***     | 1                     | BAX, CDKN1A, PIG3   | 27        |
| L254F**      | 1                     | BAX, CCNG1, CDKN1A, CON, GADD45A, IGFBP3, MDM2, RGC | 35        |
| T256A**      | 3                     | CON                 | 28        |
| D259N        | 1                     | BAX, CDKN1A, PIG3, RGC | 27, 38    |
| G266E        | 1                     | BAX, CDKN1A, PIG3   | 27        |
| V272M***     | 1–3                   | BAX, CDKN1A, CON, GADD45A, MDM2, PIG3, RGC | 19, 28, 38, 41 |
| R273H        | 3                     | CON                 | 28        |
| R273L        | 1, 3                  | BAX, CDKN1A, CON, PIG3 | 27, 28    |
| A276G        | 1                     | BAX, CDKN1A, PIG3   | 27        |
| D281Y        | 3                     | CON                 | 28        |
| R283H***     | 1                     | BAX, CDKN1A, PIG3, RGC | 38        |
| E285K**      | 1                     | RGC                 | 19        |
| E286K***     | 1                     | BAX, CDKN1A, PIG3   | 27        |
| 286K/287D*    | 1                     | BAX, CDKN1A, PIG3   | 27        |

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a The meaning of the asterisk symbols used in this column is as follows: *, ts mutants not constructed in this study; **, ts mutants also isolated in this study; ***, distinct substitution(s) at the same residue were ts mutants in this study.

b The meaning of the numbers used in this column is as follows: 1, yeast system; 2, mammalian cell system; 3, cell-free system.

c All but three of the gene names used in this column refer to those used in the Online Mendelian Inheritance in Man (OMIM) site (www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=OMIM). The three exceptions are: CON, p53-binding consensus sequence; GAL4, yeast GAL4-binding sequence; and RGC, human ribosomal gene cluster sequence. The study on the GAL4 was performed by GAL4-binding domain and p53 fusion protein.
were mapped on 82 residues of p53 (82 of 393; 20.9%) and 131 (92%) were within the core DNA-binding domain (residues 97–286).

Mapping of ts p53 Mutants on p53 Core DNA-binding Domain—To characterize the isolated ts mutants in the structural context, we mapped the 131 mutants on the core DNA-binding domain (Fig. 4, A and B). Among these, 70 mutants (53.4%) were mapped on the β-strands. The frequency of ts mutants in the constructed mutants differed among the 10 β-strands; the frequency was relatively higher in S4 (14 of 49, 28.6%), S7 (11 of 34, 32.3%), S9 (16 of 49, 32.7%), and S10 (15 of 61, 24.6%), whereas it was lower in S1 (1 of 17, 5.9%), S2 (1 of 24, 4.2%), S3 (1 of 33, 3.0%), S5 (2 of 22, 9.1%), S6 (2 of 24, 8.3%), and S8 (7 of 43, 16.3%). In particular, residues 158 to 161, 211 to 217, 251 to 256, and 268 to 272 were hot areas for ts mutants because there were 10 or more mutants in every four contiguous residues. Three or more ts mutants were observed in residues Thr158, Arg159, Val172, His214, Ser215, Pro223, Thr231, Thr253, Ile254, Thr256, Ser269, Glu271, and Glu285, and those residues should be designated ts hot spots.

Amino Acid Substitutions of the ts Mutants—Amino acid residues before and after substitution of the ts mutant are summarized in Table II. Isoleucine (21.4%), threonine (21.1%), and tryptophane (20%) were the most frequent residues among the original p53 residues. Glycine (20%), isoleucine (18%), alanine (16.6%), leucine (16.2%), and proline (13.2%) were the most frequent residues among the residues after substitution.
whereas aspartic acid (3.5%) and glutamic acid (0%) were found less frequently.

**Evaluation of the ts p53 Mutants in Mammalian Cells**—To evaluate whether the isolated p53 mutants in yeast were also ts for sequence-specific transactivation in mammalian cells, we randomly chose 54 p53 mutants from the 142 ts mutant p53 cDNA clones (Fig. 3B), and constructed expression vectors for mammalian cell experiments. Each mutant p53 was expressed in a p53-deficient human osteosarcoma cell line, Saos-2, and examined for the sequence-specific transactivation at both 32 and 37 °C by luciferase assay. When the values of the three independent experiments relative to the wild-type p53 at 32 °C were significantly (p < 0.001; t test) different from those at 37 °C in at least one of the six promoters (p21WAF1, MDM2, BAX, 14-3-3σ, p53R2, and GADD45), the mutant clone was defined as a ts mutant in mammalian cells. Among the 54 mutants, 48 (89%) were ts mutants in at least one of the six promoters. The results indicated that most ts mutants isolated in the yeast assay are also ts mutants in mammalian cells, suggesting that many of the remaining 88 clones may also be ts mutants in mammalian cells. Among the 48 clones, 16 were ts in all 6 promoters, whereas 32 clones were ts in a limited number of promoters, although many retained weak ts phenotypes for other promoters (data not shown).

**Epitope Analyses of the p53 Protein Expressed in Saos-2 Cells Using Conformation-sensitive Antibodies**—To examine whether the ts mutants display ts changes in their epitopes against conformation-sensitive antibodies, PAb1620 for wild-type-like conformation and PAb240 for denatured mutant conformation, six randomly selected ts mutants, M160R, H193Y, T211A, P219S, T253I, and V274A, were expressed in Saos-2 cells at both 32 and 37 °C. The cell lysates were immunoprecipitated using the two antibodies, detected by Western blot analysis using an HRP-conjugated anti-p53 antibody, and quantitatively analyzed using a lumino-image analyzer. In the case of wild-type p53, the PAb1620 epitope was exclusive, and only a trace of the PAb240 epitope was detected (Fig. 5A). Similar to wild-type p53, the PAb1620 epitope was dominant in R273H, although the PAb240 epitope was also detected. On the other hand, the PAb240 epitope was dominant, and the PAb1620 epitope was less abundant in R175H. The ratios of the epitope expressions of PAb1620 to PAb240 are shown in Fig. 5B. R175H and R273H were not ts because there were no significant differences in the ratios between 32 and 37 °C. Among the ts mutants, P219S and T253I showed an obvious ts increase in ratio. The remaining ts mutants showed no change or only a slight change in ratio.

**DISCUSSION**

**Comparing 142 ts p53 Mutants with the Previously Reported p53 Mutant**—Among the 142 ts mutants, 131 were previously unreported mutants. In our survey of previous papers, including our own, 61 human ts p53 mutants have been reported (Table I). These obviously include ts mutants not isolated in our system. We speculate that there are two reasons for the discrepancy. First, they were isolated using experimental systems different from those in our study, including a reporter assay for sequence-specific transactivation in mammalian cells, similar yeast assays with different p53 binding sites, an electrophoretic mobility shift assay (EMSA) in a cell-free system, and monitoring changes in structure-sensitive antibody reactivity. Therefore, it is possible that there are many potential ts mutants not isolated by the method adopted in this study. For example, a known ts mutant, V143A, did not appear as ts in the yeast cells because the ts phenotype may be mediated by ts interaction with human ASPP2 (p53BP2), a positive modulator of p53 transactivation (23, 24) that does not exist in yeast cells. Obviously, there may be mechanisms not directly affecting p53 binding to DNA. We are now planning to screen such novel ts mutants by using protein-protein interactions that may modify
p53 structure by their post-translational mechanisms. Second, as shown in Fig. 2, there is distinct strength in ts transactivation, and some reported ts mutants have been eliminated from our criteria because of a weak ts phenotype. In fact, several mutants clustered in Fig. 1 were not selected in our defined criteria. We also note that many previously identified ts mutants had a weak ts phenotype in our yeast screening (data not shown).

Promoter Specificity of the ts p53 Mutants—We have shown that several p53 mutants differ in transactivity spectrum in different p53 binding sites (18). Similarly, ts mutants differed in the ts transactivity spectra in different p53 binding sites (Fig. 3). We speculate that there are subtle differences in structural alterations caused by specific mutations and temperatures and that such alterations are responsible for the partial inactivation or reactivation of p53-binding to the distinct DNA sequences. In fact, there are similarities in the transactivity spectra among mutants in the same or contiguous residues (Fig. 3A), suggesting similar structural alterations. In particular, some showed ts in only one or two promoters, suggesting the possible application of such mutants in the conditional transactivation of specific promoters to study p53 downstream gene functions. Various ts transactivity spectra on different p53-responsive promoters were also observed in mammalian cells (Fig. 3B). The promoter selectivity of wild-type p53 by Ser46 phosphorylation has been shown as the mechanism of p53AIP1 transactivation (25). Overall, from the results of this study and our previous observations (18), we propose that there may be other unknown potential mechanisms determining the promoter selectivity of wild-type p53 on p53 downstream promoters other than the p53AIP1 gene. The ts transactivity against different promoters was similar in part but significantly different between human and yeast cells (data not shown). We speculate that there are several reasons for this discrepancy. First, the p53 binding elements, other than p21WAF1 and MDM2 used in the yeast study, were three copies of the specific p53-binding elements and differed from the genomic sequences used in the mammalian cell study. Second, the temperature for the identification of ts mutants in yeast was 30 °C, whereas it was 32 °C in mammalian cells. Third, post-translational modification and the interaction of other proteins may differ in yeast cells and mammalian cells. Finally,
the criteria to define ts mutants were strict and differed between yeast and mammalian cell systems. Therefore, there may be ts mutants defined by the yeast system but not by the mammalian cell system and vice versa, in addition to those showing ts phenotypes in both systems.

**Ts Mutants and the Structure of the p53 Protein—** We isolated 142 (6.1%) ts mutants from 2,314 p53 missense mutations. Most were mapped in the core DNA-binding domain (131, 91.5%), and a few were in the NH$_2$-terminal (4, 3.5%) or COOH-terminal (7, 4.9%) domains. The results indicated that the ts mutants isolated in this study may be mutants that directly affect sequence-specific DNA binding rather than mutants affecting the p53 function through post-translational modifications and protein-protein interactions. Within the DNA-binding domain, 50% (71 of 142) of the ts mutants were mapped on $\beta$-strands (S1–S10). As only 16% of the residues in the DNA-binding domain form $\beta$-strands, it is clear that the $\beta$-strands are more susceptible to ts isomerization.

**TABLE II**

Frequency of residue of ts mutant p53 in the DNA-binding domain before and after substitution

| Residue | No. of constructed mutations | No. of ts mutants | Frequency of ts mutant |
|---------|------------------------------|-------------------|-----------------------|
| Ile     | 42                           | 9                 | 21.43                 |
| Thr     | 76                           | 16                | 21.05                 |
| Trp     | 5                            | 1                 | 20.00                 |
| Met     | 36                           | 7                 | 19.44                 |
| Glu     | 66                           | 12                | 18.18                 |
| Ala     | 42                           | 7                 | 16.67                 |
| His     | 49                           | 8                 | 16.33                 |
| Arg     | 99                           | 14                | 14.14                 |
| Pro     | 78                           | 11                | 14.10                 |
| Phe     | 30                           | 4                 | 13.33                 |
| Val     | 76                           | 9                 | 11.84                 |
| Ser     | 86                           | 9                 | 10.47                 |
| Asn     | 63                           | 6                 | 9.52                  |
| Leu     | 75                           | 6                 | 8.00                  |
| Gln     | 36                           | 2                 | 5.56                  |
| Tyr     | 48                           | 2                 | 4.17                  |
| Gly     | 78                           | 3                 | 3.85                  |
| Asf     | 56                           | 2                 | 3.57                  |
| Cys     | 60                           | 2                 | 3.33                  |
| Lys     | 36                           | 0                 | 0                     |

| Residue | No. of constructed mutations | No. of ts mutants | Frequency of ts mutant |
|---------|------------------------------|-------------------|-----------------------|
| Gly     | 75                           | 15                | 20                    |
| Ile     | 50                           | 9                 | 18                    |
| Ala     | 84                           | 14                | 16.67                 |
| Leu     | 74                           | 12                | 16.22                 |
| Pro     | 68                           | 9                 | 13.24                 |
| Asn     | 49                           | 6                 | 12.24                 |
| Met     | 33                           | 4                 | 12.12                 |
| Val     | 99                           | 12                | 12.12                 |
| Thr     | 67                           | 8                 | 11.94                 |
| Tyr     | 47                           | 5                 | 10.64                 |
| Arg     | 88                           | 9                 | 10.47                 |
| Phe     | 43                           | 4                 | 9.30                  |
| His     | 55                           | 5                 | 9.09                  |
| Ser     | 98                           | 7                 | 7.14                  |
| Lys     | 42                           | 3                 | 7.14                  |
| Gln     | 38                           | 2                 | 5.26                  |
| Tyr     | 21                           | 1                 | 4.76                  |
| Cys     | 43                           | 2                 | 4.65                  |
| Asp     | 56                           | 2                 | 3.57                  |
| Glu     | 35                           | 0                 | 0.0                   |

**FIG. 5.** Expression of conformation-sensitive epitopes. A, the indicated p53 proteins were expressed from the corresponding p53 expression vectors in the human osteosarcoma cell line, Saos-2, at 32°C and 37°C. The crude cell lysate was separated by SDS-polyacrylamide gel electrophoresis immediately or after immunoprecipitation (IP) using PAb240 or PAb1620. The p53 proteins were detected using an HRP-conjugated anti-p53 polyclonal antibody. WT, wild-type. B, the ratio of PAb240 expression to PAb240 expression at 32and 37°C. The expression of either PAb1620- or PAb240-reactive p53 proteins (shown in panel A) was quantitatively analyzed, and the ratio of the values are shown as graphs. n.c., not calculated.

Thr$^{253}$, Ile$^{254}$, Thr$^{256}$, Ser$^{269}$, Glu$^{271}$, and Glu$^{285}$ are ts hot spots (Fig. 4A). The ts hot spot mutants are spatially located relatively far from the p53-DNA interface, whereas the tumor-derived 10 hot spot mutations (except Thr$^{229}$) are formed or located close to the interface (Fig. 4C). Although the structural reason why the second $\beta$-sheet was more susceptible to ts is still unclear, this observation indicated that the $\beta$-sheet was a key structural element controlling the p53 function, suggesting the existence of a potential intramolecular mechanism in normal p53 regulation of the promoter selectivity after post-translational modification such as damage-sensitive phosphorylation.

The consideration of ts-specific amino acid residues before and after substitution is of great interest, because such information may provide a better understanding of the structure of ts mutants. In p53, the majority of the sensitive residues before substitution comprised isoleucine, threonine, and tryptophane, all of which are hydrophobic residues preferentially used in the $\beta$-strands of many other proteins (26). On the other hand, the most frequent residues after substitution were glycine, isoleucine, alanine, leucine, and proline. Because these residues are smaller hydrophobic residues, it is possible that the structure of the $\beta$-strands is largely undisrupted. Less frequent residues were negatively charged, i.e. aspartic acid and glutamic acid. This result was reasonable, because these are known to be $\beta$-strand-disrupting residues (26). An unexpected result was frequent proline substitution, because proline is known to be a structurally stable residue and, therefore, should not be vulnerable to subtle structural change due to temperature shift.

The results of immunoprecipitation using conformation-sensitive antibodies were unexpected, because only a limited num-
ber of the mutations examined showed ts changes in the expression of epitopes. We speculate that most ts mutants partially recovered their structural alteration, but their structure and transactivation function were not completely restored. It will be interesting to examine whether such partial restoration of p53 function is sufficient to suppress tumor formation and/or progression when expressed under physiological conditions.

Frequency of ts Mutants in TP53 Mutation Databases—According to the latest International Agency for Research on Cancer (IARC) data base for tumor-derived somatic mutations (17), 1,135 distinct missense mutations, including 1,066 missense mutations with a single nucleotide substitution, are registered. These mutations have been reported 12,032 times in total. Among them, 10.3% (110 of 1,066) of mutants were thought to be ts mutants, and such ts mutations comprised 10.4% (1,254 of 12,032) of the total number of mutations. Therefore, we conclude that ts p53 mutation is not as rare as it was previously thought to be (19), and it may be a molecular target for the pharmacological rescue of p53 protein.

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