Structures of p53 Cancer Mutants and Mechanism of Rescue by Second-site Suppressor Mutations*

Received for publication, January 6, 2005
Published, JBC Papers in Press, February 11, 2005, DOI 10.1074/jbc.M500179200

Andreas C. Joerger, Hwee Ching Ang‡, Dmitry B. Veprintsev, Caroline M. Blair, and Alan R. Fersht§

From the Centre for Protein Engineering, Medical Research Council, Cambridge, CB2 2QH, United Kingdom

We have solved the crystal structures of three oncogenic mutants of the core domain of the human tumor suppressor p53. The mutations were introduced into a stabilized variant. The cancer hot spot mutation R273H simply removes an arginine involved in DNA binding without causing structural distortions in neighboring residues. In contrast, the “structural” oncogenic mutations H168R and R249S induce substantial structural perturbation around the mutation site in the L2 and L3 loops, respectively. H168R is a specific intragenic suppressor mutation for R249S. When both cancer mutations are combined in the same molecule, Arg140 mimics the role of Arg249 in wild type, and the wild type conformation is largely restored in both loops. Our structural and biophysical data provide compelling evidence for the mechanism of rescue of mutant p53 by intragenic suppressor mutations and reveal features by which proteins can adapt to deleterious mutations.

The tumor suppressor p53 is a tetrameric multidomain transcription factor that has an essential role in maintaining the genomic integrity of the cell. In response to DNA damage, UV irradiation, hypoxia, and other stresses, p53 activates the expression of a variety of genes that encode proteins responsible for G1 and G2 cell cycle arrest and apoptosis (1–3). p53 is inactivated in virtually all human cancers. In about 50% of all human cancers, this inactivation is a direct result of mutations inactivated in virtually all human cancers. The core domain constitutes the DNA binding core domain of the protein (p53C).1 Six hot spot sites are most frequently mutated in human cancers (Arg175, Gly245, Arg248, Arg249, Arg273, and Arg282) (4).

The crystal structure of the human p53 core domain in complex with consensus DNA has provided a framework for understanding the deleterious effect of many cancer mutants (5). A large immunoglobulin-like β-sandwich, forming a compact barrel-like structure, provides the basic scaffold for the conserved DNA binding surface (Fig. 1A). This extended surface, rich in basic amino acids, consists of a loop-sheet-helix motif and two large loops (L2 and L3). These two loops are stabilized by a zinc ion, which is coordinated by three cysteines and a histidine (Cys176, His179, Cys238, and Cys242) (Fig. 1B). On the basis of this structure, common cancer mutations have been classified as “structural” mutations resulting in structural perturbations of the core domain or “contact” mutations resulting in the loss of a DNA-contacting residue.

A more detailed picture has emerged from quantitative stability and DNA binding studies for wild type p53 and several cancer-associated mutants (6, 7). The core domain of human p53 has low intrinsic thermodynamic stability, and its melting temperature is only slightly above body temperature. Hence, even weakly destabilizing mutations can have a detrimental effect and result in a large population of unfolded protein under physiological conditions. On the basis of these studies, cancer-associated mutants in the core domain can be subdivided into three classes: (i) DNA contact mutations, such as R273H, with little effect on the overall stability of the protein but impaired function because of the loss of a residue mediating DNA contacts. (ii) Mutations causing local distortions that destabilize the core domain by <2 kcal/mol. Many mutations in the DNA binding surface such as the hot spot mutations G245S and R249S fall into this category. (iii) Mutations that are highly destabilizing (>3 kcal/mol) and result in global unfolding of the protein at body temperature.

The deleterious effect of some cancer mutants can be reversed by intragenic second-site suppressor mutations. Using a yeast selection system in combination with mammalian reporter gene and apoptosis assays, Brachmann et al. (8) have identified several such second-site suppressor mutations. Some of these second-site suppressor mutations are not specific for one cancer mutation, but restore activity in a whole subset of cancer mutants albeit at different levels. Remarkably, H166R, which in conjunction with T123A is reported to rescue the cancer hot spot mutant R249S, is oncogenic when on its own. In a more recent study, it was shown that 16 of 30 common cancer mutants are rescued by mutations in codons 235, 239, and 240, either alone or in combination (9). Double-mutant cycles have shown that some of these second-site suppressor mutations rescue mutant p53 by increasing its thermodynamic stability and thus compensate for the stability loss induced by the oncogenic mutation (10).

By adopting a semirational approach based on the molecular
Structures and Rescue of p53 Cancer Mutants

Evolution of p53 and Known Second-Site Suppressor Mutations
Nikolova et al. (11) designed a superstable quadruple mutant (M133L/V203A/N239Y/N268D) of human p53 core domain (T-p53C). The four mutations stabilize the core domain by 2.65 kcal/mol without impairing its DNA binding activity. The effects of the four point mutations on stability are nearly additive. The main contributions come from the N239Y and N268D mutations, which are both known to reduce activity in several cancer-associated mutants (8). The crystal structure of T-p53C in its DNA-free form reveals that the four point mutations cause only local structural changes, whereas the overall structure of the β-sandwich and the DNA binding surface is conserved (12). The observed local structural changes caused by the mutations, however, seem to affect the flexibility of the protein and reduce the plasticity of the core domain structure both locally and globally. The N268D mutation, for instance, resulted in an altered hydrogen bond pattern, which links the two sheets of the β-sandwich in an energetically more favorable way than in the wild type structure. This provided a first insight into how the two second-site suppressor mutations might rescue some of the common structurally destabilized cancer mutants such as G245S and the temperature-sensitive Arg249S, and Arg282, which are most frequently mutated in human cancers (colored in orange). The four mutation sites in the superstable quadruple mutant M133L/V203A/N239Y/N268D (T-p53C) are highlighted as green spheres. B, close-up view of loops L2 and L3 in the DNA binding surface including the zinc coordination sphere in the structure of wild type in complex with consensus DNA (PDB code 1TSR, molecule B). The orientation is different from the one shown for the whole molecule in A. The zinc ion is depicted as a green sphere. Specific interactions mediated via the guanidinium group of Arg248 are highlighted with dotted lines. These include hydrogen bonds with backbone oxygens of residues Gly245 and Met246 on the same loop and a salt bridge with Glu171 on the L2 loop. DNA contacts are made via Arg248. Selected DNA residues in the proximity of Arg248 are shown in magenta and cyan.

Experimental Procedures
Mutagenesis, Protein Expression, and Purification—The DNA coding for human p53 core domain mutant M133L/V203A/N239Y/N268D (residues 94–312) in vector pRSET(A) (11) was subcloned into the polylinker region of vector pET-24a (+) (Novagen) using the Ndel and EcoRI restriction sites. Additional point mutations were introduced using the QuikChange Site-directed Mutagenesis kit (Stratagene). All mutants were expressed in Escherichia coli BL21 and purified following published protocols (11, 13). Protein samples were flash frozen and stored in liquid nitrogen for further use.

Urea Denaturation—Samples for urea denaturation experiments were prepared using a Hamilton Microlab dispenser from stock solutions of urea, buffer, and protein to contain 1 µM protein in 25 mM sodium phosphate buffer, pH 7.2, 150 mM KCl, 5 mM DTT, and increasing concentrations of urea. Samples were incubated at 10 °C for 14 h prior to measurement. The intrinsic fluorescence spectra of the p53 core domain, excited at 280 nm, were recorded in the range of 300–400 nm on a PerkinElmer Life Sciences LS50B spectrophurometer equipped with Waters 2700 sample manager and controlled by laboratory software. The data were analyzed as described previously (6).

Determination of DNA Binding Affinities by Analytical Ultracentrifugation—DNA binding studies by analytical ultracentrifugation (AUC) were performed with fluorescein-labeled DNA containing the gadd45 recognition element. The oligonucleotides used were 5′-fluorescein-GAGCAGACATGTCTAAGCATGCTGGGCTC-3′ and an unlabeled oligonucleotide with the complementary sequence. Annealing and purification of double-stranded DNA were carried out as described previously (14). All AUC experiments were performed at 10 °C using a Beckman Optima XL-I centrifuge and a 60Ti rotor. Samples of 5 µM gadd45 DNA and 100 µM p53 core domain variant were made up in 25 mM sodium phosphate buffer, pH 7.2, 150 mM KCl, and 5 mM DTT. To measure the stoichiometry and dissociation constant for protein binding to fluorescein-labeled DNA, absorbance at 495 nm and interference data were recorded, and data were analyzed using UltraSpin software (www.mrc-cpe.cam.ac.uk). The size of the complex, the concentrations of free protein, free DNA, and complex formed at the reference position were calculated from the absorbance and interference data. The dissociation constant was calculated as shown in Equation 1.
$K_p = \frac{[\text{free DNA/complex}]}{[\text{free protein}]}$ (Eq. 1)

**Crystalization and Data Collection**—Crystals were grown at 17 °C using the sitting drop vapor diffusion technique. The drops typically contained 3 μl of protein solution (5.8–7.7 mg/ml protein in 150 mM NaCl, 10 mM DTT, 20 mM Tris, pH 7.6, or 150 mM KCl, 10 mM DTT, 25 mM sodium phosphate, pH 7.2) and 3 μl of the appropriate reservoir solution above a reservoir solution of 400 μl. The crystals used for data collection were obtained with the following reservoir conditions: T-p53C-R273H: 50 mM MgSO$_4$, 19% (w/v) polyethylene glycol (PEG) 3350, 20 mM DTT, T-p53C-R249S: 100 mM Tris, pH 7.5, 25% (w/v) PEG 2000 monomethyl ether (MME), 0.8 m sodium formate, 10 mM DTT; T-p53C-H168R: 100 mM Tris, pH 7.5, 26% (w/v) PEG 2000 MME, 0.3 m sodium formate, 10 mM DTT; T-p53C-H168R/R249S and T-p53C-T123A/H168R/R249S: 33 mM Tris, pH 7.5, 67 mM Mes, pH 6.4, 26% (w/v) PEG 2000 MME, 0.8 m sodium formate, and 10 mM DTT. Crystals of T-p53C-R273H were isomorphous to those obtained for the previously published structure of T-p53C (12). The remaining mutants crystallized in the hexagonal space group P6$_3$22 with a rather long c-axis of 10 m (w/v) PEG 2000 MME, 0.8M sodium formate, pH 7.2) and 3 μl of the Ramachandran plot and that there were no residues in forbidden regions. Using PROCHECK (19) was used to check the stereochemistry of the final models and revealed that for all structures more than 90% of the non-glycine residues were in the most favored regions of the Ramachandran plot and that there were no residues in forbidden regions. Model refinement statistics are summarized in Table III. The figures were generated using MOLSCRIPT (20), BOBSCRIPT (21), and RASTER3D (22).

**RESULTS**

**Effects of Mutations on the Stability of T-p53C—T-p53C was chosen as a framework for studying the structural effects of cancer hot spot mutations by x-ray crystallography because its higher thermodynamic stability should make it more amenable for crystallographic studies, in particular in the case of highly destabilizing mutations.**

The effect of common cancer mutations on the thermodynamic stability of wild type p53 has been well studied by urea denaturation (6, 7, 10). We have performed the same experiments for some of these cancer mutants in the context of the superstable quadruple mutant T-p53C, which is stabilized by 2.65 kcal/mol, to assess whether they have any effect on wild type p53. Our results show that this is exactly the case independent of the type of cancer mutation (Table I). The mutation R273H only marginally affects the stability of both wild type and T-p53C, whereas the R249S cancer mutation in the L3 loop destabilizes the protein by almost 2 kcal/mol in both cases. A much larger effect is observed for mutation H168R, which destabilizes the core domain by 3 kcal/mol. If the two mutations did not interact, then the double mutant would be destabilized by the sum of their individual values (10, 23). The destabilizing effects of the H168R and R249S mutations, however, are not additive, and their combined value is 1.88 kcal/mol, considerably less than the 5 kcal/mol expected for structurally independent mutations. The lack of additivity shows that there are interacting structural changes upon mutation (10, 23).

**DNA Binding Properties of T-p53C Mutants**—We analyzed the binding of p53 core domain to specific double-stranded DNA containing the gadd45 recognition element by AUC. For wild type, we measured a dissociation constant $K_D = 14$ μM in 25 mM sodium phosphate buffer, pH 7.2, 150 mM KCl, and 5 mM DTT. Measurements were performed with 5 μM gadd45 fluorescein-labeled DNA and 100 μM p53 core domain. Data were analyzed as described under “Experimental Procedures.”

**Table I**

| Mutation        | $\Delta G_{U}^{\text{H}_2\text{O}}$ | kcal/mol | $\Delta G_{U}^{\text{H}_2\text{O}}$ | kcal/mol |
|-----------------|-----------------------------------|---------|-----------------------------------|---------|
| T-p53C          |                                   |         | Wild type (p53C)                  |         |
| H168R           | 3.07 ± 0.12                       | 2.75 ± 0.21 |
| R249S           | 1.98 ± 0.12                       | 1.95 ± 0.05 |
| R273H           | 0.09 ± 0.02                       | 0.34 ± 0.04 |
| H168R/R249S     | 1.88 ± 0.08                       | 1.72 ± 0.21 |
| T123A/H168R/R249S | 2.18 ± 0.09                    | 1.91 ± 0.21 |

$\Delta G_{U}^{\text{H}_2\text{O}}$ represents the change in the free energy of urea-induced unfolding caused by mutations in either T-p53C or wild type (wt; p53C) and is defined as $\Delta G_{U}^{\text{H}_2\text{O}} = \Delta G_{U}^{\text{H}_2\text{O}-\text{wt}} - \Delta G_{U}^{\text{H}_2\text{O}-\text{p53C}}$ and $\Delta G_{U}^{\text{H}_2\text{O}-\text{p53C}} = \Delta G_{U}^{\text{H}_2\text{O}-\text{wt}} - \Delta G_{U}^{\text{H}_2\text{O}-\text{mut}}$, respectively. Data were collected at 10 °C in 25 mM sodium phosphate, pH 7.2, 150 mM KCl, 5 mM DTT.

$^a$ Data for mutations in the wild type context are taken from Refs. 6 and 10.

**Table II**

| Binding of p53 core domain mutants to gadd45 DNA |
|-----------------------------------------------|
| p53 variant | $K_p$ \( \mu \text{M} \) |
| p53C         | 14.0 ± 0.9 |
| p53C-R249S   | 210 ± 30 |
| T-p53C-H168R | 10 ± 3 |
| T-p53C-R249S | 27 ± 2 |
| T-p53C-R273H | 56 ± 3 |
| T-p53C-H168R/R249S | 51 ± 4 |
| T-p53C-T123A/H168R/R249S | 13.4 ± 0.7 |
| T-p53C-T123A/H168R/R249S | 13 ± 1 |

$^a$ $K_p$ values were determined by AUC at 10 °C in 25 mM sodium phosphate buffer, pH 7.2, 150 mM KCl, and 5 mM DTT. Measurements were performed with 5 μM gadd45 fluorescein-labeled DNA and 100 μM p53 core domain. Data were analyzed as described under “Experimental Procedures.”

**DNA Binding Properties of T-p53C Mutants**—We analyzed the binding of p53 core domain to specific double-stranded DNA containing the gadd45 recognition element by AUC. For wild type, we measured a dissociation constant $K_D = 14$ μM in 25 mM sodium phosphate buffer, pH 7.2, and 150 mM KCl (Table II). The $K_D$ for T-p53C under these conditions was slightly lower ($K_D = 10$ μM). The DNA contact mutant T-p53C-R273H had a much reduced but still appreciable affinity, with a dissociation constant $K_D = 51$ μM. The affinity for T-p53C-H168R ($K_D = 27$ μM) was between that of wild type and the contact mutant T-p53C-R273H. Most interestingly, DNA binding is gradually restored along the series p53C-R249S → T-p53C-R249S → T-p53C-H168R/R249S and T-p53C-T123A/H168R/R249S. The $K_D$ for gadd45 binding is decreased along this series from ~200 μM → 56 μM → 15 μM, which brings it back to the wild type level, despite the presence of the cancer hot spot mutation R249S.

**Crystallographic Studies**—We solved high resolution crystal structures of the cancer mutants H168R, R249S, R273H, and the combined mutants H168R/R249S and T123A/H168R/R249S in the structural context of the superstable quadruple mutant T-p53C (Table III). The resolution for all five structures was in the range of 1.80–1.98 Å. Crystals of mutant T-p53C-R273H were isomorphous to those of the previously published structure of the superstable quadruple mutant T-p53C and contained two molecules/asymmetric unit. The structures of the four remaining mutants were all solved in a new crystal form belonging to the hexagonal space group P6$_3$22 with one molecule in the asymmetric unit. In all structures, the
same positions as the equivalent atoms of Arg273 in the wild
distance of the aromatic ring of Phe134 and occupy almost the
linked to a water molecule and the hydroxyl of Ser240. A second
the carboxylate group of Asp281 on the C-terminal helix, and
phate backbone of the DNA. Further, it forms a salt bridge with
C-terminal residues beyond 291 were disordered, which is
consistent with previously reported structures of p53 core
domain (5, 12).

**Mutant T-p53C-R273H**—The cancer hot spot mutation R273H is the classic example of a DNA contact mutant. The mutation significantly impairs DNA binding but has no noteworthy effect on the thermodynamic stability of the core domain (7). In the structure of p53 wild type in complex with consensus DNA (5), the positively charged guanidinium group of Arg273 is in close contact with the negatively charged phosphate backbone of the DNA. Further, it forms a salt bridge with the carboxy terminal group at a similar resolution, are almost identical. A superimposition of the Cα atoms gives a r.m.s. deviation of 0.13 Å (molecule A) and 0.16 Å (molecule B) for individual chains and a

**TABLE III**

|                  | T-p53C* | T-p53C- R273H | T-p53C- H168R | T-p53C- R249S | T-p53C- H168R/R249S | T-p53C- T123A/H168R/R249S |
|------------------|--------|---------------|---------------|---------------|---------------------|--------------------------|
| Data collection  |        |               |               |               |                     |                          |
| Space group      | P2₁,2,2₁ | P2₁,2,2₁ | P6₂,2₂ | P6₂,2₂ | P6₂,2₂ | P6₂,2₂ | P6₂,2₂ |
| a                | 64.5   | 64.5          | 45.05         | 45.05         | 45.40              | 45.48                    |
| b                | 71.0   | 71.0          | 45.05         | 44.58         | 45.40              | 45.48                    |
| c                | 104.8  | 104.7         | 331.8         | 331.1         | 331.4              | 331.1                    |
| Monomers/ASU     | 2      | 2             | 1             | 1             | 1                   | 1                         |
| Resolution (Å)   | 35.6–1.90 | 15.6–1.98 | 55.3–1.90 | 26.6–1.90 | 39.3–1.80 | 39.4–1.80 |
| Unique reflections | 38,078 | 33,826        | 16,936        | 16,731        | 19,672              | 20,201                   |
| Completeness (%) | 98.7 (96.5) | 98.8 (93.8) | 98.6 (94.4) | 99.5 (98.9) | 98.4 (97.2) | 99.6 (97.9) |
| Multiplicity     | 6.1 (5.6) | 5.1 (4.7) | 10.2 (9.1) | 7.4 (7.6) | 7.0 (3.0) | 9.3 (5.7) |
| R<sub>sym</sub> (%) | 8.3 (29.1) | 6.6 (20.6) | 6.9 (19.7) | 9.0 (21.5) | 6.6 (18.1) | 8.7 (25.0) |
| Wilson B-factor (Å²) | 16.4 (5.5) | 15.9 (7.3) | 25.5 (8.6) | 16.5 (6.5) | 21.4 (3.9) | 22.1 (4.3) |
| Reﬁnement       |        |               |               |               |                     |                          |
| Residues in model | 96–290 | 96–290        | 97–116        | 97–116        | 96–114              | 96–114                   |
| No. water molecules | 391   | 386           | 128           | 89            | 112                | 119                      |
| R<sub>cryst</sub> (%) | 19.2  | 18.0          | 19.8          | 21.0          | 21.7               | 21.4                     |
| R<sub>free</sub> (%) | 23.0  | 22.3          | 23.6          | 23.4          | 23.3               | 25.5                     |
| R.m.s.d. bonds (Å) | 0.007 | 0.008         | 0.009         | 0.008         | 0.009              | 0.009                    |
| R.m.s.d. angles (°) | 1.4   | 1.5           | 1.5           | 1.5           | 1.5                | 1.5                      |
| B-factor (Å²)    | 16.5   | 18.5          | 29.6          | 33.1          | 30.8               | 28.9                     |
| Zinc             | 11.8   | 15.8          | 24.2          | 32.0          | 29.2               | 28.3                     |

* Data for T-p53C are taken from Ref. 12.

Mutant T-p53C-R249S—The cancer hot spot mutation R249S is only increased 5-fold relative to T-p53C, under our experimental conditions (Table II). On the basis of the crystal structure of T-p53C-R273H, this effect can be ascribed exclusively to the loss of interactions with DNA mediated by the guanidinium group of Arg273 (thin gray lines). Also shown are the two thymidylate moieties in the structure of DNA-bound wild type which are in close contact to Arg273 (magenta spheres). Asp281 (transparent light gray). Selected water molecules in the structure of T-p53C-R273H are represented by magenta spheres. Asp281 (transparent light gray). Selected water molecules in the structure of T-p53C-R273H are represented by magenta spheres. Asp281 (transparent light gray). Selected water molecules in the structure of T-p53C-R273H are represented by magenta spheres.
T-p53C-R249S now reveals the reason for the deleterious effect of this mutation. Superposition of the Ca atoms in the structures of T-p53C-H168R (magenta) and T-p53C-R249S (yellow) on the structure of T-p53C (PDB code 1UOL, molecule A; black). Ca atoms before and after chain breaks are marked with spheres in the color of the respective chain. B, structure of T-p53C-R249S (yellow) superimposed on the structure of p53 wild type (PDB code 1TSR, molecule A; light grey). The zinc ion in both structures is shown as a grey or yellow sphere. * denotes wild type residues. Cys238 in the structure of T-p53C-R249S was refined in two alternative conformations, both contacting the zinc ion. Only the conformation that was refined with higher occupancy (0.7) is shown. C, stereo view of the final (2Fo − Fc) electron density map at 1.9 Å resolution for mutant T-p53C-R249S showing the peptide segment Cys242-Met245, the zinc ion, and two residues that make contact with Met245. The orientation is the same as in B. The contour level is at 1.2σ.

T-p53C-R249S now reveals the reason for the deleterious effect of this mutation. Superposition of the Ca atoms in the structures of T-p53C-R249S and T-p53C gives an r.m.s. deviation of 1.5 Å. The structure of the β-sandwich is not affected by the mutation, whereas there are substantial structural changes in the DNA binding surface (Fig. 3, A and B). Loop L3 adopts a conformation that is very different from the one observed in wild type. Residues 244–248 next to the mutation site are highly flexible, which is reflected by the high crystallographic B-factors for the main chain atoms in this region and the absence of an ordered conformation for the side chains of Met246 and the DNA contact residue Arg248. The most striking difference is the substantial reorientation of Met243, which results in an α-helical conformation for residues 239–244. This is essentially because of a ~150° φ angle flip in the preceding residue, Cys242, relative to the structure of wild type (Fig. 3, B and C). In wild type and T-p53C, Met243 is solvent-exposed. In T-p53C-R249S, however, the side chain of Met243 is in close contact with a number of hydrophobic residues such as Val173 and Leu194. It is buried in a hydrophobic pocket that was originally occupied by Met246 and the methionine sulfur of Met243 is displaced by more than 11 Å relative to its position in T-p53C. The respective Ca displacement is 6 Å. This “methionine swap” results in a partial misfolding of loop L3, and the Ca atom of Met246, which originally occupied the hydrophobic pocket, is displaced by almost 10 Å.

There is also a small but significant shift of the backbone atoms in L2, adjacent to the mutation site. Although the overall conformation of this loop is not changed, its position has shifted away from the mutation site, probably as a direct consequence of missing interactions with the Arg249 side chain. This mainly affects residues 166 and 167 for which the Ca atoms are displaced by more than 1 Å.

Apart from the C terminus, there were two more regions of the protein for which no interpretable electron density was observed. This affected parts of the L1 loop (residues 117–120) and residues around Asp184, which in wild type forms a salt bridge with Arg175. This is a common feature of all the four mutant structures that were solved in the hexagonal crystal form, and it may, therefore, be a consequence of this particular crystal packing rather than their respective mutations (cf. Table III). It has been shown that loop L1 is flexible and adopts a different conformation depending on whether p53 is bound to DNA or not (5, 12, 24). In the hexagonal setting, the conformations found in DNA-bound wild type and DNA-free T-p53C would bring Lys120 in too close a contact with residues from a crystallographic symmetry-related molecule.

**Mutant T-p53C-H168R**—At only slightly more than 0.1% of...
all reported cases, the H168R mutation is much less frequent in human cancers than the hot spot mutations R273H and R249S (www.iarc.fr/p53; Ref. 25). It can, however, act as a second-site suppressor mutation for R249S. The H168R mutation significantly reduces the thermodynamic stability of the core domain, i.e. by \( \sim 3 \text{ kcal/mol} \) (Table I). The crystal structure of T-p53C-H168R has major distortions around the mutation site in loop L2, whereas the overall structure, especially in the \( \beta \)-sandwich region, is conserved (Fig. 3A). There was no interpretable electron density for residues 166–170, indicative of an increased mobility of these residues. The distortion also affects the side chains of Glu\(^{171} \) and Asn\(^{165} \) on both sides of the chain break, which are disordered in T-p53C-H168R. In wild type, Glu\(^{171} \) forms a well defined salt bridge with Arg\(^{249} \). Interestingly, loop L3 harboring the DNA contact residue Arg\(^{249} \) is not significantly disturbed, although it is no longer stabilized via the Arg\(^{249} \)-Glu\(^{171} \) salt bridge. The guanidinium group of Arg\(^{249} \) is at the same position as in wild type and the superstable quadruple mutant T-p53C, where it stabilizes the conformation of loop L3 via hydrogen bonds with the backbone oxygen atoms of Gly\(^{245} \) and Met\(^{246} \) (Figs. 1B and 3A). Although the L3 loop still adopts its wild type conformation, the increased relative crystallographic temperature factors compared with those in T-p53C indicate an increased flexibility of this region. The conservation of the wild type L3 loop conformation in H168R is consistent with the observation by Nikolova et al. (10) and our AUC data (Table II) that mutant H168R shows a reduced but still appreciable affinity for \( \text{gadd45} \) promoter DNA.

Mutants T-p53C-H168R/R249S and T-p53C-T123A/H168R/R249S—Mutation H168R in combination with T123A acts as an intragenic suppressor mutation that restores activity in the cancer mutant R249S (8). We have solved the structures of T-p53C-H168R/R249S and T-p53C-T123A/H168R/R249S to reveal the structural basis of this rescue mechanism. The structures of mutant T-p53C-H168R/R249S and T-p53C-T123A/H168R/R249S are almost identical, and except for the truncation of the side chain of Thr\(^{123} \) no significant differences were observed. This is consistent with previous NMR studies (10), where no substantial differences were detected. The r.m.s. deviation for Ca atoms is 0.14 Å when both structures are superimposed. There are, however, major structural differences from the H168R and R249S single mutants. The presence of both cancer mutations (H168R and R249S) in the same molecule reverses the structural changes induced by the single oncogenic mutations. The wild type conformation of the protein backbone is at least partly restored in both loops. The side chain of Arg\(^{168} \) points toward loop L3, and the position of the guanidinium group superimposes strikingly well with the position of the guanidinium group of Arg\(^{249} \) in wild type and T-p53C (Fig. 4). The only difference is that it is anchored to a different part of the protein. In wild type, the guanidinium group is contributed by Arg\(^{249} \) on loop L3; in mutants T-p53C-H168R/R249S and T-p53C-T123A/H168R/R249S, it is contributed by Arg\(^{168} \) on loop L2. Although the wild type conformations of loops L2 and L3 are largely restored, they still show signs of a higher structural plasticity than in T-p53C, and in particular the L3 loop exhibits increased flexibility (e.g. there was no interpretable electron density for residues Met\(^{243} \) and Gly\(^{244} \)). This, however, does not seem to have a significant effect on binding of specific DNA as reflected by our AUC data, which show restoration of \( \text{gadd45} \) binding to wild type levels (Table II).

**DISCUSSION**

**R273H and R249S Impair p53 DNA Binding by Different Mechanisms**—Our crystal structures allow us to look at the effects of two of the most important cancer mutations at high resolution and reveal the fundamentally different mechanisms by which contact and structural mutations inactivate p53. The structure of T-p53C-R273H confirms and reinforces that this mutant has all the trademarks of a classic contact mutant. The mutation replaces a DNA contact residue without distorting neighboring residues in the DNA binding surface. Hence, the properties of mutant R273H can be directly attributed to the loss of one particular DNA contact and are not the result of secondary effects caused by structural distortions in neighboring regions. On the basis of the structure of T-p53C-R273H, it is reasonable to assume that the other frequently cancer-associated variants of codon 273 (R273C and R273L) also induce only minor changes in adjacent residues. In contrast, R249S is a structural mutation. It disrupts a network of interactions that stabilize the L3 loop in a conformation enabling Arg\(^{249} \) to make DNA contacts. In T-p53C-R249S, this loop is highly flexible and adopts a conformation very different from wild type. It is not only more flexible, but the substantial displacement of Met\(^{243} \) in T-p53C-R249S indicates that the R249S mutation induces partial misfolding and thus favors non-native conformations of the L3 loop. This accounts for the reduced DNA binding affinity. Although the nature of the structural distortions caused by the R249S mutation is in essence the same in wild type as indicated by NMR studies (13, 26), they may be more pronounced and communicated to a larger extent to more distant regions of the protein because of the lower stability of the underlying structural scaffold.

**Effects on Binding of Regulatory Proteins**—The p53 DNA binding surface and binding sites for the 53BP1 and 53BP2 proteins partly overlap (27–29). The binding sites for many other proteins such as Rad51, which is involved in homologous recombination, are also clustered in the same region of p53 (30). The L3 loop (residues 243–249), which is substantially perturbed in T-p53C-R249S, forms an integral part of the interface with both 53BP1 and 53BP2. Hence, it is not surprising that binding of these proteins is impaired upon truncation of the Arg\(^{249} \) side chain (29, 31). The structure of T-p53C-H168R suggests that the H168R mutation may also affect binding of 53BP1, which has been reported to be involved in DNA damage.

---

**Fig. 4. Structure of T-p53C-T123A/H168R/R249S.** Stereo view of the structure of T-p53C mutant T123A/H168R/R249S (yellow) superimposed on the structure of wild type (PDB code 1TSR, molecule A; light gray). Ca traces with selected side chains for parts of the L2 and L3 loops including the zinc binding site are shown. In the case of Gly\(^{245} \) and Met\(^{246} \), all atoms of the residue are shown. The zinc ion in both structures is depicted as a gray or yellow sphere. The electron density for residues Met\(^{243} \) and Gly\(^{244} \) in T-p53C-T123A/H168R/R249S was very weak, and hence these residues were omitted from the model. Interactions mediated via the guanidinium group of Arg\(^{249} \) in wild type are shown by dotted lines. The common binding position of the guanidinium group of Arg\(^{249} \) and Arg\(^{168} \) in both structures is highlighted in magenta.
response (32, 33). Asn147, which is distorted in T-p53C-H168R, is part of the p53–53BP1 interface, where a number of contacts are made by residues on the L2 loop (27, 28). Mutant H168R may be representative of a number of cancer mutants with local distortions at the periphery of the DNA binding surface, which do not severely affect DNA binding but may significantly hamper interactions with regulatory proteins. In the case of H168R, however, this effect may be secondary to the substantially reduced thermodynamic stability of the protein. In this context, Gorina and Pavletich (29) have pointed out an interesting correlation: The frequencies of cancer-associated mutations in the DNA binding surface which have no apparent structure destabilizing effect seem to correlate with whether they affect binding to both DNA and 53BP2, or binding to only one of the two. Lys120, for example, makes contacts with DNA but is a cancer cold spot.

The Mechanism of Rescuing Cancer Hot Spot Mutant R249S—The crystal structures also show how R249S is rescued by the intragenic suppressor mutation H168R. It is not seen how the T123A mutation, which is located on the opposite end of the DNA binding surface, might contribute to the rescue of R249S. It neither significantly alters the structure nor affects the stability of the core domain. The crystal structures of T-p53C-H168R/R249S and T-p53C-T123A/H168R/R249S are almost identical, which is in agreement with earlier NMR studies (10). Possibly, T123A is only effective as a rescue mutation within the context of the p53 tetramer. In yeast-based assays using the full-length protein, mutant T123A and several other mutations in the immediate environment showed increased transactivation activity for a number of different response elements (34).

Recent in vivo studies have shown that H168R can rescue R249S in combination with mutations other than T123A, e.g. with K139R and N239Y (9). This suggests that the main contribution to rescue of R249S comes from the H168R mutation which, as our crystal structures show, mimics Arg249 (Fig. 4), although additional stabilizing mutations are also needed. This stepwise restoration of wild-type activity is also reflected by our DNA binding studies (Table II). Although T-p53-R249S shows an appreciable increase in affinity for gadd45 DNA compared with the same mutation in the wild type, the DNA binding affinity is still in the range of the classic DNA contact mutant R273H. Wild type levels are achieved, however, when the H168R mutation is included.

The mechanism of specific rescue of mutant R249S by the H168R mutation is very different from that by which global suppressors rescue mutant p53. Prime examples of such global suppressor mutations in p53 are the N329Y and N268D mutations. They increase the thermodynamic stability of the protein without compromising the structure and hence the function of the core domain (11, 12). Accordingly, these mutations can fully or partially rescue a whole subset of structurally destabilized cancer mutants.

The structure of T-p53C-R273H provides clues as to how the mutation S240R, which seems to be specific for the cancer mutants R273H and R273C (9), suppresses these mutations (Fig. 2). In T-p53C-R273H an arginine at position 240 could easily be placed in a position enabling it to make contact with the phosphate backbone of DNA and thus compensate for the loss of Arg273. Interestingly, S240R is a cancer mutant when one of its own. The current version (R9) of the p53 mutation data-base reports 12 cases in which S240R is found in tumors (www.iarc.fr/p53; Ref. 25). This scenario is reminiscent of the H168R/R249S rescue pair, where one arginine replaces another arginine. The structural effects, however, are fundamentally different. In the first case, a structural distortion in the DNA binding surface is reversed, which indirectly restores DNA binding mediated via a different arginine (Arg249). In the second case, activity would be restored by providing a direct substitute for a missing DNA contact residue.

CONCLUSION

Over the past years much effort has been put into elucidating the effect of tumorigenic mutations on the structure and function of p53 in an attempt to understand their role in carcinogenesis and find possible ways of rescuing mutant p53. Our crystal structures and the presented biophysical data provide a much more detailed understanding of the structural and functional effects of the two cancer hot spot mutations R249S and R273H. This will provide useful information to assess structure-function and mutation-function relationships in p53. Further, we have established the structural basis for the role of the oncogenic H168R mutation in rescuing R249S. This highlights the fundamental differences in the action of specific and global second-site suppressor mutations. The different underlying rescue mechanisms are not restricted to p53, but are also applicable to other disease-related proteins. They represent general strategies by which proteins can compensate for deleterious mutations or adapt to a changing environment during the course of evolution.

Acknowledgments—We thank the staff at the European Synchrotron Radiation Facility Grenoble (beamline BM14) and the Synchrotron Radiation Source Daresbury (beamline 14.1) for helpful advice and assistance in data collection.

REFERENCES

1. Vogelstein, B., Lane, D., and Levine, A. J. (2000) Nature 408, 307–310
2. Ryan, K. M., Phillips, A. C., and Vousden, K. H. (2001) Curr. Opin. Cell Biol. 13, 322–327
3. Voussen, K. H., and Lu, X. (2002) Nat. Rev. Cancer 2, 594–604
4. Hainaut, P., and Hollestein, M. (2000) Adv. Cancer Res. 77, 81–137
5. Chu, Y., Gorina, S., Jeffrey, P. D., and Pavletich, N. P. (1994) Science 265, 346–355
6. Bullock, A. N., Henckel, J., DeDecker, B. S., Johnson, C. M., Nikofova, P., Proctor, M. R., Lane, D. P., and Fersht, A. R. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 14338–14342
7. Bullock, A. N., Henckel, J., and Fersht, A. R. (2000) Oncogene 19, 1245–1256
8. Brachmann, R. K., Yu, K., Eby, J., Pavletich, N. P., and Boeke, J. D. (1998) EMBO J. 17, 1847–1859
9. Barnini, T. E., Wang, T., Qian, H., Dearth, L. R., Truong, L. N., Zeng, J., Denes, A. E., Chen, S. W., and Brachmann, R. K. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 4930–4935
10. Pikofova, P. V., Pikofova, N. V., DeDecker, B., Henckel, J., and Fersht, A. R. (2000) EMBO J. 19, 370–378
11. Nikofova, P. V., Henckel, J., Lane, D. P., and Fersht, A. R. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 14675–14680
12. Joerger, A. C., Allen, M. D., and Fersht, A. R. (2004) J. Biol. Chem. 279, 1291–1296
13. Wong, K. B., DeDecker, B. S., Freund, S. M., Proctor, M. R., Bycroft, M., and Fersht, A. R. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 14338–14342
14. Weinberg, R. L., Veprinsiev, D. B., and Fersht, A. R. (2004) J. Mol. Biol. 341, 1145–1159
15. Lesin, A. G. W. (1992) Joint CCP4 and ESR-EACMB Newsletter on Protein Crystallography 26, Daresbury Laboratory, Warrington, UK
16. Collaborative Computational Project, N. (1994) Acta Crystallogr. Sect. D 50, 780–783
17. Brugger, A. T., Adams, P. D., Clore, G. M., DeLano, W. L., Gross, P., Groth, M., Kunsted, W. R., Jiang, S., Kuszewski, J., Nilges, M., Pannu, N. S., Read, R. J., Rice, L. M., Simonson, T., and Warren, G. L. (1998) Acta Crystallogr. Sect. D 54, 905–921
18. Turk, D. (1992) Weiterentwicklung eines Programms f¨ur Molekulgraphik und Elektronische Manipulation und seine Anwendung auf verschiedene Protein-Strukturaufklarungen, Ph. D. thesis, Technische Universit¨at M¨unchen, Germany
19. Laszkowski, R. A., MacArthur, M. W., Moss, D. S., and Thornton, J. M. (1993) J. Appl. Crystallogr. 26, 283–291
20. Kraulis, P. J. (1991) J. Appl. Crystallogr. 24, 946–950
21. Esnout, R. M. (1999) Acta Crystallogr. Sect. D 55, 888–940
22. Merttr, E. A., and Bacon, D. J. (1997) Methods Enzymol. 279, 505–524
23. Carter, P. J., Winter, G., Wilkinson, A. J., and Fersht, A. R. (1984) Cell 38, 835–840
24. Zhao, K., Chai, X., Johnsten, K., Clements, A., and Marmorein, R. (2001) J. Biol. Chem. 276, 12120–12127
25. Olivier, M., Eides, R., Hollestein, M., Khan, M. A., Harris, C. C., and Hainaut, P. (2002) Hum. Mutat. 19, 607–614
26. Friedler, A., DeDecker, B. S., Freund, S. M., Blair, C., Rüdiger, S., and Fersht, A. R. (2004) J. Mol. Biol. 336, 187–196
27. Joo, W. S., Jeffrey, P. D., Cantor, S. B., Fennin, M. S., Livingston, D. M., and
Pavletich, N. P. (2002) *Genes Dev.* **16**, 583–593
28. Derbyshire, D. J., Baso, B. P., Serpell, L. C., Joo, W. S., Date, T., Iwabuchi, K., and Doherty, A. J. (2002) *EMBO J.* **21**, 3863–3872
29. Gorina, S., and Pavletich, N. P. (1996) *Science* **274**, 1001–1005
30. Friedler, A., Veprintsev, D. B., Rutherford, T., von Glos, K. I., and Fersht, A. R. (2005) *J. Biol. Chem.* **280**, 8051–8059
31. Thukral, S. K., Blain, G. C., Chang, K. K., and Fields, S. (1994) *Mol. Cell. Biol.* **14**, 8315–8321
32. DiTullio, R. A., Jr., Mochan, T. A., Venere, M., Bartkova, J., Sehested, M., Bartek, J., and Halazonetis, T. D. (2002) *Nat. Cell Biol.* **4**, 998–1002
33. Wang, B., Matsuoka, S., Carpenter, P. B., and Elledge, S. J. (2002) *Science* **298**, 1435–1438
34. Resnick, M. A., and Inga, A. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100**, 9934–9939