Crystal Structure of a Superstable Mutant of Human p53 Core Domain

INSIGHTS INTO THE MECHANISM OF RESCUING ONCOGENIC MUTATIONS

Received for publication, September 2, 2003, and in revised form, October 3, 2003
Published, JBC Papers in Press, October 8, 2003, DOI 10.1074/jbc.M309732200

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Most of the cancer-associated mutations in the tumor suppressor p53 map to its DNA-binding core domain. Many of them inactivate p53 by decreasing its thermodynamic stability. We have previously designed the superstable quadruple mutant M133L/V203A/N239Y/N268D containing the second-site suppressor mutations N239Y and N268D, which specifically restore activity and stability in several oncogenic mutants. Here we present the x-ray structure of this quadruple mutant at 1.9 Å resolution, which was solved in a new crystal form in the absence of DNA. This structure reveals that the four point mutations cause only small local structural changes, whereas the overall structure of the central β-sandwich and the DNA-binding surface is conserved. The suppressor mutation N268D results in an altered hydrogen bond pattern connecting strands S1 and S10, thus bridging the two sheets of the β-sandwich scaffold in an energetically more favorable way. The second suppressor mutation N239Y, which is located in close proximity to the DNA-binding surface in loop L3, seems to reduce the plasticity of the structure in large parts of loop L3 as indicated by decreased crystallographic temperature factors. The same is observed for residues in the vicinity of the N268D substitution. This increase in rigidity provides the structural basis for the increase in thermostability and an understanding how N268D and N239Y rescue some of the common cancer mutants.

The tumor suppressor p53, which has aptly been described as the guardian of our genome, plays a key role in the prevention of cancer development (1, 2). In response to cellular stress, p53, which is active as a tetramer, induces the transcription of genes for cell cycle arrest or apoptosis (3–6). In about half of all human cancers, p53 is inactivated as a direct result of missense mutations within the p53 gene. Most of these mutations map to its DNA-binding core domain (7), and six “hot spots” stand out as most frequently associated with human cancer (R175H, G245S, R248Q, R249S, R273H, and R282W). The crystal structure of the human p53 core domain has been solved at 2.2 Å resolution in complex with a consensus DNA sequence (8). A large immunoglobulin-like β-sandwich provides the basic scaffold for the conserved DNA-binding surface consisting of a loop-sheet-helix motif and two large loops coordinating a zinc ion. Two of the three molecules in the asymmetric unit bind on the same face of the DNA duplex, forming a head-to-tail dimer with weak protein-protein interactions between the two molecules. The third molecule makes no significant contact with DNA. This structure provided a framework to subdivide common cancer mutations into two classes: “contact mutants” that directly affect DNA binding through loss of a contacting residue and “structural mutants” causing structural perturbations within the core domain.

Quantitative folding and DNA-binding studies of numerous cancer-associated core domain mutants allowed a further classification into three broad phenotypes: (i) DNA-contact mutants with only minor effects on folding and stability; (ii) substitutions resulting in local changes, mainly in proximity to the DNA-binding surface, which are destabilized by < 2 kcal/mol relative to wild type; and (iii) mutations causing global unfolding of the β-sandwich, which are destabilized by > 3 kcal/mol (9, 10). Because the thermodynamic stability of wild type p53 core domain is already rather low, it can be extrapolated that for mutants belonging to class (iii) the population of unfolded protein at 37 °C is higher than 50%. These results show that stabilization of p53 is crucial. This can be achieved by two ways, either by introducing stabilizing suppressor mutations into the p53 core domain or by addition of stabilizing agents.

The “chaperone strategy” to rescue oncogenic mutants is based on the theory that conformationally destabilized mutants can be rescued by generic small molecules binding the native but not the denatured state. Friedler et al. (11) have demonstrated that a nine-residue peptide (CDB3) derived from the crystal structure of p53 core domain in complex with 53BP2 stabilizes various oncogenic mutants. Another molecule PRI-MA-1 that increases p53 levels in vivo was found by random screening of a low-molecular-weight compound library (12).

By semirational design based on the molecular evolution of the DNA-binding domain, Nikolova et al. (13) have identified mutations that stabilize human p53 core domain. The most stable substitutions were combined in a quadruple mutant (M133L/V203A/N239Y/N268D), which is stabilized by 2.65 kcal/mol but retains wild type DNA-binding function (13). The effects of the point mutations are nearly additive, and the main contribution to stability increase comes from the N239Y and N268D substitutions. These two mutations are of particular interest as they are known to act as second-site suppressors for various cancer-associated mutations (14).

Here we present the crystal structure of this superstable p53 core domain mutant, which reveals that the overall structural characteristics of the wild type protein are retained in the quadruple mutant. 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. This provides an insight into how the two second-site suppressor mutations might rescue some of the common structurally destabilized cancer mutants.

EXPERIMENTAL PROCEDURES

Protein Purification and Crystallization—Human p53 core domain (residues 94–312) mutant M133L/V203A/N239Y/N268D was expressed and purified following published protocols (13, 15). Crystals were grown at 17 °C using the sitting-drop vapor diffusion technique. Initially, we attempted to solve the structure of the quadruple mutant with and without bound fluorescein-labeled peptide CDB3 (FL-CDB3).1 5 µl protein solution (5.2 mg/ml protein in 250 µl FL-CDB3, 150 mM NaCl, 10 mM diithiothreitol, and 20 mM Tris, pH 7.6) was mixed with 3 µl reservoir buffer consisting of 100 mM Hepes (pH 7.2), 10 mM diithiothreitol, and 19% (w/v) polyethylene glycol 4000 above a reservoir solution of 700 µl. Yellow, rod-shaped crystals appeared within a few days. Colorless crystals of the same type were obtained in the absence of FL-CDB3. Crystals were flash frozen in mother liquor containing 20% (v/v) polyethylene glycol 200. They belonged to the orthorhombic space group P2₁2₁2₁, with cell dimensions of a = 65.0 Å, b = 71.0 Å, c = 104.8 Å.

Data Collection and Structure Determination—A data set to 1.9 Å resolution was collected from a single crystal grown in the presence of FL-CDB3 at 100 K on beamline 14.2 at the Synchrotron Radiation Source, Daresbury, UK. All data were indexed and integrated with MOSFLM (16) and further processed using CCP4 (17). Data collection statistics are shown in Table I. A total of 5% of all reflections were flagged for the calculation of Rmerge and excluded from subsequent refinement.

The structure was solved by molecular replacement with the program CNS (18) using diffraction data from 15–4.0 Å. As search model we used chain A of p53 core domain wild type (Protein Data Bank code 1TSR) (8). The rotation and translation searches gave unambiguous solutions for two molecules in the asymmetric unit. All subsequent structure refinement was carried out with CNS. Water molecules were added to the structure using the waterpick option within CNS. Several cycles of refinement with CNS and rebuilding in MAIN (19) gave a final model with an R-factor of 19.2% and an Rmerge of 23.0%. The refinement statistics are summarized in Table I. Despite the intensive yellow color of the crystals used for data collection, there was no additional electron density, suggesting an ordered conformation of bound FL-CDB3. PROCHECK (20) was used to check the stereochemistry of the model and revealed that 90.1% of the residues lie in the most favored regions of the Ramachandran plot, whereas the remaining 9.9% of the residues lie in additionally allowed areas. Calculation of buried surface areas was performed with CNS using an accessible probe radius of 1.4 Å. Figures were generated with MOLSCRIPT (21) and RASTER3D (22).

RESULTS

Overall Structure of the Quadruple Mutant—We have solved the crystal structure of the quadruple mutant M133L/V203A/N239Y/N268D of the human p53 core domain at 1.9 Å resolution (Table I). Our final model of the quadruple mutant comprises residues 96–290 for both molecules in the asymmetric unit. As in the structure of wild type, the C-terminal residues up to Thr-312 are disordered. The overall structure is very similar to wild type (Fig. 1, A–C). It consists of two anti-parallel β-sheets of four and five twisted strands, forming a β-sandwich with a “Greek key” topology. This compact, barrel-like structure provides the basic scaffold for two large loops, L2 (residues 164–194) and L3 (residues 237–250), and a loop-sheet-helix motif at the same end of the β-sandwich. Loops L2 and L3 are stabilized by a zinc ion, which is tetrahedrally coordinated by Cys-176, His-179, Cys-238, and Cys-242. The loop-sheet-helix motif contains loop L1 (residues 113–123), a short β-sheet comprising the S2-S2’ turn and the C-terminal residues of the extended β-strand S10, and helix H2 (residues 278–289). To-

\[ R_{\text{merge}} = \frac{\sum_{i}^{N} \sum_{h}^{N} |F_{\text{obs}}(h) - F_{\text{calc}}(h)|}{\sum_{i}^{N} \sum_{h}^{N} F_{\text{obs}}(h)} \]

\[ R_{\text{free}} = \frac{\sum_{i}^{N} \sum_{h}^{N} |F_{\text{obs}}(h) - F_{\text{calc}}(h)|}{\sum_{i}^{N} \sum_{h}^{N} F_{\text{obs}}(h)} \]

\[ \frac{R_{\text{merge}}}{R_{\text{free}}} \]

Table I. Data collection and refinement statistics

| Data collection and processing | Resolution range, Å | 35.6–1.9 |
|------------------------------|--------------------|---------|
| Total measurements           | 231,945            |         |
| Unique reflections           | 38,078             |         |
| Completeness, %              | 98.7 (96.5)        |         |
| Rmerge, %                    | 8.3 (29)           |         |
| Rfree                        | 18.4 (5.3)         |         |

| Refinement statistics        | Resolution range, Å | 35.6–1.9 |
|------------------------------|--------------------|---------|
| Rcryst/Rfree, %              | 19.2/3.0           |         |
| Rms deviation bond lengths, Å| 0.007              |         |
| Rms deviation angles, Å      | 1.4                |         |
| Number of atoms (non-hydrogen)| 3072              |         |
| Protein                      | 391                |         |
| Water                        | 27.0               |         |
| Zinc                         | 11.8               |         |

* The numbers in parentheses refer to the 2.0–1.9 Å resolution shell.

$ R_{\text{merge}} = \frac{\sum_{i}^{N} \sum_{h}^{N} |F_{\text{obs}}(h) - F_{\text{calc}}(h)|}{\sum_{i}^{N} \sum_{h}^{N} F_{\text{obs}}(h)} $ and $ R_{\text{free}} = \frac{\sum_{i}^{N} \sum_{h}^{N} |F_{\text{obs}}(h) - F_{\text{calc}}(h)|}{\sum_{i}^{N} \sum_{h}^{N} F_{\text{obs}}(h)} $ where $ F_{\text{obs}} $ was calculated over 5% of the amplitudes chosen at random and not used in the refinement.

Together with loops L1 and L3, helix H2 forms a positively charged surface, which is known to bind DNA (8).

The Ca atoms of the two molecules in the asymmetric unit of the DNA-free quadruple mutant superimpose with a rms deviation of 0.26 Å. When molecule A is superimposed on the wild type (residues 96–289), the rms deviation for Ca atoms is 0.53 Å for wild type chain A (DNA-free), and 0.71 and 0.68 Å for chains B and C (DNA-bound), respectively. Similar rms deviations are obtained for the pair-wise superpositions of the second molecule in the asymmetric unit. The largest deviations are found in the loop regions L1, L2, and the turn between β-strands S7 and S8 (Fig. 1C). The conformation of loop L1 resembles the one found in DNA-free wild type but is different from the one found in DNA-bound wild type, where the Ca atom of Ser-121 is displaced by 3.8 Å. Such a loop conformation is not compatible with DNA binding. In the complex of wild type p53 bound to the DNA consensus sequence, Lys-120 on loop L1 makes two DNA contacts. The backbone amide interacts with a deoxyribose moiety, and the ε amino group makes two hydrogen bonds with a guanine base in the major groove. Upon DNA binding, loop L1 has to undergo an induced fit movement to place Lys-120 in a position where it can form these interactions with DNA. As observed for loop L1, the conformation found for L2 is closer to DNA-free than to DNA-bound wild type. Maximum Ca displacements are 1.5 Å when compared with DNA-free and 3.5 Å when compared with DNA-bound wild type (for Ser-183 in both cases). The observed structural changes also include a partial unwinding of the short α-helical segment H1 into a 3_10-helical turn (residues 177–179).

The S7/S8 turn is located on the opposite site of the β-sandwich. The conformation in the quadruple mutant is somewhat an intermediate between the conformations found in DNA-free and DNA-bound wild type. This turn is also one of the regions where we observe the largest Ca deviations between the two non-crystallographically related molecules in our mutant structure. It seems that the different conformations in this region are induced by the different crystallographic environment in the crystal and thus mirror the inherent flexibility of this region. This has also been observed in the structure of p53 core domain from mouse (23).

Crystal Packing—The crystal lattice of the quadruple mutant is built on the basis of a total of 4 different crystal contacts

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1 The abbreviations used are: FL, fluorescein-labeled; CNS, crystallography NMR software; rms, root mean square.
**Fig. 1.** The crystal structure of p53 core domain quadruple mutant M133L/V203A/N239Y/N268D. A, distribution of secondary structural elements in the structure of the quadruple mutant. The *four mutation sites* are highlighted in different colors. Residues contacting DNA in the wild type structure (8) are marked by asterisks. B, ribbon presentation showing the immunoglobulin like β-sandwich, which serves as basic scaffold for two large loops (encircled in blue dashed line) connected via a zinc ion (gray sphere) and a loop-sheet-helix motif (encircled in green solid line). In the wild type DNA complex, these two motifs interact with the minor and major groove of DNA, respectively (8). C, stereo view of the Ca trace of the p53 core domain quadruple mutant (black) superimposed on DNA-free wild type (Protein Data Bank code 1TSR; chain A, blue) and DNA-bound wild type (Protein Data Bank code 1TSR; chain B, orange). The zinc ion is shown as a large gray sphere. The mutation sites in the quadruple mutant are marked by *small colored spheres* to show the spatial separation of the four mutation sites. The color coding is as in A: blue, M133L; green, V203A; red, N239Y; magenta, N268D.

**Fig. 2.** The crystal packing of p53 core domain quadruple mutant M133L/V203A/N239Y/N268D. A, view on top of a layer of molecules in the *xy* and *xz* plane. The *red box* represents the dimensions of the unit cell with *P2_1212* symmetry and the roman numerals indicate different crystal contacts. Contacts I, II, and IV connect the two non-crystallographically related molecules A and B. Each of these two molecules participates in additional contacts with crystallographically related molecules via contact III. B, comparison of the orientation of the two quadruple mutant molecules connected via crystal contact I (black) and the head-to-tail dimer formed by the DNA-bound molecules in wild type (orange). Only one molecule of the dimer is superimposed; *i.e*., molecule B of the quadruple mutant and molecule B of wild type, which binds to the DNA consensus sequence. The two strands of DNA are shown in blue and magenta, respectively.
The structure of p53 core domain quadruple mutant M133L/V203A/N239Y/N268D (chain A, yellow) superimposed on the wild type structure (chain A, transparent light gray). A, mutation site M133L; B, mutation site V203A; C, mutation site N268D; D, mutation site N239Y. The zinc ion is shown as a gray sphere. The large semi-transparent red spheres indicate the location of the two cancer hot-spot sites Gly-245 and Arg-249.

(Fig. 2A). Contacts I (960 Å$^2$ buried surface area) and II (560 Å$^2$ buried surface area) connect head-to-tail orientated molecules along the crystallographic x-axis. Contact III with a total buried surface area of 1050 Å$^2$ generates chains of head-to-tail orientated molecules running parallel to the crystallographic y-axis with each molecule participating in two identical contacts. In z-direction of the crystal lattice, the molecules are connected only via a small interface (contact IV; 530 Å$^2$ buried surface area) with head-to-head orientated molecules. A total of 21% of the solvent accessible surface of both molecules in the asymmetric unit is buried in these crystal contacts. The C-terminal helix, which is known to bind to DNA, makes no contacts with neighboring molecules and is exposed to a solvent channel within the crystal.

Interestingly, crystal contact I is very similar to the interface of the head-to-tail orientated dimer formed by the two DNA-binding molecules (chains B and C) in the wild type structure (Fig. 2B). The two dimers generated by these contacts superimpose with a rms deviation of 1.7 Å (Cα atoms). When only one molecule of the dimer is used for the superposition, as shown for the molecule binding to the DNA consensus sequence in Fig. 2B, the second molecule of the quadruple mutant is slightly rotated and shifted from the DNA-binding position. It is locked in a position that results in an increase of the contacting area between the two molecules. (960 Å$^2$ versus 720 Å$^2$ buried surface area in the DNA complex). The striking similarity between these two dimers found in a different crystal lattice in the presence and absence of DNA raises the question whether this interface might also play a role in the context of the organization of the tetramer in the full-length protein. A different interface in the context of the crystal structure of p53 core domain from mouse, which is incompatible with simultaneous binding of both subunits to duplex DNA, has been attributed to a low affinity DNA state of the p53 tetramer (23).

Structural Changes at the Mutation Sites—Mutation M133L in strand S2’ of the loop-sheet-helix motif results in only minor changes in neighboring residues (Fig. 3A). In the quadruple mutant, the Cα atom of Leu-133 occupies the position of the methionine sulfur atom in wild type. The C6 atom of Leu-133 is involved in new hydrophobic interactions, namely with the Cβ atom of Tyr-126 and the aromatic ring of Phe-113.

Mutation V203A, which is located in the turn between β-strands S5 and S6, induces a re-packing of the hydrophobic core, while retaining the backbone conformation found in wild type (Fig. 3B). The cavity created by truncating the hydrophobic side chain of residue 203 is compensated by a movement of the side chain of Leu-188, which fills the gap and is now engaged in new hydrophobic contacts with Ala-203 and Tyr-205. This is achieved mainly by a 130° rotation of Leu-188 around the Cα Cβ bond, which results in a maximum displacement of more than 4 Å for the C61 atom.

The N268D mutation alters the hydrogen bonding network (Fig. 3C). It is located in β-strand S10, which is part of both the β-sandwich and the loop-sheet-helix motif. In DNA-free wild type, the Nδ atom of Asn-268 donates a hydrogen bond to the main-chain carbonyl oxygen of Phe-109. A comparison with the orientation of the amide group in the two molecules of DNA-bound wild type suggests a transient nature of this hydrogen bond fluctuating between the main-chain oxygens of Phe-109 and Arg-267. This hydrogen bond is lost in the quadruple mutant. Instead, the side chain of Asp-268 has flipped relative to the position of the original asparagine in wild type. In this orientation it uses both oxygens of its carboxylate group to form two well defined charged hydrogen bonds with backbone nitrogens: one intra-strand hydrogen bond with Ser-269 (2.7 Å distance) and one inter-strand hydrogen bond with Leu-111 from β-strand S1 (2.9 Å distance). By linking strands S10 and S1, Asp-268 connects the two sheets of the β-sandwich.

Mutation N239Y in the loop L3 region is of particular interest as this mutation is known to act as suppressor mutation for various cancer hot-spot mutations such as G245S. Contrary to the other three mutations, the N239Y substitution has so far not been found in any known p53 sequence in nature. The aromatic ring of Tyr-239 is packed onto the side chain of Leu-
Fig. 4. Polypeptide-backbone mobility in the structures of p53 core domain quadruple mutant M133L/V203A/N239Y/N268D and wild type. A, distribution of average isotropic B-factors for main-chain atoms in chains A (thick solid line) and B (thin solid line) of the quadruple mutant, and chain A (DNA-free) of wild type (dotted line). Secondary structural elements are given for reference; blocks represent β-strands and open circles indicate helical segments. B, relative backbone mobility as calculated by \((B-<B>-\alpha_{B})/\alpha_{B}\) in chains A (thick solid line) and B (thin solid line) of the superstable quadruple mutant, and chain A (DNA-free) of wild type (dotted line). \(B-<B>-\) is the deviation of the average isotropic main-chain B-factor for a particular residue from the mean for the whole chain (backbone atoms only), \(\sigma_{B}\) the standard deviation.

137, which is slightly rearranged (Fig. 3D). This hydrophobic packing shields one side of the phenol moiety from the solvent while the other side is left solvent exposed. Tyr-239 directly connects with zinc binding as it lies within a 5 Å distance to the three zinc ligands Cys-238, Cys-242, and His-179. The side chain of Cys-238 has slightly rotated, but the overall geometry of the zinc coordination sphere itself is preserved. Tyr-239 constitutes the interface between the core of the protein and the solvent. It makes contacts via its phenolic hydroxyl group with a defined network of ordered water molecules covering the protein surface. In the wild type structure of the p53 core domain DNA complex, Asn-239 is ~4 Å away from the phosphodiester backbone of the bound DNA. The rotamer found for Tyr-239 positions its hydroxyl group still ~4 Å away from the phosphate backbone when the mutant structure is superimposed on the wild type core domain DNA complex (molecule B).

Temperature Factors—We have analyzed the crystallographic thermal factors (B-factors) in the quadruple mutant and the previously published wild type structure in order to get some information on the relative flexibilities of particular regions in both proteins. The main-chain temperature factor profiles for the two molecules in the asymmetric unit of the quadruple mutant superimpose very well as shown in Fig. 4A. The overall B-factors are much lower than in the structure of DNA-free wild type (Protein Data Bank code 1TSR chain A): 16.3 Å² (molecule A) and 16.8 Å² (molecule B) for the quadruple mutant and 26.4 Å² for DNA-free wild type. There are some regions where the plasticity of the structure seems to be reduced in the quadruple mutant (Fig. 4, A and B); most notably residues 109–113, around residue 152 and residues 245–250, the region harboring the cancer hot-spot mutations G245S and R249S. His-179 in the zinc binding pocket next to the N239Y mutation site also exhibits significantly reduced B-factors, indicative of a more rigid structural framework.

DISCUSSION

The structure of our p53 core domain quadruple mutant provides the framework for understanding the molecular basis of stabilizing mutations in p53. The fact that the overall structural features of wild type are conserved makes this superstable mutant a suitable substitute for wild type in biochemical and biophysical studies where the intrinsic thermodynamic instability of the wild type protein causes experimental difficulties. Further, the structure provides direct evidence for the possible mode of action of the second-site suppressor mutations N268D and N239Y. The spatial separation of the four point mutations (the Cα atoms of residues 239 and 268, for instance, are more than 22 Å apart) and the observation that their effects on the overall stability of the protein are nearly additive allow us to look at their effects separately. As shown by urea-induced unfolding studies, mutations M133L and V203A make the smallest contribution to the stability increase in the quadruple mutant (13). The crystal structure suggests that this is achieved by only small structural changes, mainly by local re-packing of the side chains around the mutations sites in the loop-sheet-helix motif and the turn S5/S6, respectively. The main contribution to the overall stabilization in the quadruple mutant, however, comes from the N268D and N239Y substitutions.

Formation of an Alternative Hydrogen Bond Can Rescue Cancer Mutant V143A—It has been reported that the temperature-sensitive mutant V143A shows neither DNA-binding nor transactivation activity at physiological temperature, although these activities can be detected at lower temperatures (24). Furthermore, this cancer-associated mutant is globally destabilized by more than 3 kcal/mol (25). In addition, NMR studies indicate that the V143A mutation results in changes in almost all residues of the β-sandwich (15). In wild type and our quadruple mutant (Fig. 3C), the side chain of Val-143, which is located on β-strand S3, points toward the hydrophobic core of the β-sandwich, where it is completely buried (8). It lies in the very heart of a cluster of hydrophobic residues contributed from different regions of the protein. The two methyl groups of Val-143 make direct contacts with the side chains of Leu-111, Phe-113, Tyr-234, and Phe-270. Apparently, the truncation of these methyl groups in mutant V143A is detrimental to the packing of the hydrophobic core residues. It has, however, been reported that this cancer mutation can be rescued when an additional N268D substitution is introduced (14). Double-mutant cycles have further shown that this is achieved by increasing the stability of the cancer mutant by 1.1 kcal/mol (25).

The structure of our quadruple mutant provides an explanation how this stability increase may be achieved. The mechanism of rescue can be pinpointed to the formation of new hydrogen bonds. The energy loss by creating a hydrophobic cavity in mutant V143A can be partly compensated by Asp-268, which uses its two carboxylate oxygens to bridge the two sheets of the β-sandwich by hydrogen bonds in an energetically more favorable way than could be achieved by the asparagine in wild type. Apart from an effect on the overall stability of the protein, we can clearly see a local effect, which may be crucial in rescuing V143A. Leu-111, which acts as a hydrogen bond donor for Asp-268, makes direct contact with one of the methyl groups of Val-143. The hydrogen bond with Asp-268 significantly reduces the flexibility of residues Leu-111 and Phe-113 in the
immediate vicinity of Val-143 as mirrored by the B-factors in our crystal structure (Fig. 4B). This may directly counteract the loss of interactions by the V143A mutation by providing a more rigid structural framework around the mutation site.

**Tyr-239 Stabilizes Regions of p53 Harboring Cancer Hot-spot Sites**—N239Y has been reported to act as a second-site suppressor for various cancer-associated mutations, in particular the hot spot G245S in loop L3 (14). Contrary to the V143A mutation, which causes global unfolding of the β-sandwich, the two hot-spot mutations G245S and R249S in loop L3 cause only local distortions in the L2/L3 region (25). It had been suggested that Tyr-239 may come closer to the phosphodiester backbone of DNA and may consequently influence DNA binding (14). Based on the DNA-free structure of the quadruple mutant, there is no direct evidence that the N239Y substitution might produce new DNA contacts. This is consistent with the observation that the quadruple mutant shows virtually the same affinity for the gadd45 promoter DNA as the wild type core domain (13). We cannot, however, completely rule out that Tyr-239 might rearrange upon DNA binding in the context of the full-length protein, although this seems not likely on the basis of the packing of the tyrosine in the crystal structure. Equilibrium thermodynamic data (in the absence of DNA) clearly show that Tyr-239 has a major stabilizing effect on the structure of the p53 core domain (13). We cannot, however, completely rule out that the quadruple mutant shows virtually the same stabilization N239Y has not been selected by nature. Given the inherent low thermodynamic stability of p53, this might surprise at first glance. It seems that the need for a sufficient thermodynamic stability on one hand and conformational flexibility on the other hand have been carefully balanced out during the evolution of p53. Perhaps p53 has to retain a certain degree of structural plasticity, namely in the L3 region, to fulfill its various functions in vivo.

**Acknowledgments**—We thank Dr. Assaf Friedler for the peptide FL-CDB3 and Caroline Blair for assistance with protein purification.

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