Binding of Rad51 and Other Peptide Sequences to a Promiscuous, Highly Electrostatic Binding Site in p53*

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Homologous recombination is repressed by the binding of p53 to Rad51. We identified by fluorescence and NMR spectroscopy that peptides corresponding to residues 179–190 of Rad51 bind to the core domain of p53 in a site that overlaps with its specific DNA binding site. The p53 site is quite promiscuous, since it also binds peptides derived from 53BP1, 53BP2, Hif-1α, and BCL-X₁ in overlapping regions. Binding is mediated mainly by a strong, nonspecific, electrostatic component and is fine tuned by specific interactions. Competition of the different proteins with each other and with specific DNA for a single site in p53 could be a factor in regulation of its activity.

The tumor suppressor protein p53 is a transcription factor that maintains genome integrity by two means: transcriptional pathways, which trigger processes that lead to cell cycle arrest or apoptosis in response to oncogenic stress (1, 2), and nontranscriptional processes, which include binding Rad51 and thereby inhibiting homologous recombination (3). p53 is a homodimer that consists of several structural and functional domains. These include the N-terminal transactivation domain (aa1–60) (4), the proline-rich regulatory domain (aa 64–92) (5, 6), the DNA-binding core domain (p53CD; aa 94–312) (7), the tetramerization domain (aa 324–355) (8, 9), and the C-terminal negative regulatory domain (aa 360–393) (10). The core domain is responsible for the sequence-specific DNA binding of p53. It consists of a β-sandwich that serves as a scaffold for a DNA-binding interface (7). This interface is made of two flexible loops and a loop-sheet-helix motif (7). More than 100 proteins are reported to bind p53. Most of them bind at the N-terminal transactivation domain or at the C-terminal regulatory domain. Only a few proteins bind to the core domain. Of these proteins, 53BP1 and 53BP2 bind to p53CD via its DNA-binding interface (11), interacting mainly with the loops L2 and L3, in the crystal structures (12–14), as do Hif-1α (15) and Bcl-X₁ in solution (16). Amino acids 239–285 of CTF2 bind to p53CD at residues 161–223 (17), whereas residues 120–137 of Pirh2 also bind to p53CD (18).

Rad51, which is one of the most important proteins in regulation of homologous recombination, also binds to p53CD (19, 20) (reviewed in Refs. 3 and 21). Homologous recombination is a major process in which the cell repairs acute forms of DNA damage, such as double-stranded breaks (3, 21). In response to DNA damage, Rad51 mediates the first step of homologous recombination by forming large homo-oligomers along a single-stranded DNA chain at the point of the double-stranded breaks (see Refs. 3 and 21 for a review). The binding sites between Rad51 and p53CD were mapped, using deletion mutants of p53, showing that amino acids 94–160 and 264–315 in p53CD bind residues 125–220 in Rad51 (19). Binding of p53 to Rad51 inhibits Rad51 oligomerization and, as a consequence, inhibits homologous recombination (20).

Here, we have used peptide mapping in combination with biophysical techniques to characterize the interactions of p53CD with its partner proteins Rad51, CTF2, and Pirh2, interactions for which no structural information is available. We used NMR to identify the site on p53CD that binds the Rad51 peptides, which overlaps with the specific binding site for DNA, 53BP1, and 53BP2. We identified a strong electrostatic component in the binding of peptides derived from various p53CD-binding proteins by this site in p53CD. Our findings indicate that the DNA-binding interface of p53CD is a promiscuous site for protein binding, which functions via a large electrostatic component that is fine tuned by specific interactions. We discuss the implications for the regulation of the p53 response and for drug design.

EXPERIMENTAL PROCEDURES

Peptide Synthesis—Peptides were synthesized on a Pioneer peptide synthesizer (Applied Biosystems) using standard Fmoc (N-(9-fluorenylmethoxycarbonyl) chemistry. The peptides were labeled with fluorescein at the N-terminus on the Pioneer peptide synthesizer using a 4-fold excess of fluorescein-OSu (Molecular Probes, Inc., Eugene, OR) and a 4-fold excess of hydroxybenzotriazole. All other amino acids were purchased from NOVAbiochem. The peptides were purified on a Waters high pressure liquid chromatograph using a reverse-phase C8 semi-preparative column (Vydac) as described (22).

Protein Expression and Purification—Human p53 core domain wild type and mutants (residues 94–312) were cloned, expressed, and purified as described (23). N-labeled human p53 core domain was produced as described (24).

Analytical Ultracentrifugation—The equilibrium sedimentation experiments were performed on a Beckman XL-I ultracentrifuge using a Ti-60 rotor and six-sector cells at a speed of 30,000 and 40,000 rpm. All experiments were done at 10 °C. The sample volume was 50 μl. Samples were considered to be at equilibrium as was judged by comparing several scans at each speed. Buffer conditions were 50 mM Hepes, 5 mM dithiothreitol, pH 7.2. The ionic strength of the buffer was adjusted to the desired total ionic strength with a stock solution of 3 M NaCl in the same buffer. Data were processed and analyzed using UltraSpin software (available on the World Wide Web at www.mrc-cpe.cam.ac.uk).

Fluorescence Anisotropy—Fluorescence anisotropy titrations were
performed using a PerkinElmer Life Sciences LS-55 spectrofluorimeter equipped with a Hamilton Microlab 500 titrator and controlled by laboratory software, according to the procedure described (22). The fluorescein-labeled peptides were dissolved in 50 mM Heps buffer, pH 7.2, 5 mM dithiothreitol at the desired ionic strength to a final concentration of 0.05–0.1 μM. 1 ml of the peptide solution were placed in a cuvette, and p53 core domain (240 μl, ~100 μM) was titrated into the peptide in 40 steps of 6 μl. The fluorescence anisotropy was measured after each addition. The excitation wavelength was 480 nm (bandwidth 12 nm), and the emission wavelength was 525 nm (bandwidth 12 nm). Data were fit to a simple 1:1 equilibrium model: \[ R = R_c + (\Delta R^* [P] + K_d) \]
where \( R \) represents the measured fluorescence anisotropy value, \( \Delta R \) is the amplitude of the fluorescence change from the initial value (peptide only) to the final value (peptide in complex), \([P]\) is the added protein concentration, \( R_c \) is the starting fluorescence value, corresponding to the free peptide, and \( K_d \) is the dissociation constant for the complex. In some cases, a linear drift term was added to the above equation to account for nonspecific interactions.

For investigating the effects of the ionic strength, we used a starting buffer of 10 mM Hepes, pH 7.2, which has an ionic strength of 4.1 mM. The ionic strength was adjusted using NaCl. For highly charged peptides, such as poly(Glu), nonspecific binding was observed in 10 mM Heps, pH 7.2, due to very strong electrostatic interactions. Thus, the buffer used was 50 mM Heps, pH 7.2, which has a higher ionic strength of 20.5 mM.

**NMR Spectroscopy**—All 1H,15N HSQC spectra were acquired at various temperatures on a Bruker DRX 600-MHz spectrometer as described (24). Samples for NMR experiments contained 15N-labeled wild-type or mutant p53 core domain at a concentration of 180 μM and the corresponding peptides at a concentration of 1–2 mM. The low solubility of some of the peptides derived from Rad51, CTF2, and Pirh2 peptides limited their concentrations to 100–500 μM. The buffer was 150 mM KCl, 5 mM dithiothreitol, 2% D2O in 25 mM sodium phosphate, pH 7.2 (\( T = 207 \) °C). Chemical shift analysis was performed as described (22).

**RESULTS**

**Peptide Mapping of Rad51, Pirh2, and CTF2 Binding**

Domains from Rad51, Pirh2, and CTF2 are known to bind to the p53 core domain: Rad51 125–220 binds the p53 core domain residues 94–160 and 264–315 (19); Pirh2 residues 120–137 bind the core domain in an unknown site (18); and CTF2 residues 239–285 bind the core domain at residues 161–223 (17). We first analyzed which peptide fragments of Rad51, Pirh2, and CTF2 bound to p53CD. We synthesized fluorescein-labeled peptides of overlapping sequences corresponding to the putative p53-binding regions of these proteins (Table I) and studied their binding to p53CD using NMR, fluorescence anisotropy, and analytical ultracentrifugation at low ionic strength (\( I = 20.5 \) mM, no added salt). As a positive control, we used the peptide FL-CDB3, which is derived from the p53-binding loop of 53BP2 (residues 490–498) and binds at the DNA-binding interface of p53CD (22). As a negative control, we have used the peptide FL-ctrl (Table II), which does not bind p53CD (25). At \( I = 20.5 \) mM, peptides derived from Rad51 bound the p53 core domain with a reasonable affinity in the low micromolar range (Fig. 1, A–C; see below). The Pirh2 peptides bound much more weakly, with affinities between 200 and 600 μM (Fig. 1C). The CTF2 peptides bound p53CD with affinities between 75 and 150 μM (Fig. 1C).

**Defining the Sites of the Interaction between p53 and Rad51**

**Residues 179–190 of Rad51 Mediate Its Binding to p53CD**—
The initial peptide mapping revealed that residues 175–190 of Rad51 bound to p53 core domain; the peptide FL-CDB55 (Rad51 175–190) (Table I) bound the p53 core domain with a reasonable affinity in the low micromolar range. There was good agreement between the various techniques; fluorescence anisotropy studies gave \( K_d = 25 \) μM (Fig. 1A), whereas the AUC experiments gave \( K_d = 18 \) μM (Fig. 1B). Other Rad51 peptides bound p53CD much more weakly, with \( K_d \) in the range of 100–200 μM (Fig. 1, A and C).

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**TABLE I**

| Peptide     | Peptide sequence               | Derived from residues |
|-------------|--------------------------------|-----------------------|
| Rad51-derived peptides |
| FL-CDB50 | FL-MQGEFRTGKTQICHTL             | 125–140               |
| FL-CDB51 | FL-QCHTLAVTCQLPIDR             | 135–150               |
| FL-CDB52 | FL-QLP1DRGGE GGKAMYI           | 145–160               |
| FL-CDB53 | FL-GKAMYDTEGFTRPER             | 155–170               |
| FL-CDB54 | FL-TFRPERLLAV AERYGL          | 165–180               |
| FL-CDB55 | FL-AERYGLGSGDVLDNVA            | 175–190               |
| FL-CDB56 | FL-VLDNVAARAFNTD HQ            | 185–200               |
| FL-CDB57 | FL-FNTD HTQLLYQASAM            | 195–210               |
| FL-CDB58 | FL-YQASAMMVES RYALLI          | 205–220               |
| FL-CDB80 | FL-AERYGLSGS                  | 175–183               |
| FL-CDB81 | FL-CHLGSVDLID                 | 179–187               |
| FL-CDB82 | FL-GSVDLNDV                   | 182–190               |

a Derived from the mouse Pirh2 sequence.

b Derived from the human Pirh2 sequence.

To identify the precise region in Rad51 that binds the p53 core domain, we mapped FL-CDB55 (Rad51 175–190) by synthesizing three overlapping fluorescein-labeled 9-residue peptides derived from its sequence. The peptides, FL-CDB80, FL-CDB81, and FL-CDB82, were derived from Rad51 residues 175–183, 179–187, and 182–190, respectively (Table I). Binding of the peptides to p53 core domain was tested using fluorescence anisotropy (Fig. 1B). FL-CDB81 (Rad51 179–187) bound p53CD with \( K_d = 10 \) μM, whereas FL-CDB82 (Rad51 182–190) bound p53CD with \( K_d = 8 \) μM. FL-CDB80 (Rad51 175–183) had much weaker binding to p53CD, with \( K_d = 51 \) μM. We conclude that residues 179–190 are the major p53-binding residues of Rad51. These residues are from helix α10 and strand β10 (Fig. 2A), which are exposed on the Rad51 surface and are known to be involved in protein-protein interactions (26, 27) (see “Discussion” for details).

**The DNA-binding Interface of p53CD Binds the Rad51 Peptides**—We used HSQC NMR to determine the Rad51-binding site in p53CD by examining the changes in the chemical shifts of the backbone amides of 15N-labeled p53CD upon the addition of the Rad51-derived peptides. The NMR experiments were done at higher ionic strength (\( I = 207 \) °C), and their results were consistent with the fluorescence anisotropy and AUC results. Significant chemical shift changes were observed only for FL-CDB55 (Rad51 175–190), FL-CDB56 (Rad51 185–200), FL-CDB80 (Rad51 175–183), FL-CDB81 (Rad51 179–187), and FL-CDB82 (Rad51 182–190), which all contain parts of the p53CD-binding site described above. The NMR experiments did not detect binding of the other Rad51-derived peptides to p53CD. The residues in p53CD that changed their chemical shifts upon binding Rad51 peptides were mainly in the DNA-binding interface and were almost identical for the different Rad51-derived peptides. Fig. 2B shows the chemical shift changes in p53CD induced by binding of FL-CDB81 (Rad51
Promiscuous Binding Site in p53

Table II

| Peptide     | Charge | Sequence                  | \( K_d \) (\( I = 20.5 \text{ mM} \)) | \( K_d \) (\( I = 150 \text{ mM} \)) | \( \alpha^* \) |
|-------------|--------|---------------------------|--------------------------------------|-------------------------------------|----------------|
| FL-CDB3     | -5     | FL-REDEDEIEW              | 0.6                                  | 340                                 | -48            |
| Biotin-CDB3 | -5     | Biotin—REDEDEIEW          | 12                                   | 1500                                | -38            |
| Single mutants |      |                           |                                      |                                     |                |
| R490A       | -6     | FL-AEDEDEIEW              | 0.2                                  | 420                                 | -51            |
| E491A       | -4     | FL-RADEDEIEW              | 3.4                                  | 5400                                | -60            |
| D492A       | -4     | FL-REDEDEIEW              | 2.4                                  |                                     |                |
| E493A       | -4     | FL-REDADEIEW              | 2.2                                  |                                     |                |
| D494A       | -4     | FL-REDDEAEW               | 2.9                                  |                                     |                |
| E495A       | -4     | FL-REDDEADEW              | 1.8                                  |                                     |                |
| I496A       | -5     | FL-REDDEDEIEW             | 0.6                                  |                                     |                |
| E497A       | -4     | FL-REDEDEIAW              | 2.0                                  |                                     |                |
| W498A       | -5     | FL-REDEDEIEA              | 1.2                                  |                                     |                |
| Double mutants |      |                           |                                      |                                     |                |
| E491/3A     | -3     | FL-RADEDEIEW              | 8.7                                  | 9400                                | -53            |
| E491/7A     | -3     | FL-RADEDEIEW              | 8.6                                  |                                     |                |
| E493/5A     | -3     | FL-REDDEAEW               | 7.5                                  |                                     |                |
| E495/7A     | -3     | FL-REDDEAIW               | 6.1                                  |                                     |                |
| 490/6/8A    | -6     | FL-AEDEDEAE               | 0.2                                  |                                     |                |
| 496/8/6A    | -5     | FL-REDDEAE               | 1.6                                  |                                     |                |
| Triple mutants |      |                           |                                      |                                     |                |
| 490/6/8/8A  | -6     | FL-AEDEDEAE              | 0.3                                  |                                     |                |
| 491/2/3A    | -2     | FL-RADADEIEW              | 33                                   |                                     |                |
| 493/4/5A    | -2     | FL-REDDEAEW               | 28                                   |                                     |                |
| 494/5/7A    | -2     | FL-REDDEAAW              | 16                                   |                                     |                |
| Other mutants |      |                           |                                      |                                     |                |
| E491/3/5/7A | -1     | FL-RADEAIW                | 136                                  | 11,000                               | -38            |
| FL-poly (Glu) | -9    | FL-EEEEEEEEE           | 0.012                                 | 5800                                 | -101           |
| FL-ctrl (+5) |        | FL-RKSKKKITW             | ND                                   |                                     |                |

\( a^* \) are the mean activity coefficients of the ions, as described (29).

Peptide nomenclature is according to the residue numbers in the original 53BP2 sequence used for the crystal structure (12). Mutated residues are shown in bold face type. ND, no detectable binding.

Electrostatic Component of Binding

Our results for Rad51, taken together with known binding data for other proteins, suggest that the DNA-binding interface of p53CD serves as a general protein-binding site. This site contains numerous arginine residues that are also involved in DNA binding. We have calculated the electrostatic potential of p53CD (Fig. 3) and shown that the DNA-binding interface is indeed positively charged. This leads to the question of whether the DNA-binding interface of p53CD functions by a general electrostatic interaction or by specific binding to each and every target protein. We used the interaction of p53CD with the 53BP2-derived peptide FL-CDB3 (22), which is highly negatively charged, as a model to investigate the electrostatic component of peptide binding by this site in p53CD.

We mutated each of the residues in FL-CDB3 to alanine to determine the contribution of every side chain to binding to p53CD. The binding affinity of the resulting peptides to p53CD was measured at \( I = 20.5 \text{ mM} \) using fluorescence anisotropy (Table II). In general, substitution of the negatively charged Glu and Asp residues by Ala resulted in 4–7 times lower binding affinity to p53CD, with larger effects of mutation at the N terminus of the peptide. Mutation of the positively charged Arg increased the binding affinity to 0.2 \( \mu \text{M} \), whereas mutation of the hydrophobic Ile did not affect binding. Mutation of Trp reduced the affinity 2-fold (Table II). The effects of changes of charge were cumulative. Mutation of two negatively charged residues weakened the binding 10–15-fold to \( K_d \) of 6–9 \( \mu \text{M} \), whereas mutation of three negatively charged residues weakened \( K_d \) 30–70-fold, to the range of 16–33 \( \mu \text{M} \). Mutation of 4 Glu residues to Ala resulted in \( K_d \) of over 100 \( \mu \text{M} \). In all cases, the effect of mutation at the N terminus of the peptide was more pronounced than at the C terminus. Mutating groups of non-negatively charged residues did not reduce the binding affinity and in some cases even improved it (Table II).

Negatively charged residues also contribute to the binding of Rad51 to p53CD. Peptides containing Asp and Asp bound p53CD much more tightly than did the other peptides (compare FL-CDB80 with FL-CDB81 and FL-CDB82 in Fig. 1B). This indicates that the Rad51-p53CD interaction is also mediated by large electrostatic effects.

Effects of Ionic Strength—The contributions of electrostatic interactions can be detected by the effects of ionic strength, \( I \),
were screened for p53 core domain binding using AUC at 53BP2-derived peptide FL-CDB3 and a control nonbinding peptide labeled peptides derived from Rad51, CTF2, and Pirh2, as well as the fluorescence anisotropy.

mapping of the Rad51 175–190, the region that was found to bind p53 was tested using fluorescence anisotropy at Rad51 125–220 were synthesized, and their binding to p53 core domain 125–220. Overlapping 16-mer fluorescein-labeled peptides derived from Rad51, CTF2 and Pirh2 (for sequences see Table I). For peptide sequences, see Tables I and II.

Effects of Mutations in p53CD on Binding FL-CDB3—We measured the binding of FL-CDB3 to a series of p53CD mutants. These included structural hot spot mutants (G245S, R248A, R249S, and R282W), DNA contact mutants (R248A and R273H), and e-sandwich mutants (e.g. V157F, I195T, I232T, and I255F). The results could be divided into two categories. At I = 150 mM, FL-CDB3 bound p53CD extremely tightly at the lowest ionic strength, with \( K_d \) = 12 nM, 50 times lower than for FL-CDB3 (Table II). However, it had the highest ionic strength dependence (\( \alpha \)), consistent with mainly nonspecific ionic binding. The affinities of the different peptides to the p53 core at physiological ionic strength (I = 0.15 M) were extrapolated from the dependence on log \( f^* \) (Fig. 3, C and D), since the fluorescence anisotropy measurements were not feasible for values of \( K_d \) above about 100 \( \mu \)M. Of the peptides tested, FL-CDB3 had the highest affinity to 53CD core at I = 0.15 M with \( K_d \) around 340 \( \mu \)M (Table II). We measured the binding of FL-CDB3 directly by AUC at I = 0.15 M to be 210 \( \mu \)M, in good agreement with the extrapolation.

We used AUC to test for the binding of p53CD to the peptides derived from Rad51, CTF2, and Pirh2 (for sequences see Table I) directly at physiological ionic strength (I = 150 mM). \( K_d \) values for the binding of the Rad51 and Pirh2 peptides to p53CD under these conditions were greater than 1 mM, which is >100–200 times weaker than at I = 21 mM. Because of the sensitivity limits of the AUC method, we could not precisely measure such \( K_d \) values, which are in the millimolar range. At I = 150 mM, the CTF2-derived peptides bound p53CD with similar affinity to the Rad51-derived peptides. FL-CDB70 (CTF2 239–255) bound p53CD with \( K_d \) = 670 \( \mu \)M, FL-CDB72 (CTF2 260–275) bound p53CD with \( K_d \) > 1 mM, and \( K_d \) for FL-CDB73 (CTF2 270–285) binding to p53CD was 600 \( \mu \)M. Binding of the Rad51 peptides to p53CD at I = 150 mM was still highly specific, as could be seen from the HSQC NMR binding experiments (Fig. 2), which were all done at I = 207 mM. We conclude that, in general, peptide binding by the DNA-binding interface of p53CD is highly dependent on the ionic strength and is strongly electrostatic.

Effects of Mutations in p53CD on Binding FL-CDB3—We measured the binding of FL-CDB3 to a series of p53CD mutants. These included structural hot spot mutants (G245S, R248S, and R282W), DNA contact mutants (R248A and R273H), and e-sandwich mutants (e.g. V157F, I195T, I232T, and I255F). The results could be divided into two categories. At I = 20.5 mM, FL-CDB3 bound most mutants with \( K_d \) values in the range of 0.5–1 \( \mu \)M, similar to that for wild type (Fig. 4D). However, binding was weaker for all of the arginine mutants, reaching more than 3 \( \mu \)M (5–6-fold weaker binding than for wild type) for the surface-exposed mutants R248A, R248S, and R273H. This is probably due to the mutation weakening the electrostatic component of the interaction.

**Fig. 1.** Binding of peptides derived from Rad51, CTF2 and Pirh2 for p53 core domain binding. A, peptide mapping of Rad51 125–220. Overlapping 16-mer fluorescein-labeled peptides derived from Rad51 125–220 were synthesized, and their binding to p53 core domain was tested using fluorescence anisotropy at I = 20.5 mM. B, peptide mapping of the Rad51 175–190, the region that was found to bind p53 core domain. Overlapping 9-mer fluorescein-labeled peptides were synthesized, and their binding to p53 core domain was tested using fluorescence anisotropy. C, peptide screening using AUC. Fluorescein-labeled peptides derived from Rad51, CTF2, and Pirh2, as well as the 53BP2-derived peptide FL-CDB3 and a control nonbinding peptide were screened for p53 core domain binding using AUC at I = 20.5 mM. For peptide sequences, see Tables I and II.
DISCUSSION

A Multipurpose Promiscuous Binding Site—Many protein-protein interfaces contain regions of residues that are crucial for the affinity to other proteins and are promiscuous. These regions are highly adaptable, and many proteins use them to bind numerous partners. This adaptivity can be achieved by structural changes in the promiscuous areas that are induced upon binding and are different for each binding partner (reviewed in Ref. 30).

Our results for Rad51 and CTF2, taken together with published data, show that the DNA-binding interface in p53CD serves as such a multipurpose promiscuous protein-binding site. This site mediates binding of most of the p53CD-binding proteins shown in Table III, including Rad51, HIF-1α, Bcl-XL, CTF2, 53BP1, and 53BP2 (see references in Table III). In addition, this site binds heparin (31). For Pirh2, we have detected very weak binding affinity to p53CD. Our studies were done with peptides derived from the p53CD-binding sequences of the corresponding proteins. The affinity of p53CD for the full proteins could differ, therefore, from the affinity for the peptides. Nevertheless, peptide mapping is a reliable way to delineate the site in p53 that interacts with the parent proteins as, for example, with peptides derived from HIF1-α (15, 32).

\(^2\) In Ref. 30, Wells refers to these areas as binding “hotspots.” However, this terminology would be confusing when applied to p53, since it has mutational “hotspots.” Thus, we relate to these areas as promiscuous binding regions.
The DNA-binding interface of p53CD has several characteristics of a promiscuous binding area for protein-protein interactions. It contains several loop regions, which can adapt themselves to different partner proteins, and it contains many arginine residues, which are known to be frequent in such promiscuous binding areas. We have previously shown using NMR that the DNA-binding interface of p53CD is flexible, whereas the β-sandwich scaffold is much more rigid (24, 33). This makes the DNA-binding interface more adaptive to different target proteins and generally makes it more suitable for protein binding.

The promiscuous binding site in p53CD contains two subsites. 1) Loop L1 and helix H2, on one edge of the DNA-binding interface, form a well defined site that was found by NMR studies to bind Rad51, HIP-1α (32) and the 53BP2-derived peptide FL-CDB3. This subsite is shown in Fig. 5A. 2) Loops L2 and L3, situated on the other edge of the DNA-binding interface, which were found by crystallography to be involved in binding of 53BP1 (13, 14) and 53BP2 (12), were found using NMR to be involved in binding of Bcl-X<sub>L</sub> (16) and by cell biology methods to bind CTF2 (17). Interestingly, although each of these subsites is sufficient to mediate protein binding on its own, the full DNA-binding interface (containing both of the subsites) is needed for DNA binding. Indeed, mutations in DNA-binding residues in one of these subsites are enough to abolish DNA binding (31, 34).

**The Promiscuous Binding Site in p53CD Has a High Electrostatic Component**—The promiscuous binding site of p53CD mediates protein binding with a strong electrostatic component. Binding to the peptides tested was highly dependent on the ionic strength, regardless of the peptide charge or of the parent protein (peptides derived from 53BP2, Rad51, Pirh2, and CTF2 were tested) (Fig. 4). The binding of peptides to p53CD at \( I = 150 \text{mM} \) was 100–500-fold weaker than binding at \( I = 20.5 \text{mM} \) (Figs. 1 and 4 and Table II). A detailed analysis using derivatives of FL-CDB3 showed that a plot of \( \log K_d \) versus \( \log I \) was linear, demonstrating that electrostatic effects were operating and dominant (29).

There are specific binding interactions superimposed on the general electrostatic component, as seen from the stronger binding of FL-CDB3 compared with FL-poly(Glu) at physiological ionic strength. FL-poly(Glu) interacts with p53CD solely on an electrostatic basis, and as expected its binding was by far the most dependent on the ionic strength. For many protein-protein interactions, there is an early formation of a weak specific complex through electrostatic interactions, followed by a precise “fine tuning” to form the high affinity complex (29), and this might well be the case for the peptide binding of p53CD. Of the peptide derivatives tested, FL-CDB3 is the optimal for p53 core binding at \( I = 0.15 \text{m} \) and has the right balance between residues that contribute to the electrostatic association and residues that contribute to the precise docking.

DNA binding of the promiscuous binding site in p53CD has similar characteristics to protein binding (35) and is also mediated by a combination of electrostatic and specific interactions. At low ionic strength, nonspecific electrostatic interactions dominate, and it is only at high ionic strength (\( I > 150 \text{mM} \)) that the specific binding is paramount. The high electrostatic contribution dictates that even small variations in the ionic strength would lead to large variations in binding affinity. Very precise control of the ionic strength is crucial for the successful experimental measurement of p53CD interactions.

**p53 and Rad51 Binding Sites**—The interaction of p53 with Rad51 is the key element in the nontranscriptional pathway in which p53 maintains genome stability (3, 21). The Rad51 binding residues in p53 mapped to its DNA-binding interface, mainly including residues from loop L1 and helix H2 (Fig. 2, C and D). This binding site is consistent with the mapping by Buchhop et al. (19) of the binding residues to be between residues 94 and 160 (which include loop L1) and 264–315 (which include helix H2) (19). Buchhop et al. (19) described these two regions as two separate binding sites, but our NMR studies revealed that these regions are contiguous, forming one binding site (Figs. 2D and 5A). We have also found chemical shift changes for residues outside this region, such as the loop L3 residues Asn<sup>239</sup>, Met<sup>243</sup>, and Gly<sup>244</sup>. These residues might form a part of an extended binding site but would not have been apparent in the earlier studies, which used different constructs of p53 (19).

In Rad51, we have found that the p53-binding interface consists of residues 179–190. These residues form helix α<sub>10</sub> (residues 181–188) and the N-terminal half of strand β<sub>3</sub> (residues 189–190). The residues that form the p53-binding interface can be divided into two categories. The N-terminal part of the binding site, which is mainly helical, is solvent-exposed and was not shown to be involved in binding of other proteins. However, the C-terminal part of the p53-binding site overlaps the oligomerization site of Rad51 (Fig. 5B) (26) as well as its binding site to BRCA2 (Fig. 5C) (27), making it a promiscuous site for protein binding. Strand β<sub>3</sub> is a part of the oligomerization interaction between Rad51 monomers and was found to be buried in the structure of the Rad51 oligomer (26). Helix 10, the major p53-binding region, is solvent-exposed even in the context of the Rad51 oligomer (26). Similarly, residues Leu<sup>186</sup>, Val<sup>189</sup>, and Ala<sup>190</sup> participate in formation of the hydrophobic pockets that bind BRCA2, whereas Asp<sup>187</sup>, Val<sup>189</sup>, Ala<sup>190</sup>, and Tyr<sup>191</sup> form a series of hydrogen bonds with residues 1525–1528 of BRCA2 (27). In summary, the p53-Rad51 interaction is mediated by promiscuous areas in both proteins. The DNA-binding interface in p53 core domain binds a general protein-binding site in Rad51.

Linke et al. (20) demonstrated that wild-type p53 inhibits Rad51 oligomerization, and our results show that p53 does so by binding to the edge of its oligomerization site, in strand β<sub>3</sub>. Our results also provide the structural explanation to several mutational studies and binding studies reported in the literature. p53 core domain mutants at residues 175, 248, and 273 were shown to stimulate, rather than inhibit, Rad51-mediated homologous recombination (36). Our results show that these
mutations are close to the Rad51-binding interface and thus probably impair the interaction with Rad51. Similarly, the Rad51 mutant L186P exhibits reduced binding to p53 (20), and the p53 mutants R273H and R249S exhibit reduced binding to Rad51 (19, 20), because the mutations are in the binding interface.

Implications of a Promiscuous Binding Site in p53—Not only does p53CD bind its partner proteins via a general promiscuous binding site, but it also seems that some of its partner proteins use the same strategy. For example, the ankyrin repeats and Src homology 3 (SH3) domain that 53BP2 uses to bind p53CD (12) are also involved in binding other proteins such as NF-κB (37), YAP (38), Bcl-2 (39), and protein phosphatase 1 (40). 53BP1 binds p53CD via its BRCT domain, which is another general protein recognition motif (13, 14). As shown above, Rad51 uses a general protein-binding interface, close to its oligomerization interface, to bind p53CD. The use of such general binding sites in the p53 pathway means that interac-

![Graph A](image1.png)  ![Graph B](image2.png)  ![Graph C](image3.png)  ![Graph D](image4.png)

**FIG. 4.** The electrostatic component of peptide binding to p53 core domain. A, ionic strength dependence of peptide binding to p53 core domain. Binding of FL-CDB3-, FL-poly(Glu)-, and FL-CDB3-derived peptides to the p53 core domain at various ionic strengths was determined using fluorescence anisotropy. Log $K_d$ versus the log of the total ionic strength is shown for the various peptides. B, Same as A, but showing the dependence of $K_d$ on log $f^*$, where $f^*_m$ is the mean activity coefficient (29). For the results of the curve fits, see Table II. C, the dependence of $K_d$ on log $f^*$ for binding of p53CD R273H to FL-CDB3. D, binding of FL-CDB3 to p53 core domain mutants. The binding constants were determined using fluorescence anisotropy.

| Protein | Binding site in p53 core domain | Reference |
|---------|--------------------------------|-----------|
| 53BP1   | DNA-binding interface          | 13, 14    |
| 53BP2/ASPP | DNA-binding interface         | 12        |
| Hif-1α  | DNA-binding interface          | 15        |
| Bcl-Xl  | DNA-binding interface          | 16        |
| Rad51   | aa 94–160, 264–315             | 19        |
| Pirh2   | NA                            | 18        |
| CTF2    | aa 161–223                    | 17        |
| BRCA1<sup>ab</sup> | NA                    | 43, 44    |
| HPV-16 E6 | aa 66–326<sup>c</sup>               | 45        |
| MDM2<sup>d</sup> | Strands 9 and 10              | 46        |

<sup>a</sup> NA, not applicable.
<sup>b</sup> We have recently shown that the BRCT domain of BRCA1 does not interact with p53 core domain (47).
<sup>c</sup> This region of p53 also spans the proline-rich domain and extends well beyond the core domain.
<sup>d</sup> It was shown that RNA is needed to facilitate this interaction.

![Table III](image5.png)
tions in the p53 pathway should not be studied in isolation, but as competitive processes for the same binding sites.

The presence of promiscuous protein-binding sites in the p53 pathway may be crucial for the regulation (Fig. 5D). p53 can be regulated at two levels: direct, by binding of proteins to the core domain, or indirect, by binding of proteins to regulatory domains such as the C-terminal domain and by post-translational modifications of such domains (41). Most p53-binding proteins interact with it outside of the core domain and indirectly regulate the DNA binding of p53CD, with post-translational modification being a key factor for this indirect regulation. Direct regulation by binding the core domain is also efficient, since a protein bound to the core domain will inhibit its DNA-binding and hence its transcriptional activity. This could explain why only a few proteins bind the core domain and why these proteins are so distinct from each other and are involved in different pathways. One could speculate that once p53 is activated, protein binding to the core domain could be a key event in regulation of the pathway that p53 would choose. For example, binding of 53BP2/ASPP will activate apoptosis, whereas binding of Rad51 will inhibit homologous recombination and activate the nontranscriptional p53 response. The relative affinities of p53CD to its binding proteins could thus be an important factor in selecting for the p53 response. For example, different affinities of p53CD to Rad51 versus other binding proteins could determine whether the p53 response will proceed via the transcriptional or the nontranscriptional pathway. Such a model also explains the lack of post-translational modifications in the core domain. Being directly regulated by the binding of proteins that inhibit its DNA-binding, there is no need for such an indirect regulation process.

Implications for Rescue of Mutant p53 and for Drug Design—
The promiscuous protein-binding site in p53CD is frequently mutated in cancer, and the six most frequent cancer-associated mutations in p53 (“hot-spot mutations”) map to this site (7). The frequently mutated residues (Gly245, Arg248, and Arg249 from loop L3, Arg282 from helix H2, Arg175 and Arg273) are important not only for DNA binding and protein stability (34) but are also involved in protein binding. Inactivation of p53 due to mutations is then not only due to loss of DNA binding but also due to loss of the ability to bind partner proteins. Indeed, it had been shown that hot-spot mutants of p53 show reduced binding to Rad51 (19) and stimulate rather than inhibit homologous recombination (36) and that p53 mutants are unable to bind 53BP2 (11). Mutant p53 that lacks protein-binding ability will also be defective in its ability to select for its response. As a consequence, mutant p53 is unable to proceed with both its transcriptional and nontranscriptional activities.

Since p53CD is mutated in more than 50% of human cancers, rescue of mutant p53 is an important goal in cancer therapy (34). Many of the oncogenic mutations of p53 cause the core domain to denature at body temperature (23). A promising strategy is the development of small molecule chemical chaperones that stabilize, refold, and reactivate mutant p53 (22, 34). Our previous studies suggest that once p53 is denatured, it cannot be reactivated, so small molecule chaperones are effective only by binding to p53 during or immediately after biosynthesis (34, 42).

Which site in p53CD should chemical chaperones target? p53CD does not contain obvious binding pockets for small molecules, and its interactions with proteins and the DNA are mediated by a large interface. The two possible target sites for chemical chaperones are the promiscuous protein-binding site or the relatively inert β-sandwich. Chemical chaperones like FL-CDB3, which bind p53CD in its general protein-binding site, have to bind strongly enough that they can stabilize p53

![Fig. 5. Implications of promiscuous protein binding sites.](http://www.jbc.org/)
but weakly enough so that they do not inhibit DNA or protein binding. It would be advantageous to have putative chemical chaperones that bind outside the promiscuous site so that they could bind simultaneously with the partner protein or the DNA and so not inhibit. However, it appears that the DNA-binding interface in p53CD is the major surface for ligand binding, and it mediates the binding of ligands of all types: proteins, DNA, and peptides. Since it is the only site in p53 that seems to contain hot spot areas for binding, this site should serve as a target for p53-stabilizing drugs of the right affinity to stabilize but not unduly inhibit.

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Binding of Rad51 and Other Peptide Sequences to a Promiscuous, Highly Electrostatic Binding Site in p53

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