Human p53 Binds Holliday Junctions Strongly and Facilitates Their Cleavage*

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Holliday junctions in DNA are generated as a product of homologous recombination events. To test the hypothesis that human p53 may bind to Holliday junctions, synthetic junctions with four ∼75-base pair (Hol75) or ∼565-base pair (Hol565) arms were generated. As seen by electron microscopy, under conditions in which 50–61% of the Hol565 DNAs were bound by p53, 80–96% of the p53 was located specifically at the junction with, in the latter case, only 4% of the p53 visualized at the DNA ends or along the arms. Given the large number of potential binding sites, this represents very high specificity for the junctions. Gel retardation assays using the Hol75 DNA confirm these observations, and indicate that the tight junction complexes have a half-life of greater than 4 h. The binding of p53 to three-way junctions is severalfold less than to four-way junctions. Addition of p53 greatly increases the rate of resolution of the Hol75 DNA by T4 endonuclease VII and T7 endonuclease I, two enzymes known to cleave such junctions. This latter finding further confirms the interaction of p53 with Holliday junctions and suggests that p53 binding facilitates their resolution in vivo.

Holliday junctions in DNA are produced by homologous recombination events that occur spontaneously or during the repair of DNA damage. Recombination and thus Holliday junctions are seen at high frequency in meiotic cells. In mitotic cells, they are believed to appear in G1, and in S phase with the repair of replication errors (1, 2). The appearance of Holliday junctions following DNA damage is inferred from the elevated levels of sister chromatid exchanges observed following damage (3). If these junctions are not resolved prior to metaphase, then during segregation, chromosomes will remain attached, resulting in duplication, loss, or breakage. Thus these DNA junctions represent essential structural intermediates of homologous recombination, downstream products of DNA repair, and, at certain times in the cell cycle, severe lesions.

Cell cycle checkpoints exist in part to ensure that deleterious structures in DNA are resolved or repaired prior to the cell progressing to the next phase of the cell cycle. In higher eukaryotic cells, these checkpoints involve the tumor suppressor protein p53, which has been shown to play a key role in blocking the cell at the G1/S border in response to DNA damage (4). This is accomplished through transactivation of cell cycle inhibitors, such as p21 (5, 6). More recently p53 has also been shown to be responsible for a G2/M block in response to damage, possibly by the same mechanism involved in the p53-induced G1/S block (7–9).

p53 also plays a role in suppressing genomic instability in particular changes related to DNA damage. Mouse fibroblast cells deficient in p53 show a 2-fold increase in the frequency of UV-induced sister chromatid exchanges (10). Uncontrolled gene amplification also appears to be kept in check by p53. Bischoff et al. (11) observed that cultured fibroblast cells from a Li-Fraumeni syndrome patient (loss of germ line wild type p53) exhibit chromosome rearrangements and develop aneuploidy. Furthermore, Livingstone et al. (12) noted that these cells exhibit at least a 102-fold increase in gene amplification events leading to the development of aneuploid cell populations, and Yin et al. (13) showed that this phenomena could be reversed by introduction of wild type p53 in these cells.

Connections between p53 function and homologous recombination, an event likely to be closely linked to gene amplification, have come from studies showing that wild type p53 will down-regulate homologous recombination between SV-40 molecules (14), the increase in rates of homologous recombination in p53 mutant cell lines (15), the observation of a 3’ to 5’ exonuclease (16) and strand transfer (17) activities in p53, and the demonstration that p53 will directly bind to RAD51 and RecA proteins and, in doing so, can inhibit their function (18). A further link between p53 and recombination may be found in the observation of unusually high levels of p53 in certain mitotic cells such as those in the testes and in oocytes (19, 20). Here the elevated levels of homologous recombination may require higher levels of p53 to limit the extent of recombination and to monitor the level of recombination-induced errors. If p53 is able to keep a check on deleteriously high levels of homologous recombination in vivo, then identification of which steps or structures in DNA may be involved becomes a key question and may be related to its ability to sense damage in DNA.

Following damage to DNA by a variety of agents including irradiation and mutagenic drugs, the activity of p53 increases. This increase is due in part to a prolonged half-life resulting from post-translation modification, and in part to an increased translation of the protein itself, but not to increased transcription of the p53 gene (Ref. 21; reviewed in Ref. 22). The mechanism by which p53 signals the presence of damage in DNA has not been fully understood, but recent work suggests that it may in part occur at the level of direct recognition and binding of p53 to sites of damage. Recently we showed that p53 and its 14-kDa C-terminal fragment directly recognize DNA damage in the form of extra base bulges (insertion/deletion loop-type mismatches), which can arise from errors in replication or recombination between sequences that are not fully homologous (23). Additionally, Reed et al. (24) showed that p53 will bind to DNA damaged by ionizing radiation. If p53 possesses a generalized ability to directly sense unusual structures in DNA, then it...
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**EXPERIMENTAL PROCEDURES**

**Materials: DNA and Proteins**

The oligonucleotides used to construct the J12 junction with 4-base AGCC 3' overhangs were synthesized on an Applied Biosystems model 380B DNA synthesizer (Lineberger Comprehensive Cancer Center nucleic acids core facility) deprotected, purified by electrophoresis in 12% polyacrylamide gels containing 7% urea, annealed, and electrophoresed in 10% nondenaturing polyacrylamide gels to remove any single-strand DNA. The addition of 538-bp duplex arms, which contain one blunt end and one end with a GGCT 3' overhang, is described in Ref. 23. The 50-bp arms were made by direct synthesis of two complementary oligonucleotides that generated a duplex with the same sequence as the first 50 bp of one 538-bp arms beginning at the overhang. The arms were ligated to the J12 junction DNA at 21 °C for 2 h. The resulting four-armed Holliday junction DNAs were purified by electrophoresis on agarose or acrylamide gels. A 43-base oligonucleotide (5'-CAAGT CAT AGA CGA TTA CAG CCA GAA TTC GGC AGC GTC AGC C-3') was synthesized and used to generate the three-way junction DNA.

p53 protein was purified as described in Ref. 25 from nuclear extracts prepared from SF9 cells infected with a *Autographa californica* polyhedrosis virus recombinant for the human p53 gene. The purified protein was estimated to be greater than 90% pure as judged by Coomassie Blue staining on an SDS gel and Western blotting using the monoclonal antibody 421 to p53 protein. Restriction endonucleases, T4 DNA ligase, and T4 DNA Kinase were purchased from New England Biolabs and were used according to the manufacturer's specifications. T7 gene 5 protein with thioredoxin was the gift of Charles Richardson (Harvard University, Cambridge, MA). Bacteriophage T4 endonuclease VII and T7 endonuclease I were the kind gift of Dr. Brorres Kemper (University of Koln, Koln, Germany).

**Methods**

**Electron Microscopy**—p53-DNA complexes were formed by incubating 100 ng of DNA in 50 ml of 10 mM Hepes (pH 7.5), 100 mM NaCl buffer for 20 min at 21 °C using 5 mg protein tetramers/junction DNA. The complexes were fixed with 0.6% glutaraldehyde (v/v) for 10 min at 21 °C, followed by filtration through 2-mL columns of Bio-Gel A5m (Bio-Rad). To mount the DNA or the DNA-protein complexes for EM, the samples were mixed with a buffer containing 2 mM spermidine, 2 mM MgCl2, 0.15 mM NaCl, 0.1 mM EDTA, and applied for 30 s to thin carbon foils supported by 400-mesh copper grids. The grids were washed with a water/ethanol series, air-dried, and rotary shadowcast at 10°7 torr with tungsten (26). Samples were examined in a Philips CM12. For publication, images were digitized using a CoHu CCD camera attached to a Macintosh computer and the contrast adjusted using the NIH IMAGE software. Angles formed by arms in the Holliday junction DNAs were measured by capturing the molecule images with the CCD camera and using routines in the NIH IMAGE software. Measurement of the projected areas of protein-DNA complexes was done by capturing the image from the EM film with the CoHu CCD camera and using the area measurement capability of NIH IMAGE. Mass analysis was carried out by forming the p53-DNA complexes as described above, then mixing them with a dilute solution of T7 gene 5 protein complexed with thioredoxin (T7 DNA polymerase, 104 kDa mass) that had been fixed with glutaraldehyde. The mixture was mounted for EM. Micrographs were taken of fields of p53 Holliday junction complexes with the DNA polymerase molecules in the background. For each field, the average projected area of the DNA polymerase molecules was determined. This was then compared to the projected areas of the p53 Holliday junction complexes in the same field. The mass of the p53 complexes was calculated by multiplication of the mass of T7 DNA polymerase times the ratio of the projected areas to the 3/2 power.

**Gel Retardation Assays**—For the gel retardation assays, the DNAs were labeled with [γ-32P]ATP using T4 DNA kinase and then passed through G50 spin columns. Details of amounts of DNA, incubation temperature, and time are in the text and figure legends. Amounts of DNA with the same amount of radioactivity were mixed with loading dye and electrophoresed on 4% polyacrylamide gels (19:1 acrylamide: bisacrylamide) at 200 V at 4 °C in 0.5 × TBE buffer until the unbound DNAs migrated close to the bottom of the gel. The DNA was visualized using a Molecular Dynamics PhosphorImager and direct autoradiography using a Kodak scientific imaging film. Binding buffer for the gel retardation assays contained 100 mM NaCl, 10 mM Hepes (pH 7.5), 10% glycerol, 100 μg/ml BSA, 1 mM DTT, and 0.02% Nonidet P-40 to aid in stabilizing p53 at the low protein concentrations.

**RESULTS**

**Synthesis and Visualization of Synthetic Holliday Junction DNAs with 75-bp and 565-bp Arms**—DNAs with centrally located Holliday junctions were constructed from oligonucleotides based on the J12 molecule, whose physical structure and properties have been characterized in detail (27). This junction is able to branch migrate over a 12-bp core (see Fig. 1 in Ref. 28). In studies of the J12 and other junctions, it has been shown that in the presence of even low concentrations of spermidine or Mg2+ ions, or relatively high Na+ ions, the junctions adopt a stacked X configuration with the smaller angles between the DNA arms being roughly 60°. Furthermore, with the J12 junction in the presence of Mg2+ ions, the arms lie in an antiparallel manner and base pairing is fully preserved at the junction as demonstrated by chemical cleavage studies (27). In the absence of cationic metals, the junction opens into a square configuration with the arms separated by right angles (reviewed in Ref. 29; see also Refs. 30–32). Here the J12 junction was synthesized with 23–28-bp arms including a 4-base AGCC 3' overhang (termed Hol565 DNA). For the EM experiments, the arms were extended an additional 50 or 542 bp to generate molecules with 73–78-bp or 565–570-bp arms termed Hol75 and Hol565 DNAs, respectively. The sequence of the arms was from the plasmid pBR322 (see "Experimental Procedures"; see also Ref. 29). The resulting four-armed junction DNAs separated well away from the partial ligation products on agarose gels with less than 5% of the DNA, as judged by EM and gel electrophoresis, consisting of DNAs with three or fewer arms.

To attach DNA to the thin carbon foils used as EM supports by the methods utilized here the DNA is adsorbed in the presence of a mixture of 150 mM Na+, 2 mM Mg2+, and 2 mM spermidine ions (Ref. 26; see also “Experimental Procedures”). When the Hol565 DNA was mounted for EM in this way, examination of fields of DNAs revealed two forms (Fig. 1A). In one form (30%), the DNA arms were arranged side-by-side and frequently appeared wrapped about themselves. In the other (70%–79%), the arms were spread apart creating an X or + structure. Examination of the small Hol75 DNAs revealed five forms: X-shaped molecules, +-shaped molecules, “K”-shaped molecules (i.e. having unequal angles across the junction), Y-shaped molecules (created by two of the arms lying side-by-side), and finally straight rods (resulting from two pairs of arms lying side-by-side) (Fig. 1, B and C). The fraction of molecules in each class were 14%, 8%, 6%, 43%, and 29%, respectively.

1 The abbreviations used are: EM, electron microscopy; ssDNA, single-strand DNA; CT DNA, PvuII-digested calf thymus DNA; bp, base pair(s); BSA, bovine serum albumin; DTT, dithiothreitol.
51% of the DNA molecules scored (n = 233) had p53 bound somewhere on the DNA, and in 80% of these molecules p53 was bound exactly at the junction. Of the remainder, 10% contained p53 bound at the end of an arm and another 10% had p53 bound internally along an arm. When the incubation was extended to 30 min, 61% of the DNA molecules scored (n = 153) showed p53 bound, and in 96% of these molecules, p53 was bound at the junction, and only 2% each had p53 at a DNA end or along an arm. Here approximately two-thirds of the total p53-DNA complexes were not in aggregates and thus could be clearly scored.

It was not possible to determine whether the distribution of angles by which the DNA arms exited the p53 complex was more consistent with p53 holding the junction into an open cross (90°), a stacked X (60° and 120°) configuration, or some other arrangement. p53 could be seen bound to the Hol565 DNA, but only short DNA stubs exited the large protein complex (usually tetramers and higher oligomers). Inspection of the Hol565-DNA-p53 complexes showed no examples in which the DNA arms were in a Y shape or lay side-by-side. Instead, the arms were arranged into X, +, and K structures. The angular distributions, however, were broad and suggestive of structures that might be intermediate between the stacked X and open cross arrangements.

To estimate the mass of the p53 particles bound at the junction, the projected area of the p53 complexes was compared with that of T7 DNA polymerase (see “Experimental Procedures”). This analysis showed that 6% of the junctions were complexed by p53 monomers, 15% were bound by p53 dimers, 46% by p53 trimers or tetramers, and the remainder (33%) contained higher oligomeric forms of p53.

It was of interest to ask whether p53 will also recognize three-way junctions. Three-way junctions were generated using the 565-bp arms employed with the Hol565 DNAs. A separate 43-base oligonucleotide was synthesized and annealed to oligonucleotides 37 and 38 described in Table I of Alani et al., (28) used to generate the four-way junction DNAs. Following addition of the arms and purification (see “Experimental Procedures”), the resulting three-way junction DNAs were mixed with the Hol565 DNA in a 1:1 ratio. p53 was then added to molar ratios of 0.5, 1.0, or 10.0 p53 tetramers/DNA. Following a 20 min incubation at room temperature as in Fig. 2, the fraction of three-armed or four-armed DNAs with p53 bound at the junction was scored by EM. At a saturating level (10:1) of p53, 67% (n = 90) of the four-way junction DNA contained p53 at the junction as contrasted to 51% (n = 134) bound to the three-way junction DNA. Reduction of p53 to a 1:1:1 ratio resulted in scoring 23% of the four-way junction DNA with p53 bound (n = 100) as contrasted to 7.5% of the three-way junction DNAs (n = 134). Further reduction of p53 to a 0.5:1:1 ratio resulted in 9% of the four-way junction DNAs with p53 bound (n = 102) as contrasted to 3% of the three-way junction DNA (n = 120). Thus, while saturating levels of p53 resulted in only a 30% higher fraction of the four-way junction DNA bound by p53, limiting concentrations of p53 resulted in a ~3-fold difference in binding affinity under identical conditions. In these experiments the total nonspecific binding was less than 10% that of the specific junction complexes. In the EM experiments above with three-way and four-way junctions and with Hol75 or Hol565 DNAs, inclusion of 2 mM Mg2+ was not found to greatly affect p53 binding.

Gel Retardation Assays Confirm the Tight Specific Binding of p53 to Holliday Junctions—Gel retardation assays were used to confirm and quantify the binding of p53 to Holliday junctions. Hol565 DNA (1 ng, 32P-labeled) was incubated with p53 at molar ratios of 0, 5, 10, 20, or 40 tetramers/DNA in the presence of 100 ng of PvuII-digested calf thymus DNA (CT DNA) for 20 min at room temperature, and under three different salt conditions: 30 mM NaCl, 130 mM NaCl, and 30 mM NaCl with 5 mM MgCl2 (Fig. 3). The sample was then electrophoresed on a 4% polyacrylamide gel (see “Experimental Procedures”). Prominent retarded bands representing p53-Hol565 DNA complexes were apparent even at the lowest molar ratio of p53 to DNA (Fig. 3, lanes 2, 7, and 12). With increasing amounts of p53...
added, most of the DNA was shifted into the retarded positions for all three salt conditions. It was noted (data not shown) that above 300 mM NaCl, the formation of p53-Holliday junction complexes was inhibited. Concentrations of MgCl₂ above 10 mM had a similar effect. This might reflect either a direct inhibition of binding or the sequestering of protein or DNA into aggregates. Based on the EM observations, the multiple retarded bands likely represent different oligomeric states of p53 bound at the junction. Incubation of the Hol₇₅ DNA with E. coli SSB protein, a strong single-strand DNA-binding protein at a ratio of 10 SSB tetramers/DNA produced no significant retarded bands as seen by autoradiography (data not shown). The greater binding of p53 in low salt as contrasted to the two higher salt concentrations may reflect the change in structure of the Holliday junction that occurs between these two conditions. This junction has been shown to exist in a more open conformation in 30 mM NaCl, but a fully base-paired, stacked X conformation in the higher salt conditions (27, 29–32) and the latter conformation might be less effective in binding p53.

Gel retardation assays coupled with the addition of competitor DNA provide a means of examining the specificity and stability of DNA-protein complexes. Here radiolabeled Hol₇₅ DNA (1 ng) was mixed with increasing amounts (10, 35, 60, 110, and 210 ng) of CT DNA followed by the addition of p53 (2.5 p53 tetramers/Hol₇₅ DNA) in 10 mM Tris (pH 7.5), 5 mM MgCl₂, 100 μg/ml BSA, 1 mM DTT (binding buffer) for 20 min at room temperature and then electrophoresed. As shown in Fig. 4 (lanes 1–5), the retarded bands in the gel representing the p53-DNA complexes remained prominent even in the presence of a 200-fold excess of competitor DNA (lane 5). When unlabeled Hol₇₅ DNA was added as a competitor (25, 50, 100, or 200 ng with in each case 10 ng of CT DNA; lanes 7–10), the retarded bands were gradually lost, as would be expected by a simple dilution of the probe.

To examine the stability of the specific junction complexes, 1 ng of ³²P-labeled Hol₇₅ DNA was incubated with p53 (2.5 p53 tetramers/DNA) in binding buffer with 10 ng of CT DNA for 20 min, followed by the addition of 50 ng of unlabeled Hol₇₅ DNA for an additional 15, 30, 60, 120, and 240 min on ice (Fig. 5A, lanes 2–6) and then electrophoresed. As shown, ~80% of the p53-DNA complexes were lost after 15 min (Fig. 5A, lane 2, and B), followed by no significant drop through 4 h, suggesting that the tight complexes have a half-life of greater than 4 h. Biphasic decay curves such as these can be interpreted in either of two ways. First, they could result from the presence of a large number of nonspecific complexes, for example p53 bound at the DNA ends or along the arms which are rapidly lost upon challenge, and a small number of resistant complexes formed at the junction. Second, the curves could reflect two classes of (specific) complexes formed at the Holliday junctions, one that is highly resistant to challenge and another that is less stable.

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**FIG. 2.** Visualization of human p53 bound to Holliday junction DNAs.
Human p53 was incubated with Hol₇₅ DNA at a molar ratio of 5 p53 tetramers/DNA for 15–30 min at room temperature. The complexes were then fixed and prepared for EM as in Fig. 1A. Field of complexes (taken at a 40° tilt to enhance contrast) in which 5 of the 6 Hol₇₅ DNAs have p53 bound specifically at the junction. B–D, individual examples (0° tilt) with p53 bound at the junction as a dimer (C), tetramer (B), or higher oligomer (D). Images are shown in reverse contrast. Bar equals 0.06 μm (A) and 0.05 μm (B–D).

**FIG. 3.** Gel retardation analysis of p53-Holliday junction binding under different salt conditions. ³²P-Labeled Hol₇₅ DNA (1 ng, 0.25 μM) was incubated for 20 min at room temperature without p53 (lanes 1, 6, and 11) or with p53 protein at molar ratios of 5 (lanes 2, 7, and 12), 10 (lanes 3, 8, and 13), 20 (lanes 4, 9, and 14), or 40 (lanes 5, 10, and 15) p53 tetramers/DNA. The binding buffer (10 mM Tris (pH 7.5), 100 μg/ml BSA, 1 mM DTT, and 10 ng/μl PvuII-digested CT DNA) was supplemented with 30 mM NaCl (lanes 1–5), 130 mM NaCl (lanes 6–10), and 5 mM MgCl₂ with 30 mM NaCl (lanes 11–15). The samples were electrophoresed on 4% polyacrylamide gels and DNA imaged by autoradiography (see “Experimental Procedures”). The positions of free DNA (Hol₇₅) and p53 protein-DNA complexes (p53/DNA) are indicated.
and more easily lost in the presence of competitor. Here, the EM observations strongly argue for the latter interpretation since almost all of the p53 was observed bound at the junctions. The different oligomeric complexes of p53 at the junction, in particular dimers and tetramers, may differ in their stability.

\[ p53 \text{ Binding Enhances the Endonuclease-mediated Cleavage of Holliday Junctions} \]

The tight binding of p53 to Holliday junctions is well characterized (27). Both the endonucleases and Holliday junctions from resolving enzymes or possibly expose the junctions to cleavage. T4 endonuclease VII and T7 endonuclease I bind such junctions specifically and cleave four-way junctions more. The cleavage was stopped and the DNA electrophoresed on a 4% polyacrylamide gel (see "Experimental Procedures").

The kineticsof junction cleavage by T4 endonuclease VII was characterized in detail.

\[ \text{The tight binding of p53 to Holliday junctions used here was taken from Pick-sley et al. (27), where the cleavage of this particular junction by these two enzymes in a reaction requiring magnesium was characterized in detail.} \]

\[ \text{Holliday DNA (1 ng; } ^{32}\text{P-labeled) was incubated at room temperature with 1 \mu l of p53 storage buffer or 1 \mu l of p53 protein (5 ng, 5 tetramer proteins/DNA) in a 10-\mu l volume of 50 mM Tris (pH 8), 5 mM MgCl}_2, 1 \text{mM DTT, 100 } \mu \text{g/ml BSA, and 10 } \mu \text{g/ml CT DNA (cleavage buffer) for 15 min, followed by the addition of 10–90 units of T4 endonuclease VII for 15 min more. The cleavage was stopped and the DNA electrophoresed on a 4% polyacrylamide gel (see "Experimental Procedures"). The Holliday DNA was cleaved much more readily in the presence of p53 protein as contrasted to the level of cleavage in the absence of p53 (Fig. 6, A and B).} \]

\[ \text{Indeed, in the presence of p53, 10 units of T4 endonuclease VII produced at least as much cleavage as that seen with 90 units of enzyme in the absence of p53 (Fig. 6, A and B). A parallel experiment was carried out using T7 endonuclease I (1 through 5 units), with a similar outcome (Fig. 6, C and D). Under these conditions, addition of E. coli SSB protein in place of p53 (at the same molar ratio), which was not found to bind the Holliday junctions, did not alter the cleavage rates of the junctions by T4 endonuclease VII or T7 endonuclease I (data not shown). Analysis of the cleavage products showed that the DNA arms of the junctions migrated to the same position in the 4% polyacrylamide gel whether or not p53 had been present. Furthermore, analysis of the size of the cleavage products on 6% urea denaturing gels (data not shown) revealed no difference in size whether or not p53 was present.} \]

\[ \text{The kinetics of junction cleavage by T4 endonuclease VII was examined in the presence or absence of p53 (Fig. 7, A and B).} \]

\[ \text{DISCUSSION} \]

In this study we show that human p53 binds specifically and strongly to Holliday junctions. In one experiment, when p53 was incubated with a DNA containing a synthetic Holliday junction flanked by four 570-bp arms, 61% of the DNA molecules contained p53 bound and in 96% of these, the p53 was bound exclusively at the junction. Considering the large number of potential binding sites along the length of the arms and at the DNA ends, this represents a very high specificity for the junction. Gel retardation assays confirm this observation, revealing that once formed, the specific junction complexes are relatively stable to challenge by competitor DNA, and provide an estimate of the half-life of the tight junction complexes of at least 4 h. Binding to three-armed junctions is ~3-fold lower. Finally, rather than protecting the junctions from nucleolytic
attack, p53 facilitates their cleavage by T4 endonuclease VII and T7 endonuclease I.

Holliday junctions are generated in DNA as a product of homologous recombination events and during recombinational repair of DNA damage. They also represent potentially lethal structures if they pass the G2/M border. In metaphase, during chromosome formation and segregation, a Holliday junction could lead to chromosomes remaining paired, resulting in chromosome loss, duplication, or possibly breakage. Hence it seems reasonable that cellular mechanisms would exist to measure the level of unresolved junctions, halt the cell cycle until this level was appropriately low, and possibly also to facilitate their resolution. Here we have provided direct evidence that this may be accomplished by a p53-related pathway. Furthermore, given the recent links that have been found between p53 activity and homologous recombination (see Introduction and Ref. 18), the findings presented here suggest that p53 may function through direct binding to Holliday junctions in vivo.

The EM and biochemical data provide clues concerning the features of the Holliday junctions recognized by p53 and the structure of the junction following p53 binding. The images of the Hol565 and Hol75 DNAs were consistent with a fraction of the DNA being in the stacked X conformation under the mounting conditions utilized (relatively high divalent cations and or spermidine). However, the large fraction of molecules that were in other structures suggests significant distortion of the DNA upon adsorption to the support. By EM, p53 was seen to bind to the Holliday junctions with high specificity both in the presence and absence of magnesium. This was confirmed by gel retardation data (Fig. 3), which also pointed to a slightly greater binding at low protein concentrations in low salt where the DNA would exist in the more extended conformation as contrasted to higher salt (with or without magnesium) where the DNA would have been in the stacked X conformation. When p53 bound to the Hol75 DNA, the large size of the p53 complex (predominantly tetramers and octamers) obscured the angles at which the arms exited the protein complex. In complexes formed with the Hol565 DNA, analysis of the angles formed by the DNA arms suggested that most fell somewhere between the stacked X conformation (60° and 120° angles) and the extended conformation (90° angles). Indeed, few if any molecules were seen with the arms at 90° angles or folded on themselves (very shallow angles). These observations are consistent with studies by Lilley and colleagues (33, 34) on the binding of T4 endonuclease VII and T7 endonuclease I to four-way junctions, where it was concluded that these enzymes hold the junction into a

with 1 μl of p53 storage buffer (lanes 1–10) or 1 μl of p53 protein (5 ng, 5 tetramer proteins/DNA, lanes 11–20) in 10 μl of cleavage buffer (see “Experimental Procedures”) at room temperature for 15 min, and then incubated for an additional 15 min at room temperature with 10 (lanes 2 and 12), 20 (lanes 3 and 13), 30 (lanes 4 and 14), 40 (lanes 5 and 15), 50 (lanes 6 and 16), 60 (lanes 7 and 17), 70 (lanes 8 and 18), 80 (lanes 9 and 19), and 90 (lanes 10 and 20) units of T4 endonuclease VII. The samples were deproteinized, and the DNA electrophoresed on a 4% polyacrylamide gel and imaged by autoradiography (see “Experimental Procedures”). The positions of Hol565 DNA, analysis of the angles formed by the DNA arms suggested that most fell somewhere between the stacked X conformation (60° and 120° angles) and the extended conformation (90° angles). Indeed, few if any molecules were seen with the arms at 90° angles or folded on themselves (very shallow angles). These observations are consistent with studies by Lilley and colleagues (33, 34) on the binding of T4 endonuclease VII and T7 endonuclease I to four-way junctions, where it was concluded that these enzymes hold the junction into a

FIG. 6. p53 binding to Holliday junction can enhance the junction cleavage. A, 32P-labeled Hol565 DNA (1 ng, 0.25 nM) was incubated with 1 μl of p53 storage buffer (lanes 1–10) or 1 μl of p53 protein (5 ng, 5 tetramer proteins/DNA, lanes 11–20) in 10 μl of cleavage buffer (see “Experimental Procedures”) at room temperature for 15 min, and then incubated for an additional 15 min at room temperature with 10 (lanes 2 and 12), 20 (lanes 3 and 13), 30 (lanes 4 and 14), 40 (lanes 5 and 15), 50 (lanes 6 and 16), 60 (lanes 7 and 17), 70 (lanes 8 and 18), 80 (lanes 9 and 19), and 90 (lanes 10 and 20) units of T4 endonuclease VII. The samples were deproteinized, and the DNA electrophoresed on a 4% polyacrylamide gel and imaged by autoradiography (see “Experimental Procedures”). The positions of Hol565 DNA (Hol565) and the cleavage products (product) are indicated. B, the percentage of uncleaved DNA in the gel shown in A was measured using a Molecular Dynamics PhosphorImager and was calculated by dividing the intensity of Hol565 DNA by the total intensity (Hol565 plus product). Closed circles, no p53 added; open circles, with p53. C, a parallel experiment to that described in A and B was carried out using 1 (lanes 2 and 8), 2 (lanes 3 and 9), 3 (lanes 4 and 10), 4 (lanes 5 and 11), and 5 (lanes 6 and 12) units of T7 endonuclease I without p53 (lanes 1–6) or preincubated with p53 as in A and B (lanes 7–12). The positions of Hol565 DNA (Hol565) and the cleavage products (product) are indicated. D, the percentage of uncleaved DNA in the gel shown in C was measured and plotted as described in B. Closed circles, with p53; opened circles, no p53.
was found to facilitate their resolution. The combination of these two activities could strongly limit the number and extent of recombinational junctions in vivo. This conclusion is bolstered by the observation that the C-terminal domain of p53 has anti-helicase activity (17, 35), a function that might slow the progress of branch migration. Beyond maintaining a check on the level of normal recombinational events, these activities of p53 could be particularly important in homeologous recombination between more distantly related sequences. Here, the recombination products will contain numerous insertion/deletion mismatches that would present additional targets for p53, which could further aid in targeting p53 to the region of the junction. The involvement of p53 with recombinational intermediates may share features with that of MSH2, a protein in the postreplication mismatch repair pathway that plays a pivotal role in the recognition of mismatches and which has been recently shown to bind Holliday junctions (28). Several studies have suggested that the mismatch repair proteins may have a role in recombination, including suppressing homeologous recombination, inhibiting the formation of recombinational intermediates containing too many mismatches, and possibly aiding in the resolution of Holliday junctions (reviewed in Ref. 29). In these reactions MSH2 appears to interact with other members of the mismatch repair family including MSH3 and MSH6 (36).

In binding to Holliday junctions in vivo, p53 must come in contact with a multitude of proteins including those that catalyze recombination, those that resolve junctions, and others known to bind Holliday junctions such as HMG1 (37). Indeed p53 has been shown to directly bind to two proteins that catalyze homologous recombination, hRad 51 and RPA, and to the transcription factor TFIH also involved in repair (18, 35, 38, 39).

In the future it will be essential to learn how p53 interacts with these proteins at a Holliday junction.

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