THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 280, NO. 52, pp. 42568–42572, DECEMBER 30, 2005

p53 Monitors Replication Fork Regression by Binding to "Chickenfoot" Intermediates*

Received for publication, June 10, 2005, and in revised form, September 29, 2005 Published, JBC Papers in Press, October 4, 2005, DOI 10.1074/jbc.M506348200

Deepa Subramanian and Jack D. Griffith[1]

From the Lineberger Comprehensive Cancer Center and Department of Microbiology and Immunology, University of North Carolina, Chapel Hill, North Carolina 27599

The tumor suppressor protein, p53, utilizes multiple mechanisms to ensure faithful transmission of the genome including regulation of DNA replication, repair, and recombination. Monitoring these pathways may involve direct binding of p53 to the DNA intermediates of these processes. In this study, we generated templates resembling stalled replication forks and utilized electron microscopy to examine p53 interactions with these substrates. Our results show that p53 bound with high affinity to the junction of stalled forks, whereas two cancer-derived p53 mutants showed weak binding. Additionally, some of the templates were rearranged to form "chickenfoot" structures in the presence of p53. These were mostly formed due to p53 trapping intermediates of spontaneous fork regression; however, in a small population, the protein appeared to be promoting their formation. Collectively, these results demonstrate the importance of sequence-independent binding in p53-mediated maintenance of genomic integrity.

As the "guardian of the genome," p53 acts as a link between upstream signaling cascades and downstream response pathways. Genotoxic stress results in p53 activation, and the protein primarily functions as a sequence-specific transcriptional activator of genes involved in cell cycle arrest and apoptosis (1–3). However, there are several lines of evidence that show that p53 also participates in other cellular processes in a transcription-independent manner. Certain p53 mutants that have lost their transcription-dependent properties are still able to arrest the cell cycle (4–6), whereas others show transcriptional regulation but are deficient in growth suppression function (7, 8). Treatment with hydroxyurea, a replication inhibitor, results in p53 stabilization without stimulating transcriptional functions (9). p53 also possesses a second DNA binding domain that recognizes damaged or altered DNA, such as heteroduplexes and Holliday junctions (10–12), and could be directly involved in DNA replication, recombination, and repair pathways.

Several reports have shown that wild type p53 acts as a negative regulator of spontaneous as well as radiation-induced homologous recombination (13–15). Inactivation of the protein, either using p53-deficient mice or mutations at certain hot spot residues, results in increased occurrence of recombination-related genomic abnormalities such as deletions, inversions, and translocations (15–18). Regulation of recombination by p53 has been shown to occur in a transcription-independent manner (14, 19–21). p53 can physically associate with several recombination-specific proteins including hRad51 (22, 23) and hRad54 (23) and the BLM (24) and WRN helicases (25). Functionally, p53 suppresses hRAD51-induced recombination events (23) and specifically inhibits hRad51-mediated strand exchange and fork regression reactions (26). p53 can also modulate BLM and WRN helicase activity on Holliday junction templates (27).

Strand exchange between two duplexes is a key step in homologous recombination creating a four-stranded Holliday junction intermediate. Branch migration of the Holliday junction results in further exchange between the two strands; this process can be spontaneous or protein-induced. As a result, heteroduplexes can be created if the exchanged strands are not completely complementary. p53 has been shown to bind both heteroduplexes and Holliday junctions as well as increase junction resolution by resolvases (11). Thus, p53 regulation of homologous recombination could involve interactions with recombination-specific proteins or direct association with the DNA intermediates. By recognizing and binding to these structures, the protein can ensure that exchange between imperfectly matched sequences does not occur.

p53 also plays an important role in monitoring the various steps of DNA replication to ensure faithful transmission of genetic material. p53 blocks replication initiation if DNA damage is present upstream of the origin. Using templates containing the polyoma virus origin (which contains an internal p53 binding site) and upstream damage sites, Zhou and Prives (28) showed that replication was inhibited and proposed a model of DNA looping between the two p53 sites, thus sterically hindering replication initiation or elongation. Once replication has been initiated, fork arrest can result from a variety of factors such as the presence of DNA lesions on the parental strand or bound proteins that act as a barrier to fork progression. Recombinational repair can be used to restart these stalled forks in which double strand breaks are created followed by strand invasion by the broken strand and duplication from the intact chromatid. However, this process may generate small insertions or deletions in the daughter strand. A second mechanism of fork reactivation is replication fork reversal in which reverse migration causes the newly synthesized strands to anneal to each other and extrude a fourth strand, thus creating a "chickenfoot" intermediate that resembles Holliday junctions. Once the lesion on the parental strand is repaired, reverse branch migration can restore the fork to the initial position by a non-recombinogenic method. Alternately, endonucleolytic cleavage of the chickenfoot structure and recombinational repair can also restart the fork. Yoon *et al.* (26) have shown that hRAD51 can promote regression of stalled replication forks and that this activity is inhibited by p53. However, the exact mechanism of p53-dependent hRad51 inhibition was not clear. These authors (26) proposed either interference with hRad51 promoted nucleofilament formation or branch migration could be responsible for p53 inhibition. p53 also modulates BLM helicase activity on stalled replication forks, but the mechanism involved is unknown.

To understand the exact role of p53 in processing stalled replication forks, we generated large templates that resemble these structures and

* This work was supported by the Ellison Medical Foundation and National Institutes of Health Grants CA19014 and GM31819. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] To whom correspondence may be addressed: 119 Lineberger Comprehensive Cancer Center, CB 7295, Mason Farm Rd., Chapel Hill, NC 27599. Tel.: 919-966-2151; Fax: 919-966-3015; E-mail: jdg@med.unc.edu.

used electron microscopy (EM)[2] to examine the interactions of p53 with these substrates. The results show that p53 bound with high affinity at the junction of stalled replication forks. Additionally, some of the bound templates were found to be rearranged to form four-stranded or chickenfoot structures. These appear to be formed as a result of p53 trapping intermediates of spontaneous fork regression. In some cases p53 can also induce fork regression reactions. Additionally, two cancer-related p53 mutants bound very poorly to these templates. These observations suggest that p53 functions in the processing of stalled replication forks as well as branch migration of Holliday junctions containing heteroduplexes by binding to functional intermediates, thus preventing further strand movement until any DNA damage present is repaired.

EXPERIMENTAL PROCEDURES

Plasmid Construction—The 400-bp G-less cassette (29) was cloned into the EcoRI and BamHI sites of pBluescript KS (plasmid pBSGLess). Complementary oligomers 1) 5′-AATTCGCTAAGGACCGACGCTTAAGTAGGTTAAGGGTTAA**GCTGAGG**-3′ and 2) 5′-CCTCAGCTTAACCCTTAACCTACTTAAGCGTCGGTCCTTAGCGC-3′ were annealed to create a 35-bp duplex that has a 5′-overhang complementary to EcoRI and a 3′-blunt end. The duplex contains a site for the nicking endonuclease, N.BbvCIA, indicated in bold on oligonucleotide 1. The 35-bp duplex was cloned into the EcoRI/SmaI site of pBSGless to create pGLGAP.

Preparation of Replication Fork Templates—Plasmids (10 μg) were digested with N.BbvCIA (New England Biolabs, Beverley, MA) using the manufacturer's conditions to introduce a single nick at the start of the G-less cassette. Single strand tails were created by strand displacement using 5 units of the Klenow fragment (exo$^-$) (New England Biolabs) in 20-μl reactions containing 100 mM Tris, pH 7.5, 50 mM $MgCl_2$, 0.1 M dithiothreitol, and 5 mM each dATP, dTTP, and dGTP at 37 °C for 30 min. Samples were purified using DNA Clean and Concentrator spin columns (Zymo Research, Orange, CA) following the manufacturer's conditions. To create a double strand tail, primer 25 (5′-CTTCCTCCATCTATACCACC-3′) was annealed to the 3′-end of the displaced single strand at a 10-fold molar excess at 37 °C for 30 min in 40-μl reactions containing 100 mM Tris, pH 7.5, 50 mM $MgCl_2$, 0.1 M dithiothreitol, and 5 mM each dATP, dTTP, and dCTP followed by the addition of 5 units of Klenow fragment (exo$^-$) and further incubation at 37 °C for 30 min. The templates were purified using DNA Clean and Concentrator spin columns and quantified. This results in a duplex circle with a double strand tail that has a 25-nucleotide gap at the junction of the circle and tail. In some cases the plasmids were linearized with XmnI (New England Biolabs), placing the double strand tails asymmetrically.

DNA Probes—Holliday junction probes were synthesized as described previously (30). Briefly, one strand of the probe was first end-labeled using [γ-^{32}P]ATP and T4 polynucleotide kinase (New England Biolabs) followed by the addition of the other three strands to create a four-way junction. The samples were heated to 65 °C for 10 min in the presence of 0.4 M NaCl and allowed to slowly cool to room temperature overnight. Annealed products were purified on 10% non-denaturing polyacrylamide gels.

Proteins—Wild type p53 as well as mutants, R175H and R273H (obtained from Dr. Arnold Levine), were purified from baculovirus-infected insect cells as described previously (31).

Electron Microscopy—Reactions (20 μl) containing 45 fmol of DNA, 180 fmol of p53 (tetramers), 20 mM Hepes (pH 7.8), 100 mM KCl, and 1 mM dithiothreitol were incubated at room temperature for 10 min and fixed with gluteraldehyde (0.6% final concentration) for an additional 10 min. Protein-DNA complexes were separated from free proteins on a 1-ml gel filtration column (4% agarose, Agarose Bead Technologies, Tampa, FL) and mounted on carbon-coated copper grids followed by ethanol dehydration and rotary shadowcasting with tungsten as described previously (32). Samples were examined on a Phillips CM12 electron microscope, and images were captured on a Gatan multiscan 794 digital camera (Pleasanton, CA) or on sheet film. Image contrast was adjusted, and the panels were arranged used Adobe Photoshop. Molecule lengths were measured using Gatan Digital Micrograph software. For linear templates, the length of the short segment and total DNA were measured for each molecule in nm and then converted to bp by the following equation.

$$\text{short arm(bp)} = \left(\frac{\text{short arm (nm)}}{\text{total length (nm)}}\right) \times 3400 \text{ bp} \quad \text{(Eq. 1)}$$

The total length of the templates was 1172.2 ± 49.8 nm (n = 550); therefore variations due to measurement errors are 144 bp.

Mobility Shift Assays—End-labeled probes (0.18 pmol) were incubated with proteins (1.8 or 3.6 pmol) in 20-μl reactions containing 20 mM Hepes (pH 7.8), 100 mM KCl, 1 mM dithiothreitol, 10% glycerol, and 25 ng of salmon sperm DNA as nonspecific competitor for 20 min at room temperature. Reaction products were separated on 5% non-denaturing polyacrylamide gels in 0.5× TBE (45 mM Tris borate, 1 mM EDTA) and were run at 200 V for 2.5 h at 4 °C. Gels were dried and analyzed by autoradiography.

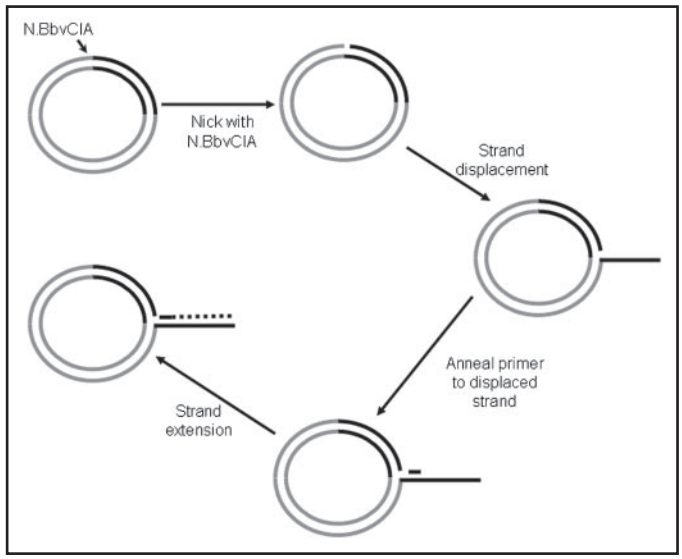

FIGURE 1. **Schematic representation of replication fork templates.** Plasmid pGLGAP was nicked in front of the G-less cassette (indicated in *black*) with N.BbvCIA followed by strand displacement with the Klenow fragment (exo$^-$) in reactions excluding dCTP thus generating a single strand tail. A primer was annealed to the 3′-end of the displaced strand, and the second strand was synthesized with the Klenow fragment (exo$^-$) to generate a 400-bp ds tail on a 3.4-kb duplex circle.

RESULTS

Preparation of Replication Fork Templates—To create templates that mimic replication forks, we generated plasmids that contain a 400-bp G-less cassette (see "Experimental Procedures") as well as an N.BbvCIA recognition site at the 5′-end of this cassette (Fig. 1). Supercoiled plasmids were nicked at the N.BbvCIA site, and strand displacement reactions were carried out with the Klenow fragment (exo$^-$) in the absence of dCTP so that

[2] The abbreviations used are: EM, electron microscopy; ds, double strand.

polymerization stopped at the end of the cassette when the first guanine was encountered. Additionally, nonspecific strand displacement reactions were prevented by the exclusion of dCTP. Primers were annealed to the 3′-end of the single strand displaced tail leaving a 25-nucleotide gap at the fork junction followed by strand extension using the Klenow fragment (exo^-) thus generating a 400-bp double strand (ds) tail on a 3.4-kb duplex circle (Fig. 1). The ds tail represents the lagging strand where one arm of the circle is the leading strand, and the other arm is the parental strand. By EM, we found that 95% of the molecules scored had ds tails that had an average length of 390 ± 80 bp ($n = 280$).

p53 Binds to the Junction of Replication Forks—To examine p53 binding to replication forks, ds tailed templates were incubated with the protein (1:4 molar ratio of DNA/p53 tetramers), and the reaction products were analyzed by EM. The results showed that 69.4 ± 1.7% of the DNA molecules were bound by p53 ($n = 240$, three independent experiments). Binding to the junction of replication forks (Fig. 2, *A* and *B*) was observed in 69.7 ± 6.2% of the protein-bound molecules, whereas 11.6 ± 4.0% had protein associated with the circle or tail. Interestingly, a fraction of the molecules (10.6 ± 4.1%) were p53-bound but had 2 shorter ds tails associated with the complex at the replication fork junction (Fig. 2, *C* and *D*). The sum of the length of the 2 short tails measured ~400 bp, which is the same as the tail length of the initial substrate. These structures were not observed in the absence of p53. Incubation with the checkpoint protein, claspin, as well as the Rad9-Rad1-Hus1 complex did not produce these double-tailed molecules (Ref. 33, data not shown). We believe these structures to be products of fork regression reactions thus creating chickenfoot intermediates. One possible mechanism of chickenfoot formation would involve p53-induced regression in molecules that it recognizes as stalled replication forks. Alternately, the substrates could undergo spontaneous branch migration resulting in products where the ds tail has moved to a new position that is 400 bp from the original site. In the absence of p53 this may occur rapidly, and the only molecules observed would be the initial template and the final product. Addition of p53 could result in trapping chickenfoot intermediates formed during the migration, thus preventing further strand movement. Both processes represent mechanisms by which p53 can signal the presence of stalled replication forks. On circular molecules, the products of complete regression cannot be distinguished by EM from the original templates, and therefore these substrates cannot be used to determine the total fraction of regressed forks, whether spontaneous or p53-induced.

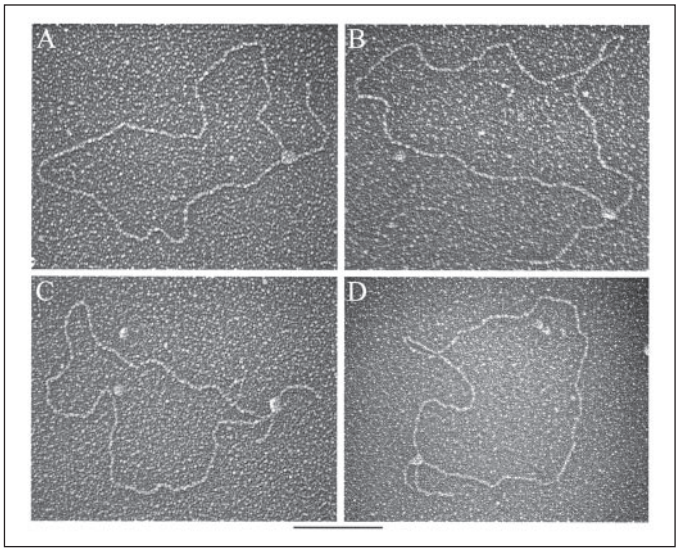

FIGURE 2. **p53 interactions with stalled replication forks.** Circular replication forks were incubated with p53, and products were visualized by electron microscopy. Samples were mounted onto thin carbon-coated copper grids and rotary shadowcasted with tungsten. *A* and *B*, p53 bound to the junction of replication forks. *C* and *D*, p53 bound to chickenfoot intermediates. Images are shown in reverse contrast. The *bar* is equivalent to 400 bp.

p53 Induces Regression of Stalled Replication Forks—By converting the duplex circles into linear molecules, the position of the ds tail can be determined by measuring the length of the segments on either side of the tail. Templates were synthesized as described above followed by digestion of the circle with XmnI to produce asymmetrical arms that measure 1.05 kb (short) and 2.35 kb (long). If complete regression occurs, then the position of the ds tail should move by 400 bp, and the segments will measure 1.45 and 1.95 kb, respectively. Control reactions were carried out in the absence of p53, and the length of the short segment was measured to determine the position of the tail. Molecules were sorted into three categories: unregressed (short segment = 1.05 ± 0.075 kb), fully regressed (short segment = 1.45 ± 0.075 kb), and partially regressed (short segment with intermediate lengths between unregressed and fully regressed). The results show that 81% of the molecules measured ($n = 100$) had short arms of 1.05 ± 0.075 kb (that is, they had not regressed), whereas 17% were fully regressed. The remaining 2% had short segments of intermediate length, but no double-tailed structures were observed in these molecules presumably because one of the tails was too short to be visualized by EM. These results indicate that a fraction of the templates undergoes spontaneous regression; however, in the absence of p53, regression mostly progresses to completion. This is similar to the observation by Yoon *et al.* (26) that approximately 10–20% of their replication fork templates underwent reverse branch migration.

Linear templates were incubated with p53 and scored for binding efficiency as well as tail position. Binding to linear templates showed patterns similar to circular templates, with 76.3 ± 4.9% of total molecules ($n = 300$, three independent reactions) bound by p53 (TABLE ONE). Most of the binding occurred at the fork junction (65%), whereas 8% of the molecules showed chickenfoot structures. The lengths of the short arms of p53-bound molecules ($n = 100$) were determined and sorted based on the tail position. Addition of p53 resulted in an increase in the number of completely regressed molecules from 17 to 26%

TABLE ONE

p53 binding to stalled replication forks

Linear replication fork templates were incubated with p53, and samples ($n = 300$, three independent reactions) were scored for total p53 binding as well as protein location on the template. Binding at various positions was calculated as a percentage of total bound molecules.

Template	Unbound	Bound	p53			
			Junction	Chickenfoot	Circle or tail	Multiple proteins
	% total	*% total*	*% bound*			
$RF25_{37}$	23.7 ± 4.9	76.3 ± 4.9	64.9 ± 5.5	8.0 ± 3.0	21.1 ± 7.1	6.1 ± 2.9
$RF25_{42}$	35.7 ± 4.6	64.3 ± 4.6	63.3 ± 3.3	10.3 ± 1.7	17.1 ± 0.8	9.3 ± 3.8
$RF25_{55}$	27.7 ± 10.8	71.7 ± 10.4	62.5 ± 4.2	16.0 ± 2.3	17.1 ± 4.0	4.4 ± 2.2

TABLE TWO

Fork regression as a function of p53 binding

Linear replication forks were incubated in the presence or absence of p53, and the position of the 400-bp double strand tail was determined by measuring the length of the short segment of linear molecules. Molecules were sorted into three categories: unregressed, fully regressed, and partially regressed.

Molecules	Tail position					
	$RF25_{37}$		$RF25_{42}$		$RF25_{55}$	
	−p53	+p53	−p53	+p53	−p53	+p53
	% total					
Unregressed (1.05 ± 0.075 kb)	80.6	54.3	67.7	60.2	66.7	57.6
Fully regressed (1.45 ± 0.075 kb)	17.2	26.1	22.6	26.1	23.3	22.8
Partially regressed (1.125–1.325 kb)	2.2	19.6	9.7	13.6	8.9	19.6

(TABLE TWO). Furthermore, there was also a greater number (20%) of partially regressed molecules, which included chickenfoot structures. Not all partially regressed molecules had 2 visible tails, most likely because they were either obscured by the protein mass or the second strand was too small to be seen by EM. Indeed, the visible strand of these partially regressed molecules measured 311 ± 70 bp, making the second tail ~80 bp in length, which would be easily missed by EM. Overall, the total number of regressed molecules (complete and partial) increased by 2.5-fold with the addition of p53 indicating that the protein can actively promote fork regression. However, we cannot exclude the possibility that some of the partially regressed molecules are a result of p53 trapping intermediates of spontaneous regression.

p53 Binds Intermediates of Spontaneous Regression to Prevent Further Strand Movement—We observed that the amount of spontaneous regression in the templates could be altered by changing the temperature at which the second strand primer was annealed. The substrates used in the above experiments were synthesized by annealing the primer at 37 °C ($RF25_{37}$). Additional templates were synthesized by increasing the annealing temperature to 42 °C ($RF25_{42}$) and 55 °C ($RF25_{55}$) followed by second strand synthesis and digestion with XmnI. In the absence of p53, both $RF25_{42}$ and $RF25_{55}$ showed greater levels of spontaneous regression (TABLE TWO). Completely regressed molecules increased to 23% in both cases (compared with 17% with $RF25_{37}$), whereas partially regressed molecules increased to ~10% (compared with 2% with $RF25_{37}$). p53 bound to these new templates with the same efficiency as $RF25_{37}$ and showed similar binding patterns (TABLE ONE) except for increased chickenfoot structures (16%) with $RF25_{55}$. Measurement of the short segment following p53 addition showed that ~25% of both templates had tails positioned at 1.45 ± 0.075 kb on the linear template, *i.e.* they were completely regressed. This is not significantly different from the untreated templates. However, an increase in the number of partially regressed molecules (including chickenfoot molecules) was observed with the addition of p53, from 10 to 14% with $RF25_{42}$ and from 9 to 20% with $RF25_{55}$. In the case of $RF25_{42}$, the total amount of regression (complete and partial) increased from 33 to 40% with the addition of p53, whereas with $RF25_{55}$, it increased from 32 to 42%. These data suggest that with these two templates, the majority of observed chickenfoot molecules resulted from p53 trapping intermediates of spontaneous regression, and the contribution from active fork regression by the protein was not as great. However, with the $RF25_{37}$, where spontaneous regression is not as high, p53 appears to switch functions and induce fork regression.

Mutants of p53 Do Not Recognize Stalled Replication Forks—Certain mutations in p53 can result in an increase in genomic rearrangements *in vivo*, making cells more cancer-prone. We tested two such p53 mutants (175H and 273H) for their ability to recognize stalled replication forks as well as the four-stranded intermediates. To examine the ability of the two p53 mutants to bind four-stranded structures, mobility shift assays were performed using probes resembling Holliday junctions. We have shown previously that wild type p53 binds to these structures with high affinity (30). In contrast, the two p53 mutants bound poorly to these probes (Fig. 3) with the 273H mutant showing slightly higher affinity than the 175H mutant.

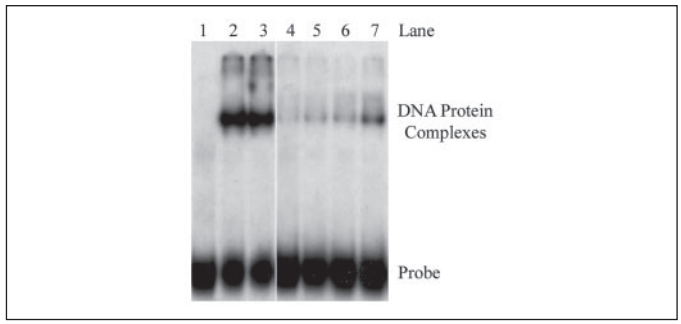

FIGURE 3. **Binding of p53 mutants to Holliday junctions.** End-labeled probes (9 nM) were incubated with either 90 or 180 nM wild type p53 (*lanes 2* and *3*), mutant 173H (*lanes 4* and *5*), or mutant 273H (*lanes 6* and *7*), and reaction products were separated on 5% non-denaturing polyacrylamide gels followed by autoradiography. *Lane 1*, untreated probe.

TABLE THREE

Binding of p53 mutants to stalled replication forks

Wild type p53 and mutants 175H and 273H were incubated with linear $RF25_{37}$ templates, and samples were scored (n = 100) for total p53 binding as well as protein location on the template. Binding at various positions was calculated as a percentage of total bound molecules.

Protein	Unbound	Bound	p53 position			
			Junction	Chickenfoot	Circle or tail	Multiple proteins
	% total	*% total*	*% bound*			
Wild type	24	76	65	8	21	6
175H	75	25	56	4	40	0
273H	72	28	54	7	32	7

The ability of the two mutants to bind replication forks was tested by EM using the $RF25_{37}$ template and mutant proteins at the same molar ratio as wild type p53. Our results show that both mutants were deficient in recognizing stalled replication forks (TABLE THREE). Only 25% of the total molecules (n = 100) were bound by the 175H mutant and 28% by the 273H mutant. Of the bound molecules, ~55% of the molecules were bound at the junction. In both cases, there was also increased nonspecific binding along the circle or tail. A reduction in chickenfoot structures was observed with the 175H mutant but not the 273H. These results provide further evidence that p53 functions by binding to stalled forks, intermediates of fork regression, and Holliday junctions to signal their presence and by preventing recombinogenic activities at these sites to ensure genomic integrity.

DISCUSSION

In this study we have shown that p53 binds with high affinity to the junctions of stalled replication forks. By EM, we were also able to observe that a fraction of the p53-bound replication forks were rearranged to form four-stranded chickenfoot structures. Spontaneous regression was observed in 19–33% of the replication fork templates depending on the conditions of synthesis. These would result in the formation of chickenfoot intermediates to which p53 would bind with high affinity. Indeed, a greater number of chickenfoot molecules were observed when p53 was added to templates that showed increased spontaneous regression. However, the total number of regressed molecules only increased slightly with p53 addition. In this situation, p53 appears to function largely to protect stalled replication forks by binding to the junctions and chickenfoot intermediates thus preventing excessive movement in these molecules. Interestingly, we found that in templates where spontaneous fork movement is slow ($RF25_{37}$), there was a significant increase in the total number of regressed molecules when p53 was added suggesting that the protein can also actively promote the formation of chickenfoot intermediates. Here, p53 may function to catalyze the initial steps in reactivating replication arrest via fork regression mechanisms thus favoring this pathway over recombinational repair. Alternately, spontaneous regression could continue even after p53 has bound to the replication fork junction, converting the three-stranded structures into four-way junctions. p53 could have a greater affinity for the chickenfoot intermediates over replication fork junctions, which would result in preferential binding to the four-stranded structures. Regardless of the mechanism by which p53 stabilizes chickenfoot intermediates, the overall outcome is the protection of stalled forks by preventing further fork movement until the cause of the replication arrest is repaired, thus avoiding deleterious rearrangements.

In vivo, fork arrest can be induced by hydroxyurea that results in the formation of replication foci containing multiprotein complexes, which include BRCA1 (34), the Rad50-MRE11-NBS1 complex (34), BLM helicase (34), and hRad51 (35). More recently p53 (36) has also been identified at the replication foci. Recruitment of p53 to these foci was shown to be BLM-dependent, as cells deficient in the helicase do not show p53 localization to sites of stalled forks (36). These authors propose that p53 can regulate further processing of replication forks by modulating BLM action (reverse branch migration) and hRAD51-hRad54 (homologous recombination) activities. A similar model by Janz and Wiesmuller (37) suggests that p53 acts as a surveillance factor of recombination events at stalled replication forks. More recently, it was also shown that p53 specifically inhibits hRad51-mediated strand exchange and fork regression activities (26). Based on our data, p53 could function at the replication foci to prevent fork restart prior to lesion repair by binding to stalled forks as well as trapping chickenfoot intermediates in molecules undergoing spontaneous fork movement. Once fork progress is completely halted, the protein could assess the level and types of damage and activate the appropriate downstream proteins. As a suppressor of homologous recombination, p53 would activate non-recombinogenic pathways, such as lesion repair and reverse branch migration, which would involve the BLM helicase (36). Additionally, by binding to the stalled fork, p53 could inhibit hRad51-induced fork resolution, thus avoiding recombinogenic events. Using these model templates, we will be able to visualize the activity of the BLM helicase as well as hRad51 on stalled replication forks in the presence and absence of p53. Finally, p53 mutants that are deficient in preventing genomic instability bound very poorly to stalled replication forks as well as four-stranded intermediates. These mutants would not be able to prevent progression of unrepaired replication forks nor would they be able to block hRad51 activities on stalled forks. Collectively, our observations further demonstrate the importance of the sequence-independent DNA binding properties of p53 in regulating the steps subsequent to replication fork arrest.

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