p53 Directly Enhances Rejoining of DNA Double-Strand Breaks with Cohesive Ends in \( \gamma \)-Irradiated Mouse Fibroblasts

Wei Tang, Henning Willers, and Simon N. Powell

Laboratory of Molecular and Cellular Radiation Biology, Department of Radiation Oncology, Massachusetts General Hospital, Harvard Medical School, Charlestown, Massachusetts 02129 [W. T., S. N. P.], and Institute for Biophysics and Radiobiology, University of Hamburg, 20246 Hamburg, Germany [H. W.]

Abstract

The p53 gene regulates the cell cycle response to DNA damage, which may allow time for adequate DNA repair. We asked whether p53 could directly increase the repair of defined double-strand breaks (DSBs) by homologous and nonhomologous end-joining in \( \gamma \)-irradiated mouse embryonic fibroblasts with differing p53 status. By using an episomal plasmid reactivation assay, we found that presence of wild-type p53 enhanced rejoining of DSBs with short complementary ends of single-stranded DNA. p53 appeared to be directly involved in this regulation, because rejoining enhancement was dependent on the presence of nonspecific DNA binding activity as mediated by the COOH-terminal domain and was independent of transactivation function. We hypothesize that tumor cells lacking p53 and normal cells with wild-type p53 may use different pathways for repair of radiation-induced DSBs.

Introduction

The tumor suppressor gene p53 is considered the guardian of the genome that regulates the cellular response to various stress signals, most notably DNA damage. Following DNA damage, p53 becomes activated to up-regulate the transcription of several downstream genes. In many cell types, the dominant cellular response pathway is the p21-mediated G1 cell cycle arrest, which is assumed to allow for adequate repair of damaged DNA before entering S phase (1–3). In addition to its role in controlling the cell cycle, accumulating in vitro and in vivo evidence suggests that p53 may directly act in pathways of DNA repair. p53 has been shown to bind nonspecifically to single-stranded and double-stranded DNA, detect regions of damaged DNA, and promote annealing of single-stranded nucleic acids, all via its COOH-terminal domain (reviewed in Refs. 1 and 2). Mummenschaubner et al. (4) recently identified an intrinsic exonuclease activity of p53. p53 also interacts with components of the excisional (1) and recombination repair machinery. Importantly, with regard to recombinational repair of DSBs,\(^3\) p53 appears to be linked to both principal repair pathways, nonhomologous and homologous recombination (5–9). In vivo data have suggested that wild-type p53 can enhance nucleotide excision repair after UV irradiation (10–13). However, it is unclear under which circumstances reduced repair capability upon loss of wild-type p53 function translates into decreased survival after UV irradiation (11, 14). The relationship between p53 status, repair of DNA damage induced by IR, and corresponding clonogenic survival in cell lines that have no gross DNA repair defect is even less understood (11). In contrast to repair of UV damage, there has not yet been any indication that induction of DNA repair after treatment with IR may also be positively influenced by p53. Because DSBs are a critical component of IR-induced DNA damage, studies have tested whether the rejoining of DSBs is influenced by cellular p53 status. Interestingly, in some experiments, loss of wild-type p53 did not appear to lead to decreased but rather to increased levels of DSB repair (15–17).

In summary, it seems likely that p53 may modify DNA repair in a pleiotropic manner dependent on the type of DNA damage and its subsequent repair and the cell type under investigation (11, 16). The purpose of this study was thus to determine the influence of p53 on a defined type of DSB repair after \( \gamma \)-irradiation of rodent fibroblasts. We used isogenic cell pairs of untransformed MEFs, which differed only in their cellular p53 status. Repair of defined DSBs through nonhomologous end-joining was measured by using an episomal plasmid reactivation assay similar to experimental systems used previously (15, 17). The following questions were addressed: (a) Is the rejoining of DSB in \( \gamma \)-irradiated cells influenced by p53 status? (b) If DSB rejoining is enhanced by p53, is this regulation (i) dependent on the transactivation function of p53, (ii) dependent on nonspecific DNA binding activity as mediated by the COOH-terminal domain, and (iii) restricted to certain types of break repair?

Materials and Methods

Cell Lines. We studied two pairs of MEFs and one REF line: (a) primary MEFs with wild-type p53 and (b) MEFs from a p53-null mouse as described (18); (c) the 10.1/Val5 line, which is an established mouse BALB/c 3T3 line exogenously expression a temperature-sensitive p53 protein. This protein is in mutant conformation at 37–39°C (Val135 mutation) and assumes a wild-type conformation at 32°C incubation temperature; (d) the 10.1/VASSk1 line, which expresses the same temperature-sensitive protein, except that the COOH-terminal 26 amino acids (NS, residues 364–390) are exchanged by 17 different residues (AS; Ref. 19); and (e) primary REFs with wild-type p53 (18). Cell tissue culture conditions have been described (7, 18).

DNA Plasmid Substrates. In vivo DSB repair capacity was assessed by parallel transfections of a reporter plasmid that was either circular or had been linearized by restriction enzyme digest (Fig. 1). This reporter plasmid, pSV2-LUC, contains the firefly luciferase gene under control of an early SV40 promoter. Before transfection, the plasmid was linearized between the promoter and the luciferase gene by HindIII restriction enzyme digest, generating cohesive ends with a 5-bp overhang of 4-bp single-stranded DNA. Alternatively, blunt-end linear plasmids were generated by end-filling of HindIII-cleave plasmids using deoxynucleotide triphosphates and Klenow fragment (New England Biolabs). End-filling was verified by blunt-end ligation and the subsequent failure of restriction enzyme digestion. In linearized plasmids the luciferase gene cannot be expressed. Only after DSB rejoining with recircularization of the plasmid, transcription of the reporter gene can proceed. To exclude any potential assay-related bias, experiments were repeated using an analogous CAT reporter plasmid, which was linearized by BanHI cleavage (pCMV-CAT, Promega).
P53 ENHANCES DOUBLE-STRAND BREAK REJOINING

Fig. 1. Schematic representation of the plasmid rejoining assay used. A. plasmid substrate pSV2-LUC was linearized between the SV40 promoter and the luciferase gene by HindIII cleavage producing DSBs with cohesive ends (overhang of 4-bp single-stranded DNA). This cleavage prevented cellular expression of luciferase after plasmid transfection without prior repair of the break. B. after rejoining of the plasmid ends, luciferase enzyme activity can be measured. Break rejoining was thus assessed by comparison of luciferase enzyme activities detected in cells transfected with linearized plasmid relative to cells transfected in parallel with circular plasmid.

Fig. 2. DSB repair activities measured by rejoining of breaks with cohesive ends in a linearized plasmid compared with a circular control (%L/C). After exposure to IR, DSB rejoining was enhanced in primary MEFs with endogenous wild-type p53 (wt) by 2.9-fold, in primary REFs with wild-type p53 (wt) by 1.7-fold, and in MEFs with exogenous wild-type p53 (wt) by 2.8-fold. No enhancement was seen in wt null MEFs. Bars, 95% confidence intervals of the mean.

Fig. 3. Repair of DSBs analogous to Fig. 2. A, after treatment with IR, DSB rejoining was enhanced in cells with wild-type p53 by 2.8-fold. The same enhancement was seen in cells expressing the mutant protein p53-Val135, which has been deleted. In contrast to cells with wild-type p53, there appeared to be a somewhat higher basal level of repair (mean, 29%), but no enhancement was observed after irradiation (19%).

Results

Wild-Type p53 Enhances Rejoining of DSBs in Irradiated Cells. DSB repair activities were assessed by measuring the rejoining of cohesive double-stranded ends in a linearized episomal reporter plasmid coding for the luciferase gene (Fig. 1). Rejoining activity was first measured in primary MEFs with endogenous wild-type p53. In unirradiated cells, the relative portion of rejoined linear plasmid was 15% compared with the circular plasmid control. In parallel, cells were exposed to IR prior to transfection of plasmid substrate. In the presence of activated wild-type p53, rejoining activities increased by about 3-fold from baseline levels (Fig. 2). Testing immortalized MEFs that expressed exogenous wild-type p53 and primary REFs with endogenous p53 revealed the same results; mean basal rejoining activities were ~18%, and following IR treatment, a 2–3-fold increase was detected. The enhancement of DSB rejoining in cells with wild-type p53 was statistically highly significant (P = 0.002; Table 1). We next studied DSB rejoining in MEFs in which both p53 alleles had been deleted. In contrast to cells with wild-type p53, there appeared to be a somewhat higher basal level of repair (mean, 29%), but no enhancement was observed after irradiation (19%).

Enhancement of DSB Rejoining Is Independent of Transactivation. To investigate whether IR-induced DSB repair was dependent upon an intact transactivating function of p53, we used a well-characterized p53 mutant that contains an amino acid exchange at codon 135 (Ala to Val). It has been shown that this mutant protein cannot transcriptionally up-regulate downstream genes, but there are data to suggest that p53-Val135 may have retained regulatory prop-
cells with wild-type status were: primary MEFs (LUC) and REFs (LUC), 10.1/Val5 (LUC, CAT; see "Materials and Methods").

The enhancement is statistically significant at \( P = 0.002 \) (Wilcoxon signed-rank test, two-sided).

**Enhancement of DSB Rejoining Depends on the COOH-Terminal Domain.** Because regulation of DSB rejoining was not dependent on transactivation function, we next wanted to identify the domain of p53 that might have been directly involved in repair of DSB. We used MEFs that expressed an AS form of p53 that had lost its ability to bind nonspecifically to DNA due to inactivation of the basic COOH-terminal domain (residues 364–390). Importantly, the COOH-terminal domain has been found to participate in promoting the annealing of complimentary single-stranded nucleic acids. Thus, this domain was hypothesized to be particularly responsible for reannealing the cohesive ends of restriction enzyme-cleaved plasmid DNA. Consistent with this hypothesis, p53-AS failed to confer increased DSB rejoining after exposure to IR, in contrast to wild-type p53 with an intact COOH-terminus (Fig. 3b). Rejoining activities were 33 and 27% before and after irradiation, respectively. By analogy, when the Val135 mutant (Fig. 3a) was compared with a derivative that contained the same COOH-terminal alteration, the enhancement of DSB rejoining after irradiation was also lost, with rejoining activities being 56 and 24%, respectively (Fig. 3b). This high basal repair level was confirmed by the CAT reporter system (see “Discussion”).

**Rejoining of Blunt-Ended DSBs Is Not Enhanced by p53.** Finally, we wanted to test whether the DSB rejoining enhancement observed was dependent on the specific type of DSB present, i.e., breaks with cohesive ends, as could be inferred from the COOH-terminal strand-annealing function. We therefore used the same plasmid substrate as before but with the cohesive ends transformed into blunt ends. Rejoining of this DSB substrate was assessed in MEFs expressing exogenous wild-type p53 or the p53-Val135 mutant, analogous to Fig. 3a. There was no enhancement of rejoining activity after irradiation, neither in the presence of wild-type nor of mutant p53 (Fig. 3c). Note that the y-axis in Fig. 3c was altered because the basal levels of blunt-ended repair were found to be considerably lower than the rejoining activity observed for breaks with cohesive ends, i.e., ∼3% versus 20%, respectively.

**Discussion**

We showed here that the presence of p53 could enhance nonhomologous end-joining of DSBs with cohesive ends in γ-irradiated rodent embryonic fibroblasts (Fig. 2; Table 1). Our data suggest that p53 was directly involved in this regulation, because enhancement of DSB rejoining was dependent on the presence of nonspecific DNA binding activity as mediated by the basic COOH-terminal domain and was independent of transactivating function (Fig. 3). On the basis of the known strand-annealing property of the COOH-terminus and the observation that blunt-ended DSB repair was not modulated by p53, we conclude that p53 was enhancing DSB rejoining specifically by increasing the ability to reanneal short complementary strands of single-stranded DNA.

DSB repair was assessed in vivo by indirectly measuring the rejoining of an episomal linear reporter plasmid that had been transfected following exposure of cells to IR. Transfection of mammalian cells with plasmid substrates has been a common approach to study DSB repair activities (13, 17). It can be assumed that repair of DSBs generated by restriction enzymes at least partially involves the same pathways as used for repair of IR-induced DSBs (21). Furthermore, the use of a linearized plasmid substrate has the distinct advantage of introducing a uniform type of damage needing repair. Modifying the specific type of DSB present may allow us to draw conclusions regarding the underlying repair mechanisms, as indicated in this study by comparing cohesive versus blunt ends. In our assay, we avoided any bias that could result from plasmid amplification by the host cell, because the plasmids were not designed to replicate episomally. Forty-eight h were allowed for maximal plasmid repair and expression of reporter enzyme before determining rejoining activities, thereby excluding possible bias from any different repair kinetics after irradiation. By measuring enzymatic reporter activities based on parallel transfections with either an “undamaged” circular or a “damaged” linear reporter plasmid (including a β-galactosidase control), confounding variables should have been minimized under the assumption that linear and circular plasmid were taken up into cells with equal efficiency.

Rejoining enhancements obtained with the luciferase reporter were corroborated using another analogous reporter system (CAT; Table 1). It is emphasized that it was the enhancement that depended on p53. It was unclear whether p53 status also impacted on absolute levels of rejoining, which seemed to be somewhat assay related. In a few instances, loss of wild-type p53 did appear to result in higher basal rejoining levels (Figs. 2 and 3); however, it is unknown whether this finding has a biological significance. Interestingly, Bill et al. (15) found that loss of wild-type p53 function led to a 2-fold higher plasmid end-joining activity in total cellular extracts from unirradiated human lymphoblastoid lines. The authors interpreted these data to be consistent with the high spontaneous genetic instability seen in the p53-mutated cell line. However, comparison with our data may be complicated by the different assay and cell systems used (see below).

Given the nonspecific DNA binding properties of p53 and its various interactions with other repair proteins (1, 2), it was not surprising to find that the observed enhancement of DSB rejoining by p53 appeared to be independent of transactivation function. This conclusion was based on the finding that the p53-Val135 mutant, despite being unable to act as a transcription factor, was still capable

**Table 1 Comparison of DSB rejoining enhancements**

<table>
<thead>
<tr>
<th>Cellular p53 status</th>
<th>Fold enhancement after IR&lt;sup&gt;b&lt;/sup&gt;</th>
<th>LUC assay</th>
<th>CAT assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.5&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.4</td>
<td></td>
</tr>
<tr>
<td>Val135</td>
<td>2.2</td>
<td>1.4</td>
<td></td>
</tr>
<tr>
<td>Null</td>
<td>0.6</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Wild-type AS</td>
<td>0.8</td>
<td>0.9</td>
<td></td>
</tr>
<tr>
<td>Val135 AS</td>
<td>0.4</td>
<td>0.6</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Mean relative enhancements in rejoining activity of DNA DSBs with cohesive ends. LUC, luciferase reporter system; ND, not determined; AS, alternatively spliced COOH terminus.

<sup>b</sup> Cells with wild-type status were: primary MEFs (LUC) and REFs (LUC), 10.1/Val5 (LUC, CAT; see “Materials and Methods”).

<sup>c</sup> The enhancement is statistically significant at \( P = 0.002 \) (Wilcoxon signed-rank test, two-sided).

of modulating DSB rejoining (Fig. 3a). The notion that the p53-Val135 mutant has retained transactivation-independent regulatory functions is supported by other data. For example, Guillouf et al. (20) reported that p53-Val135 could mediate apoptosis after γ-irradiation of murine myeloblastic leukemia cells. Our laboratory found that p53-Val135 was able to suppress homologous recombination in MEF similar to the effect of endogenous wild-type p53.4

Our observation of a positive regulation of radiation-induced DSB repair by p53 was based on the comparison of wild-type p53 versus p53-null status in untransformed MEFs. In other cellular systems, different observations have been made. Bristow et al. (16) reported increased levels of DSB repair, together with increased radioresistance if wild-type p53 function was abrogated by expression of dominant-negative mutants in transformed REF clones. Mallya and Sikpi (17) observed a 2-fold higher activity of rejoining linear plasmid substrates after irradiation when they compared an EBV-transformed human lymphoblastoid line expressing a p53 mutant to a closely related line with wild-type p53. However, comparison of these and our data may be only done with caution: (a) different genetic backgrounds may determine how DSB are processed or responded to; (b) presence of mutant p53, as compared with wild-type status, may confer a gain-of-function phenotype (11); and (c) cell cycle profiles may impact on the way of how DSBs are processed (22). For example, p53-mediated G1 arrest after irradiation of untransformed fibroblasts (7, 9) presumably functions to allow DNA repair, including nonhomologous end-joining of DSB. However, in cells lacking G1 arrest such as the lymphoblastoid lines mentioned, p53 may trigger alternative responses.

What could be the physiological significance of an enhancement of DSB rejoining by p53? It appears unlikely that p53 increases total repair capacity for DSBs in response to γ-irradiation. Support for this view comes from the observation that there appears to be no difference in cellular radiosensitivity when comparing untransformed MEFs that are either wild-type or null for p53 (18). Of note, p53-dependent apoptosis was not found to play a role in untransformed MEFs exposed to IR (7, 23). Although p53 may not alter total levels of DSB repair, it may be involved in determining the optimal repair in the respective physiological context. DSBs can be repaired via two principal pathways, nonhomologous end-joining and homologous recombination. It appears that in mammalian cells, nonhomologous end-joining constitutes the dominant repair pathway (24) compared with homologous recombination. In our working model, p53 is an important determinant of the relative contributions of these pathways to break repair, in addition to other factors such as cellular genetic background and the cell cycle phase in which breaks occur (22). The data presented here indicate that p53 can positively modulate nonhomologous end-joining, at least the rejoining of breaks with short complementary ends of single-stranded DNA, and conversely we and others have shown p53 to restrict homologous recombination processes (5, 7). In turn, the absence of wild-type p53, up-regulated homologous recombination would be expected to make an increased contribution to DSB repair. In conclusion, our hypothesis is that DSB are predominantly repaired by nonhomologous end-joining in the presence of wild-type p53, such as in normal untransformed cells, but that homologous recombination becomes the preferred pathway when wild-type p53 function has been lost, such as in the majority of human tumor cells (2).

**Acknowledgments**

The authors are grateful to contributors of cells, Drs. A. Levine and T. Jacks, and to Dr. J. Wang for technical assistance.

**References**

p53 Directly Enhances Rejoining of DNA Double-Strand Breaks with Cohesive Ends in γ-Irradiated Mouse Fibroblasts

Wei Tang, Henning Willers and Simon N. Powell


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/59/11/2562

Cited articles
This article cites 22 articles, 11 of which you can access for free at:
http://cancerres.aacrjournals.org/content/59/11/2562.full#ref-list-1

Citing articles
This article has been cited by 9 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/59/11/2562.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.