The EMBO Journal vol.15 no.8 pp.1992–2002, 1996

p53 is linked directly to homologous recombination processes via RAD51/RecA protein interaction

Horst-Werner Stürzbecher[1], Beate Donzelmann, Wilhelm Henning, Uwe Knippschild and Sabine Buchhop

Heinrich-Pette-Institut für Experimentelle Virologie und Immunologie an der Universität Hamburg, Martinistrasse 52, D-20251 Hamburg, Germany

[1]Corresponding author

The tumour suppressor p53 prevents tumour formation after DNA damage by halting cell cycle progression to allow DNA repair or by inducing apoptotic cell death. Loss of wild-type p53 function renders cells resistant to DNA damage-induced cell cycle arrest and ultimately leads to genomic instabilities including gene amplifications, translocations and aneuploidy. Some of these chromosomal lesions are based on mechanisms that involve recombinatorial events. Here we report that p53 physically interacts with key factors of homologous recombination: the human RAD51 protein and its prokaryotic homologue RecA. *In vitro*, wild-type p53 inhibits defined biochemical activities of RecA protein, such as three-way DNA strand exchange and single strand DNA-dependent ATPase activity. *In vivo*, temperature-sensitive p53 forms complexes with RAD51 only in wild-type but not in mutant conformation. These observations suggest that functional wild-type p53 may select directly the appropriate pathway for DNA repair and control the extent and timing of the production of genetic variation via homologous recombination. Gene amplification and other types of chromosome rearrangements involved in tumour progression might occur not only as result of inappropriate cell proliferation but as a direct consequence of a defect in p53-mediated control of homologous recombination processes due to mutations in the p53 gene.

Keywords: homologous recombination/p53/protein–protein interaction/RAD51/RecA

Introduction

The genomic integrity of cells is controlled by a network of protein factors that assess the status of the genome at any given point in time and cause either progression of proliferation or induce a halt of the cell cycle. p53 has been identified as participating in this pathway controlling genomic stability (Lane, 1992, 1993). Mutations in the p53 gene ultimately lead to the disruption of chromosomal integrity. Chromosomal lesions include gene amplifications, translocations, aneuploidy and others. Some of these lesions are based on mechanisms that involve recombinatorial processes (Livingstone *et al.*, 1992; Yin *et al.*, 1992; Nelson and Kastan, 1994).

Recombination is a fundamental process that is essential for all living cells, as is the repair of damaged DNA. In *Escherichia coli*, the major pathway for homologous recombination absolutely requires the activities of the *recA* gene product. RecA protein searches for homologous regions between two double-stranded DNA (dsDNA) molecules and promotes strand exchange between them. It is also involved in recombinatorial repair of double strand breaks (DSBs) and controls the SOS response after DNA damage by UV radiation (Radding, 1991; Dunderdale and West, 1994; West, 1994). Recently, several proteins with remarkable structural homology to RecA, the RAD51 proteins, have been identified in eukaryotic organisms from yeast to man (Morita *et al.*, 1993; Ogawa *et al.*, 1993; Shinohara *et al.*, 1993; Yoshimura *et al.*, 1993). To secure a controlled realization of recombinatorial processes, the functional interplay of key factors like the RecA/RAD51 proteins with a panel of accessory factors that contribute to these reactions, via intrinsic biochemical activities or by physical interaction with key protein factors, is required (Hays *et al.*, 1995).

It is generally agreed that in mammalian cells processes creating genetic diversity must be kept at a tolerable level in order for cells to survive. A prominent factor which regulates DNA repair processes and guarantees genomic stability of cells is p53. The wide variety of chromosomal defects in cells without wild-type p53 and the extremely short period of time in which such lesions occur after loss of p53 function raises an important question as to the molecular mechanisms responsible for the genomic instabilities observed. The problem that has to be addressed, therefore, is whether such lesions are a mere passive consequence of cell cycle progression of p53-negative cells with DNA damage or whether there might be a more direct link between p53 and processes determining genomic stability or variability via recombinatorial DNA repair and homologous recombination. These considerations motivated us to study the direct interconnection between p53 function in maintaining chromosomal integrity and processes of homologous recombination and recombinational DNA repair.

We have used immunochemical assays *in vivo* and *in vitro* to detect direct interactions between p53 and key factors of homologous recombination, and functional *in vitro* assays to determine the quantitative and qualitative effects of p53 on biochemical recombination-associated activities. Using these techniques, we see a specific protein–protein association between p53 and human RAD51 or *E.coli* RecA recombination proteins. We find striking differences in the ability of p53 in wild-type or tumour mutant form to interact with RAD51 *in vivo* and we detect profound inhibitory activity of p53 towards RecA-catalysed three-strand exchange reactions and ATPase activity.

© Oxford University Press

Results

Purified p53 interacts with human RAD51 and E.coli RecA proteins in vitro

Any direct implication of p53 in regulating processes that maintain or modulate the genomic stability of cells might include either its function as a transcriptional regulator of relevant genes or, alternatively, its ability to interact physically with protein factors important in maintaining genomic stability. To test whether p53 and human RAD51, one of the key factors in DNA repair and recombination processes, might form heterologous protein complexes, *in vitro* translated wild-type human RAD51 protein and *E.coli*-produced purified human p53 proteins were analysed for physical interaction (Stürzbecher *et al.*, 1992). Purified wild-type or mutant p53 proteins were immobilized on Ni^{2+}–agarose beads via six histidine residues at the N-terminal end of the respective p53 polypeptides and incubated with *in vitro* translated [^{35}S]methionine-labelled human RAD51 protein. The results of these experiments show that full-length wild-type p53 and mutant I p53 (aa 365H→A 372K→L 379R→A 386K→L) carrying multiple point mutations in the C-terminal basic domain readily bind RAD51 protein (Figure 1A, h and i). To test for the specificity of the interaction observed, other protein factors were also reacted with purified p53. Irrelevant proteins like β-actin did not develop any p53 binding activity. Simian virus 40 (SV40) large T antigen formed complexes with full-length wild-type p53 but not with p53 fragments lacking the published T antigen binding region (Jenkins *et al.*, 1988; data not shown). Consequently, these *in vitro* binding experiments imply a specific physical interaction between p53 and the human RAD51 recombination factor.

Further controls showed that binding between p53 and RAD51 is not mediated by some nucleic acid intermediate, since nuclease treatment (DNase I, RNase A) of the purified proteins either before or after the binding reaction did not change the outcome of the experiment (data not shown). Additionally, Figure 1A also reveals that C-terminal fragments of wild-type p53 encompassing amino acids 264–393 and p53 fragments of identical size carrying point mutations in the basic region of p53 (mutant I p53: aa 365H→A 372K→L 379R→A 386K→L; mutant II: aa 373K→L 380H→L 387T→L) readily bind to *in vitro* translated RAD51 protein (Figure 1A, b, d and f). Shorter fragments, however, starting at amino acid 334 (Figure 1A, c and e) and amino acids 264–393 of a monomeric p53 mutant (mutant III: aa 341F→K 344L→E 348L→E 355A→K) (Figure 1A, g) only marginally interact with RAD51 compared with the control (Figure 1A, a). These results again argue for a direct protein–protein interaction between p53 and RAD51, since C-terminal p53 fragments starting at amino acid 334 do interact with nucleic acids in a non-sequence-specific manner, but are not able to form complexes with RecA (Figure 1B) or RAD51 (Figure 1A).

To determine whether complex formation with p53 is an evolutionarily conserved biochemical property of RAD51 homologous proteins, *E.coli* RecA protein was tested for p53 binding. In these experiments, the C-terminal p53 fragments described above were reacted with purified RecA protein (Boehringer, Mannheim). Again, the wild-type p53 fragment starting at amino acid 264 binds to purified *E.coli* RecA protein (Figure 1B, a, g and h), as

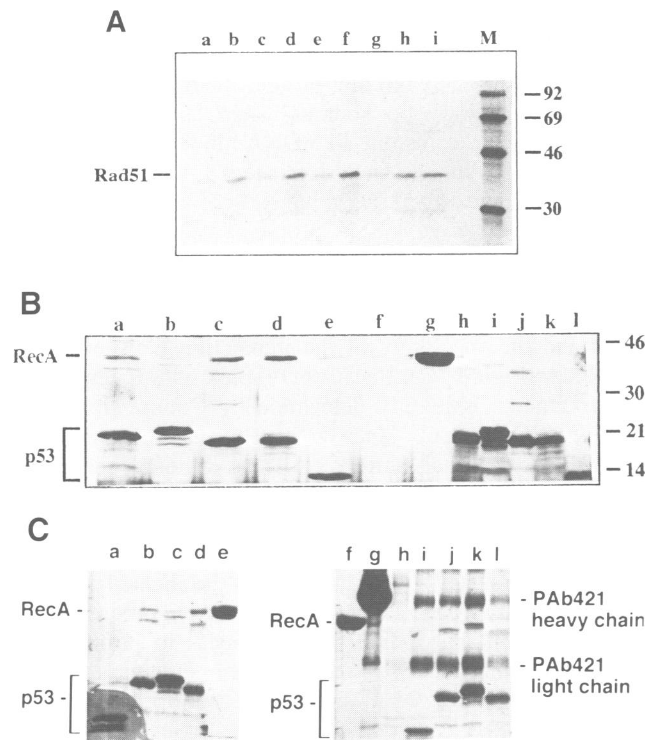

Fig. 1. *In vitro* association between p53 and RAD51/RecA proteins. (**A**) Purified human p53 binds to *in vitro* translated human RAD51 protein. (a) Control: interaction of *in vitro* translated RAD51 protein with Ni^{2+}-NTA agarose resin; (b–i) binding reactions of RAD51 with p53 proteins loaded on the Ni^{2+}-NTA agarose resin via six histidine residues at the N-terminus of the respective polypeptides. (b) Amino acids (aa) 264–393 of wild-type p53; (c) aa 334–393 of wild-type p53; (d) aa 264–393 of mutant I (aa 365H→A 372K→L 379R→A 386K→L); (e) aa 334–393 of mutant I; (f) aa 264–393 of mutant II (aa 373K→L 380H→L 387T→L); (g) aa 264–393 of mutant III (aa 341F→K 344L→E 348L→E 355A→K); (h) aa 1–393 of wild-type p53; (i) aa 1–393 of mutant I. (**B**) Purified human p53 fragments bind to purified *E.coli* RecA protein. (a–e) Binding reactions of purified RecA protein (Boehringer Mannheim) to p53 proteins loaded on the Ni^{2+}-NTA agarose resin; (f) control: interaction of purified RecA protein with the Ni^{2+}-NTA agarose resin; (g) 2 μg of purified RecA; (h–l) control: 2 μg of purified p53 fragments, no incubation with purified RecA protein; (a and h) aa 264–393 of wild-type p53; (b and i) aa 264–393 of mutant III; (c and j) aa 264–393 of mutant I; (d and k) aa 264–393 of mutant II; (e and l) aa 334–393 of mutant I. (**C**) Monoclonal anti-p53 antibody PAb421 blocks interaction between human p53 fragments and *E.coli* RecA protein. (a–d) Binding reactions of purified RecA protein (Boehringer, Mannheim) to p53 proteins loaded on the Ni^{2+}-NTA agarose resin; (e and f) 2 μg of purified RecA; (g) affinity chromatography of purified PAb421 antibody (4 μg); (h) control: interaction of purified PAb421 antibody with the Ni^{2+}-NTA agarose resin; (i to l) inhibition of p53–RecA interaction by introducing PAb421 into the binding reaction; (a and i) aa 334–393 of wild-type p53; (b and j) aa 264–393 of wild-type p53; (c and k) aa 264–393 of mutant III; (d and l) aa 264–393 of mutant II. The protein bands below the indicated RecA protein band in lanes b–d and j–l represent covalently linked dimeric oxidation products of the respective p53 fragments created during purification of recombinant protein. They are also present in the absence of RecA protein and react with PAb421 in Western blot analysis (data not shown).

do p53 fragments carrying point mutations in the basic region of p53 (Figure 1B, c, d, j and k). Monomeric p53 (Figure 1B, b and i) and fragments encompassing amino acids 334–393 (Figure 1B, e and l) bind poorly or not at all. In control experiments, several purified *E.coli* proteins, such as the integration host factor (IHF), the histone-like

DNA binding protein (HU) (gifts from Dr C.Vorgias, EMBL outstation DESY, Hamburg, Germany) and the single-stranded DNA binding protein (SSB), and proteins from other prokaryotic sources, such as the *Bacillus stearothermophilus* histone-like DNA binding protein and the gene 32 product of bacteriophage T4, were tested for p53 binding. The only protein that showed a weak positive signal was the *E.coli* SSB protein (data not shown). Antibody blocking experiments using the monoclonal anti-p53 antibody PAb421, recognizing a C-terminal epitope on p53 (aa 372–381; Wade-Evans and Jenkins, 1985), confirmed the specificity of the interaction between p53 and RecA (Figure 1C). RecA in complex with C-terminal p53 fragments is readily detectable by Coomassie Blue staining (Figure 1C, lanes b–d). Introducing purified PAb421 monoclonal antibody in the binding reaction completely prevents physical association between the p53 fragments and RecA protein (Figure 1C, lanes i–l).

Taken together, these data suggest that the region between amino acids 264 and 334 is implicated in the interaction with RAD51/RecA. Hence, the oligomerization and basic domains of p53 do not appear to participate directly in physical contact between the molecules. Blocking the binding by PAb421 must therefore be based on steric hindrance by the antibody molecule or the induction of conformational changes rather than direct interference with the binding site for RecA on the p53 molecule. Further mapping experiments performed in collaboration with Dr C.C.Harris (NIH, Bethesda) revealed that the requirements for human RAD51 binding of p53 are even more complex, with a second binding region within the core domain of p53 (M.K.Gibson, X.W.Wang, S.Buchhop, H.-W.Stürzbecher and C.C.Harris, manuscript in preparation).

As for the heterologous partner, the central domain between amino acids 33 and 240 of RecA and amino acids 96 and 314 of human RAD51, respectively, is considered to be required for the formation of a helical nucleoprotein filament with ATP and dsDNA (Shinohara *et al.*, 1993; Story *et al.*, 1993). Since both the *E.coli* and human protein interact with p53, this highly conserved central region of the respective proteins might be involved in complex formation with p53.

p53 interacts with RAD51 in vivo

To gain a better understanding of the interrelationship between p53 function and genomic integrity under *in vivo* conditions, we investigated whether p53 might form complexes with RAD51 in living cells.

The clone6 cell line originates from transformation of rat embryo fibroblasts (REFs) with activated *ras* and the temperature-sensitive mouse p53 mutant val135 (ts-p53) (Michalovitz *et al.*, 1990). At 32°C, clone6 cells express ts-p53 in wild-type conformation which leads to cell cycle arrest in G_1. At 38°C, ts-p53 adopts tumour-mutant conformation and develops oncogenic potential. The cells establish a transformed phenotype. To detect p53 in complex with RAD51 protein in these cells, Western blot analyses were performed using a polyclonal anti-RAD51 serum for immunoprecipitation of RAD51 immunocomplexes and PAb122 monoclonal anti-p53 antibody as secondary antibody to detect co-precipitating p53. Figure 2A demonstrates that p53 readily forms complexes with RAD51 in clone6 cells at 32°C but not at 38°C.

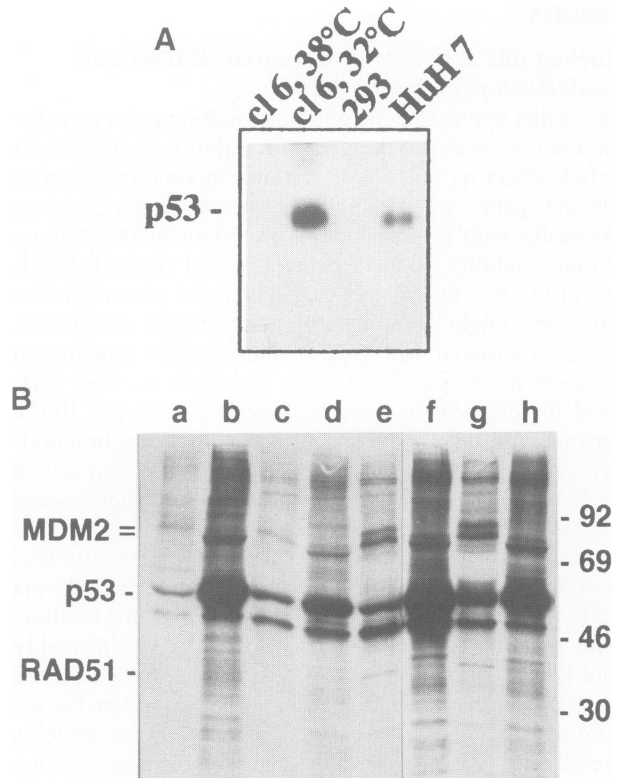

Fig. 2. Interaction between p53 and RAD51 *in vivo*. (**A**) Detection of co-precipitating p53 in anti-RAD51 immunocomplexes from different cell lines by Western analysis. cl 6: clone6 cells originated from rat embryo fibroblasts (REFs) transformed by the temperature-sensitive mouse p53 mutant val135 and *ras*. p53val135 adopts a wild-type-like conformation at 32°C and a mutant-like (PAb240 positive) conformation at 38°C. 293: adenovirus 5 (Ad5)-transformed human cell line, expressing PAb240-negative p53 in complex with Ad5 $p55^{E1b}$ protein. HuH7: human hepatocellular carcinoma cell line, expressing mutant p53. Unlabelled cell lysates were immunoprecipitated with polyclonal anti-RAD51 serum and analysed by SDS–PAGE. Subsequently, proteins were transferred to nitrocellulose. Co-precipitated p53 was detected using anti-p53 monoclonal PAb122 as second antibody. (**B**) Analysis of p53–RAD51 complexes from clone6 cells with a panel of anti-p53 monoclonal antibodies. Clone6 cells cultivated at 32°C (lanes a, c, e and g) or at 38°C (lanes b, d, f and h), respectively, were labelled for 2 h with $[^{35}S]$Trans-label. p53 was immunoprecipitated from the cell extracts with PAb240 (lanes a and b), PAb242 (lanes c and d), PAb246 (lane e and f) and PAb248 (lane g and h). Proteins were analysed by SDS–PAGE and fluorography. The subline of clone6 cells used continues to express some PAb246-positive p53 even at 38°C.

To substantiate this finding and to characterize the requirements for complex formation in more detail, p53 was immunoprecipitated from $[^{35}S]$methionine-labelled clone6 cells with a panel of anti-p53 monoclonals. Among those, the monoclonal PAb246 (Yewdell *et al.*, 1986) recognizes predominantly the wild-type form of p53 while PAb240 (Gannon *et al.*, 1990) reacts specifically with p53 in mutant conformation. Figure 2B shows that co-precipitating RAD51 is detectable in PAb246 (Figure 2B, e) and PAb248 (Figure 2B, g) immunocomplexes only at 32°C and not at 38°C (Figure 2B, lanes f and h) although RAD51 protein is expressed equally well at both temperatures. p53–RAD51 complexes are undetectable in PAb240 (Figure 2B, a and b) or PAb242 (Figure 2B, c and d)

immunoprecipitates from clone6 cells cultivated at either 32 or 38°C. The surprising result that p53–RAD51 complexes are virtually undetectable by PAb242 might indicate that this epitope on p53 might be masked upon binding to RAD51.

These results imply that p53–RAD51 complexes are formed in living cells and that there is a requirement for p53 in wild-type conformation for stable interaction with RAD51 *in vivo*. Since the level of RAD51 protein expressed in these fibroblast cell lines is very low compared with the overexpressed p53, co-precipitating RAD51 appears to be underrepresented in anti-p53 immunocomplexes as judged from the autoradiograph. Cascade immunoprecipitations revealed that in these cells there is always a subpopulation of RAD51-free p53, but also some RAD51 that is not bound to p53 (data not shown). Therefore, one has to assume that complex formation between these two proteins is regulated tightly in living cells.

Other cell lines were also examined for p53–RAD51 complex formation. Surprisingly, the Western blot analysis shown in Figure 2A reveals that p53 from 293 cells is not detectable in anti-RAD51 immunoprecipitates although RAD51 protein is readily detectable by immunoprecipitation (data not shown). Line 293 is an adenovirus-transformed human cell line expressing the adenovirus early region proteins (Graham and Smiley, 1977). p53 in these cells adopts wild-type conformation and is complexed to $p55^{E1b}$ protein. The N-terminus of the p53 polypeptide has been implicated in direct interaction with $p55^{E1b}$ (Kao *et al.*, 1990; Braithwaite *et al.*, 1991). This binding site partially overlaps with the epitope recognized by PAb242 anti-p53 monoclonal antibody (Yewdell *et al.*, 1986). Consequently, in analogy to the findings for PAb242 in clone6 cells, complex formation with $p55^{E1b}$ protein might interfere with RAD51 binding due to the conformational state of p53 required for this interaction, since the N-terminus of p53 is not implicated directly in RAD51 binding (M.K.Gibson, X.W.Wang, S.Buchhop, H.-W. Stürzbecher and C.C.Harris, manuscript in preparation).

The third cell line examined for p53–RAD51 complexes was the human hepatocarcinoma cell line HuH7. HuH7 cells express a mutant form of p53, in which the tyrosine residue at position 220 is substituted by cysteine (Hsu *et al.*, 1993). Figure 2A demonstrates that, although genetically mutant, a subpopulation of p53 from HuH7 cells is found in complex with RAD51. Immunoprecipitations of radiolabelled cell lysates clarify that the bulk of HuH7 p53 adopts a PAb240-precipitable mutant conformation. However, a significant amount of p53 is found in wild-type conformation detectable by monoclonal PAb1620. This subpopulation forms stable complexes with RAD51 (Figure 3A, 1, lane c). To test whether this difference in the ability of various forms of p53 to bind to RAD51 is maintained *in vitro*, parallel experiments were performed, in which p53 immunocomplexes from unlabelled HuH7 precipitated either with PAb1620, representing p53 in wild-type conformation (Figure 3A, 2, c), or PAb240, representing the mutant form (Figure 3A, 2, d), were tested for interaction with *in vitro* translated RAD51 protein. To obtain a quantitative measure of the capacity of the different forms of p53 to bind *in vitro* translated RAD51, the amount of PAb1620- and PAb240-immunoreactive p53 from long-term [^{35}S]methionine-labelled cells was calculated by densitometric analysis as was the relative amount of RAD51 bound to these different forms of p53 in the *in vitro* binding reaction. The ratio of these calculations indicates that there is an ~10-fold difference in the ability of p53 in the wild-type as compared with the mutant conformation to form stable complexes with RAD51 *in vitro*.

To illustrate the identity of p53 and RAD51 as partners in protein–protein complexes, two experimental approaches were chosen. The identity of co-precipitating RAD51 in anti-p53 immunocomplexes from HuH7 cells was verified by proteolytic cleavage of excised co-precipitating bands compared with RAD51 from anti-RAD51 immunocomplexes and authentic *in vitro* translated RAD51. The *N*-chlorosuccinimide (NCS) cleavage pattern of *in vitro* translated RAD51 (Figure 3B, lane b) appears identical to the sample of RAD51 immunoprecipitated either directly with polyclonal anti-RAD51 serum (Figure 3B, lane c) or as a co-precipitate from anti-p53 immunocomplexes (Figure 3B, lane a). These results verify the identity of RAD51 in anti-p53 immunocomplexes and the specific complex formation between p53 and RAD51 *in vivo*. As an alternative approach, two-dimensional phosphopeptide analysis was performed to verify the identity of co-precipitating p53 in RAD51 immunocomplexes and to ask whether complex formation with different partner proteins might be reflected in differences in post-translational modifications of p53. As a control for p53 in complex with a known cellular partner, the human MDM2 protein, which binds to the N-terminal region of p53, was chosen (Oliner *et al.*, 1992; Picksley *et al.*, 1994). The phosphorylation state of p53 immunoprecipitated directly with monoclonal PAb421 was compared with p53 in complex with either MDM2 or RAD51, detectable via polyclonal anti-MDM2 serum or anti-RAD51 serum. The results are shown in Figure 3C. Phosphopeptides were identified and labelled according to the nomenclature first published by Steenenga *et al.* (1995). Phosphopeptide M1 corresponds to amino acids 386–393 which include the CKII phosphorylation site at Ser392, M2 represents amino acids 307–320 and contains Ser315, which is phosphorylated by CDK2, and M3-1 and M3-3, both of which correspond to amino acids 1–24 and include target amino acids for DNA-dependent protein kinase (DNA-PK) (Ser4, 6 and 15) and CKI (Ser4, 6 and 9). Peptide M3-3 is the highly phosphorylated variant of peptide M3-1. All of these peptides can be identified in total p53 (Figure 3C, a) as well as in p53 in complex with MDM2 (Figure 3C, c) and RAD51 (Figure 3C, b). In addition, Figure 3C shows at least three as yet uncharacterized phosphopeptides (a, b and c), one of which (a) may correspond to a more highly phosphorylated form of peptide M2. Thus, these phosphopeptide maps confirm the identity of co-precipitating p53 in anti-RAD51 immunocomplexes. Secondly, the maps clearly demonstrate qualitative differences between total p53 and p53 in complex with MDM2 and RAD51. Complex formation between p53 and different cellular partners might therefore be reflected in a specific phosphorylation state of p53. However, phosphorylation of p53 *per se* is not a prerequisite for stable complex formation with RAD51, since *E.coli*-produced unphosphorylated p53 readily interacts with RAD51 *in vitro*.

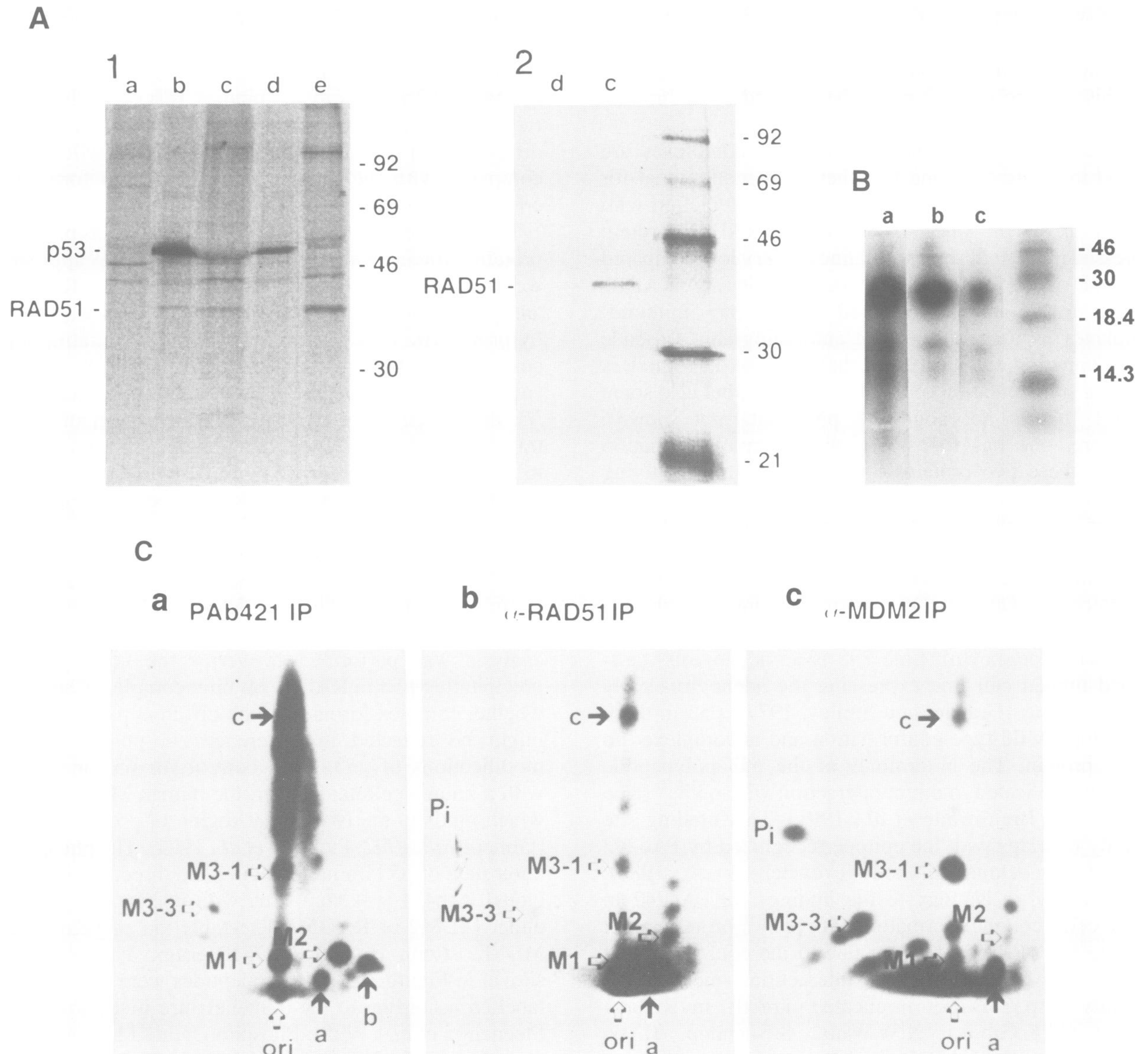

Fig. 3. Characterization of p53–RAD51 complexes from HuH7 cells. (**A**) (1) Analysis of p53 and RAD51 from [^{35}S]methionine-labelled HuH7 cells. (a) Rabbit pre-immune serum; (b) PAb122; (c) PAb1620; (d) 240; (e) polyclonal anti-RAD51 serum. (2) Binding of *in vitro* translated, [^{35}S]methionine-labelled RAD51 to unlabelled p53 protein from HuH7 cells, obtained in a parallel experiment to that shown in 1. (d) p53 was immunoprecipitated with PAb240; (c) p53 was immunoprecipitated with PAb1620. Proteins were analysed by SDS–PAGE and fluorography. (**B**) Partial proteolysis map of authentic RAD51 and RAD51 in complex with p53 from HuH7 cells. (a) Co-immunoprecipitated RAD51 protein derived from anti-p53 immune complexes (PAb421) from HuH7 cells; (b) authentic *in vitro* translated human RAD51 protein; (c) RAD51 from HuH7 cells derived from immunoprecipitation using polyclonal anti-RAD51 serum. Proteins were analysed by SDS–PAGE and fluorography. For details of partial proteolysis see Materials and methods. (**C**) Two-dimensional phosphopeptide analysis of p53 from HuH7 cells. Tryptic digests of ^{32}P-labelled PAb421 (a), anti-RAD51 (b) and anti-MDM2 (c) serum-precipitated p53 excised from SDS–PAGE gels were separated at pH 1.9 (horizontal direction) and by chromatography (vertical direction) as described in the text. About 1800, 1200 and 1800 c.p.m. were loaded on the (a), (b) and (c) plates, respectively. Autoradiograms were exposed for 4 days (a and c) and 6 days (b) at –70°C with an intensifying screen. P_i, inorganic phosphate; ori, origin of two-dimensional separation of phosphopeptides; M1, M2, M3-1 and M3-3, characterized phosphopeptides according to Steenenga *et al.* (1995) as described in the text; a, b and c, additional as yet uncharacterized phosphopeptides.

In summary, the findings presented clearly demonstrate that p53–RAD51 protein complexes are formed *in vivo* and that the formation of such complexes in living cells is dependent upon aspects of the wild-type conformation of p53 molecules. Although it is not a prerequisite for complex formation, the phosphorylation pattern of p53 might be indicative of the subpopulation of p53 molecules in complex with either RAD51 and/or MDM2 proteins.

p53 inhibits RecA-mediated transfer of complementary DNA strands

Recent data provide evidence that *Saccharomyces cerevisiae* RAD51 protein develops single-stranded DNA (ssDNA)-dependent ATPase and strand exchange activity similar to *E.coli* RecA protein (Sung, 1994). Human RAD51 protein binds to single- and double-stranded DNA, exhibits DNA-dependent ATPase activity and is capable

of underwinding duplex DNA (Benson *et al.*, 1994). A summary of the known biochemical activities of eukaryotic RAD51 proteins and *E.coli* RecA is given in Table I. For the strand exchange reaction, *E.coli* RecA as well as eukaryotic RAD51 proteins require the concomitant action of ssDNA binding protein. It is known that p53 itself binds to the mammalian ssDNA binding protein RPA and inhibits its interaction with ssDNA (Dutta *et al.*, 1993). Consequently, any effects of p53 on RAD51-promoted strand exchange in a homologous system using mammalian proteins might, at least in part, be caused by p53–RPA interaction. On the other hand, p53 does not bind to the ssDNA binding gene 32 protein of bacteriophage T4. Considering these arguments, the sets of experiments described below concentrate on the effects of human p53 on the biochemical activities of *E.coli* RecA protein. For this set of experiments, wild-type and mutant p53 proteins were expressed in *E.coli* and purified to near homogeneity as described earlier (Stürzbecher *et al.*, 1992). The identity of the purified protein was established by immunoblotting, and correct folding was verified by *in vitro* binding studies with SV40 large T antigen. Purified full-length human wild-type (Figure 4A, a, b and c) and mutant I p53 (Figure 4A, e, f and g) were tested for modulation of the RecA-promoted strand exchange reaction between circular single-stranded φX174 and double-stranded (ds) linear φX174 phage DNA (Morita *et al.*, 1993; Ogawa *et al.*, 1993; Yoshimura *et al.*, 1993) in the presence of T4 gene 32 protein. At a ratio of 0.5 p53 to RecA protein molecules, p53 completely inhibits the formation of nicked circular dsDNA (Figure 4A, replicative form II DNA). Under identical conditions, p53 itself does not catalyse this strand transfer reaction (Figure 4A, d and h). The aa 264–393 fragment of wild-type p53 suppresses the reaction in the same way as the full-length protein (Figure 4B, a, b and c). The shorter aa 334–393 fragment (Figure 4B, e, f and g) or irrelevant proteins like the *B.stearothermophilus* histone-like protein do not cause any measurable effect (data not shown). These results clearly demonstrate that p53 directly interferes with RecA-catalysed strand exchange between long stretches of homologous DNA sequences. Suppression of the reaction appears to be specific for p53 and correlates with its ability to bind to RecA/RAD51. The observed inhibition is not due simply to competition for DNA binding, since the aa 334–393 p53 fragment, although able to interact with nucleic acids, does not block the strand exchange reaction.

Table I. Biochemical activities of *E.coli* RecA protein and its eukaryotic homologues

	E.coli RecA	*S.cerevisiae* RAD51	*H.sapiens* RAD51
Rec homologue		+	+
DNA binding	+	+	+
ssDNA-dependent ATPase	+	+	+
Renaturation of cDNA	+	+	+
Nucleoprotein filament formation	+	+	+
Strand transfer reaction	+	+	+
5′ to 3′ exonuclease activity	–	–	

For references, see Radding (1991), Benson *et al.* (1994), Dunderdale and West (1994), Sung (1994) and Sung and Robberson (1995).

p53 suppresses RecA-catalysed ssDNA-dependent ATP hydrolysis

The initiation of homologous recombination requires the generation of a region of ssDNA for binding of RecA molecules. Assembly of the nucleoprotein filament by the co-operative binding of RecA to dsDNA in the presence of ATP causes an extension of the DNA helix. ATP cleavage occurs late during the strand exchange reaction and may be required for the release of the DNA products and disassembly of the nucleoprotein filaments (Radding, 1991; Dunderdale and West, 1994; West, 1994). The ssDNA-dependent ATPase activity of RecA protein was determined *in vitro* as a function of increasing amounts of p53. Figure 5A illustrates that full-length wild-type and mutant I p53 severely suppress RecA-promoted ATPase activity. Only equimolar amounts of full-length p53 and RecA proteins are required for 90% inhibition. Wild-type and mutant aa 264–393 p53 fragments blocked the RecA ssDNA-dependent ATPase reaction almost completely at a molar ratio between p53 and RecA of 4 to 7.5 (Figure 5B). Although the protein–protein interaction between the wild-type p53 fragment and RecA appears to be less stable than for the variants tested (Figure 1), it inhibits the ATPase reaction at a lower molar ratio than the better binding variants. aa 334–393 p53 fragments do not affect the RecA ATPase activity at all (Figure 5B). This latter result clearly argues against the interpretation that suppression might be due to competition between p53 and RecA

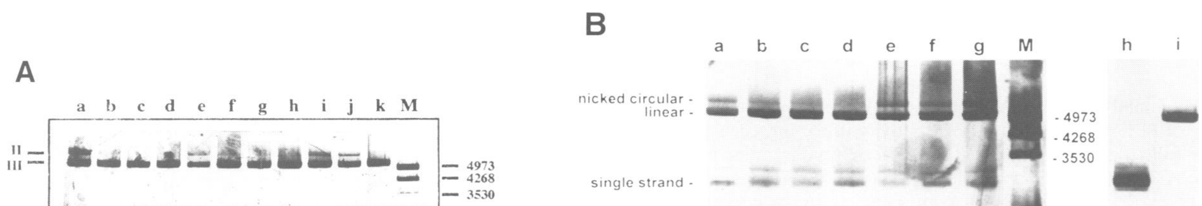

Fig. 4. p53 inhibits three-strand exchange reactions catalysed by RecA protein. (**A**) Lanes a–c represent 0.2, 0.4 and 0.6 μg of purified full-length human wild-type p53 included in the RecA-catalysed strand exchange reaction; lane d contains 0.6 μg of wild-type p53 but no RecA protein; lanes e–g represent 0.2, 0.4 and 0.6 mg of purified full-length mutant I p53; lane h contains 0.6 mg of mutant I but no RecA protein; lane i represents the buffer control containing all reaction components plus 6 μl of p53 buffer devoid of p53; lane j is the positive control without any p53; lane k represents the negative control without added RecA protein; M: molecular weight marker; II: circular duplex DNA containing a nick (form II); III: linear duplex DNA (form III). (**B**) Lanes a–c represent 0.2, 0.4 and 0.6 μg of purified aa 264–393 human wild-type p53 fragment included in the RecA-catalysed strand exchange reaction; lane d contains 0.6 μg of aa 264–393 wild-type p53 fragment but no RecA protein; lanes e–g represent 0.2, 0.4 and 0.6 μg of purified aa 334–393 wild-type p53 fragment included in the RecA-catalysed strand exchange reaction; lane h contains 0.2 μg of single-stranded, circular φX174 viral input DNA; and lane i 0.2 μg of *Xho*I-digested double stranded linear φX174 input DNA.

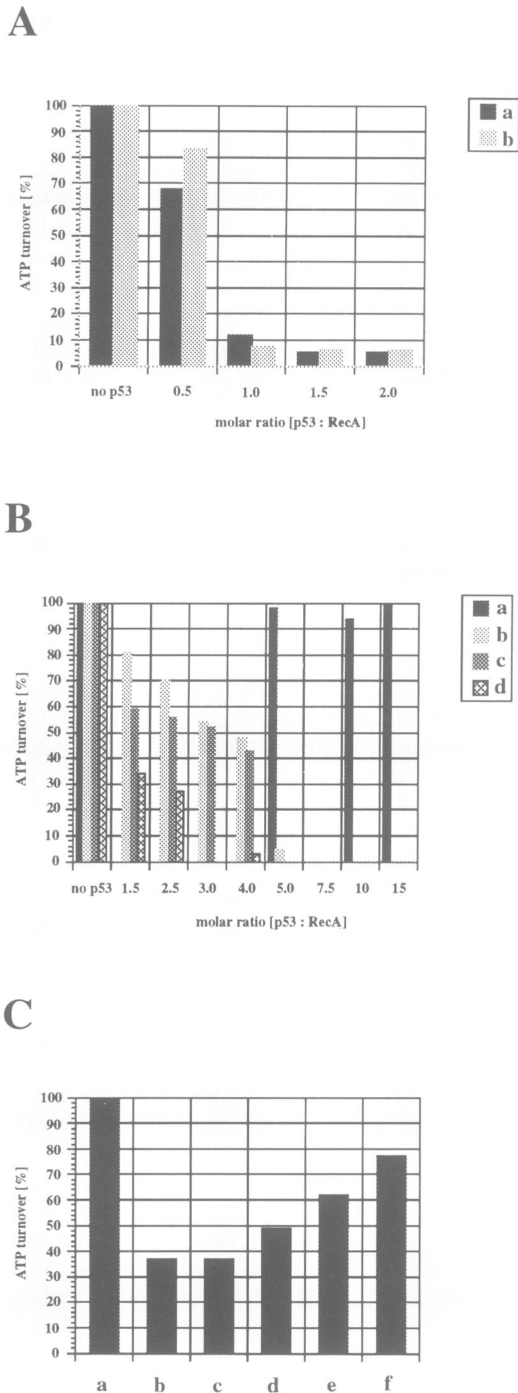

Fig. 5. p53 inhibits the single-stranded DNA-dependent ATPase activity of RecA protein. (**A**) ATPase activity in the presence of purified full-length p53 proteins at the indicated molar ratios to RecA protein; ATP turnover (%) represents a measure of ATP degradation catalysed by RecA protein; 100% corresponds to the reaction in the absence of p53. (a) Full-length wild-type p53; (b) full-length mutant I p53. (**B**) ATPase activity in the presence of purified C-terminal fragments of p53. (a) aa 334–393 of mutant I p53; (b) aa 264–393 of mutant I p53; (c) aa 264–393 of mutant II p53; (d) aa 264–393 of wild-type p53. (**C**) Release of the p53-induced inhibition of ATPase activity by SV40 T antigen. (a) RecA-catalysed ATP degradation; (b) ATP turnover in the presence of full-length wild-type p53 at a molar ratio (p53:RecA) of 0.8; (c–f) ATP turnover under conditions identical to (b) with increasing amounts of purified SV40 T antigen: (c) 0.2 μg; (d) 0.4 μg; (e) 0.6 μg; (f) 0.8 μg.

for DNA binding, since the aa 334–393 p53 fragment readily interacts with DNA but does not cause any effect on RecA ATPase activity. The block caused by C-terminal p53 fragments (aa 264–393) can be overcome by including the anti-p53-specific monoclonal PAb421 in the reaction (data not shown).

The large tumour antigens of polyomavirus and SV40 are known to promote recombination events very efficiently (St-Onge *et al.*, 1990; St-Onge and Bastin, 1993). T antigen has been proposed to facilitate recombinatorial processes by melting and unwinding of DNA at the viral origin of replication, so as to create a favourable substrate for homologous recombination (St-Onge *et al.*, 1990; St-Onge and Bastin, 1993). To test whether, in addition to these mechanisms, SV40 T antigen would cause a direct effect on p53-controlled recombinatorial activities, increasing amounts of purified T antigen were added to the RecA ATPase reaction under p53 block. Figure 5C clearly shows that T antigen is capable of releasing the p53-dependent suppression of RecA-catalysed ATP degradation. In control experiments, the T antigen intrinsic ATPase activity was tested under RecA reaction conditions resulting in negligible ATP turnover rates (1–5%) compared with the RecA activity. These findings suggest that SV40 T antigen, in addition to the mechanisms described above, might promote homologous recombination through interference with p53-dependent suppression of RecA/RAD51-catalysed reactions.

Discussion

The studies reported here demonstrate for the first time the direct physical interaction between the tumour suppressor p53 and RAD51, the key factor of homologous recombination and recombinatorial DNA repair. Complex formation with p53 appears to be accomplished by direct protein–protein interaction and is a property conserved between RAD51 homologues from *Homo sapiens* to *E. coli* (RecA). Binding can be observed *in vitro* and also in living cells. Here, complex formation appears to be dependent on aspects of p53 'wild-type' like conformation. *In vitro*, p53 exerts inhibitory effects on such biochemical activities of RecA protein as the three-way DNA strand exchange reaction and the RecA-catalysed degradation of ATP. This suppression of ATPase activity can be released by SV40 T antigen.

Major functions of wild-type p53 protein include the regulation of a G_1–S cell cycle checkpoint activated by DNA strand breaks and the mediation of apoptosis in certain situations in response to DNA damage (Kastan *et al.*, 1991; Lane, 1992; Livingstone *et al.*, 1992; Yin *et al.*, 1992). Strand breaks may predispose to genomic alterations associated with neoplastic transformation and progression (Nelson and Kastan, 1994). Broken DNA ends are potentially recombinogenic, resulting in chromosome segment translocations, deletions or amplifications in cells that traverse the cell cycle (Livingstone *et al.*, 1992; Yin *et al.*, 1992). The problem for mammalian cells in dealing with such a threat appears to be solved at least partially by the creation of the p53-dependent DNA damage response pathway. The results presented here directly link p53 *in vivo* and *in vitro* with the major pathways for homo-

logous recombination and/or recombinatorial DNA repair conserved from bacteria to man.

Physical interaction between p53 in wild-type conformation and the *RAD51* gene product might play a major role in controlling the extent and timing of homologous recombination processes, thereby influencing the genomic stability of cells. Some variants of p53 in mutant conformation (Figure 2) display highly reduced interaction with RAD51 *in vivo*. It is tempting to speculate that this difference might be the molecular basis for genomic instability of cells without functional wild-type p53 protein. The mechanism by which p53 inhibits RecA/RAD51-promoted recombinational events is not understood in detail at present. p53 might impair the biochemical activities of RecA/RAD51 simply by physical protein–protein contact. Although binding to RAD51/RecA appears necessary, this interpretation does not explain entirely the observed experimental data. Wild-type p53 blocks RecA activities at a much lower molar ratio than the variant p53 proteins tested. However, some of the carboxy-terminal mutants of p53 interact much more strongly with RecA/RAD51 yet they appear to interfere less strongly with RecA-catalysed biochemical activities. The simplest model to explain the observed effects of p53 would suggest that p53 may prevent the dissociation of RecA/RAD51 from DNA by blocking its ATPase activity. This activity is needed for the release of RecA from the DNA, thus fixing it onto one target DNA molecule and preventing the recruitment of other potential targets for homologous recombination. This block by p53 might then be released either by interaction of p53 with viral proteins like SV40 large T antigen (see Figure 5) or by complex formation with cellular factors. One potential candidate for such a cellular 'release factor' is certainly the MDM2 protein. Experiments to address this question directly are being performed currently in the laboratory. Biochemical activities of p53 itself, such as the renaturation of short single-stranded complementary DNA molecules, the preferred binding of p53 to ssDNA ends with transition to double-stranded segments and the p53-catalysed DNA strand transfer between short duplex DNA and complementary ssDNA (Oberosler *et al.*, 1993; Bakalkin *et al.*, 1994, 1995), have also to be taken into account.

With respect to the role of p53 in tumour biology, the findings presented here argue that p53 participates directly in certain pathways of DNA repair (Wang *et al.*, 1994) but inhibits others such as the recombination-dependent repair of DSBs. In yeast, the repair of DSBs is mediated almost exclusively by *RAD52* epistasis group-dependent recombination. Within this group of genes, mutations in *RAD51*, *RAD52* and *RAD54* result in the most severe recombination and repair defects, suggesting that these genes may encode the central components critical for recombinatorial repair (Game, 1983). There is, however, genetic evidence for an antagonistic interaction between proteins involved in recombinatorial repair and the product of the *srs2* gene. Removal of SRS2 allows recombinational repair of lesions that otherwise would be processed by the post-replication repair machinery (Milne *et al.*, 1995). RAD51 on the other hand, appears to work as a positive regulator of recombinatorial repair (Milne *et al.*, 1995). Additionally, mismatch correction acts as a barrier to homologous recombination in *S.cerevisiae* (Selva *et al.*, 1995), arguing that recombinatorial processes appear to be tightly controlled and that there exist regulatory mechanisms that decide which one of several possible pathways to use to deal with lesions in the DNA. The mechanisms by which mammalian cells repair DSBs are not clearly understood, but it is likely that the majority of such repair involves the rejoining of the broken ends without the intervention of homologous recombination. Recently it has been shown that the repair of DSBs in DNA and the recombination of antibody gene V(D)J segments share a common pathway involving the Ku protein, which binds to DNA ends, and its associated protein, the DNA-PK. For V(D)J recombination, a scenario is emerging in which the Ku antigen binds to pre-formed DSBs. Subsequent complex formation and activation of DNA-PK then triggers the assembly of proteins at this aggregate, resulting in the accessibility of the DNA ends to a nuclease and the rejoining of DNA ends with limited complementary sequences (for review, see Roth *et al.*, 1995).

The possibility that DNA-PK is also involved in cell cycle control is appealing. Activation of DNA-PK by binding to broken DNA ends would provide a logical sensing mechanism that could prevent cells from initiating DNA replication before they had repaired damage in their DNA. DNA-PK is one of the enzymes that phosphorylates p53 (Wang and Eckhart, 1992). Mutation of this phosphorylation site of human p53 reduces the ability of p53 to inhibit cell cycle progression (Fiscella *et al.*, 1993). Thus, the wild-type p53-dependent G_1 checkpoint might not only prevent DNA replication of damaged template DNA, but also might be the means by which a cell favours one pathway of DNA repair and, at the same time, actively prevents the exchange of damaged DNA via homologous recombination at inappropriate time points of the cell cycle. Loss of wild-type p53 function might lead directly to uncontrolled exchange of genomic material via homologous recombination and ultimately cause chromosomal disruption. Meyn and co-workers (1994) have obtained a first piece of evidence that this interpretation might reflect the *in vivo* situation. In order to interfere with the G_1–S cell cycle checkpoint, they transfected a normal cell line with vectors expressing either a dominant-negative p53ala143 mutant or a human papilloma virus E6 gene. These transformants showed 10- to 80-fold elevations in spontaneous recombination rates when compared with their parent.

In *S.cerevisiae*, RAD51 protein is expressed in a cell cycle-dependent manner with maximum expression occurring in late G_1 to early S (Basile *et al.*, 1992). It will be interesting to see whether p53–RAD51 complex formation might be restricted to a particular time in the cell cycle, thus determining the quantity and perhaps quality of genome integrity. Elevated p53 protein levels have been detected in developing spermatocytes which undergo meiotic recombination events (Almon *et al.*, 1993; Schwartz *et al.*, 1993). Here again, p53 might not only minimize non-homologous meiotic recombinations and other heritable DNA template alterations, but also regulate the extent and timing of homologous meiotic recombination. Determining the exact nature of the physical and functional involvement of p53 in RAD51-controlled recombinatorial processes might provide a deeper under-

standing of the interrelationship between p53-induced G_1 cell cycle arrest and the control of genomic integrity by p53.

Materials and methods

Recombinant plasmids

A cDNA of the *rad51* gene was amplified by PCR from a human testis cDNA library. The nucleotide sequence was verified by dideoxy nucleotide sequencing and the cDNA cloned into the pCR^{TM}II plasmid vector under the control of the SP6 promoter. All p53 cDNAs were cloned into the pET19b prokaryotic expression plasmid (AGS GmbH, Heidelberg). p53 mutants I, II and III, as well as the respective C-termini of the p53 proteins, were created by PCR-directed mutagenesis of wild-type human p53 as described previously (Stürzbecher *et al.*, 1992).

Sera and monoclonal antibodies

The hybridomas PAb122 (Gurney *et al.*, 1980), PAb421 (Harlow *et al.*, 1981), PAb242, PAb248 (Yewdell *et al.*, 1986) and PAb240 (Gannon *et al.*, 1990) were provided by D.P.Lane. Antibody PAb1620 (Ball *et al.*, 1984) was provided by G.Brandner. The polyclonal anti-RAD51 and anti-MDM2 (S.Buchhop, T.Kolzau and H.-W.Stürzbecher) sera were raised against purified full-length human RAD51 and MDM2 proteins produced in *E.coli*.

In vitro transcription/translation

For *in vitro* expression of the RAD51 protein, the pCR^{TM}II plasmid containing the human *rad51* cDNA insert was linearized with *Xba*I. Transcription was performed using the commercial 'Transcription *in vitro* system' (Promega) in 100 µl reactions containing 40 mM Tris–HCl, pH 7.5, 6 mM $MgCl_2$, 10 mM NaCl, 2 mM spermidine, 10 mM dithiothreitol (DTT), 100 U of RNasin, 0.5 mM rNTPs, 40 U of SP6 polymerase and 2–4 µg of DNA template. After 2 h incubation at 37°C the template DNA was removed by digestion with DNase. RNAs were extracted with phenol–chloroform, ethanol precipitated and used for *in vitro* translation. Protein synthesis was performed in a messenger-dependent rabbit reticulocyte lysate (Promega) containing 0.8 mCi/ml [^{35}S]methionine according to the supplier's instructions.

Protein purification

For p53 purification, plasmid-transformed BL21(DE3) *E.coli* colonies were cultured overnight, diluted 10-fold with fresh LB medium and incubated at 30°C to an OD_{600} of 0.6 prior to induction with 1 mM IPTG. After 3 h, cells were harvested by centrifugation and lysed in 6 M guanidinium-hydrochloride, 50 mM Tris–HCl, pH 8.0 with shaking for 1 h at 4°C. The lysate was cleared by centrifugation (10 000 *g*, 20 min, 4°C) and the supernatant reacted overnight at 4°C with Ni^{2+}-NTA agarose resin followed by extensive washing with 6 M guanidinium-hydrochloride, 50 mM Tris–HCl, pH 8.0, and stepwise dialysis against p53 buffer (25 mM Tris–HCl, pH 8.0, 2 mM EDTA, 10 mM 2-mercaptoethanol, 0.1% Triton X-100, 7.5% glycerol, 300 mM NaCl; Oberosler *et al.*, 1993) containing 1, 0.1 and 0 M guanidinium hydrochloride. The resin was transferred to a column and washed twice with p53 buffer, pH 6.3. p53 proteins were eluted with p53 buffer, pH 5.0. The pH was immediately readjusted to 8.0 and the protein stored in aliquots under liquid nitrogen.

In vitro protein–protein binding

For *in vitro* protein binding reactions, 20 µl of Ni^{2+}-NTA agarose resin, equilibrated in NET-N buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% NP-40) (Ewen *et al.*, 1989) were incubated for 45 min at room temperature with 2 µg of purified p53 protein. After washing twice with NET-N, either 5 µl of *in vitro* translated RAD51 or 2 µg of purified RecA protein were added. The reactions were incubated with shaking for 30 min at room temperature and washed three times with NET-N buffer. Retained proteins were analysed by SDS–PAGE, followed by either fluorography or staining with silver or Coomassie Brilliant Blue. With ~90% binding the efficiency of binding to the Ni^{2+} matrix was similar for the different p53 proteins used. Binding reactions were carried out with equal amounts of input p53-carrying Ni^{2+} matrix. Under the conditions used, ~15–20% of input RecA protein interacted with wild-type p53.

Radiolabelling, extraction of cells and immunoprecipitation

A total of 10^6 cells was labelled with 200 µCi of [^{35}S]Trans-label (ICN Radiochemicals) for 2 h. The cells were then washed and lysed for 15 min on ice with 500 ml of EBC buffer (50 mM Tris–HCl, pH 8.0, 120 mM sodium chloride, 0.5% NP-40) containing 10 µg/ml each of aprotinin (Sigma) and phenylmethylsulfonyl fluoride (PMSF; Sigma) (Ewen *et al.*, 1989). Lysates were cleared by centrifugation (4°C; 30 min; 13 000 *g*). Supernatants were mixed with 0.5 ml of NET-N (Ewen *et al.*, 1989) containing the appropriate antibody. The mixture was rocked for 1 h at 4°C before addition of 20 µl of washed protein A–Sepharose (Pharmacia). After 30 min incubation, beads were washed five times with NET-N, immunocomplexes were eluted from the beads, and resolved using SDS–PAGE. Bacterially expressed proteins were stained with Coomassie Brilliant Blue. Transfer of proteins to nitrocellulose filters and Western blotting were performed using the ECL detection system (Amersham) according to the protocol of the manufacturer with modifications to prevent interactions of the secondary antibodies with the heavy and light IgG chains of the primary antibody.

Three-strand exchange reaction

Three-strand exchange reactions were carried out as follows: 0.15 µg of *Xho*I-linearized φX174 DNA and 0.04 µg of circular single-stranded φX174 DNA were mixed in incubation buffer (25 mM Tris–HCl, pH 7.2; 10 mM $MgCl_2$, 1 mM DTT, 5% glycerol) and pre-incubated for 10 min at 37°C. The following components were then added (final concentrations): 1 mM ATP, 6 mM phosphocreatine, 10 U/ml creatine phosphokinase, 14 ng/µl T4 gene 32 protein, 75 ng/µl RecA protein. The final volume was 40 µl. The mixture was incubated for 60 min at 37°C. After incubation with proteinase K, the reaction products were analysed by electrophoresis on a 0.8% agarose gel. DNA was visualized by staining with silver.

ATPase activity assay

For five ATPase reactions, 2 µg of RecA protein and 2 µg of heat-denatured activated calf thymus DNA in reaction buffer (25 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 5% glycerol) were pre-incubated at 25°C for 5 min. The reaction was started by adding an equal volume of 0.5 mM [γ-^{32}P]ATP in reaction buffer. After 4 min incubation at 25°C, the reactions were quenched with 40 µl of 0.5 M HCl and subsequently incubated for 5 min at room temperature with 250 µl of acid-washed activated charcoal (7.5 % w/v). The charcoal was pelleted twice by centrifugation and the supernatant was counted for free $^{32}P_i$ by Cerenkov radiation analysis. Under these conditions, the ATP turnover catalysed by RecA was linear for up to 20 min with a turnover rate of ~120 pmol/min.

Partial proteolysis mapping

N-Chlorosuccinimide (NCS) cleavage was performed essentially as described elsewhere (Stürzbecher *et al.*, 1990). After analysis of [^{35}S]methionine-labelled immunoprecipitated proteins by SDS–PAGE, the wet gel was exposed to autoradiography overnight, the relevant bands were excised and rinsed several times in a 0.1% solution of urea in acetic acid and water (1:1). The gel slice was then exposed for 30 min to 40 mM NCS in the same solution. After further washes, the slices were equilibrated in Laemmli buffer and loaded directly onto a 17% SDS–polyacrylamide gel. The gel was fixed, dried and analysed by autoradiography. To circumvent potential problems with minor amounts of contaminating proteins of similar size from the cell lysate in the immunocomplexes, this protocol of cleavage at high molar excess of NCS was used. Although the primary amino acid sequence of RAD51 does not contain tryptophan residues, some cleavage does occur (Lischwe and Sung, 1977). Any tryptophan-containing contaminating protein would have been detected by this approach.

Phosphopeptide analysis

HuH7 cells were labelled for 4 h with 1 mCi $^{32}P_i$ (carrier free; Hartmann Analytics) in P_i-free DMEM supplemented with 5% P_i-free fetal calf serum (FCS). Whole cell extracts were prepared by lysis in a buffer containing 20 mM Na_2HPO_4 (pH 9.0), 120 mM NaCl, 1% NP-40, 5 mM DTT, 30 µg of aprotinin (Trasylol, Bayer), 10 µg of leupeptin and 20% FCS, followed by immunoprecipitation with polyclonal anti-MDM2 or anti-RAD51 serum or monoclonal anti-p53 antibody PAb421. Immunoprecipitated proteins were purified by SDS–PAGE. Relevant proteins were extracted from the gel and oxidized with performic acid as described previously (Pátschinsky, and Bister, 1988; Patschinksy and Deppert, 1990).

Phosphopeptides of p53 were prepared by digestion with TPCK-trypsin (Fluka) and analysed on cellulose thin-layer plates by electrophoresis at pH 1.9 for 30 min at 6 kV followed by ascending chromatography in

n-butanol:pyridine:acetic acid:H_2O (15:10:3:12; v/v). The phosphopeptides were visualized by autoradiography.

Acknowledgements

We wish to thank W.Deppert for continuing support and for providing laboratory space at the Heinrich-Pette-Institut für Experimentelle Virologie und Immunologie an der Universität Hamburg, Germany and Dr C.Vorgias for purified prokaryotic control proteins and helpful discussions. This work was supported (Grant Stu 178/2-1) by Deutsche Forschungsgemeinschaft and is part of the Ph.D. thesis of S.Buchhop. The Heinrich-Pette-Institut is supported by Freie und Hansestadt Hamburg and Bundesministerium für Gesundheit.

References

Almon,E., Goldfinger,N., Kapon,A., Schwartz,D., Levine,A.J. and Rotter,V. (1993) Testicular tissue-specific expression of the p53 suppressor gene. *Dev. Biol.*, **156**, 107–116.

Bakalkin,G., Yakovleva,T., Selivanova,G., Magnusson,K.P., Szekely,L., Kiseleva,E., Klein,G., Terenius,L. and Wiman,K.G. (1994) p53 binds single-stranded DNA ends and catalyzes DNA renaturation and strand transfer. *Proc. Natl Acad. Sci. USA*, **91**, 413–417.

Bakalkin,G., Selivanova,G., Yakovleva,T., Kiseleva,E., Kashuba,E., Magnusson,K.P., Szekely,L., Klein,G., Terenius,L. and Wiman,K.G. (1995) p53 binds single-stranded DNA ends through the C-terminal domain and internal DNA segments via the middle domain. *Nucleic Acids Res.*, **23**, 362–369.

Ball,R.K., Siegl,B., Quellhorst,S., Brandner,G. and Braun,D.G. (1984) Monoclonal antibodies against simian virus 40 nuclear large T tumour antigen: epitope mapping, papovavirus cross-reaction and cell surface staining. *EMBO J.*, **3**, 1485–1491.

Basile,G., Aker,M. and Mortimer,R.K. (1992) Nucleotide sequence and transcriptional regulation of the yeast recombinational repair gene *RAD51*. *Mol. Cell. Biol.*, **12**, 3235–3246.

Benson,F.E., Stasiak,A. and West,S.C. (1994) Purification and characterization of the human Rad51 protein, an analogue of *E.coli* RecA. *EMBO J.*, **13**, 5764–5771.

Braithwaite,A.W., Blair,G.E., Nelson,C.C., McGovern,J. and Bellett,A.J. (1991) Adenovirus E1b-58 kDa antigen binds to p53 during infection of rodent cells: evidence for an N-terminal binding site on p53. *Oncogene*, **6**, 781–787.

Dunderdale,H.J. and West,S.C. (1994) Recombination genes and proteins. *Curr. Opin. Genet. Dev.*, **4**, 221–228.

Dutta,A., Ruppert,J.M., Aster,J.C. and Winchester,E. (1993) Inhibition of DNA replication factor RPA by p53. *Nature*, **365**, 79–82.

Ewen,M.E., Ludlow,J.W., Marsilio,E., DeCaprio,J.A., Millikan,R.C., Cheng,S.H., Paucha,E. and Livingston,D.M. (1989) An N-terminal transformation-governing sequence of SV40 large T antigen contributes to the binding of both p110Rb and a second cellular protein, p120. *Cell*, **58**, 257–267.

Fiscella,M., Ullrich,S.J., Zambrano,N., Shields,M.T., Lin,D., Lees-Miller,S., Anderson,C.W., Mercer,W.E. and Appella,E. (1993) Mutation of the serine 15 phosphorylation site of human p53 reduces the ability of p53 to inhibit cell cycle progression. *Oncogene*, **8**, 1519–1528.

Game,J. (1983) Radiation-sensitive mutants and repair in yeast. In Spencer,J.F.T., Spencer,D. and Smith,A.R.W. (eds), *Yeast Genetics: Fundamental and Applied Aspects*. Springer-Verlag, New York, pp. 105–137.

Gannon,J.V., Greaves,R., Iggo,R. and Lane,D.P. (1990) Activating mutations in p53 produce a common conformational effect. A monoclonal antibody specific for the mutant form. *EMBO J.*, **9**, 1595–1602.

Graham,F.L. and Smiley,J. (1977) Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *J. Gen. Virol.*, **36**, 59–72.

Gurney,E.G., Harrison,R.O. and Fenno,J. (1980) Monoclonal antibodies against simian virus 40 T antigens: evidence for distinct subclasses of large T antigen and for similarities among non-viral T antigens. *J. Virol.*, **34**, 752–763.

Harlow,E., Crawford,L.V., Pim,D.C. and Williamson,N.M. (1981) Monoclonal antibodies specific for simian virus 40 tumor antigens. *J. Virol.*, **39**, 861–869.

Hays,S.L., Firmenich,A.A. and Berg,P. (1995) Complex formation in yeast double-strand break repair: participation of Rad51, Rad52, Rad55, and Rad57 proteins. *Proc. Natl Acad. Sci. USA*, **92**, 6925–6929.

Hsu,I.C., Tokiwa,T., Bennett,W., Metcalf,R.A., Welsh,J.A., Sun,T. and Harris,C.C. (1993) p53 gene mutation and integrated hepatitis B viral DNA sequences in human liver cancer cell lines. *Carcinogenesis*, **14**, 987–992.

Hupp,T.R., Meek,D.W., Midgley,C.A. and Lane,D.P. (1992) Regulation of the specific DNA binding function of p53. *Cell*, **71**, 875–886.

Jenkins,J.R., Chumakov,P., Addison,C., Stürzbecher,H.-W. and Wade-Evans,A. (1988) Two distinct regions of the murine p53 primary amino acid sequence are implicated in stable complex formation with simian virus 40 T antigen. *J. Virol.*, **62**, 3903–3906.

Kao,C.C., Yew,P.R. and Berk,A.J. (1990) Domains required for *in vitro* association between the cellular p53 and the adenovirus 2 E1B 55K proteins. *Virology*, **179**, 806–814.

Kastan,M.B., Onykwere,O., Sidransky,D., Vogelstein,B. and Craig,R.W. (1991) Participation of p53 protein in the cellular response to DNA damage. *Cancer Res.*, **51**, 6304–6311.

Lane,D.P. (1992) Cancer. p53, guardian of the genome. *Nature*, **358**, 15–16.

Lane,D.P. (1993) Cancer. A death in the life of p53. *Nature*, **362**, 786–787.

Lischwe,M.A. and Sung,M.T. (1977) Use of *N*-chlorosuccinimide/urea for the selective cleavage of tryptophanyl peptide bonds in proteins. *J. Biol. Chem.*, **252**, 4976–4980.

Livingstone,L.R., White,A., Sprouse,J., Livanos,E., Jacks,T. and Tlsty,T.D. (1992) Altered cell cycle arrest and gene amplification potential accompany loss of wild-type p53. *Cell*, **70**, 923–935.

Meyn,M., Strasfeld,L. and Allen,C. (1994) Testing the role of p53 in the expression of genetic instability and apoptosis in ataxia-telangiectasia. *Int. J. Radiat. Biol.*, **66**, S141–S149.

Michalovitz,D., Halevy,O. and Oren,M. (1990) Conditional inhibition of transformation and of cell proliferation by a temperature-sensitive mutant of p53. *Cell*, **62**, 671–680.

Milne,G.T., Ho,T. and Weaver,D.T. (1995) Modulation of *Saccharomyces cerevisiae* DNA double-strand break repair by SRS2 and RAD51. *Genetics*, **139**, 1189–1199.

Morita,T., Yoshimura,Y., Yamamoto,A., Murata,K., Mori,M., Yamamoto,H. and Matsushiro,A. (1993) A mouse homologue of the *Escherichia coli recA* and *Saccharomyces cerevisiae RAD51* genes. *Proc. Natl Acad. Sci. USA*, **90**, 6577–6580.

Nelson,W.G. and Kastan,M.B. (1994) DNA strand breaks: the DNA template alterations that trigger p53-dependent DNA damage response pathways. *Mol. Cell. Biol.*, **14**, 1815–1823.

Oberosler,P., Hloch,P., Ramsperger,U. and Stahl,H. (1993) p53-catalysed annealing of complementary single-stranded nucleic acids. *EMBO J.*, **12**, 2389–2396.

Ogawa,T., Yu,X., Shinohara,A. and Egelman,E.H. (1993) Similarity of the yeast RAD51 filament to the bacterial RecA filament. *Science*, **259**, 1896–1899.

Oliner,J.D., Kinzler,K.W., Meltzer,P.S., George,D.L. and Vogelstein,B. (1992) Amplification of a gene encoding a p53-associated protein in human sarcomas. *Nature*, **358**, 80–83.

Patschinsky,T. and Bister,K. (1988) Structural analysis of normal and transforming mil (raf) proteins: effect of 5′-truncation on phosphorylation *in vivo* or *in vitro*. *Oncogene*, **3**, 357–364.

Patschinsky,T. and Deppert,W. (1990) Phosphorylation of p53 in primary, immortalised and transformed Balb/c mouse cells. *Oncogene*, **5**, 1071–1076.

Picksley,S.M., Vojtesek,B., Sparks,A. and Lane,D.P. (1994) Immunochemical analysis of the interaction of p53 with MDM2: fine mapping of the MDM2 binding site on p53 using synthetic peptides. *Oncogene*, **9**, 2523–2529.

Radding,C.M. (1991) Helical interactions in homologous pairing and strand exchange driven by RecA protein. *J. Biol. Chem.*, **266**, 5355–5358.

Roth,D.B., Lindahl,T. and Gellert,M. (1995) Repair and recombination: how to make ends meet. *Curr. Biol.*, **5**, 496–499.

Schwartz,D., Goldfinger,N. and Rotter,V. (1993) Expression of p53 protein in spermatogenesis is confined to the tetraploid pachytene primary spermatocytes. *Oncogene*, **8**, 1487–1494.

Selva,E.M., New,L., Crouse,G.F. and Lahue,R.S. (1995) Mismatch correction acts as a barrier to homologous recombination in *Saccharomyces cerevisiae*. *Genetics*, **139**, 1175–1188.

Shinohara,A., Ogawa,H., Matsuda,Y., Ushio,N., Ikeo,K. and Ogawa,T. (1993) Cloning of human, mouse and fission yeast recombination genes homologous to *RAD51* and *recA*. *Nature Genet.*, **4**, 239–243.

Steenenga,W.T., Shvarts,A., van Laar,T., van der Eb,A. and

Jochemsen,A.G. (1995) Altered phosphorylation and oligomerization of p53 in adenovirus type 12-transformed cells. *Oncogene*, **11**, 49–57.
St-Onge,L. and Bastin,M. (1993) Amplification mediated by polyomavirus large T antigen defective in replication. *J. Virol.*, **67**, 5025–5029.
St-Onge,L., Bouchard,L., Laurent,S. and Bastin,M. (1990) Intrachromosomal recombination mediated by papovavirus large T antigens. *J. Virol.*, **64**, 2958–2966.
Story,R.M., Bishop,D.K., Kleckner,N. and Steitz,T.A. (1993) Structural relationship of bacterial RecA proteins to recombination proteins from bacteriophage T4 and yeast. *Science*, **259**, 1892–1896.
Stürzbecher,H.-W., Maimets,T., Chumakov,P., Brain,R., Addison,C., Simanis,V., Rudge,K., Philp,R., Grimaldi,M., Court,W. and Jenkins,J.R. (1990) p53 interacts with $p34^{cdc2}$ in mammalian cells: implications for cell cycle control and oncogenesis. *Oncogene*, **5**, 795–801.
Stürzbecher,H.-W., Brain,R., Addison,C., Rudge,K., Remm,M., Grimaldi,M., Keenan,E. and Jenkins,J.R. (1992) A C-terminal alpha-helix plus basic region motif is the major structural determinant of p53 tetramerization. *Oncogene*, **7**, 1513–1523.
Sung,P. (1994) Catalysis of ATP-dependent homologous DNA pairing and strand exchange by yeast RAD51 protein. *Science*, **265**, 1241–1243.
Sung,P. and Robberson,D.L. (1995) DNA strand exchange mediated by a RAD51-ssDNA nucleoprotein filament with polarity opposite to that of RecA. *Cell*, **82**, 453–461.
Wade-Evans,A. and Jenkins,J.R. (1985) Precise epitope mapping of the murine transformation-associated protein, p53. *EMBO J.*, **4**, 699–706.
Wang,X.W., Forrester,K., Yeh,H., Feitelson,M.A., Gu,J.-R. and Harris,C. (1994) Hepatitis B virus X protein inhibits p53 sequence-specific DNA binding, transcriptional activity, and association with transcription factor ERCC3. *Proc. Natl Acad. Sci. USA*, **91**, 2230–2234.
Wang,Y. and Eckart,W. (1992) Phosphorylation sites in the amino-terminal region of mouse p53. *Proc. Natl Acad. Sci. USA*, **89**, 4231–4235.
West,S.C. (1994) The processing of recombination intermediates: mechanistic insights from studies of bacterial proteins. *Cell*, **76**, 9–15.
Yewdell,J.W., Gannon,J.V. and Lane,D.P. (1986) Monoclonal antibody analysis of p53 expression in normal and transformed cells. *J. Virol.*, **59**, 444–452.
Yin,Y., Tainsky,M.A., Bischoff,F.Z., Strong,L.C. and Wahl,G.M. (1992) Wild-type p53 restores cell cycle control and inhibits gene amplification in cells with mutant p53 alleles. *Cell*, **70**, 937–948.
Yoshimura,Y., Morita,T., Yamamoto,A. and Matsushiro,A. (1993) Cloning and sequence of the human RecA-like gene cDNA. *Nucleic Acids Res.*, **21**, 1665.

Received on June 7, 1995; revised on December 8, 1995