p53 protein interacts specifically with the meiosis-specific mammalian RecA-like protein DMC1 in meiosis

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The tumor suppressor protein p53 is specifically expressed during meiosis in spermatocytes. Subsets of p53 knockout mice exhibit testicular giant cell degenerative syndrome, which suggests p53 may be associated with meiotic cell cycle and/or DNA metabolism. Here, we show that p53 binds to the mouse meiosis-specific RecA-like protein Mus musculus DMC1 (MmDMC1). The C-terminal domain (amino acid 234–340) of MmDMC1 binds to DNA-binding domain of p53 protein. p53 might be involved in homologous recombination and/or checkpoint function by directly binding to DMC1 protein to repress genomic instability in meiotic germ cells.

Introduction

The tumor suppressor protein p53 is a multifunctional molecule that regulates cell-cycle progression, DNA repair and apoptosis by interacting with other molecules responding to DNA damage (1–7). A lack of p53 function results in elevated levels of gene amplification, and homologous recombination and repair (8–10). Furthermore, p53 protein interacts directly with human Rad51 protein (11,12), which is a homolog of Escherichia coli RecA and yeast RAD51 gene, and catalyzes DNA strand transfer and recombination (13–15).

p53 gene is expressed in tetraploid primary spermatocytes at the meiotic pachytene stage of spermatogenesis, and its expression is enhanced by γ-irradiation (16,17). Impairment of p53 expression results in giant cell degenerative syndrome (18). This suggests that p53 protein may be involved in meiotic cell-cycle progression and/or recombinational repair. We speculated that during meiosis, p53 interacts with recombinational machinery as in the case of mitosis.

In meiotic cells of eukaryotes, two RecA homologs are expressed: Rad51 and Dmc1 (19,20). Both proteins have similar structures to the central core region of the RecA protein (domain II), including two nucleotide-binding motifs. An in vitro study revealed that purified human DMC1 protein has DNA-dependent ATPase activity, single-stranded DNA-binding activity, and strand exchange activity (21). Thus, DMC1 protein is expected to catalyze DNA mediated recombination in meiosis, as observed for RecA and Rad51 proteins. Another in vivo study showed that mouse DMC1 protein is expressed in leptotene-to-zygotene spermatocytes, and that a defect in the Dmc1 gene results in meiotic arrest caused by the impairment of homologous chromosome synopsis (22,23).

Here, we examined the interaction of p53 protein with the recombinaton proteins RAD51 and DMC1 during meiosis. We demonstrate that the RecA-homolog, which interacts with p53 in spermatocytes is DMC1. This suggests, that p53 and DMC1 proteins are involved in meiotic cell-cycle progression, homologous synopsis or recombinational repair to maintain genomic integrity during meiosis.

Materials and methods

Protein extraction and immunoprecipitation

Cell extracts from mouse testes or 293T cells transfected with appropriate expression plasmids were prepared as follows. Seminiferous tubules from adult Balb/c mouse testes were lysed in 1 ml of Harlow buffer (50 mM HEPES, pH 7.5, 0.2 mM EDTA, 10 mM NaF, 0.5% Nonident–40, 250 mM NaCl), and kept on ice for 10 min. Cell debris was spun down at 10 000 r.p.m. for 5 min, and the supernatant was used in an immunoprecipitation assay. Transfected 293T cells were washed twice with ice-cold PBS, and lysed with 1 ml of Harlow buffer. After storing on ice for 10 min, cell debris was spun down at 10 000 r.p.m. for 5 min. The protein concentration of each extract was measured with a Protein Assay Kit (Bio-Rad, CA). Equal amounts (15 μg) of each extract were analyzed by SDS-PAGE and western blotting followed by detection with an ECL detection system according to the manufacturer’s instructions (Amersham Pharmacia Biotech, Buckinghamshire, UK). For immunoprecipitation, non-specific binding in the cell extracts was removed by adding 50 μl of protein G-Sepharose beads (Amersham Pharmacia Biotech) equilibrated with Harlow or RIPA buffer to the extracts and rocking for 2 h at 4°C. After removing the beads, cell extract containing 15 μg of protein was incubated with anti-p53 monoclonal antibody (pAb1; CALBIOCHEM, CA), anti-Mus musculus DMC1 (MmDMC1) rabbit polyclonal antibody or anti-Mus musculus RAD51 (MmRad51) polyclonal antibody. Then, 10 μl of protein G-Sepharose beads equilibrated with the same buffer was added to each reaction mixture and rocked for 4 h at 4°C. The beads were washed five times with Harlow buffer, and analyzed by western blotting using anti-p53 monoclonal antibody (pAb1), anti-MmDMC1 polyclonal antibody, or anti-MmRad51 polyclonal antibody.

Expression plasmids

Expression plasmids for wild-type (WT393) N-terminal and C-terminal deletion mutants (CT213, NT115) of p53 were constructed by PCR, and subsequently cloned into pcDNAI vector (Invitrogen, CA) under the CMV promoter. The primers we used for PCR of WT393 were: 5'-GGAAATTCCATGGAAGGAGGCCTGATCA-3' and 5'-GCTTCTAGAATTTGACTTATGCTCAAGCCCTTCTC-3' for primers for CT213, 5'-GGAAATTAATGGAAGGCCGAGTCAAGAG-3' and 5'-GCTTCTAGAATTTGACTTATGCTCAAGCCCTTCTC-3; and for NT115, 5'-GGAAATTAATGGAAGGCCGAGTCAAGAG-3' and 5'-GCTTCTAGAATTTGACTTATGCTCAAGCCCTTCTC-3'. Each primer was linked with an EcoRI or XhoI site for cloning into pcDNAI vector.

For in vivo expression of MmDMC1 and MmRad51 in mammalian cells, we amplified MmDMC1 and MmRad51 coding sequences using mouse cDNAs

Abbreviations: GST, glutathione S-transferase; MmDMC1, Mus musculus DMC1.
as templates. An EcoRI or XbaI site was artificially inserted into the primer sequences for cloning into pcDNAI.

The primer sequences for MmRAD51 were 5'-GGAATTCATGGCTATGCAAAATGCAGCT-3' and 5'-GCTCTAGATCAGTCTTTGGCATCGCCCA-3', and for DMC1 were 5'-GGAATTCATGAAGGAGGATCAAGTTGT-3' and 5'-GCTCTAGACTACTCCTTGGCATCCCCGA-3', respectively.

To make glutathione S-transferase (GST)-fusion proteins with full-length p53 and N- and C-terminal deletion proteins, PCR-generated DNA fragments were digested by BamHI and EcoRI restriction enzymes, and subcloned into the BamHI and EcoRI sites of pGEX-2T (Amersham Pharmacia Biotech).

**Purification of GST-fusion protein**

To purify GST-fusion proteins, the GST-capture procedure was carried out following the manufacturer's instructions (Amersham Pharmacia Biotech). Escherichia coli HB101 (recA') induced to produce GST, GST115-213 (A3), GST115-190 (A2), GST115-165 (A1), GST141-213 (B3), GST165-213 (B2) or GST165-190 (B1) proteins was lysed by the freeze-thawing procedure in Start buffer (PBS, pH 7.4, plus 5 mM DTT). After sonication, lysates containing A2, B3, B1 or GST protein were directly applied to 1 ml of glutathione--Sepharose 4B (Amersham Pharmacia Biotech) equilibrated by Start buffer. After washing with the buffer, GST-fusion proteins were eluted by elution buffer (10 mM Tris-HCl, pH 8.0, 10 mM glutathione, 5 mM DTT). The other proteins (GST-p53, A3, A1, B2) were insoluble in Start buffer, so they were separated by SDS-PAGE and extracted from the gels with denaturing buffer (PBS, pH 7.4, plus 6 M guanidine-HCl and 5 mM DTT) for 20 h at 4°C followed by sequential dialyses against denaturing buffer containing 3, 2, 1 and 0 M guanidine-HCl. Solubilized GST-fusion proteins were dialyzed against store buffer (PBS, pH 7.4, 50% glycerol and 5 mM DTT) and applied to a glutathione--Sepharose column. After washing, GST-fusion proteins were eluted with elution buffer. Each eluted fraction was dialyzed twice against

![Fig. 1.](image-url)

**Fig. 1.** p53 protein interacts with MmDMC1 protein, but not with MmRAD51 protein in the testis. (A) Co-immunoprecipitation analysis of p53 and MmDMC1 proteins using mouse testis extracts. Mouse testis extracts were immunoprecipitated with the indicated antibodies. Immunoprecipitated proteins were separated on SDS-polyacrylamide gels and analyzed by western blotting using anti-p53 monoclonal antibody (lanes 2 and 4), anti-DMC1 (lanes 6 and 7), anti-Immunoe-precipitation antibody (lane 7). p53 (53 kDa), MmDMC1 (37 kDa) and MmRAD51 (37 kDa) proteins are indicated by arrow heads. (B) Transient expression of p53, MmDMC1 and MmRAD51 proteins in 293T cells. The proteins were separated on SDS-polyacrylamide gel and detected by western blotting with anti-p53 monoclonal antibody, anti-MmDMC1 or anti-MmRAD51 polyclonal antibody. (C) Co-immunoprecipitation analysis of p53, MmDMC1, and MmRAD51 proteins in 293T cells. Immunoprecipitated proteins were detected with anti-p53 monoclonal antibody, anti-MmDMC1 polyclonal antibody, and anti-MmRAD51 polyclonal antibody.
store buffer for 12 h at 4°C. To remove non-specific binding materials, cell extracts containing DMC1 protein were incubated with glutathione-Sepharose beads (Amersham Pharmacia Biotech) for 2 h at 4°C. After removing the beads, GST-fusion proteins were added to 100 μl of cell extract and rocked for 6 h at 4°C. Then, 10 μl of glutathione-Sepharose beads was added to the reaction mixture and rocked for 6 h at 4°C. The beads were washed five times by Harlow buffer, and directly analyzed by SDS-PAGE and western blotting using anti-MmDMC1 polyclonal antibody.

Results and discussion

We examined the interaction between MmDMC1 and p53 protein in mouse testicular cells. Using anti-p53 monoclonal antibody, MmDMC1 protein was co-immunoprecipitated from the whole protein lysate of the testis (Figure 1A, lane 2). In contrast, MmRAD51 protein was not detected (Figure 1A, lane 4) by anti-p53 immunoprecipitation. Anti-MmDMC1 polyclonal antibody co-precipitated p53 protein (Figure 1A, lane 6), whereas anti-MmRAD51 antibody did not (Figure 1A, lane 7). These results indicated that MmDMC1 and p53 proteins formed a complex that did not involve the MmRAD51 protein in testicular cells.

To confirm the interaction in cultured cells, we transiently co-expressed p53, MmDMC1 and MmRAD51 proteins (Figure 1B) in the human embryonic kidney 293T cells (Figure 1B). We examined the expression of p53, RAD51 and DMC1 proteins in the cells (lanes 1–4) in various combinations. We
extracted whole cell lysates and performed co-immunoprecipitation followed by western blot analysis. When p53 was expressed with MmRAD51 or MmDMC1 protein, p53 co-immunoprecipitated with MmRAD51 or MmDMC1, respectively (Figure 1C, lanes 2 and 4). When MmDMC1 and MmRad51 were expressed simultaneously, p53 protein co-precipitated with MmDMC1, but not with MmRAD51 protein (Figure 1C, lane 1). This may reflect the small amount of RAD51 protein produced in transfected cells. Alternatively, it might indicate that p53 forms a protein complex exclusively with MmDMC1, but not with MmRAD51 protein, when the three proteins (p53, MmDMC1 and MmRad51) are expressed together as in the testis in vivo.

To define the region of p53 responsible for MmDMC1 binding, we first used two deletion mutants of p53 (CT213 and NT115) (Figure 2A). They were expressed transiently in 293T cells along with wild-type MmDMC1 protein. Whole cell proteins were immunoprecipitated with anti-DMC1 antibody and detected by western blotting using anti-p53 antibody. When MmDMC1 protein was expressed with wild-type p53 (WT393), we detected normal-sized p53 protein in the precipitate. By transfection with the mutant p53 genes, two p53 deletion mutant proteins (CT213 and NT115) were detected together with endogenous wild-type p53 protein (Figure 2B) by anti-DMC1 co-immunoprecipitation. This suggested that the MmDMC1 bound between amino acids (aa) 115 and 213 in p53. To more precisely define the interaction domain, we employed a pull-down assay for six GST–p53 mutant proteins (A1–A3 and B1–B3) (Figure 2C and D). We analyzed their interaction with purified MmDMC1 in vitro using the GST-pull-down assay. We tested six GST–p53 deletion mutants, and found that A1, A2, A3 and B3 mutant proteins of p53 bound to purified MmDMC1 protein (Figure 2E). B1 and B2 proteins were negative in this experiment (Figure 2E). These results indicated that the region between aa 141 and 165 in p53 protein where the sequence-specific DNA-binding domain was located was responsible for MmDMC1 binding. However, weak binding of the B3 fragment suggests the region between 115 and 141 aa was necessary for full binding activity.

The p53-binding site on MmDMC1 protein was examined using a series of C-terminal deleted MmDMC1 proteins (CT234, CT89 and D-CT228) (Figure 3A) together with alternatively spliced forms of DMC1 (DMC1-D; D-284) (20), which lacks a highly conserved region, the homologous core. This region is shared by members of the RecA family and is critical for their biological and biochemical activities in homologous recombination (24–26). DMC1 mutant proteins were transiently expressed in 293T cells with wild-type p53 protein and co-immunoprecipitated with anti-p53 antibody (Figure 3B). Wild-type DMC1 (WT340) and DMC1-D (D-284) proteins co-immunoprecipitated with p53. However, the C-terminal deleted proteins (CT234, CT89 and D-CT228) did not co-immunoprecipitate with p53 protein (Figure 3B). From these results, we concluded that the region in DMC1 protein involved in p53 interaction was the C-terminal part (234–340 aa region). This result differs from those obtained for the RAD51 protein. Sturzbecher et al. reported that the region of RAD51 responsible for p53-binding resides within the homologous core (125 and 220 aa for Homo sapiens RAD51 and HsRAD51 proteins) (11,12). Thus, p53 targets a different portion of DMC1 compared with RAD51 for its binding, indicating a different mode of involvement of p53 in recombination and/or biochemical activities (27,28).

Since DMC1 protein is involved in homologous synopsis during meiosis (22), we suggest that the direct interaction between p53 and DMC1 proteins plays a role in a cell–cycle checkpoint to detect chromosome aberrations in meiotic synopsis or recombination (29–31). Alternatively, p53 protein may directly influence meiotic recombination or synaptonemal complex formation by binding to DMC1 protein (32–35).

p53 inhibits tumorigenesis through a variety of functions such as the mediation of cell-cycle arrest, premature senescence and apoptosis. p53 also associates with proteins involved in homologous recombination such as Rad51 and Rad54 (36). These proteins are involved in DNA damage repair induced by environmental factors and replication during the cell cycle. If the system is not properly regulated, genetic instability and carcinogenesis may occur. In fact, dominant-negative Rad51 stimulates tumorigenesis in mammalian cells defective in p53 (37). Our result that meiosis-specific homologous recombination protein DMC1 binds to p53 protein suggests that DMC1 plays a role similar to RAD51 in DNA repair during meiosis to repress carcinogenesis in testicular and ovarian cells.

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References


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