Interaction with p53 Enhances Binding of Cisplatin-modified DNA by High Mobility Group 1 Protein*

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A nonhistone chromosomal protein, high mobility group (HMG) 1, is ubiquitous in higher eukaryotic cells and binds preferentially to cisplatin-modified DNA. HMG1 also functions as a coactivator of p53, a tumor suppressor protein. We investigated physical interactions between HMG1 and p53 and the influence of p53 on the ability of HMG1 to recognize damaged DNA. Using immunochemical coprecipitation, we observed binding of HMG1 and p53. Interaction between HMG1 and p53 required the HMG A box of HMG1 and amino acids 363–376 of p53. Cisplatin-modified DNA binding by HMG1 was significantly enhanced by p53. An HMG1-specific antibody that recognized the A box of this protein also stimulated cisplatin-modified DNA binding. These data suggest that an interaction with either p53 or antibody may induce conformational change in the HMG1 A box that optimizes DNA binding by HMG1. Interaction of p53 with HMG1 after DNA damage may promote activation of specific HMG1 binding to damaged DNA in vivo and provide a molecular link between DNA damage and p53-mediated DNA repair.

The cytotoxic effect of cisplatin is believed to result from formation of covalent adducts with DNA (1). Cisplatin treatment induces a tumor suppressor protein, p53, to suppress cell proliferation through p21 induction (2). Interestingly, p53 possesses 3’ to 5’ exonuclease activity (3). The recent observation that another protein, HMG1, can enhance p53-mediated transactivation suggests links between HMG1 and p53 in other physiologic settings (4). The p53 protein has been reported to accumulate in cisplatin-resistant cells (5). In addition to transactivation, p53 is involved in recognition and repair of DNA damage (5). HMG1 has three domains. The N-terminal domain (A box) and the central domain (B box) are positively charged and bind to DNA, whereas the acidic C-terminal domain interacts with histones (6). The amino acid sequences of the A and B boxes of HMG1 are homologous to a segment of about 70 amino acid residues called the HMG box. HMG1 does not bind to DNA in a sequence-specific manner; rather, it functions as an architectural protein for structuring chromatin. Notably, HMG1 binds preferentially to cisplatin-modified DNA (7). Yeast mutants lacking the HMG domain protein Ixr1 have been found to be significantly more resistant to cisplatin than wild type cells (8). On the other hand, distribution of HMG1 protein is changed after cisplatin treatment (9), and HMG1 protein genes usually are up-regulated in cisplatin-resistant human cancer cells. Thus, uncertainty prevails as to whether cellular levels of HMG proteins correlate with cellular resistance to cisplatin. HMG proteins enhance sequence-specific DNA binding of a variety of transcription factors, but whether these factors can modulate HMG1 binding to damaged DNA is not known. We therefore investigated the effect of p53 on the ability of HMG1 to recognize damaged DNA.

EXPERIMENTAL PROCEDURES

Cells—MCF-7 cells were grown in Dulbecco’s modified Eagle’s medium (Nissui, Tokyo, Japan) supplemented with 10% fetal calf serum. Antibodies—Antibodies to p53 (Do-1) and Sp1 (PEP 2) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody to p53 (Pab421) was purchased from Calbiochem (San Diego, CA). Antibodies to HMG1 and HMG2 were generated from synthetic peptides KGTTKKKKDFPNAP (K plus amino acids 83–95), and KSEAGKKG-PQRTPTG (K plus amino acids 188–189) respectively, as described previously (10). Antibodies to HMG1 and HMG2 were purified using protein A/G-agarose (Qiagen, Hilden, Germany) (11).

Plasmid Preparation—A full-length cDNA for human p53 was amplified from total RNA from a human epidermoid cancer lines, KB cells, by reverse transcription-polymerase chain reaction using the primer pairs 5’-CCATGGGAGGCGCCGAGTCAGATCC-3’ and 5’-GAATTG-GAGAATGTCAGTCTGAGTCAGGCC-3’. The polymerase chain reaction product was cloned into the pGEM-T Easy vector (Promega, Madison, WI). The cDNA fragment then was gel purified after digestion with NolI and cloned into the pThioHis vector (Invitrogen) for a ThioHis fusion construct or the pGEX-4T vector (Amersham Pharmacia Biotech) for a GST fusion construct. GST-p53 deletion mutants were prepared as follows. For construction of GST-p53 N124, GST-p53 plasmid was digested with BglII, filled in, and self-ligated. For GST-p53 160C, GST-p53 plasmid was digested with NcoI and circularized by self-ligation. ThiHis-p53 deletion mutants were constructed from a ThiHis-p53 plasmid by digestion with AccI for N376, BanII for N362, and EcoR11 for 224C. Plasmids for GST-HMG1 and GST-Y box-binding protein 1 were described previously (10). GST-HMG1 deletion mutants were prepared as follows. For construction of GST-HMG1ΔA and GST-HMG1ΔB, Stu-
FIG. 1. Interaction of HMG1 with p53 in vitro and in vivo. A, interaction of HMG1 or p53 with MCF-7 cell nuclear extract. GST, GST-HMG1 or p53 on glutathione-Sepharose beads was incubated with 35S-labeled nuclear extract from MCF-7 cells treated with or without cisplatin. Bound proteins were detected by SDS-polyacrylamide gel electrophoresis and autoradiography. Molecular mass markers are indicated as well as positions of proteins that likely correspond to p53 (open arrowhead) and HMG1 (closed arrowhead). HMG1 and p53 expression in vivo is shown. Western blots of fractionated protein (150 μg/lane) extracted from MCF-7 cells treated with 20 μm of cisplatin for 0, 12, or 24 h are shown. p53 was detected with the monoclonal antibody Do-1. HMG1 and Sp1 were detected with polyclonal antibody. B, coimmunoprecipitation assay. A nuclear extract prepared from MCF-7 cells treated with cisplatin (20 μm) for 24 h was incubated with pre-immune serum or antibodies to HMG1 or p53. The immune complexes and 10% of input were electrophoresed and analyzed by immunoblotting with antibody to p53 or HMG1. C, pull-down assay using nuclear extract. A nuclear extract from cisplatin-treated MCF-7 cells was incubated with immobilized GST fusion proteins. Bound protein samples representing 10% of input were electrophoresed and analyzed by immunoblotting with antibody to p53 (middle panel) or antibody to HMG1 (right panel). Purified GST fusion proteins used in this assay were stained with Coomassie Brilliant Blue (left panel). Asterisks indicate full-length GST fusion protein.
Half of each labeled oligonucleotides was treated with 0.3 mM cisplatin at 37 °C for 12 h and then was purified by ethanol precipitation. Numbers of platinum atoms bound to oligonucleotides (32-mer) were determined by atomic absorption spectroscopy (polarized Zeeman atomic absorption spectrophotometer Z-8200; Hitachi). The mean amount of platinum bound to DNA was about 6.7 platinum atoms/oligonucleotide under our conditions. GST fusion proteins were incubated with ThioHis-p53 expressed in bacteria, and the bound protein samples representing 10% of input were electrophoresed and analyzed by immunoblotting with anti-Thio antibody (bottom panel). 

**RESULTS**

**HMG1 Interacts with p53**—HMG1 has been shown to interact directly with p53 to enhance p53 DNA binding in vitro (4). We initially examined the spectrum of nuclear proteins bound to GST fusion proteins (32-mer) were determined by atomic absorption spectroscopy (polarized Zeeman atomic absorption spectrophotometer Z-8200; Hitachi). The mean amount of platinum bound to DNA was about 6.7 platinum atoms/oligonucleotide under our conditions. GST fusion proteins binding glutathione-Sepharose 4B were eluted with 50 mM Tris-HCl, pH 8.0, and 20 mM reduced glutathione according to the manufacturer's protocol (Amersham Pharmacia Biotech). GST fusion proteins were used directly for EMSA.

**Fig. 2.** Direct interaction of HMG1 with p53 and localization of the portion of the HMG1 molecule that binds to p53. A, schematic representation of GST-HMG1 fusion protein and pull-down assay using ThioHis-p53 fusion protein. The upper panel provides a schematic illustration of the HMG1 protein and their deletion mutants used in the assay. Purified GST fusion proteins were stained with Coomassie Brilliant Blue (right panel). Asterisks indicate full-length GST fusion protein. Immobilized fusion proteins were incubated with ThioHis-p53 expressed in bacteria, and the bound protein samples representing 10% of input were electrophoresed and analyzed by immunoblotting with anti-Thio antibody (bottom panel). B, effect of DNaseI treatment on pull-down assay. GST fusion or ThioHis fusion protein was treated with (+) or without (−) DNaseI. Immobilized GST fusion proteins were incubated with ThioHis-p53, and bound proteins were analyzed by immunoblotting with anti-Thio antibody.

HMG1 on the other hand, a 28-kDa protein likely to correspond to HMG1 was observed by pull-down with GST-p53. No significant difference of protein profile was seen between MCF-7 cells treated with cisplatin and those treated without cisplatin. To determine whether these proteins respond to treatment with cisplatin, extracts from nuclei and cytoplasm were prepared from logarithmically growing MCF-7 cells and analyzed by Western blotting in Fig. 1A (right panel). When cells were treated with cisplatin, p53 was rapidly up-regulated. Both HMG1 and p53 were localized mainly in nuclei. Sp1, a nuclear transcription factor, showed no change in amount after cisplatin treatment. Sp1 could not be detected in the cytosol, indicating that the cytosolic fraction was not contaminated with nuclear protein. Basal expression of wild type of p53 was relatively high in MCF-7 cells. When MCF-7 cells were treated with cisplatin for 24 h, nuclear levels of p53 increased 4-fold compared with untreated MCF-7 cells. When MCF-7 cells were treated with cisplatin for 24 h, nuclear levels of p53 increased 4-fold compared with untreated MCF-7 cells. Cellular amounts of HMG1 and p53 were essentially similar at base lines, but relative in vivo levels of p53 following cisplatin treatment significantly exceeded levels of HMG1. To assess the intracellular association of p53 with HMG1, coimmunoprecipitation was performed using either anti-HMG1- or anti-p53 antibodies with MCF-7 cells treated with cisplatin. Immunoblotting analysis showed that p53 interacted with HMG1 and the interaction of HMG1 with p53 was reproducible (Fig. 1B). To further confirm this association, we performed a pull-down assay using immo-
bilibized GST fusion proteins and nuclear extracts. Immunoblotting of the bound nuclear proteins demonstrated that p53 interacted with HMG1 but not with GST alone (Fig. 1C). Y box-binding protein 1 also has been found to interact with p53 (13) (Fig. 1C).

We mapped the domain of the HMG protein required for interaction with p53 by a pull-down assay using GST fusion proteins containing deletion mutants of HMG1 and ThioHis fusion proteins with p53 (Fig. 2A). Immobilized GST fusion proteins were shown by staining. Fusion protein deleting the A box of HMG1 was unable to bind p53, whereas fusion protein lacking the B box of HMG1 showed intact binding (Fig. 2A). To exclude the possibility that p53 and HMG1 might associate to form a ternary complex via genomic DNA, we also tested the fusion proteins after treatment with DNaseI, which completely destroys bacterial DNA under the conditions (data not shown). As shown in Fig. 2B, an association between HMG1 and p53 could be demonstrated when DNaseI-treated fusion protein was assayed, indicating that HMG1 interacted with p53 directly and that the HMG A box of HMG1 was sufficient for interaction with p53.

We next mapped the domain of p53 responsible for interaction with HMG1, using GST fusion proteins to capture ThioHis-p53 or its deletion derivatives (Fig. 3A). This assay demonstrated that the C-terminal region of p53 participated in the interaction with HMG1 (Fig. 3B). A region containing 14 amino acids (residues 363–376) in this region of p53 is critical for the interaction.

p53 Enhances Cisplatin-modified DNA Binding by HMG1—HMG1 can stimulate sequence-specific DNA binding of p53 (4). We examined the converse, the influence of p53 on DNA binding of HMG1, because HMG1 is well known to bind preferentially to cisplatin-modified DNA (7). We previously have shown that HMG1 cannot bind effectively to cisplatin-modified oligonucleotides of a length limited to 20 base pairs (10). We prepared oligonucleotides of two different lengths, a 32-mer and a 63-mer, and examined HMG1 binding. As shown in Fig. 4A, a single band with retarded migration was detected when the 32-mer was modified with cisplatin, whereas two retarded bands were observed with the cisplatin-modified 63-mer. These results indicated that the 32-mer length is sufficient for strong binding of HMG1, whereas with the 63-mer, the slowly migrating bands appear to represent attachment of two HMG1 molecules. We therefore used the 32-mer for the following experiments.

We next analyzed the DNA-binding activity of GST fusion protein using synthetic oligonucleotides with or without cisplatin modification. As shown in Fig. 4B, HMG1 and HMG1AB can significantly bind to double-stranded oligonucleotides modified with or without cisplatin modification. As shown in Fig. 4B, HMG1 and HMG1AB can significantly bind to double-stranded oligonucleotides modified with cisplatin but not to single-stranded oligonucleotides. On the other hand, p53 alone could not bind to these probes. We next examined the effect of p53 on cisplatin-modified DNA binding of HMG1 by EMSA (Fig. 5). Cisplatin-modified DNA binding by HMG1 was significantly enhanced by p53 in a dose-dependent manner (Fig. 5A). To avoid HMG1 aggregation in the EMSA, we diluted the HMG1 protein and examined the effect of p53 on DNA binding activity. Mobility of the HMG1-DNA complex was not affected by the concentration of HMG1. Under our conditions, addition of p53 to the HMG1-DNA binding reaction resulted in 5–10-fold activation of the DNA binding activity of HMG1. Mutant p53, which cannot interact with HMG1, was unable to stimulate DNA binding by HMG1 or GST alone (Fig. 5B). However, p53 did not alter the electrophoretic mobility of the HMG1 complex formed with cisplatin-modified DNA, and p53-induced HMG1-DNA complex could not be supershifted by the anti-p53 antibody Pab421 (data not shown). Further, we found that HMG1-specific antibody also promoted formation of the cisplatin-modified DNA HMG1 complex but HMG2- or p53-specific antibody did not

**Fig. 3.** Mapping of the HMG1 binding sites of p53. A, schematic representation of the ThioHis-p53 and its deletion mutants used in this assay. A schematic representation of the functional domains of p53 also is shown (top panel). B, pull-down assay using ThioHis-p53 and its deletion mutants. Full-length and truncated forms of ThioHis-p53 were expressed in bacteria and used for pull-down experiments with GST or GST-HMG1. Bound protein samples representing 10% of input were electrophoresed and analyzed by immunoblotting with anti-Thio antibody. ThioHis tag was used alone as a negative control.
Addition of anti-HMG1 antibody did not stimulate formation of DNA-protein complex when HMG1 DB was used, because the HMG1 antibody epitope is not present in HMG1 DB. Addition of anti-HMG1 antibody to the binding reaction resulted in a further shift in mobility (Fig. 5C, right panel).

**DISCUSSION**

We demonstrated here that p53 directly interacts with HMG1 in cell extracts and in intact cells to enhance the cisplatin-modified DNA binding activity of HMG1. Both HMG1 and HMG2 are nonhistone chromosomal proteins that are abundant and highly conserved in eukaryotic cells (14). Although the function of these proteins is not fully understood, they play an important role in chromatin structure and function including DNA replication, DNA repair, transcription, and chromatin assembly. The most distinctive feature of the HMG box is a basic domain of about 70 amino acids that contains three α-helical regions including well conserved hydrophobic amino acids. The HMG A box and the HMG B box are about 30% identical in amino acid sequence. A phenylalanine residue at position 37 is critical for binding to cisplatin-modified oligonucleotides (15). However, no phenylalanine residue is present in the HMG B box, so the two linked HMG boxes are not functionally equivalent in DNA binding. Under our conditions, only the HMG A box can bind to cisplatin-modified DNA, whereas the HMG B box cannot. Although the conditions under which EMSA is performed can greatly influence the results obtained, our result is consistent with the previous report that the HMG1 A box has a higher affinity than the HMG1 B box for cisplatin-modified DNA (16). Further, the HMG A box has been proposed to be involved mainly in structure-specific DNA binding, whereas the B box may be a target for protein-protein interactions (17).

Interestingly from that viewpoint, the HMG A box is involved in p53 binding, but the HMG B box is not. The lack of p53 binding of the HMG B box may reflect a difference in protein structure between this region and the HMG A box. Interaction between the HMG A box and p53 required amino acids 363–376 of p53. This region of the highly basic C terminus of p53 is particularly “sticky” and appears to associate with many cellular proteins. The HMG A box also is highly basic, suggesting that interaction is not of a simple electrostatic nature. This view of interactions is supported by the finding that DNA binding by HMG1 also is enhanced by addition of anti-HMG1 antibody (Fig. 5C). Participation of the DNA-binding domain in the HMG1-p53 interaction implies that the mode of DNA binding and the binding specificity of resulting heterodimer might be altered.

Normal cells contain p53 protein in a latent form that can be activated by DNA damage. Post-translational modifications of the C-terminal domain of p53 have been shown to be important in p53-specific DNA binding such as phosphorylation, antibody binding, and acetylation (18–20). HMG1 has been shown to significantly stimulate the sequence-specific DNA binding of p53 (4). Small peptides derived from the negative regulatory
domain can activate the latent sequence-specific DNA binding function of p53 (21). In the present study, we found that the C-terminal negative regulatory domain of p53 is involved in HMG protein binding (Fig. 3). Interaction of HMG1 with p53 may be modulated by the post-translational modifications of p53.

HMG proteins have been further shown to enhance the sequence-specific DNA binding of a wide variety of transcription factors such as sex-hormone receptor (22, 23), HOX proteins (24), POU domain-containing factors (25), and p53, functioning as a coactivator for transcription of their target genes (4). However, no evidence has been reported to indicate whether cisplatin-modified DNA binding activity of HMG proteins is modulated by these cellular proteins. Therefore, we examined whether damaged DNA binding of HMG1 is shown in the right panel (lane 1, 0 ng of antibody; lane 2, 20 ng of antibody; lane 3, 60 ng of antibody; lane 4, 180 ng of antibody; and lane 5, 540 ng of antibody). Arrowhead indicates DNA-protein complex. Arrow indicates supershifted complex.

Four classes of damage detector proteins have been identified that bind preferentially to damaged sites: repair-related proteins, HMG box-containing proteins, transcription factors, and linker histones (7). Both the p53 protein and a damage detector protein possibly might bind at sites of DNA damage and repair. In this context, determination of whether other
damage detector proteins can interact directly with p53 is important. The p53 protein not only recognizes structurally altered DNA such as single-stranded DNA (26) and mismatching DNA (27) but also exhibits 3' to 5' exonuclease activity (3). Increased p53 protein after DNA damage might be associated with active participation in a DNA repair process, because p53 can associate with a number of proteins involved in excision repair. Thus, interaction of p53 with HMG proteins may have an important role in p53-dependent DNA repair. Overexpression of HMG1 in response to the steroid hormones have been recently shown to sensitize MCF-7 cells to cisplatin and carboplatin (28). HMG1 often is up-regulated in cisplatin-resistant cell lines (data not shown). These observations indicate that HMG proteins could be important in modulating the cellular toxicity of cisplatin. Further study is needed to explore the role of HMG1 in cisplatin resistance.

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