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Binding of Wild-Type P53 by Topoisomerase II and Overexpression of Topoisomerase II in Human Hepatocellular Carcinoma

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In order to study the mechanisms by which p53 function is regulated, human wild-type p53 cDNA was cloned into a vaccinia virus vector and the expressed p53 protein was used to investigate binding of the p53 by cellular proteins from a cDNA expression library from human liver. One protein that bound wild-type p53 had >99% homology with DNA topoisomerase IIb. p53 protein was coimmunoprecipitated from topoisomerase II-rich cell lysates (but not from topoisomerase II-deficient cell lysates) by an antibody to topoisomerase IIa and IIb. This binding was shown to occur without a dsDNA intermediary. Hepatocellular carcinomas (HCCs) and adjacent nontumorous liver tissues from ten patients were studied to determine the level of expression of topoisomerase II and p53. Overexpressed topoisomerase II proteins were detected by western blot in six of ten HCCs (60%), including several in which presumed wild-type p53 was detected by immunohistochemistry. No topoisomerase II expression was detectable in the ten nontumorous liver tissues from the same patients or in a sample of normal human liver. © 1997 Academic Press

The p53 protein plays an important role in the negative control of transformation (1, 2, 3). The functions of p53 may be abolished by a deletion or mutation in the p53 gene, or by the binding of certain viral or cellular proteins to wild-type p53 (4, 5). Inactivation of p53 by any of these mechanisms thereby leads to a selective growth advantage, which can take the form of tumor progression. Despite extensive data linking mutations in the p53 gene to human cancer (6), little is known about cellular regulators and mediators of p53 protein.

To gain additional insight into the mechanisms by which p53 protein is regulated, proteins that interact with p53 were sought using wild-type human p53 produced by a vaccinia virus vector. By screening a cDNA library from human liver, DNA topoisomerase II was found to form a complex with wild-type p53. The significance of this in carcinogenesis was evaluated by screening human hepatocellular carcinomas (HCCs) for overexpression of topoisomerase II.

MATERIALS AND METHODS

Cell lines and viruses. HCC cell lines HLE and HLF (both derived from the HCC of one Japanese patient (Japanese Cancer Research Resources Bank, Tokyo), monkey kidney cell line CV-1 (American Type Culture Collection [ATCC], Rockville, MD), HeLa cells (ATCC), and $P388D_1$ cells (mouse lymphoid tumor/leukemia) (ATCC) were maintained in modified Eagle's MEM plus 10% fetal bovine serum, 1% non-essential amino acids (Life Technologies, Gaithersburg, MD), 500 μg/ml spectinomycin, 60 μg/ml tylocine, and 0.25 μg/ml fungizone at 37°C in 5% CO_2.

Recombinant vaccinia virus vv65, derived from vaccinia virus WR strain by adding the bacterial β galactosidase gene, was kindly provided by Dr. Bernard Moss of the National Institute for Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD. Recombinant vaccinia virus vv105 was constructed from vv65 by inserting a 1.2 kb human wild-type p53 cDNA (7).

Antibodies. Polyclonal antibodies to p53 were used for immunohistochemistry (CM1, Novocastra Laboratories, Newcastle, UK), and for immunoprecipitation and western blot (p53 Kit, Oncogene Science, Uniondale, NY). Monoclonal antibodies to p53 (DO-1 and PAb 1801, Oncogene Science), to mdm2 (IF2, Oncogene Science), and to topoisomerase II (reactive to both topoisomerase IIa and topoisomerase IIb) (SWT3D1, Oncogene Science) were used for immunoprecipitation. Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG and goat anti-rabbit IgG (DAKO, Carpinteria, CA) were used for immunohistochemistry, immunoprecipitation, and western blot.

Tissue samples. HCCs and adjacent nontumorous liver tissues were obtained by surgical resection from 10 patients from Qidong, China, an area with a high incidence of HCC. Of the 10 patients, 9 were male and 1 was female. The median age was 47 years (mean = 49 ± 9 years; range = 38 to 65 years). One normal liver tissue was from a 55 year old American patient who died of causes unrelated to liver disease. All resected samples were snap-frozen in liquid nitrogen and kept at −70° C until use.

cDNA library screening. A cDNA expression library made from normal human liver (Clontech, Palo Alto, CA) was screened and

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purified by three rounds of plaque purification. These bacteriophage (10^6 pfu/150mm petri dish in the first round, 10^4 pfu/150mm petri dish in the second round, and 10^2 pfu/150 mm petri dish in the third round), grown in E.coli strain $Y1090^{r-}$, were induced in isopropyl-D-thiogalactopyranoside (IPTG) and transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with cell lysate prepared from CV-1 cells infected with recombinant vaccinia virus containing the β galactosidase gene (vv65). A lysate from 10^7 CV-1 cells infected with recombinant vaccinia virus containing wild-type human p53 (vv105) was applied to each membrane along with 10 μg of monoclonal antibody to p53 (DO-1) in the first round of screening. The antibody was reduced to 1 μg in the second round and 0.1 μg in the third round to eliminate the direct binding between bacteriophage-encoded p53 protein and the monoclonal antibody to p53. In the third round, the membrane was cut into two pieces; one part was stained with a complex of recombinant p53 protein (produced by vaccinia virus vv105) and the antibody to p53; the other part was stained with the antibody to p53 alone to show that any positive staining was not due to the antibody binding to bacteriophage containing the p53 gene insert from the cDNA library. The membrane was then incubated with HRP-conjugated goat anti-mouse IgG, treated with enhanced chemiluminescence (ECL, Amersham, Arlington Heights, IL) and exposed to X-ray film. The cDNA inserts from purified bacteriophage were later amplified by PCR with the primer set for λgt 11 (Clontech) and inserted into TA cloning vector (Invitrogen, San Diego, CA). The DNA sequences were determined by using Sequenase 2.0 (USB, Cleveland, O) and analyzed by comparison with DNA sequences in GenBank using the program WORDSEARCH included in the University of Wisconsin Genetics Computer Group (UWGCG) package (Version 8.0-UNIX).

Immunoprecipitation. Studies were conducted using cell monolayers infected with vaccinia viruses vv105, vv65, and WR strain at 10 PFU per cell, or uninfected cell monolayers. Two hours after infection, each monolayer was washed once with methionine-free Eagle's MEM and the cells were then labeled with [^{35}S] methionine (1,000 Ci/mmol, Amersham, Arlington Heights, IL) at 1 μCi/10^5 cells for two hours in 2 ml of methionine-free Eagle's MEM, followed by a chase with complete Eagle's MEM plus 10% fetal bovine serum for 22 hours. The monolayer was washed once with phosphate-buffered saline, and 1.0 ml of cell lysate buffer (1% NP-40, 100mM Tris-HCl [pH 8.0], 100 mM NaCl, 0.2 mM phenylmethylsulfonylfluoride [PMSF]) was added. The cell lysates were kept on ice for 10 minutes, transferred to microcentrifuge tubes and centrifuged at 5,000 rpm for two minutes to remove the cellular debris. The supernatants were mixed with 10 μl of protein G agarose (Life Technologies, Gaithersburg, MD) and maintained at 4°C for two hours with slow rotation. The mixture was centrifuged, 5 μl of antibody to p53 and 10 μl of protein G agarose were added to the supernatant and kept at 4°C for two hours with rocking. The immune complexes were washed twice with washing buffer (0.3 M NaCl, 50 mM Tris-HCl [pH 7.4], 0.1% SDS, 0.1% sodium deoxycholate) and the aragose beads were heated for five minutes at 90°C to release the complexes; the complexes were separated on 10% polyacrylamide gel with SDS (SDS-PAGE). The gel was soaked in Enhance solution (Du Pont NEN, Boston, MA), vacuum dried, and exposed to X-ray film.

Immunohistochemistry. Formalin-fixed, paraffin-embedded sections of human tissues were stained with a rabbit polyclonal antibody to recombinant human p53 protein (CM1) using an avidin-biotin-peroxidase technique (Vectastain Elite ABC Kit, Vector Laboratories, Inc., Burlingame, CA). Mutant p53 was differentiated from wild-type by the observation of nuclear p53 staining, because the short half-life of wild-type p53 usually prevents its detection by immunohistochemistry (8).

Western blot. Frozen human tissues were homogenized in lysate buffer (100 mM Tris-HCl [pH 8.0], 50mM NaCl, 0.5% Triton X100, 0.2mM PMSF) (10 μl/mg of tissue). After three cycles of freezing and thawing, the cell lysates were centrifuged at 800g for three minutes to remove the cellular debris. Cellular proteins contained in 5 μl of the lysate (equivalent to 0.5 mg of the original tissues) and cell lysates from 10^4 HeLa cells and $P388D_1$ cells were subject to 10% SDS-PAGE. Separated proteins were transferred to a PVDF membrane that was then blocked with blocking solution (Life Technologies, Gaithersburg, MD), stained with antibody to topoisomerase II (SWT3D1) and HRP-conjugated goat anti-mouse IgG, and exposed to X-ray film after ECL treatment.

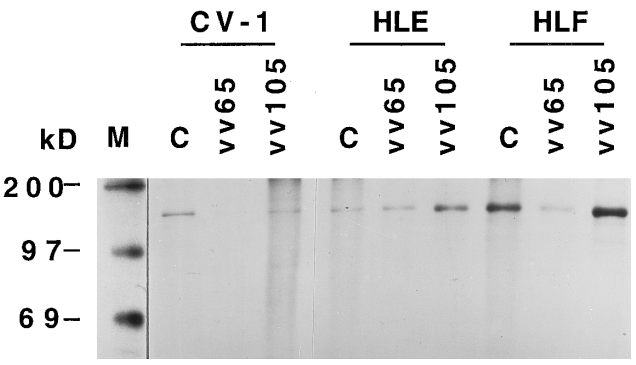

FIG. 1. Coimmunoprecipitation of labeled cellular proteins and wild-type p53 expressed in a vaccinia virus vector. HCC cell lines HLE and HLF, and monkey kidney cell line CV-1 were labeled with [^{35}S]methionine prior to virus infection (C = uninfected; vv65 = recombinant vaccinia virus with β galactosidase gene; vv105 = recombinant vaccinia virus with β galactosidase gene and p53 cDNA). Cell lysates from 10^4 cells were mixed with monoclonal antibody to p53 (PAb 1801) and protein G agarose. The precipitated proteins were subjected to 10% SDS-PAGE and autoradiographed. Molecular weight standard in kilodaltons is shown on the left.

RESULTS

In cells infected with the recombinant vaccinia virus containing the gene for wild-type human p53 (vv105), three cellular polypeptides (p40 and p50, previously reported [7], and p150-180 [Figure 1]) could be coimmunoprecipitated by a monoclonal antibody to p53 (PAb 1801). A cDNA expression library from normal human liver was therefore screened to elucidate the nature of these binding proteins by using p53 proteins expressed in the recombinant vaccinia virus and the monoclonal antibody to p53. After three rounds of plaque purification, 12 bacteriophage were isolated and individually purified. The nucleotide sequences of their cDNA inserts were determined. By comparison with a DNA data base, one of the 12 cDNA sequences matched at all codons to cDNA encoding human mdm2, a cellular protein known to bind to p53 (5). Another sequence (bacteriophage clone 242, containing a 1.9 kb insert) had >99% homology in both the nucleotide and amino acid sequences to the 1.4 kb 3′-end of the published cDNA sequence encoding DNA topoisomerase IIb, a 180 kD nuclear protein (GenBank accession number U54831).

Interaction between p53 and topoisomerase II was further confirmed *in vitro* by coimmunoprecipitation of

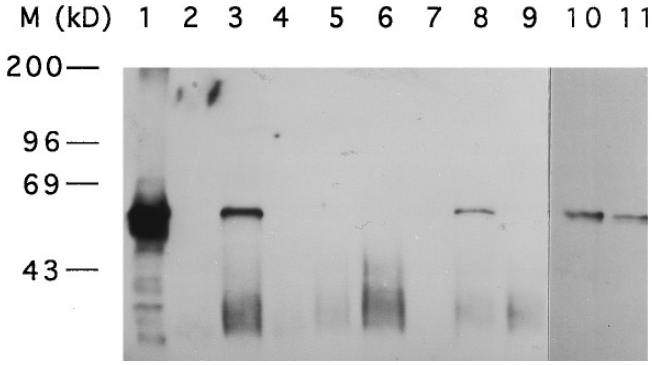

FIG. 2. Binding of p53 to topoisomerase II. Cell lines were infected with the recombinant vaccinia virus (vv105) containing the gene for wild-type human p53 (lanes 1, 3, 6, 7, 8, and 9) or with vaccinia virus without the p53 insert (vv65) (lane 2). Uninfected cells were also used as additional controls (lanes 4 and 5). Cell lines used were HeLa cells (lanes 1, 2, 3, 4, 5, 7, and 8) and $P388D_1$ cells (lanes 6 and 9). Cell lysates were prepared and immunoprecipitated with a monoclonal antibody to p53 (DO-1) (lanes 1 and 5), a monoclonal antibody to topoisomerase II (SWT3D1) (lanes 2, 3, 4, and 6), a monoclonal antibody to mdm2 (IF2) (lanes 8 and 9), and PBS (lane 7). Lysates from HeLa cells infected with vv105 were incubated at 37°C for four hours with one unit/μl of DNase I (lane 10) or without this treatment (lane 11) prior to coimmunoprecipitation with antibody to topoisomerase II. The immunoprecipitants were then separated on 10% SDS-PAGE and blotted onto a PVDF membrane, which was then blocked, stained with a polyclonal antibody to p53 (p53 Kit, Oncogene Science), and visualized with ECL. M, prestained molecular weight standard.

p53 protein by a monoclonal antibody to topoisomerase II followed by western blot with a polyclonal antibody to p53 (Figure 2). p53 protein could be immunoprecipitated only from lysates of topoisomerase II-rich (9) HeLa cells, not from lysates of the topoisomerase II-deficient (10) and mdm2-deficient (unpublished observations) $P388D_1$ cells (Figure 2, lanes 1 to 9). A reciprocal experiment attempting to coimmunoprecipitate topoisomerase II with a polyclonal antibody to p53 was unsuccessful, possibly due to the limited selection of antibodies to topoisomerase II available (data not shown).

Since double-stranded DNA theoretically could contribute as a mediator to such protein binding, the HeLa cell lysate was treated with DNase I to eliminate cellular DNA prior to coimmunoprecipitation (data not shown), after which there was no apparent difference between treated and untreated samples in the western blot with antibody to p53 (Figure 2, lanes 10 and 11).

In order to determine whether topoisomerase II expression could interfere with wild-type p53 and possibly contribute to carcinogenesis in the liver, studies were conducted on HCCs and adjacent nontumorous liver tissue from 10 HCC patients. In normal liver, as well as in adjacent nontumorous liver tissues from the 10 HCC patients, there was no topoisomerase II expression detectable by western blot and only wild-type p53 was present, based on no detectable nuclear staining in immunohistochemistry (Table I). In contrast, topoisomerase II proteins were detected by western blot in six of 10 HCC tissues. Probable wild-type p53 (as determined by the absence of detectable p53 by immunohistochemistry) was present in four of the six cases (Table I).

DISCUSSION

Topoisomerase II, one of the two types of DNA topoisomerases (I and II), is a nuclear enzyme that is active in chromosome segregation during mitosis (11, 12). The topoisomerases perform their functions by introducing transient protein-bridged DNA breaks on one (type I) or both (type II) DNA strands, to permit conformational changes required for DNA replication and RNA transcription (13). Cellular levels of topoisomerase II increase rapidly when cells are induced to proliferate, and levels decrease when cells are induced to differentiate (14, 15). Overexpressed topoisomerase II has been observed in cancers of the human cervix, lung and colon (16). Autoantibody to topoisomerase II has been reported in a patient with HCC, possibly due in part to overexpression of topoisomerase II (17). A number of antineoplastic drugs have been designed to target topoisomerase II by forming complexes with it (13). Binding of p53 by topoisomerase II, as shown in the study reported here, suggests that p53-triggered apoptosis may be inhibited by overexpression of topoisomerase II, leading to unrestricted cell growth even in the presence of wild-type p53.

p53 plays an important role in the negative control of cell growth and malignant transformation (1, 2, 3). The functions of p53 can be abolished by a deletion or mutation in the p53 gene, or by the binding of wild-type p53 by certain viral or cellular proteins such as the E6 protein of HPV (4) and human mdm2 (6). In the present study, topoisomerase II could be detected in 6 of 10 HCCs but not in adjacent nontumorous

TABLE 1

Expression of p53 and Topoisomerase II in Human Hepatocellular Carcinoma and Adjacent Nontumorous Liver Tissue

Tissue type	Number of cases	p53	Topoisomerase II
Normal liver	1	WT	−
HCC patients:			
nontumorous liver	10	WT	−
Hepatocellular			
carcinoma	4	WT	+
	4	MUT	−
	2	MUT	+

Note. Abbreviations: WT, wild-type; MUT, mutant

liver tissues from the same patients or in a normal liver. The fact that topoisomerase II can bind wild-type p53, as shown here, may be a mechanism by which p53 proteins are regulated and may help to explain the early stages of hepatocarcinogenesis, when mutant p53 is an uncommon finding, although the possibility that other cellular proteins could mediate the binding between topoisomerase II and p53 cannot be ruled out at present.

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