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(54) Title: p53CP, A PROTEIN THAT SPECIFICALLY BINDS TO CONSENSUS p53 DNA BINDING SITES

(57) Abstract

p53 Tumor suppressor protein negatively regulates cell growth, mainly through the transactivation of its downstream target genes. As a sequence specific DNA binding transcriptional factor, p53 specifically binds to a 20 bp consensus motif 5'–PuPuPuC(A/T)(T/A)GPyPyPuPuPuC(A/T)(T/A)GPyPyPy–3'. We have now identified, partially purified and characterized an additional nuclear protein, p53CP (p53 competing protein) that specifically binds to consensus p53 binding sites found in several p53 downstream target genes including Waf–1, Gadd45, Mdm–2, Bax, and RGC.

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p53CP, A PROTEIN THAT SPECIFICALLY BINDS TO CONSENSUS p53 DNA BINDING SITES

FIELD OF THE INVENTION

This invention relates to p53CP, a protein that binds to consensus p53 DNA binding sites.

BACKGROUND OF THE INVENTION

p53, a 53 kDa nuclear protein is one of the most fascinating molecules in the field of cancer research. The multiple biochemical and biological functions of p53 can be mainly defined from its protein primary structure (Arrowsmith C.H. and Morin P., *Oncogene*, 1996;12:1379-1385). Structurally, the p53 protein consists mainly of three distinct domains: a transactivation domain at the N-terminal, a central specific DNA binding domain, and the oligomerization domain at the C-terminal of the molecule (Ko L.J. and Prives C., *Genes & Develop.*, 10:1054-1072 and references therein). As a transcription factor, p53 either transactivates or transrepresses gene expression. Other p53 biochemical activities include inhibition of DNA helicase (Wang X.W., Yeh H., Schaeffer L., et al., *Nature Genet.*, 1995;10:188-193), binding to single-stranded DNA and stimulating their annealing (Bakalkin G., Yakovleva T., Selivanova G., et al., *Proc. Natl. Acad. Sci. USA.*, 1994;91:413-417; Selivanova G. and Wiman K.G., *Adv. Cancer Res.*, 1995;66:143-180; and references therein), and action as exonuclease (Mummenbrauer T., Janus F., Muller B., Wiesmuller L., Deppert W., and Grosse F., *Cell*, 1996;85:1089-1099). As a typical tumor suppressor, p53 has been shown to inhibit tumor cell growth and suppress transformation by either inducing G1 arrest or apoptosis (Ko L.J. and Prives C., *Genes & Develop.*, 10:1054-1072; Levine A.J., *Cell*, 1997;88:323-331; and references therein). As a "genome guard", p53 is induced upon DNA damage to prevent gene amplification and preserve genetic stability (Ko L.J. and Prives C., *Genes & Develop.*,

10:1054-1072; Levine A.J., *Cell*, 1997;88:323-331; Livingstone L.R., White A., Sprouse J., Livanos E., Jacks T., and Tlsty T.D., *Cell*, 1992;70:923-935; Yin Y., Tainsky M.A., Bischoff F.Z., Strong L.C., and Wahl G.M., *Cell*, 1992;70:937-948). In addition, p53 may play a role in differentiation, senescence, and angiogenesis (Rotter V., Aloni-Grinstein R., Schwartz D., et al., *Semin. Cancer Biol.* , 1994;5:229-236; Vojta P.J. and Barrett J.C., *Biochim. Biophys. Acta*, 1995;1242:29-41; Bouck N., *Biochim. Biophys. Acta*, 1996;1287:63-66).

Many of p53 functions are mediated by its transactivation activity. As a transcription factor, p53 binds specifically to its consensus DNA sequence consisting of two copies of the 10 bp motif 5′-PuPuPuC(A/T)(T/A)GPyPyPy-3′, separated by 0 to 13 bp (El-Deiry W.S., Kern S., Pietenpol J.A., Kinzler K.W., and Vogelstein B., *Nature Genet.*, 1992;1:45-49). This sequence has been found in many p53 regulatory genes including Waf-1/p21 (El-Deiry W.S., Tokino T., Velculescu V.E., et al., *Cell*, 1993;**75:**817-825), Mdm-2 (Barak Y., Juven T., Haffner R., and Oren M., *EMBO J.*, 1993;12:461-468), Bax (Miyashita T. and Reed J.C., *Cell*, 1995;80:293-299), Gadd45 (Kastan M.B., Zhan Q., El-Deiry W.S., et al., *Cell*, 1992;71:587-597), PCNA (Morris G.F., Bischoff J.R., and Mathews M.B., *Proc. Natl. Acad. Sci. USA*, 1996;93:895-899), thromspodin (Dameron K.M., Volpert O.V., Tainsky M.A., and Bouck N., *Science*, 1994;265:1582-1584), and type IV collagenase (Bian J. and Sun Y., *Mol. Cell. Biol.* 1997;17:6330-6338) among the others. Those are the genes involved in regulation of cell growth and differentiation, apoptosis, DNA damage/replication, and angiogenesis. Due to its biological significance in cell growth control, p53 becomes inactivated by many ways during human carcinogenesis (Chang F., Syrjanen S., and Syrjanen K., *J Clin. Oncol.*, 1995;13:1009-1022 and references therein). The most common way to inactivate p53 in cells is by point mutations in its DNA binding domain, which were detected in about 50% of all human cancers. Mutant p53 proteins often lose DNA binding and transactivation activity, while some of them gain oncogenic activity (Greenblatt M.S., Bennett W.P., Hollstein M., and Harris C.C., *Cancer Res.*, 1994;54:4855-4878 and references therein). p53 can also be inactivated by binding to and being inhibited by several viral proteins such as SV40 large T antigen, E1B, and HPV E6 (Ko L.J. and

Prives C., *Genes & Develop.*, 10:1054-1072; Levine A.J., *Cell*, 1997;88:323-331; and references therein). In some sarcomas, oncoprotein Mdm-2 was overexpressed (Oliner J.D., Kinzler K.W., Meltzer P.S., George D.L., and Vogelstein B., *Nature*, 1992;358:80-83) which binds to and inactivates p53 (Ko L.J. and Prives C., *Genes & Develop.*, 10:1054-1072; Levine A.J., *Cell*, 1997;88:323-331; and references therein). The third way to inactivate p53, as seen in some breast cancers (Moll U.M., Riou G., and Levin A.J., *Proc. Natl. Acad. Sci. USA*, 1992;89:7262-7266), is through the nuclear exclusion by which p53 is excluded from the nucleus where it normally functions as a transcription factor. Recently, p53 was found to be subjected to redox regulation both *in vitro* and *in vivo* (Sun Y. and Oberley L.W., *Free Rad. Biol. Med.*, 1996;21:335-348, 1996 and references therein, Sun Y., Bian J., Wang Y., and Jacobs C., *Oncogene*, 1997;14:385-393;).

We have recently cloned the gene encoding mouse tissue inhibitor of metalloproteinases-3 (mTIMP-3) and its promoter (Sun Y., Hegamyer G. and Colburn N.H., *Cancer Res.*, 1994;54:1139-1144 and Sun Y., Hegamyer G., Kim H., Sithanandam K., Li H., Watts R. and Colburn N.H., *J. Biol. Chem.*, 1995;270:19312-19319), and identified in the promoter a putative p53 binding site: 5′-**GGGCTTGCTT** GACGTCCA **GAACAGGGTC**-3′ (SEQ ID NO. 1). This site contains two copies of the p53 binding motif (bold) separated by 8 base pairs with two mismatches (underlined) in the second motif (Sun Y., Hegamyer G., Kim H., Sithanandam K., Li H., Watts R. and Colburn N.H., *J. Biol. Chem.*, 1995;270:19312-19319). We have conducted extensive studies as to whether mTIMP-3 is a p53 downstream target gene and have concluded that TIMP-3 is not subjected to p53 regulation (Bian J., Jacobs C., Wang Y., and Sun Y., *Carcinogenesis*, 1996;17:2559-2562). During the course of this study, we identified a nuclear protein which also specifically bound to the p53 sites. We named this protein p53CP (p53 competing protein) for its potential competition with p53 for p53 DNA binding sites. We report here the identification, partial purification and characterization of p53CP, and propose a novel mechanism for p53 inactivation. Identification of such a protein opens new avenues in study of p53 inactivation and regulation during human carcinogenesis.

SUMMARY OF THE INVENTION

This invention provides a protein having a molecular weight of about 40 kDa that specifically binds to the consensus p53 binding sites.

DESCRIPTION OF THE DRAWINGS

Figure 1. Identification of a nuclear protein that binds to p53 binding sites: (A) binding of p53CP to T3SF, and (B) binding of p53CP to Gadd45, Waf1 and p53CON: The nuclear extract was prepared from H-Tx mouse liver tumor cells and subjected to gel retardation assay as described herein with a poly(dI/dC) concentration of 10 μg/mL. The oligonucleotides used are the following: (a) T3 5′-A**GGGCTTGCTT** GACGTCCA **GAACAGGGTC**T-3′ (SEQ ID NO. 2), the sequence found in the promoter of mouse TIMP-3; (b) T3SF 5′-A**GGGCTTGCTTGAACAGGGTC**T-3′ (SEQ ID NO. 3); (c) T3MD 5′-A**GGGCTTGCTT**GACGTCCA**GGTC**T -3′ (SEQ ID NO. 4); (d) GADD 5′-GAACATGTCTAAGCATGCTG-3′ (SEQ ID NO. 5); (e) WAF 5′ GAACATGTCCCAACATGTTG-3′ (SEQ ID NO. 6); and (f) p53CON 5′-AGACATGCCTAGACATGCCT-3′ (SEQ ID NO. 7). The protein complexes bound to T3 oligo are indicated by arrows. Also indicated are p53-Ab complex, p53CP (band 3) and free probe.

Figure 2. p53CP is not another form of p53: Nuclear extract was prepared from p53 negative lines including human Saos-2 (A), PC-3 and MEF cells from p53 knockout mice (p53-/-MEF) (B), and subjected to gel retardation assay as described herein. The oligonucleotide probes used were WAF, T3SF, and p53CON. The positions of p53CP and free probe are indicated.

Figure 3. p53CP and p53-Ab complex showed differential binding specificity to p53 binding consensus sequences: Nuclear extract was prepared from mouse H-Tx cells and subjected to gel retardation assay with increasing amounts of the nonspecific competitor, poly(dI/dC). All gel shift reactions contained p53 antibody, pAb421, to enhance p53 binding. The oligonucleotides

used were WAF, GADD, and T3SF. The positions of p53-Ab complex and p53CP are indicated.

Figure 4. p53CP also binds to single stranded DNA: Two complementary strands of T3SF (T3SF.S1 and T3SF.S2) were labeled separately and included individually or in combination in the gel retardation reaction (containing 50 μg/mL poly dI/dC) with the nuclear extract isolated from H-Tx cells. The p53CP-oligo (ds) and p53CP-oligo (ss) are indicated by arrows.

Figure 5. Mapping the minimal sequence required for p53CP binding: A series of deletion oligonucleotides based upon T3SF sequence were synthesized and labeled to define the minimal p53CP binding site. The sequences of these oligonucleotides are listed in Table 1. The gel retardation assay was performed as described in Figure 1 with a poly(dI/dC) concentration of 50 μg/mL; (A) binding of T3SF deletion mutants with p53CP and competition of the binding by unlabelled wildtype T3SF; and (B) binding of p53CP with T3SF and competition by unlabelled deletion mutants. The position of p53CP-DNA complex is indicated by the arrow.

Figure 6. Partial purification of p53CP by DNA affinity chromatography: T3SF (A) or T3SF-Core (B) concatamer-coupled Sepharose 4B DNA affinity columns were prepared as detailed herein. The nuclear extract was prepared from either H-Tx (A) or p53-/-MEF (B) cells, dialyzed with cold binding buffer and loaded onto the column. After washing with binding buffer, the nuclear proteins were eluted with increasing amount of salt concentration. An aliquot of each fraction was subjected to gel retardation assay using labeled T3SF (A) or T3SF5 (B) as probes. The position of p53CP complexed with oligonucleotides is indicated. BC stands for before passing through the column, and FT stands for flow-through.

Figure 7. Determination of the molecular weight of p53CP: Molecular weight of both mouse and human p53CP was determined by Southwestern analysis (A), *in vivo* labeling coupled with gel retardation assay (B), and UV-crosslinking (C). Nuclear extracts from mouse H-Tx or human PC-3 (A), or p53-/-MEF (B) were used. For UV crosslinking (C), T3SF was used as the probe

for gel retardation assay with nuclear extract from H-Tx cells. As a positive control, partial affinity purified p53 was used with p53CON as the probe. The position of p53CP, as well as p53 complexed with oligonucleotide, is indicated by arrows. The molecular weight markers are included on the left of each figure.

Figure 8. Coordinate regulation of p53 and p53CP in response to cell cycle blockers: Mouse H-Tx cells (A) and human PC-3 cells (B) were subjected to treatment with cell cycle blockers for 24 hours. The concentrations used were DMSO (for control, 0.1%), aphidicolin (10 μg/mL), thymidine (2.5 mM), nocodazole (4 μg/mL), and lovastatin (1 μg/mL). Nuclear extracts were prepared after treatment and subjected (2 μg) to gel retardation assay. To measure p53CP binding activity, T3SF was used with a poly(dI/dC) concentration of 50 μg/mL (lanes 1-6). To determine p53 binding, p53CON oligo was used with a poly(dI/dC) concentration of 10 μg/mL in the presence of p53Ab (lanes 7-12). The p53CP and p53-Ab complexed with oligo are indicated by arrows.

DETAILED DESCRIPTION OF THE INVENTION

Cell Cultures and Reagents: The mouse H-Tx liver tumor cells (Sun Y. Oberley L.W., Oberley T.D., Elwell J.H., and Sierra-Rivera E., *Carcinogenesis*, 1991;14:1457-1463) were grown in 10% DMEM with 1 mM sodium pyruvate. The human Saos-2 cells and p53-/-MEF (mouse embryonic fibroblast) cells were both grown in 10% DMEM. The human PC-3 cells were grown in 10% RPMI. All synthetic oligonucleotides were made by BRL. CNBr-activated Sepharose 4B and poly (dI/dC) were purchased from Pharmacia. Aphidicolin, thymidine, and nocodazole were from Sigma. Lovastatin was made according to known procedure. For treatment with cell cycle blockers, H-Tx or PC-3 cells were either serum starved or incubated with DMSO (control), aphidicolin (10 μg/mL), thymidine (2.5 mM), nocodazole (4 μg/mL) and lovastatin (1 μg/mL) for 24 hours.

Nuclear Extract Preparation and Gel Retardation Assay: Nuclear extract from confluent cells were prepared and subjected to gel retardation assay (Sun Y., Bian J., Wang Y., and Jacobs C., *Oncogene*, 1997;14:385-393). Briefly, cells were lysed in cold lysis buffer (25 mM Hepes, pH 7.8, 50 mM KCl, 0.5% NP-40, 1 mM PMSF, 10 μg/mL leupeptin, 20 μg/mL aprotinin, and 100 μM DTT) centrifuged. The cell pellet was resuspended in cold extraction buffer (25 mM Hepes, pH 7.8, 500 mM KCl, 10% glycerol, 1 mM PMSF, 10 μg/mL leupeptin, 20 μg/mL aprotinin, and 100 μM DTT) followed by incubation on ice for 20 minutes with frequent mixture. Cell lysate was then centrifuged at 14,000 rpm for 5 minutes or at 40,000 rpm for 40 minutes in the large scale preparations for subsequent affinity purification. The resulting supernatant was used as nuclear extract. For gel retardation assay, the synthetic oligonucleotide was annealed and labeled with ^{32}P using T4 polynucleotide kinase and [γ-^{32}P]ATP. The labeled oligonucleotide was purified with a Quick spin column (BMB). A DNA-binding reaction mixture of 20 μL contained 20 mM Tris-HCl, pH 7.5, 4% Ficoll-400, 2 mM EDTA, 0.5 mM DTT, 10-50 μg/mL of poly (dI/dC), ^{32}P-labeled oligo (20,000 cpm) was incubated with 1 to 5 μg of protein-containing nuclear extract (assayed by a Bio Rad protein assay kit). In some experiments, the anti-p53 antibody, pAb421 (Oncogene Science, 2 μL) was included. To determine the binding specificity, 50-fold excess of unlabeled oligonucleotide was included in some reactions. The mixture was incubated for 45 minutes and then loaded onto a 3.5% polyacrylamide gel. The gel was run in 0.5 X TBE buffer at 60 V for 3.5 hours, dried, and exposed to film.

DNA Affinity Purification of p53CP: DNA affinity chromatography was performed as described (Kadonaga J.T. and Tjian R., *Proc. Natl. Acad. Sci. USA*, 1986;83:5889-5893) with modifications. Two concatamers of T3SF or T3SF-Core were made by annealing the following complementary oligonucleotides: 5′-GGGCTTGCTTGAACAGGGTC-3′ (SEQ ID NO. 8) and 5′-GCCCGACCCTGTTCAAGCAA-3′ for T3SF (SEQ ID NO. 9), and 5′-CTTGCTTGAACAGG-3′ (SEQ ID NO. 10) and 5′-CAAGCCTGTTCAAG-3′

(SEQ ID NO. 11) for T3SF-Core. Both strands of oligonucleotides (440 μg each) were mixed in 130 μL TE buffer, boiled in a water bath for 5 minutes, and then annealed overnight. The annealed oligonucleotides were 5′-phosphorylated by T4 nucleotide kinase followed by phenol extraction and ethanol precipitation. The resulting oligonucleotides were then ligated by T4 DNA ligase at 15°C overnight. The concatamers (from dimer all way up to 20 mers) were again phenol extracted, ethanol precipitated, and resuspended in 100 μL H_2O. To conjugate concatamers to Sepharose 4B, 3 g of CNBr-activated Sepharose 4B was washed with 500 mL of 1 mM HCl, 100 mL of H_2O, and 100 mL of potassium phosphate (10 mM), pH 8.0 and then resuspended in 4 mL of 10 mM potassium phosphate, pH 8.0. Sepharose 4B was then incubated with 100 μL of concatamer oligonucleotides prepared above on a rotator overnight. The resin was washed with 100 mL of water twice and 100 mL of ethanolamine hydrochloride (1 M, pH 8.0) and then incubated with 5 mL of 1 M ethanolamine hydrochloride (pH 8.0) for 4 hours on a rotator. The final washing included 100 mL of the following solution sequentially: 10 mM potassium phosphate (pH 8.0), 1 M potassium phosphate (pH 8.0), 1 M KCl, water, and column storage buffer (10 mM Tris-HCl, pH 7.8, 1 mM EDTA, 0.3 M NaCl, 0.04% sodium azide). The resin was resuspended in 5 mL of column storage buffer at 4°C. For DNA affinity purification, 5 mL of nuclear extract from H-Tx or p53-/-MEF cells were dialyzed in cold binding buffer (20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 2.5 mM $MgCl_2$, 0.5 mM DTT), at 4°C for 3 hours in a Slide-A-Lyzer (Pierce). Poly (dI/dC) was added at 50 μg/mL. The resulting sample was passed through a T3SF or T3SF-core-Sepharose 4B affinity column (0.5 mL in volume), pre-equilibrated with the binding buffer. After washing with 10 mL of binding buffer, a series of elutions were made with 0.5 mL of binding buffer containing various NaCl or KCl concentrations (0.1 to 1 M). The presence of p53CP was monitored in gel retardation assays using 2 μL of each fraction.

Southwestern Analysis: Each strand of T3SF oligonucleotides (10 pmol) used in preparation of T3SF concatamer was labeled separately with ^{32}P, annealed,

ligated, and purified. Nuclear extracts of H-Tx and PC-3 cells (20 μg of protein) were run on 10% to 20% SDS tricine gel (Novax) and transferred to nitrocellulose membrane. The membrane was incubated sequentially with cold Buffer A (50 mM KCl, 10 mM Tris-HCl, pH 7.5, 2 mM EDTA, 0.1 mM DTT, 1 mM PMSF) containing 4 M urea for 1 hour, Buffer A with 2 M urea for 1 hour, and then Buffer A without urea for 15 minutes at 4°C. The membrane was blocked with 5% dry milk in Buffer A for 1 hour and washed with the binding buffer used in gel retardation assay. The membrane was cut into strips marked by the prestained molecular weight standard and incubated with labeled T3SF concatamer probe (5×10^6 cpm) in 1 mL of gel retardation buffer for 4 hours with gentle rocking. The strips were washed with the gel retardation buffer 3 times (5 minutes per wash), exposed to a Kodak film.

Detection of ^{35}S-labeled p53CP: p53-/-MEF cells with 80% confluency in T75 flask were methionine/cysteine starved at 37°C for 1 hour, labeled with ^{35}S-TransLabel (5 mCi, ICN) for 4 hours. Nuclear extract was then prepared and gel retardation assay performed using ^{32}P-T3SF as probe. The resulting wet gel was covered with Saran Wrap and exposed to two x-ray films on one side of the gel overnight. The band corresponding to p53CP-oligo complex (which appeared in both films) due to the presence of ^{32}P signal was cut from the gel. The excised gel piece was smashed into small pieces and soaked in 2 × SDS protein sample buffer for 48 hours. The extract from the gel piece was run on a 10% to 20% SDS tricine gel and exposed to two films to distinguish the ^{32}P and ^{35}S signals.

UV-Crosslinking of p53CP With Oligonucleotides: Gel retardation assay was performed using nuclear extract of H-Tx (200 μg) or partial purified recombinant p53 (200 μg) (29) and ^{32}P-T3SF (for p53CP) and ^{32}P-p53CON (for p53) oligonucleotides, respectively. The wet gel was exposed, and the bands corresponding to p53CP- and p53-DNA complexes were excised from the gel, wrapped with Saran Wrap, placed on ice, and exposed to UV for 30 minutes from

a UV illuminator placed about 4 cm away from the gel. The gel slice was then cut into small pieces and soaked in 2 × SDS protein sample buffer for 48 hours. The gel extracts were run on a 10% to 20% SDS tricine gel followed by autoradiography.

Procedures for p53CP Purification

1. Ammonium Sulfate Precipitation

In a pilot experiment, we have performed ammonium sulfate precipitation of 20 mL of nuclear extract. Briefly, 0.33 g of $(NH_4)_2SO_4$ per milliliter of nuclear extract was slowly added with gentle stirring. After this addition, the solution was stirred for an additional 20 minutes and then centrifuged at 12,000 g for 40 minutes. The pellet is suspended in buffer 1 (20 mM HEPES, pH 7.9, 20 mM KCl, 1 mM MgCl, 17% (v/v) glycerol, and 2 mM DTT), and the resulting suspension was dialyzed against two changes of 100 volume each of buffer 1 for 5 hours. The dialyzate was centrifuged at 10,000 g for 10 minutes to remove insoluble material. The supernatant was measured for protein concentration, and an aliquot was assayed by gel retardation assay to show p53CP activity. Activity of p53CP retained after ammonium sulfate precipitation.

2. Sephacryl S-100 Gel Filtration

The soluble protein extract was applied to a Sephacryl S-100 column equilibrated with buffer 2 (50 mM Tris-HCl, pH 7.9, 12.5 mM $MgCl_2$, 1 mM EDTA, 20% glycerol, 0.1 M KCl). Protein elution was monitored by absorbance at 280 nm, and p53CP activity was determined by gel retardation assay of column fractions. Fractions 83 to 102 contained p53CP activity.

3. DEAE Sepharose CL-6B Column Chromatography

The fractions containing p53CP activity were pooled and applied to a DEAE-Sepharose CL-6B column equilibrated with buffer 2. The flow-through will be tested for p53CP activity, and fractions containing activity will be pooled.

4. Heparin-Agarose Column Chromatography

The pooled DEAE flow-through containing p53CP activity was applied to a heparin agarose column, equilibrated with buffer 2, and was eluted with the buffer 2 series containing, successively, 0.2 M, 0.3 M, and 1.0 M KCl. The eluent will be tested for p53CP activity, and fractions with activity will be pooled.

5. FPLC Mono S Column Chromatography

The pooled fractions will be applied to a 1 mL FPLC Mono S column. The p53CP will be eluted with a 20 mL linear gradient of KCl (60 to 400 mM). Fractions will be collected and assayed for p53CP activity. The fractions with activity will be pooled.

6. DNA Affinity Chromatography

The following is a detailed protocol for DNA affinity purification of p53CP.

Materials: oligonucleotides: 440 μg of each strand synthetic T3SF01 and T3SF12
T4 nucleotide kinase (Boehringer Mannheim)
T4 ligase (New England Biochemicals)
CNBr-activated Sepharose 4B (Pharmacia)

Major Steps: (a modification of protocols in Current Protocols in Molecular Biology, Unit 12.10)

1. Annealing and 5′-phosphorylation of oligonucleotide T3SF:
   (1) take 440 μg T3SF01 and 440 μg T3SF12 in 130 μL 1X TE buffer
   (2) add 20 μL 10X phosphorylation buffer
   (3) boil in a water bath for 5 minutes and turn off the power to let it cool down overnight
   (4) add 20 mM ATP 30 μL and nucleotide kinase 20 μL (200 units)
   (5) incubate at 37°C for 2 hours

(6) separate into two 100 µL tubes for the convenience in centrifuge balance

(7) add 50 µL 10 M NH_4OAc and 100 µL H_2O to each tube

(8) add 750 µL 100% ethanol to each tube, mix, sit on dry ice for 10 minutes

(9) spin at 14,000 rpm for 10 minutes, discard the sup, wash with 70% ethanol once

(10) dry the pellet on speedvac, resuspend pellet in each tube in 225 µL TE

(11) extract with 250 µL of phenol/chloroform/isoamyl alcohol (25:24:1) and spin

(12) take top phase, add 250 µL of chloroform, mix, spin

(13) take top phase, add 25 µL 3 M NaOAc, 750 µL 100% ethanol to each tube

(14) sit on dry ice for 10 minutes, spin, wash the pellet with 70% ethanol, and dry the pellet

2. Ligation to form concatamers of T3SF

(1) dissolve the pellet combined from both tubes into 130 µL H_2O

(2) add 20 µL 10X ligation buffer, 40 µL 20 mM ATP (pH 7.0), and 10 µL ligase

(3) incubate at 15°C overnight

(4) take 0.5 µL, run on 2.5% agarose gel to verify the quality of formed concatamers

(5) separate to two 100 µL samples and spin

(6) add 100 µL phenol to each tube, vortex 1 minute, spin at 14,000 rpm for 5 minutes

(7) take the top phase, add 100 µL chloroform, mix and spin

(8) take the top phase, add 33 µL 10 M NH_4OAc to each tube, vortex

(9) add 133 µL isopropanol, mix by inversion

(10) sit on dry ice for 10 minutes, spin for 10 minutes, keep the pellet

(11) add 225 µL TE to each tube and dissolve the pellet

(12) add 25 µL 3 M NaOAc to each tube, mix, and add 750 µL 100% ethanol, mix

(13) spin 15 minutes, wash the pellet in each tube twice with 70% ethanol, dry on speedvac

(14) dissolve the combined pellet from both tubes in 100 µL H_2O

3. Coupling of oligo with Sepharose 4B

(1) weigh out 3 g CNBr-activated Sepharose 4B in a 15-mL conical polypropylene tube

(2) add 10 mL of 1 mM HCl, and mix gently to produce an even slurry within 5 minutes

(3) transfer the whole slurry to a 60-mL coarse-sintered glass funnel

(4) pour 500 mL of 1 mM HCl through the funnel

(5) wash the resin with 100 mL H_2O and then with 100 mL of 10 mM potassium phosphate buffer, pH 8.0

(6) transfer the resin to a 15-mL conical tube and add 4 mL of 10 mM potassium phosphate, pH 8.0, mix gently

(7) add 100 µL oligo prepared above, incubate at room temperature on a rotating wheel for overnight

(8) transfer to 60-mL coarse sintered glass funnel, and wash 2X with 100 mL water

(9) wash with 100 mL 1 M ethanolamine hydrochloride, pH 8.0, transfer to a conical tube

(10) add 5 mL 1 M ethanolamine hydrochloride, ph 8.0, rotate on wheel for 4 hours at room temperature

(11) transfer the resin back to 60 mL sintered glass funnel

(12) wash with 100 mL of 10 mM potassium phosphate (pH 8.0) once
wash with 100 mL of 1 M potassium phosphate (pH 8.0) once
wash with 100 mL of 1 M KCl once

wash with 100 mL water once

wash with 100 mL column storage buffer once (10 mM Tris-HCl, pH 7.8, 1 mM EDTA, 0.3 M NaCl, 0.04% sodium azide)

(13) store the resin at 4°C

4. Affinity Purification of p53CP

(1) take 5 mL nuclear extract of H-TX prepared by Ghosh or Nicolas methods

(2) dialyze in cold Suretrack binding buffer (20 mM Tris-HCl pH 7.5, 50 mM NaCl, 2.5 mM MgCl2, 0.5 mM DTT, 4°C, 3 hours in Slide-A-Lyzer (Piece)

(3) add 0.5 mL 1 μg/μL dI/dC

(4) pack a small column of 0.5 mL T3RF-Sepharose 4B, add 10 mL binding buffer to equilibrate

(5) apply the 5 mL sample through the column, collect the flow-through

(6) wash with 10 mL binding buffer

(7) elute with 0.5 mL binding buffer containing various NaCl concentrations, collect eluent

(8) take 2 μL from each fraction in gel-shift assay

Characterization of Purified p53CP

We have determined that p53CP is a 40-kDa nuclear protein. To purify and sequence p53CP, the following procedure can be used: (a) purified p53CP will be electroblotted onto and excised from a PVDF membrane; (b) protein digestion with trypsin; (c) HPLC purification/separation; (d) mass spectrometer analysis; and (e) automated protein sequencing.

cDNA Cloning of p53CP Encoding Gene

Based upon p53CP protein sequence, degenerate PCR primers will be designed to amplify cDNA encoding the p53CP protein from a cDNA library according to standard protocol. The PCR product will be used as a probe to screen cDNA library to clone the full-length cDNA. Upon the cloning of the gene,

activity of p53CP will be assessed as to (a) whether it competes with p53, or (b) whether it has p53-like activity.

1. If p53CP competes with p53 for sequence-specific DNA binding:

Mutational analysis as well as protein expression determination of p53CP will be performed in human cancers, particularly those harboring an endogenous wildtype p53. The prediction is that p53CP should be mutated (activated) or overexpressed in these cancers to inhibit p53 activity. If that is the case, genetic or pharmaceutical manipulations to disrupt p53CP activity will be used for cancer therapy, particularly with cancers having wildtype p53.

2. If p53CP does not compete with p53, but has p53-like activity:

p53CP will be another typical tumor suppressor gene. Mutational analysis, as well as protein expression determination, will be again performed to see whether p53CP is altered in particular type of human cancers or in cancers whose p53 gene is rarely mutated (such as nasopharyngeal carcinomas). Genetic or pharmaceutical manipulations to restore p53CP activity can be used for cancer therapy.

Identification of a Nuclear Protein (p53CP), Other Than p53, That Binds to p53 Consensus Sequence: We have previously identified a p53 binding site in the promoter of the gene encoding mouse TIMP-3. This site, named as T3 (5′-**AGGGCTTGCTT** *GACGTCCA* **GAACAGGGTC**T-3′) (SEQ ID NO. 12) consists of two copies of the p53 binding motif (bold) separated by 8 base-pair spacer (italic) with two mismatches (underlined) in the second motif (Sun Y., Hegamyer G., Kim H., Sithanandam K., Li H., Watts R., and Colburn N.H., *J. Biol. Chem.*, 1995;270:19312-19319). To test whether p53 binds to this sequence, we performed a gel retardation assay using the nuclear extract prepared from a mouse liver tumor line, H-Tx. This is a spontaneously transformed line established by conventional subcultivation of an immortalized liver line (Sun Y. Oberley L.W., Oberley T.D., Elwell J.H., and Sierra-Rivera E., *Carcinogenesis*, 1991;14:1457-1463, 1993). H-Tx cells contain a high level of wildtype p53 (Sun Y., Hegamyer G., Nakamura K., Kim H., Oberley L.W., Colburn N.H.,

Int. J. Cancer, 1993;55:952-956). As shown in Figure 1A, T3 did not bind to p53 since p53 antibody did not induce a supershift (lanes 1 and 2). It, however, binds to 5 nuclear proteins as indicated by arrows. The binding is sequence specific since they can be largely blocked by unlabelled T3 oligo (lane 3). A closer examination of T3 sequence revealed within the spacer a consensus sequence (TGACGT) for a cellular transcription factor ATF, a cAMP-responsive element (CRE) binding protein (Lin Y.-S. and Green M.R., *Proc. Natl. Acad. Sci. USA*, 1988;85:3396-3400 and Deutsch P.J., Hoeffler J.P., Jameson J.L., Lin J., and Habener J.F., *J. Biol. Chem.*, 1988;263:18466-18472). Some of these multiple bands may be, therefore, resulted from the binding of CREB proteins. To test this hypothesis, we deleted the spacer sequence and made the oligo T3SF (spacer free), 5′-A**GGGCTTGCTT GAACAGGGTC**T-3′ (SEQ ID NO. 3) and performed the same gel retardation assay. T3SF is a typical p53 binding site, without the spacer, but with two mismatches (underlined). As shown in lanes 4 and 5, T3SF binds to band 3 strongly, as well as to p53 as evident by a supershift induced by p53 antibody. The band 3, however, cannot be supershifted by p53 antibody. Again, the binding of the T3SF to the band 3 and p53 is sequence specific, since it can be blocked completely by cold T3SF (lane 6) but not at all by a mutant T3SF (5′-A**GGGGTTCCTTGAAGAGCGTC**T-3′) (SEQ ID NO. 13) that contained a substitution in both motifs of the C to the G and the G to the C at conserved positions 4 and 7 (underlined), respectively. The disappearance of the bands 2, 4, and 5 indicates that they bind to 8 bp spacer sequence. The identity of the band 1 is not clear. It seems to have similar migration to p53 but should not be p53 since (a) T3 did not bind to purified p53 as described previously (Bian J., Jacobs C., Wang Y., and Sun Y., *Carcinogenesis*, 1996;17:2559-2562) and (b) it cannot be supershifted by p53 antibody (lane 2). It is noteworthy that T3SF also binds to a nuclear protein shown as a fast migration band (the band above the free oligo, lanes 4 and 5). The binding is, however, nonspecific since it cannot be blocked by cold T3SF (lane 6, compared to lanes 4 and 5). To further test the binding specificity of the band 3 to p53 consensus sequence , we made another oligo (T3MD, 5′-A**GGGCTTGCTT***GACGTCCA***GGTC**T-3′) (SEQ ID NO. 14) for gel

retardation assay which retained the spacer sequence (CRE site) but had the first 6 nucleotides deleted in the second p53 binding motif. As shown in lanes 7-9, T3MD binds specifically to bands 2, 4, and 5, but not to the band 3 and p53, further confirming that (a) bands 2, 4, and 5 belong to CREB proteins, and (b) two 10 bp motifs are needed for both p53 and the band 3 to bind. These experiments indicate that a nuclear protein, present in H-Tx cells, specifically binds to an artificial p53 binding site, and the binding is dependent upon the integrity of p53 binding motif.

We next examined whether the nuclear protein (revealed as the band 3 after complexed with T3SF) also binds to the p53 consensus binding sequences found in genes encoding Gadd45 and Waf-1. Again, the nuclear extract from H-Tx cells was used. As shown in Figure 1B, indeed, both p53 and the band 3 bind Gadd45 and Waf-1 (lanes 1 and 5). The binding to p53, but not to the band 3, was enhanced and supershifted by p53 Ab (lanes 2 and 6). The binding to both p53 and the band 3 is sequence specific. It can be blocked by cold Gadd45 (lane 4) or Waf (lane 8), respectively, as well as by cold T3SF (lanes 3 and 7). We have included another artificial p53 consensus sequence (p53CON, without mismatch) and found that it binds to both p53 and the band 3 (lanes 9 and 10) with a stronger binding to p53 in the presence of p53Ab (lane 10). Again, binding is specific, it can be specifically blocked by cold p53CON and T3SF (lanes 11 and 12). The band 3 also binds to p53 site found in the genes encoding Mdm-2, Bax, and RGC (data not shown), and the binding was detected only in nuclear, not in cytoplasmic fractions (data not shown). Since this nuclear protein binds to all tested p53 consensus DNA binding sequences found in p53 regulated genes, it may compete with p53 for specific DNA binding *in vivo*, we have named it p53CP (p53 competing protein).

p53CP Is Not Another Form of p53: To exclude the possibility that p53CP observed in the gel retardation assay is an alternate form of p53, we used antibody against alternatively spliced p53 (Kulesz-Martin M., Lisafeld B. Huang H., Kisiel N.D., and Lee L., *Mol. Cell. Biol.*, 1994;14:1698-1708 and Wu Y., Liu Y., Lee L., Miner Z. and Kulesz-Martin M., *EMBO J.*, 1994;13:4823-4830) and found

that the antibody neither blocked nor supershifted p53CP. We next used nuclear extract from human Saos-2 cells, a sarcoma cell line with the p53 gene deleted (Masuda H., Miller C., Koeffler H.P., Battifora H. and Cline M.J., *Proc. Natl. Acad. Sci. USA*, 1987;84:7716-7719). If p53CP is another form of p53, it should not be detected in Saos-2 cells. As shown in Figure 2A, p53CP, but not p53 can be detected with both the Waf or T3SF DNA binding oligonucleotides, and again, the presence of p53 antibody did not induce a supershift of p53CP. This result indicates that p53CP is not another form of p53, and that p53CP is also present in human cells (also detectable in human cell lines Du145, LNCap, HT1080, HeLa, and Rhek-ras). We further confirmed this by using nuclear extract prepared from MEF cells derived from p53 knockout mice and from PC3 human prostate carcinoma cells, having one nucleotide deletion in the p53 coding region (Isaacs W.B., Carter B.S., and Ewing C.M., *Cancer Res.*, 1991;51:4716-4720 and Borner M.B., Myers C.E., Sartor O., Sei Y., Toko T., Trepel J.B., and Schneider E., *Cancer Res.*, 1995;55:2122-2128). Only p53CP, not p53, showed binding (Figure 2B, lanes 2 and 3). The level of p53CP appears to be quite low in p53-/-MEF cells. Binding of p53CP can only be seen when T3SF, but not p53CON (good for p53 binding, Figure 1B, lane 9, and Bian J., Jacobs C., Wang Y., and Sun Y., *Carcinogenesis*, 1996;17:2559-2562), was used (compared lanes 1 and 2). Again, a fast migrating nonspecific binding band was visualized with T3SF oligo (lanes 2 and 3, compared to lanes 4-6 in Figure 1A). All these experiments confirmed that p53CP is not p53, but specifically binds to p53 consensus sequences.

P53 CP and p53 Have Different Binding Specificity in a p53 Binding Site Dependent Manner: In order for p53CP to compete with p53 *in vivo* efficiently, p53CP should have a comparable binding specificity to the p53 DNA binding sites as p53 does. We therefore examined binding specificity of p53CP and p53 using several p53 consensus sequences. We used oligonucleotides of p53 binding sites found in the genes encoding Waf-1 and Gadd45 as well as of T3SF by which we originally identified p53CP. The nuclear extract from H-Tx cells which contains high levels of both p53CP and p53 was used as a protein source. Since p53 binds

weakly to these oligonucleotides in the absence of p53-Ab and since p53 antibody did not influence the binding of p53CP (see Figure 1), we included p53 antibody in the assay. To determine specificity, we used increasing amounts of poly(dI/dC) as nonspecific competitor. As shown in Figure 3, binding of all three oligonucleotides to both p53CP and p53-Ab complex decreases as the amount of dI/dC increases. No significant binding difference between p53CP and p53-Ab can be seen for Waf binding site (lanes 1-5). However, some differential binding specificity revealed when Gadd and T3SF were used. Compared to p53CP, p53-Ab complex requires more poly(dI/dC) to compete its binding with Gadd, suggesting that p53-Ab complex binds to the Gadd site more specifically (lanes 6-10). On the other hand, when the T3SF site was used, p53CP requires more poly(dI/dC) for competition, suggesting a tighter binding (lanes 11-15). These results demonstrated a sequence dependent difference in binding specificity between p53CP and p53. It implies that some *in vivo* p53 target genes (such as Gadd45) may be subjected to differential regulation by p53 and p53CP, while others (such as Waf-1) may be regulated by the two proteins in a competitive way.

Like p53, p53 CP Binds to Both dsDNA and ssDNA, But With a Higher Preference for dsDNA: p53 has been shown previously to bind to both double stranded DNA (dsDNA) as well as single stranded DNA (ssDNA) (Bakalkin G., Yakovleva T., Selivanova G., et al., *Proc. Natl. Acad. Sci. USA.*, 1994;91:413-417 and Selivanova G. and Wiman K.G., *Adv. Cancer Res.* , 1995;66:143-180, and references therein). To determine whether p53CP also binds to ssDNA, we performed a gel retardation assay using a single stranded oligo T3SF.S1 (top strand) and T3SF.S2 (complementary/bottom strand) separately or in combination. To avoid interference with p53 binding, we increased poly(dI/dC) concentration to 50 μg/mL and excluded addition of p53 antibody. As shown in Figure 4, T3SF binds to p53CP (lane 1) which cannot be blocked by either single stranded T3SF.S1 or T3SF.S2 (lanes 2 and 3), but can be blocked by dsT3SF (lane 4). When T3SF.S1 was used as a labeled probe, p53CP binding was visualized as a fast-migrating band (lane 5), which can be blocked by cold T3SF.S1 (lane 6) or dsT3SF (lane 8). Interestingly, inclusion of cold T3SF.S2 caused the formation of

dsT3SF and a typical p53CP band was observed (lane 7). Similarly, when T3SF.S2 was used as a labeled probe, it binds to p53CP but little weaker (lane 9, compared to lane 5). The binding could be blocked by cold T3SF.S2 or T3SF (lanes 11 and 12). Again, addition of cold T3SF.S1 formed dsT3SF and a strong p53CP band revealed (lane 10). Moreover, we have labeled both T3SF.S1 and T3SF.S2 individually and added them to reaction mixture without pre-annealing. It was found that both strands annealled rapidly and bound to p53CP in a double stranded form (lane 13). The binding can only be blocked by ds T3SF (lane 16), not by either its single stranded oligo (lanes 14 and 15). The results clearly demonstrated that like p53, p53CP can bind both single and double stranded DNA with a high preference for dsDNA binding. These data point out an additional interesting feature of p53CP. If it functions like p53, it could be involved in both transactivation (dsDNA binding) and DNA repair (ssDNA binding). On the other hand, if it competes with p53, it may compete with p53 for both of these functions.

Mapping of the p53CP Binding Core Sequence: 14 Nucleotides Residing at the Center of p53 Binding Site: The minimal sequence required for p53 binding is a 20 mer consisting of PuPuPuC(A/T)(T/A)GPyPyPyPuPuPuC(A/T)(T/A) GPyPyPy (El-Deiry W.S., Kern S., Pietenpol J.A., Kinzler K.W., and Vogelstein B., *Nature Genet.*, 1992;1:45-49). To further define the biochemical features of p53CP, we performed a deletion/mutation mapping analysis to identify the minimal sequence requirement for p53CP binding. The oligonucleotides used in this assay were listed in Table 1. To keep the oligonucleotides at a length of 20 base, each deletion was compensated by the addition of a T, either in the 5′ end or the 3′ end. To increase the binding specificity and avoid possible interference from p53 binding, we have increased poly(dI/dC) concentration to 50 μg/mL. Under these conditions, p53 will not bind to T3SF (data not shown). Again, nuclear extract from H-Tx was used. As shown in Figure 5A, deletion of two purine residues from the 5′ end of the p53 site decreased p53CP binding (lane 3, T3SF1), and one additional purine deletion further decreased the binding (lane 5, T3SF2, and lane 9, T3SF4). Deletion of C at position 4 completely abolished

binding, indicating its importance (lane 7, T3SF3). Strong binding restored by the replacement of the first three purine residues by three pyrimidines (lane 11, T3SF5), indicating that the first three purine residues in the p53 DNA binding site are not required for p53CP binding. Deletion of the G at the position 7 or deletion of both the G at the position 7 and the C at position 4 from the 3′ end completely abolished p53CP binding (lanes 13 or 15, T3SF6, and T3SF7). All the p53CP binding can be blocked by cold T3SF (lanes 2, 4, 6, 10, 12), indicating that the binding is specific and the band does represent p53CP as we identified previously. We further examined the potential blockage of p53CP binding to T3SF by these deletion mutants. As shown in Figure 5B, a good correlation was found between binding to labeled oligonucleotide and unlabeled oligonucleotide competition. That is, the stronger is the binding, the better is the competition. The binding can be completely blocked by cold T3SF, partially blocked by T3SF5, slightly blocked by T3SF1, T3SF2, and T3SF4, and not at all by T3SF3, T3SF6, and T3SF7. The binding and competition data were summarized in Table 1. From these data, particularly T3SF5, we deduced that the minimal sequence requirement for the p53CP binding is a 14 oligonucleotide 5′-CTTGCTTGAACAGG-3′. We have named this core sequence as T3SF-core. The nucleotides C and G at the positions 1, 4, 11, and 14 (underlined) are critical for the binding. Substitution of the C to the G or vice versa at these positions will abolish the binding. We, however, did not perform a systematic alterations of each nucleotide (other than these four) to define other sequence requirement. This 14 oligonucleotides may represent a consensus sequence of 5′-C(A/T)(T/A)GPyPyPyPuPuPuC (A/T)(T/A)G-3′, which is the typical p53 DNA binding sequence with the deletions at the first three purines and the last three pyrimidines. It is worth noting that three non-specific nucleotides are needed in the 5′ end of this core sequence (perhaps at 3′ end also, not tested) to ensure a strong p53CP binding. The T3SF5 (three nucleotides) has a stronger binding than T3SF1 or T3SF4 (two nucleotides) than T3SF2 (one nucleotide) (compared lane 11 to lanes 3 and 9 and to lane 5). As expected, the binding of p53 to T3SF5 (the first three purines was replaced by three pyrimidines) is significantly reduced even in the presence of p53 antibody.

Names	Oligo Sequences		P53CP Binding	T3SF Competition
	1 4 7 1 4 7			
T3SF	AGGGCTTGCTTGAACAGGGTCT	SEQ ID NO. 3	+++	+++
T3SF1	TGCTTGCTTGAACAGGGTCT	SEQ ID NO. 15	++	++
T3SF2	TCTTGCTTGAACAGGGTCTT	SEQ ID NO. 16	+	+
T3SF3	TTTTGCTTGAACAGGGTTTT	SEQ ID NO. 17	-	-
T3SF4	TTCTTGCTTGAACAGGGTTT	SEQ ID NO. 18	+	+
T3SF5	TTTCTTGCTTGAACAGGTTT	SEQ ID NO. 19	+++	+++
T3SF6	TTTTCTTGCTTGAACAGTTTT	SEQ ID NO. 20	-	-
T3SF7	TTTTTCTTGCTTGAATTTTT	SEQ ID NO. 21	-	-

Arabic numerals labeled on the top of sequence indicate the position of each nucleotide in p53 consensus sequence. The two 10 nucleotide motifs are underlined and the critical C and G at the positions 4 and 7 are in bold.

Partial Purification of p53CP From Nuclear Extract: As an initial attempt to purify p53CP and to clone the encoding gene, we prepared a sequence-specific DNA affinity column using oligonucleotide concatamer (made of either T3SF or T3SF-Core oligo)-conjugated Sepharose 4B. Nuclear extract prepared from either H-Tx or p53-/-MEF cells was dialyzed to reduce the salt concentration and passed through the column. After washing, the proteins were eluted by an increasing concentration of salt. The presence of p53CP in collected fractions was monitored by a gel retardation assay using ^{32}P-T3SF or ^{32}P-T3SF5 oligonucleotide as labeled probes. As shown in Figure 6 (6A for H-Tx nuclear extract and 6B for p53-/-MEF), both DNA affinity chromatography resulted in elution of p53CP in the fractions of 0.3 M and 0.4 M salt. The weaker p53CP binding seen in Figure 6B reflected a lower abundance of p53CP in p53-/-MEF cells (Figure 2B). The results clearly demonstrate partial purification of p53CP through DNA affinity chromatography coupled with the gel retardation assay as a monitor.

A 40 kDa Nuclear Protein is p53CP or Its DNA Binding Component: It is well-known that p53 binds to its consensus sequence in a tetrameric form

(Jeffrey P.D., Gorina S., and Pavletich N.P., *Science*, 1995;267:1498-1502). We knew that the p53CP-DNA complex migrates faster than the p53-DNA complex in previous gel retardation assays. This suggested that the molecular weight of p53CP be smaller, provided that it also forms a tetramer with DNA. We performed a native PAGE analysis and found that p53CP-DNA complex had a similar migration to the catalase, a 240 kDa tetrameric brown protein (Sun Y., *Free Rad. Biol. Med.*, 1990;8:583-599) (data not shown). This estimation is, however, not accurate, since migration of a protein in a native PAGE was determined by molecular weight, charge, and shape. To determine the actual molecular weight of p53CP, we used three different approaches. First, we performed Southwestern analysis. The nuclear extracts from mouse H-Tx cells or human PC-3 (a prostate carcinoma line) was run in a SDS denaturing gel and transferred onto a nitrocellulose membrane. Proteins were refolded and incubated with ^{32}P-labeled T3SF. As shown in Figure 7A, a strong T3SF binding band with a MW of ~40 kDa was observed in both cell lines. Secondly, we labeled MEF cells from p53-/-mice with ^{35}S-methionine, prepared nuclear extract, and ran a gel retardation assay using ^{32}P-T3SF as the probe. We then cut out the band corresponding to p53CP-DNA complex and resolved the p53CP in a SDS gel. Again, as shown in Figure 7B, a major 40 kDa band was observed. Other minor bands could result from their co-migration with p53CP-oligo complex in the native gel, or they are part of p53CP protein complex. Thirdly, we performed a UV cross-linking assay and found a 46 kDa p53CP-DNA complex. As a positive control, partial purified p53 protein was included in the assay, and a 59 kDa protein-DNA complex was observed. These three independent determinations suggest that p53CP or its DNA binding component has a denatured molecular weight of about 40 kDa. We, however, cannot exclude the possibility that p53CP is a multicomponent protein with the 40 kDa protein being its DNA binding subunit and other bands shown in Figure 7 being the other members of this complex.

p53 Activation and p53CP Inactivation by Cell Cycle Blocking Reagents: Finally, we determined the potential co-regulation of the activities of p53 and p53CP. If cross-talk exists between these two proteins, they may be regulated in a coordinate way. Since p53 is actively involved in regulation of the various phases of the cell cycle (Ko L.J. and Prives C., *Genes & Develop.*, 1996;10:1054-1072 and references therein), we examined the effect of cell cycle blockers on DNA binding activity of p53 and p53CP. H-Tx cells (with a doubling time of about 10 hours) were synchronized by incubating with the following cell cycle blockers for 24 hours: (a) serum starvation for quiescence G0 phase; (b) lovastatin for G1 arrest; (c) aphidicolin and thymidine for S phase arrest; and (d) nocodazole for G2/M arrest. Nuclear extract was then prepared and subjected to gel retardation assay. We used p53CON and T3SF as binding oligonucleotides, since our previous experiments showed that they bound the best (among the oligonucleotides tested) to p53 and p53CP, respectively (Figure 1 and Bian J., Jacobs C., Wang Y., and Sun Y., *Carcinogenesis*, 1996;17:2559-2562). As shown in Figure 8A, treatment of H-Tx cells with cell cycle blockers induced p53 DNA binding activity (lanes 7-12). The highest activation was seen with aphidicolin treatment (lane 9). In contrast, cell cycle blocker treatment inactivated p53CP binding (lanes 1-6). A nearly complete inactivation was observed in aphidicolin-treated cells (lane 3). We have also treated cells with DNA damaging reagents (camptothecin and etoposide) and found activation of p53 binding and inactivation of p53CP binding (data not shown). To further examine whether p53CP inactivation by cell cycle blockers was p53-dependent, we performed the same treatment with PC-3 cells, a human prostate carcinoma line with a deletion mutation in the p53 gene (Isaacs W.B., Carter B.S., and Ewing C.M., *Cancer Res.*, 1991;51:4716-4720 and Borner M.B., Myers C.E., Sartor O., Sei Y., Toko T., Trepel J.B., and Schneider E., *Cancer Res.*, 1995;55:2122-2128). As shown in Figure 8B, in this p53 nonfunctional cell line, cell cycle blockers did not induce an inactivation of p53CP-DNA binding activity, they (except nocodazole), in contrast, activate p53CP binding to some extent. The result demonstrated that p53CP inactivation is dependent upon p53 activation. Overall, the data presented here highly suggest a coordinate regulation of p53 and p53CP in response to

external stimuli. It will be of great interest to identify the reagents which could activate p53CP activity and to see whether they would, at the same time, inactivate p53 activity.

We have identified a nuclear protein (p53CP) that is not p53, but has p53-like DNA binding activity. There are two alternative mechanisms by which p53CP may effect p53 functions. *First of all,* p53CP has p53-like functions and regulates a group of p53 target genes to which it has high binding specificity. This is supported by the fact that (a) p53 and p53CP showed differential binding specificity in a target sequence dependent manner; (b) both proteins bind dsDNA, as well as, ssDNA, and (c) for sequence-specific binding, p53CP only requires 14 bp motif, 5′-CTTGCTTGAACAGG-3′ (SEQ ID NO. 22) which located in the center of p53 consensus sequence. This suggested that p53CP may regulate both p53 responsive and nonresponsive genes. *Alternatively*, p53CP may compete with p53 for specific sequence DNA binding. This is supported by (a) the minimal sequence requirement for p53CP binding is a 14 bp motif, which centered in the typical p53 binding site, providing a physical basis for its potential competition; (b) there is coordinate regulation between p53CP and p53 binding activity upon external stimuli, which is p53 dependent. In the situations where p53 is activated, the binding of p53CP is dramatically decreased in p53 positive H-Tx cells, but not in p53 negative PC3 cells; and (c) it is noteworthy that determinations of the binding specificity were made based upon p53CP vs. p53-antibody complex rather than p53 alone due to the technical reason. The fact that p53 has very weak binding in the absence of p53 antibody may suggest that it can be easily competed away by p53CP.

Competition between two transcription factors for specific DNA binding site with biological consequence has been previously reported (Khachigian L.M., Lindner V., Williams A.J., and Collins T., *Science*, 1996;271:1427-1431). The binding site of Sp-1 and Egr-1 (early-growth-response gene) overlaps in the PDGF-B (platelet-derived growth factor B-chain) promoter. In unstimulated cells, Sp-1 occupies the binding site. During acute mechanical injury, Egr-1 expression increased, which displaced Sp-1 from the binding site in the PDGF-B promoter, and induced expression of PDGF-B (Khachigian, supra., 1996). The overlapping

binding site of Sp-1 and Egr-1 was also found in IL-2Rβ (interleukin-2 receptor-β chain) promoter. In this promoter, however, Sp-1 (responsible mainly for constitutive expression) and Egr-1 (for induced expression) cooperate physically and functionally to mediate maximal IL-2Rβ expression (Lin J.-X. and Leonard W.J., *Mol. Cell. Biol.*, 1997;17:3714-3722). Another case of transcription factor competition was recently reported in hepatitis B virus X promoter (Choi C.Y., Choi B.H., Park G.T., and Rho H.M., *J. Biol. Chem.*, 1997;272:16934-16939). The binding site for ATF2 (activating transcription factor 2) and AP-1 (activating protein 1) overlaps in the hepatitis B virus E element. The basal transcription mediated by AP-1 was inhibited by ATF2 through the competition for the AP-1 binding site as well as the formation of the ATF2-jun heterodimer (Choi C.Y., Choi B.H., Park G.T., and Rho H.M., *J. Biol. Chem.*, 1997;272:16934-16939).

p53 Has been involved in many cellular functions including growth arrest and apoptosis, tumor cell growth inhibition, genome guardian, differentiation, senescence, and angiogenesis (Ko L.J. and Prives C., *Genes & Develop.*, 1996;10:1054-1072; Levine A.J., *Cell*, 1997;88:323-331; Rotter V., Aloni-Grinstein R., Schwartz D., et al., *Semin. Cancer Biol.* 1994;5:229-236; Vojta P.J. and Barrett J.C., *Biochim. Biophys. Acta*, 1995;1242:29-41; Bouck N., *Biochim. Biophys. Acta*, 1996;1287:63-66; and references therein). Inactivation of p53 was found to be the most frequent genetic alterations in human cancers. About 50% of human cancers contain p53 mutations (Greenblatt M.S., Bennett W.P., Hollstein M., and Harris C.C., *Cancer Res.*, 1994;54:4855-4878). Competition with p53 for its specific DNA binding by p53CP, as proposed here, may provide a novel mechanism of p53 inactivation. Alteration or overexpression of p53CP may play an important role in the other 50% of human cancers harboring a wildtype p53. Inactivation of p53 by p53CP through the competition for DNA binding can render p53 mutation unnecessary in these cancers. Disruption of p53CP activity through therapeutic manipulation may therefore restore wildtype p53 function and reverse the tumor cell phenotype. Alternatively, p53CP may have p53-like functions and serves as a novel member of p53 family to negatively regulate cell

growth and proliferation. Indeed, Kaghad M., Bonnet H., Yang A., et al., (*Cell*, 1997;90:809-819) have recently reported the cloning of p73, a p53 homolog with 63% identity to p53 in DNA binding domain (Kaghad, supra., 1997). Although DNA binding of p73 to p53 consensus DNA binding sites was not examined, p73 did transactivate luciferase/CAT reporters driven by the p53 site-containing promoters as well as induce expression of endogenous p21, a well-known p53 target gene (Jost C.A., Marin M.C., and Kaelin Jr. W.G., *Nature*, 1997;389:191-194). Cloning of p73 indicates that like other tumor suppressor genes, there exists a family gene for p53 (Oren M., *Cell*, 1997;90:829-832). Apparent size difference between p73 and p53CP (a 40 kDa protein) suggests that p53CP could be the third member of p53 family.

CLAIMS

What is claimed is:

1. A protein having a molecular weight of about 40 kDa that specifically binds to the consensus p53 binding sites.

2. An isolated DNA fragment comprising the sequence GGGCTTGCTTGAACAGGGTC (SEQ ID NO. 8).

3. An isolated DNA fragment comprising the sequence CTTGCTTGAACAGG (SEQ ID NO. 10).

4. An isolated DNA fragment comprising the sequence GGGCTTGCTTGGGCTTGCTT (SEQ ID NO. 23).

SEQUENCE LISTING

<110> Warner-Lambert Company

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<151> 1997-11-17

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INTERNATIONAL SEARCH REPORT

Intern ial Application No
PCT/US 98/23992

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/11 C07K14/47 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95 17213 A (SLOAN KETTERING INST CANCER) 29 June 1995	1
A	see page 21 - page 26 see figures 1A and 1B ---	2-4
X	BIAN ET AL: "Characterisation of a putative p53 binding site in the promoter of the mouse tissue inhibitor of metalloproteinases-3 (TIMP-3) gene: TIMP-3 is not a p53 target gene" CARCINOGENESIS, vol. 17, no. 12, 1996, pages 2559-2562, XP002098171 cited in the application	2,3
A	see abstract see page 2560, right-hand column, paragraph 2 - page 2561, left-hand column, line 4 ---	1,4
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
29 March 1999	13/04/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Jansen, K-S

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INTERNATIONAL SEARCH REPORT

Inter ›nal Application No
PCT/US 98/23992

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	KAGHAD ET AL: "Monoallelically Expressed Gene Related to p53 at 1p36, a Region Frequently Deleted in Neuroblastoma and Other Human Cancers" CELL, vol. 90, 22 August 1997, pages 809-819, XP002098172 cited in the application see abstract ---	1
A	EL-DEIRY ET AL: "Definition of a consensus binding site for p53" NATURE GENETICS, vol. 1, 1992, pages 45-49, XP002098173 cited in the application ---	2-4
A	WO 96 01907 A (SQUIBB BRISTOL MYERS CO) 25 January 1996 see page 38, SEQ ID NO.:3 ---	4
P,X	BIAN AND SUN: "p53CP, a putative p53 competing protein that specificlly binds to the concensus p53 DNA binding sites: A third member of the p53 family?" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES USA, vol. 94, December 1997, pages 14753-14758, XP002098174 see the whole document -----	1-4

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

Intern ıal Application No
PCT/US 98/23992

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9517213 A	29-06-1995	AU 1440695 A	10-07-1995
WO 9601907 A	25-01-1996	US 5667987 A	16-09-1997
		EP 0804609 A	05-11-1997