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J Clin Invest. 1998;101(1):120-127. https://doi.org/10.1172/JCI1140.

Research Article

The clinical potential of the p53 tumor suppressor gene is being evaluated currently for gene therapy of cancer. We have built a variant of wild-type p53, chimeric tumor suppressor 1 (CTS1), in which we have replaced the domains that mediate its inactivation. CTS1 presents some very interesting properties: (a) enhanced transcriptional activity; (b) resistance to the inactivation by oncogenic forms of p53; (c) resistance to the inactivation by MDM2; (d) lower sensitivity to E6-induced degradation; (e) ability to suppress cell growth; and (f) faster induction of apoptosis. Thus, CTS1 is an improved tumor suppressor and an alternative for the treatment of wild-type p53-resistant human tumors by gene therapy.

CTS1: A p53-derived Chimeric Tumor Suppressor Gene with Enhanced In Vitro Apoptotic Properties

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Abstract

The clinical potential of the p53 tumor suppressor gene is being evaluated currently for gene therapy of cancer. We have built a variant of wild-type p53, chimeric tumor suppressor 1 (CTS1), in which we have replaced the domains that mediate its inactivation. CTS1 presents some very interesting properties: (*a*) enhanced transcriptional activity; (*b*) resistance to the inactivation by oncogenic forms of p53; (*c*) resistance to the inactivation by MDM2; (*d*) lower sensitivity to E6-induced degradation; (*e*) ability to suppress cell growth; and (*f*) faster induction of apoptosis. Thus, CTS1 is an improved tumor suppressor and an alternative for the treatment of wild-type p53–resistant human tumors by gene therapy. (*J. Clin. Invest.* 1998. 101:120–127.) Key words: p53 • mdm2 • apoptosis • cancer • gene therapy

Introduction

Numerous genetic alterations are involved in the tumorigenic process. Among these, *ras* and p53 constitute major susceptibility genes (35 and 50% of human cancers, respectively) (1, 2). Gene therapy has been considered for these two types of genetic defects, but, despite ongoing clinical trials with p53 (3, 4), we are still facing numerous challenges. In addition to the design of more efficient and specific vectors, the transgene to be expressed in the tumor may have to be modified for increased efficacy. Such is the case for wild-type p53 which is known to be inactivated in cells by several mechanisms. The most frequent targets for p53-based gene therapy are tumors in which one p53 allele is mutated (missense mutation) and the other one is deleted. The resulting p53 mutant is stabilized, adopting a conformation which in some instances has been shown to act as a neutralizing dominant negative molecule by heterooligomerization with wild-type p53 (5, 6). A similar situation can occur in a very large number of human cancers which overexpress HDM2 (7–9) which has been shown to inactivate p53 by binding to its NH_2-terminal domain (10–12) and inducing its degradation (13, 14). Lastly, the high-risk human papilloma viruses, which are clearly implicated in the pathogenesis of cervical cancers, produce the E6 protein, which stimulates the ubiquitinylation-mediated degradation of p53 (15, 16). Clinically efficient p53 molecules for gene therapy should ideally be resistant to inactivation and have reinforced apoptotic properties.

Wild-type p53 protein consists of five domains, each involved in a specific function of the protein. First, the NH_2 terminus (amino acids 1–73) interacts with the transcription machinery (17, 18) (e.g., TBP) and is the target for neutralizing viral or cellular proteins such as MDM2, EBNA5, or E1B (10–12, 19, 20). Second, the core domain (amino acids 91–306) mediates sequence-specific DNA-binding (21–23) and is the major target for point mutation in human cancers (2, 24). Third, the major nuclear localization signal (NLS;[1] amino acids 315–325) is necessary for the translocation of p53 to the nucleus. Fourth, the oligomerization domain (amino acids 326–355) adopts a loop/β-sheet/loop/α-helix structure (25–27), that mediates tetramerization and neutralization by interactions with some p53 mutants (5, 6). This region has been shown recently to be responsible for the physical association with the adenovirus E4orf6 protein leading to its inactivation (28, 29). Fifth, the COOH terminus (amino acids 356–393) negatively regulates the sequence-specific DNA-binding activity of p53 (30). Here we describe a p53 analogue, chimeric tumor suppressor 1 (CTS1), in which the NH_2- and COOH-terminal domains have been substituted with heterologous sequences. CTS1 binds DNA as efficiently as wild-type p53, although it is dimeric in solution. It retains the ability to act as a transcriptional transactivator and as a cell growth suppressor in contexts where wild-type p53 is nonfunctional, and it induces both growth arrest and apoptosis. This synthetic tumor suppressor gene should not only increase the efficacy of gene therapy but also should expand the range of cancers amenable to treatment.

Methods

Construction of CTS1. The p53 core domain (amino acids 75–325) was amplified by PCR, using a SalI containing 5′ primer and a BamHI containing 3′ primer: 5′-AAGCTTGGGCCGGGTCGACCTGCACCAGCAGCTCCT-3′, 5′-CGGATCCCCATCCAGTGGTTTCTT-3′. The leucine-zipper (with a STOP codon containing 3′-end) was obtained by hybridization of six 5′-end phosphorylated synthetic oligonucleotides in order to create a double-strand DNA sequence that contains the nucleotide sequence of the leucine-zipper flanked with a BglII-cohesive extremity at 5′-end and with a NotI-cohesive extremity at 3′-end: 5′-GATCTGAAGGCCCTCAAGGAGAAGCTGAAGGCC-3′, 5′-CTGGAGGAGAAGCTGAAGGCCCTGGAGGAGAAGCTG-3′, 5′-AAGGCACTAGTGGGGGAGCGATGATGAATCGATATCGC-3′, 5′-GGCCGCGATATCGATT-

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Received for publication 8 July 1997 and accepted in revised form 29 October 1997.

J. Clin. Invest.

0021-9738/98/01/0120/08 $2.00
Volume 101, Number 1, January 1998, 120–127
http://www.jci.org

1. *Abbreviations used in this paper:* CAT, chloramphenicol acetyl transferase; CTS1, chimeric tumor suppressor 1; NLS, nuclear localization signal; RE, responsive element.

CATCATCGCTCCCCCAC-3′, 5′-TAGTGCCTTCAGCTTCTCCT-CCAGGGCCTTCAGCTT-3′, 5′-CTCCTCCAGGGCCTTCAGCTT-CTCCTTGAGGGCCTTCA-3′. The resulting amino acid sequence of the leucine-zipper is: D L K A L K E K L K A L E E K L K A L E E K L K A L V G E R. The hybridization product was ligated to the 3′-end of the p53 core domain using the BamHI site. The VP16 activation domain (amino acids 412–490) was amplified by PCR, using a Kozak sequence containing 5′ primer and a SalI containing 3′ primer: 5′-AAGCTTGAATTCGTTAACGCCACCATGGCCAC-GGCCCCCCCGACC-3′, 5′-GGTCGACCACCGTACTCGTCAAT-3′. It was ligated to the 5′-end of the [p53 core domain/leucine-zipper] fusion using the SalI site.

Gel retardation assay. Duplex substrate was prepared and end-labeled as described (30) from two complementary oligonucleotides containing *waf1* promoter–derived 20 mer p53 DNA-binding site (31) with BamHI/HindIII-compatible ends: 5′-GATCC*GAACATGT-CCCAACATGTTG*A-3′, 5′-AGCTT*CAACATGTTGGGACATGG-TC*G-3′. A 25-μl reaction volume containing DNA-binding buffer (20 mM Tris-HCl, pH 7.5, 0.1 M NaCl, 5 mM $MgCl_2$, 0.05 mM $ZnCl_2$, 5 mM dithiothreitol, 0.1 mg/ml BSA, 10% glycerol, 1% NP-40, 2 μg/ml aprotinin, 2 μg/ml E-64, 2 μg/ml leupeptin, and 2 μg/ml pepstatin) was mixed with end-labeled duplex substrate (2.4×10^{-9} M), 1.2×10^{-6} M AP2 (Promega Corp., Madison, WI) as DNA competitor and 30 ng purified wild-type p53 or histidine-tagged CTS1 in the presence or absence of 300 ng PAb421 monoclonal antibody. After a 30-min incubation on ice, reaction products were analyzed by electrophoresis as described (30). Sequences of the complementary oligonucleotides leading to a mutated *waf1* promoter–derived 20 mer p53 DNA-binding site are: 5′-GATCC*GAA**T**ATA**T**ATCCCAA**T**ATA**T**TTG*A-3′, 5′-AGCTT*CAA**T**ATA**T**TTGGGA**T**ATA**T**TTC*G-3′. Wild-type p53 was purified from baculovirus-infected Sf9 cells by affinity chromatography on agarose-coupled PAb421 (32). Histidine-tagged CTS1 was purified from baculovirus-infected Sf9 cells by chromatography on nickel chelate agarose (QIAGEN Inc., Chatsworth, CA). Recombinant baculovirus for expression of histidine-tagged CTS1 was generated by using the Baculogold kit (PharMingen, San Diego, CA) after insertion of CTS1 cDNA into the baculovirus transfer vector pAcHLT-A (PharMingen).

Plasmids. p53 and CTS1 cDNAs were cloned in the pcDNA3 vector (Invitrogen Corp., San Diego, CA) between the HindIII and NotI sites. The responsive element (RE)-chloramphenicol acetyl transferase (CAT) vector was constructed by inserting a single consensus p53 response element sequence (33) upstream from a minimal *tk* promoter driving the CAT gene. The MDM2 expression vector was obtained from B. Wasylyk (34).

Cell culture and transfection. All cell lines were maintained in medium supplemented with 10% heat-inactivated FCS and grown at 37°C. The SAOS-2, EB, HCT116, H322, and HeLa cell lines were maintained in DME and H358a and OsA-CL cell lines in RPMI 1640. For transfection, 3×10^5 cells were seeded per 6-cm plate and transfected with 3 μg of plasmid or plasmid mixtures preincubated for 30 min with 10 μl lipofectAMINE reagent (GIBCO BRL, Gaithersburg, MD).

Western blot analysis. Cells (10^6) were lysed for 15 min at 4°C with 100 μl of RIPA buffer and centrifuged at 15,000 rpm for 15 min. The supernatants were collected, loaded onto 10 or 14% SDS-PAGE, and blotted onto PVDF membranes (New England Nuclear, Boston, MA). WAF1 and HDM2 were detected with rabbit polyclonal antibodies together with an anti–rabbit, horseradish peroxidase–conjugated secondary antibody and p53 and CTS1 were detected with the PAb240 monoclonal antibody together with an anti–mouse, horseradish peroxidase–conjugated secondary antibody. Proteins were visualized by chemiluminescent detection (ECL) (Amersham Corp., Arlington Heights, IL).

CAT assay. Cells were collected 48 h after transfection, rinsed twice in PBS, then resuspended in 200 μl 0.25 M Tris-HCl, pH 8, lysed by freezing three times in dry ice/ethanol, clarified by centrifugation, and the soluble material was assayed for CAT activity by incubation for 1 h at 37°C in 0.18 M Tris-HCl, pH 8, 0.4 mM acetyl-coenzyme A, 23 μM chloramphenicol,D-threo-(dichloroacetyl-1,2-^{14}C) (200 nCi) (Amersham Corp.). CAT activity was quantified by counting the acetylated chloramphenicol forms in an Instantimager (Packard Instruments, Meriden, CT).

Selection of drug-resistant colonies. 48 h after transfection, cells were seeded in 10-cm plates, cultured in 400 μg/ml G418 containing medium for 2–3 wk, and then colored with fuchsin.

E6-induced degradation. p53, CTS1, and E6 proteins were in vitro translated (final volume: 40 μl) using the TNT Coupled Reticulocyte Lysate System (Promega Corp.) with (p53 and CTS1) or without (E6) 44 μCi [^{35}S]methionine (1,175 Ci/mmol) (Amersham Corp.). Subsequently, 2 μl of p53 and CTS1 lysates were incubated for various times at 30°C with 10 μl reticulocyte lysate and with or without 2 μl of E6. The reaction was stopped by adding Laemmli loading buffer (35) (vol/vol). Samples were then loaded onto 10% SDS-PAGE and p53 and CTS1 were quantified with an Instantimager (Packard Instruments).

Apoptosis detection. Cells (10^6) were induced for various times with 200 μM $ZnCl_2$, and analyzed for apoptosis either by FACS® or by DNA-ladder analysis. For FACS® analysis, cells were fixed and permeabilized for 40 min with 1 ml Permeafix (Ortho Diagnostic Systems Inc., Westwood, MA), washed twice with PBS (GIBCO BRL), incubated for 1 h at 25°C with 100 μl PBS, 2% BSA (PBS-BSA), and 1 μg PAb240, washed twice with PBS-BSA, incubated for 1 h at 25°C with 100 μl PBS-BSA and 1 μg GAM-FITC (Immunotech, Marseilles, France), washed twice in PBS-BSA, and incubated for 1 h at 25°C with 1 ml PBS-BSA, 5 μg propidium iodide, and 1 mg RNase (DNase-free) before FACS® analysis. For DNA-ladder analysis, cells were treated as described previously (36).

Results

Construction of CTS1. CTS1 (GenBank accession No. AF021816) was constructed by fusions of the VP16 activation domain (amino acids 412–490), and of an optimized leucine-zipper domain (see Methods), respectively, at the amino and carboxy termini of the p53 core domain (amino acids 75–325) (Fig. 1). Based on the available crystallographic data of the p53 COOH terminus (25–27), the leucine-zipper domain fusion places the leucine residues facing each other for efficient dimerization. These fusions were carried out according to standard PCR-based molecular biology techniques. The CTS1 construct was characterized biochemically and functionally as described in the following paragraphs.

CTS1 binds specifically to the p53 response DNA element as a dimer. Gel retardation assays were performed with wild-type p53 and CTS1 expressed from baculovirus (Fig. 2) and a synthetic oligonucleotide corresponding to the p53 RE found in the *waf1* promoter (31). The CTS1 retarded band (lane *6*) migrated faster than the wild-type p53 band (lane *2*), consistent with CTS1 binding as a dimer. As expected, in contrast to p53 (lane *3*), it was not supershifted by the addition of the PAb421 monoclonal antibody (30) (lane *7*), which recognizes a p53 COOH-terminal epitope absent in CTS1. CTS1 bound as efficiently as wild-type p53 and did not heterooligomerize with wild-type p53 as shown by incubations with equal amounts of the two proteins (lanes *10* and *11*). CTS1 is displaced by a four-fold excess of p53 (lane *12*), and this displacement is enhanced when p53 is activated by PAb421 (lane *13*). Finally, as with wild-type p53, the CTS1 retarded band disappeared when an excess of unlabeled competitor RE was added (lanes *4* and *8*), an effect not observed with a DNA element bearing point mutations known to be critical for p53-binding (lanes *5* and *9*)

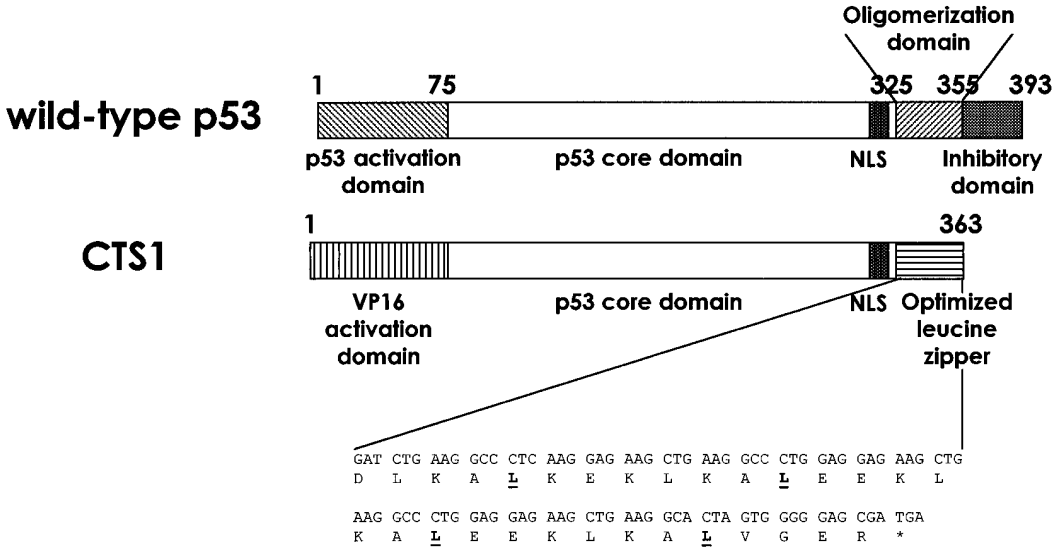


Figure 1. Schematic comparison of CTS1 and wild-type p53. The p53 core domain (amino acids 75–325) containing an NLS is common to both proteins. In CTS1, VP16 activation domain has replaced p53 NH_2-terminal activation domain, while a dimerization leucine-zipper domain has substituted the p53 oligomerization and inhibitory domains. The sequence of the leucine-zipper is shown.

(37). Taken together, these data establish that CTS1 has kept the same binding specificity as p53.

Gel permeation chromatography on TSK 3000 gel filtration column (Bio-Rad Laboratories, Richmond, CA) gave apparent molecular weights of ~ 500,000 for purified tetrameric p53 known to be an unusually shaped tetramer (38) and ~ 200,000 for CTS1, confirming that CTS1 is dimeric in solution (data not shown).

CTS1 has enhanced transcriptional activity. We derived from the EB colon carcinoma cells (p53−/−) a clone called EB-CTS1, which expresses CTS1 under the control of the MT-1–inducible metallothionein promoter. It resembles EB-1, which expresses wild-type p53 from the same promoter (39). As shown by Western blot analysis of both cell lines after 200 μM Zn^{2+} induction (Fig. 3 *A*), the time courses of expression of p53 and CTS1 were very similar, as were those of HDM2 and WAF1, two known p53 responsive gene products (31, 40).

The transcriptional activity of CTS1 was also tested in transient transfection assays in SAOS-2 and H358a tumor cell lines which do not express endogenous p53 (3, 41). The reporter has the CAT gene downstream from a promoter containing a consensus p53 RE (33). CTS1 activated transcription more efficiently than wild-type p53 in both cell lines (Fig. 3 *B*). Moreover, when this activity was tested in a dose-dependent manner (SAOS-2), transactivation by wild-type saturated at low doses, whereas it continued to increase with CTS1. These effects were not merely due to more efficient expression of CTS1 compared with wild-type p53 as shown by Western blotting (Fig. 3 *B*, *inset*). Similar results were obtained with the natural *mdm2*, *waf1*, *bax*, and *cyclin G* promoters (data not shown).

CTS1 retains wild-type p53 growth suppressor activity. We examined the growth suppression capacity of CTS1 by selecting drug-resistant colonies after transfection of various tumor-derived cell lines and nontransformed cells (NIH3T3) (Table I). Both CTS1 and wild-type p53 inhibited tumor cell growth and had a very minimal effect on nontransformed cells, as demonstrated previously for wild-type p53 (4). pcDNA3 plasmids encoding CTS1 or p53 were microinjected at several concentrations (0.1–1 mg/ml) into normal human dermal fibroblasts (Clonetics Corp., San Diego, CA). Overexpression of both proteins was demonstrated by PAb240 immunofluorescence. DAPI staining revealed no apoptotic cells among microinjected cells and no detectable cell death was observed during 72 h.

CTS1 is insensitive to the dominant negative effect of mutant p53. p53 mutants can exert a dominant negative effect on the transcriptional activity of wild-type p53 through the formation of heterooligomers in which they induce a conformation switch on the wild-type protein (5, 6). CTS1, unlike wild-type p53, was insensitive to inactivation by the H175 mutant (Fig.

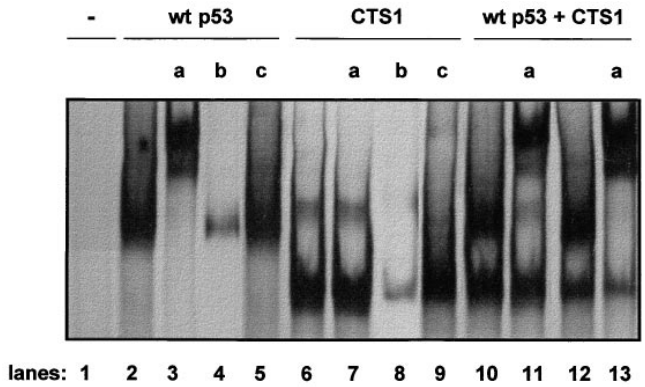


Figure 2. DNA-binding of wild-type p53 and CTS1 to a RE derived from the *waf1* promoter. Autoradiography of gel electrophoresis of purified recombinant proteins incubated as described in Methods with ^{33}P-end-labeled *waf1* oligonucleotide: no protein: lane *1*; p53: lanes *2–5*; CTS1: lanes *6–9*; 30 ng p53 + 30 ng CTS1: lanes *10* and *11*; 120 ng p53 + 30 ng CTS1: lanes *12* and *13*. *a*, 300 ng PAb421 was added to the incubation mixtures. *b*, 50×10^{-9} M cold *waf1* oligonucleotide (×20 excess) was added to the incubation mixtures. *c*, 50×10^{-9} M mutated cold *waf1* oligonucleotide (×20 excess) described in the Methods section was added to the incubation mixtures.

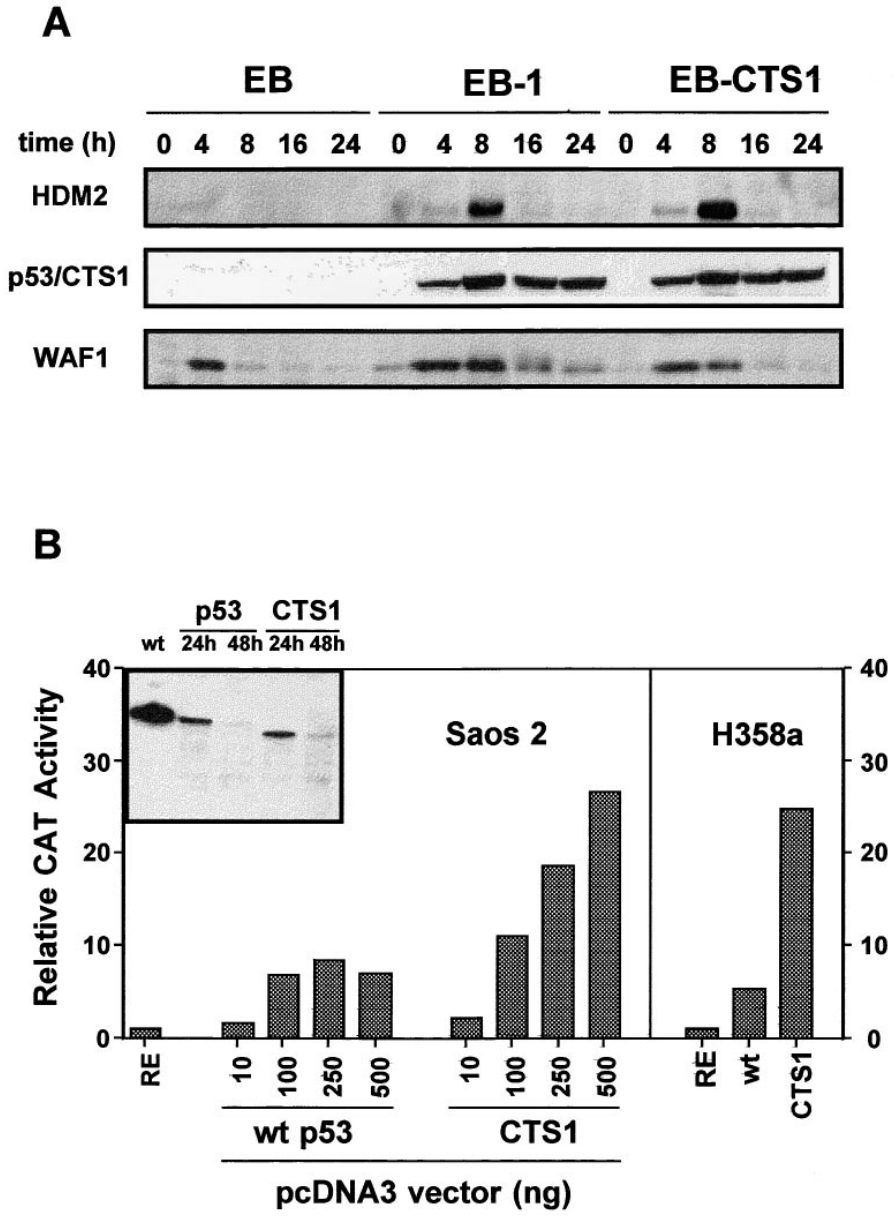


Figure 3. Activation of transcription by wild-type p53 and CTS1. (*A*) EB, EB-1, and EB-CTS1 cells (10^6) were treated with 200 μM $ZnCl_2$ for various times and HDM2, WAF1, wild-type p53, and CTS1 were detected by Western blotting (14% SDS-PAGE). (*B*) Cells (3.5×10^5) were cotransfected with 500 ng RE-CAT reporter and various quantities of p53 or CTS1 expression vectors and activation of transcription was quantified by CAT assay. The amount of expression vectors varied in SAOS-2 cells whereas a single dose (50 ng) was used in H358a cells. Wild-type p53 and CTS1 were detected by Western blotting (10% SDS-PAGE).

Table I. Effect of Wild-Type p53 and CTS1 on Cell Growth

Cell	p53 status	Vector	Wild-Type p53	CTS1
Tumor cell lines				
SAOS-2	(−/−)	134	5	7
HCT116	(wt/wt)	112	8	9
H322	(−/L248)	93	5	4
H460	(wt/wt)	153	15	16
H358a	(−/−)	53	2	1
EB	(−/−)	37	5	7
Nontransformed cell line				
NIH 3T3	(wt/wt)	172	128	135

Cells (3×10^5) were transfected with 3 μg of DNA (pcDNA3 vector) and selected with G418 after transfection as described in Methods. These data are the results of a duplicate experiment representative of three different ones. p53 status is according to previous data (3, 39, 41).

4). Surprisingly, it was activated by H175 in a dose-dependent manner possibly due to the titration of a negative regulator.

CTS1 is less sensitive to E6-induced degradation. Since HPV16 and HPV18 E6 proteins inhibit wild-type p53 (42) by the induction of ubiquitin-dependent p53 degradation (15, 16), CTS1 was tested for its susceptibility to E6-mediated degradation. Transactivation by CTS1, in contrast to wild-type p53, was insensitive to E6 inactivation (Fig. 5 *A*). In HeLa cells that express endogenous E6, CTS1 exhibited dose-dependent transcriptional activation, in contrast to wild-type p53 (data not shown). The susceptibility of CTS1 to E6-mediated degradation was tested in vitro. CTS1 was clearly less sensitive to E6-induced degradation than wild-type p53, which would explain the transactivation data (Fig. 5, *B* and *C*).

CTS1 is not inhibited by MDM2. MDM2 inhibits the transcriptional (10–12) and apoptotic (43, 44) activities of wild-type p53 through interactions with its NH_2 terminus and subse-

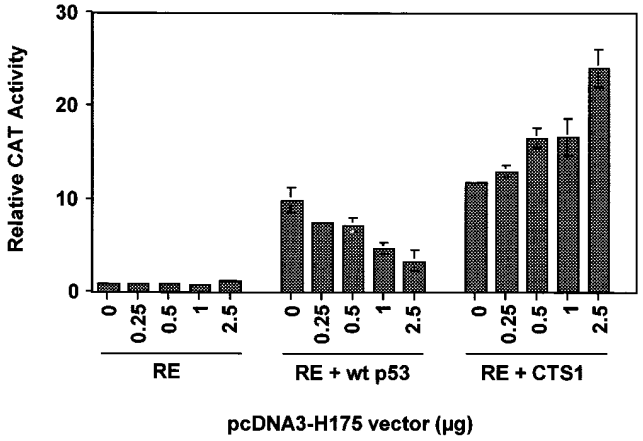


Figure 4. Dominant negative effect of p53 mutant (H175) on wild-type p53 and CTS1. SAOS-2 cells (3.5×10^5) were cotransfected with 500 ng RE-CAT reporter, 50 ng of p53 or CTS1 expression vectors, and various quantities of the p53 mutant (H175) expression vector and activation of transcription was quantified by CAT assay.

quent degradation (13, 14). We tested the susceptibility of CTS1 to inhibition by MDM2 (Fig. 6). Transactivation by CTS1 was insensitive to inactivation by MDM2, in contrast to wild-type p53 (Fig. 6 *A*). Growth suppressor activity was measured with the G418-resistant colony formation assay in OsA-CL cells, a human osteosarcoma cell line that overexpresses HDM2 (7, 8). CTS1 exhibited strong suppressed growth whereas wild-type p53 was inactive (Fig. 6 *B*).

CTS1 induces apoptosis. We investigated the possibility that CTS1 inhibited cell growth by inducing apoptosis. FACS® analysis was used to detect sub-G_1 cells undergoing apoptosis (43). The EB-1 and EB-CTS1 clones that express either wild-type p53 or CTS1 under the control of the inducible metallothionein promoter were induced for various times and double-labeled with propidium iodide and PAb240 (45) to monitor DNA content and p53 expression, respectively (Fig. 7). Apoptotic cells, with a high content of either p53 or CTS1, appeared after 16 h (Fig. 7 *A*, region *a*). A quantitative analysis revealed that CTS1 induced apoptosis faster and more efficiently than p53 (Fig. 7 *A*, compare regions *a* and *b*, and Fig. 7 *B*). With a different assay, DNA-ladder analysis, CTS1 was also found to be more efficient than wild-type p53 at inducing apoptosis (Fig. 7 *C*).

Discussion

CTS1 has several essential features that differ from wild-type p53. Although both are specifically toxic for tumor cells, CTS1 is resistant to MDM2 inactivation and to the dominant negative effects of p53 mutants, has reduced sensitivity to E6-induced degradation, inhibits cell growth of p53-insensitive tumor cell lines, and is more efficient at inducing apoptosis in tumor cells.

CTS1 was designed by exchanging the domains of p53 involved in various inactivation mechanisms (e.g., interaction with cellular or viral proteins or degradation) with heterologous sequences. Various p53 modifications have been reported previously. The NH_2 terminus has been replaced already by the VP16 activation domain (amino acids 409–490), fused to

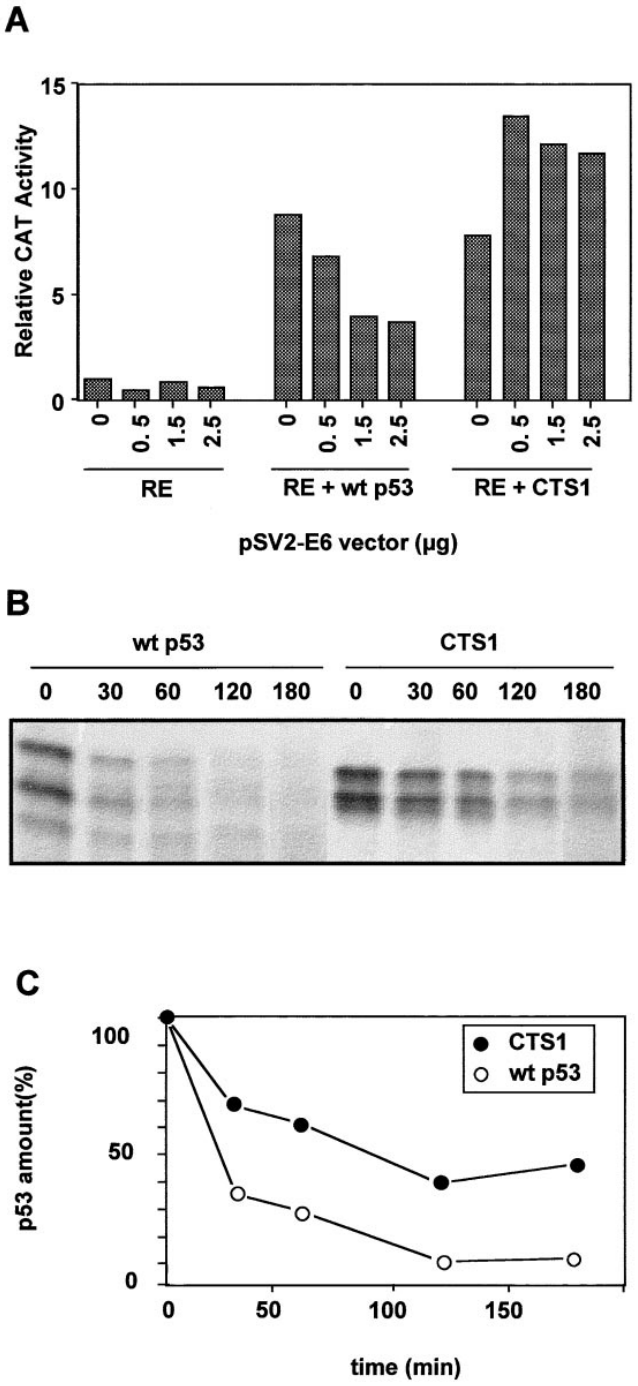
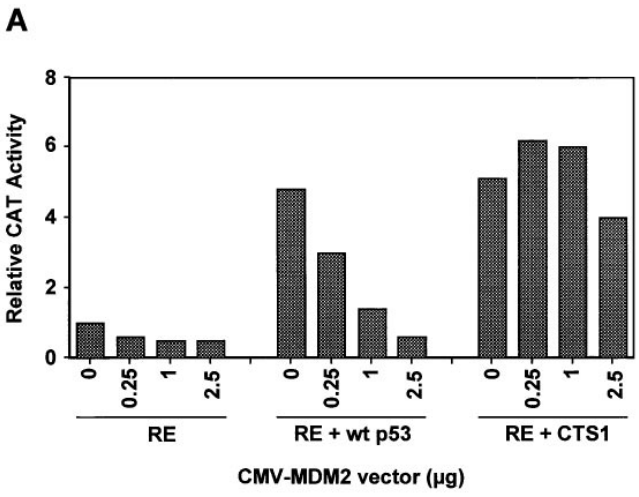


Figure 5. E6-induced inactivation and degradation of wild-type p53 and CTS1. (*A*) SAOS-2 cells (3.5×10^5) were cotransfected with 500 ng RE-CAT reporter, 50 ng of p53 or CTS1 expression vectors, and various quantities of the E6 expression vector and activation of transcription was quantified by CAT assay. (*B*) In vitro translated p53 and CTS1 ^{35}S-labeled proteins were incubated for various times with in vitro translated E6, and analyzed by SDS-PAGE. In control incubations without in vitro translated E6, no detectable degradation of both p53 and CTS1 was observed (data not shown). (*C*) Quantification of relative p53 and CTS1 amounts (*B*) with an Instantimager (Packard Instruments).



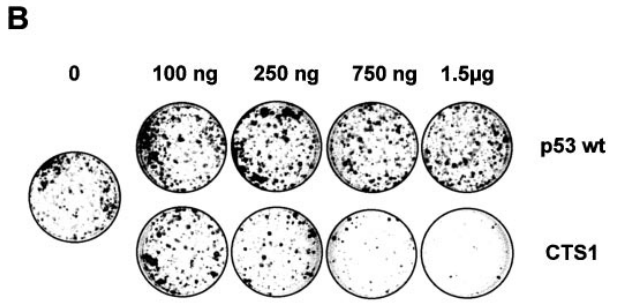
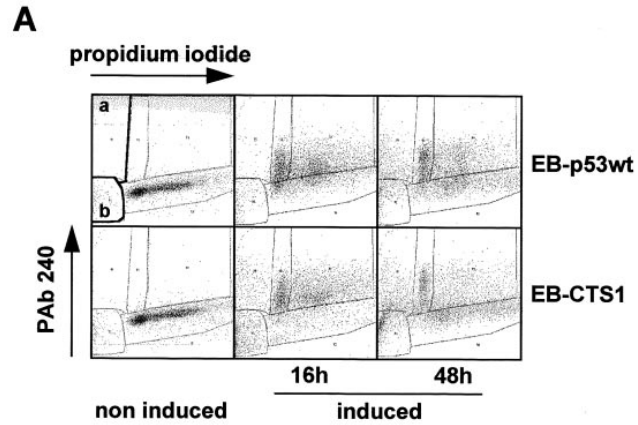
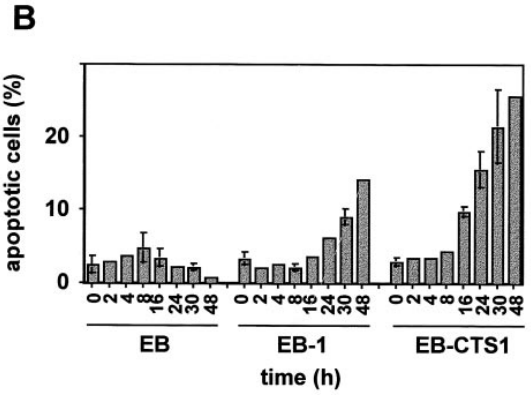


Figure 6. Effect of MDM2 on wild-type p53 and CTS1. (*A*) SAOS-2 cells (3.5×10^5) were cotransfected with 500 ng RE-CAT reporter, 50 ng of p53 and CTS1 expression vectors, and various quantities of the MDM2 expression vector and activation of transcription was quantified by CAT assay. (*B*) OsA-CL cells (3.5×10^5) were transfected with various quantities of p53 and CTS1 expression vectors and selected on G418 medium before fuchsin coloration.

amino acid 83 or 100 of p53 (46). The position 83 fusion still mediated specific transcription activation, G_1 arrest, growth suppression, and induction of apoptosis, but to a lesser extent than wild-type p53 (46, 47). The position 100 fusion lacked all of the p53-associated tumor suppressor properties (46, 47). Substitution of the p53 COOH terminus (amino acids 343–393) by the GCN4 coiled–coil domain, also led to a less efficient tumor suppressor (46–48). Finally, the combination of these two modifications (VP16 fusion to amino acid 83 and COOH-terminal domain swapping by GCN4) was even less efficient than singly substituted chimeras (46, 47), contrary to CTS1. This discrepancy is presumably due to the choices of fusion of both heterologous domains and/or to differences in the structure of the oligomerization domain. The COOH-terminal substitution is very different from the other constructs, both in position and type of substitution. In CTS1, the leucine-zipper was fused to position 325 which corresponds to the beginning of the β-sheet of the oligomerization domain (25–27). This fusion position may be important for the enhanced activity of CTS1, since fusions at positions 336 and 343 yielded less active proteins (data not shown). The leucine-zipper domain used in CTS1 has been designed to form dimers only. Unlike previously described p53 dimers reported to be "weak tumor suppressors" (48), CTS1 behaves as efficiently as wild-type p53 in specific DNA-binding and cell growth inhibition assays. These results strongly suggest that the dimeric state is sufficient for some of the ma-





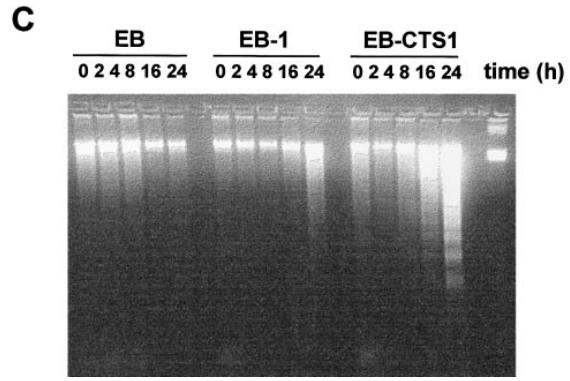


Figure 7. Induction of apoptosis by wild-type p53 and CTS1. Cells (10^6) were induced for various times with 200 µM $ZnCl_2$ and analyzed as described in Methods. (*A*) FACS® analysis of EB-1 and EB-CTS1 treated cells. The *y*-axis (PAb240) corresponds to p53 and CTS1 expression. The *x*-axis (propidium iodide) corresponds to DNA content. The sub-G_1 population is in region ($a + b$). (*B*) Kinetics of apoptosis detected by FACS® analysis (sub-G_1 population) (average of two independent experiments). (*C*) Kinetics of apoptosis detected by DNA-ladder analysis.

jor functions associated with p53, namely, transcription activation, cell growth inhibition, and induction of apoptosis.

This study clearly demonstrates that CTS1 is at least as efficient as wild-type p53 in cellular backgrounds where the natural protein is active, but that it is also efficient in cellular contexts where wild-type p53 is inactive (e.g., OsA-CL). Overexpression of HDM2 is clinically relevant in human cancers, such as sarcomas (7, 8) and bronchogenic carcinomas (9). These types of human cancers are potential targets for CTS1-based gene therapy. CTS1 has all the properties of a viable alternative to wild-type p53, for tumors in which it is likely to be inactive, expanding the range of tumors amenable to gene therapy.

In addition, recent data indicate that the adenovirus E4orf6 protein can inhibit p53-dependent transcriptional activation and apoptosis (28, 29). This suggests that a recombinant adenoviral vector deleted from the E4 region would be a more appropriate vector for p53 gene therapy. However, ΔE4 adenoviruses are still associated with lower expression levels of the therapeutic gene they would carry, due to a lower number of templates (49). CTS1 should be insensitive to E4orf6, as the p53 domain mediating this interaction (amino acids 318–360) is almost entirely deleted in the chimeric construct. Thus, CTS1 may prove advantageous for adenoviral mediated gene transfer (e.g., by reducing the therapeutic dose to be administered to the patient).

Acknowledgments

We gratefully acknowledge Dr. Bohdan Wasylyk for fruitful discussions and critical reading of the manuscript, Dr. Mike Jaye for critical reading of the manuscript, Dr. Phil Shaw and Dr. Roland Sahli for providing us with EB and EB-1 cells and the plasmid pmlMT1-i for expression of proteins under the control of the MT-1 metallothionein inducible promoter, Dr. Isabelle Rey-Delumeau for expertise in microinjection, and Christine Dureuil and Marie-Noëlle Mary for expert technical assistance.

This project was funded by the Bioavenir Program (Ministère de la Recherche et de l'Industrie).

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