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► **To cite this version:**

Ascensión Cuesta, Alberto Zambrano, María Royo, Angel Pascual. The tumor suppressor p53 regulates the expression of the beta-amyloid precursor protein. *Biochemical Journal*, 2009, 418 (3), pp.643-650. 10.1042/BJ20081793 . hal-00479095

HAL Id: hal-00479095

<https://hal.science/hal-00479095>

Submitted on 30 Apr 2010

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The tumor suppressor p53 regulates the expression of the beta-amyloid precursor protein

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Running Title:

p53 regulates APP gene expression

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Abstract

The expression of the β -amyloid precursor protein (APP), which plays a key role in the development of Alzheimer's disease, is regulated by a variety of cellular mediators in a cell-dependent manner. In the present study, we present evidence that p53 regulates the expression of the APP gene in neuroblastoma cells. Transient expression of ectopic p53, as well as the activation of endogenous p53 by the DNA-damaging drug camptothecin or MDM2 depletion, decrease intracellular levels of APP in murine N2a β neuroblastoma cells. This effect was also observed in primary cultures of rat neurons as well as in SH-SY5Y cells, a human neuroblastoma cell line. Transient transfection studies using plasmids which contain progressive deletions of the 5' region of the gene, demonstrate that p53 represses APP promoter activity through a mechanism that is mediated by DNA sequences located downstream of the transcription start site (+55/+101). Accordingly, expression of a dominant negative p53 mutant significantly increases the transcriptional activity of the APP promoter. In addition, data obtained in gel mobility shift assays show that p53 does not bind to the +55/+101 APP region, although reduces binding of the transcription factor Sp1. Reduction of Sp1 binding after activation of p53 with camptothecin was also observed in chromatin immunoprecipitation assays. All together our results strongly suggest a mechanism by which p53 precludes binding of Sp1 to DNA, and therefore the stimulation of the APP promoter by this transcription factor.

INTRODUCTION

Alzheimer's disease is a degenerative disorder of the central nervous system which causes mental deterioration and progressive dementia, and is accompanied by neuropathologic lesions including the presence of senile plaques of which the β -amyloid protein ($A\beta$), a hydrophobic 39-43 residues amino acid peptide, is the major component [1, 2]. The β -amyloid protein is proteolytically derived from a set of alternatively spliced β -amyloid precursor proteins (APP), which are encoded by a single gene located on human chromosome 21. APP plays a central role in Alzheimer's disease, and it has been suggested that an increase in the production of this protein might actively contribute to the development of this pathology [3, 4], likely by inducing the synthesis and deposition of β -amyloid [4, 5], and the subsequent neurotoxicity [3]. In contrast, it has been also reported that physiological APP levels appear to be involved in neurotrophic events [1], thus protecting cells against neurodegeneration. APP is ubiquitously expressed in mammalian tissues, and its expression has been proved to be regulated by a variety of cellular mediators, including growth factors [6, 7], phorbol esters [8, 9] or ligands of the superfamily of steroid/thyroid nuclear hormone receptors [10, 11].

On the other hand, it has been described that p53 levels are significantly higher in Alzheimer's brains than in aged-matched controls [12], and that p53-associated apoptosis may be directly involved in the development of this pathology. Thus, β -amyloid deposits are associated with local neuritic degeneration, and it has been suggested that intracellular β -amyloid would be selectively cytotoxic to human neurons through a p53-associated cell death pathway [13]. In addition, intracellular β -amyloid may directly activate the p53 promoter and the apoptosis process induced by this factor [14], and a correlation between amyloid accumulation and p53 activation in neurons has been also previously described [15].

Physiological levels of APP protect neuronal cells against degeneration induced by several apoptosis-controlling factors including p53, an effect that has been proposed to involve the control of p53-mediated gene transactivation [16]. Furthermore, it has been more recently reported that AICD, a secretase-derived β -amyloid intracellular c-terminal domain, controls p53 at a transcriptional level, in vitro and in vivo [17, 18].

In the present study we present evidence that ectopic expression of p53 or activation of endogenous p53 by treatment with camptothecin, or MDM2 depletion, regulate APP expression, and specifically decrease the intracellular levels of APP in N2a β cells, a subclone of the mouse N2a neuroblastoma cell line that has been previously used to analyze the effect of thyroid hormones on APP [10]. The effect of p53 on APP expression appears to be mediated at least in part at the transcriptional level, since transient transfection studies demonstrate that p53 inhibits APP promoter activity. This inhibition requires DNA sequences located within the first exon of the APP gene, between positions +55 and +101, a region that nevertheless does not bind p53. In contrast, this fragment contains sequences that bind Sp1, and p53 appears to inhibit binding of this factor. Taken together, our results suggest a novel mechanism by which the tumor suppressor

p53 might decrease APP gene expression by interfering with the activation induced by the transcription factor Sp1, which binds sequences of DNA located downstream the transcriptional initiation site.

MATERIALS AND METHODS

Chemicals and antibodies

Dulbecco's modified Eagle's medium (DMEM) was purchased from Biowhittaker (Verviers, Belgium). Fetal bovine serum was from Gibco.Life technologies Ltd. (Paislay, UK). Polyclonal antibody 369A against the cytoplasmic domain of APP [19] was a generous gift of Dr. Samuel E. Gandy (Mount Sinai School of Medicine, N.York). Polyclonal antibody against phospho-p53 was from Cell Signaling Technology, Inc (USA). Anti β -catenin antibody was from BD Biosciences (Belgium). The second biotinylated anti-rabbit antibody and the peroxidase-conjugated streptavidine used in Western blot analysis were from Amersham International plc (England). Camptothecin was from Sigma-Aldrich (USA). siRNA against mRNA encoding Mdm2 was obtained from Dharmacon Research (Lafayette, CO). Sequence analyses and primer design were performed on-line by using the nucleotide sequence databases provided by the EMBL (<http://www.ebi.ac.uk/embl>).

Cell culture

Murine N2a β neuroblastoma cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum as previously described [20, 21]. Human SH-SY5Y neuroblastoma cells were cultured in RPMI medium containing 10% fetal bovine serum. Primary cultures of rat cortical neurons were kindly prepared by Dr. Diaz-Guerra's group as previously described [22]. Briefly, the plates were treated with poly-L-lysine (100 μ g/ml) and laminin (4 μ g/ml) overnight at 37 °C before seeding. Cerebral cortices from 18-day-old rat embryos were dissected and mechanically dissociated in culture medium (Eagle's minimum medium supplemented with 28.5 mM NaHCO₃, 22.2 mM glucose, 0.1 mM glutamine, 5% fetal bovine serum, and 5% donor horse serum). The cells were seeded at a density of 0.3X10⁵ cells/cm² in the same medium.

Reporter plasmids and expression vectors

The chloramphenicol acetyl transferase (CAT) reporter plasmid containing the -1099/+101 fragment of the human APP gene has been previously described [10, 23]. Progressive 5' deletions to -487, -307, -15 bp were prepared by polymerase chain reaction from the original -1099/+105 bp fragment, kindly provided by Dr. Lahiri's laboratory, and subcloned into the BamH1 site of pBL-CAT8, a plasmid which lacks the AP-1 binding site present in the pUC backbone. The reporter construct +55/+101 consists of a single copy of this APP fragment inserted in front of a tk promoter driving the expression of the CAT gene [10]. Wild-type and mutant (V143A) human p53 plasmids, respectively, utilize a pCMV-Neo-Bam or a T7 polymerase-transcribed pBluescript vector [24].

DNA transfection

For transient transfection assays cells were incubated with expression vectors for wild-type or mutated p53, or an empty vector, together with chloramphenicol acetyl transferase (CAT) reporter plasmids [10]. Cells were transfected with Lipofectamine 2000 following the instructions of the manufacturer (Invitrogen, Carlsbad, CA), and one hundred nanograms of a luciferase reference vector were simultaneously used as an internal control of the transfection efficiency. Transfected cells were then incubated for an additional 48 hours period. Assays were performed in duplicate cultures that normally showed less than 5-15% variation in CAT activity which was determined by incubation of [¹⁴C]-chloramphenicol with cell lysate protein. After autoradiography, the non-acetylated and acetylated [¹⁴C]-chloramphenicol were quantified and the data expressed as the mean \pm standard deviation (SD) of the percent of acetylated forms after each treatment. Each experiment was repeated at least 2-3 times with similar relative differences in regulated expression.

Western blot analysis

Cells transiently transfected 48 hours earlier with the p53 expression vector, or treated for six hours with 10 μ M camptothecin, were washed twice with PBS and cellular proteins extracted by lysis with a buffer (150 mM NaCl, 50 mM Tris pH8, 2 mM EDTA, 1% Triton, 0.1% SDS) containing the protease inhibitors PMSF (1 mM) and leupeptin (10 μ g/ml). The protein content of cells was determined by using the BCA assay (Pierce, Illinois), following the manufacturer's instructions. Equal amounts, 40 μ g, of cell extracts were then electrophoresed in an 8% SDS-polyacrylamide gel and transferred to an immobilon polyvinylidene difluoride membrane. Non specific binding was blocked with 5% nonfat dried milk in TBS-T (Tris buffered saline, 0.1% Tween 20) for 2-3 h at room temperature and the cellular APP detected with a 1/1,500 dilution of the rabbit polyclonal antibody 369A, raised against the carboxi-terminal domain of APP. After 1 hour incubation at room temperature, the membrane was washed and incubated with a second biotinylated anti-rabbit antibody (1/2,000) for one additional hour, washed again and finally incubated for 1 hour with 1/2,000 peroxidase-conjugated streptavidine. All incubations took place at room temperature, and detection by enhanced chemiluminescence (ECL, Amersham International plc, England) was carried out according to manufacturer's indications. The loading control β -catenin was detected with a 1/1500 dilution of a specific antibody obtained from BD Biosciences. Phospho-p53 was detected by using a 1/1000 dilution of the corresponding antibody and a 1/5000 dilutions of a second biotinylated anti-rabbit antibody.

The apparent molecular mass (kDa) of the detected bands was always determined by using a wide range protein standard (Mark 12 from Novex.-S. Diego, CA).

Quantitative real-time PCR assays.

Total RNA was extracted using Tri Reagent (Sigma) and mRNA levels analyzed by quantitative RT-PCR. RT was performed with 2 μ g of RNA following

specifications of SuperScript™ First-Strand Synthesis System (Invitrogen Life Technologies). PCR reactions were performed using a MX3005P instrument (Stratagene) and detected with Sybr Green. Data analysis was done using the comparative CT method and data were corrected with the GAPDH mRNA levels, which were not affected by any of the treatments used. The following PCR primers were used: APP, 5'-GGCCCTCGAGAATTACATCA-3', and 5'-GTTTCATGCGCTCGTAGATCA-3'); GAPDH, 5'-ACAGTCCATGCCATCACTGCC-3', and 5'-GCCTGCTTACCACCTTCTTG-3').

Small Interfering RNA (siRNA).

Mdm2 (mouse double minute 2) expression was lowered using siRNA targeting mouse MDM2 mRNA. MDM2 siRNA and a control non-targeting siRNA (L-041098-00 and D-001206-13, respectively) were purchased from Dharmacon (Lafayette, CO). Cells were seeded in six well plates and the siRNAs were transfected into cells using Lipofectamine 2000. RNA was extracted after 72 hours and APP-mRNA levels were then analyzed by using quantitative RT-PCR.

Electrophoretic mobility shift assays

Synthetic oligonucleotides containing the +55 to +101 sequence of the human APP promoter, or a consensus p53 binding site [25] were end-labeled with [³²P]ATP using T4-polynucleotide kinase and then incubated with either nuclear extracts obtained from p53-transfected cells or with *in vitro* translated p53 and/or recombinant Sp1 proteins. Nuclear extracts, obtained by the method of Andrews and Faller [26], or p53 and Sp1, were incubated on ice for 15 min in a buffer (20 mM Tris HCl pH=7.5, 75 mM KCl, 1mM DTT, 5 µg/ml BSA, 13% glycerol) containing 3 µg poly (dl-dC), and then for 15-20 min at room temperature with approximately 70,000 cpm of the double-stranded labeled oligonucleotide.

For competition experiments, a 25 or 50 excess of unlabeled probe, the oligonucleotide containing the consensus sequence for p53 binding or an unrelated DNA were added together with the nuclear extracts to the binding reaction mixture.

For gel retardation assays with p53 and Sp1, cDNA for p53 was transcribed and translated *in vitro* with the TNT kit (Promega) following the manufacturer's recommendations, and the Sp1 recombinant protein obtained from Promega (USA). In these assays an unprogrammed reticulocyte lysate was used as a control for nonspecific binding.

DNA-protein complexes were resolved on 7% nondenaturing polyacrylamide gels containing 0,5% TBE buffer, and the gels were dried and autoradiographed at -70°C.

Chromatin immunoprecipitation (ChIP) and PCR amplification

For ChIP assays, cells were fixed with formaldehyde lysed and subsequently sonicated. Twenty 30 seconds bursts of sonication were applied with 30 seconds pauses to avoid overheating. Chromatin was precipitated with 2 µg of antibodies against Sp1 (Santa Cruz Biotechnology, Inc. USA), p53 (Cell Signaling Technology), or a control IgG, purified and subjected to PCR analysis. Primers used to detect binding to the -99 to +213 region of the APP gene were as follows:

5'-AGGCTCCGCTAGGGGTCTCTG-3' (forward) and 5'-
CCTCCAGAGCCCGAACCGTCC-3' (reverse).

Statistics

Unless indicated otherwise, all the data points are the mean of two independent experiments performed in duplicate. Significance of the differences was calculated with the Student's test, and is indicated in the figures with an asterisk: * ($p < 0.05$)

RESULTS

Effects of p53 on cell-associated APP isoforms

Cells were transiently transfected with an expression vector for p53, or with a control noncoding vector. Forty eight hours later, the cell-associated APP isoforms were detected by Western blot using the polyclonal antibody 369A. Previous to that, we first confirmed that transfection with the expression vector for p53 is actually followed by an increase in the activation of this transcription factor. As shown in figure 1A, the levels of phosphorylated-p53 (P-p53) were significantly higher in lysates obtained from p53-transfected cells. In addition, transient expression of p53 induces a reproducible decrease in the levels of intracellular APP. As previously reported [10, 27-29], the various bands observed in the western blots represent the mature and immature forms of the three major APP isoforms, APP₇₇₀, APP₇₅₁ and APP₆₉₅, which result by alternative splicing from the APP gene. The faster migrating bands representing immature APP and the slower migrating the mature isoforms. A densitometric quantification of the total bands is illustrated in the panel B of the figure and clearly evidenced that p53 significantly reduces the total level of APP isoforms.

Depletion of MDM2 decreases APP gene expression

The inhibitory effect of the tumor suppressor p53 on APP gene expression was also analyzed in cells depleted of MDM2, the major negative regulator of p53 in cells. MDM2 acts as an E3 ligase to ubiquitinate p53 and drive its proteasomal degradation. Thus, knock-down of MDM2 should be followed by a significant increase of cellular p53, and therefore by a reduced expression of APP in the cells. To prove this, cells were depleted of MDM2 by means of siRNA. Seventy-two hours after transfection, RNA was extracted and MDM2- and APP-mRNA levels were analyzed by using quantitative RT-PCR. Results are shown in figure 2. As expected, MDM2-mRNA levels were drastically reduced (panel A), p53 was accumulated in the cells (panel B), and APP-mRNA levels were significantly reduced (panel C) in cells exposed to 30 nM MDM2-siRNA, thus confirming the inhibitory effect of p53 on APP expression.

Activation of p53 by camptothecin reduces APP expression

To further confirm that p53 activation reduces APP expression, we also analyzed the influence of the topoisomerase-1 inhibitor camptothecin, a DNA-damaging agent that induces a strong accumulation and activation of p53 [31, 32], on APP

levels. Cells were incubated for 6 hours in the presence or in the absence of 10 μ M camptothecin, and the levels of activated (phosphorylated)-p53, APP and β -catenin (as a loading control) were determined by Western blot. The results obtained are illustrated in figure 3. As expected, camptothecin induced a strong activation of endogenous p53 that was accompanied by a marked decrease in the levels of intracellular APP, an effect that was even more evident than that induced by p53 transfection (figure 1). Additionally, the effect of camptothecin on APP mRNA levels was also analyzed by quantitative real time PCR. APP mRNA was significantly reduced in camptothecin treated cells, thus confirming that activation of p53 indeed leads to a reduced expression of the APP gene.

Moreover, to confirm whether or not these results obtained in N2a cells, a tumor cell line of murine origin, can be replicated in primary cultures and also in samples of human origin, we have analyzed the effect of camptothecin in primary cultures of rat cortical neurons, as well as in SH-SY5Y human neuroblastoma cells. As shown in figure 4, the results were in both cases very similar to those obtained in N2a cells, thus discarding a cell type specific response.

p53-mediated repression of APP promoter activity

Transient transfection assays were carried out to determine whether or not activation of p53 affects the transcriptional activity of the APP gene in neuroblastoma cells. N2a β cells were transiently cotransfected with a chimeric plasmid containing the -1099 to +101 bp fragment of the APP gene linked to the CAT reporter gene, and an expression vector for p53, a dominant negative p53 mutant, or an empty noncoding vector. Cells were collected 48 hours later, and CAT activity was determined. As shown in figure 5, reporter activity was significantly inhibited in p53-transfected cells and increased in those cells transiently expressing the dominant negative p53 mutant, thus demonstrating that p53 indeed represses the transcriptional activity of the APP gene.

Identification of the DNA region mediating the repression of APP promoter activity by p53

To map the DNA sequences involved in the p53-induced response, progressively deleted fragments (-1099, -487, -307, -15 and +55) of the APP promoter were linked to the upstream region of the CAT gene and transfected into N2a β cells. As shown in panel A of figure 6, the negative effect of p53 was maintained even in cells transfected with the shortest (+55/+101 bp) fragment, which only contains sequences located in the first exon of the gene. In addition, we also analyzed the effect of p53 in cells cotransfected with a -15 to +101 promoter fragment either wild-type or mutated in positions 81 to 84 (AACT instead of GGGC) or 86 to 90 (AACTC instead of GAGCA) which correspond to the proposed p53 responsive region. As shown in panel B of the figure the inhibitory effect of p53 was observed in cells expressing the wild-type fragment but not in cells transfected with the mutant constructs. All together these results suggest the existence of a p53 response element located downstream of the transcription start site of the gene. To further confirm that sequences located within the +55 to +101 bp fragment mediate the effect of p53 on the APP promoter activity, the cells were transiently cotransfected with the +55 to +101 bp fragment of the APP gene and

an expression vector for p53, a p53 dominant negative mutant, or an empty noncoding vector. As shown in figure 7, CAT activity determined 48 hours later was significantly inhibited in p53-transfected cells and increased in those cells transiently expressing the dominant negative mutant, thus demonstrating that this DNA fragment indeed mediates the repression of APP promoter activity by p53. Of interest, a computer-assisted study revealed that the +55/+101 responsive fragment that, as previously reported contains a Sp1-binding site [21], also contains a sequence that largely resembles a half-site of the consensus core sequence established for the p53 binding site [25, 30].

p53 interferes with binding of Sp1 to the +55 to +101 bp fragment of the APP gene

Despite the results presented in the previous figures, this fragment was unable to bind p53 as proved by gel mobility shift assays performed with a radiolabeled probe containing the +55 to +101 responsive sequence of the APP gene and nuclear extracts obtained from p53-transfected cells or *in vitro* translated p53. Unexpectedly, no bands compatible with p53-containing mobility complexes were detected in any of the samples analyzed. As shown in figure 8A, the retarded bands obtained in a representative assay carried out with nuclear extracts were specifically competed by an excess of unlabelled probe. However, they were not displaced by competition with an unrelated oligonucleotide or in the presence of an oligonucleotide containing the consensus p53 binding site 5'-AGCTTGAACATGTCCCAACATGGTGA-3' [25, 30]. Furthermore, as illustrated in figure 8B, p53 binds very efficiently to the oligonucleotide containing the consensus p53 binding site. However, no specific bands compatible with p53-containing complexes were detected when the radiolabeled +55/+101 probe was exposed to *in vitro* translated p53, whereas as expected [21] a specific retarded band running in a position compatible with a Sp1-containing complex was detected in assays performed with recombinant Sp1. Remarkably, although p53 does not bind to this fragment, it was very effective decreasing the binding of Sp1 to this region of the APP gene. The intensity of that band was clearly reduced in the presence of p53, thus suggesting a mechanism in which p53 decreases the accessibility of Sp1 to DNA, and consequently the constitutive stimulation of the APP promoter induced by this factor. In addition, it should be mentioned that the mobility of the Sp1-associated band was not altered in the presence of p53, thus discarding "in vitro" binding of a p53-Sp1 complex to the Sp1-binding site.

The effect of p53 on "in vivo" binding of Sp1 to the APP gene was analyzed in ChIP assays performed in cells treated with camptothecin to activate endogenous p53. As shown in figure 8C, binding of p53, which was essentially undetectable in control cells, was increased in camptothecin-treated cells. In addition, binding of Sp1 to the APP responsive region was markedly decreased after camptothecin-induced p53 activation, confirming the reduction of Sp1 binding observed in the *in vitro* assays.

DISCUSSION

In this report we present evidence that activation of the tumor suppressor p53 decreases the intracellular APP levels in a murine neuroblastoma cell line. Transient expression of p53 results in a reliable increase in the cell content of total and phosphorylated p53 that in turn generates a decrease in the intracellular levels of APP isoforms. This effect of p53 on APP was confirmed in cells depleted of MDM2, a negative regulator of p53, and also in cells exposed to the DNA-damaging agent camptothecin, which activates the endogenous p53. Furthermore, the inhibitory effect of p53 activation on APP expression has been proved to occur not only in murine N2a β cells, but also in neuronal primary cultures and in neuroblastoma cells of human origin, thus discarding a potential cell type specific response. Our results together with the increase of p53 levels observed in Alzheimer brain [12, 33] suggest a role of this protein in Alzheimer's disease.

The reduction of intracellular levels that follows the p53 activation could be secondary to a p53-mediated reduction of the transcriptional activity of the APP gene, and this possibility has been actually proved in transient transfection assays performed with a CAT reporter plasmid containing the -1099/+101 fragment of the human APP gene. Transient expression of p53 significantly reduces CAT activity, while expression of a dominant negative mutant of p53 increases activity above that observed in control cells.

Additional assays carried out with pBL-CAT plasmids containing progressive deletions of the APP promoter evidenced a mechanism of p53 repression mediated by DNA sequences located downstream of the transcription initiation site, in the +55/+101 region of the APP gene. Surprisingly, this region was unable to bind p53 suggesting that, as reported by other repressed promoters lacking a p53 consensus binding site [34], the repressive effect of this protein could be mediated by a functional interaction with other specific DNA-binding factor/s that stimulate the promoter through those sequences. At this respect, we have previously reported that the basal transcription factor Sp1 can stimulate APP promoter activity by binding to sequences located in the region of the gene responsible for the effect of p53 [21], and the existence of a physical interaction between p53 and Sp1 has been also demonstrated [35, 36]. The tumor suppressor p53 appears to cooperate with Sp1 in the activation of several promoters, such as p21, hTERT or IGF-IR [37-39]. However, it has not been yet clarified how that interaction may contribute to control promoter activity. As previously reported Sp1-p53 complexes could bind to Sp1-recognition sites in the promoters of different genes [40]. Nevertheless, it has been also reported that interaction of both transcription factors may result in a reduced ability of Sp1 to bind to DNA [41].

Our results demonstrate that although p53 does not bind directly to the +55/+101 region of the APP gene, indeed decreases the binding of Sp1 to that region of DNA, thus suggesting a mechanism involving a functional interaction between the tumor suppressor p53 and the basal transcription factor Sp1. Moreover, immunoprecipitation of fragmented chromatin with an anti-p53 antibody reflects the presence of this factor in the protein complex associated to the responsive APP region. Therefore, p53 may affect expression of APP by

interacting with Sp1 and/or other unidentified factor, which would mediate its recruitment to chromatin.

All together these results open a new and interesting link between a neurodegenerative pathology and the tumor suppressor p53. Data obtained from *in vivo* and *in vitro* models have previously demonstrated that p53 plays an important role in the neuronal death induced by the β -amyloid protein in Alzheimer's disease brains. It has been described that $A\beta_{1-42}$ activates the p53 promoter, thus inducing p53-dependent apoptosis and neurotoxicity [13-15]. On the other hand, the β -amyloid precursor APP appears to play a dual function in this pathology since it can prevent neuronal apoptosis by inhibiting p53 activation [16], but it can also contribute to neurodegeneration if overexpressed [3, 4]. In the present work we demonstrate that p53 activation represses APP expression and that therefore this tumor suppressor could also play two different and opposite functions in the neurodegenerative processes that characterize the Alzheimer's disease. According to our results, p53, a transcription factor that induces neuronal apoptosis could also play a positive role in preventing Alzheimer's disease, since it can reduce APP expression and therefore the production and accumulation of $A\beta_{1-42}$, the most neurotoxic isoform of the β -amyloid protein [13].

In summary, our results suggest that in addition to the previously reported function of p53 in the $A\beta$ -induced neurotoxicity, this protein could also reduce the $A\beta_{1-42}$ generation by decreasing APP gene expression. These functions very likely may depend on the cellular levels of p53, and it is evident that new experiments should be performed to fully understand the role of this protein in Alzheimer's disease and the precise mechanisms that mediate the repression of APP by p53.

Acknowledgments

This study has been supported by grants SAF2003-01646 and SAF2006-05577 from the Spanish Comision Interministerial de Ciencia y Tecnologia. We thank Prof. Diaz- Guerra by the preparation of the primary culture of cortical neurons.

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LEGEND TO THE FIGURES

Fig. 1 Western blot analysis of phosphorylated-p53 and cell-associated APP isoforms.

Cell lysates obtained from N2a cells transfected with an expression vector encoding p53 (Trans), or with an empty noncoding vector (C), were analyzed for p53 activation (phosphorylation) and for cell-associated APP proteins. A) Representative results obtained in Western blots performed with the specific antibodies mentioned under Experimental Procedures. β -catenin was used as a loading control. B). Densitometric quantification of the intracellular APP bands. Data are the average \pm SD from two separate experiments performed in duplicate and are expressed relative to the corresponding control values.

Fig. 2 Depletion of MDM2 decreases APP gene expression.

MDM2 mRNA (panel A), phosphorylated-p53 (panel B) and APP mRNA levels (panel C) were analyzed in control untransfected cells (C), and in cells transfected with a control siRNA (siC) or with siRNA for MDM2 (siMDM2). Data represent the mean \pm SD from two independent experiments performed in duplicate. The inset in panel B shows a representative blot of phosphorylated p53 (P-p53)

Fig. 3 DNA damage induces activation of p53 and decreases APP expression in N2a neuroblastoma cells.

Activated (phosphorylated)-p53 and cell-associated levels of APP, as well as the levels of APP mRNA, were analyzed in protein or RNA extracted from cells treated for six hours with or without 10 μ M camptothecin (CPT). Panel A shows representative blots of phosphorylated-p53, β -catenin, used as a loading control, and cell-associated APP. The quantitation of the APP bands is illustrated in panel B. Panel C shows an estimation of APP mRNA levels as determined from two micrograms of total RNA by quantitative real time PCR. Data in panels B and C represent the mean \pm SD from two independent experiments performed in duplicate.

Fig. 4 Activation of p53 inhibits APP expression in primary cultures of cortical neurons and in human neuroblastoma cells.

p53 phosphorylation and cell-associated levels of APP were analyzed in protein extracts obtained from cells treated with or without 10 μ M camptothecin (CPT). Representative blots obtained from primary neuronal cultures and SH-SY5Y cells are illustrated in the upper panel. The densitometric quantification of the intracellular APP bands shown in the lower panel represents the mean \pm SD from two independent experiments performed in duplicate.

Fig. 5 Negative regulation of APP promoter activity by p53.

CAT activity was determined in N2a β cells transiently cotransfected with a pBL-CAT plasmid containing, or not, the -1099 to +101 bp fragment of the APP gene promoter and an expression vector encoding wild type p53, a p53 dominant

negative mutant or the empty non coding vector. Data, expressed as % of CAT activity obtained in control cells transfected with the -1099/+101 fragment, are the mean \pm SD activities obtained from two independent assays performed in duplicate.

Fig. 6 Identification of DNA sequences that mediate the negative effect of p53 on the APP gene promoter.

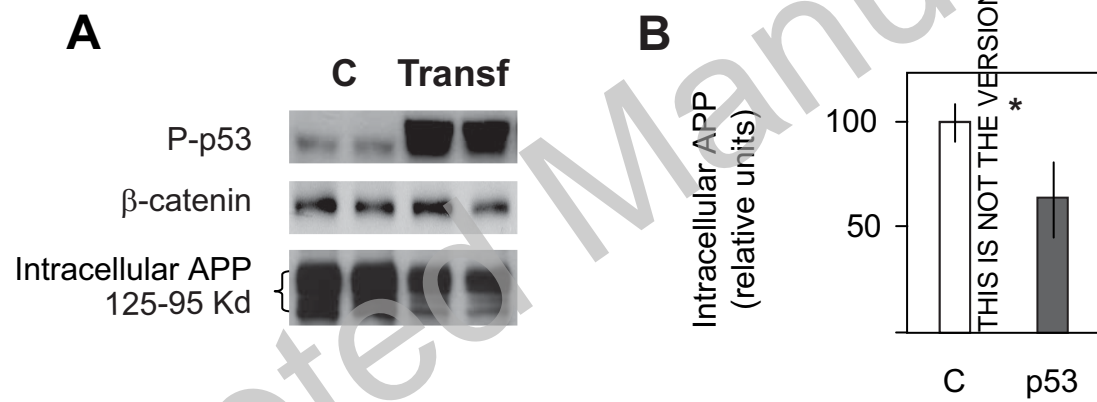
A) N2a β cells were transiently transfected with pBL-CAT plasmids containing progressive deletions of the APP promoter. CAT activity was determined 48 hours after these plasmids were cotransfected with an expression vector encoding the tumor suppressor p53, or the empty noncoding vector. B) Cells were transiently transfected with a pBL-CAT plasmid containing the wild type -15/+101 fragment of the APP promoter, or two mutants in positions +81 to +84 (mut 1) or +86 to +90 (mut 2). Data are expressed relative to CAT activity obtained with the largest -1099/+101 (panel A), or the wild type -15/+101 (panel B) fragments, and are the mean \pm SD of CAT activities obtained from three separate experiments performed in duplicate.

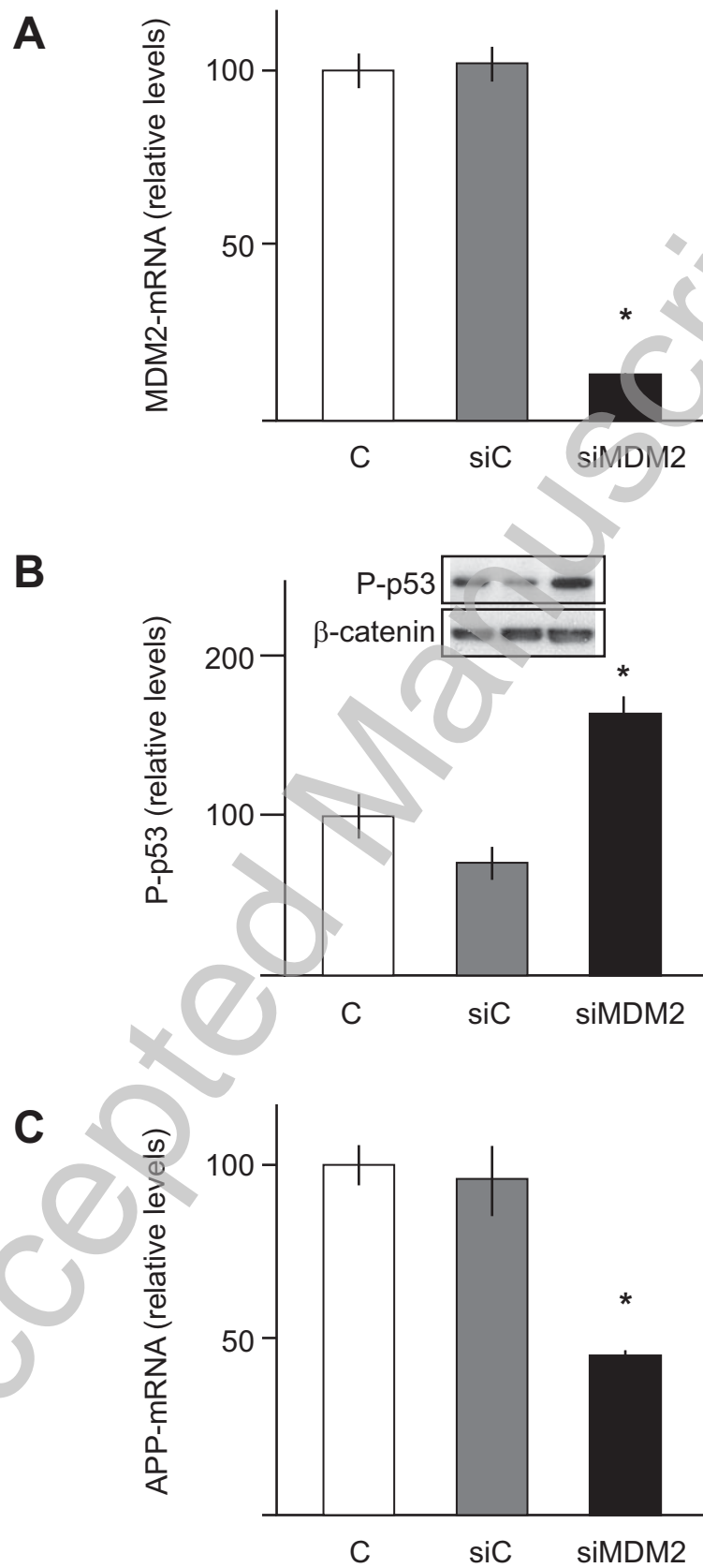
Fig. 7 The +55 to +101 bp fragment contains DNA sequences that mediate the effect of p53 on the APP promoter.

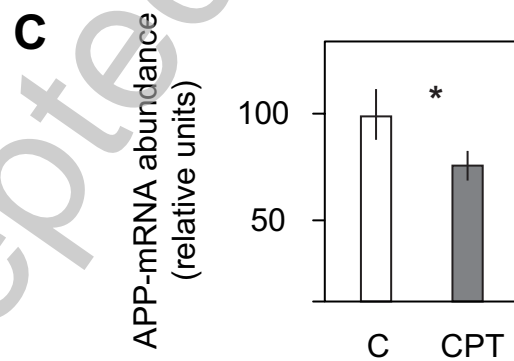
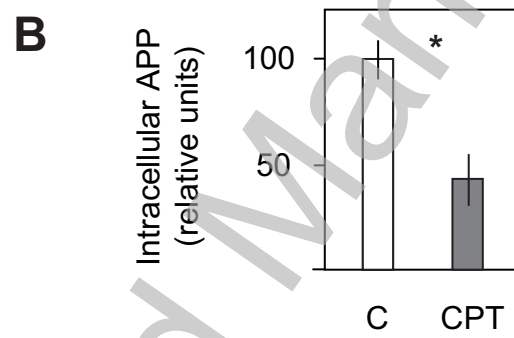
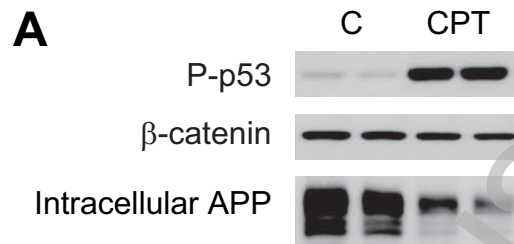
Cells were transiently cotransfected with a pBL-tkCAT plasmid containing, or not, the +55 to +101 bp fragment of the APP gene promoter and expression vectors encoding wild type p53, a dominant negative p53 mutant or the empty non coding vector. CAT activity was determined 48 hours later. Data are the mean \pm SD activities obtained from two independent assays performed in duplicate, and are expressed relative to the CAT activity obtained in control cells transfected with the +55/+101 fragment.

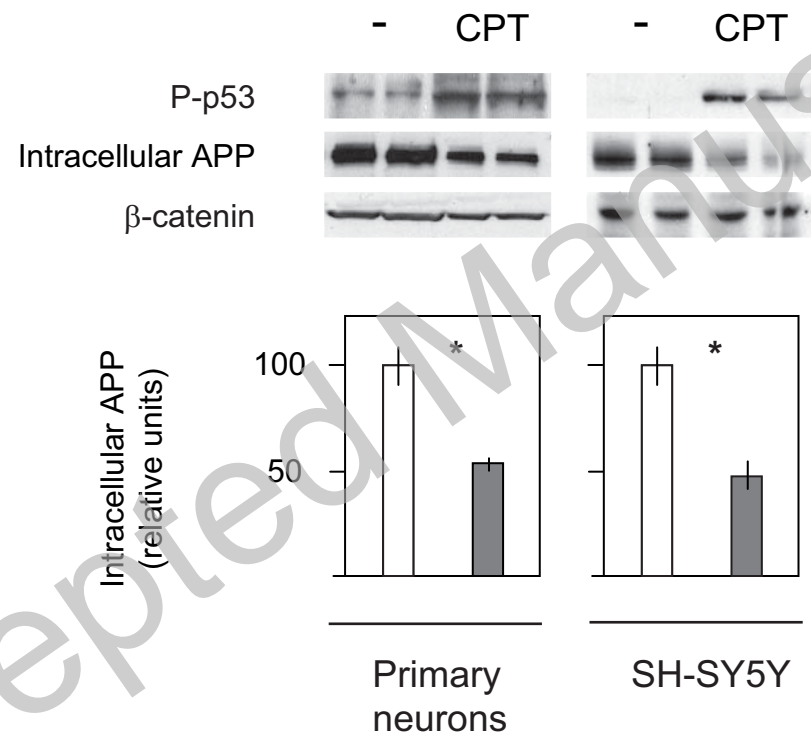
Fig. 8 Gel retardation analysis of p53 and Sp1 binding to the +55 to +101 region of the APP promoter.

Illustrated at the top of the figure are the nucleotide sequence of the +55 to +101 bp region, and the positions of the Sp1 binding site and a putative p53 half-site. A) Gel retardation assay performed with 10 μ g of nuclear extracts in the absence of competitor (C) or in the presence of increasing amounts of a consensus p53 binding site, the +55/+101 oligonucleotide, or an unrelated oligonucleotide. B) At the left, a consensus p53 binding site was incubated with or without 2 μ l of in vitro translated p53. At the right, the +55/+101 oligonucleotide was incubated with 2 μ l of in vitro translated protein and 0,2 μ l of recombinant Sp1 alone or in combination. The same amount (6 μ l) of unprogrammed reticulocyte lysate was always present in the binding reaction. C) ChIP assay of the APP promoter in cells treated with or without camptothecin (CPT). Chromatin was immunoprecipitated with specific antibodies against p53 and Sp1, and analyzed by PCR. At the top is a scheme of the amplified fragment. The corresponding inputs as well as a negative control (IgG antibody) are also included in the figure.

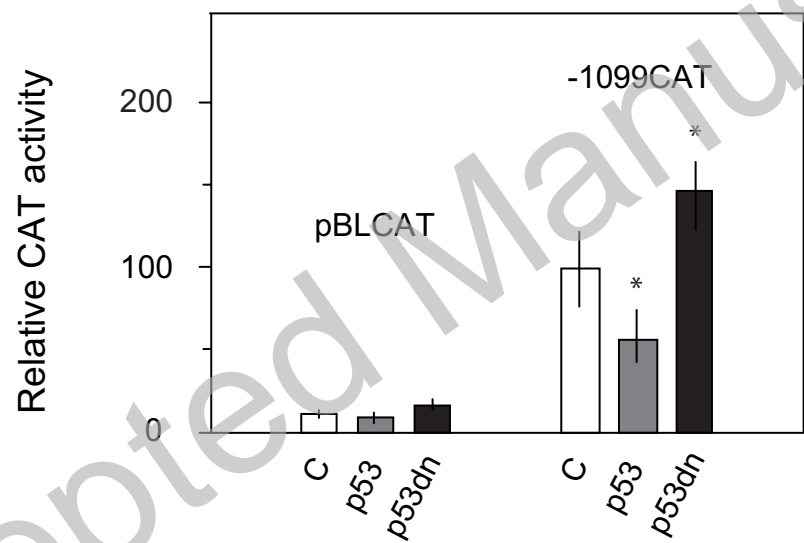




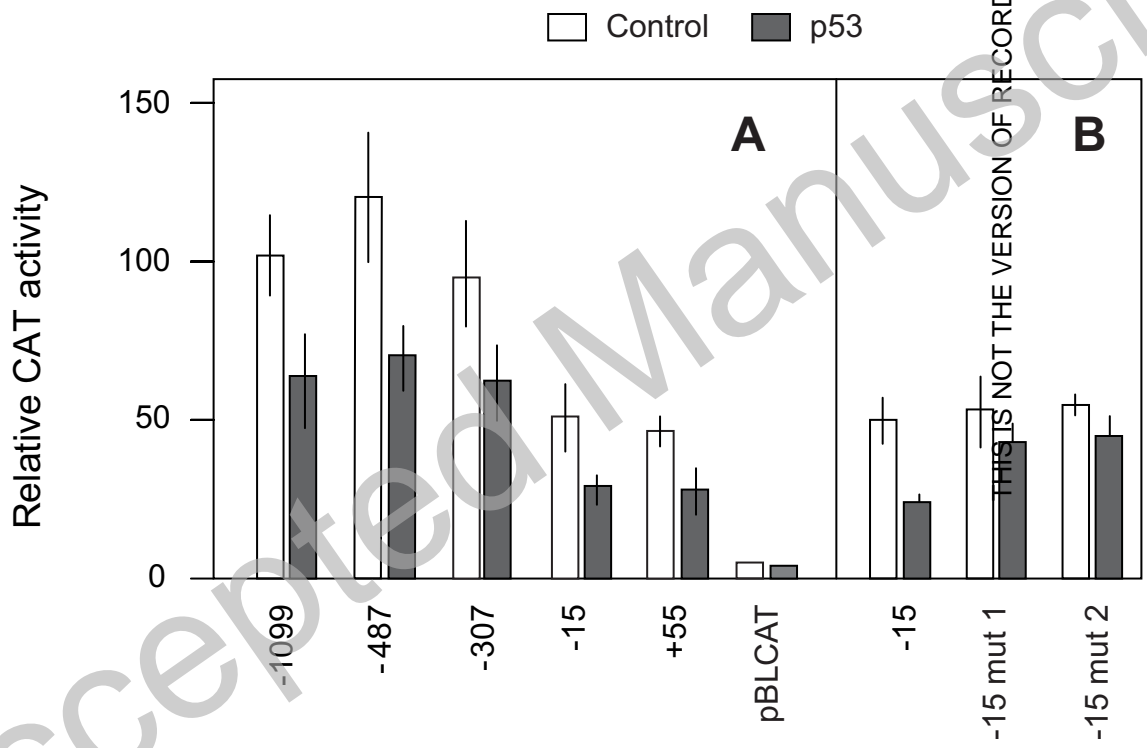




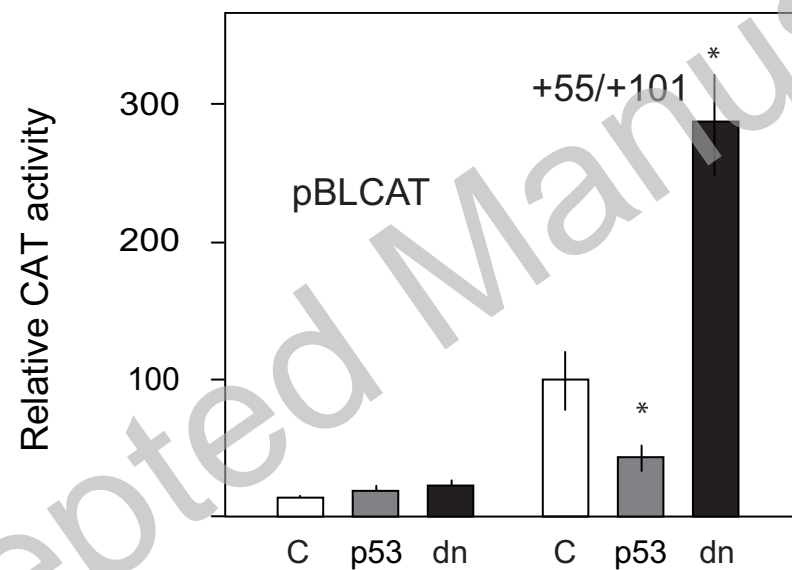
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