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p53-dependent suppression of the human calcyclin gene (*S100A6*): the role of Sp1 and of NFκB

Weronika Króliczak[1], Maciej Pietrzak[2] and Monika Puzianowska-Kuznicka[1,2]✉

[1]*Department of Biochemistry, Medical Center of Postgraduate Education, Warszawa, Poland;* [2]*Department of Biochemistry and Molecular Biology, Medical Research Center, Polish Academy of Sciences, Warszawa, Poland*

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Calcyclin (S100A6) is believed to participate in cell cycle control. It was, however, unclear if its expression depends on p53, a key regulator of apoptosis and cell cycle. We therefore performed transcription regulation assays in HeLa cells and found that wild type p53 suppressed the *S100A6* promoter up to 12-fold in a dose-dependent manner. In contrast, the well-characterized V143A, R175H, R249S, and L344A p53 mutants cloned from human cancers suppressed this promoter with a 6 to 9-fold lower efficiency. All the sites mediating the p53-dependent suppression were contained in the –167 to +134 fragment of the *S100A6* promoter. Separate overexpression of either Sp1 or of NFκB only partially counteracted the p53 inhibitory effect on the *S100A6* promoter, while simultaneous overexpression of both these transactivators resulted in a complete abolishment of the p53 inhibitory effect on this promoter. Sp1 and NFκB binding to the probes resembling their putative binding sites present in the *S100A6* promoter was decreased in the presence of wild type p53. We propose that the suppression of *S100A6* is yet another mechanism by which p53 inhibits proliferation. Insufficient suppression of this gene by p53 mutants could well be responsible for calcyclin overexpression and cell cycle deregulation observed in cancer tissues.

Keywords: calcyclin gene (*S100A6*), wild type and mutant p53, gene suppression, Sp1, NFκB

INTRODUCTION

Calcyclin (also known as S100A6) seems to be involved in early phase of cell differentiation (Tonini *et al*., 1995), exocytosis (Thordarson *et al*., 1991; Okazaki *et al*., 1994) and, above all, in cell cycle regulation, but there are still some doubts about the precise function of this protein. Calcyclin belongs to the S100 family of calcium-binding proteins (Heizmann *et al*., 2002; Donato, 2003; Santamaria-Kisiel *et al*., 2006) and under physiological conditions it is mainly expressed in epithelial cells and fibroblasts (Kuznicki *et al*., 1992), in neurons (Filipek *et al*., 1993), and in lymphocytes (Ferrari *et al*., 1992). Its putative role in cell cycle regulation is supported by the following findings: first, the amount of calcyclin mRNA in the cell undergoing mitosis is not uniform: it is high in the late G1 phase and decreases in the S phase of the cell cycle (Hirschhorn *et al*., 1984); second, during late prophase, calcyclin relocates from the nuclear envelope to the cytoplasm and then it disappears (Stradal & Gimona, 1999; Tomas & Moss, 2003; Farnaes & Ditzel, 2003); third, calcyclin mRNA and protein levels increase in the renal cortex during recovery after acute tubular necrosis, and the protein co-localizes with the proliferating cell nuclear antigen (PCNA) (Cheng *et al*., 2005). In addition, calcyclin is overexpressed in many different human cancers, such as melanoma (Weterman *et al*., 1993),

✉Corresponding author: Monika Puzianowska-Kuznicka, Department of Endocrinology, Medical Research Center, Polish Academy of Sciences, Stefana Banacha 1a, 02-097 Warszawa, Poland; tel: (48 22) 599 1755; fax: (48 22) 599 1975; e-mail: monika@amwaw.edu.pl

Abbreviations: BSA, bovine serum albumin; C/EBP, CCAAT/enhancer binding protein; DMEM, Dulbecco modified eagle's medium; EGFP, enhanced green fluorescent protein; FBS, fetal bovine serum; NFκB, nuclear factor kappa B; PBS, phosphate-buffered saline; p53, tumor suppressor p53; Sp1, stimulatory protein 1; S100, family of calcium binding proteins; S100A6, calcyclin; TBS, Tris-buffered saline.

squamous cell carcinoma of the mouth (Berta *et al*., 1997), cholangiocarcinoma (Kim *et al*., 2002), colorectal adenocarcinoma (Komatsu *et al*., 2000; Alvarez-Chaver *et al*., 2007), stomach adenocarcinoma (Jang *et al*., 2004), pancreas cancer (Vimalachandran *et al*., 2005; Ohuchida *et al*., 2007), breast cancer (Cross *et al*., 2005), and papillary thyroid cancer (Brown *et al*., 2006). It has been suggested that the nuclear localization of calcyclin is associated with poor survival in pancreatic cancer patients (Vimalachandran *et al*., 2005), and high concentration of calcyclin significantly correlates with advanced stages of colorectal carcinoma (Komatsu *et al*., 2000) and with melanoma clinical stage and metastatic potential (Weterman *et al*., 1992; 1993).

Expression of the gene encoding calcyclin, *S100A6*, is activated by growth factors. Serum-inducible and platelet-derived growth factor-responsive sequences are contained within a 164 bp promoter fragment just upstream of the transcription start site (Ghezzo *et al*., 1988). To further support the hypothesis on calcyclin involvement in cell cycle control, we decided to establish if the activity of its gene depends on the action of p53, one of the major regulators of proliferation and apoptosis (Szymańska & Hainaut, 2003). While p53-dependent activation of transcription is mediated by direct p53 binding to its recognition sequences within the target promoters, transcription suppression by p53 is exerted *via* a number of different mechanisms: direct p53 binding to target promoter (St Clair *et al*., 2004), competition with transactivators for binding to DNA (Subbaramaiah *et al*., 1999; Li & Lee, 2001), or formation of complexes between p53 and other transcriptional activators leading to their sequestration or to a change in their activity or affinity for DNA (Xu *et al*., 2000; Sengupta *et al*., 2005). The transcription factor most commonly involved in the latter mechanism of transrepression by p53 is Sp1 (Webster *et al*., 1996; Bargonetti *et al*., 1997; Ohlsson *et al*., 1998; Xu *et al*., 2000; Zhang *et al*., 2000). Other transcription factors include C/EBP (Webster *et al*., 1996), NFκB (Bargonetti *et al*., 1997), as well as members of the basal transcriptional machinery, such as the TATA-binding protein (Seto *et al*., 1992; Truant *et al*., 1993; Subbaramaiah *et al*., 1999) and other general transcription factors (Ragimov *et al*., 1993; Xiao *et al*., 1994; Farmer *et al*., 1996).

In this paper we show that the promoter of the *S100A6* gene encoding calcyclin is suppressed by wild type p53 in a dose-dependent manner, while suppression by p53 mutants cloned from human cancers is less efficient. We also provide evidence that this is a result of a dual mechanism: p53 interference with Sp1 and with NFκB function on the *S100A6* promoter.

MATERIALS AND METHODS

Cloning of Sp1 transcription factor. The 2355 bp human Sp1 coding sequence (GeneBank NM138473) was cloned from human cDNA (made from healthy thyroid mRNA) with Platinum *Pfx* polymerase (Invitrogen Life Technologies, Carlsbad, CA, USA) and using the following primers: 5′Sp1 5′-CA**GGTACC**ATGAGCGACCAAGATCACTC-3′ (incorporated *Kpn*I site in bold), 3′Sp1 5′-CA**CTCGAG**TCAGAAGCCATTGCCACTGA-3′ (incorporated *Xho*I site in bold) in a PCR reaction supplemented with $MgSO_4$ up to 2 mM. After a 3 min initial denaturation at 94°C, five cycles of 94°C for 20 s, 55°C for 30 s and 68°C for 3 min, and then 30 cycles of 94°C for 20 s, 58°C for 30 s and 68°C for 3 min were performed, followed by a final extension at 68°C for 5 min. Once the PCR reaction was completed, 2 units of *Taq* polymerase was added to generate A-overhangs, and the sample was incubated at 72°C for 20 min. The PCR product was then cloned into the pGEM-T vector (Promega, Madison, WI, USA), cut with *Kpn*I and *Xho*I endonucleases and re-cloned into the pcDNA3.1(+) expression vector (Invitrogen Life Technologies, Carlsbad, CA, USA) prepared with the same enzymes. The Sp1 coding sequence was verified by restriction and sequencing.

Cloning of the –66 to +134 deletion mutant of the *S100A6* promoter. The shortest, 200 bp long deletion mutant of the *S100A6* promoter (pGL2-CACY(–66/+134)) was cloned by PCR on a template of the vector containing the –1731 to +134 fragment of the *S100A6* promoter (pGL2-CACY(–1731/+134) (a gift from Dr. Wiesława Lesniak, Nencki Institute of Experimental Biology, Warszawa, Poland; Lesniak *et al*., 2000), with proofreading Platinum *Pfx* polymerase, the forward primer 5′TA**CACGAG**CTTGGCCGAGCTGGCCT3′ (*Xho*I restriction site in bold) and the reverse primer 5′-AG**AAGCTT**GATCCAGCGGCTGAACTGG-3′ (*Hin*dIII restriction site in bold). The cycles were as follows: 94°C for 3 min, five cycles of 94°C for 20 s, 56°C for 30 s, 68°C for 1 min, 30 cycles of 94°C for 20 s, 58°C for 30 s, 68°C for 1 min, final extension at 68°C for 5 min. To produce A-overhangs at 3′ ends of PCR product, 2 units of *Taq* polymerase was then added, and the sample was incubated at 72°C for 20 min. The product of the reaction was cloned into the pGEM-T vector, restricted out with *Xho*I and *Hin*dIII enzymes, and recloned into the pGL2-Basic vector (Promega, Madison, WI, USA) cut with the same enzymes.

Cloning of the pEGFP-CACY(–1731/+134) reporter plasmid. The –1731 to +134 fragment of the *S100A6* promoter was amplified on the template of pGL2-CACY(–1731/+134) with Platinum *Pfx* polymerase, and with 5′-TCGAACGTAG**AGTACT**CGGTGTTCCTGAG-3′ forward and 5′-TCGAACGTAG-

GGATCCAGCGGCTGAAC-3′ reverse primers (*Sca*I and *Bam*HI restriction sites in bold, respectively). The cycles were: 94°C for 2 min, 20 cycles of 94°C for 20 s, 68°C for 2 min, and the final extension at 68°C for 5 min. To produce A-overhangs at the 3′ ends of the PCR product, 2 units of *Taq* polymerase was added, and the sample was incubated at 72°C for 20 min. The product of the reaction was cloned into pGEM-T vector, and restricted out with *Sca*I and *Bam*HI. pEGFP-N1 vector (Clontech Laboratories, Mountain View, CA, USA) was restricted with *Ase*I, and the overhanging ends were blunted with the Klenow enzyme. After gel purification, the vector was restricted with *Bam*HI. The PCR product was ligated into this vector, producing a pEGFP-CACY(–1731/+134) reporter plasmid.

Mutagenesis of the *S100A6* promoter. A 38 bp GC-rich fragment located at positions –100 to –63 of the *S100A6* promoter (5′-GGGTGGGGCGCG-GGCGGGACTTGGGCGGGCGGGGTGGG-3′), containing putative Sp1 binding sites, was removed by PCR from the 1.5 kb promoter fragment. Initially, two fragments of this promoter were cloned. The first one flanking the GC-rich sequence from the 5′- side was cloned with the following primers: 5′-cacy 5′-TCGAACGTAG**AGTACT**CGGTGTTCCT-GAG-3′ (*Sca*I restriction site in bold) and -3′cacyint 5′-AGCTCGGCCAAG//TGCGCCGTGGCTTACAC-3′ consisting of the sequences flanking the GC-rich promoter fragment from both sides (// shows the border of the two sequences), while the second one located at the -3′ side of the GC-rich fragment was cloned with the following primers: 5′-cacyint 5′-AGCCACG-GCGCA//CTTGGCCGAGCTGGCCT-3′ consisting of the sequences flanking promoter fragment to be removed (// shows the border of the two sequences) and -3′cacy 5′-TCGAACGTAG**GGATCC**AGCGGCT-GAAC-3′ (*Bam*HI restriction site in bold). The products of these PCRs were resolved on 1% agarose gel. The bands of approx. 1.25 kb (the first *S100A6* promoter fragment) and 200 bp (the second fragment) were excised and incubated separately in 100 µl of dH_2O for 4 h. Aliquots of 4 µl from each fragment were pooled and used as a template in a second round of PCR performed with 5′-cacy and -3′cacy primers. All PCRs were performed with proofreading Platinum *Pfx* polymerase. The cycles of the first round of PCR were as follows: 94°C for 2 min, five ycles of 94°C for 20 s, 58°C for 30 s, 68°C for 2 min, 25 cycles of 94°C for 20 s, 68°C for 2 min, and the final extension at 68°C for 5 min. The cycles of the second PCR were: 94°C for 2 min, 30 cycles of 94°C for 20 s, 68°C for 2 min, and the final extension at 68°C for 5 min. The product of the second round of PCR was cut with *Sca*I and *Bam*HI enzymes, while pGL2 basic vector was cut with *Sma*I and *Bgl*II enzymes. Both DNAs were ligated producing pGL2-CACY(–1371/+134)Δ38 plasmid. pGL2-CACY(–167/+134)Δ38 was prepared from pGL2-CACY(–1371/+134)Δ38 by restriction of a 263 bp long promoter fragment (–167 to +134) with *Sma*I and *Hin*dIII enzymes, and its ligation into the pGL2-basic vector prepared with the same enzymes.

Cell culture. HeLa cells were grown in high glucose Dulbecco Modified Eagle's Medium (DMEM, Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS), with or without 100 u/ml penicillin G and 100 µg/ml streptomycin. Cells were incubated in a humidified incubator at 37°C in 5% CO_2.

Transient transfections. HeLa cells were seeded 1:2 onto a 24-well dish and maintained without antibiotics for 24 h before transfection. Cells were transfected with 1.5 µl Metafectene (Biontex Laboratories GmbH, Munich, Germany), with 250 ng reporter vector pGL2-CACY containing the *S100A6* promoter fragments –1371 to +134, –588 to +134, –365 to +134, –167 to +134 (a gift from Dr. Wiesława Lesniak, Nencki Institute of Experimental Biology, Warszawa, Poland), or –66 to +134, 20 ng phRL-CMV internal control vector (Promega, Madison, WI, USA), and with 20–250 ng CMV-p53 expression vector encoding wild type human p53, or with 100 ng of the vector encoding mutant p53 (a gifts from Professor Maciej Zylicz, International Institute of Molecular and Cell Biology, Warszawa, Poland). When necessary, co-transfections were performed with 20–250 ng pcDNA3.1(+)-Sp1 expression vector encoding human Sp1, or with 50–150 ng pcDNA3.1(+)-NFκB p50 and 2.5–100 ng pcDNA3.1(+)-NFκB p65 (provided by Dr. Jochen Seufert, Medical Policlinic of the University of Wuerzburg, Germany). Control transfections were performed with pGL2-basic and pcDNA3.1(+) vectors. Metafectene and DNA were diluted separately in 20 µl of plain DMEM, mixed, incubated at room temperature for 20 min, and then added to HeLa cells. After 24 h incubation, the cells were washed with phosphate buffered saline (PBS) and lysed at room temperature for 20 min with 100 µl passive lysis buffer (Promega, Madison, WI, USA) added directly to the cells. Firefly luciferase and *Renilla* luciferase activities were measured in a microplate luminometer (BMG Labtech, Offenburg, Germany). Each experiment was repeated 9 to 12 times.

Immunofluorescence. HeLa cells (10^4) were seeded onto glass coverslips (soaked in 70% ethanol for 3 h and air-dried) placed into a 24-well dish. The cells were transfected 24 h later with 250 ng pEGFP-CACY(–1371/+134) reporter plasmid containing EGFP reporter gene placed under the control of the –1371 to +134 fragment of the *S100A6* promoter, and with 100 ng CMV-p53 plasmid or with 100 ng of 'empty' pcDNA3.1(+) vector. After 24 h incubation, the cells were washed 3 times with PBS, fixed for 10

min at 4°C with 2% paraformaldehyde, incubated for 10 min at room temperature in 0.1% Triton X-100 in PBS, and washed again 3 times in PBS for 1 min. The cells were then incubated in 2% BSA in PBS for 1 h at room temperature, incubated for 1 h at room temperature in a humidified chamber with a primary anti-p53 mouse monoclonal antibody (1:500 in 2% BSA in PBS; DO1, Oncogene Science, Inc., Cambridge, MA, USA), washed 3 times in 2% BSA in PBS, and incubated for 1 h in a humidified chamber in the dark with Alexa Fluor 594 goat anti-mouse secondary antibody (1:1000 in 2% BSA in PBS; Invitrogen Molecular Probes, Eugene, OR, USA). After 3 further washes with 2% BSA in PBS (twice for 40 min at room temperature, and once for 16 h at 4°C), the coverslips were mounted with SlowFade Light Antifade Reagent (Invitrogen Molecular Probes, Eugene, OR, USA) and sealed with acetone-free nail polish. The cells were analyzed under a fluorescence microscope.

Whole-cell protein isolation from HeLa cells and Western blotting. HeLa cells (2×10^5) were suspended in 70 μl of lysis buffer consisting of 75 mM Tris/HCl, pH 8.0, 2% SDS, 15% glycerol, and boiled for 5 min. Forty micrograms of protein extract was supplemented with β-mercaptoethanol to 5% and bromophenol blue to 0.01%, boiled, and loaded onto a 10% polyacrylamide gel. Proteins were transferred onto a nitrocellulose membrane (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The protein loading and integrity were monitored by Ponceau Red staining. The destained membrane was blocked overnight at 4°C in 5% nonfat dry milk solution in TBS-T (Tris-buffered saline (TBS) supplemented with 0.1% Tween 20), washed at room temperature in TBS-T once for 15 min and twice for 5 min, incubated with a mouse monoclonal anti-p53 antibody (1:10000 in TBS-T; DO1, Oncogene Science, Inc., Cambridge, MA, USA), or a rabbit polyclonal anti-Sp1 antibody (1:2500 in TBS-T; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), or a rabbit polyclonal anti-p65 NFκB antibody (1:5000 in TBS-T; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), or a monoclonal anti-β-actin antibody (1:10000 in TBS-T; Sigma-Aldrich, St. Louis, MO, USA) for 1 h at room temperature, washed as before, then incubated at room temperature for 1 h either with a goat anti-mouse horseradish peroxidase-conjugated polyclonal antibody (1:10000 in TBS-T; Calbiochem, San Diego, CA, USA), or a goat anti-rabbit horseradish peroxidase-conjugated polyclonal antibody (1:10000 in TBS-T; Calbiochem, San Diego, CA, USA), respectively. Specific bands were visualized by chemiluminescent reaction performed with an ECL kit (Amersham Biosciences UK Limited, Little Chalfont, England). The blots were exposed against the film (Biomax MS, Eastman Kodak Company, Rochester, NY, USA) for 15 s to 3 min. Relative amounts of the receptor proteins were estimated from the densitometric measurements of the intensity of the specific bands normalized against the intensity of β-actin band.

Isolation of nuclear proteins. A 10 cm-diameter dish with HeLa cells (approx. 5×10^6 cells) was washed twice with PBS. The cells were then scrapped off, suspended in 500 μl of buffer A consisting of 10 mM Hepes, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, supplemented with a protease inhibitors mix (Complete Protease Inhibitor Cocktail, Roche Applied Science, Basel, Switzerland) and PMSF (phenylmethylsulfonyl fluoride) to 400 μM, and incubated on ice for 1 h. After incubation, the cells were homogenized in an ice-cold glass-teflon homogenizer. The resulting homogenate was transferred to an Eppendorf tube and centrifuged at $400\times g$ for 5 min at 4°C. The pellet was resuspended in an equal volume of buffer A, centrifuged as before, and again resuspended in two volumes of buffer B consisting of 20 mM Hepes, pH 7.9, 10% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, supplemented with protease inhibitors as above. After a 30 min incubation on ice, the sample was centrifuged at $15000\times g$ for 20 min at 4°C, then the supernatant was transferred to a new tube and supplemented with an equal volume of buffer C consisting of 20 mM Hepes, pH 7.9, 30% glycerol, 1.5 mM $MgCl_2$, 0.2 mM EDTA, and protease inhibitors. The sample containing soluble nuclear proteins was aliquoted into pre-chilled Eppendorf tubes, flash-frozen in liquid nitrogen, and stored at –80°C.

Electrophoretic mobility shift assay. The assays were performed with control HeLa nuclear extracts and with nuclear extracts made from HeLa cells transfected with p53 expression plasmid 24 h before protein isolation, or HeLa cells treated with camptothecin (6 μM final) for the same period. The probes were identical to the *S100A6* promoter fragments containing putative p53, Sp1 and NFκB binding sites (TESS, Transcription Element Search Software, Technical Report CBIL-TR-1997-1001-v0.0). The probes were made by hybridization of two complementary oligos as follows:
for p53 DR-1: 5′-TAGGCAGGCAGGCCTGACTTGTC-3′ and 5′-CTGTGGACAAGTCAGGCCTGCC-3′,
for p53-DR13: 5′-GGCCTGACTTGTCCACAGCT-CACCCGGAGGCCACC-3′ and 5′-GCTGCCAAG-GTGGCCTCCGGGTGAGCTGTGGACAAGTC-3′,
for p53-DR17: 5′-CCGGAGGCCACCTTGGCAGCAC-CTGTAGGAAGGGCATG-3′ and 5′-GGCCGACAT-GCCCTTCCTACAGGTGCTGCCAAGGTGGCC-3′,
for p53-DR0: 5′-AGGTGGGCTTGGCCGAGCTGGCC-3′ and 5′-CCGGAGGCCAGCTCGGCCAAGCCC-3′,
for 1Sp1: 5′-CGGCGCAGGGTGGGGCGCGGGCG-GG-3′ and 5′-GCCCAAGTCCCGCCCGCGCCCCAC-CC-3′,

for 2Sp1: 5′-GGACTTGGGCGGGCGGGGTGG-3′ and 5′-GCCAAGCCCACCCCGCCCGCC-3′,
for NFκB: 5′-GGAGCCCTGGGTACTTTCCAGG-3′ and 5′-GCAGCTGGCCCTGGAAAGTACCCAGG-3′.

Double stranded DNA was labeled by fill-in reaction with the Klenow fragment and [α-^{32}P]dCTP. Two to five micrograms of each nuclear extract was incubated at room temperature for 20 min in binding buffers consisting of: 10 mM Tris, pH 7.9, 50 mM NaCl, 2 mM $MgCl_2$, 1 mM DTT (dithiothreitol), 0.1 mM EDTA, 0.05% Nonindet P-40, 5% glycerol for p53 binding; 20 mM Hepes, pH 7.9, 50 mM NaCl, 2 mM $MgCl_2$, 1 mM DTT, 0.5 mM EDTA, 0.01% Nonindet P-40, 5% glycerol for Sp1 binding; 10 mM Tris, pH 7.5, 50 mM KCl, 1 mM $MgCl_2$, 0.5 mM DTT, 0.5 mM EDTA, 0.1% Triton X-100, 5% glycerol for NFκB binding, in the presence of 250 ng dIdC, 0.5 ng probe, and protease inhibitors. If required, the samples were additionally supplemented with 25-fold excess of a specific competitor (cold probe, or, for p53 binding, a consensus p53 binding site 5′-GAGTTAGACATGCCTAGACATGCCTAACTC-3′), or of a non-specific competitor (5′-CCTGCTGATCTATCAGCACAGATTAG-3′). For supershift experiments, the reactions were incubated with 200 ng monoclonal anti-p53, or 2 μg anti-Sp1, or anti-NFκB p65 antibodies in the respective binding buffer in the presence of dIdC. After 50 min on ice, the respective probes were added and the incubation was continued for an additional 20 min at room temperature. The products of the reaction were resolved on a 5% native gel. Gels were then dried and exposed against the film (Biomax MS, Eastman Kodak Company, Rochester, NY, USA) for 4–24 h at –80°C.

RESULTS

p53 suppresses the human *S100A6* promoter

Initially, to verify that p53 expressed from CMV-p53 plasmid was functional in the transfected HeLa cells that weakly express endogenous p53 (Hoppe-Seyler & Butz, 1993), we performed immunoblot analysis of the cells transfected with this plasmid (Fig. 1A), and control transcription regulation assays with this plasmid and with pGL3-p53BS reporter plasmid containing a p53BS synthetic promoter with consensus p53 binding sites (a gift from Professor Maciej Zylicz, International Institute of Molecular and Cell Biology, Warszawa). They showed that in the presence of overexpressed p53, the activity of the reporter gene increased approx. 16-fold (Fig. 1B).

To address the question of regulation of the human *S100A6* promoter by p53, the pGL2-CACY(–1371/+134) reporter plasmid, containing a 1505 bp fragment of the human *S100A6* promoter, was transfected into HeLa cells together with increasing amounts of the CMV-p53 expression vector. These experiments showed that the inhibition of the

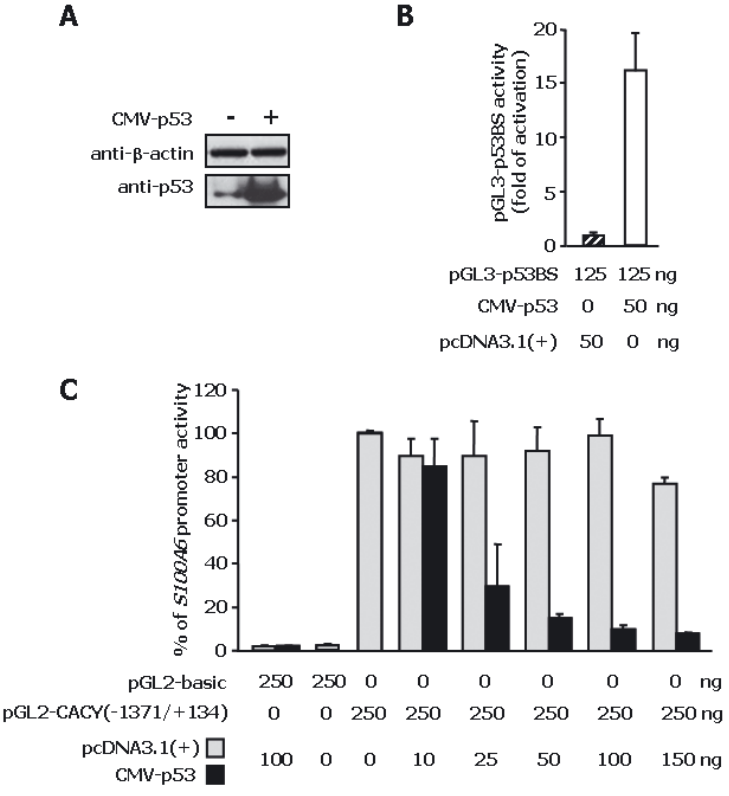

Figure 1. Human calcyclin (*S100A6*) promoter is inhibited by p53.
(**A**) Immunoblots of whole-cell extracts made from control HeLa cells, and from HeLa cells overexpressing p53. The blots were probed with anti-p53 antibody and re-probed with anti-β-actin antibody. (**B**) HeLa cells transfected with CMV-p53 vector overexpress transcriptionally active p53 protein. HeLa cells were transfected with pGL3-p53BS reporter vector with the promoter containing consensus p53 binding sites, as well as with CMV-p53 expression vector encoding wild type p53. (**C**) The *S100A6* promoter is inhibited by p53 in a dose-dependent manner. HeLa cells were transfected with pGL2-CACY(–1371/+134) reporter vector, with increasing amounts of CMV-p53 expression vector or with equal amounts of 'empty' pcDNA3.1(+) vector, and with phRL-CMV internal control vector. Firefly luciferase activity was measured 24 h post-transfection, and the results normalized against *Renilla* luciferase activity. Bars represent the mean result (± standard deviation) of 9 experiments performed with two different DNA preparations.

S100A6 promoter was p53 dose-dependent and that the *S100A6* promoter was suppressed up to 12-fold (8% of its activity without p53). In the presence of an 'empty' pcDNA3.1(+) vector, the *S100A6* promoter activity remained unaltered, providing that no more than 100 ng of this vector was used (Fig. 1C). Transfection with higher amounts of the expression construct resulted in a slight, non-specific promoter suppression. Based on the above results, we decided to use 100 ng of CMV-p53 in all subsequent experiments. This amount of expression vector caused an 8-fold suppression of the *S100A6* promoter after 24 h incubation.

To corroborate the above result with a different method, we designed an experiment in which HeLa cells were transfected with pEGFP-CACY(–1731/+134) reporter vector encoding EGFP protein under the control of the *S100A6* promoter, and either with the CMV-p53 expression vector, or with an equal amount of 'empty' pcDNA3.1(+). The relative amount of EGFP protein was assessed 24 h later under a fluorescence microscope in the cells transfected with the reporter and pcDNA3.1(+) vectors *vs*. cells transfected with the reporter and p53 expression plasmids. A direct comparison of both cell types was not possible, because the single and double transfections had to be performed separately, and, for this reason, the cells were grown on coverslips placed in separate wells. However, both coverslips were identically processed for immunofluorescence. Therefore, both cell types had identical controls, i.e. neighboring, not-transfected cells. Using them as go-between controls, we found that the cells transfected with the EGFP reporter vector and with pcDNA3.1(+) expressed high levels of EGFP protein in the cytoplasm (Fig. 2B, black arrows). In contrast, the cells transfected with the EGFP reporter vector and the CMV-p53 expression plasmid, and, therefore, overexpressing p53 in the nucleus (Fig. 2C, black arrows), did not express EGFP, judging by the absence of a specific immunofluorescent signal in their cytoplasm (Fig. 2D, black arrow), a situation identical to that observed in control cells that had not been transfected (Fig. 2D, white arrows).

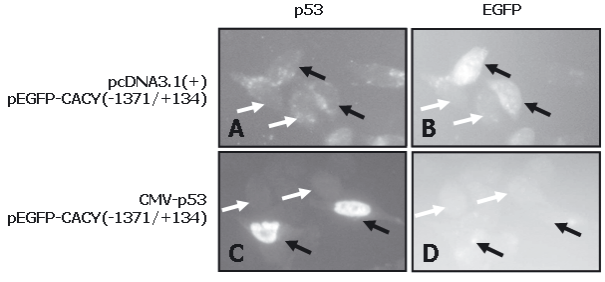

Figure 2. Overexpressed p53 inhibits EGFP expression driven by the *S100A6* promoter.
HeLa cells were transfected with pEGFP-CACY(–1371/+134) reporter vector, either with CMV-p53 expression vector, or with an 'empty' pcDNA3.1(+) vector, probed with anti-p53 antibodies, and analyzed under a fluorescence microscope for the presence of p53 and EGFP proteins. (**A**) Cells transfected with pEGFP-CACY(–1371/+134) and with pcDNA3.1(+) vectors, analyzed for the presence of p53. (**B**) The same cells analyzed for the presence of EGFP. (**C**) Cells transfected with pEGFP-CACY(–1371/+134) and CMV-p53 vectors, analyzed for the presence of p53. (**D**) The same cells analyzed for the presence of EGFP. Black arrows, transfected cells. White arrows, non-transfected control cells.

p53 mutants cloned from human cancers weakly suppress the *S100A6* promoter

We then proceeded to establish whether common p53 mutants cloned from human cancers suppress the *S100A6* promoter. HeLa cells were therefore transfected with pGL2-CACY(–1371/+134) reporter vector and expression vectors encoding either wild type p53 or well-characterized DNA-binding domain mutants (V143A, R175H, and R249S), or a tetramerization domain mutant (L344A) (Hernandez-Boussard *et al*., 1999; Strano *et al*., 2007; Petitjean *et al*., 2007). All examined mutants suppressed the *S100A6* promoter activity only to 60–90% of its activity without p53, showing that they were 6 to 9-fold less effective than the wild type p53 (Fig. 3).

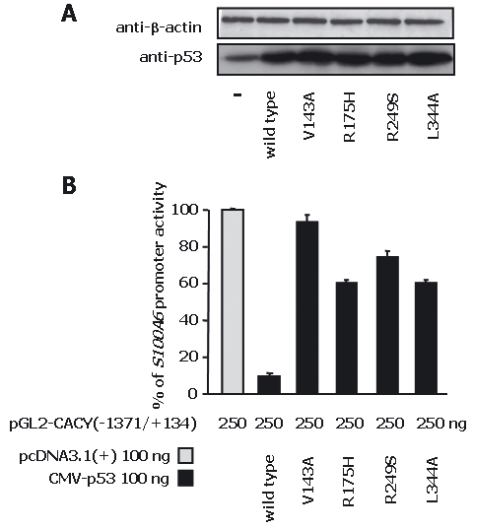

Figure 3. p53 mutants suppress the *S100A6* promoter 6 to 9-fold less than wild type p53.
(**A**) Immunoblots of protein extracts made from control HeLa cells as well as HeLa cells overexpressing wild type and mutant p53. The blots were probed with anti-p53 antibody and re-probed with anti-β-actin antibody. (**B**) p53 mutants weakly inhibit the *S100A6* promoter. HeLa cells were transfected with pGL2-CACY(–1371/+134) reporter plasmid, with CMV-p53 expression plasmid encoding either wild type or mutant p53, and with phRL-CMV internal control vector. Firefly luciferase activity was measured 24 h post-transfection, and the results were normalized against *Renilla* luciferase activity. Bars represent the mean result (± standard deviation) of 6 experiments.

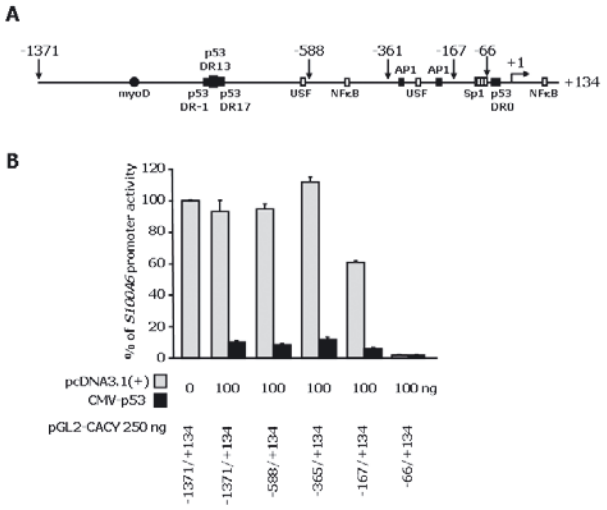

Figure 4. Deletion of three out of four promoter fragments similar to p53-specific sequences has no effect on *S100A6* promoter inhibition by p53.
(**A**) Schematic presentation of the –1371 to +134 *S100A6* promoter fragment and the localization of the selected putative transcription factor binding sites. The arrows point to 5′ ends of the deletion mutants. +1, transcription start site. (**B**) The –167 to +134 fragment contains all sites mediating p53 inhibitory effect on the *S100A6* promoter. HeLa cells were transfected with pGL2 reporter construct containing different deletion mutants of the *S100A6* promoter, with CMV-p53 expression plasmid encoding wild type human p53 or 'empty' pcDNA3.1(+) vector, and with phRL-CMV internal control vector. Firefly luciferase activity was measured 24 h post-transfection and the results were normalized against *Renilla* luciferase activity. Bars represent the mean result (± standard deviation) of 9 experiments performed with two different DNA preparations.

The –167 to +134 fragment of the *S100A6* promoter contains all sites mediating p53-dependent suppression

In order to find the region of the *S100A6* promoter mediating the suppression exerted by p53, we used promoter deletion mutants (–1371/+134, –588/+134, –365/+134, –167/+134, and –66/+134, Fig. 4A) in transcription regulation assays. The assays were performed as described above. Deletion of subsequent fragments from the 5′ end of the *S100A6* promoter had a weak influence on the promoter's activity; only shortening of the promoter to the –167 position resulted in a 40% decrease of its activity. Deletion of the subsequent 101 bp to the –66 position resulted in the complete silencing of the *S100A6* promoter. With the exception of the inactive –66 to +134 fragment, each promoter fragment was suppressed by p53 to the same extent, i.e. approx. 8-fold (Fig. 4B). This data indicate that the three deleted putative p53-binding sites (DR-1, DR13, DR17, located at positions –846 to –828, the –837 to –805, and –814 to –778, respectively, Fig. 4A) did not mediate the *S100A6* promoter inhibition by p53. Furthermore, lack of a direct involvement of p53 in this process was confirmed by the negative result of electrophoretic mobility shift assays performed with HeLa nuclear extracts containing overexpressed p53, and with the probes resembling the four putative p53-binding sites present in the *S100A6* promoter (the three described above, and the fourth located at position –65 to –46, DR0, Fig. 4A). No specific binding was observed (not shown).

Taken together, these results indicate that the –167 to +134 promoter fragment contains all the sequences mediating the p53-dependent suppression of the *S100A6* transcriptional activity.

Deletion of a GC-rich region located at position –100 to –63 decreases the level of p53-induced *S100A6* promoter suppression

The –167 to +134 *S100A6* promoter fragment differs from the –66 to +134 fragment by 101 bp including the 38 bp long GC-rich sequence located at the –100 to –63 position, containing six overlapping putative Sp1-binding sites. To determine if this sequence is important for p53-dependent suppression of the *S100A6* activity, transcription regulation assays were performed with pGL2-CACY(–1371/+134)Δ38, and with pGL2-CACY(–167/+134)Δ38 plasmids devoid of this sequence (Fig. 5). The deletion of the GC-rich sequence decreased the overall promoter activity by approx. 50%. Importantly, deletion of the putative Sp1-binding sites decreased

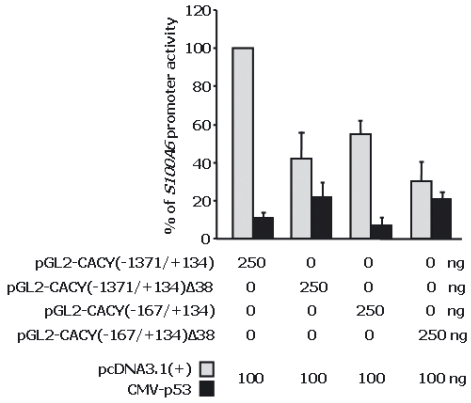

Figure 5. Deletion of the 38 bp GC-rich region located at position –100 to –63 weakens p53-dependent inhibition of the *S100A6* promoter.
HeLa cells were transfected with pGL2-CACY(–1371/+134) or pGL2-CACY(–167/+134) plasmids, or with pGL2-CACY(–1371/+134)Δ38 or pGL2-CACY(–167/+134)Δ38 plasmids bearing the *S100A6* promoter fragments devoid of the 38 bp-long GC-rich region, with CMV-p53 expression construct encoding wild type p53 or 'empty' pcDNA3.1(+) vector, and with phRL-CMV internal control vector. Firefly luciferase activity was measured 24 h post-transfection and the results were normalized against *Renilla* luciferase activity. Bars represent the mean result (± standard deviation) of 6 experiments.

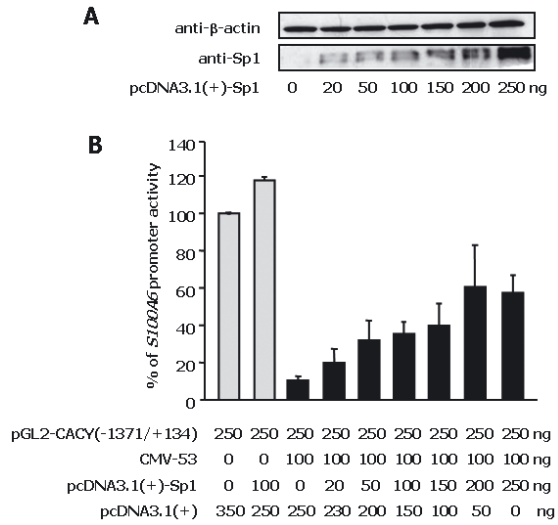

Figure 6. Overexpression of Sp1 partially counteracts the p53 inhibitory effect on the *S100A6* promoter.
(**A**) Immunoblots of whole-cell extracts made from control HeLa cells as well as HeLa cells overexpressing p53 and different amounts of Sp1 were probed with anti-Sp1 antibody. Duplicate blots were re-probed with anti-β-actin antibody. (**B**) Overexpression of Sp1 counteracts the p53-dependent inhibition of the *S100A6* promoter in a dose-dependent manner. HeLa cells were transfected with pGL2-CACY(–1371/+134) reporter plasmid, with CMV-p53 expression plasmid encoding wild type human p53, with different amounts of pcDNA3.1(+)-Sp1 expression plasmid encoding wild type human Sp1, with phRL-CMV internal control vector, and, to equalize the total amount of DNA in each sample, with different amounts of pcDNA3.1(+). Firefly luciferase activity was measured 24 h post-transfection and the results were normalized against *Renilla* luciferase activity. Bars represent the mean result (± standard deviation) of 9 experiments.

the level of promoter suppression by p53 from 8 to 2-fold.

Sp1 only partially counteracts the p53-dependent suppression of the *S100A6* promoter

The role of Sp1 in the *S100A6* promoter suppression by p53 was further analyzed using transactivation assays performed in the presence of overexpressed p53 and of increasing amounts (20–250 ng) of the pcDNA3.1(+)-Sp1 expression construct. As shown before (Fig. 1C), in the presence of 100 ng of the CMV-p53 expression plasmid, the activity of the *S100A6* promoter was inhibited 8-fold. In the presence of overexpressed Sp1 (Fig. 6A) only a partial relief of the p53-dependent suppression of the *S100A6* promoter was achieved. This effect was dose-dependent (Fig. 6B). In the presence of 200 ng of the Sp1 expression construct, the p53-dependent suppression of the *S100A6* promoter activity was only 1.8-fold (to 55–60% of the promoter activity without p53). This effect was not magnified by further increase of the amount of the Sp1 expression vector.

NFκB only partially counteracts the p53-dependent suppression of the *S100A6* promoter

We then turned our attention to the role of the NFκB transcription factor (another factor indicated by others as mediating the p53 inhibitory effect on transcription) in the *S100A6* promoter suppression by p53. Transcription regulation assays were performed as described before, in the presence of increasing amounts of pcDNA3.1(+)-NFκB p50 (5–150 ng) and of pcDNA3.1(+)-NFκB p65 (2.5–100 ng) expression plasmids. Transfection performed with 25 ng or higher amounts of the NFκB p65 expression construct resulted in a similar production of the encoded protein. Again, in the presence of overexpressed NFκB (Fig. 7A), only a partial, dose-dependent reversal of the p53-dependent suppression of the *S100A6* promoter was achieved. In the presence of optimal amounts of the NFκB expression

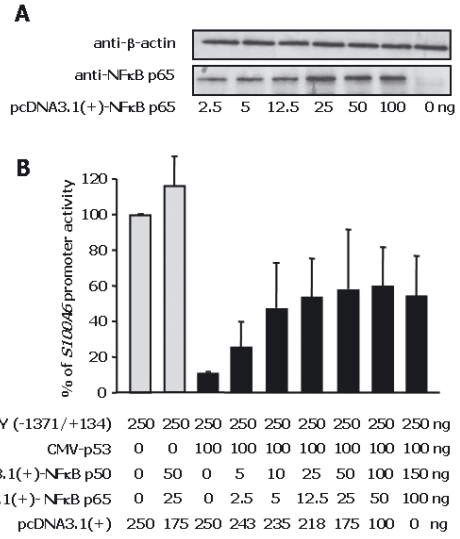

Figure 7. Overexpression of NFκB partially counteracts the p53 inhibitory effect on the *S100A6* promoter.
(**A**) Immunoblots of whole-cell extracts made from control HeLa cells as well as HeLa cells overexpressing p53 and NFκB p50 and p65. The blots were probed with anti-NFκB p65 antibody. Duplicate blots were probed with anti-β-actin antibody. (**B**) Overexpression of NFκB counteracts the p53-dependent inhibition of the *S100A6* promoter in a dose-dependent manner. HeLa cells were transfected with pGL2-CACY(–1371/+134) reporter plasmid, with CMV-p53 expression plasmid encoding wild type human p53, with different amounts of pcDNA3.1(+)-NFκB p50 and of pcDNA3.1(+)-NFκB p65 expression plasmids encoding wild type human NFκB p50 and p65 proteins, respectively, with phRL-CMV internal control vector, and, to equalize the total amount of DNA in each sample, with different amounts of 'empty' pcDNA3.1(+). Firefly luciferase activity was measured 24 h post-transfection and the results were normalized against *Renilla* luciferase activity. Bars represent the mean result (± standard deviation) of 9 experiments.

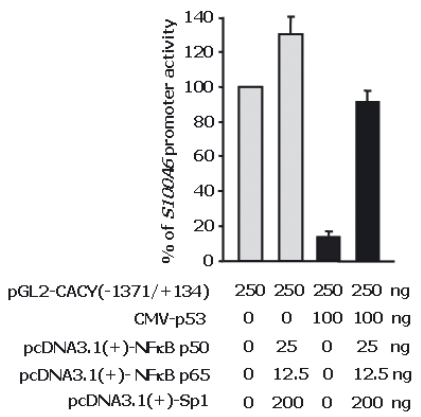

Figure 8. Simultaneous overexpression of Sp1 and NFκB completely counteracts the p53 inhibitory effect on the *S100A6* promoter.
HeLa cells were transfected with pGL2-CACY(–1371/+134) reporter plasmid, with CMV-p53 expression plasmid encoding wild type human p53, with pcDNA3.1(+)-Sp1, pcDNA3.1(+)-NFκB p50 and pcDNA3.1(+)-NFκB p65 expression plasmids encoding wild type human Sp1, NFκB p50 and p65 transcription factors, respectively, with phRL-CMV internal control vector, and, to equalize the total amount of DNA in each sample, with 'empty' pcDNA3.1(+). Firefly luciferase activity was measured 24 h post-transfection and the results were normalized against *Renilla* luciferase activity. Bars represent the mean result (± standard deviation) of 9 experiments.

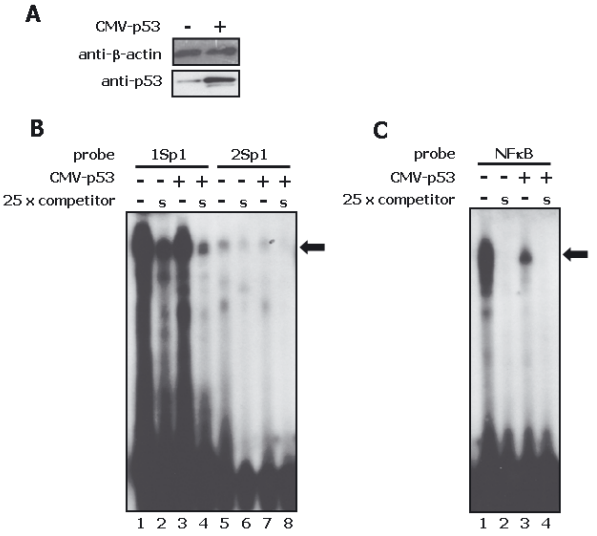

Figure 9. p53 decreases Sp1 and NFκB binding to their recognition sites in the *S100A6* promoter.
(**A**) Immunoblot of protein extracts made from control HeLa cells as well as HeLa cells overexpressing p53. The blot was probed with anti-p53 antibody and re-probed with anti-β-actin antibody. (**B**) Electrophoretic mobility shift assays performed with control HeLa nuclear extracts and with nuclear extracts made from HeLa cells transfected with p53 expression plasmid and the two overlapping probes identical to the *S100A6* promoter fragment containing putative Sp1-binding sites (GC-rich region located at –100 to –63 position). (**C**) Electrophoretic mobility shift assays performed with control HeLa nuclear extracts or with nuclear extracts made from HeLa cells transfected with p53 expression plasmid, and with the probe identical to the putative NFκB-binding site located at +66 to +77 position of the *S100A6* promoter. S, specific competitor (cold probe). Arrows, specific shifted bands.

constructs, the activity of the *S100A6* promoter was suppressed by p53 only 1.8-fold (to 55–60% of its activity without p53). The plateau of de-inhibition was reached with 25 ng of NFκB p50 and 12.5 ng of NFκB p65 expression vectors (Fig. 7B).

Simultaneous action of Sp1 and NFκB fully counteracts the *S100A6* promoter suppression by p53

To establish if Sp1 and NFκB are the only factors mediating p53-dependent suppression of the *S100A6* promoter, its activity was analyzed in the presence of overexpressed p53 and of the lowest amounts of expression plasmids pcDNA3.1(+)-Sp1, pcDNA3.1(+)-NFκB p50 and pcDNA3.1(+)-NFκB p65 (200 ng, 25 ng and 12.5 ng, respectively) that, as shown in the previous experiments, maximally counteracted the p53-dependent suppression of the *S100A6* promoter. Simultaneous overexpression of both transcription factors completely abolished the inhibitory action of p53 on the *S100A6* promoter (Fig. 8).

Sp1 and NFκB binding to the *S100A6* promoter is decreased in the presence of p53

To further elucidate the role of Sp1 and NFκB in the p53-dependent suppression of the *S100A6* promoter, electrophoretic mobility shift assays were performed using probes identical to the putative Sp1-binding sites present in the GC-rich region of the *S100A6* promoter (two overlapping probes covering together the whole GC-rich region), and to the putative NFκB-binding site located downstream of the transcription start site (+66 to +77). The other NFκB-binding site located at the –460 to –451 position (Joo *et al.*, 2003) was not included in this analysis, since we had previously shown that deletion of this site had no influence on the *S100A6* promoter suppression by p53. Experiments were performed with HeLa nuclear extracts made from cells transfected with p53 expression vector and from control cells weakly expressing endogenous p53 (Fig. 9A). A weak-to-strong binding of endogenous transcription factors to the putative Sp1-binding sites was detected (Fig. 9B, lanes 1 and 5, black arrow). The presence of overexpressed p53 in the binding reaction decreased the amount of Sp1-DNA complexes (Fig. 9B, lanes 1 *vs.* 3, 5 *vs.* 7, black arrow). No other changes in the binding pattern were observed in the pres-

ence of p53. A specific binding to the NFκB putative binding site was also detected (Fig. 9C, lane 1, black arrow). As described above for Sp1 binding to DNA, in the presence of overexpressed p53 the binding of NFκB to its recognition site was decreased (Fig. 9C, lane 1 *vs*., 3, black arrow). The results of the assays performed with HeLa cell extract containing high amount of endogenous p53 induced by 6 μM camptothecin (Houser *et al*., 2001) were identical to these described above (not shown).

DISCUSSION

In this work we show that the promoter of the *S100A6* gene encoding calcyclin, a member of the S100 family of Ca^{2+}-binding proteins, is suppressed by p53 in a dose-dependent manner. Such dependence is an important additional argument in the discussion concerning the role of calcyclin in cell cycle regulation.

Calcyclin is overexpressed in many types of human cancers, and sometimes is considered a tumor marker and diagnostic factor (Wojda & Kuznicki, 1993; Filipek & Kuznicki, 1993; Weterman *et al*., 1993; Berta *et al*., 1997; Komatsu *et al*., 2000; Kim *et al*., 2002; Jang *et al*., 2004; Vimalachandran *et al*., 2005). Since p53 accumulates in more than 50% of all cancers, the elevation of p53 should be accompanied by a corresponding decrease of calcyclin. However, as shown by us in this paper, such correlation might be true only for the cases with accumulation of the wild type p53, which is not common. This could be so because p53 is mutated in more than 50% of malignant tumors (Hernandez-Boussard *et al*., 1999; Strano *et al*., 2007; Petitjean *et al*., 2007). The majority of these mutations are missense mutations located in the DNA-binding domain (Martin *et al*., 2002; Olivier *et al*., 2004). Since trans-repression by p53 usually does not require DNA binding, these mutants may retain their ability to suppress transcription. However, the majority of the mutants analyzed so far by other authors have lost, at least in part, this ability (Unger *et al*. 1993; Harris *et al*., 1996; Wang & Beck, 1998; Subbaramaiah *et al*., 1999; Blagosklonny, 2000). Our data is consistent with their results, as we also showed that certain p53 mutants suppress the *A100A6* promoter 6 to 9-fold weaker than the wild type p53. Therefore, an absence of correlation should be expected in cancer tissues, rather than an inverse correlation between p53 and calcyclin levels. In accordance with this expectation, in pancreas cancer, the only cancer type analyzed so far for the association between p53 and calcyclin, there was no correlation between nuclear calcyclin expression and p53 (Vimalachandran *et al*., 2005). This is consistent with the fact that in 50–80% of cancers of this organ, p53 mutants have been reported (Redston *et al*., 1994; Iwao *et al*., 1998).

It has been shown by other authors that the suppression of transcription by p53 might be a result of the diverse mechanisms listed in the introduction (Subbaramaiah *et al*., 1999; Xu *et al*., 2000; Li & Lee, 2001; St Clair *et al*., 2004; Sengupta *et al*., 2005), and might be mediated by different proteins (Seto *et al*., 1992; Truant *et al*., 1993; Ragimov *et al*., 1993; Xiao *et al*., 1994; Farmer *et al*., 1996; Webster *et al*., 1996; Bargonetti *et al*., 1997; Ohlsson *et al*., 1998; Subbaramaiah *et al*., 1999; Xu *et al*., 2000; Zhang *et al*., 2000). Our data indicate that the *S100A6* promoter is suppressed by p53 due to its interference with the function of Sp1 and NFκB. Therefore, the *S100A6* promoter suppression by p53 is, in fact, the result of insufficient activation by the Sp1 and NFκB transcription activators. Electrophoretic mobility shift assays point to disturbed binding of these transcription factors to the *S100A6* promoter in the presence of p53: lower amounts of the Sp1–DNA and NFκB–DNA complexes are formed in the presence of p53 than in its absence. This indicates that either Sp1 and NFκB decrease their affinity for DNA or are sequestered by p53, most possibly due to the formation of p53–Sp1 and p53–NFκB heterocomplexes. Such interaction between p53 and Sp1 has been previously described by other authors studying suppression of transcription by p53 (Bargonetti *et al*., 1997; Ohlsson *et al*., 1998; Xu *et al*., 2000; Sengupta *et al*., 2005).

In conclusion, we propose that the suppression of the *S100A6* promoter and the resulting decrease of the amount of calcyclin could well be a mechanism by which p53 inhibits cell proliferation. The weak or no suppression of the *S100A6* promoter by mutant p53 may be unable to initiate such a mechanism in cancers, leading to overexpression of calcyclin and, possibly, to the deregulation of cell cycle control. However, to confirm this hypothesis, a detailed analysis of p53-calcyclin interrelationship throughout the cell cycle should be performed.

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