Sp1 is involved in the transcriptional activation of $p16^{INK4}$ by $p21^{Waf1}$ in HeLa cells

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Abstract Both $p16^{INK4}$ and $p21^{Waf1}$ are very important negative regulators of the cell cycle. In this study we examined the effects of $p21^{Waf1}$ on the transcription of $p16^{INK4}$. We determined that $p21^{Waf1}$ can activate the transcription of $p16^{INK4}$, and that this effect is GC-box dependent. We also found that the transcription factor Sp1 plays a key role in this event. Up-regulation of Sp1 contributes to the transcriptional activation and protein level of $p16^{INK4}$ mediated by $p21^{Waf1}$, and is a potential point of cooperation between the p16/pRb and p14 (ARF)/p53 tumor suppressor pathways.

Key words: $p16^{INK4}$; $p21^{Waf1}$; Transcription regulation; Sp1

1. Introduction

$p16^{INK4}$ gene is a tumor suppressor involved in regulating cell cycle checkpoints. It is frequently inactivated by methylation or mutation [1]. The $p16^{INK4}$ protein binds specifically to and inhibits the cyclin-dependent kinases 4/6 (CDK4/6), which regulate cell cycle progression in G1 through phosphorylation of the retinoblastoma protein (pRb) [2,3]. $p21^{Waf1}$ is one key regulator of the cell cycle and acts as a universal inhibitor of CDKs [4]. It plays an important role in cellular senescence, proliferation, differentiation, as well as DNA damage and repair [5–7]. Significant data have shown that $p16^{INK4}$ and $p21^{Waf1}$ have very similar biological functions and it has been reported that $p21^{Waf1}$ is an effector for $p16^{INK4}$ [8]. However, much less is known about the effect of $p21^{Waf1}$ on the expression of $p16^{INK4}$, especially with regards to transcriptional regulation. Whether they can influence each other and whether there is crosstalk between these two pathways (p16/pRb and ARF/p53) remain open questions. In this report, we present our study of the influence of $p21^{Waf1}$ on the expression of $p16^{INK4}$ at the transcription level.

2. Materials and methods

2.1. Plasmids

The $p21^{Waf1}$ cDNA was a gift from Dr. David Beach (Howard Hughes Medical Institute, Cold Spring Harbor, NY, USA). The full-length 2.1-kb $p21^{Waf1}$ cDNA was inserted, in both antisense and sense orientation, into the cloning site of the retroviral vector pDOR-neo [9]. The pDOR-neo vector without an insert served as the control [10]. The Sp1 cDNA expression vector was a gift from Dr. Robert Tjian (University of California, Berkeley, CA, USA) and the Sp3 expression vector was a gift from Dr. G. Suske (IMT, Marburg, Germany). The pCMV vector without an insert also served as the control. The plasmids pSIR-0-EGFP, pSIR-620-EGFP and pSIR-870-EGFP acted as the reporter reconstructions, into which the different fragments of the promoter of $p16^{INK4}$ were inserted (pSIR-0-EGFP, no fragment of the promoter was inserted; pSIR-620-EGFP, 620 bp upstream of ATG was inserted; pSIR-870-EGFP, 870 bp upstream of ATG was inserted), all of them were gifts from Dr. W. Wang in our lab [11].

2.2. Cell culture

A human epithelioid cervical carcinoma cell line (HeLa cells) was cultured in Dulbecco's modified Eagle's medium (DMEM). Stable transfection using 1 μg pDOR-$p21^{Waf1}$ was performed with lipofectamine (Invitrogen). 400 μg/ml G418 was added into the medium for drug selection 48 h after transfection, until the stable positive cell clones were obtained. Mithramycin A (Sigma) (50, 100 nM) was added to the cells 24 h before harvest [12,13].

2.3. Construction of promoter-Luc constructs and luciferase activity assay

To make pGL3-870, an 870-bp 5′-fragment of human $p16^{INK4}$ promoter was digested with *Xho*I and *Hin*dIII from pSIR-870-EGFP, and then inserted into the same sites in pGL3-basic (Promega). pGL3-620 was generated with a 620-bp 5′-fragment of human $p16^{INK4}$ promoter digested with *Xho*I and *Hin*dIII from pSIR-620-EGFP. A 126-bp 5′-fragment of human $p16^{INK4}$ promoter was cloned into the *Sac*I site of the same vector by site-directed mutagenesis of the −620-bp $p16^{INK4}$ promoter (Quickchange™ site-directed mutagenesis methods, Stratagene). A synthetic double-strand oligonucleotide was used to create a new *Sac*I restriction site at position −126 bp (5′-GGGTCGGAGGGAGCTCTTCCGCCAGCAC-3′). Mutation of Sp family binding sites was performed with the same kit as mentioned above. They were called −449 m (5′-GGGGCGGATT-3′ to 5′-GGGGCaGATc-3′), −459m (5′-AAACGGGGCGGGG-3′ to 5′-AAACGGatCcGGG-3′) and dm (double mutation 5′-AAACGGGGCGGGGGCGGATT-3′ to 5′-AAACGGatCcGGGGCaGATc-3′). All plasmids were purified with QIAGEN Plasmid Midi Kits (Qiagen). Transfections were performed in 24-well plates using lipofectamine 2000 (Invitrogen). HeLa cells were harvested 48 h post-transfection and assayed for luciferase and β-galactosidase activities according to standard procedures. All transfections were carried out in triplicate, and all experiments were performed twice for confirmation.

2.4. Western blot

Cell extracts were prepared following standard procedures. Briefly, three to five volumes of lysis buffer (50 mM Tris–HCl, pH 7.4, 0.25 M

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NaCl, 0.1% Triton X-100, 1 mM ethylenediamine tetraacetic acid (EDTA), 50 mM NaF, 1 mM dithiothreitol (DTT), 0.1 mM Na_3VO_4) were added to a cell pellet. Then the following protease inhibitors were added: 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml leupeptin, 1 μg/ml aprotinin. After incubation on ice for 30 min, samples were centrifuged at 14 000 rpm for 5 min at 4°C to recover the supernatant. After proteins were electrophoresed in a 15% (for Id1 and p16) or 8% (for Sp1, Sp3, β-actin and E47) denaturing polyacrylamide gel and transferred to a polyvinylidene difluoride (PVDF) membrane, the membrane was blocked in 5% non-fat milk–Tris-buffered saline (TBS)–0.25% Tween 20 for 1 h and incubated with the primary antibody in TBS–0.25% Tween 20 for 1 h at room temperature. Complexes were detected with horseradish peroxidase-linked secondary antibody and enhanced chemiluminescence (SuperSignal, Pierce). The primary and secondary antibodies used in this study were all from Santa Cruz Biotechnology (Santa Cruz).

2.5. Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared from cells, following the previous described procedures [11]. Sp1 and Sp3 binding probes were annealed by heating the following single-stranded oligonucleotides: forward: 5′-AAGGAAACGGGGCGGGGGCGGATTTCTTTTTAACAGAG-3′ and reverse: 5′-CTCTGTTAAAAAGAAATCCGCCCCCGCCCCG-TTTCCTTCC-3′. Probes were labeled using Klenow at room temperature for 15 min. The labeled probe was purified with QIAquick Nucleotide Removal Kit (Qiagen). ^{32}P-labeled probe DNA (30 000–50 000 cpm) was incubated in DNA binding buffer (10 mM Tris–HCl, pH 7.5, 2.5 mM $MgCl_2$, 50 mM KCl, 1 mM DTT, 10% glycerol, and 25 μg/ml poly dI:dC) for 20 min at room temperature. DNA–protein complexes were analyzed on 6% polyacrylamide gels. Gels were dried and exposed to X-ray film for 24 h.

3. Results

3.1. p21^{Waf1} activates the expression of the p16^{INK4} in both the promoter-luc constructs and the protein in HeLa cells

Transient cotransfection studies with pGL3-870 and the p21^{Waf1} expression vector were performed in HeLa cells (it has previously been shown that fragments longer than 870 bp have no additional promoter activity [14]). The results showed that p21^{Waf1} augmented the p16^{INK4} promoter activity (Fig. 1A). The same tendency was shown in the stably transfected cells using the EGFP as reporter gene (Fig. 1B), the percentage of the positive cells is 10% higher than the control. To determine the location of the DNA sequences associated with the activation, the p21^{Waf1} vector was cotransfected with shorter sequences (620 and 126 bp) of the p16^{INK4} promoter DNA. Marked activation was found with the 620 bp construct (it is nearly 4-fold). The promoter region −127–−620 bp seemed particularly important (Fig. 1A), suggesting that p21^{Waf1} interacts with p16^{INK4} regulatory region through certain transcriptional regulators whose binding sites are located within this region.

To further demonstrate that whether the endogenous p16^{INK4} could be upregulated by p21^{Waf1}, Western blot was used to detect the protein level of p16^{INK4} after the transfection of p21^{Waf1}-sense and p21^{Waf1}-antisense. The expression of p16^{INK4} showed a slight increase of nearly 20% in the p21^{Waf1}-sense group and a dramatic decrease of nearly 50% in the p21^{Waf1}-antisense group, through the analysis of density versus control (Fig. 1C and D).

We then examined the character of the sequence from −127 to −620 bp of p16^{INK4} promoter in more detail. This region contains three GC-boxes (GGGCGG), two E-boxes (CANNTG) and some other regulatory elements. Among these elements, the GC-box is a prominent feature of the p16^{INK4} promoter. To determine whether the effect of

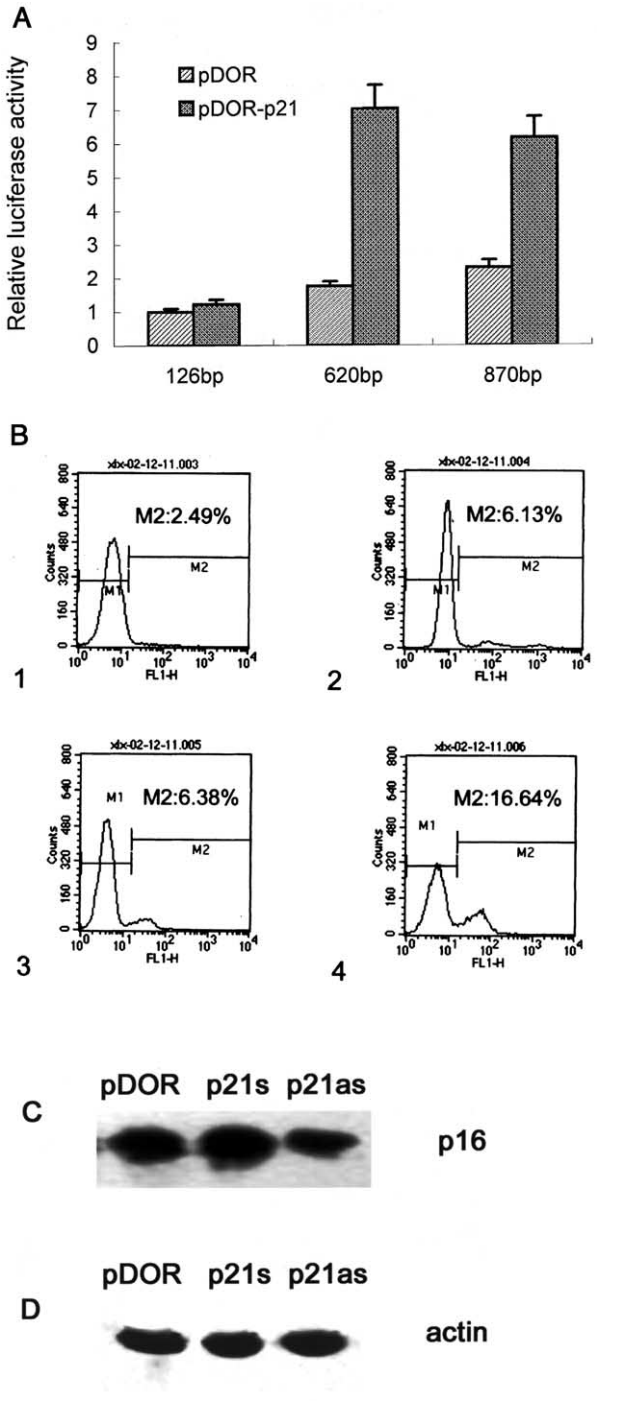

Fig. 1. The effect of transcriptional activation of p16^{INK4} by p21^{Waf1} A: Effect of p21^{Waf1} on expression of the p16^{INK4} promoter-luc constructs (126, 620 and 870 bp). Various deletions of the p16^{INK4} constructs (0.4 μg) were cotransfected with p21^{Waf1} (0.4 μg). Relative promoter activity (luciferase units/β-gal activity) following transfection is shown. B: The effect of p21^{Waf1} on transcriptional activity of p16^{INK4} transfected stably with p21^{Waf1} and pSIR-EGFP of the promoter of p16^{INK4}. Lane 1, pDOR+pSIR-0-EGFP; lane 2, pDOR-p21+pSIR-0-EGFP; lane 3, pDOR+pSIR-870-EGFP; lane 4, pDOR-p21+pSIR-870-EGFP. The intensity of the reporter EGFP was detected by flow cytometry. C, D: The effect of p21^{Waf1} on protein level of p16^{INK4} transfected stably with pDOR-p21^{Waf1}-sense and pDOR-p21^{Waf1}-antisense by Western blot analysis.

$p21^{Waf1}$ on $p16^{INK4}$ expression is mediated through these GC-boxes, we mutated two sites (−449 and −459 bp) individually and then in combination. Luciferase reporter constructs, containing different combinations of wild-type and mutated sites, were generated and transiently cotransfected with the $p21^{Waf1}$ expression vector (Fig. 2). Mutation of the −449 bp binding site reduced the expression of the $p16^{INK4}$-Luc reporter to the basal level while the mutation at −459 bp reduced the expression of the $p16^{INK4}$-Luc reporter by 80–90%. It could not promote its activities even when transfected with $p21^{Waf1}$. These results suggested that the GC-box at −449 bp is the key constitutive element of the $p16^{INK4}$ promoter and the GC-box at −459 bp is also significant.

3.2. *Sp1 is involved in the activation of $p16^{INK4}$ promoter by $p21^{Waf1}$*

The results of the $p16^{INK4}$-Luc experiment showed that $p21^{Waf1}$ can activate the transcription of $p16^{INK4}$, but $p21^{Waf1}$ itself is not a transcription factor and cannot bind the $p16^{INK4}$ promoter directly. To find which transcription factor plays the most important role, we used Western blot analysis to examine the protein level of transcription factors Sp1, Sp3, E47 and Id1, which have been demonstrated to participate in the transcriptional regulation [15,16]. The results showed that the expression of Sp1 increased significantly after $p21^{Waf1}$ transfection (Fig. 3A). There was no difference in the expression of Sp3 (Fig. 3A) or E47 (Fig. 3B) after transfection with $p21^{Waf1}$, while a small reduction in the expression of Id1 was found (Fig. 3C). These results suggested that among these transcription factors, Sp1 is the key factor in affecting the activation of $p16^{INK4}$ by $p21^{Waf1}$. While E47–Id1 heterodimeric complex may also have an effect, however, it may not be important to this event.

3.3. *Sp1 activates the expression of $p16^{INK4}$-Luc and promotes the protein level of $p16^{INK4}$*

To investigate whether Sp1 can activate the $p16^{INK4}$ promoter, an Sp1 expression vector was transfected with $p16^{INK4}$-Luc reporter in HeLa cells. The results showed that Sp1 can activate the $p16^{INK4}$ promoter and exerts its effects through the promoter region −127 to −620 bp (Fig. 4A, B). Transfection with Sp3 expression vector showed that Sp3 acts as a negative regulator of the $p16^{INK4}$ promoter in this system (Fig. 4A, B).

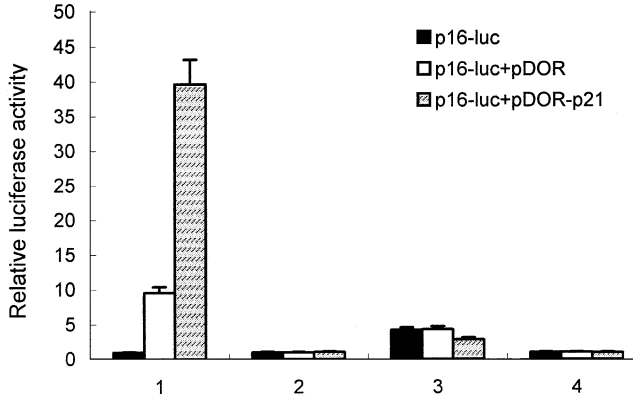

Fig. 2. The effect of GC-box mutations on the transcriptional activity of $p16^{INK4}$ promoter measured by luciferase assay. Lane 1, pGL3-basic negative control (□ 0-bp $p16^{INK4}$ promoter fragment) and positive control (■▨ −620-bp $p16^{INK4}$ promoter fragment); lane 2, 620-bp promoter fragment with mutation within the GC-box at position −449 bp; lane 3, 620-bp promoter fragment with mutation within the GC-box at position −459 bp; lane 4, 620-bp promoter fragment with mutation at both −449 and −459 bp. These were transfected into either HeLa cells only or HeLa cells transiently transfected with pDOR or pDOR-p21 construct.

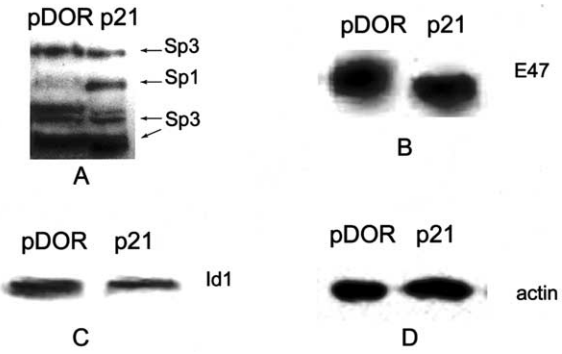

Fig. 3. Western blot analysis of HeLa cells transfected stably with pDOR-$p21^{Waf1}$. A: Sp1 and Sp3, B: E47, C: Id1, and D: β-actin.

To further support the results above, mithramycin A, a potent inhibitor of Sp1 binding to GC-box in DNA, was added to the cells to detect whether the protein level of $p16^{INK4}$ could be decreased when the activity of Sp1 was blocked. The result indicated that the addition of mithramycin A to the cells led to a concentration-dependent inhibition of the protein level of $p16^{INK4}$ with 40% inhibition at 50 nM and nearly 70% inhibition at 100 nM mithramycin A concentration (Fig. 4C, D).

3.4. *Sp1 and Sp3 bind to the GC-box*

Using nuclear extracts of HeLa cells with double-stranded oligo DNA containing GC-box showed three specific bands (Fig. 5). Excess amounts of oligo DNA (specific competitor) diminished the specific bands (Fig. 5, lane 3). Preincubation with Sp1 antibodies supershifted the top band (Fig. 5, lane 4), whereas preincubation with Sp3 antibodies diminished the bottom band (Fig. 5, lane 5). Coincubation with Sp1 and Sp3 antibodies supershifted the top band and diminished the bottom band (Fig. 5, lane 6). These experiments suggested that Sp1 and Sp3 could bind to the GC motif and activate or inactivate $p16^{INK4}$ promoter activity.

4. Discussion

$p21^{Waf1}$- as well as $p16^{INK4}$-mediated inhibition of CDKs will result in non- phosphorylation of Rb. Due to this E2F could not be released to activate a certain gene to regulate the progression from G1 phase to S phase of the cell cycle. This is the classic pathway. In addition, CDK inhibitors, for example $p21^{Waf1}$, can also affect certain transcription factors directly to activate or inactivate target genes [17,18]. There is much evidence to support the fact that $p21^{Waf1}$ is an effector of $p16^{INK4}$, which is associated with a posttranscriptional induction of $p21^{Waf1}$ [19]. In addition, $p21^{Waf1}$-mediated inhibition of CDK2 is a common element of the ARF/p53 and $p16^{INK4}$/pRb response pathways, as well as a potential point of cooperation between them. However, the question of what is the effect of $p21^{Waf1}$ on $p16^{INK4}$ remains. Recently, there is a hypothesis that $p21^{Waf1}$ can initiate the expression of

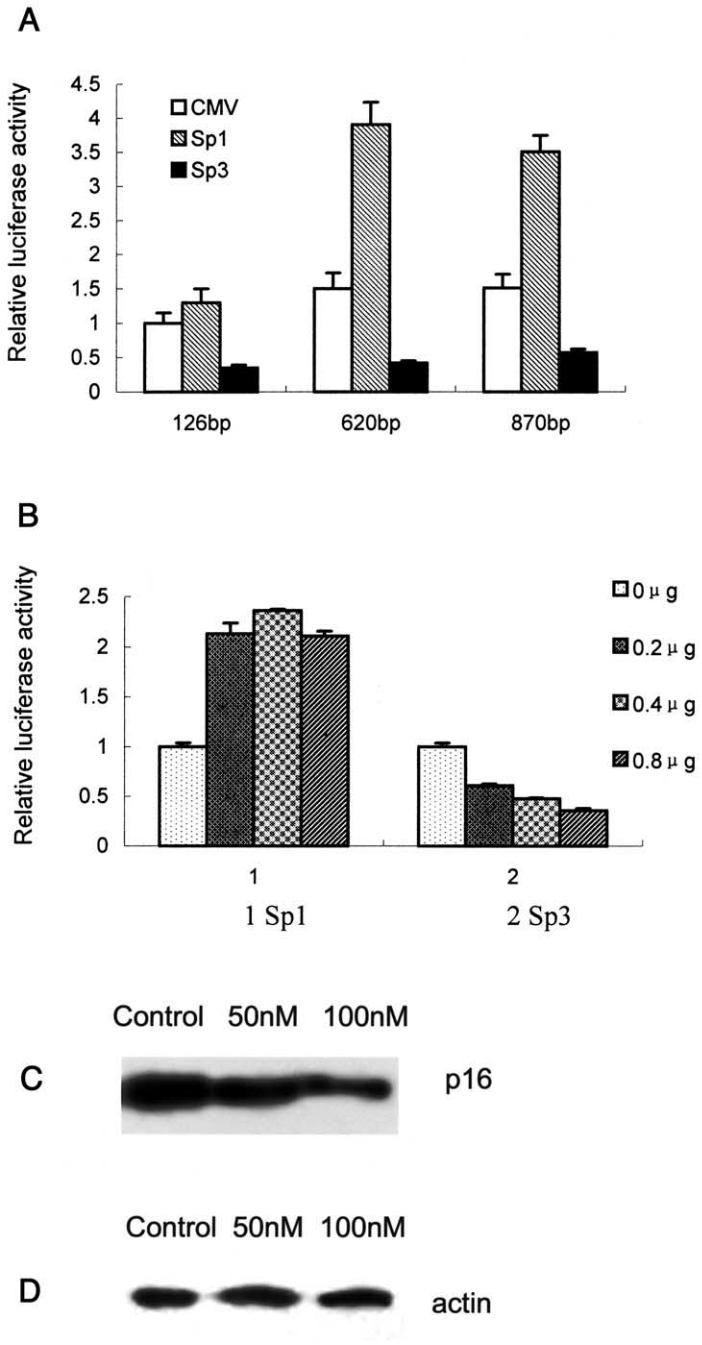

Fig. 4. Transcriptional activities of the $p16^{INK4}$ promoter deletions with Sp1 or Sp3. A: The different $p16^{INK4}$ promoter pGL3-Luc constructs (0.4 μg) were cotransfected with 0.4 μg of pCMV-Sp1, pCMV-Sp3 or pCMV only as control, and 0.2 μg of pSV-β-gal. The luciferase and β-gal activity values were determined. B: Various doses of the pCMV-Sp1 or pCMV-Sp3 (0, 0.2, 0.4, 0.8 μg) were cotransfected with 0.2 μg of pGL3-Luc constructs. Luciferase and β-gal activity values were determined. C, D: The effect of Sp1 on protein level of $p16^{INK4}$ in HeLa cells treated with mithramycin A by Western blot analysis.

$p16^{INK4}$ [20]. How does $p21^{Waf1}$ behave in this case? The aim of this paper is to further explore the relationship between $p21^{Waf1}$ and $p16^{INK4}$ at the transcription level and search for the potential transcription factors involved in this process.

HeLa cell line, a typical cell line that has high transfection efficiency, was reported to maintain low p53 levels (from the website of ATCC, www.atcc.org), and in this cell line the functions of p53 and pRb were deficient [21]. Therefore, $p21^{Waf1}$, as the downstream target of p53 pathway, will not be affected by p53, and $p16^{INK4}$ will not be regulated by pRb. Thus this cell line is an ideal model for studying the biological effect of $p21^{Waf1}$ expression on $p16^{INK4}$.

The complexity of tissue- or stage-specific gene regulation normally results from well-defined interactions between the promoter and the transcription factors. The promoter region of a gene contains multiple binding sites for transactivators. Some elements, like the E-box, GC-box and AP-1-like site, which are all the binding sites of E-proteins, Sp family proteins and the transcription factor JunB respectively, are distributed in the $p16^{INK4}$ promoter. The transactivation effect of

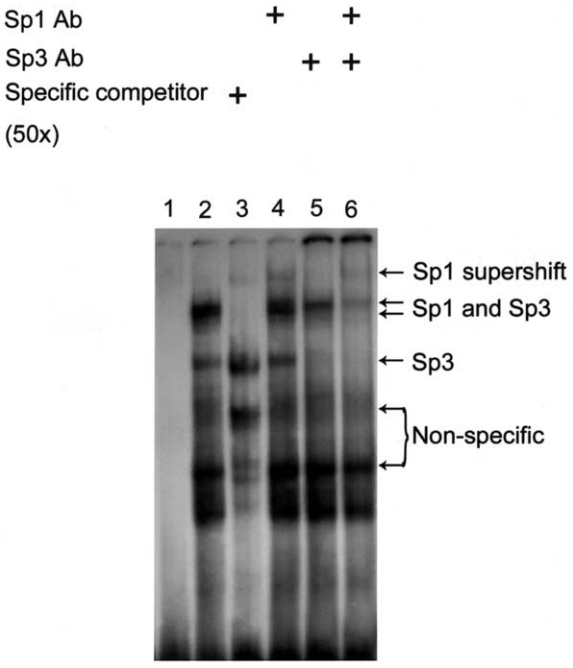

Fig. 5. EMSA using nuclear extracts of HeLa cells with double-stranded oligo DNA containing GC-box (−435 to −474 bp). Excess amounts of oligo DNA (specific competitor) diminished the specific bands (lane 3). Anti-Sp1 antibodies supershifted the top band (lane 4), anti-Sp3 antibodies diminished the bottom band (lane 5). Anti-Sp1 and anti-Sp3 antibodies supershifted the top band and diminished the bottom band (lane 6). The free probe without nuclear extracts (lane 1) and the free probe with nuclear extracts (lane 2) were as control.

$p21^{Waf1}$ on the $p16^{INK4}$ promoter raises the question which transcription factor makes the most significant contribution to the event.

Among these transcription factors, Sp1 can stimulate the transcription not only from proximal promoters, but also from distal enhancers [22]. Although Sp3 was found to be highly homologous to Sp1 with similar affinities for GC- and GT-boxes, there are some striking functional differences between them. In some cell lines, it can activate transcription [23–26]. However, under other circumstances Sp3 is only weakly active, and in some cases Sp3 can repress transcription driving by Sp1 or other transcription factors [27–29]. In this research, the effect of the expression of Sp1 upregulated by $p21^{Waf1}$ may be relevant to the mitogen-activated protein/extracellular signal-regulated kinase pathway, as well as other potential mechanisms. It was reported that the phosphorylation of Sp1 in known mitogen-activated protein kinase residues (threonine 453 and 739) was promoted, when operated with agents that could arrest the cell cycle, thereby leading to an Sp1 binding increase and transcription enhancement [30]. In addition, expression of Sp1 can be increased by low concentrations of CDK inhibitors such as UCN-01 (the chemical inhibitor) and overexpression of $p21^{Waf1}$ by increasing the levels of Sp1 mRNA [17]. The effects that Sp1 activates $p16^{INK4}$ were further conformed using mithramycin A, an inhibitor of Sp1 binding, other than the luciferase assay. The effect of activation on $p16^{INK4}$ promoter is dependent on the integrity of the regulating sequences, such as the region −449 to −459 bp where Sp1/Sp3 bind to in $p16^{INK4}$ promoter, the mutation within this region caused the opposite effect, as a new negative element may be formed or the spatial structure could be changed, which could then alter the combination between the transcription factor and the element. Although Sp3 acts as a suppressor of $p16^{INK4}$, its level is not significantly affected by $p21^{Waf1}$.

E47 belongs to the basic helix-loop-helix (bHLH) family of proteins. It could bind DNA as homodimers or as heterodimers with the class B or tissue-specific bHLH transcription factors and activate transcription of target genes that contain the E-box motif CANNTG in their promoter [31]. Id1, a member of Id HLH protein family, acts by associating with bHLH transcription factors, such as E47, preventing these factors from forming functional hetero- or homodimeric DNA binding complexes; thus suppressing the activation of transcription induced by E-proteins. We have shown that $p21^{Waf1}$ expression has no significant effect on E47 and only leads to a slight decrease of Id1. This indicates that E47–Id1 is not the major effector in the activation of $p16^{INK4}$ transcription induced by $p21^{Waf1}$.

Recently, great progress has been made in the understanding of transcriptional regulation of different CDK inhibitors. However, most studies have focused on the roles of the element of the promoter and transcription factors in individual CDK inhibitors. The interactions between them have rarely been reported. This study begins to unravel the complex relationships between two of the most important negative regulators of the cell cycle, $p16^{INK4}$ and $p21^{Waf1}$, at the level of transcriptional regulation. This knowledge is needed to fully understand cell cycle progression, as well as the mechanisms of tumorigenesis and senescence. Nevertheless, further work should be carried out, in normal and other cell lines, to increase general information from multiple perspectives.

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