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Increased Expression of Integrin β1 Subunit Enhances $p21^{WAF1/Cip1}$ Transcription Through the Sp1 Sites and p300-Mediated Histone Acetylation in Human Hepatocellular Carcinoma Cells

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Abstract Integrins, a class of membrane receptors, are major players in transmitting the mechanical force across the plasma membrane. We previously reported that overexpression of integrin β1 subunit imposed a growth inhibitory effect on the hepatocellular carcinoma cell line SMMC-7721 through transcriptional activation of $p21^{WAF1/CIP1}$ gene. In this study, we further determined the molecular mechanisms underlying $p21^{WAF1/CIP1}$ expression induced by integrin β1 overexpression. We report herein that overexpression of integrin β1 subunit upregulates $p21^{WAF1/Cip1}$ transcription through a p53-independent pathway. The overexpressed integrin β1 activates the $p21^{WAF1/Cip1}$ promoter through the Sp1/Sp3 sites and makes more transcription factor Sp1 recruited to the proximal p21 promoter region. In addition, it makes the acetylation value of histone proteins increased across some parts of the $p21^{WAF1/Cip1}$ gene, especially in the promoter region. The transcriptional co-activator p300, which possesses intrinsic histone acetyltransferase, was found to be involved in the integrin β1-mediated histone acetylation and p21 transcriptional activation. Therefore, these findings presented the mechanisms by which integrin β1 induced the elevated p21 expression in hepatic cancer cells. J. Cell. Biochem. 101: 654–664, 2007.

Key words: integrin; $p21^{CIP1}$; transcriptional regulation; promoter activity; histone acetylation; p300/CBP

Integrins are a family of cell surface molecules mediating cell–cell and cell–extracellular matrix (ECM) interactions. Together with growth factor receptors, integrins could upregulate cyclins D and E and downregulate CKIs $p21^{cip1}$, $p27^{kip1}$, and $p57^{kip2}$ [Giancotti and Ruoslahti, 1999; Sherr and Roberts, 1999]. This action allows cells to pass through the G1/S transition and complete the cell cycle. However, many studies have demonstrated that integrins give rise to growth inhibition rather than growth stimulation [Giancotti and Ruoslahti, 1990; Giancotti and Mainiero, 1994; Wang et al., 1999; Zhou et al., 2000]. Integrin a5β1 has been often observed to be lost in cancerous areas other than in its normal counterpart tissues [Su et al., 2002]. It is apparent from these studies that integrin signaling may play a major role in negative control of cell growth, which may be lost in some cancer cells, and the mechanisms of this effect are not completely known yet.

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In our previous study, after the human hepatocellular cancer cell line SMMC-7721 was stably transfected with integrin β1 subunit, its cell cycle was suppressed due to a S-phase delay [Liang et al., 2003]. In the following study, we found that in this integrin β1 subunit-overexpressed cells (β1-77221 cells) the expression level of cyclin dependent kinase (Cdk) inhibitor $p21^{WAF1/Cip1}$ was markedly increased. This might partly explain the negative control of cell cycle by the overexpression of integrin proteins. And we also found that the p21 mRNA level was increased as well, suggesting the transcriptional activation of p21 by integrin β1 subunit [Liang et al., 2004].

$p21^{WAF1/Cip1}$ was first cloned and characterized as a mediator of p53-induced growth arrest and its expression is usually regulated at the transcription level [el-Deiry et al., 1993; Gu et al., 1993; Dulic et al., 1994]. p21 can negatively regulate cell cycle progression from G1 to S phase by inhibiting the catalytic activity of Cdk [Gu et al., 1993; Harper et al., 1993; Xiong et al., 1993; Dulic et al., 1994]. Although p21 has been shown to be expressed in a p53-dependent fashion in response to DNA damage [Macleod et al., 1995], its expression could also be upregulated p53-independently by various stimuli including transforming growth factor TGF-β [Hu et al., 1999], tamoxifen [Lee et al., 2000], progesterone [Owen et al., 1998], and nerve growth factor. Accumulating evidence suggests that the p53-independent induction of p21 by these stimuli could be influenced by signal transduction pathways that mediate cell growth, differentiation, and stress response.

Of particular importance has been the elucidation of the transcriptional mechanisms that operate during p21 gene induction by the above listed factors. For many such factors, including p53, retinoic acid, vitamin D3, interferon g, and others, specific cis-acting DNA motifs have been identified on the p21 promoter in a region that extends between positions 22,300 and 2,210 relative to the transcriptional initiation site. On the other hand, an increasing number of regulatory factors including TGF-β, progesterone, phorbol esters, and phosphatase inhibitors mediate their effects on p21 gene expression via the proximal region of the promoter (2,210 to 11 base pairs). The proximal promoter contains characteristic GC-rich motifs that serve as binding sites for members of the Sp1 family of ubiquitous transcription factors [Datto et al., 1995].

The present work is part of our effort to understand the linkage between integrin β1 subunit overexpression and $p21^{WAF1/Cip1}$ transcriptional activation. $p21^{WAF1/Cip1}$ expression is regulated by a rapidly growing list of physiological and pathological factors. And the study of transcriptional regulation of p21 has been moving its focus to chromatin level recently. The molecules involved in chromatin transcription of p21 include DNA (promoter, enhancer, or silencer), histones, and non-histone proteins. In this study, we showed that integrin β1 subunit could upregulates $p21^{WAF1/Cip1}$ expression through the activation of Sp1 binding sites, and the p300-mediated acetylation of histone proteins is required in the integrin β1 subunit induced activation of $p21^{WAF1/Cip1}$ expression.

MATERIALS AND METHODS

Cell Lines and Cell Culture

Human hepatocellular carcinoma cell lines SMMC-7721, Mock-7721 (mock plasmid stably transfected SMMC-7721), and β1-7721(integrin β1 subunit stably overexpressed SMMC-7721) cells were introduced previously [Liang et al., 2004]. Cells were cultured in RPMI1640 (Gibco, Invitrogen) supplemented with 10% fetal bovine serum (HyClone Laboratories, Logan, UT) and 1% penicillin/streptomycin (Life Technologies, Inc.).

Plasmid Construction

The human wild-type $p21^{WAF1/Cip1}$ promoter-luciferase reporter construct, PGL3-luc, was a kind gift from Dr. Bert Vogelstein (Howard Hughes Medical Institute, Johns Hopkins University). The full-length human $p21^{WAF1/Cip1}$ promoter-luciferase reporter constructs, pGL3-1908 was introduced in our previous studies [Liang et al., 2004]. The 5′deletion construct of pGL3-1908 containing only the proximal region of p21 promoter, pGL3-217, was also described before [Liang et al., 2004]. The wild-type (wt) p300 expression vector (pCI-p300) and its HAT deletion derivative (pCI-p300HAT △ 1,472–1,522) are kind gifts from Dr. Joan Boyes (Institute of Cancer Research, London, UK). The E1A expression vector (pCDNA3-E1A) and its dominant negative structure (E1A △ 2-63) are kind gifts from Dr. Elizabeth Moran

(Department of Biochemistry, Temple University School of Medicine).

Reagents and Antibodies

Sodium butyrate (NaB) was obtained from Calbiochem. Antibodies against p21 (Antibody Diagnostica, Inc.), p53 (Pab 1801, Santa Cruz Biotechnology), GAPDH (Sigma), were used for immunoblotting. Antibodies against Sp1 (UPSTATE) and acetylated histone H3 (UPSTATE) were used for chromatin immunoprecipitations.

Dual-Luciferase Reporter Assays

Mock-7721 and β1-7721 were cultured in 24-well plates. Cells were co-transfected with the appropriate luciferase-reporter plasmids (1 μg/ml) and SV40 renilla luciferase plasmids (1 μg/ml) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Cell lysates were obtained using 250 ml per well of passive cell lysis buffer (Promega). Luciferase activity was measured using 20 ml of cell lysate per assay tube in an Autolumat machine (LB 953; EG&G Berthold, Oak Ridge, TN). Relative luciferase activity (RLU, relative luciferase unit) was obtained through normalization of the firefly luciferase activity by renilla luciferase activity. The reporter constructs used here included pGL3-1908 containing full length 1908 bp of the $p21^{WAF1/Cip1}$ gene promoter; pGL3-217 containing proximal 217 bp region of the $p21^{WAF1/Cip1}$ promoter; pGL3-217m1-2, in which the firs two Sp1 sites of pGL3-217 were mutated; pGL3-217m3, in which the third Sp1 sites of pGL3-217 was mutated; pGL3-217m4, in which the fourth Sp1 sites of pGL3-217 was mutated; and the pGL3-217m5-6, in which the last two Sp1 sites of pGL3-217 were mutated. The latter four constructs were generated by digesting pGL3-217 with *Kpn* I and *Hin*d III and the following ligation of the above digested plasmids with the synthesized DNA fragments containing the appropriate mutant Sp1 binding sites. These constructs were confirmed by digestion and sequencing.

RT-PCR

Total RNAs were isolated using the Trizol system (Watson Biotechnologies, Shanghai, China) according to the manufacturer's guidelines. RT-PCR was performed to quantify the mRNA level of the p21 gene. Oligo dT primer and MMLV-RTase were used for first strand synthesis. cDNA products (2 μl) were mixed with Taq DNA polymerase (SABC, Luoyang, China), 50 pmol/l of each appropriate primer, 200 μmol/l each dNTP in a reaction buffer containing 10 mmol/l Tris-HCl (pH 8.3), 50 mmol/l KCl, 0.01% (W/V) bovine serum albumin (BSA), 2 mmol/l $MgCl_2$ in a final volume of 100 μl. The primer pair for p21 was: 5′-CAG TGG ACA GCG AGC AGC TG (sense) and 5′-TAC AAG ACA GTG ACA GGT CC (antisense). Primers for β-actin were described before (17), which were used as the internal control. The expected product sizes were as follows: p21, 282 bp; β-actin, 412 bp. The samples were amplified for 32 cycles at cyclic temperatures of 94°C 45 s, 61°C 45 s, 72°C 45 s. PCR products were analyzed through 1% agarose gel electrophoresis and following ethidium bromide staining. The band area of p21 was measured and normalized by that of β-actin, and then the specific p21 mRNA level was estimated.

Western Blot Analyses

Cells were plated at a density of 5×10^5 cells/ml of medium in flasks and grown for 24 h. Cultured cells were then harvested with trypsinization and centrifugation and rinsed twice in ice-cold PBS, and lysed in 1U sodium dodecyl sulfate (SDS) lysis bufer (50 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 100 μg/ml phenylmethylsulfonyl) fluoride, 10 μg/ml leupeptin, and 5 mM Na_3VO_4) for 10 min on ice. The samples were boiled and clarified by centrifugation at 12,000*g* for 8 min at 4°C. The supernatants were transferred to a microcentrifuge tube and stored at −20°C. Protein lysates were resolved by SDS–polyacrylamide gel electrophoresis (PAGE) and transferred onto polyvinylidene difluoride membranes. The membranes were blocked in 5% non-fat dry milk in PBST (PBS+0.05% Tween 20) and incubated with mouse anti-human integrin β1 subunit McAb (BD Transduction Laboratories, 1:500∼1:1,000) or rabbit anti-human p21 polyclonal antibody (Santa Cruz, 1:500) diluted in 5% milk in PBST overnight at room temperature. Following three washes in PBST, the blots were incubated with the appropriate horseradish peroxidase-conjugated secondary antibody (Calbiochem) diluted in 1:500. Finally, these blots were washed three times in PBST, and developed by enhanced chemiluminescence (Amersham).

ChIP Assays

The Chromatin immunoprecipitation assays were carried out using the Chromatin immunoprecipitations (ChIP) assay kit (UPSTATE). When cells were grown to 1×10^6 on a 10 cm dish, formaldehyde was added to the cells to a final concentration of 1%, and the cells were then incubated at 37°C for 10 min. Cells were collected and suspended in 1 ml of ice-cold PBS containing protease inhibitors (Complete, Boehringer Mannheim). Cells were pelleted, resuspended in 0.5 ml of SDS lysis buffer (1% SDS, 10 mMEDTA, 50 mM Tris-HCl, pH 8.1), and incubated on ice for 10 min. The lysate was sonicated to shear the chromatin to an average length of <2 kb. Samples were diluted 10-fold with the immunoprecipitation dilution buffer (1% v/v Triton X-100, 16.7 mM Tris, pH 8, 1.2 mM EDTA, 167 mM NaCl plus the protease inhibitors). A 75 μl slurry of salmon sperm DNA/protein A-agarose was added to reduce nonspecific binding, and the mixture was rotated for 1 h at 4°C then centrifuged at 500*g* for 1 min. Precleared chromatin solutions were incubated with antibody (anti-sp1/anti-AcH3)or with no antibody (negative control) and rotated at 4°C for 12 h. Immune complexes were collected by adding 60 μl of salmon sperm DNA/protein A-agarose slurry for 4 h with rotation. Samples were washed and the immunoprecipitated material was eluted by three successive 5 min incubations with 150 μl of elution buffer (1% (w/v) SDS, 50 mM $NaHCO_3$). To reverse the formaldehyde-induced crosslinking, the eluates were pooled, NaCl was added at a final concentration of 0.3 M and the samples were incubated at 65°C for 4 h. Immunoprecipitated DNA (both immunoprecipitation samples and Input) was recovered by phenolychloroform extraction and ethanol precipitation and analyzed by PCR. Specific primers spanning overall promoter and part of the coding region of $p21^{WAF1/Cip1}$ were used, and the optimal reaction conditions were also determined for each primer pair. The primer pairs used for p21 ChIP analysis were described previously [Richon et al., 2000]: 5′-ACC AAC GCA GGC GAG GGA CT-3′ (uP1), 5′-CCG GCT CCA CAAGGAACT GA-3′ (dP1), 5′-GGT GTC TAG GTG CTC CAG GT-3′ (uP2), 59-GCA CTC TCC AGG AGG ACA CA-39 (dP2), 59-CGT GGT GGT GGT GAG CTA GA-3′ (uP3), 5′-CTG TCT GCA CCT TCG CTC CT-3′ (dP3), 5′-GAG GCC CAC AAG GAC TCT CA-3′ (uP4), 5′-GCT GAG ATC ATG CCA CCT GC-3′ (dP4), 5′-CGG TGC TTG GTC TCT ATG AA-3′ (uP5), 5′-TGG CCA CAC TGA GGA ATG AT-3′ (dP5), 5′-AGG AAT CCC TGG TCA CGC TC-3′ (uI1), 5′-GTG GTG GAC ACA GTG GCG TA-3′ (dI1), 5′-AGC TGA GCC GCG ACT GTG AT-3′ (uE2), 5′-CTG AGG AGA GAC AGC AGA AG-3′ (dI2), 5′-AAT CGT CCA GCG ACC TTC CT-3′(uE3), and 5′-ACA TGG GGA GCC GAG AGA AA-3′ (dE3).

RESULTS

Integrin β1 Subunit Activates $p21^{WAF1/Cip1}$ Transcription Through a p53 Independent Pathway

Previously, we demonstrated that p21, functioning as a cell cycle inhibitor, was remarkably upregulated in β1-7721 human hepatic cancer cell line where integrin β1 subunit was stably overexpressed [Liang et al., 2004]. As shown in Figure 1A,B, both protein and mRNA levels of p21 were significantly higher in β1-7721 than in Mock-7721 cells.

We next investigated whether overexpression of integrin β1 subunit could stimulate the p21 gene promoter activity. Both Mock-7721 and β1-7721 cells were transiently transfected with pGL3-1908 containing the full-length wild-type p21 promoter region. As shown in Figure 1C, relative luciferase activity controlled by wild-type full-length p21 promoter was increased to more than three folds in β1-7721. According to the previous literature [Archer and Hodin, 1999], p21 gene is regulated mainly through p53-dependent and p53-independent signaling pathway. So we also transiently transfected a 5′ deletion construct of the $p21^{WAF1/Cip1}$ promoter, pGL3-217, into SMMC-7721 or β1-7721 cells, and examined which pathway was involved in p21 upregulation induced by overexpression of integrin β1. It was found that the luciferase activity driven by pGL3-217 lacking two p53 binding sites was also increased to threefold in β1-7721 than in Mock-7721 cells (Fig. 1C). Altogether, these findings indicated that β1 integrin might activate the p21 promoter in a p53-independent manner. Actually, overexpression of β1 integrin could not affect the p53 expression level in SMMC-7721 cells (L02 as a positive control) (Fig. 1D), which further confirmed that p53 was not involved in the integrin

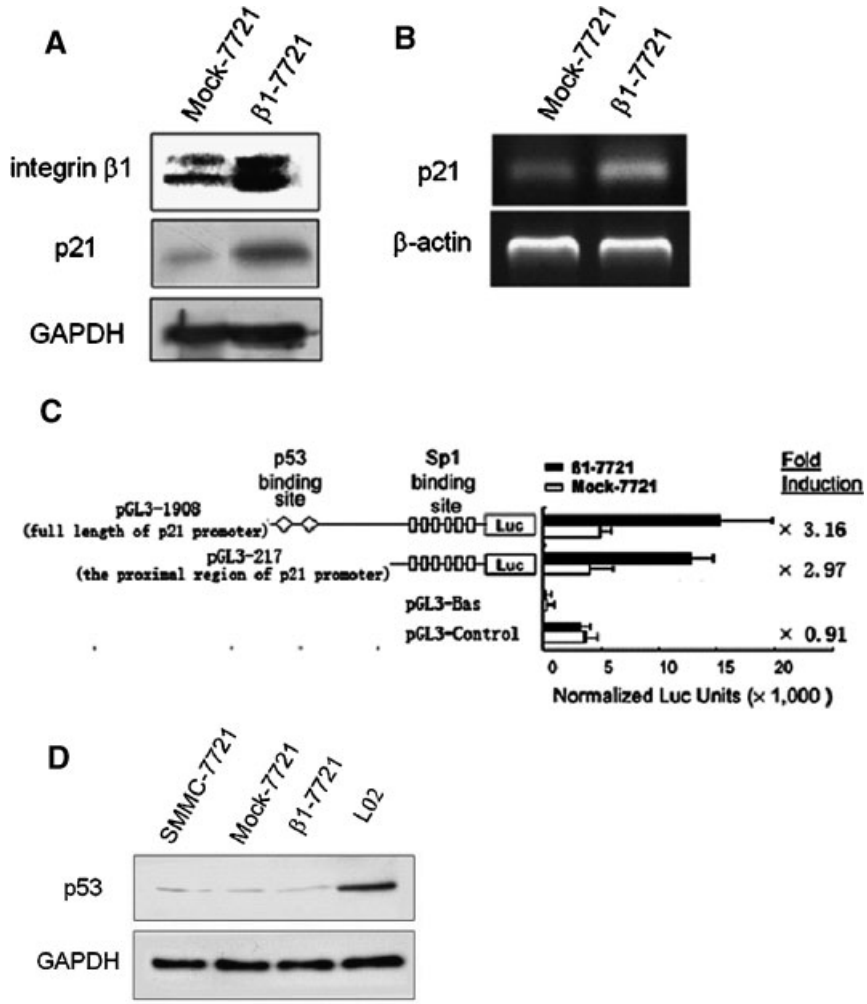

Fig. 1. Overexpression of integrin β1 activates $p21^{WAF1/Cip1}$ transcription through a p53-independent pathway. **A**: The protein levels of p21 and integrin β1 subunit in Mock-7721 and β1-7721 were investigated by immunoblotting analysis as described under ''Materials and Methods'' section. The expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was regarded as a loading control. **B**: The mRNA levels of p21 in Mock-7721 and β1-7721 were analyzed using RT-PCR, and normalized with the amount of β-actin. **C**: Luciferase activity assays of 5′ deletion constructs of the p21 promoter. The firefly luciferase reporter constructs, pGL3-Bas, pGL3-Control, pGL3-217, and pGL3-1908 were transiently co-transfected with renilla luciferase reporter into the two cell lines, and the firefly luciferase activities were analyzed and normalized with renilla luciferase activity. NLU, normalized luciferase units. **D**: p53 expression levels were detected by Western blot analysis in the four cell lines SMMC-7721, Mock-7721, β1-7721, and L02. The L02 used here was a positive control for p53 expression.

β1-induced p21 expression. So the next focus will be on the proximal promoter region of p21.

Integrin β1 Subunit Upregulates $p21^{WAF1/Cip1}$ Expression Through the Sp1/Sp3 Sites

It is well known that p21 expression was controlled by various transcription factors together with some co-activators or corepressors, especially Sp1/Sp3 and its co-activators [Biggs et al., 1996; Billon et al., 1999]. In our previous study, we showed that the proximal region of p21 promoter (between −189 and +28 bp) might harbor the core elements that controlled p21 gene expression in hepatic cancer cells [Liang et al., 2004]. There are six potential Sp1/Sp3-binding sites in the very limited proximal region of p21 promoter (Fig. 2A). To determine whether the Sp1 binding sites were involved in transcriptional activation of p21 by integrin β1 subunit, we constructed a series of Sp1-binding-site mutation plasmids of pGL-217 as introduced in the "Materials and Methods" section. Identification of these p21 promoter mutants were shown in Figure 2B. These constructs were used to assay the luciferase activity in Mock-7721 or β1-7721. We found that

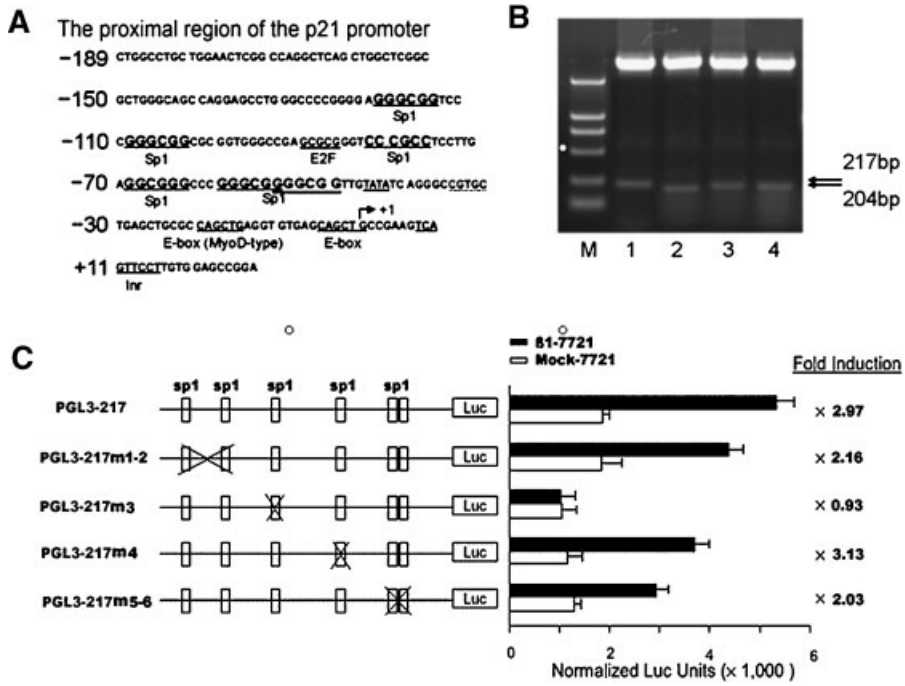

Fig. 2. Integrin β1 subunit upregulates $p21^{WAF1/Cip1}$ expression through activation of Sp1 binding sites. **A**: Sequence representation of the proximal region of the p21 gene promoter (−189 to +28). Some putative binding sites for transcription factors regulating the p21 promoter activation are indicated and underlined. The dotted line represents the mutant-binding site for E2F transcription factor, and +1 indicates the transcription start site, Inr, initiator. **B**: Identification of the p21 promoter mutants. The four constructs were digested with the same two restriction enzymes *Kpn* I/*Hin*d III and resolved by 0.8% agarose gel electrophoresis. **Lane 1**, pGL3-217m1-2; **lane 2**, pGL3-217m3; **lane 3**, pGL3-217m4; **lane 4**, pGL3-217m5-6; M, DL-2000 DNA marker (TaKaRa). The length of the insert in each lane is as follows. Lane 1, 204 bp; lane 2, 217 bp; lane 3, 217 bp; lane 4, 214 bp. **C**: Mutation analysis of the p21 promoter. Four different mutants shown at the left are identical to the pGL3-217 except for the Sp1 binding site mutations indicated in the picture. These constructs identified above were transiently transfected into the Mock-7721 and β1-7721 cells. The firefly luciferase activity of each construct was analyzed and the data were normalized to the activity of renilla luciferase and are shown as means ± SD. NLU, NLU, normalized luciferase units.

mutation of the 1st and 2nd Sp1 sites did not significantly affect the proximal promoter activity of p21 in the Mock-7721 or β1-7721 cells. Because the luciferase activity is still higher about twofold in β1-7721 than in Mock-7721. Similar results were found for mutations of the 4th, 5th, and 6th Sp1 binding sites of p21 proximal promoter (Fig. 2C). However, when the 3rd Sp1 binding site was mutated, the activity of the p21 promoter in both mock-7721 and β1-7721 cells was markedly reduced to the basal promoter activity over the control, suggesting that the 3rd Sp1 site (−82∼−77 bp) plays an important role in the p21 promoter activity induced by β1 integrin.

More Ubiquitous Transcription Factor Sp1 Were Recruited Into the p21 Promoter in β1-7721 Cells

Sp1 belongs to a zinc finger family of transcription factors that recognizes the GC-rich DNA sequences. We have shown above that Sp1/Sp3 sites played a crucial role in integrin-induced p21 promoter activation. But we did not have any direct evidence to support the hypothesis that increased expression of integrin β1 could alter the binding ratio of Sp1 to these sites and thereafter affect the promoter activity of p21. In fact, the protein and mRNA levels of Sp1 expressed in β1-7721 have no difference with that in Mock-7721 (Fig. 3A,B). Here we wonder whether more Sp1 physically binds to the proximal region of p21 promoter when integrin β1 subunit was overexpressed. To resolve this issue, we performed ChIP assays and the following polymerase chain reaction. The primer pair used here spans the proximal 324 bp region (−324 to −1) of p21 gene which contains all of the six Sp1-binding Fig. 2A). The result showed that more transcription factor Sp1 was recruited into the proximal region of p21 promoter in β1-7721

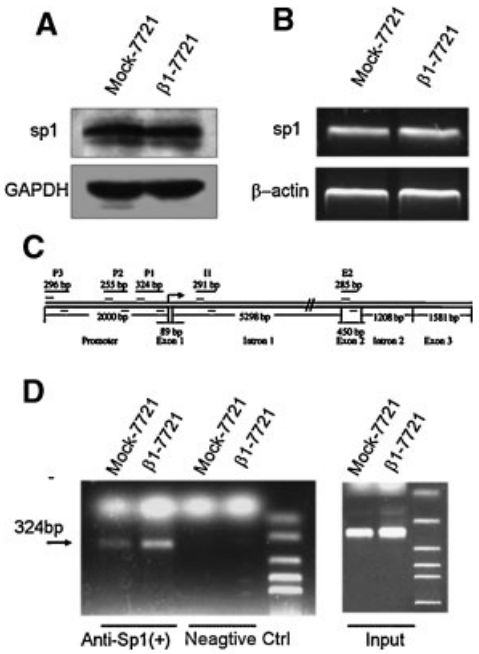

Fig. 3. Increased accumulation of Sp1 was present at the $p21^{WAF1/Cip1}$ promoter in β1-7721 cells. **A**: Sp1 protein levels were investigated using the immunoblotting analysis. The GAPDH was regarded as a loading control. **B**: The Sp1 mRNA levels in Mock-7721 and β1-7721 were analyzed using RT-PCR, and normalized with the amount of β-actin. **C**: Schematic representation of the partial human p21 gene. The primer set indicated here as P1 was used for chromatin immunoprecipitation (ChIP) assays in (**D**). Soluble chromatin was immunoprecipitated with anti-Sp1 antibodies from the Mock-7721 and β1-7721 cells. The following PCR was used to amplify the DNA isolated from the immunoprecipitated chromatin, and the primers used here were P1 set mentioned above.

cells (Fig. 3C). This further proved the importance of the ubiquitous transcription factor Sp1 in integrin β1-induced p21 expression.

Acetylation Value of Histone Proteins Across the $p21^{WAF1/Cip1}$ Gene Was Increased After the Integrin β1 Subunit Is Overexpressed

The Sp1 family of transcription factors regulates the expression of a variety of genes by binding to the GC/GT box through three highly conserved Cys-His-type zinc fingers [Philipsen and Suske, 1999; Bieker, 2001]. Members of this family have been shown to regulate target gene expression through recruiting co-activators and co-repressor complexes such as histone acetylation system and chromatin remodeling complexes [Grunstein, 1997; Wade and Wolffe, 1997; Kadonaga, 1998; Struhl, 1998; Workman and Kingston, 1998]. Acetylation of histone proteins is important modifications in chromatin structure and chromosomal architecture that facilitate transcription, which is necessary for binding of transcription factors like Sp1 to DNA [Hassig and Schreiber, 1997]. Recently, evidence was accumulating that histone acetylation and deacetylation plays an important role in transcriptional regulation of p21 [Archer and Hodin, 1999; Shin et al., 2000]. So we wonder whether histone acetylation was involved in integrin β1-mediated induction of p21 transcription. As shown in Figure 4A, Sodium butyrate (NaB, 5.0 mM) could markedly induce p21 expression in SMMC-7721 and Mock-7721, while there is no apparent change in β1-7721 cells. NaB, one of the classic histone deacetylation inhibitors, could induce p21 expression through acetylation of p21 gene and activation of Sp1 sites in various cell types [Nakano et al., 1997]. This implied that similar mechanisms were shared by integrin β1 signaling with the HDAC inhibitors regarding the induction of p21, and histone acetylation around p21 gene might have already been increased in β1-7721 cells.

Next, to determine the extent of histone acetylation localized to p21-associated chromatin, we carried out ChIP assays using an

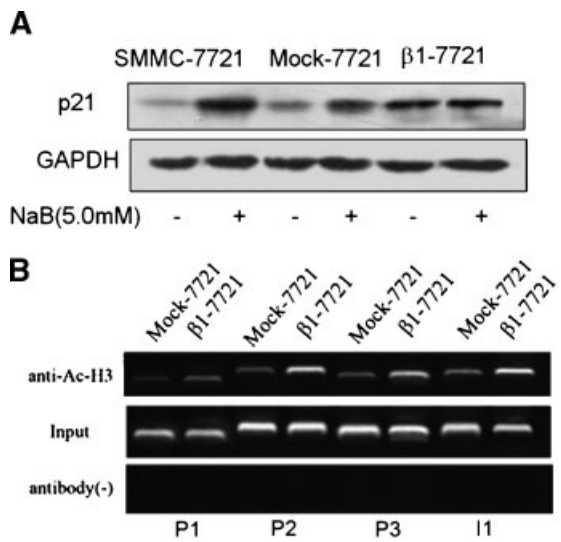

Fig. 4. Acetylation value of histone proteins across the $p21^{WAF1/Cip1}$ gene was increased after integrin β1 subunit was overexpressed. **A**: The SMMC-7721, Mock-7721 and β1-7721 cells were treated with 5.0 mM sodium butyrate for 24 h. The endogenous p21 protein levels were detected and the GAPDH protein was regarded as a loading control. **B**: Soluble chromatin from the Mock-7721 or β1-7721 cells was immunoprecipitated with anti-acetylated histone H3 antibodies. The primer pairs used here were P1, P2, P3, and I1, and the locations of these primers are indicated in Figure 3C.

anti-AcH3 antibody and the following PCR in Mock-7721 and β1-7721. The primer sets encompassing the promoter region and first several exons/introns of p21 gene were used here (Fig. 4B). The result demonstrated that the increased accumulation of acetylated histones was mainly present in the p21 promoter region in β1-7721 cells, and the fold increase of acetylated histones in β1-7721 cells was not the same for the areas of the p21 gene we examined (Fig. 4C). So the different extent of acetylated histones might be responsible for the discrepancy of p21 expression between Mock-7721 and β1-7721. This finding also possibly reflected location of acetylated histones within the gene or, alternatively, regions with a higher density of nucleosomes.

p300/CBP Is Involved in the Integrin β1 Subunit-Induced p21 Promoter Activation

It is well known that acetylation status of histone proteins is mainly regulated by histone acetyltransferase (HAT) and histone deacetylase (HDAC). Xiao et al. [2000] found that transcriptional coactivator p300/CBP which possesses intrinsic histone acetylation activity was required for Sp1- or sp3-mediated transactivation of p21 promoter. We considered that the increased histone acetylation and thereafter activation of Sp1 sites in this study might result from the function of p300. As the first step to issue this question, we examined the effect of p300 on p21 promoter in Mock-7721 and β1-7721 cells. As shown in Figure 5A, transfection of wild-type p300 expression construct, PCI-p300, into Mokc-7721 cells enhanced proximal promoter (pGL3-217) activity, while there is no apparent changes in β1-7721 (Fig. 5A). This suggested the positive effect of p300 on p21 transcription. While in β1-7721 cells this kind of effect may already be sufficient.

The interrelationship of p300 and integrin β1 subunit-induced promoter activation was further evidenced by co-transfection of a dominant-negative mutant of p300 expression construct (PCI-p300 HAT △ 1,472–1,522) in the two cell lines. The product of PCI-p300 HAT △ 1,472–1,522 encodes mutant p300 with a 50-amino-acid deletion (1,472–1,522) in the HAT domain (p300-hm) that abrogates HAT activity [Boyes et al., 1998]. In our experiment, this mutant lost the upregulating effect of wild-type p300 on the p21 promoter in Mock-7721 cells. Meanwhile, in the β1-7721 cells expressing this mutant p300, the p21 proximal promoter (pGL3-217) activity induced by integrin β1 subunit was obviously suppressed (Fig. 5A).

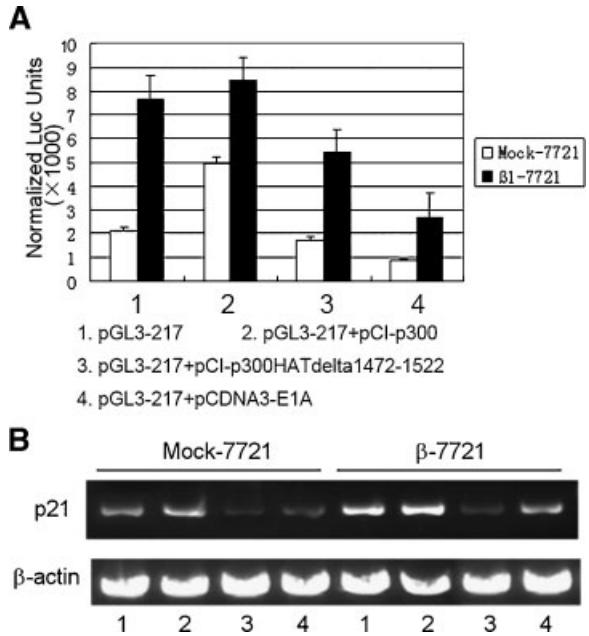

Fig. 5. p300/CBP was involved in the integrin β1-induced p21 promoter activation. **A**: Mock-7721 and β1-7721 cells were transiently co-transfected with the plasmids indicated below. **Lane 1**: pGL3-217; **lane 2**, pGL3-217 + pCI-p300; **lane 3**: pGL3-217 + pCI-p300HAT △ 1,472–1,522; **lane 4**, pGL3-217 + pCDNA3-E1A. The firefly luciferase activities were analyzed and the data were normalized to the activity of SV-40 renilla luciferase and were shown as means ± SD. **B**: Mock-7721 and β1-7721 cells were transiently co-transfected with the plasmids as below. Lane 1, no plasmid used; lane 2, pCI-p300; lane 3, pCI-p300HAT △ 1,472–1,522; lane 4, pCDNA3-E1A. The p21 mRNA levels were determined using RT-PCR and the β-actin mRNA levels were used as a control.

The adenoviral E1A oncoprotein can repress the transcription of many p300/CBP-dependent genes including p21 through its direct interaction with p300/CBP [Dorsman et al., 1995; Song et al., 1995; Lee et al., 1996; Smits et al., 1996; Somasundaram and El-Deiry, 1997]. After transfected with E1A expression vector (pCDNA3-E1A), the activity of p21 proximal promoter in both Mock-7721 and β1-7721 cells was markedly reduced to a relatively low level (Fig. 5A). These results suggest that the p300 was involved in the integrin-induced p21 promoter activation.

We next carried out the semi-quantitative reverse transcription-PCR (RT-PCR) to analyze the p21 mRNA level in Mock-7721 and β1-7721, which were transfected with plasmids

mentioned above. As shown in Figure 5B, the level of p21 mRNA was increased after transfected with PCI-p300 in Mock-7721 while there is no apparent change in β1-7721 cells. And the integrin β1 subunit-induced p21 mRNA expression was suppressed by the dominant-negative mutant of p300. When the adenoviral E1A oncoprotein was overexpressed, the mRNA levels of p21 in both Mock-7721 and β1-7721 were robustly reduced. These findings further proved involvement of p300 in the regulation of p21 induced by integrin β1 subunits.

DISCUSSION

For the transcription factor that involved in the integrin β1 subunit-mediated p21 transcription, we first consider the ubiquitous transcription factor Sp1. Sp1/Sp3 was involved in many p53-independent p21 activation pathways. Various stimuli, including TGF-β [Hu et al., 1999], tamoxifen [Lee et al., 2000], the PKC activator phorbolester [Biggs et al., 1996], the phosphatase 1 and 2A inhibitor okadaic acid, the geranyltransferase I inhibitor GGTI-298 [Adnane et al., 1998], progesterone [Owen et al., 1998], and nerve growth factor [Billon et al., 1999] could upregulate p21 transcription through the activation of the Sp1 sites, indicating the essential role of Sp1 sites in the transcriptional activation of $p21^{WAF1/Cip1}$ gene. In this study, we first found that the ubiquitous transcription factor Sp1 also played an important role in this integrin-mediated $p21^{WAF1/Cip1}$ transcription. When the certain Sp1 site was mutated in $p21^{WAF1/Cip1}$ proximal promoter, overexpression of integrin β1 could not activate $p21^{WAF1/Cip1}$ promoter any more. Then the direct evidence was found that more of this sequence-specific DNA-binding factor Sp1 was recruited into the proximal region of $p21^{WAF1/Cip1}$ promoter in this β-7721 cells. It seemed that overexpression of β1 integrin subunit made the DNA of $p21^{WAF1/Cip1}$ gene more accessible for the Sp1-mediated transcription machinery.

The transcriptional competence of eukaryotic chromatin requires access both for the activating gene-specific factors and for RNA polymerase II and its associated protein complexes. When a specific gene is activated, a cascade of chromatin modifications mediated by the ATP-dependent remodeling complexes and HATs makes the DNA accessible for transcription factors, including general transcription factors such as Sp1. The reorganization of chromatin associated with activation can be mediated by large remodeling complexes and is also strongly correlated with histone hyperacetylation [Grunstein, 1997; Struhl, 1998; Workman and Kingston, 1998] Regarding the correlation of histone acetylation and $p21^{WAF1}$ gene expression, HDAC inhibitors that induce $p21^{WAF1/Cip1}$ transcriptional activity are involved in most studies. Most HDAC inhibitors could induce $p21^{WAF1/Cip1}$ expression through the hyper-acetylation of histone proteins and activation of Sp1-binding-sites on p21 gene [Nakano et al., 1997; Sowa et al., 1999; Huang et al., 2000]. In this study, the classic HDAC inhibitor, sodium butyrate, could not induce $p21^{WAF1/Cip1}$ transcription in integrin β1 overexpressed SMMC-7721 cells as it did in the mock cells, suggested the similar pathway that HDAC inhibitors and integrin β1 subunit might share in the induction of p21. Then we found the direct evidence that the acetylation value of histone proteins was increased in β1-7721 cells comparing to Mock-7721 cell, which might make chromatin structure of p21 gene changed and be easier to be accessed by the transcription machinery.

The acetylation value of histone proteins is determined by the states of HAT and deacetylase. In view of recent progress in transcriptional regulation of p21, we assumed that some kinds of HAT and/or deacetylase may be altered physically or functionally after integrin β1 subunit is overexpressed and thus influence function of Sp1 family transcription factors. In working to try to identify these involved HAT and/or deacetylase, we found that p300, one of the HATs, could upregulates p21 promoter activity in Mock-7721 cells, while it made little changes in β1-7721 cells. A dominant negative mutant of p300 could block the integrin β1 subunit-induced p21 transcription, and the result from co-transfecting with E1A further supported the involvement of p300 in this integrin-mediated transcription.

In summary, we demonstrated here that overexpression of integrin β1 subunit upregulates $p21^{WAF1/Cip1}$ transcription through a p53-independent pathway. Integrin β1 subunit overexpression activates the p21 promoter through the Sp1/Sp3-binding sites and makes more transcription factor Sp1 recruited to proximal p21 promoter region. In addition, it

makes the acetylation value of histone proteins increased across some parts of the p21 gene. The transcription co-activator p300, which possess intrinsic histone acetylation, was involved in this integrin β1 subunit-mediated p21 transcription.

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