

c-Abl Tyrosine Kinase Activates p21 Transcription Via Interaction with p53

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c-Abl non-receptor tyrosine kinase has been implicated in many cellular processes including cell differentiation, stress response and regulating gene transcription. The mechanism by which c-Abl is involved in the regulation of gene transcription remains to be elucidated. In this study, we investigated the functions of c-Abl in the activation of p21 promoter. Our results showed that overexpression of c-Abl tyrosine kinase activated p21 promoter and endogenous p21 transcription in U2OS cells. We found that p53 is involved in the activation of p21 promoter by c-Abl, and integrative structure of p53 is required for regulating p21 transcription. In addition, the chromatin immunoprecipitation study demonstrated that c-Abl and p53 can be recruited to the region containing p53 binding site of p21 promoter, and c-Abl increases the DNA binding activity of p53 to the p21 promoter. Furthermore, not only the activation of p21 promoter but also the recruitment to p21 promoter by c-Abl is dependent on the interaction between c-Abl and p53 protein.

Key words: c-abl tyrosine kinase, gene transcription, p21, p53.

The c-Abl proto-oncoprotein is a member of the Src family of non-receptor tyrosine kinases. The evolutionarily conserved c-Abl was identified as the cellular homologue of the Abelson murine leukaemia virus, and its transforming variants have been implicated in tumorigenesis and in many important cellular processes (1, 2). c-Abl is ubiquitously expressed in cells and localized both to nucleus and cytoplasm where it plays distinct roles (3). The c-Abl function is required for the normal growth and development because c-Abl deficient mice exhibited the phenotype of embryonic or neonatal lethality (4–6). c-Abl has been implicated in a wide variety of cellular processes, including the regulation of cell growth (7), oxidative stress and DNA damage responses (8), signal transduction (9) and gene transcription (10). It has been reported that overexpression of c-Abl activates HIV promoter and up-regulates c-myc and c-fos transcription (10, 11). c-Abl has been demonstrated to contribute to the regulation of many genes that are involved in the control of cell cycle (12). It has been proposed that c-Abl may be involved in the G1/S checkpoint. Cells expressing a c-Abl kinase mutant and c-Abl nullizygous fibroblasts are impaired in their ability to down-regulate Cdk2 or undergo G1 arrest in response to ionizing radiation (13).

P21 gene, also known as Waf1/Cip1, encodes a CDK inhibitor which is able to induce G1 cell cycle arrest. As a transcription factor, p53 recognizes a specific consensus DNA sequence of p21 promoter and activates the expression of p21 gene, which involved in either growth arrest or apoptosis. Previous studies suggest a role for

p53 in the process of c-Abl regulating cell growth arrest (14). It has been reported that c-Abl binds p53 *in vivo* and enhances p53-dependent transcription from a promoter containing p53 DNA binding sites (15). Although it has been known that c-Abl participates in the regulation of p53-mediated gene transcription, the mechanism by which c-Abl regulates gene transcription remains in large elusive, and the knowledge of c-Abl-involved transcriptional regulation needs to be expanded. In this study, we showed that overexpression of c-Abl tyrosine kinase activated p21 promoter in U2OS cells. Not only the activation of p21 promoter but also the recruitment to p21 promoter by c-Abl is dependent on the interaction between c-Abl and p53 protein.

MATERIALS AND METHODS

Cell Culture and Transfection—U2OS cells and Saos2 cells were cultured in DMEM medium (Gibco, Grand Island, NY) with 10% fetal calf serum. Cells were grown at 37°C with 5% CO₂ and supplemented with 2 mM glutamine and 100 units/ml of penicillin. Transient transfections of U2OS and Saos2 cells were performed by the calcium–phosphate method. The transfected cells were cultured for 48 h and harvested for luciferase activity assay and chromatin immunoprecipitation assay.

Plasmids and Antibodies—Expression plasmids containing wild-type (WT) c-Abl and c-Abl mutated in kinase domain were kind gifts of Dr Jean Y. J. Wang (University of California, San Diego), and subcloned into pcDNA3 vector. pcDNA3-p53, pcDNA3-p53(175H), pcDNA3-p53(248W), pcDNA3-p53(273H) and the luciferase reporter plasmid carrying the p21 promoter (p21-LUC) were obtained from Dr Bert Vogelstein (Hopkins University, Maryland). The pREP7-Rluc

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vector was kindly provided by Dr Keji Zhao (NIH, Maryland). Anti-c-Abl, anti-p53, and anti- β -actin antibodies were obtained from Santa Cruz Biotechnology. Horseradish peroxidase (HRP)-conjugated secondary antibody against mouse IgG or rabbit IgG was purchased from Jackson Laboratory (Bar Harbor, ME).

Dual-luciferase Assay—U2OS or Saos2 cells were transfected with 3 μ g of c-Abl expression plasmid. All the aliquot of cells were cotransfected with both 1 μ g of p21-LUC and 300 ng pREP7-Rluc vector. The relative luciferase activity was analysed after 48 h by using a Turner Designs TD20/20 Luminometer (Sunnyvale, CA) with the dual-luciferase assay system (Promega). Firefly luciferase activity was normalized to Renilla luciferase activity.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) and Quantitative Real Time PCR Analysis (qReal Time PCR)—The transfected U2OS cells were cultured at 37°C for 48 h. The total RNA (2 μ g) isolated by using TRIZOL reagent (Invitrogen) was reversely transcribed by using an M-MLV reverse transcriptase kit (Promega). Quantitative real time PCR mix included the following: diluted cDNA sample, 0.5 μ M primers, nucleotides, Taq DNA polymerase and buffer included in the SYBR Green I Mastermix (PE Applied Biosystems). In the ABI Prism 7000 sequence detection system, PCR cycling conditions were as follows: 50°C for 2 min, 95°C for 10 min, 40 cycles at 94°C for 15 s, and 60°C for 1 min. Sequence Detector Software was used to extract the PCR data, which were then exported to Excel for further analysis. Expression of p21 gene was measured in triplicate and normalized to Homo sapiens glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression levels. GAPDH primers: 5-aggggggagccaaaaggg-3, 5-gaggagtgggtgctgctgttg-3. p21 primers: 5-gagcctcctccatcctatg-3, 5-ctcccagcacactcac-3.

Chromatin Immunoprecipitation—U2OS cells or Saos2 cells were cross-linked with 1% formaldehyde at 37°C for 20 min. The cells were then washed and scraped into 10 ml of phosphate-buffered saline with protease inhibitors. The cells were collected by a 5 min spin at 1500 rpm at 4°C, resuspended in 200 μ l of SDS lysis buffer supplemented with 1 mM PMSF and sonicated three times for 30 s at 60% maximum power. Soluble chromatin was collected by centrifugation at 13,000 $\times g$ for 10 min and diluted in chromatin immunoprecipitation (ChIP) IP buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris, pH 8.1, 167 mM NaCl and 1 mM PMSF). Approximately 10% of the lysate was retained as an input control. The chromatin solution was then pre-cleared with 100 μ l of protein G beads, which had been pre-adsorbed with sonicated salmon sperm DNA. Immunoprecipitation was performed at 4°C overnight by using 2 μ g of either non-specific IgG or antibodies to c-Abl or p53. The immune complexes were then captured with 40 μ l of protein G beads, and the beads were then washed with 1 ml of ChIP wash buffer 1 (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl [pH 8.1], 150 mM NaCl), ChIP wash buffer 2 (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, 500 mM NaCl), ChIP wash buffer 3 (0.25 M LiCl, 1% NP-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-HCl [pH 8.1]) and finally twice with Tris-EDTA (10 mM Tris-HCl [pH 8.1] mM EDTA). The protein-DNA complexes were then eluted

by adding 250 μ l of ChIP elution buffer (1% SDS-0.1 M NaHCO₃) to the beads. Following reverse crosslinking at 65°C for 6 h, DNA was purified by proteinase K digestion, phenol-chloroform extraction and ethanol precipitation, and then resuspended in 20 μ l of 1 \times TE. One-fifth of the immunoprecipitated DNA and 1% of input DNA were analysed by PCR. The following primers were used for detecting p21 promoter sequences: 5-cccagtgacagcgcagcagc-3; 5-ctcccctgagcagcagcacaag-3.

RESULTS

c-Abl Promotes p21 Gene Transcription—Previous study has shown that v-Abl, c-Abl oncogene homologue, up-regulates p21 gene transcription in cell cycle arrested and proliferating myeloid cells (16). To investigate whether c-Abl functions in the regulation of p21 gene transcription, we conducted dual-luciferase assay by cotransfecting U2OS cells with the c-Abl expression vector pcDNA3-c-Abl and a luciferase reporter driven by the p21 promoter (p21-LUC). As a result, the cells transfected with the c-Abl expression vector showed a 4–5-fold increase in the luciferase activity compared with those transfected with the empty vector (Fig. 1A), indicating that c-Abl up-regulates p21 promoter activity.

To confirm the function of c-Abl in regulating p21 gene transcription, we transiently transfected the c-Abl expression vector pcDNA3-c-Abl into U2OS cells and analysed the endogenous p21 gene transcription. The RT-PCR and Realtime PCR results showed that overexpression of c-Abl augmented the endogenous p21 gene expression about 4.5-fold (Fig. 1B), consistent with the luciferase assay results. Taken together, these data demonstrated that c-Abl, with intact kinase activity, promotes p21 gene transcription.

The Kinase Activity of c-Abl is not Required for p21 Gene Transcription—As a tyrosine kinase, the kinase activity of c-Abl is essential for the induction of growth arrest and apoptosis by c-Abl in response to different signals (7, 14, 17). It has been reported that c-Abl with intact kinase can transactivate the c-myc gene promoter (10). To determine whether the activation of p21 promoter by c-Abl is dependent on its tyrosine kinase activity, we conducted dual-luciferase assay by cotransfecting U2OS cells with WT c-Abl, kinase-deficient c-Abl (KD), and p21-LUC. The results showed that the kinase-defective c-Abl, which lost the tyrosine kinase activity, still stimulated the activation of p21 promoter (Fig. 2A), indicating that c-Abl does not depend on its tyrosine kinase activity for activating p21 promoter. To further confirm this result, U2OS cells transfected with wt c-Abl were treated with STI571 (formerly known as CGP57148B), a specific c-Abl tyrosine kinase inhibitor which competitively binds to the ATP-binding site of c-Abl and inhibits protein tyrosine phosphorylation (18, 19). The results showed that the relative p21-luc activity did not change in contrast to the untreated cells (Fig. 2B). These results indicated that kinase activity of c-Abl is not required for regulating p21 gene transcription.

c-Abl Cooperated with p53 to Activate p21 Promoter—The cyclin-dependent kinase inhibitor p21/waf1 is one of

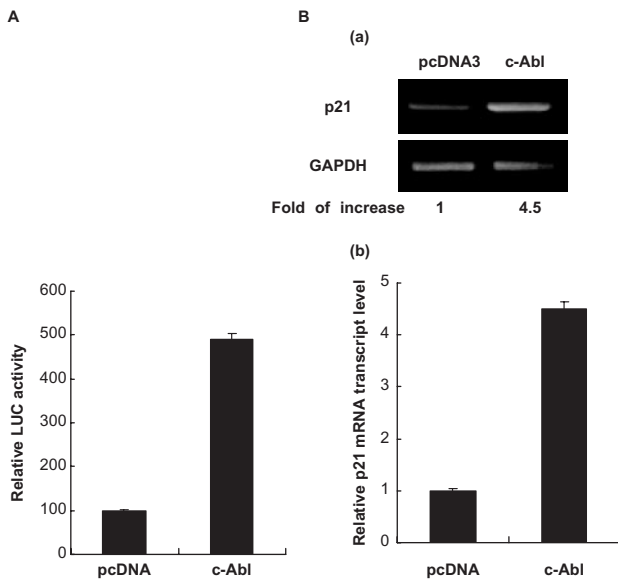


Fig. 1. c-Abl promotes p21 gene transcription. (A) U2OS cells were transfected with 3 μ g of the indicated expression plasmids along with 1 μ g of luciferase reporter driven by p21 promoter. The luciferase activity was analysed by the dual-luciferase system at 48 h post-transfection by using 200 ng of pREP7-RL reporter as an internal control. Results shown are the mean \pm SD of three independent experiments performed in triplicate. (B) U2OS cells were transfected with 3 μ g of c-Abl expression vector or pcDNA3 empty vector. The total RNA (2 μ g) was reversely transcribed with an oligo(dT) primer, followed by PCR with either p21 primers or GAPDH primers. Products were analysed by agarose gel electrophoresis followed by ethidium bromide staining and photodensitometry analysing. (a) mRNA level of the cells transfected with c-Abl expression vector and pcDNA3 empty vector. (b) Quantitative real time PCR analysis of the endogenous p21 gene transcription activated by c-Abl. Total RNA was isolated from U2OS cells transfected with indicated plasmids for 48 h, and 2 μ g of total RNA was used for reverse transcription. PCR products were measured continuously with an ABI PRISM 7000 Sequence Detection System. Transcript abundance was measured in triplicate as the ratio of control or experimental sample values normalized to GAPDH expression levels.

the targeted genes of p53 protein (20). It has been reported that c-Abl binds p53 *in vivo* and enhances p53-dependent transcription (15). To test whether c-Abl cooperate with p53 to activate p21 promoter, we conducted dual-luciferase assay by cotransfecting U2OS cells with c-Abl, p53 and p21-LUC. The results showed that in contrast to pcDNA3 vector, pcDNA3-c-Abl and pcDNA3-p53 expression plasmids can activate the luciferase activity of p21, about 4.5-fold and 7-fold, respectively. Co-overexpression of c-Abl and p53 can increase p21-luc activity about 10-fold, indicating that c-Abl and p53 can cooperate to activate the p21 promoter. In contrast, overexpression of the p53 conformational mutant 175H or DNA binding mutant 273H, 248W cannot cooperate with c-Abl to activate p21 promoter (Fig. 3A). Taken together, these data indicated that c-Abl cooperated with the WT p53, but not the mutants to promote the p21 gene transcription.

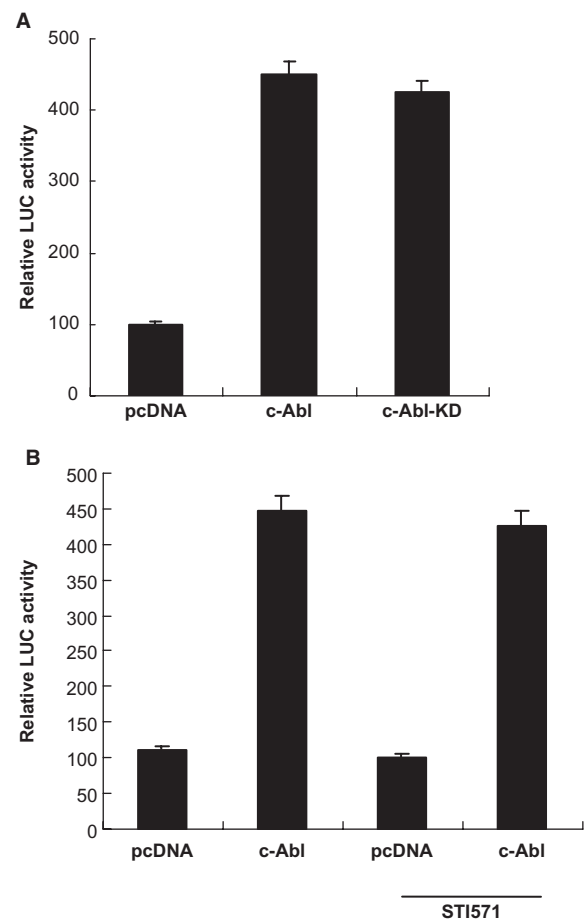


Fig. 2. The kinase activity of c-Abl is not required for the activation of p21 promoter. (A) U2OS cells were transfected with 3 μ g of the indicated expression plasmids along with 1 μ g of p21 promoter driven luciferase plasmid. The luciferase activity was analysed by the dual-luciferase system at 48 h post-transfection by using 200 ng of pREP7-RL reporter as an internal control. Results shown are the mean \pm SD of three independent experiments performed in triplicate. (B) U2OS cells were transfected with 3 μ g of the indicated expression plasmids along with 1 μ g of p21 promoter driven luciferase plasmid. After 48 h, cells were treated with 5 μ M STI571 for 2 h. The luciferase activity was analysed by the dual-luciferase system. Results shown are the mean \pm SD of three independent experiments performed in triplicate.

To ensure that the activation of p21 promoter by c-Abl was not due to the increase of the p53 protein levels induced by overexpression of c-Abl, western blotting analysis was performed. The results showed that transfected c-Abl had no effect on the expression of endogenous p53 protein (Fig. 3B).

c-Abl Controls p53 Transcription Activity by Stimulating its DNA Binding to p21 Promoter— Previous studies have shown that although c-Abl lacks intrinsic transcriptional activity, c-Abl can be recruited to DNA-cis element by complexing with the transcription factors, such as CREB, E2F and RFXI, to modulate their transcriptional activity (12, 21, 22). A ChIP assay was performed to determine whether c-Abl can be recruited to

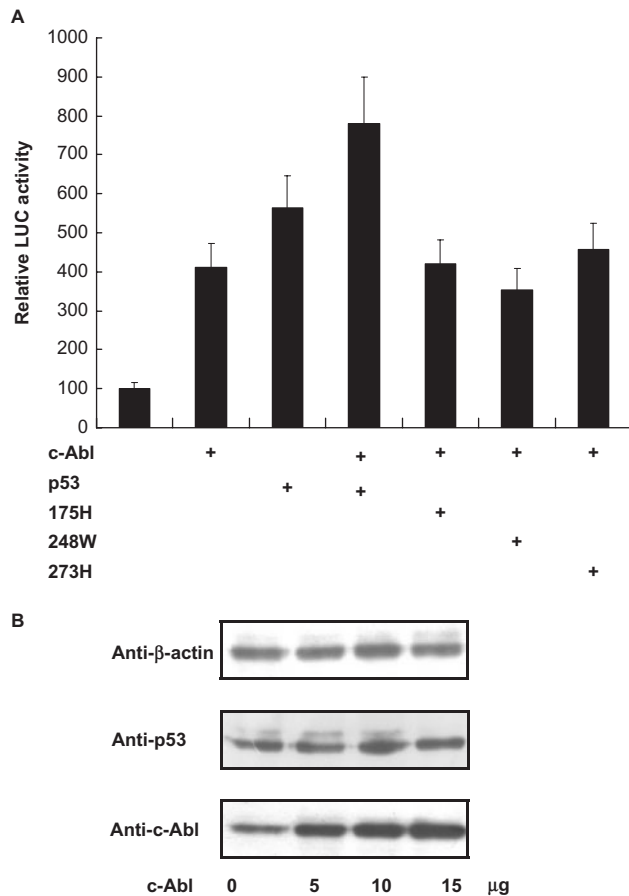


Fig. 3. c-Abl cooperated with p53 to activate p21 promoter. (A) U2OS cells were transfected with indicated plasmids: 3 μ g of c-Abl, 0.5 μ g of p53, p53-175H, p53-248W and p53-273H, along with 1 μ g of p21-LUC. The luciferase activity was analysed by the dual-luciferase system at 48 h post-transfection by using 200 ng of pREP7-RL reporter as an internal control. Results shown are the mean \pm SD of three independent experiments performed in triplicate. (B) U2OS cells were transfected with empty vector or 5, 10 and 15 μ g of c-Abl expression plasmid. After 48 h, cell lysates from the transfected cells were fractionated by SDS-PAGE and detected by anti- β -actin antibody (up-panel), anti-p53 (middle-panel) anti-c-Abl antibody (bottom-panel).

the region containing p53-binding site of p21 promoter *in vivo*. U2OS cells were cross-linked and the DNA-protein complexes were immunoprecipitated with antibodies against c-Abl and p53, respectively. The results showed that c-Abl antibody and p53 antibody were both able to immunoprecipitate the p21 promoter region, compared with that of no antibody (Fig. 4A). These results indicated that p53 and c-Abl could be recruited to the region of p21 promoter *in vivo*.

To show that c-Abl can enhance p53-DNA binding activity *in vivo*, we performed a ChIP assay by using the p21 promoter as a target of p53 antibody. U2OS cells were transfected with c-Abl. After 48 h, cells were cross-linked and the DNA-protein complexes were immunoprecipitated with p53 antibodies. The results showed that c-Abl increase the binding activity of the

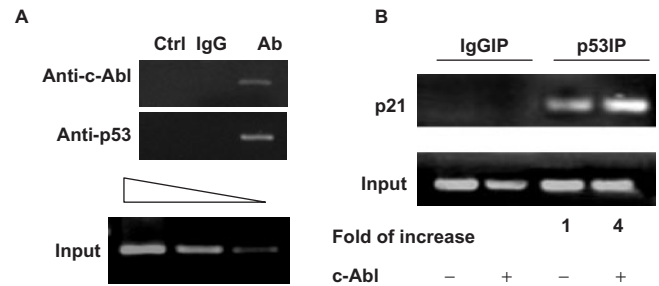


Fig. 4. c-Abl controls p53 transcription activity by modulating p53-DNA binding to p21 promoter. (A) c-Abl is recruited to the region of p21 promoter *in vivo*. U2OS cells were cross-linked and the DNA-protein complexes were immunoprecipitated with antibodies to c-Abl (up panel), p53 (middle panel) or non-specific IgG as a control. The following were primers for the CSF1 upstream control sequence: -5234R, 5'CTCTTCTCCTGATAGCTCCATGA3'; -5436F, 5'CACTATGTTAGCCAGGATGGTCTC3'. DNA was subjected to PCR amplification by using *Taq* polymerase in the following conditions: 94°C, 30 s; 55°C, 30 s; 72°C, 30 s; repeat 35 times. Input represents a 1:10 dilution series of the genomic DNA used (bottom panel). (B) c-Abl enhances DNA binding activity of p53 in U2OS cells. U2OS cells were transfected with pcDNA3-c-Abl. After 48 h, cells were cross-linked and the DNA-protein complexes were immunoprecipitated with antibody to p53 or to non-specific IgG as a control. PCR analysis (using primers to p21) on input DNA or p53 immunoprecipitated DNA are shown. The fold of increase in the presence of c-Abl was quantified.

endogenous p53 to the p21 promoter about 3.5-fold (Fig. 4B). These data suggested that c-Abl regulates p53 transcriptional activity by stimulating its DNA binding activity.

Activation of p21 Promoter by c-Abl is Dependent on its Interaction with p53—To further investigate whether the activation of p21 promoter by c-Abl is dependent on its interaction with p53 protein, we conducted dual-luciferase assay by cotransfecting pcDNA3-c-Abl, pcDNA3-p53 and p21-LUC into p53-negative Saos2 cells. As shown in Fig. 5A, in the p53-negative Saos2 cells pcDNA3-c-Abl expression plasmid alone almost cannot activate p21 promoter activity, while co-overexpression of p53 can restore the ability of c-Abl to enhance p21 promoter activity, indicating that the activation of p21 promoter by c-Abl is dependent on its interaction with p53.

To further confirm this result, a ChIP assay was performed to determine whether c-Abl can be recruited to the region containing p53-binding site of p21 promoter in Saos2 cells. Saos2 cells or Saos2 cells transfected with p53 were cross-linked and the DNA-protein complexes were immunoprecipitated with antibodies against c-Abl and p53, respectively. The p21 promoter sequences from -2359 to -2041 and from -1219 to -1401, which contain the p53 binding site, in the immunoprecipitated chromatin were analysed by PCR. The results showed that c-Abl antibody and p53 antibody were not able to immunoprecipitate the p21 promoter region containing p53 binding site in Saos2 cells, while in Saos2 cells transfected with p53 plasmid, c-Abl antibody was able to immunoprecipitate the p21 promoter region (Fig. 5B). These results indicated that c-Abl cannot be recruited to the

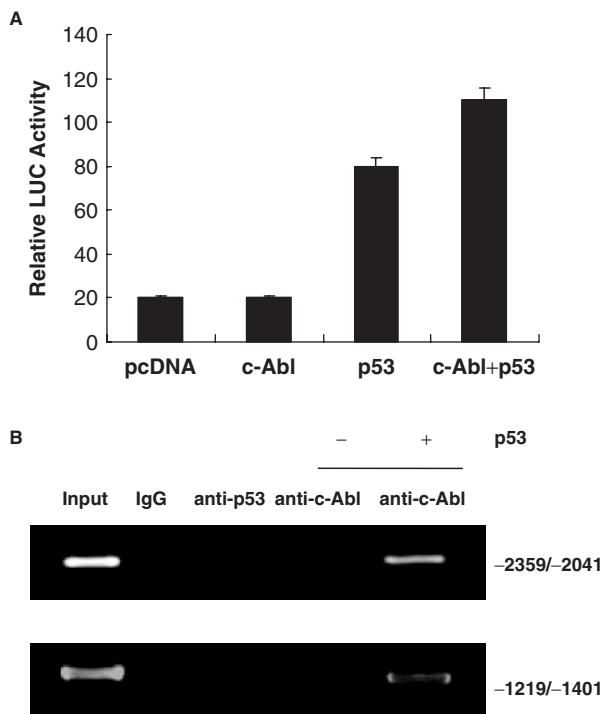


Fig. 5. Activation of p21 promoter by c-Abl is dependent on its interaction with p53. (A) Saos2 cells were transfected with the indicated expression plasmids along with 1 μ g of p21-LUC. The luciferase activity was analysed by the dual-luciferase system at 48 h post-transfection by using 200 ng of pREP7-RL reporter as an internal control. Results shown are the mean \pm SD of three independent experiments performed in triplicate. (B) Saos2 cells were transfected with 3 μ g pcDNA3.0 empty vector or c-Abl expression plasmid and the cells were cross-linked at 48 h post-transfection. The DNA-protein complexes were immunoprecipitated with antibodies to c-Abl, p53 or non-specific IgG as a control. DNA was subjected to PCR amplification by using *Taq* polymerase in the following conditions: 94°C, 30 s; 55°C, 30 s; 72°C, 30 s; repeat 35 times. Input represents a 1:10 dilution series of the genomic DNA used. A region of the p21 downstream sequence without p53 binding sites served as negative control. p21 primers: from -2359 to -2041: 5'-GAGCCTCCCTCCATCCCTATG-3', 5'-CTCCCAGCACACTCACAC-3'; from -1219 to -1401: 5'-TGCGTTAGAGGAAGAAGACT-3', 5'-CTGTGCCTGAAACATTGTC-3'.

region containing p53 binding site of p21 promoter in p53 negative cells, but can be recruited to the region containing p53 binding site via the interaction with p53 protein. Taken together, these data suggested that activation of p21 promoter by c-Abl is dependent on the interaction with p53.

DISCUSSION

c-Abl gene, which encodes a member of the Src family of non-receptor tyrosine kinases, has been identified as the cellular homologue of the Abelson murine leukaemia virus. A number of studies have shown that c-Abl may be involved in the G1/S checkpoint. Cells expressing a c-Abl kinase mutant and c-Abl nullizygous fibroblasts are impaired in their ability to down-regulate Cdk2 or undergo G1 arrest in response to ionizing radiation

(13). c-Abl has been demonstrated to contribute to the regulation of many genes that are involved in the control of cell cycle, including *c-myc*, *c-fos*. In this study, based on the relative luciferase assay and Real time PCR analysis, we showed that overexpression of c-Abl enhances p21 promoter activity and endogenous p21 transcription. Interestingly, the activation of p21 promoter by c-Abl does not require its tyrosine kinase activity, indicating a kinase-independent function for c-Abl.

The cyclin-dependent kinase inhibitor p21/waf1 is one of the targeted gene of p53 protein (20). The carboxyl terminus of p53 is a target of a variety of signals for regulation of p53 DNA binding. Growth suppressor c-Abl interacts with p53 in response to DNA damage (13, 23, 24) and overexpression of c-Abl leads to G1 growth arrest in a p53-dependent manner (25). It has been reported that c-Abl binds directly to the carboxyl-terminal regulatory domain of p53 and the binding is necessary for c-Abl stimulation (26). Here, we showed that c-Abl cooperated with the WT p53, but not the mutants to promote the p21 gene transcription. A number of study showed that the p53 protein is subject to Mdm2-mediated degradation by the ubiquitin-proteasome pathway. c-Abl can prevent the ubiquitination and nuclear export of p53 by Mdm2, or by human papillomavirus E6. Overexpression of c-Abl may increase the expression and accumulation of p53 protein. As shown in Fig. 3B, the effect observed in this study that c-Abl promotes p21 transcription is not due to the increased expression of endogenous p53 protein by the transfected c-Abl.

Despite our wealth of knowledge on the genes that are targeted by p53 in growth arrest and apoptosis, relatively little is known about the mechanism by which p53 is involved in the processes for the activation of p21 by c-Abl. The region of human p21 promoter contains binding sites for transcription factors such as p53, C/EBP, BRCA1, E2F, AP1 and Sp1 (20). It has been reported that c-Abl may interact with E2F and AP1 to activate gene transcription. Previous studies have shown that although it lacks intrinsic transcriptional activity, c-Abl can be recruited to DNA-cis element by complexing with the transcription factors CREB, E2F and RFX1 to modulate their transcriptional activity (12, 21, 22). Here we showed that p53 and c-Abl could be recruited to the region of p21 promoter *in vivo*, and the interaction of the two proteins promotes p53 binding to p21 promoter, and as a consequence c-Abl up-regulates p21 transcription. Furthermore, our ChIP assays showed that c-Abl cannot be recruited to the region (from -2359 to -2041 or from -1219 to -1401) which includes p53 binding site of human p21 promoter in p53 negative cells, but can be recruited to the region containing p53 binding site via the interaction with p53 protein. Therefore, activation of p21 promoter by c-Abl is dependent on its interaction with p53.

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