Abstract. Zbtb7, a member of the POK protein family, is involved in tumorigenesis and cellular differentiation by acting as a crucial transcription factor, but its role in cell cycle modulation remains uncharacterized. In the present study, CDK2 and E2F4, two cell cycle regulators, are shown to be downregulated at the mRNA and protein levels by Zbtb7 in HepG2 and QGY7703 cells. Moreover, we demonstrate that the activities of CDK2 and E2F4 promoters were suppressed by the modulation of Zbtb7 levels and that Zbtb7 represses promoter activities through a mechanism involving direct binding of Zbtb7 to the promoters. Furthermore, it was identified that the site at -259 to -252 within the CDK2 promoter is responsible for Zbtb7-induced repression of the promoter activity. It was found that siRNA-induced knockdown of Zbtb7 resulted in the suppression of cell cycle progression in HepG2 and QGY7703 cells. Collectively, these data indicate that CDK2 and E2F4 are the downstream targets of Zbtb7, and Zbtb7 may be a cell cycle modulator by regulating the expression of cell cycle-associated genes in liver cancer cells.

Introduction

Zbtb7, also known as LRF10, OCZF11 and FBI-1 (1-3), is a POK protein family member originally isolated as a cellular factor that binds to the inducer of short transcripts of human immunodeficiency virus-1 and the proximal GC-rich sequence of ADH5/FDH (4,5). Zbtb7 has a critical function in cellular differentiation and its aberrant expression has been shown to be implicated in tumorigenesis in various organs including the lung, liver, spleen and thymus (2,6). It has also been revealed that Zbtb7 plays a vital role in the late stage of osteoclastogenesis (3) and that Zbtb7 is involved in instructing early lymphoid progenitors in mice to develop into B lineage cells by repressing T cell-instructive signals produced by the cell-fate signal protein, Notch (7). Zbtb7 was found to be a modulator of a series of genes including Egr-1, c-Krox, cyclinA, E2F4, ADH5, Rb, FANS, p107 and ARF (2,5,8-11), suggesting a significant role for Zbtb7 in a range of biological activities.

Cyclin-dependent kinase 2 (CDK2) is thought to be a major cell cycle regulator functioning during the G1/S transition and S phase, and CDK activity is largely regulated positively through association with the appropriate cyclins and negatively by CDK inhibitors. CDK2 expression has been shown to be regulated at the transcription level by MITF in melanoma and the sequence spanning -100 to +108 was found to be sufficient for the basal activity of CDK2 promoter in NIH3T3 cells (12). Since both Zbtb7 and CDK2 are involved in tumorigenesis and potential cis elements for Zbtb7 have been found within the promoter of CDK2, we investigated whether Zbtb7 regulates the expression of CDK2 in liver cancer cells. We demonstrated that this is indeed the case, using real-time PCR measurements of CDK2 mRNA and western blot analysis of CDK2 protein upon modulation of the Zbtb7 expression level, chromatin immunoprecipitations (ChIPs) and luciferase reporter assays.

Materials and methods

Cell lines and cell culture. Human liver cancer HepG-2 cells and QGY7703 cells (American Type Culture Collection, Manassas, VA, USA) were cultured in DMEM medium containing 10%
fetal bovine serum (Hyclone, USA), L-glutamine (2 mM), streptomycin (0.1 mg/ml) and penicillin (100 U/ml) at 37°C in a humidified incubator supplied with 5% CO₂.

**Plasmid preparation.** Various 5'-3' deletion constructs of DNA fragments relative to the translation start site (ATG) were generated from HepG-2 cells. For construction of the putative CDK2 promoter deletion constructs, the reverse primer for PCR was GAAAGGCTTGAAGTCTCCATGAA, and the forward primers were 5'-GAAAGATCTAAATTTAACCCTTAG TGCAGATA-3' (528 bp) and 5'-GAAAGATCTCAATAGTGA AAGCATC-3' (1046 bp). For the construction of the putative E2F4 promoter deletion constructs, the reverse primer for PCR was 5'-GCAAGACTTTAGGTGGTGAACAGTC-3', and the forward primers were 5'-GCAAGACTCTGTCTTTAGCAG-3' (301 bp), 5'-GCAAGACTCTAGTGGGAGGCTTTAG-3' (600 bp) and 5'-GCAAGACTCTTATAGTGAGACCCACATCTC-3' (1152 bp). PCR-amplified products were digested with enzymes BglII and HindIII, and cloned into the corresponding sites of the pG04.10-basic firefly luciferase expression plasmid (Promega). The internal control pRL-TK Renilla Luciferase vector was purchased from Promega Corp. The Zbtb7 expression construct was generated by inserting a cDNA fragment obtained by reverse transcription PCR from HepG-2 cells into the pcDNA3.1 (-) vector at the HindIII sites. The PCR oligonucleotide primer pair used for the Zbtb7 cDNA amplification was as follows: 5'-CTTGAAGCTTGGC-3' and 5'-GTCAGAATCTTCACCAG-3'. All constructs were verified by sequencing.

**Site-directed mutagenesis.** Zbtb7 putative recognition site mutations of the CDK2 promoter were obtained by preparing the PMS1, PMS2, PMS3 and PDS2 constructs, using mutated plasmids. The plasmids with the mutation were generated by using a site-directed mutagenesis system (Promega) according to the manufacturer's protocol and pLuc528 construct was used as a template. The oligonucleotides used for mutagenesis (mutations indicated in bold italics) are as follows: GCCTGGGCGGATCACCTTTGC (PMS1 -329 to -322 bp), GGGCTGGGAGGGAGCCAGGG (PMS2 -259 to -252 bp), AGCAAGGCGGAGGCTCTGTGG (PMS3 -227 to -218 bp), AGCCAGTGGAA (PDS2 -259 to -252 bp). DNA sequencing was performed to confirm that the sequence of the PCR products was correct as compared with the CDK2 promoter published in the Human Genome database.

**Transient transfections and luciferase assay.** HepG-2 and QGY7703 cells (1x10⁵) were seeded into 24-well plates, and 2 μl of Lipofectamine-2000 (Invitrogen, Carlsbad, CA, USA) was used for plasmid transfection. At 48 h following transfection, the cells were harvested, lysed and luciferase activities were measured in triplicate using Dual Luciferase™ Reporter Assay System (Promega, Wallisellen, Switzerland).

**Chromatin immunoprecipitation (ChIP) assay.** ChIP assay was carried out to analyze the physical interaction between Zbtb7 and the CDK2 promoter in the HepG-2 cells. Formaldehyde was added at 1% to the culture medium for 10 min at 37°C to cross-link protein to DNA. Cells were washed twice with ice-cold 1X PBS, scraped and resuspended in ChIP sonication buffer (% Triton-100, 0.1% deoxycholate, 50 mM Tris 8.1, 150 mM NaCl, 5 mM EDTA) containing protease inhibitors. The cells were sonicated to shear DNA to lengths between 500 and 2000 bp. The sonicated supernatant was diluted with ChIP dilution buffer, and incubated with antibody against Zbtb7 (Abcam) overnight at 4°C with rotation. The chromatin-antibody complexes were collected by Dynabeads Protein G (Invitrogen). Following three washes with pH 5.0 TE buffer (10 mM Tris, 1 mM EDTA), the pellet was dissolved with pH 3.0 TE buffer. Following precipitation with ethanol, the pellets were resuspended and treated with proteinase K. The supernatant was extracted with saturated NaCl and precipitated with 2-propanol to recover the DNA. The primers for ChIP PCR were designed to amplify potential Zbtb7 binding sites in the CDK2 and E2F4 core promoter sequences. The primers used were as follows: 5'-GAAAGATCTTAACTTAGGTGCATA-3', 5'-GAAAGCTTATAGTGAGACCCACATCTC-3'.

**siRNA transfection.** Cells were transfected with Zbtb7-targeting siRNAs (5'-UCGGUACCUUGUAACUAA-3', 5'-UUGAUUCAACAGGGUCG-3') using a modified Oligofectamine protocol (Invitrogen). In brief, 1 day prior to transfection, cells were seeded without antibiotics to a density of 50-60%. In all experiments, cells were transfected with siRNAs at a concentration of 50 nM. Control cells were treated with scramble RNA. Cells were harvested 48 h following siRNA treatment for cell cycle analysis and RT-PCR.

**Real-time PCR analysis.** Total RNA was prepared from cells using TRizol reagent (Invitrogen Corp.) according to the manufacturer's instructions. Following isolation, RNA was reversely transcribed to cDNA using random primers (Promega). The reaction mixture containing SYBR Green PCR Master Mix (Takara) was run in a 7500 Real-Time PCR system (Applied Biosystems). The PCR primers used for quantitative RT-PCR were: 5'-ATTCTATGTTGGTCTCGG-3', 5'-GCTCGGCTTACCTCCATGAA-3' (CDK2); 5'-CATCCAGTGGAAGGTGTG-3', 5'-TTGGACGTGAGGCTTCCTG-3' (E2F4); 5'-TGCAAGGTCCCTGGTACCAG-3', 5'-TGCAAGGTCCCTGGTACCG-3' (Zbtb7). Real-time PCR parameters used were as follows: 95°C for 10 sec; 95°C for 5 sec, 60°C for 34 sec for 40 cycles; 95°C for 15 sec, 60°C for 1 min and 95°C for 15 sec.

**Western blot analysis.** HepG2 cells transfected with Zbtb7-targeting siRNA and scramble RNA were lysed on ice for 30 min with lysis buffer (10 mM N-(2-hydroxyethyl) piperoxane-N'-2-ethane sulfonic acid, 10 mM KCl, 1 mM ethylendiaminetetraacetic acid (EDTA) (pH 8.0), 0.1% NP-40, 1 mM DTT, 1 mM PMSF and 0.5 mM Na₂VO₃). Soluble protein (30 μg) was separated on a 12% sodium dodecyl sulfate (SDS)-polyacrylamide electrophoresis gel and blotted onto a pure nitrocellulose membrane (Bio-Rad, Hercules, CA) at 180 mA for 2 h. Following blocking with 5% skim milk in phosphate-buffered saline at room temperature for 45 min, membranes were incubated with the Zbtb7 antibody (1:500; Sigma, St. Louis, MO, USA) and the CDK2 antibody (1:300;...
Abcam) in the same buffer for 2 h, followed by incubation with goat anti-rabbit IgG (1:2000) for 1 h. Antibody binding was detected using an enhanced chemiluminescence system (Pierce, Rockford, IL). To correct protein loading amounts, the membranes were reprobed with β-actin monoclonal antibody (1:40,000) (Sigma).

Cell cycle analysis. For cell cycle analysis, at 48 h following siRNA transfection, cells were harvested, washed with PBS and fixed in 70% chilled ethanol at 4°C for 30 min, then treated with 1 mg/ml of RNase A (Sigma-Aldrich) and stained with 100 μg/ml of propidium iodide for 30 min. The DNA content was determined with a fluorescent-activated cell sorter FACScan (Beckman Coulter, Inc, Miami, FL, USA). The data were analysed with cell cycle analysis software ModFit LT 2.0 (Verity Software House, Topsham, ME, USA). Most of the experiments were performed in triplicate.

Statistical analysis. Statistical analysis was performed using the Student's t-test with INSTAT statistical analysis package (Graph Pad Software). Significance was defined as p<0.05.

Results

Zbtb7 downregulates the expression of CDK2 and E2F4 in hepatocarcinoma cells. To determine whether CDK2 and E2F4 are the potential downstream targets of the Zbtb7 gene in liver cancer cells, HepG2 and QGY7703 cells, two liver cancer cell lines, were utilized as a cell model. As shown in Fig. 1A and B, the overexpression of Zbtb7 resulted in downregulation of CDK2 and E2F4 mRNA levels in both HepG2 and QGY7703 cells. To further confirm the observation at the mRNA level, Zbtb7-targeting siRNA was used to knock down the expression of Zbtb7 in HepG2 cells, a cell line with high endogenous Zbtb7 expression. As shown in Fig. 1C, the Zbtb7-targeting siRNA-induced downregulation of Zbtb7 protein level led to an increase in CDK2 and E2F4 protein levels in the HepG2 cells. These observations suggest that CDK2 and E2F4 may be the downstream targets of Zbtb7 and that Zbtb7 functions as a negative modulator of CDK2 and E2F4 gene expression in liver cancer cells.

Zbtb7 represses the promoter activity of CDK2 and E2F4 genes in hepatocarcinoma cells. To elucidate the mechanism of Zbtb7-induced downregulation of the CDK2 and E2F4 genes, the core promoter sequences of the CDK2 and E2F4 genes were cloned and ligated to luciferase reporter plasmids (Fig. 2A and B). The delivery of the Zbtb7 expression plasmid into HepG2 and QGY7703 cells obviously repressed the promoter activities of the CDK2 and E2F4 genes (Fig. 2C and D) which indicates that Zbtb7 downregulated the expression of CDK2 and E2F4 by suppressing the promoter activities in liver cancer cells. To further investigate whether Zbtb7 represses the promoter activities of CDK2 and E2F4 genes by a direct mechanism, ChIP PCR was carried out in the HepG2 cells. As observed in Fig. 2E, Zbtb7 was shown to bind with the core promoter sequences of the CDK2 and E2F4 genes. Those results indicate that Zbtb7 downregulated the expression of CDK2 and E2F4 genes by repressing the promoter activities by a direct mechanism that involved the direct binding of Zbtb7 to the corresponding promoter sequences in the liver cancer cells.

Zbtb7 suppresses CDK2 promoter activity via an element at -259 to -249. To determine the Zbtb7 regulatory elements within the CDK2 promoter sequence, site-directed mutagenesis was performed. The consensus Zbtb7 regulatory elements were identified as either a single guanine-rich site or a site with the consensus sequence G (A/G)GGG (T/C)(C/T)(T/C)(C/T) (11). Searching for the Zbtb7 regulatory consensus sequences resulted in identification of three potential elements within the CDK2 promoter: -329 to -322, -259 to -252, and -227 to -218 bp. As shown Fig. 3A, those sites were replaced with G or T by mutagenesis. It was found that the mutation at -259 to -249 (PMS2, PDS2) restored Zbtb7-induced repression of CDK2 promoter activity in both HepG2 and QGY7703 cells, while no such observation was obtained when mutation occurred at the sites -329 to -322 and -227 to -218 (Fig. 3B and C). These results suggest that the site at -259 to -249 within the
Zbtb7 induces G1/S arrest in hepatocarcinoma cells. Since CDK2 acts as a regulator of G1/S in cell cycle progression, it may be postulated that Zbtb7 might affect cell cycle distribution. To test this hypothesis, HepG2 and QGY7703 cells were treated with 50 nM Zbtb7 siRNA for 48 h, and the cell cycle distribution was analyzed by using a FACScan flow cytometer. Following incubation with 50 nM Zbtb7 siRNA for 48 h, 61.08% of HepG2 cells and 53.12% of QGY7703 cells were in G0/G1 phase compared to 65.06% of the scramble siRNA-treated HepG2 cells and 68.32% of the scramble siRNA-treated QGY7703 cells were in the G0/G1 phase (Fig. 4A and B). These results suggest a role of Zbtb7 in the regulation of the G1/S phase of the cell cycle in liver cancer cells.

Discussion

CDK2 was thought to be a regulator of G1/S transition in cell cycle progression (13), and its activity is regulated positively through an association with cyclins and negatively by CDK2 inhibitors. The TATA-less CDK2 promoter has been studied in NIH3T3 mouse fibroblasts (12) and multiple transcription factor binding sites have been identified. It was identified that the sequence spanning -100 to +108 is sufficient for basal promoter activity in NIH3T3 cells, specifically attributed to two SP1 sites within this region (12). In addition, a serum-responsive cis-element was found to be present 700 bp upstream of the transcriptional start. It was revealed that the transcriptional start site of the melanocyte-specific gene SILV/PMEL17 resides just 796 nucleotides away from the translational start site of the CDK2 gene (14), posing an interesting regulatory challenge.

In the present study, it was demonstrated that Zbtb7 acts as a negative regulator of CDK2 gene expression. Zbtb7-induced downregulation of CDK2 was confirmed at both the mRNA and protein level by gene delivery and siRNA transfection in HepG2 and QGY2203 cells. To further elucidate the mechanism underlying Zbtb7-induced downregulation of CDK2, the core promoter sequences of the CDK2 gene were cloned from HepG2 cells and ligated to luciferase reporter plasmids. A luciferase activity assay showed that the expression of Zbtb7 significantly repressed promoter activity of the CDK2 gene.

Figure 2. Zbtb7 represses the promoter activities of the CDK2 and E2F4 genes in hepatocarcinoma cells. (A and B) Schematic representation of CDK2 and E2F4 promoter luciferase reporter plasmids: pLuc-1046, pLuc-528, pLuc-1152, pLuc-600 and pLuc-301, containing a series of 5'-3' deleted promoters; (C and D) HepG2 and QGY7703 cells were transfected with promoter luciferase reporter plasmids and PCDNA3.1 or PCDNA3.1/Zbtb7, and cells were harvested for luciferase activity assay as described in the text at 48 h following transfection. The values are expressed as the means ± SD from three independent experiments. *p<0.05; **p<0.01. (E) Sheared chromatin from HepG2 cells was immunoprecipitated using Zbtb7 antibody and ChIP-PCR was performed using primers targeting CDK2 (+1 to -528 bp) and E2F4 (+1 to -301 bp) promoter, respectively.
Figure 3. Zbtb7 suppresses CDK2 promoter activity via an element at -259 to -252. (A) Schematic representation of CDK2 promoter sequences for site-directed mutagenesis. (B and C) HepG2 and QGY7703 cells were transfected with the indicated promoter luciferase reporter plasmids and PCDNA3.1 or PCDNA3.1/Zbtb7, and the cells were harvested for luciferase activity assay as described in the text at 48 h after transfection. The values are expressed as the means ± SD from three independent observations. *p<0.05, **p<0.01.

Figure 4. Zbtb7 induces G1/S arrest in hepatocarcinoma cells. (A) HepG2 cells were treated with Zbtb7-targeting siRNA or scramble siRNA for 48 h, and cell cycle analysis was performed with a FACScan flow cytometer. (B) QGY7703 cells were treated with Zbtb7-targeting siRNA or scramble siRNA for 48 h, and the cells were harvested for cell cycle analysis. Right panels: measurements using a FACScan flow cytometer. Left panels: quantification of cell cycle distribution from the right panels. *p<0.05.
in both HepG2 and QGY2203 cells. ChIP PCR revealed that Zbtb7 suppressed promoter activity of the CDK2 gene through its binding with the core promoter sequences. To further determine the cis elements within the core promoter sequences of the CDK2 gene which are responsible for Zbtb7-induced repression of promoter activity, three potential Zbtb7 binding elements were utilized for mutagenesis analysis. The mutation or deletion at -259 to -249 (PMS2, PDS2) of the CDK2 promoter resulted in diminished Zbtb7-induced repression of CDK2 promoter activity, which suggests that the site at -259 to -249 within the CDK2 promoter is responsible for Zbtb7-induced repression of promoter activity.

E2F4, one of the E2F family, has been implicated in cell cycle regulation, and its deregulation can lead to neoplastic transformation of certain immortalized cell lines (15-18). E2F4 was identified as a downstream target of Zbtb7, and it was shown that Zbtb7-induced downregulation of E2F4 results in promotion of terminal preadipocyte differentiation (8). We also demonstrated Zbtb7 to be a negative regulator of E2F4 expression in two liver cancer cell lines, HepG2 and QGY7703 cells. Zbtb7 was shown to repress E2F4 promoter activity through direct binding with the promoter sequence in human liver cancer cells which is consistent with the observation in preadipocytes (8).

In the present study, we provide evidence that Zbtb7 acts as a negative regulator of both CDK2 and E2F4 expression by a direct mechanism that involves binding of Zbtb7 with the promoter sequences in liver cancer cells. CDK2 and E2F4 were shown to be regulators of the G1/S phase in cell cycle progression, so it can be postulated that Zbtb7 might have a role in cell cycle regulation. To test this hypothesis, cell cycle analysis was performed in HepG2 and QGY7703 cells. Notably, it was found that the siRNA-induced Zbtb7 knockdown led to a lower percentage of cells in G1 phase which suggests a negative role for Zbtb7 in the regulation of G1/S entry in liver cancer cells and the repression of CDK2 and E2F4 induced by Zbtb7 might explain our observation. The mechanism underlying Zbtb7-induced cell cycle arrest at G1/S remains to be further characterized.

In conclusion, in the present study CDK2 and E2F4 were identified as downstream targets of Zbtb7 in liver cancer cells. Zbtb7 was shown to negatively regulate the expression of both CDK2 and E2F4 genes through a mechanism involving the direct binding of Zbtb7 to promoter sequences. The evidence for the role of Zbtb7 in the regulation of cell cycle progression was also provided, and our finding might have important implications for understanding the role of Zbtb7 in carcinogenesis, development and differentiation.

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References


