Y-box Binding Protein 1 Is Involved in Regulating the G₂/M Phase of the Cell Cycle

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Abstract. *Background/Aim:* The transcription factor Y-box-binding protein 1 (YB1) is overexpressed in many types of human cancers. YB1 regulates the G₁ phase of the cell cycle by controlling transcription of G₁ regulators. Here, we report that YB1 is also involved in regulating G₂/M phase. *Materials and Methods:* YB1-depleted TKO cells were subjected to quantitative reverse transcription-polymerase chain reaction and cell-cycle analysis. RNA immunoprecipitation (RIP)-chip assay was performed using anti-YB1 antibodies. Precipitated RNAs were subjected to microarray analysis. *Results:* Silencing YB1 inhibited the proliferation of TKO cells, which lost the machinery required for G₁ phase arrest. Cell-cycle analysis showed that silencing YB1 caused G₂/M phase cell-cycle arrest. RIP-chip assay showed that YB1 associated with mRNA of multiple cell-cycle-related genes, including G₂/M phase regulators. *Conclusion:* YB1 positively regulates not only the G₁ phase but also G₂/M phase by regulating multiple cell-cycle-related genes.

Y-box-binding protein 1 (YB1) is a member of the cold shock domain (CSD) protein superfamily. YB1 binds to DNA and RNA through its CSD domain and regulates the expression of multiple genes. YB1 is involved in fundamental gene expression processes including transcription, translation and mRNA stabilization (1). YB1 is highly expressed in actively proliferating tissues and in a wide range of human cancers, including breast, ovary, lung and colon cancer (2, 3). Transgenic mice overexpressing YB1 in mammary epithelia develop breast carcinomas, supporting the hypothesis that YB1 functions as an oncogene (4).

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Biochemical studies have shown that YB1 binds directly to the promoters of cell-cycle-related genes, including *EGFR*, *HER-2* and *CDC6*, inducing their transcription and thereby promoting cell proliferation (5, 6). We have also shown that YB1 directly binds to and activates the *Cyclin D1* promoter, thereby activating *cyclin D1* transcription (7). YB1 and cyclin D1 have been found to be highly expressed in non-small-cell lung cancer clinical specimens (7), supporting the hypothesis that YB1 positively regulates cell proliferation by activating *cyclin D1* transcription. Mouse embryonic fibroblasts derived from YB1-knockout mice have been shown to exhibit retarded cell growth, enhanced cellular senescence (8, 9) and increased levels of the cyclin-dependent kinase (CDK) inhibitors p16 and p21. Consistent with this, YB1 has been shown to bind at the promoter of the CDK inhibitor *p16* and represses its transcription, suppressing cellular senescence (10, 11).

pRB family proteins bind to and repress E2F transcription factors, thereby arresting cells in the G₁ phase (12). In cells entering cell cycle, a mitogenic signal induces expression of cyclin Ds, which bind to and activate CDK4 and CDK6, leading to phosphorylation of pRB family proteins. This phosphorylation can release pRB family proteins from E2F transcription factors, allowing transcriptional activation of S-phase related genes and initiating G₁-S phase transition. The CDK inhibitor p16 binds to CDK4/6 complex and inhibits its kinase activity (13). YB1 negatively regulates pRB family proteins by repressing p16 transcription and activating *Cyclin D1* transcription, which promotes G₁-S phase progression (7, 10). In this study, we investigated the role of YB1 in the regulation of cell-cycle phases other than the G₁ phase.

Materials and Methods

Cell culture. Mouse embryonic fibroblasts carrying triple deletion of all pRB family genes (*pRB*, *p107* and *p130*), designated TKO cells, were kindly provided by Julien Sage (14). TKO, HCT116 (human colorectal cancer) and WI38 (human foetal lung fibroblast) cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) supplemented 10% foetal bovine

Figure 1. Silencing *YB1* decreases levels of cyclin *D1* mRNA, but not of *p16* mRNA in TKO cells. (A) TKO cells were transfected with control (ctr-i) or *YB1*-targeting siRNA oligonucleotides (*YB1*-i). At 72 h after transfection, cells were harvested and subjected to q-RT-PCR to confirm *YB1* silencing. The effects of *YB1* silencing on cyclin *D1* (B) and *p16* (C) mRNA levels were determined by q-RT-PCR. Results are expressed relative to the corresponding values for ctr-i-transfected TKO/cells. Mean values and standard deviations were calculated from triplicates of representative experiments.

serum (GIBCO, Grand Island, NY, USA) and maintained at 37°C in an atmosphere containing 5% CO₂.

Cell-cycle analysis. HCT116 cells (1×10⁶ cells) were fixed with 70% ethanol and kept overnight at -20°C. Fixed cells were stained using a Muse™ Cell Cycle Kit (Merck Millipore, Darmstadt, Germany) according to the manufacturer's instructions. The DNA content was analysed using a Muse Cell Analyzer and analysis software (Merck Millipore).

RNA interference. TKO and HCT116 cells were transfected with siRNA targeting *YB1* or control siRNA oligonucleotides by RNAiMAX (Invitrogen), according to the manufacturer's protocol. The nucleotide sequence of the siRNA targeting mouse *YB1* was 5'-CAACGUCGGUAUCGCCGAAACUUC-3', and the nucleotide sequence of the siRNA targeting human *YB1* was 5'-GGUCCCCACCUACUACAU-3', with 3' dTdT overhangs.

Quantitative reverse transcription-polymerase chain reaction (q-RT-PCR). Total RNA was extracted from cells using an RNeasy Plus kit (Qiagen, Tokyo, Japan) according to the manufacturer's instructions, and subjected to reverse transcription using SuperScript Reverse Transcriptase II (Invitrogen). The synthesized cDNA was amplified using SYBR Green PCR master mix (Qiagen) and specific primers. The primer sequences were as follows: mouse *YB1*, 5'-CGGAGACCCTATGCGCGTCG-3' and 5'-TTTGGCGAGGAGGGCCCTT-3'; mouse *p16*, 5'-GTACCCCGATTGAG GTGATG-3' and 5'-ACCAGCGTGTCAGGAAG-3'; mouse *Cyclin D1*, 5'-GAGCTGCTGCAAATGGAAGT-3' and 5'-AAAGAAAGTGC GTTGTGCGG-3'; and mouse *glyceraldehyde-3-phosphate dehydro-*

genase (GAPDH), 5'-GGTGAAGGTC GGTGTGAACG-3' and 5'-TGTAGACCATGTAGTTGAGG-3'.

Assays were performed in triplicate using an Mx3000P Real-Time q-PCR System (Agilent Technologies, Santa Clara, CA, USA).

RNA immunoprecipitation (RIP)-chip. RIP-chip assay was performed by MBL (Medical and Biological Laboratories Co., Ltd., Nagoya, Japan). Briefly, 1×10⁶ WI38 cells were lysed and subjected to RNA immunoprecipitation with anti-*YB1* antibodies (RN015P, MBL) using a RIP assay kit (MBL). The precipitated mRNA was reverse transcribed into T7-cDNA using T7-oligo(dT) primers and *in vitro* transcribed to synthesize biotinylated-cRNA. The biotinylated-cRNA was hybridized to a DNA microarray (Illumina BeadArray, Human HT-12 v3.0; Illumina, San Diego, USA). The microarray data were analysed using GeneSpring software (Agilent Technologies).

Results

Our team previously reported that *YB1* positively regulates cell proliferation by promoting G₁-S phase transition *via* repression of *p16* and activation of *Cyclin D1* (7, 10). In this study, we examined whether *YB1* may regulate other cell cycle phases than the G₁ phase. *YB1* was knocked-down in TKO cells using specific siRNA oligonucleotides. TKO cells carry a triple deletion of *pRB*, *p107* and *p130* and therefore lack the capacity for G₁ phase arrest (14). Depletion of *YB1* expression in knocked-down TKO cells was confirmed by q-

Figure 2. Silencing YB1 causes G₂/M phase cell cycle arrest. (A) 5×10^5 TKO cells were seeded then incubated overnight, then transfected with control or YB1-targeting siRNA oligonucleotides. At 72 h after transfection, the cells were observed by phase-contrast microscopy, and viable cells were counted by trypan blue staining. Results are expressed relative to the corresponding values for ctr-i-transfected TKO cells in (B). (C) HCT116 cells were transfected with control and YB1 siRNA-targeting oligonucleotides. At 72 h after transfection, the cells were harvested and subjected to cell cycle analysis. The percentages of cells in each cell cycle phase are shown (D). The statistical analyses were performed using two-tailed Student's *t*-test. * $p < 0.05$; n.s., not significant.

RT-PCR (Figure 1A). *Cyclin D1* mRNA levels were decreased in YB1 knocked-down TKO cells (Figure 1B), but *p16* mRNA levels were not affected (Figure 1C) indicating that YB1 is involved in transcriptional regulation of *Cyclin D1*, but not *p16* in TKO cells. YB1 depletion caused in TKO cells (Figure 2A) and inhibited cell growth as measured by trypan blue assay 72 hours after transfection (Figure 2B). However, the morphological change of TKO cells was not observed by YB1 depletion. This may indicate that YB1 is involved in regulating cell cycle phases other than the G₁ phase, since TKO cells don't have the capacity for G₁ phase arrest. Consistent with this hypothesis, silencing YB1 in HCT116 (human colorectal cancer cells) induced pronounced accumulation of cells in the G₂/M phase compared to control cells (Figure 2C and D). This result suggests that YB1 may positively regulate G₂/M phase progression.

Because these results suggested that YB1 is involved in regulating more than one cell cycle phase, we then used global analysis to search for target genes of YB1 that are

involved in cell cycle regulation. Recently, it has been reported that YB1 binds to 3' untranslated regions of pro-oncogenic transcripts, leading to their stabilization (15). It has also been shown that YB1 binds to several transcription factors involved in epithelial mesenchymal transition, such as SNAI1, leading to activation of the cap-dependent translation pathway (16). We therefore globally searched for YB1-associated mRNAs involved in cell cycle regulation by RIP-chip assay. We used YB1 antibodies to probe the cytosolic fraction of WI38 cells (Figure 3A). The precipitated RNA were analysed using a Bioanalyzer instrument and visualized as a gel electrophoresis image. The electrophoresis image shows that specific RNAs were precipitated by RIP assay with YB1 antibodies (Figure 3B). Following the RIP assay, DNA microarray analysis was performed. The DNA microarray data showed that mRNAs of multiple genes are co-immunoprecipitated in the RIP sample with YB1. We identified and focused on the cell cycle-related genes among the genes detected by gene

Figure 3. *YB1* binds to multiple RNAs. (A) RNA immunoprecipitation was performed on cytoplasmic extracts of WI38 cells using anti-*YB1* antibodies. The precipitated *YB1* proteins were confirmed by western blotting. IgG-1 was used as a negative control. (B) Precipitated RNAs were detected using a Bioanalyzer instrument and visualized as a gel electrophoresis image.

ontology analysis. The data show that *YB1* binds to many cell cycle-related genes (Table I). Interestingly, *YB1* associated with *cyclin D1* mRNA, suggesting that *YB1* regulates *cyclin D1* at both the transcriptional and post-transcriptional levels. Notably, *YB1* associated with several mRNAs of genes involved in G_2/M phase regulation, such as *SEPT2*, *NUDC*, *CDC25B*, *BUB3*, *AURKA* and *CDC20*. These results suggest that *YB1* is involved in the positive regulation of G_2/M phase progression via modulation of these G_2/M phase regulators.

Discussion

YB1 is involved in regulating the expression of multiple genes, and has been found to be up-regulated in various cancers, including breast, ovary, lung and colon cancer (2, 3). Overexpression of *YB1* activates transcription of various genes, including *EGFR*, *HER-2* and *CDC6*, thereby promoting cell proliferation (1, 5, 6). Moreover, we previously showed that *YB1* activates *cyclin D1* transcription and represses *p16* transcription, thereby promoting G_1 -S phase cell-cycle transition (7, 10). In this study, we demonstrated that *YB1* is also involved in regulating the G_2/M phase. We found that *YB1* depletion decreased *cyclin D1* mRNA levels in TKO cells carrying triple deletion of all pRB family genes. Interestingly, silencing *YB1*

did not affect *p16* mRNA levels in TKO cells, suggesting that pRB family proteins are required for *YB1*-dependent repression of *p16* transcription. This supports previous reports that showed that pRB protein represses *p16* transcription (17). *YB1* depletion inhibited TKO cell proliferation even though the cells lost the capacity for G_1 phase arrest, which suggests that *YB1* is involved in regulating other cell cycle phases in addition to the G_1 phase. Supporting this hypothesis, silencing *YB1* in HCT116 cells caused G_2/M phase arrest, suggesting that *YB1* positively regulates the G_2/M phase.

Recent studies have shown that *YB1* binds to mRNAs of various oncogenes involved in metastasis to stabilize them or activate cap-dependent translation, thereby promoting metastasis (15, 16). Here, we also showed by RIP-chip assay that *YB1* binds to mRNAs of several genes that regulate the G_2/M phase, such as *SEPT2*, *NUDC*, *CDC25B*, *BUB3*, *AURKA* and *CDC20*, supporting the involvement of *YB1* in the G_2/M phase. We therefore postulate that *YB1* stabilizes these mRNAs or activates their cap-dependent translation to promote G_2/M phase progression. The involvement of *YB1* in regulation of these mRNAs and the molecular mechanism underlying this process are important issues that require further investigation. *YB1* overexpression may lead to irregular expression of multiple genes involved in the cell cycle and metastasis, resulting in the progression of various cancers.

Table I. List of cell cycle-related genes whose mRNAs associated with YB1.

Rank	Symbol	Description	Score
1	<i>UBA52</i>	Ubiquitin A-52 residue ribosomal protein fusion product 1	30,11
2	<i>CCND1</i>	Cyclin D1	29,4
3	<i>SEPT9</i>	Septin 9	29,3
4	<i>EIF4G2</i>	Eukaryotic translation initiation factor 4 gamma, 2	27,74
5	★ <i>SEPT2</i>	Septin 2	27,43
6	<i>CCND3</i>	Cyclin D3	27,27
7	<i>NME1</i>	NME/NM23 nucleoside diphosphate kinase 1	27,22
8	<i>S100A6</i>	S100 calcium binding protein A6	26,82
9	<i>BRMS1</i>	Breast cancer metastasis suppressor 1	26,72
10	<i>UBE2C</i>	Ubiquitin-conjugating enzyme E2C	26,71
11	<i>DCTN2</i>	Dynactin 2	26,44
12	★ <i>NUDC</i>	NudC nuclear distribution protein	26,4
13	<i>TUBG1</i>	Tubulin, gamma 1	26,35
14	★ <i>CDC25B</i>	Cell division cycle 25B	26,3
15	<i>PFDN1</i>	Prefoldin subunit 1	26,23
16	★ <i>BUB3</i>	BUB3 mitotic checkpoint protein	25,79
17	<i>PTTG1</i>	Pituitary tumor-transforming 1	25,76
18	★ <i>AURKA</i>	Aurora kinase A	25,68
19	<i>PPP1R15A</i>	Protein phosphatase 1, regulatory subunit 15A	25,64
20	<i>TFDP1</i>	Transcription factor Dp-1	25,63
21	<i>CETN2</i>	Centrin, EF-hand protein, 2	25,57
22	<i>MAPRE1</i>	Microtubule-associated protein, RP/EB family, member 1	25,49
23	<i>GSPT1</i>	G ₁ to S phase transition 1	25,44
24	<i>EXT1</i>	Exostosin glycosyltransferase 1	25,35
25	<i>GPS1</i>	G protein pathway suppressor 1	25,31
26	<i>BAX</i>	BCL2-associated X protein	25,3
27	<i>PPM1G</i>	Protein phosphatase, Mg ²⁺ /Mn ²⁺ dependent, 1G	25,17
28	★ <i>CDC20</i>	Cell division cycle 20	25,04
29	<i>CKS1B</i>	CDC28 protein kinase regulatory subunit 1B	24,95
30	<i>PPP1CA</i>	Protein phosphatase 1, catalytic subunit alpha	24,9

Genes identified by RIP-chip assay are ranked according to signal intensity. Score refers to the sum of the signal intensity of two independent experiments. Asterisk (★) indicates a G₂/M phase-related gene.

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