Activity of the upstream TATA-less promoter of the $p21^{Waf1/Cip1}$ gene depends on transcription factor IIA (TFIIA) in addition to TFIIA-reactive TBP-like protein

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Introduction

Transcriptional regulation of an RNA polymerase II driven gene is governed by a particular set of gene-specific DNA-reactive transcription regulatory factors and their associating transcriptional cofactors. Moreover, general transcription factors such as TFIID (transcription factor IID) and TFIIA are assembled at a promoter region to conduct transcriptional initiation [1–3], which is enhanced by gene-specific transcription regulatory factors through functional interaction.

Structured digital abstract

- TLP physically interacts with TFIIA beta and TFIIA alpha by anti tag coimmunoprecipitation (View interaction)
- TFIIA alpha/beta physically interacts with TLP by anti bait coip (View interaction)

Abbreviations
ChIP, chromatin immunoprecipitation; qPCR, quantitative PCR; siRNA, short interfering RNA; TBP, TATA-binding protein; TFIIA, transcription factor IIA; TLP, TBP-like protein.
TATA-binding protein (TBP) is an essential component in TFIIH that binds to the TATA-box promoter element [2,4–6]. TBP-like protein (TLP, also called TRF2) has been identified as one of the TBP family proteins [7–9] and has been shown to enhance expression of TATA-less genes such as NF-1, Cyclin-G2, Tap63 and Wee1 [10–12]. TLP is unable to bind to the TATA-box, and a consensus TLP-binding sequence has not been determined so far. In the Droso phila PCNA gene, TLP is engaged in transcriptional activation as a cofactor for a transcription regulatory factor called DREF [13]. Although TLP is usually concentrated in the cytoplasm, it translocates to the nucleus in a particular cell-cycle period or when activated by a genotoxin such as etoposide [12]. Hence, TLP is thought to be involved in gene regulation related to growth control and DNA damage response. Recently, we have identified p21 (p21Waf1/Cip1) as one of the TLP-target genes [14].

p21 is a CDK inhibitor and causes cell-cycle arrest at G1 or G2 phase [15,16]. Since p21 also participates in apoptosis, DNA repair and tumor suppression in some cases [17], p21 is regarded as a major gene for cell growth regulation. The amount of intracellular p21 is regulated at the transcription level, and its expression level is enhanced by multiple transcription factors [18,19]. The promoter-enhancer region of p21 contains several binding sites for p53, which enhances the promoter activity. p53, which works for genome homeostasis, is a typical tumor suppressor and major regulator of the p21 gene [20–22]. The human p21 gene has two major promoters: a TATA-containing downstream promoter and an upstream TATA-less promoter [23,24]. TFIIH is recruited to the TATA-box of the downstream promoter together with p53 upon UV irradiation, but it does not participate in regulation of the upstream promoter [24]. We have found that activity of the upstream promoter absolutely depends on TLP and p53 [14], and these two factors form a complex in cells [14,25].

The most attractive property of TLP is its potent TFIIA-binding ability. TFIIA is another member of the family of general transcription factors [3]. Although TFIIA binds to TBP to some extent in order to potentiate TFIIH-dependent promoters, it is also used for TATA-less promoters [3,26,27]. In higher eukaryotes, TFIIA consists of three subunits including TFIIA α, β and γ [3]. TFIIAαβ is encoded by a single gene and is cleaved into individual α and β subunits [3,28]. We have found that TLP binds more strongly to TFIIA than to TBP [29]. Although TLP is mainly localized in the cytoplasm, mutant TLPs with impaired TFIIA-binding ability display a diffuse localization pattern [29]. However, the significance of the TFIIA-binding ability of TLP in transcriptional regulation has remained to be clarified.

In this study, we investigated the contribution of TLP-TFIIA interaction to p21 gene regulation, and we found that mutant TLPs with weakened TFIIA-binding ability exhibit decreased transcription stimulation activity. Moreover, etoposide, which stimulates p21 gene expression, facilitated binding of the upstream promoter to TFIIA and TFIIA-reactive TLP. One reason why TLP possesses a strong TFIIA-binding ability may be elucidated through this study.

Results

Transcriptional activation function of mutant TLPs for the p21 promoter

We previously constructed various kinds of mutant TLPs [29]. Among them, N37E and R52E have weakened binding ability to TFIIA, and N37E is a more severe mutant than R52E for TFIIA binding, whereas R55E binds to TFIIA as strongly as does wild-type TLP. In this study, we first investigated in detail the intracellular TFIIA-binding strength of these mutants by co-immunoprecipitation assays. It was confirmed that wild-type TLP and R55E exhibited significant binding to TFIIA, whereas N37E did not bind to TFIIA (Fig. 1). We further investigated processed and unprocessed forms of TFIIAαβ and found that R52E binds to the α and β subunits of TFIIA but does not bind to uncleaved TFIIAαβ (Fig. 1, lane 12).

The human p21 gene produces mainly alt-a and variant-1 transcripts from the upstream and downstream promoters, respectively (Fig. 2A) [14,23]. Knockdown

![Fig. 1. TFIIA-binding ability of TLP. Co-immunoprecipitation to detect the interaction between TLP and TFIIA. Extracts of HCT116 cells into which FH-TLP (TLP) and its mutants (R55E, R52E and N37E) had been introduced were immunoprecipitated with M2 beads (M2) and examined for indicated proteins by western blotting using specific antibodies. Inp, input.](image)
of endogenous TLP resulted in decreased production of whole \(p21\) transcripts, mainly due to the decreased level of alt-a transcripts (Fig. 2B). An overexpression experiment confirmed that alt-a is specifically dependent on intracellular TLP level. Next, we investigated the transcriptional activation function of the above-mentioned TLP mutants. R55E, which has a native TFIIA-binding ability, considerably enhanced alt-a production (Fig. 2C–a, lane 3), whereas R52E and N37E exhibited almost no effect (lanes 5 and 6). R52E and N37E showed decreased transcription stimulation activity for the upstream promoter compared with that of wild-type TLP and R55E (Fig. 2C). To obtain direct evidence that TFIIA-reactive TLP potentiates the upstream promoter, we performed a luciferase reporter assay in mutant TLP-overexpressed cells. It was demonstrated that R55E enhanced promoter activity as much as wild-type TLP did, whereas R52E and N37E had less effect on promoter activity than did wild-type TLP (Fig. 2D). These results suggest that TFIIA-binding ability is required for the transcription stimulation function of TLP.
TFIIA sensitivity of the upstream promoter

Since TFIIA-binding ability of TLP was found to affect the transcriptional activation function of the upstream promoter (Fig. 2C,D), we investigated how TFIIA works for p21 promoters. Overexpression of TFIIAαβ considerably stimulated the upstream promoter (Fig. 3A-a, lane 3). Since TFIIAγ exhibited little effect (Fig. 3A-a, lane 4), the concentration of TFIIAγ in cells seemed to be sufficient for the upstream promoter. We further investigated the cooperative effect directed by TLP and TFIIA in transcriptional regulation through co-overexpression of TFIIAαβ and TLP or N37E. Although the activation degree of TLP for the upstream promoter was 2.6-fold, co-overexpression of both TLP and TFIIAαβ yielded 4.0-fold activation (Fig. 3A-b, lanes 1-3). However, this additive effect was relatively small (1.8-fold) when N37E and TFIIAαβ were used (Fig. 3A-b, lanes 4 and 5). A dose-responsive effect of TFIIA on TLP-dependent promoter activation was observed (Fig. 3A-c), suggesting physical and functional interactions between TLP and TFIIA. To exclude a possibility that overexpressed TFIIA increases the amount of TLP protein, we examined the expression level of TLP and TFIIA and confirmed that TFIIA does not exhibit a significant effect on the expression of both endogenous and exogenous TLP (Fig. 3A-d). The downstream promoter was potentiated only slightly by TFIIA (Fig. 3B). Therefore it is suggested that the upstream promoter is much more sensitive to the

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Fig. 3. Activation of p21 promoter by TFIIA. The promoter activity in a reporter plasmid was examined in response to TFIIA in normal HCT116 cells. Cells were co-transfected with TFIIA expression plasmids for TFIIAαβ (IIAαβ), IIA and TFIIAγ (IIAγ), and activities of the upstream promoter in p21up/GL4 (A) and downstream promoter in p21down/GL4 (B) were determined. (A-b) Cells were transfected with TLP and TFIIAαβ or N37E and TFIIAαβ to investigate the additive effect of the transcription factors. (A-c) Cells were transfected with a constant amount of TLP and an increasing amount of TFIIAαβ. (A-d) Expression levels of TFIIA and TLP protein were determined by western blotting. (C) Cells were co-transfected with TFIIAαβ siRNA (+) or control siRNA (−) and the indicated reporter plasmids. (C-a) Luciferase activities were determined for the upstream (up) and downstream (down) promoters; core, core region of the downstream promoter without a TATA-box in p21core/GL4 plasmid. (C-b) Effect of TFIIA knockdown was checked by western blotting.
concentration of TFIIA and that TFIIA-binding activity of TLP is involved in this process. Knockdown experiments revealed that the upstream promoter is much more dependent on TFIIA than is the downstream promoter (Fig. 3C–a). The effect of downregulation of TFIIA by short interfering RNA (siRNA) was evaluated by western blotting (Fig. 3C–b). We then investigated whether TFIIA-dependent activation of the upstream promoter occurs in p53-deficient cells. As shown in Fig. 4A, however, TFIIA did not activate the upstream promoter in p53-deficient cells. Moreover, the upstream promoter harboring a mutant p53RE did not respond to TFIIA in addition to TLP (Fig. 4B).

**Recruitment of TFIIA to the upstream promoter of the endogenous p21 gene**

It has been reported that TFIIA can be recruited to some TATA-less promoters as well as TFIID-dependent TATA-containing promoters [26,27,30,31]. In this study, we demonstrated that TFIIA activates the upstream promoter additively with native TLP (Fig. 3A-b,c). We investigated whether TFIIA is associated with p21 promoters. Although we detected chromatin-bound TFIIA in the upstream promoter region (p53RE) as well as the downstream promoter region (TATA-box) (Fig. 5B), the amount of TFIIA was larger for the downstream promoter (Fig. 5B-c), possibly due to TFIID-assisted recruitment. We performed TFIIA knockdown experiments to examine TFIIA function for p21 gene regulation, and we found that the production of alt-a mRNA was dependent on the amount of TFIIA (Fig. 5C). The amount of p21 variant-1 (Fig. 5C) and the total amount of p21 mRNA (data not shown) were also decreased in TFIIA-depressed cells.

**Recruitment of TFIIA and TFIIA-reactive TLP to the upstream promoter in etoposide-treated cells**

Previously, we demonstrated that p53 and TLP are recruited to the same region of the p21 gene in etoposide-treated cells [14]. In this study, we confirmed that p53 (Fig. 6A) and TLP (Fig. 6D, lanes 1 and 2) were substantially recruited to the upstream promoters in cells treated with etoposide, which is one of the typical genotoxins. Figure 6B shows that large amounts of TFIIA bind to the upstream p53RE-containing region. Furthermore, we found that exogenous TLP but not N37E increased the amount of upstream promoter-bound TFIIA (Fig. 6C). These results indicate that TFIIA-binding ability of TLP is required for recruitment of TFIIA to the upstream promoter. We next determined how many mutant TLPs are recruited to the promoter in etoposide-treated cells. Exogenously expressed TLP and R55E clearly bound to the p53-responsive element (Fig. 6D, lanes 2 and 3). On the other hand, R52E and N37E showed decreased binding signals (lanes 4 and 5) although these proteins were substantially present in cells. A further chromatin immunoprecipitation (ChIP) assay demonstrated that the amount of promoter-bound wild-type TLP was significantly increased by etoposide, whereas that of N37E did not change and was lower than TLP, even though amounts of N37E were higher than those of TLP in nuclei of control and etoposide-treated cells (Fig. 6E). Consequently, R52 and N37E, whose chromatin-binding results were overestimated (Fig. 6D,E), were demonstrated to have weakened promoter-binding abilities compared with wild-type TLP. We then examined the effect of etoposide on TLP–TFIIA interaction. A co-immunoprecipitation experiment revealed that TLP and TFIIA form an intracellular complex in etoposide-treated cells upon DNA damage (Fig. 6F).
The same result was obtained when exogenously expressed FH-TFIIA was examined (data not shown).

Lastly, we investigated whether activity of the upstream promoter is modulated by TLP in etoposide-treated cells. TLP knockdown resulted in a decrease in upstream promoter-driven mRNA production (Fig. 6G, left two columns). We found that the net quantity of knockdown-directed decreased mRNA production in etoposide-treated cells was twice as much as that obtained in normal cells (Fig. 6G, right two columns). We have confirmed that etoposide enhances transcription from the endogenous p21 upstream promoter [14,23]. These situations suggest that etoposide-augmented promoter-recruited TLP, perhaps together with TFIIA, works for activated transcription from the p21 upstream promoter.

**Discussion**

Previously, we demonstrated that TLP, which is one of the TBP family proteins, is involved in regulation of the upstream promoter [14]. The most attractive property of TLP is its stronger TFIIA-binding ability than that of TBP [29,30], although the functional significance of this property has not been elucidated. Bryant et al. [31] reported that mutant TBP with decreased TFIIA-binding ability showed decreased transcription activation function in vitro. In this study, we demonstrated that Asn37 and Arg52 of TLP, which correspond to TFIIA-reactive Asn189 and Arg205 of TBP, respectively [31], are required for TFIIA binding in human cells (Fig. 1). N37E and R52E exhibited decreased transcriptional activation functions for the endogenous upstream promoter, while R55E, which exhibited a slight but significant growth-inhibitory effect compared with wild-type TLP (Fig. 7B). Knockdown of TFIIA also resulted in acceleration of the cell proliferation rate (Fig. 7C). We next examined the effects of TLP and TFIIA on the profile of etoposide-triggered cell death by knockdown experiments (Fig. 7D), and we found that both TLP (Fig. 7D–a) and TFIIA (Fig. 7D–b) accelerated cell death rate in a DNA-damaged condition. Since TLP and TFIIA play a negative role in cell growth and since association of the two factors is implicated from the results shown in Fig. 7B, these factors might modify the expression of growth- and apoptosis-related genes including p21.
has substantial TFIIA-binding capacity, exhibited native function (Fig. 2C). These mutant TLPs also exhibited decreased transcriptional activation function for the upstream promoter in an exogenous reporter plasmid (Fig. 2D). Consequently, TFIIA-binding ability of TLP is thought to be required for TLP-dependent transcriptional activation. Although R52E had binding ability to processed TFIIA (Fig. 1, lane 12), it exhibited little transcriptional function. Because unprocessed TFIIA has been reported to be transcriptionally
active [28], it is possible that binding to unprocessed TFIIA is required for TLP function to activate p21 upstream promoter.

TFIIA activates RNA polymerase II promoters via interaction with various transcription factors. As is generally known, TFIIA indirectly associates with the TATA-box promoter element via TBP [1–3, 6]. Li et al. [24] showed that TFIID is recruited to the TATA-box of the p21 downstream promoter and that p53 is associated indirectly with the TATA-box via TFIID. Indeed, abundant chromatin-bound TFIIA was detected at the downstream promoter (Fig. 5B). However, overexpressed TFIIA enhanced the endogenous downstream promoter only slightly (Fig. 3B). On the other hand, the upstream promoter was significantly activated by TFIIA (~3.0-fold) (Fig. 3A-a). Moreover, results shown in Fig. 3C and maybe Fig. 5C suggest that the upstream promoter requires a high concentration of intracellular TFIIA for its maximal activity. It has remained a question for a long time why TFIIA is an essential factor for cell growth [32], despite the fact that it works just as a cofactor. We speculate that some essential TATA-less genes need TFIIA as well as TLP. Results shown in Fig. 3A-b also demonstrate an additive effect between TFIIA and TFIIA-interactive TLP but not mutant ones, suggesting a functional interaction of these two factors for the upstream promoter. As already stated, the human p21 gene has two major promoters: a TATA-less upstream promoter and a TATA-containing downstream promoter [23,24]. Although the mechanism by which TFIIA exhibits different responses to the two promoters of the p21 gene is not fully understood, the TATA-box element seems to be one of the determinants. Existence of multiple promoters of the p21 gene might have an advantage to maximize the level of gene expression, which is governed by different sets of transcription factors, when cells are exposed to different kinds of stimuli and stresses.

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**Fig. 7.** Inhibitory effect of TLP and TFIIA on cell growth. HCT116 cells were treated with dimethylsulfoxide (A) or etoposide (D). (A) Normal (a) and p53−/− cells (b) were transfected with TLP siRNA (siTLP) or control siRNA (scr). The cells were replated and cultured. Then cell numbers were counted at the indicated times. (B) Growth profile of TLP-overexpressing cells. Cells transfected with an effector plasmid expressing TLP or its mutants were replated and cell numbers were counted. (C) Growth profile of TFIIAαβ knockdown cells. Cells were transfected with TFIIAαβ siRNA (siIIA) or control siRNA (scr), and the growth profile was analyzed. (D) Knockdown of TLP (a) or TFIIA (b) of etoposide-treated cells. Cells transfected with siRNAs were cultured in an etoposide-containing medium and viable cells were counted at the indicated times.
It has been confirmed that the upstream promoter is basically driven by p53 [23]. Activity of the upstream promoter was almost inert in p53-deficient cells (Fig. 4A). Moreover, TLP does not exhibit a transcription activation function for the upstream promoter in p53-null cells. We found that the upstream promoter is upregulated by TFIIA and is dependent on TFIIA in addition to p53 and TLP (Figs 3 and 4). The results shown in Figs 5 and 6 demonstrate that these three transcription factors are recruited to the upstream promoter, and etoposide, which stimulates p21 gene expression, increased this recruitment. Drosophila TLP works as a co-activator for DREF transcription factor of the PCNA gene [13]. Moreover, TFIIA can work as a co-activator of several activators [33–36] and binds to p53 [37]. We have observed intracellular binding of TLP and TFIIA [29]. Furthermore, we showed interaction between TLP and p53 [25]. We therefore speculate that TLP can form a triple complex with TFIIA and p53, and TLP and TFIIA coordinate function as a binary co-activator complex for p53 on the p21 upstream promoter. The fact that the native, but not N37E, TLP stimulates the upstream promoter additively with TFIIA (Fig. 3A-b) supports this hypothesis.

In addition to p21, TLP and p53 are widely involved in growth repression and apoptosis of cells. The present study revealed that TFIIA is also associated with the function of TLP. The results presented in Fig. 7A show that TLP-mediated growth repression is dependent on p53. Since TLP with decreased TFIIA reactivity exhibited a weaker growth-inhibitory effect (Fig. 7B), some parts of TLP-mediated growth repression can be governed by at least TLP- and TFIIA-dependent transcription from the upstream promoter of the anti-mitotic p21 gene. Furthermore, we observed that TLP and TFIIA are also involved in etoposide-mediated cell death (Fig. 7D). We believe that TLP and TFIIA contribute to this phenomenon through interaction with the p21 upstream promoter.

Materials and methods

Cell culture, drug treatment and DNA transfection

Human HCT116 cells (wild-type and p53-deficient mutant cells) [27] and HeLa cells were maintained in Dulbecco’s modified MEM with high glucose and low glucose respectively (Sigma-Aldrich, St. Louis, MO USA) at 37 °C in the presence of 10% fetal bovine serum. Cell numbers were counted by the trypsin blue dye-exclusion method with a hemocytometer. Etoposide dissolved in dimethylsulfoxide was added to the medium to 30–50 μM. Transfection of nucleic acids was performed by using Lipofectamine and Plus Reagent (Invitrogen, Carsbad CA, USA).

Expression plasmids for mammalian cells

pCIneo-FH-TLP, which is an expression plasmid of flag/oligohistidine (FH) tagged mouse TLP, was described previously [14]. Mouse and human TLPs have an identical amino acid sequence. Plasmids for mutant TLPs (R55E, R52E and N37E) were described previously [29]. TFIIA expression plasmids, pCIneo-FH-TFIIAab and pCIneo-FH-TFIIAγ, have an open reading frame of human TFIIAab and TFIIAγ with an FH-tag at their amino termini.

Reporter plasmids for luciferase assay

pGL4.10 vector (Promega, Madison WI, USA) was used for construction of luciferase reporter plasmids. A reporter plasmid (designated p21up/GL4) containing a human p21 promoter region encompassing from −2266 to −1875 was described previously [14]. The +1 position represents the transcription start site of the downstream promoter. In this study, we constructed two new luciferase reporter plasmids, p21down/GL4 and p21core/GL4, that contain a downstream promoter region from −168 to +66 and a short DNA stretch from −5 to +66 of the p21 downstream promoter, respectively. These constructs were generated by a PCR-based strategy using a reporter plasmid encompassing from −2677 to +66, which has been named p21luc1 as previously described [14]. Primer sets to amplify DNA fragments from −168 to +66 and from −5 to +66 sequences were as follows: −168 to +66 forward, 5'-CTCGAGGCCCTGTGGAATCCGCGC-3' and 5'-GATGGGCAGGTGGAAGAAG-3'; −5 to +66 forward, 5'-CTCGAGGCCCTGTGGAATCCGCGC-3' and 5'-GATGGGCAGGTGGAAGAAG-3'; and common reverse, 5'-AGATCTCGGCGAATCCGCGC-3'.

RNA interference

siRNAs were prepared by a Silencer siRNA Construction Kit (Ambion, Carlsbad, CA, USA). Sequences for target human TFIIAab were 5'-GATGGGCAGGTGGAAGAAG (sense) and 5'-CTCGAGGCCCTGTGGAATCCGCGC-3' (antisense). The sequence for human TLP was described previously [14]. A scrambled sequence of a part of TFIIAab was used as a control siRNA. Cells were transfected with 50–100 nM of siRNA and cultured for an appropriate period.

PCR

Total cellular RNAs were prepared using an RNeasy Mini Kit (Qiagen, Chatsworth, CA, USA), and RT-PCR was performed as described previously [38]. Amplified products were analyzed by agarose gel electrophoresis. Quantitative
determination of the PCR products (qPCR) was performed using a Thunderbird qPCR Mix (Toyobo, Osaka, Japan) and 7300 Real Time PCR System (Applied Biosystems, Foster City, CA, USA). All reactions were performed in triplicate. Primer sets to detect p21 transcripts were as follows: total p21 forward, 5'-GGGTGA; reverse, 5'-GGGTGA. Primer sets to detect TLP transcripts were as follows: total TLP forward, 5'-GGGTGA; reverse, 5'-GGGTGA.

Luciferase assay

Cells were inoculated into a 24-well plate (8 x 10^4 cells-well^-1). Twenty-four hours later, cells were transfected with the indicated amount of a reporter plasmid and an effector plasmid and cultured for 24 h. Total amounts of transfected DNA were adjusted with pRL-TK (Promega). Luciferase activity in lysates was determined by a Dual Luciferase Reporter Assay System (Promega).

Chromatin immunoprecipitation (ChIP)

Cells transfected with plasmids were treated with 50 µM etoposide for an appropriate time. After fixation of cells, ChIP assay was performed as described previously [14]. Endogenous and exogenous FH-proteins were precipitated with a specific antibody and Protein G-Sepharose 4 Fast Flow (GE Healthcare Bioscience) and α-Flag M2 Affinity Gel (Sigma-Aldrich), respectively. Immunoprecipitated DNAs and control input DNAs were analyzed by semi-quantitative PCR or qPCR using p21 promoter-specific primer sets. Primer sets for ChIP analysis were as follows: p53RE forward, 5'-CAC TTTCACATTTCCCCTTtg; reverse, 5'-AC AACAAAATAG; TATA-box forward, 5'-TGTTGGAAC TCGGCGAGCTCAGCTG; reverse, 5'-CCAGCTC CG GCTCCACAGGAGACT; control forward, 5'-GGGTGA; reverse, 5'-GGGTGA.

Statistical analysis

Data in this study are shown as mean ± standard error of the mean obtained from at least three independent experiments. Statistical significance of quantitative data was determined using Bonferroni’s method with R CONSOLE (ver. 3.0.3). The number of experiments used for statistical analysis was at least three (n = 3). P < 0.05 was considered to be statistically significant. Statistical significance of differences between samples is shown in the figures with asterisks: *P < 0.05; **P < 0.01; ***P < 0.001.

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Author contributions

HS, RM and TN performed experiments. HS and TT prepared the manuscript. TT organized and conducted the study.

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