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Characterization of Bcl10 as a potential transcriptional activator that interacts with general transcription factor TFIIB☆

Yingle Liu,[1] Wei Dong,[1] Lu Chen, Peng Zhang, and Yipeng Qi*

Laboratory of Molecular Virology (Key laboratory of Education Ministry), College of Life Sciences, Wuhan University, Hubei, Wuhan 430072, PR China

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Abstract

The importance of aberrant Bcl10 nuclear expression implicated in lymphomagenesis is becoming increasingly apparent. Our previous works indicate that Bcl10 can transactivate gene expression in yeast, nevertheless, little is known about the activities of nuclear Bcl10 in the mammalian cells and the mechanisms by which it modulates transcription. To understand it better, we mapped the location of the activation domain of Bcl10. This was done in the context of its interaction with TFIIB, as well as its ability to activate transcription as a fusion protein linked to the DNA-binding domain of Gal4 in the mammalian cells. Both approaches demonstrated that Bcl10 contains an activation domain in its N-terminal 13 amino acids. Together, these findings suggest that Bcl10 nuclear expression may modulate gene expression and Bcl10 is a potential transcriptional activator apart from its traditional roles that have been found.

Keywords: Bcl10; Transcriptional activator; TFIIB

Bcl10 is an intracellular NF-κB activator that was originally found to be involved in its translocation in a case of mucosa-associated lymphoid tissue (MALT) lymphoma [1,2]. This rearrangement leads to the juxtaposition of the entire *bcl10* gene to chromosome under the control of the Ig enhancer element, resulting in its deregulated expression [1]. Bcl10 contains a caspase recruitment domain and was found to weakly promote apoptosis, whereas truncation of Bcl10 beyond the caspase recruitment domain failed to induce apoptosis [1].

Based on the fact that Bcl10 is closely correlated with the pathogenesis of MALT lymphomas, several working models have been postulated and discussed for during these years. In one opinion, it is hypothesized that deregulation of Bcl10 might induce MALT tumorigenesis via constitutive NF-κB activation that provides both anti-apoptotic and proliferative signals by up-regulating transcription of specific targets [3,4].

At the other side, the initial report and following works describe a high frequency of truncating *bcl10* mutations in a variety of tumors including several kinds of lymphomas [1,5,6]. It is therefore representing another opinion that Bcl10 mutation may be implicated in the pathogenesis and progression of the lymphoma through the loss of its proapoptotic function. In a series of other studies, however, a comparable rate of *bcl10* mutation was not confirmed when genomic DNA from a wide range of tumors was analyzed [7–9]. So the role of Bcl10 mutation in tumorigenesis remains controversial and it seems that *bcl10* mutation cannot be responsible for the development or the progression of MALT-type lymphoma.

Besides those opinions, other important findings resulted from the current study concern the Bcl10 cellular localization. It was found that some lymphoma cases, including several MALT-type lymphoma and nasal NK/T-cell lymphoma, displayed aberrant Bcl10 nuclear expression [10–13]. Although until now it is unknown

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* Corresponding author. Fax: +86-27-87661831.
E-mail address: mvlwu@whu.edu.cn (Y. Qi).
[1] Equal contribution to this work.

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whether Bcl10 might take in charge of other functions apart from NF-κB activation, it has been shown that a panel of regulators in the NF-κB pathway may have unexpected functions in nucleus. For instance, IKKα, IKKγ, NIK, IκB, and p65 have been demonstrated to shuttle between the nucleus and cytoplasm [14,15]. Recently, IKKα, and IKKγ have been found to be involved in CBP- and P65-dependent transcriptional activation near NF-κB responsive promoters [16]. Notably, Bcl10 was ever reported co-precipitated in the IKK complex [17]. Thus by analogy, the activity of nuclear Bcl10 is an interesting point worthy to be investigated, if we want to get some clues into the tumorigenesis of certain correlated lymphoma cases.

In our previous work, we found Bcl10 can activate gene expression in yeast [18]. Herein, we demonstrate that Bcl10 fused with Gal4DBD can activate the promoter containing copies of Gal4 binding sites. Moreover, we found Bcl10 interacts with TFIIB, which is critical to assembly of Bcl10 with transcriptional preinitiation machinery.

Materials and methods

Antibodies and reagents. Anti-RNApolII, anti-TFIIB, and anti-TFIID (TBP) were from Santa Cruz biotechnology. Anti-GST was from Ptglab (America). Anti-HA was from Roche.

Cells and plasmids. The HeLa cells were obtained from CCTCC (China) and maintained in DMEM with 10% FCS (Sigma Chemical). The constructs including p17X4-luc, p17X4-CAT, pGEX4-Bcl10, pGEX4-Bcl10 (1–13), pGEX4-Bcl10 (1–90), pGEX4-Bcl10 (14–90), pGEX4-Bcl10 (14–233), pGEX4-Bcl10 (91–233), pcDNA3.1-TFIIB, pcDNA3.1-Bcl10, pcDNA3.1-GalBcl10, pcDNA3.1-GalBcl10 (1–13), pcDNA3.1-GalBcl10 (1–90), pcDNA3.1-GalBcl10 (14–90), pcDNA3.1-GalBcl10 (14–233), pcDNA3.1-GalBcl10 (91–233), pcDNA3.1-Gal4DBD, and pHM6-Bcl10 were cloned in our laboratory.

Preparation of Bcl10 affinity column. GST-fused Bcl10 proteins were expressed in *Escherichia coli* by induction for 3 h at 30 °C with 0.4 mM isopropyl-β-D-thiogalactopyranoside. Cells were harvested and sonicated in buffer A [20 mM Tris–HCl (pH 7.4), 0.2 mM EDTA, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride] containing 1 M NaCl. After centrifugation, the extracts (supernatants) were incubated with glutathione–agarose resin (Pharmacia Biotech) for 1 h at room temperature. The protein–agarose beads were precipitated and washed three times in buffer A containing 1 M NaCl and another three times in buffer A containing 0.1 M NaCl. The protein-agarose resins were stored in buffer A containing 0.1 M NaCl and 0.03% sodium azide at 4 °C.

Affinity chromatography. Affinity chromatography was as described [19]. Ten milliliters of nuclear extract was loaded onto Bcl10 affinity column preequilibrated with buffer D [20] for two successive incubations. After collecting the second flowthrough fraction, the column was washed with the equilibration buffer and eluted with buffer D containing 1 M KCl. Respectively, 9 ml flowthrough and 2 ml elute were dialyzed against buffer D containing 100 mM KCl.

Preparation of nuclear extracts. Nuclear extracts were prepared from HeLa cells untreated or transfected with Bcl10 and TFIIB expression vectors. After 48 h, three 100-mm tissue culture dishes transfected with each construct were washed, and the cells were collected and spun at 1000*g* for 5 min. The pellet was resuspended in 4 ml lysis buffer (10 mM Hepes, pH 7.6, 100 mM KCl, 0.1 mM EDTA, 3 mM $MgCl_2$, 10% glycerol, 1 mM dithiothreitol, 1% trasylol, and 1 μg/ml leupeptin/pepstatin A) and ruptured with 10 strokes of a Dounce homogenizer. The mixture was spun at 500*g* for 5 min, and the pellet was resuspended in 1 ml of the above lysis buffer. Cells were again pelleted for 2 min at 15,000*g* in a microcentrifuge, and the protein was extracted by resuspension in 150 μl of lysis buffer with 0.27 M KCl. This mixture sat on ice for 15 min with intermittent mixing, was spun for 15 min at 15,000*g*, and the protein-containing supernatant was stored at −70 °C.

Immunodepletions. Antibodies directed against the various RNA polymerase II GTFs were incubated with protein A–Sepharose beads for 1 h at room temperature, washed in phosphate-buffered saline, and cross-linked. The antibody beads were washed in buffer containing 20% glycerol, 20 mM Hepes (pH 7.9), 50 mM KCl, 0.2 mM EDTA, 3 mM DTT, 0.05% Tween 20, and 0.5 mM phenylmethylsulfonyl fluoride, and used for depletions. Nuclear extracts (approximately 20 μg/μl) were subjected to two successive incubations with antibody beads, each for 25 min at room temperature with agitation. After the second incubation, the supernatants were used for in vitro transcription reactions and depletions were monitored by immunoblotting.

In vitro transcription and primer extension. The in vitro transcription was carried out by incubating different combinations of nuclear extract, nuclear extracts immunodepleted of special GTFs, elute from Bcl10 affinity chromatography and flowthrough from Bcl10 affinity chromatograph at 30 °C for 30 min in transcription buffer [100 mM KCl, 25 mM Hepes (pH 7.9), 12.5 mM $MgCl_2$, 0.1 mM EDTA, 10% glycerol, and 1 mM dithiothreitol]. Transcription was initiated by addition of 5 mM nucleoside triphosphate (NTP) mixture. The reaction was terminated after 1 h by the addition of stop solution (0.2 M NaCl, 20 mM EDTA, 1% SDS, 0.25 mg yeast tRNA per ml, and 50 μg proteinase K per ml) and incubation at room temperature for 2 min.

The RNA was isolated by phenol–chloroform (1:1) and subsequent chloroform extraction followed by ethanol precipitation. The RNA products were detected by extension of γ-^{32}P 5′-end-labeled primer (5′-CTCAAAATGTTCTTTACGATG-3′) complementary to the CAT coding region.

In vitro transcription–translation. Coupled in vitro transcription–translation of TFIIB was performed with TNT-T7 (Promega) and labeled with [^{35}S]methionine (Amersham–Pharmacia Biotech).

GST-resin pull-down assay. Twenty microliters of glutathione–Sepharose beads coated with bacterially expressed GST fusion proteins was mixed with equal amounts of in vitro translated TFIIB and rocked at 4 °C for 1 h in modified GBT buffer containing 1% bovine serum albumin. Beads were collected by low speed centrifugation and washed extensively in modified GBT buffer. Bound proteins were eluted in sample buffer and analyzed by 10% SDS–PAGE and Western blotting. Input lanes show one-tenth of loaded lysate.

Immunoprecipitation. For precipitation of TFIIB from nuclear extracts, 25 μg of HeLa nuclear extract transfected with pHM6-Bc10 was prepared. After reaction with the 3 μg anti-HA antibody for 1 h at 4 °C, 10 μl of 50% swollen protein A–Sepharose resin was added. Samples were rotated for another hour followed by three washes with washing buffer (10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.1% SDS, and 1 mM EDTA). The bound proteins were eluted with elution buffer (10 mM Tris–HCl, pH 7.4, 2% SDS, 0.3 M NaCl, and 1 mM EDTA). The Western blotting was carried out with anti-TFIIB for detection.

Results

To examine a possible function for Bcl10 in transcription in mammalian cells, we constructed fusion proteins of Bcl10 with the DNA-binding domains of yeast transcription factor Gal4. Gal4 fusion proteins have been used widely to identify transactivation activ-

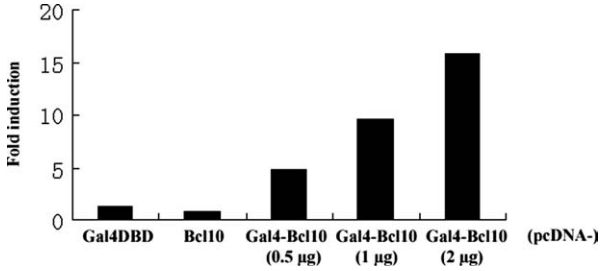

Fig. 1. Activation of luciferase reporter gene transcription by Gal4-Bcl10 fusion protein. HeLa cells were co-transfected with 1 μg p17X4-luc (firefly luciferase) alone or in combination with the indicated amounts (μg) of pcDNA-GalBcl10 or 1 μg pcDNA-Bcl10 (control) or 1 μg pcDNA-Gal4DBD in 6 cm plates. Extracts from transfected cells were subjected to luciferase activity assays. Results represent the average value for relative luciferase activity from three experiments. An expression plasmid pRL-TK *Renilla* reporter (0.1 μg) was used for normalization in all transfections. Results of transfections are presented as fold induction over the expression of p17X4-luc alone. p17X4-luc is a reporter plasmid containing four copies of the Gal4 DNA-binding site upstream of a minimal promoter element TATA box driving the firefly *luciferase* gene. The luciferase activity was measured 48 h after transfection using a dual-luciferase reporter gene assay system, according to the procedures provided by the manufacturer (Promega). All the experiments in this paper were repeated at least three times with reproducible results.

ity of transcription factors. Gal4/Bcl10 fusion constructs were co-transfected into HeLa cells with the luciferase reporter plasmid, containing four copies of the Gal4 binding site upstream of a minimal promoter element TATA box and expressing luciferase. All cells were co-transfected with a *Renilla* luciferase expression plasmid to standardize the transfection efficiency. We found that expression of Bcl10 induced considerable transcriptional activation on reporter gene luciferase (Fig. 1). These results are consistent with the notion that Bcl10 may be responsible for transactivation of transcription.

To map the activation domains of Bcl10, a series of fusion proteins containing the DBD of Gal4 and various regions of Bcl10 was constructed. Bcl10 contains a CARD motif from amino acids 14–90 and is entirely serine–threonine rich from amino acids 119 to C-terminal. In this study, various Gal4 fusion constructs were co-transfected into HeLa cells with the luciferase reporter plasmids. The wild type Bcl10 exhibited maximum transactivation at the concentration indicated. Strikingly, the constructs lacking N-terminal 13 amino acids (amino 14–90, 14–233, and 91–233) failed to transactivate, whereas the constructs containing this region (amino 1–13 and 1–90) exhibited considerable transactivation activity (Fig. 2). This suggests that the region containing N-terminal 13 amino acids is necessary for transactivation.

Activation of a Gal4-luciferase reporter by Gal4 fusion Bcl10 and its deletion mutants addresses the potential role of Bcl10 in the process of transactivation. Thus, it is interesting to investigate whether Bcl10 interacts through direct protein–protein binding with components of the transcriptional pre-initiation complex. We prepared an affinity column (Bcl10-column) containing covalently linked GST-Bcl10 proteins using the glutathione–agarose beads and passed the nuclear extract from HeLa cells through the Bcl10-column. The eluted proteins or the "column elute" that contained bound proteins was tested for the ability to sustain GAL4-dependent transcription in the presence of individual transcription-factor-deleted nuclear extract. Since GAL4-activated transcription basically requires RNAPolII, TFIIB, and TFIID (TBP), we prepared the nuclear extract immunodepleted with these indicated GTFs. We observed that in the presence of the column elute, only leaving out TFIIB would not impair the transcription level, which suggested that the column elute contained TFIIB and complemented full transcription with TFIIB-deleted nuclear extract (Fig. 3A). So one of the factors that are bound to the Bcl10 on the column is TFIIB.

To confirm the interaction specificity between TFIIB and Bcl10-column, the flowthrough fractions (those that did not bind to the column) from the Bcl10-column and control column containing only GST proteins were compared for their ability to support transcription. We found the flowthrough fraction from the control column supported Bcl10 stimulated transcription at a similar level to that of the crude nuclear extract, indicating there is no contamination between TFIIB and the

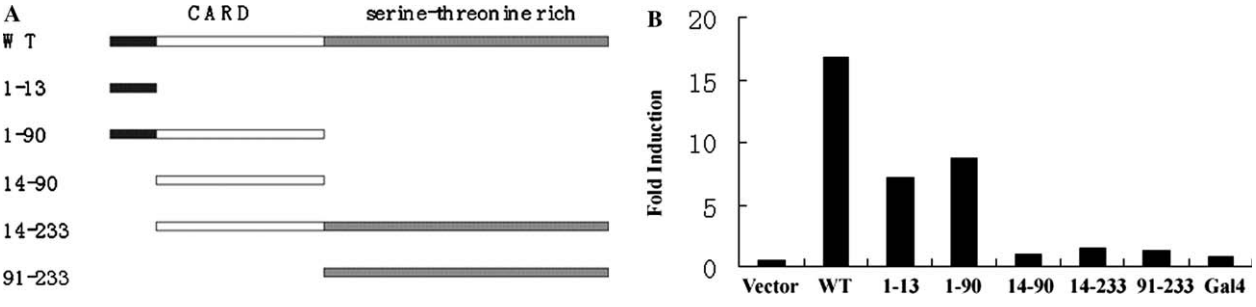

Fig. 2. The N-terminal 13 amino acids in Bcl10 are required for Gal4-Bcl10-dependent transactivation. (A) Schematic representation of the structure of Bcl10 deletion mutants. The N-terminal 13 amino acids (N13), caspase recruitment domain (CARD), and the serine- and threonine-rich region 1 (STRR1) are indicated. All deletions were ligated with a Gal4 DBD and then subcloned into pcDNA3.1. (B) HeLa cells were co-transfected with 0.5 μg p17X4-luc and 1 μg pcDNA-GalBcl10 or its mutants. Extracts from transfected cells were subjected to luciferase activity assays.

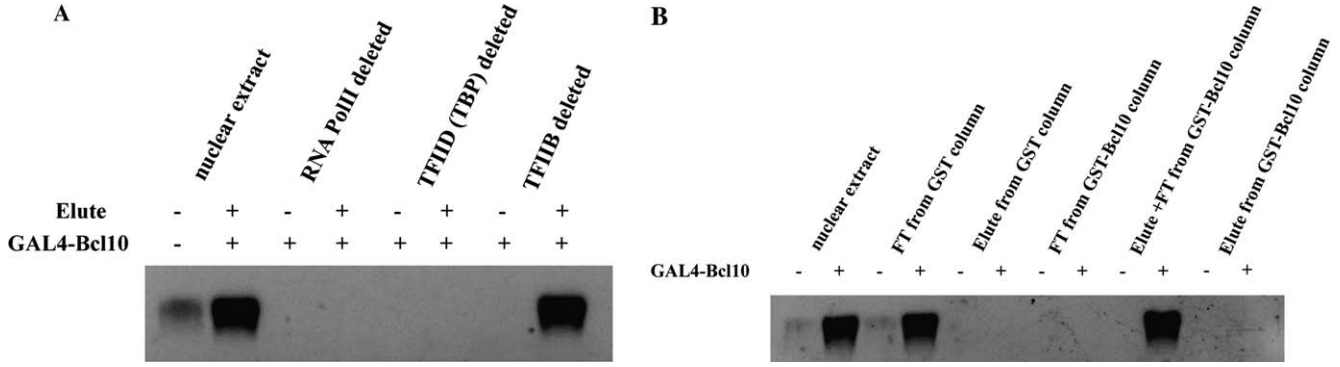

Fig. 3. TFIIB is identified as a Bcl10-associated general transcription factor. (A) The 15 μl nuclear supernatant immunodepleted with indicated general transcription factors and 10 μl of 0.2 M KCl elute from the GST-Bcl10 column were tested for transcription activity in total 50 μl reaction mixture containing 5 μg GAL4-Bcl10 and 0.5 μg template DNA p17X4-CAT. The in vitro transcription and primer extension assays were carried out. In the case while the immunodepleted supernatant was omitted, 10 μl of 0.2 M KCl elute from the GST-Bcl10 column was tested for transcription activity in total 25 μl reaction mixture containing 2.5 μg GAL4-Bcl10 and 0.25 μg template DNA. As the positive control, 10 μl crude nuclear extract was also tested for transcription activity in total 25 μl reaction mixture containing 2.5 μg GAL4-Bcl10 and 0.25 μg template DNA. (B) The 10 ml nuclear extract was chromatographed in 0.2 M KCl on 5 ml GST column or GST-Bcl10 affinity column. The 15 μl flowthrough fraction or 10 μl elute from affinity chromatograph or GST column was tested for transcription activity in total 30 μl reaction mixture containing 3 μg GAL4-Bcl10 and 0.3 μg template DNA p17X4-CAT. A mixture of flowthrough and elute was tested in total 60 μl reaction mixture containing 6 μg GAL4-Bcl10 and 0.6 μg template DNA. As the positive control, 10 μl crude nuclear extract was also tested for transcription activity in total 25 μl reaction mixture containing 2.5 μg GAL4-Bcl10 and 0.25 μg template DNA (FT: flowthrough).

glutathione–agarose beads. Consistent with above findings, transcription activity was lost in the flowthrough fraction from the Bcl10-column, whereas restored upon addition of Bcl10-column elute, suggesting one or more general transcription factors were lacking in the flowthrough fraction (Fig. 3B).

To further verify the interaction between Bcl10 and TFIIB, we carried out GST pull-down assay, and the results suggest that there is a specific interaction between Bcl10 and TFIIB (Fig. 4). Moreover, in a co-immunoprecipitation assay, we found that TFIIB formed a stable complex with Bcl10 that allowed it to be precipitated with antibody recognizing HA-tagged Bcl10 (Fig. 5). Our results suggest that transcriptional activation activity of Bcl10 may involve recruitment of the transcriptional pre-initiation machinery to the promoter DNA through binding with TFIIB.

From the above conclusion, the parts of Bcl10 responsible for activation of transcription appear to be strongly correlated with intact N-terminal 13 amino acids. To investigate whether this domain is also necessary for binding with TFIIB, we carried out GST pull-down assay. The results indicated that Bcl10 interacted with TFIIB in a manner dependent on the integrity of N-terminal 13 amino acids. A truncated mutant form of Bcl10 containing only these 13 amino acids is capable of binding with TFIIB, whereas CARD motif plus serine–threonine rich region (amino 14–233) seems to be dispensable for binding with TFIIB (Fig. 4).

Given the localization of the amino acid sequences interacting with TFIIB, we found the Bcl10-dependent transactivation effect could be enhanced to an extent by over-expression of TFIIB in the co-transfection experiments. Moreover, this TFIIB effect also depends on

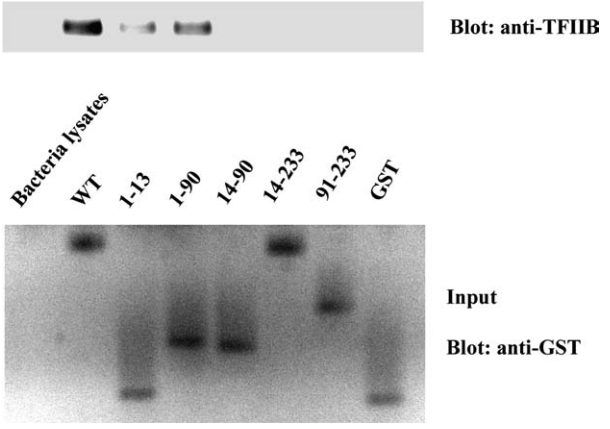

Fig. 4. Bcl10 and its N-terminal amino acids (N13) interact with TFIIB in vitro. Human general transcription factor TFIIB was synthesized, ^{35}S-labeled by in vitro translation, and tested for binding to immobilized GST-Bcl10 or its mutants in a pull-down assay. Loaded in the input lane is 5% of the protein used in the binding reaction. GST-Bcl10 or its mutants were immobilized on glutathione–Sepharose beads, and control beads are glutathione–Sepharose without protein.

N-terminal 13 amino acids, and was lost when it was deleted (Fig. 6). Together, our data support that Bcl10-dependent transactivation is mediated through interaction with TFIIB and the region responsible for association with TFIIB is located in the N-terminal 13 amino acids of Bcl10.

Discussion

In this study, we demonstrate that Bcl10 exerts transactivation activity in in vitro assay and has a direct interaction with a component of the basal transcription

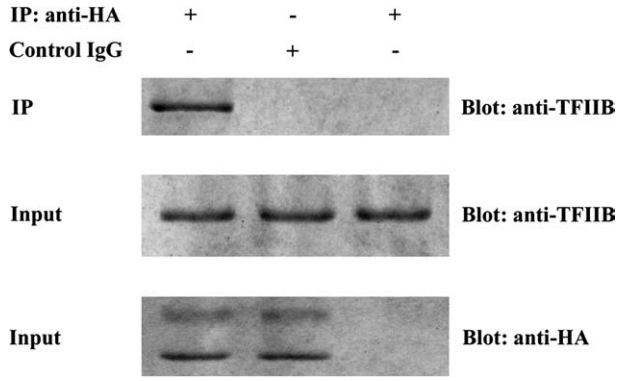

Fig. 5. TFIIB is co-immunoprecipitated with Bcl10. Lysates from HeLa infectants that expressed ectopic HA-tagged Bcl10 were immunoprecipitated with anti-HA antibody or control IgG. The immunoprecipitates were separated by SDS–PAGE and analyzed by immunoblotting with the anti-TFIIB antibody.

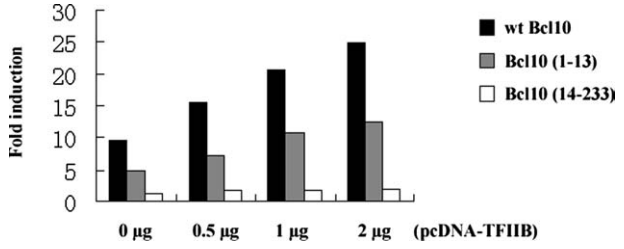

Fig. 6. Bcl10-dependent transactivation is enhanced by over-expression of ectopic TFIIB. HeLa cells were co-transfected with 1 μg p17X4-luc alone or in combination with 1 μg pcDNA-GalBcl10 or its mutant pcDNA-GalBcl10 (1–13) or pcDNA-GalBcl10 (14–233), together with indicated amount of pcDNA-TFIIB. Extracts from transfected cells were subjected to luciferase activity assays. 0.1 μg pRL-TK *Renilla* reporter was used to normalize the transfection efficiency. Results of transfections are presented as fold induction over the expression of p17X4-luc alone.

machinery TFIIB. The association with TFIIB is necessary to allow assembly of transcriptional activators with transcriptional pre-initiation machinery [21,22]. Our study mapped the region within N-terminal 13 amino acids that is responsible for the interaction with TFIIB as activation domain. Activation domain may serve multiple functions by binding to the components of the basic transcription machinery, to bring an activation domain over large distances into close proximity of the initiation complex near the transcription start site [19]. Besides general transcription factor TFIIB, our study could not exclude the possibility that there may be other components that are essential for Bcl10-dependent transactivation. On the other hand, Bcl10 may also act as a co-activator that associates with general transcription factors and bridges its interaction with other activators. Moreover importantly, whether Bcl10 contains other typical domains for a transactivator, such as DNA-binding domain, nucleus location sequence (NLS), and nucleus export sequence (NES), is still to be investigated.

A panel of preliminary studies has revealed a strong correlation between MALT lymphomas genesis and aberrant nuclear Bcl10 expression. Furthermore, aberrant Bcl10 nuclear location was also found in nasal NK/T-cell lymphoma [10–13]. Therefore, it raises the possibility that nuclear localization of Bcl10 can occur as the result of some cytogenetic events, and may signal in a disparate way from its activity in the cytoplasm. Given the fact that in several cases such as in normal B cell follicles including germinal center B cells and marginal zone B cells, Bcl10 is expressed predominantly in the cytoplasm [12], thus, Bcl10 shuttling from cytoplasm to nucleus seems to be in the tight control in response to cytogenetic signals. It is well established that Bcl10 is a NF-κB activator whose function is theoretically confined in the cytoplasm. However, it is still to be questioned whether Bcl10 plays a duplicated role in the nucleus just as that in the cytoplasm.

Recently it has been found that nuclear IKKα and IKKγ, components of the IKK complex, are involved in NF-κB activation [16]. Traditionally, IKK complex has been thought to play a key role in regulating the NF-κB pathway as cytoplasmic proteins and all of processes it may be involved are likely restrained exclusively in the cytoplasm. However, additional studies have defined novel roles of IKKα that is capable of increasing NF-κB-dependent gene expression in nucleus [15]. By analogy, nuclear Bcl10 may resemble these two proteins to play a role in regulation of certain promoters, which takes place in a functionally different mechanism from the signaling in the cytoplasm.

Together, the transactivation effect of Bcl10 is evident from our experiments and it involves at least a biologically important nuclear protein TFIIB. In this respect, it is interesting to determine the precise role of nuclear Bcl10 and how this contributes to lymphomagenesis. Our work perhaps provides some insight into this unknown field. Particularly in a series of lymphoma cases, deregulation of Bcl10 expression may lead to an oncogenic event through over-activated responsive promoters, which originally transfer the surviving signals in response to a variety of stress stimuli.

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