Interaction between the Hematopoietic Ets Transcription Factor Spi-B and the Coactivator CREB-binding Protein Associated with Negative Cross-talk with c-Myb

Hitomi Yamamoto, Fumiko Kihara-Negishi, Toshiyuki Yamada, Mitsuhiro Suzuki, Tohru Nakano, and Tsuneyuki Oikawa

Department of Cell Genetics, Sasaki Institute, Tokyo 101-0062, Japan [H. Y., F. K.-N., T. Y., M. S., T. O.], and Department of Molecular Cell Research Institute for Microbial Diseases, Osaka University, Osaka 565-0871, Japan [T. N.]

Abstract
We have previously shown that the hematopoietic Ets transcription factor PU.1 interacts with the transcriptional coactivator CREB-binding protein (CBP). In this study, we further investigated whether Spi-B, another hematopoietic Ets transcription factor, also interacts with CBP. Direct physical interaction of Spi-B with CBP was demonstrated by glutathione S-transferase binding assay. Analysis using several deletion mutants of Spi-B and CBP revealed that the NH₂-terminal region including the activation domain of Spi-B interacted with the region spanning amino acid residues 1283–1915 of CBP in vitro. The interaction of Spi-B with CBP was also observed in vivo. CBP potentiated Spi-B-mediated transcription of the reporter gene driven by the multimerized PU.1/Spi-B binding sites. This transcriptional activation by Spi-B and CBP was inhibited by expression of c-Myb, and the transcriptional activation by c-Myb and CBP was inhibited by expression of Spi-B, suggesting competition for CBP between these two transcription factors. Our results suggest that CBP acts as a transcriptional coactivator of Spi-B and mediates synergistic or antagonistic interactions between other transcription factors.

Introduction
Members of the Ets family of transcription factors are characterized by a highly conserved Ets domain that mediates binding to the purine-rich element of 5′-GGA(A/T)-3′ (1). This family of transcription factors consists of approximately 30 different proteins, some of which play critical roles in hematopoietic cell growth and development. PU.1 is a hematopoietic Ets transcription factor that controls the expression of many B-cell- and macrophage-specific genes (2). Spi-B is a B-cell-specific Ets transcription factor. It has a DNA binding domain that shares 67% homology with that of PU.1 and a transcriptional activation domain that is divergent from that of PU.1 (3). PU.1 and Spi-B are believed to be important for the development of certain lineages of hematopoietic cells. PU.1-deficient mice die in utero and exhibit defects in the development of B cells, monocytes, and neutrophils (4, 5), whereas Spi-B-deficient mice are viable and possess mature B and T lymphocytes but exhibit severe abnormalities in B-cell function (6).

We reported previously that PU.1 interacts with transcriptional coactivator CBP via the glutamine-rich region of its activation domain (7). Although there is little homology between the activation domains of PU.1 and Spi-B, it is of interest to examine whether Spi-B could also interact with CBP, considering that PU.1 and Spi-B belong to the Ets family of transcription factors and regulate hematopoietic cell development.

In this study, to address this question, we initially investigated the physical interaction of Spi-B with CBP by GST binding assays, and then we analyzed the functional cooperation between Ets family transcription factors and CBP.

Whereas Spi-B and PU.1 are up-regulated during B-cell and macrophage differentiation, respectively (8, 9), c-Myb is down-regulated during hematopoietic cell maturation (10). Because CBP is a shared coactivator for several transcription factors including c-Myb (11) and the amounts of CBP are limited in cells (12), it is reasonable to speculate that the hematopoietic Ets transcription factors and c-Myb could use limited amounts of CBP reciprocally in hematopoiesis. We therefore also investigated transrepression between Spi-B or PU.1 and c-Myb via competitive usage of CBP by the transient luciferase reporter assays.

Results
CBP Interacts with Spi-B in Vitro. To prove the physical association between hematopoietic Ets transcription factor Spi-B and CBP, we performed GST binding assays. A GST fusion protein containing human Spi-B (GST-Spi-B) was used as the affinity reagent (Fig. 1A). The GST-Spi-B fusion protein was attached to glutathione-agarose beads and then
mixed with extracts prepared from mouse 293T cells transfected with an expression plasmid of the mouse CBP gene. Bound proteins were eluted and then analyzed by SDS-PAGE followed by Western blot analysis. CBP bound to GST-PU.1 used as a positive control but did not bind to GST alone (used as a negative control; Fig. 1B). This was consistent with our previous report (7). CBP protein was found to bind to GST-Spi-B. PU.1 and Spi-B also interact with the CBP-related p300 protein \textit{in vitro} (Fig. 1B).

**Fig. 1.** A, schematic diagram of Ets transcription factors, PU.1 and Spi-B, fused to GST. B, in vitro association of the Ets family transcription factors with CBP. PU.1 and Spi-B were expressed as GST fusion proteins. The fusion proteins were bound to glutathione-agarose and incubated with extracts prepared from mouse CBP-transfected 293T cells. Specifically associated proteins were resolved by SDS-PAGE, transferred to PVDF membranes, and detected with anti-CBP or anti-p300 antibody. All of the GST-fused proteins were confirmed by SDS-PAGE followed by Coomassie Brilliant Blue staining.

**Fig. 2.** A, schematic diagram of the CBP functional domains fused to GST. B, identification of Spi-B sequences required for binding to CBP. CBP-(1–312), CBP-(313–1098), CBP-(1283–1915), and CBP-(1916–2441) were expressed as GST fusion proteins. These proteins were bound to glutathione-agarose and incubated with whole cell extracts from MEL cells expressing PU.1 and Spi-B, respectively. Specifically associated proteins were resolved by SDS-PAGE, transferred to PVDF membranes, and detected with anti-PU.1 or anti-Spi-B antibody. All of the GST-fused proteins (asterisks) were confirmed by SDS-PAGE followed by Coomassie Brilliant Blue staining.

**Fig. 4.** As shown in Fig. 4, GST binding assays using \textit{in vitro}-translated [35S]methionine-labeled CBP-(1283–1915) with specific band for Spi-B was detected mainly in the eluted proteins bound to GST-CBP-(1283–1915) (Fig. 2B), suggesting that Spi-B strongly interacted with the CBP fragment that encompasses the E1A-binding domain (Fig. 2A). These results indicate that CBP interacts with Spi-B via the region spanning amino acids 1283–1915, which is overlapped for PU.1 binding.

To delineate the regions of Spi-B required for interaction with CBP, we prepared extracts from CBP-transfected 293T cells and used them in GST binding assays with some of the GST-Spi-B deletion mutants (Fig. 3A). CBP protein was retained on the full-length Spi-B (GST-Spi-B-WT), confirming the result shown in Fig. 1B. Analysis using three kinds of deletion mutants of Spi-B revealed that CBP bound strongly to the NH2-terminal region (GST-Spi-B-N) and weakly to the activation domain (GST-Spi-B-AD) but did not bind at all to the COOH-terminal region containing the Ets domain (GST-Spi-B-C) of the protein (Fig. 3B). These results suggest that the NH2-terminal region including the activation domain of Spi-B is responsible for binding with CBP.

As shown in Fig. 4, GST binding assays using \textit{in vitro}-translated [35S]methionine-labeled CBP-(1283–1915) with
GST-Spi-B or in vitro-translated [35S]methionine-labeled Spi-B with GST-CBP-(1283–1915) showed the similar results obtained by using extracts from MEL cells expressing Spi-B and extracts from 293T cells expressing CBP, supporting the argument that the interaction between Spi-B and CBP may be direct.

CBP Interacts with Spi-B in Vivo. To prove that the interaction between Spi-B and CBP we detected in vitro could also be observed in vivo, we then performed the co-immunoprecipitation experiments. An expression plasmid of Spi-B or PU.1 was introduced with that of FLAG-tagged CBP-(1283–1915) into 293T cells. Extracts from the transfected cells were immunoprecipitated with anti-FLAG antibody, and the presence of Spi-B or PU.1 in the precipitates was ascertained by Western blot analysis with antibody against Spi-B or PU.1, respectively. Spi-B as well as PU.1 was found to be coimmunoprecipitated with FLAG-tagged CBP-(1283–1915) (Fig. 5). These results indicate that Spi-B formed a complex with CBP in vivo.

CBP Is a Coactivator for Spi-B. Because Spi-B and CBP physically interact with each other in vivo, we next investigated the functional consequence of this interaction. HeLa cells were cotransfected with their corresponding expression plasmids and a reporter construct recognized by both PU.1 and Spi-B whose transcription is driven by trimerized PU box. As shown in Fig. 6A, overexpression of Spi-B increased the promoter activity by 2.3-fold. CBP synergistically enhanced the activity of Spi-B by 3.9-fold, although overex-
expression of CBP alone had no effect on this promoter. Because HeLa cells do not express Spi-B and PU.1 endogenously, the synergistic effect was easily detected. Control experiments with a luciferase reporter construct devoid of PU.1/Spi-B binding sites demonstrated that the observed effects were binding site specific (data not shown). Cotransfections of the Spi-B expression plasmid with increasing quantities of the CBP expression plasmid resulted in a dose-dependent increase of luciferase activity (Fig. 6A). These results indicate that CBP functions as a coactivator for the transcription factor Spi-B. Moreover, we investigated whether CBP is functionally important for PU.1 and Spi-B activity in vivo by studying the effect of CBP-(1283–1915), a subdomain of CBP that contains the PU.1- and Spi-B-interacting domain. As shown in Fig. 6B, expression of CBP-(1283–1915) (dnCBP) caused a marked reduction in the PU.1- and Spi-B-dependent activation of the reporter construct. These data suggest that CBP-(1283–1915) acts in a dominant negative fashion by competing with endogenous CBP and that CBP is an important coactivator for Spi-B-dependent transcription in vivo.

**Competition between c-Myb and PU.1 or Spi-B for CBP.** Among the hematopoietic transcription factors, c-Myb is the first factor found to be regulated by CBP (11). c-Myb is expressed in immature hematopoietic cells and down-regulated during cell maturation (10). PU.1 is highly expressed in B cells and macrophages and is up-regulated in macrophage differentiation (1, 2). Similarly, Spi-B is highly expressed in B cells and up-regulated during B cell maturation (8). Therefore, it is reasonable to postulate that there may be trans-repression between Myb and PU.1 or Spi-B as a result of competition for the limited amounts of CBP. We then examined whether the transcriptional activity of PU.1 or Spi-B is affected by the presence of c-Myb. As shown in Fig. 7A, the reporter plasmid with trimmerized PU box [PU(x3)-Luc] was positively regulated by PU.1 and Spi-B in HeLa cells, whereas it was not affected by c-Myb or CBP alone. Overexpression of c-Myb decreased the promoter activity induced by PU.1 or Spi-B. Next, we examined the promoter activity of the 6MBS-Luc reporter gene, which is driven by six tandem repeats of the Myb binding site. This promoter was positively regulated by c-Myb but was not affected by PU.1 or Spi-B alone. Expression of PU.1 or Spi-B inhibited the promoter activity of 6MBS-Luc augmented by c-Myb. Both the transcriptional inhibition of the promoter activity of PU(x3)-promoter-luc by c-Myb and the transcriptional inhibition of the promoter activity of 6MBS-Luc by PU.1 or Spi-B were decreased by exogenous expression of CBP (Fig. 7, A and B). The expression levels of Spi-B, PU.1, and c-Myb proteins were not altered, even when their expression plasmids were introduced into the cells in combination (data not shown). These results suggest that the competitive utilization of CBP by c-Myb and PU.1 or Spi-B may mediate negative cross-talk between these two transcription factors.

**Discussion**

Transcriptional regulation requires the participation of several classes of proteins including the basic transcription factors, sequence-specific transcriptional activators or repressors, and transcriptional coactivators or corepressors through protein-protein interaction (13). CBP and its related protein, p300, serve as a transcriptional adapter for several transcription factors by direct bridging between basic transcription factors and several sequence-specific transcriptional activators (14, 15). In the present study, we have shown that Spi-B, a B-cell-specific Ets transcription factor, interacts with CBP/p300 in a manner similar to that reported previously for PU.1 (7), although PU.1 and Spi-B do not share much similarity in their activation domains (3), which are necessary for interaction with CBP/p300. We have evidence showing that Fli-1, another Ets protein, also interacts with CBP (data not shown). Direct interactions have also been reported between Ets-1 and CBP by others (16). Thus, all results, considered together, suggest that several Ets family transcription factors interact with the coactivator CBP/p300.
CBP/p300 has an intrinsic histone acetyltransferase activity, which is thought to be important in regulating chromatin assembly, and is thereby responsible for enhanced transcription by increasing the accessibility of RNA polymerase II holoenzyme to the transcription machinery (17). Our results of luciferase assays demonstrated that CBP acts as a transcriptional coactivator of Spi-B. Thus, binding of CBP to Spi-B protein appears to be essential for high levels of transcription.

Mice with monoallelic inactivation of the CBP gene develop severe defects in hematopoietic development and vasculo-angiogenesis (18) and have an increased incidence of hematopoietic malignancies (19), suggesting the importance of CBP in hematopoiesis. The biological functions of CBP/p300 in hematopoiesis are linked to the functions of the transcription factors with which they interact at various steps of hematopoietic development (15). In the B-cell lineage, several Ets transcription factors such as Ets-1 (16), PU.1 (2), and Spi-B (3) are expressed. We demonstrated that CBP physically and functionally interacts with PU.1 (7) and Spi-B (this study), as well as with Ets-1 (16). Furthermore, CBP-related p300 also forms a stable complex with E47 that is an essential regulator of B cells (20). These findings suggest an important role for CBP/p300 in the expression of B-cell-specific genes.

CBP/p300 possesses intrinsic acetyltransferase activity for histone and nonhistone proteins, including transcription factors. Recent studies have demonstrated that hematopoietic transcription factors such as GATA-1, EKLF, and c-Myb were acetylated by CBP/p300 or pCAF (21–24). We have a preliminary result that PU.1 and Spi-B are acetylated at their Ets domain containing the conserved lysine residues in vivo (data not shown), suggesting that they might be acetylated by CBP/p300 or other acetyltransferases. Acetylation of the proteins may alter DNA binding and/or the transcriptional activities of these transcription factors (17). More extensive studies are required in the future to determine the effect of acetylation of PU.1 and Spi-B.

The Ets transcription factors act synergistically with a variety of other transcription factors to regulate many cellular promoters and enhancers (25). Transcription factor AP-1 interacts with a variety of Ets proteins. Direct protein-protein interactions have already been reported between Jun and the Ets domain of Ets-1 and PU.1 (26). Thus, the Ets transcription factors mediate expression of hematopoietic genes in combination with other transcription factors and CBP. CBP may act as a bridging factor between sequence-specific transcription factors and components of the basal transcription machinery (14).

Differentiation of hematopoietic cells is thought to be achieved by stochastic and/or hierarchical programmed cascades of the expression of several hematopoietic transcription factors (27). c-Myb is expressed primarily in immature hematopoietic progenitor cells (10), and its overexpression may therefore block both erythroid and myeloid differentiation. c-Myb binds CBP in a phosphorylation-dependent manner at a site that overlaps with the CREB-binding domain of CBP (11). Binding of c-Myb to the corresponding sequence in the c-fms promoter inhibits its activity (28), whereas PU.1 contributes as a positive factor to the cellspecific potential of the same promoter (29). Additionally, expression of c-Myb and hematopoietic Ets transcription factors PU.1 and Spi-B appears to be reciprocal during hematopoietic cell differentiation. Therefore, it can be speculated that there might be a negative cross-talk between c-Myb and PU.1 or Spi-B. Indeed, in the present study, we demonstrated that overexpression of c-Myb inhibits PU.1- and Spi-B-dependent transactivation and that overexpression of PU.1 or Spi-B inhibits c-Myb-dependent transactivation. This inhibition appeared possibly due to the competitive utilization of limited amounts of CBP between these transcription factors because overexpression of CBP weakened the mutual transrepression. It is possible but must be proven in future that c-Myb inhibits chromatin-embedded Spi-B target genes via competition for CBP in vivo. Similar mutual transrepression mediated via CBP has been reported be-
Functional Interaction between Spi-B and CBP

GAACTTGGTG-3

sequence in the SV40 enhancer (5).

constructed by inserting the double-stranded trimerized 27-mer of CBP (1283–1915) was also constructed by using pCMV-genes, such as the c-Myb and Ets transcription factors when their relatively immature hematopoietic cells. In these cases, it is speculated that CBP/p300 may act as a bridging factor between c-Myb and Ets transcription factors when their binding sites exist nearby in the same promoter in cis. Whether CBP acts as a competitive target of two transcription factors or as a bridging factor between them may depend on the context of the promoters, expression levels and modification of the transcription factors, and cooperation with other transcriptional activators during maturation of hematopoietic cells.

Our data presented in this study, along with data from previous studies, suggest that positive and negative cross-talks between Ets family transcription factors and other cellular factors are important for hematopoietic gene expression and that CBP may function as a key factor during the process of growth and differentiation of hematopoietic cells.

Materials and Methods

Plasmids and Constructs. The plasmids containing full-length mouse CBP (pRc/RSV-CBP), mouse PU.1, human Spi-B, and c-Myb were kindly provided by Drs. R. H. Goodman, D. Kabat, F. Moreau-Gachelin, and S. Ishii, respectively. Deletion mutants of CBP and Ets transcription factors were constructed by subcloning in frame into pGEX plasmids (Pharmacia Biotech). The pGEX-PU.1 plasmid encodes a fusion protein of GST and PU.1 amino acids 1–272. The pGEX-Spi-B-WT, pGEX-Spi-B-N, pGEX-Spi-B-AD, and pGEX-Spi-B-C plasmids encode fusion proteins of GST and Spi-B amino acids 1–262, 1–159, 1–98, and 160–262, respectively. The pGEX-CBP-(1–312), pGEX-CBP-(313–1098), pGEX-CBP-(1283–1915), and pGEX-CBP-(1916–2441) plasmids encode fusion proteins of GST and CBP amino acids 1–312, 313–1098, 1283–1915, and 1916–2441, respectively. For construction of expression plasmids, fragments of full-length PU.1, Spi-B, and CBP were inserted under the elongation factor-1α promoter of pEF-BOS provided by Dr. S. Nagata. A FLAG-tagged expression plasmid of CBP (1283–1915) was also constructed by using pCMV-Tag2 plasmid (Stratagene).

The reporter plasmid pGL3-PU(x3)-promoter-luc was constructed by inserting the double-stranded trimerized 27-mer oligonucleotides corresponding to the PU.1-binding sequence in the SV40 enhancer (5′-gatcTTCTCTGTAAAGAGGAACTTGGTG-3′) at the BglII site of plasmid pGL3-promoter-luc (Promega). The reporter plasmid p6MBS-Luc was constructed as reported previously (33).

GST Binding Assay. GST fusion proteins were expressed in Escherichia coli DH5α and purified according to the manufacturer’s protocol (Pharmacia Biotech). The expression plasmid of CBP or PU.1 was transiently transfected into 293T cells by the CaPO4 method, and then whole cell extracts were prepared in lysis buffer [1% Triton X-100, 150 mM NaCl, 10 mM Tris (pH 7.4), 1 mM EDTA, 1 mM EGTA, 0.2 mM sodium vanadate, 0.2 mM phenylmethylsulfonyl fluoride, and 0.5% NP40]. Approximately 500 μg of fusion proteins were immobilized onto glutathione-agarose, washed extensively in binding buffer (the same as the above-mentioned lysis buffer), and incubated with 150 μl of 293T or MEL cell extracts at 4°C for 2 h. Bound proteins were washed four times with binding buffer, eluted with reduced glutathione, boiled for 2 min in 10 μl of 2× SDS gel sample buffer, and subjected to SDS-PAGE. Eluted proteins were detected by Western blot analysis as described previously (35). Specific antibody for Spi-B was a kind gift from Dr. F. Moreau-Gachelin. Specific antibodies for PU.1, CBP, and p300 were purchased from Santa Cruz Biotechnology.

DNA Transfection and Luciferase Assay. Plasmid DNA was transiently transfected into 2 × 105 HeLa cells by LipofectAMINE PLUS (Life Technologies, Inc.). For each transfection, 0.1 μg of pGL3-PU(x3)-luc or 6MBS-Luc reporter construct was used with 0.5 μg of each expression plasmid and the corresponding empty plasmid to adjust total amounts of DNA to 2 μg. All transfection experiments were performed in duplicate. The cells were harvested for luciferase assay 36 h after transfection. Preparation of extracts and the enzyme assay were carried out using the Luciferase Assay System (Wako, Osaka, Japan). The results represent at least three independent experiments.

IP and Western Blot Analysis. The expression plasmid of Spi-B or PU.1 (10 μg) was transfected into 293T cells with that of CBP. Cells were harvested 36 h after transfection and lysed by sonication in the IP buffer [150 mM NaCl, 10 mM Tris (pH 7.4), 1% Triton X-100, 0.5% NP40, 1 mM EDTA, 1 mM EGTA, 2 mM sodium orthovanadate, and 0.2 mM phenylmethylsulfonyl fluoride]. The extracts (700 μg) were immunoprecipitated with 4 μg of anti-FLAG (M2; Stratagene) antibody, and the precipitates were washed four times with IP buffer containing 350 mM NaCl. Each immunoprecipitate was run in the Nu-PAGE Tris-bis system (Novex) and then transferred onto PVDF membrane (Millipore) by a semi-dry transfer system (Bio-Rad). Western blot analyses were done using standard procedures with anti-PU.1 antibody (T-21; Santa Cruz) or anti-Spi-B antibody. Specific bands were visualized using an ECL-PLUS kit (Amersham).

References


