The Carboxyl-terminal Fragment of Pro-HB-EGF Reverses Bcl6-mediated Gene Repression*

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Yumi Kinugasa 1,†, Miki Hieda 2, Masatsugu Hori 3, and Shigeki Higashiyama 4,††

From the 1 Department of Biochemistry and Molecular Genetics, Ehime University Graduate School of Medicine, Shitsukawa, To-on, Ehime 791-0295, Japan, the 2 Division of Internal Medicine, Department of Cardiovascular Medicine, Osaka University Graduate School of Medicine, Osaka 565-0871, Japan, and the 3 PRESTO, Japan Science and Technology Agency, 4-1-8 Honcho Kagawuchi, Saitama 332-0012, Japan

Heparin-binding epidermal growth factor-like growth factor (HB-EGF), a member of the EGF family, is synthesized as a type I transmembrane precursor (pro-HB-EGF). Ectodomain shedding of pro-HB-EGF yields an amino-terminal soluble ligand of EGF receptor (HB-EGF) and a carboxyl-terminal fragment (HB-EGF-CTF) consisting of the transmembrane and cytoplasmic domains. We previously showed that the HB-EGF-CTF translocates from the plasma membrane to the nucleus and plays a role as a signaling molecule. Immunoprecipitation showed that HB-EGF-CTF can associate with Bcl6, a transcriptional repressor in mammalian cells. A glutathione S-transferase pulldown assay revealed that HB-EGF-CTF interacted efficiently with zinc fingers 4–6 of Bcl6. A luciferase reporter assay showed that the nuclear translocation of HB-EGF-CTF following shedding reversed transcriptional repression of cyclin D2 by Bcl6. Additionally, the level of cyclin D2 protein increased and Bcl6 interaction with the cyclin D2 promoter decreased in parallel with the shedding of pro-HB-EGF at all endogenous levels. These findings suggest that HB-EGF-CTF is a potent regulator of gene expression via its interaction with the transcriptional repressor Bcl6.

Heparin-binding EGF2-like growth factor (HB-EGF), a member of the EGF family, directly binds to and activates the EGFR (ErbB1/HER1) and can indirectly transactivate ErbB2 (HER2/neu), ErbB3 (HER3), and ErbB4 (HER4) by forming a heterodimer with the EGFR (1). HB-EGF is synthesized as a 20- to 30-kDa type I transmembrane precursor (pro-HB-EGF) (2, 3). Pro-HB-EGF, when expressed on the cell surface, is bio-

logically active as a juxtacrine growth factor that signals neighboring cells via cell-cell contact (4, 5). When cells are subjected to various extracellular stimuli, such as treatment with the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA), growth factors, cytokines, G-protein-coupled receptor agonists, stress, and wounding (1, 6–8), pro-HB-EGF is transiently cleaved at the juxtamembrane domain by a disintegrin and metalloprotease (ADAM) 9, 12, 10, or 17 (6, 9, 12), a process called ectodomain shedding, resulting in the release of HB-EGF into the extracellular space and the production of a plasma membrane-associated remnant (pro-HB-EGF carboxyl-terminal fragment (HB-EGF-CTF)).

Recent studies have shown that the shedding of pro-HB-EGF by ADAMs is required for EGFR transactivation by G-protein-coupled receptor signaling (13), aids in mediating the mitogenic effects of arachidonic acid metabolites (14, 15), and plays important roles in various biological processes, such as cardiac hypertrophy (6), cystic fibrosis (10), and cutaneous wound healing (8, 16). In a previous study, we showed that HB-EGF-CTF is translocated from the plasma membrane to the nucleus and binds to the promyelocytic leukemia zinc finger (PLZF) protein, which promotes entry into the S-phase of the cell cycle (17–19).

It has been reported that PLZF and B-cell lymphoma 6 (Bcl6) co-localize and heterodimerize and that they share many functional properties, i.e. both inhibit cell growth, concentrate into punctuated nuclear subdomains, and are sequence-specific transcriptional repressors recruiting a histone deacetylase-repressing complex (20). Bcl6 is a proto-oncogene that was identified in t(3, 22) chromosomal translocations in B-cell non-Hodgkin lymphoma (21–24). The Bcl6 protein is a 92- to 98-kDa nuclear phosphoprotein that is produced at low levels in multiple tissues and is expressed at high levels exclusively in germinal center B cells (25, 26). Bcl6 is highly conserved among vertebrate species, and the mouse and human Bcl6 proteins are 94% conserved (27). Bcl6 contains a BTB/POZ domain in the amino terminus and six Krüppel-type (C2H2) zinc finger (ZnF) motifs in the carboxyl terminus. The BTB domain of Bcl6 autonomously represses transcription by recruiting various corepressors, including SMRT, NCoR, BCoR, and class I or II histone deacetylases (28, 29). The ZnF motifs of Bcl6 bind to the consensus core sequence TTTC/T(A/C)GAA found in several Bcl6 target genes (30). These target genes are cyclin D2, CD69, and MIP-1α (31). PLZF contains two functional domains: (i) an amino-terminal self-interacting BTB/POZ domain and (ii) a
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carboxyl-terminal domain containing multiple Krüppel-like C2H2 ZnFs, which mediate binding DNA and HB-EGF-CTF binding. Bcl6 and PLZF have similar domain structures and amino acid sequences, suggesting that Bcl6 may functionally interact with HB-EGF-CTF. Our study was, therefore, directed at whether HB-EGF-CTF interacts with and regulates Bcl6. To accomplish this, we characterized the properties of HB-EGF-CTF as a transcriptional regulator. Our results indicate that HB-EGF-CTF interacts with Bcl6 and reverses the transcriptional repression of the cyclin D2 gene expression by Bcl6. Our results also suggest that HB-EGF-CTF acts as a regulator of cell growth.

EXPERIMENTAL PROCEDURES

Antibodies—The mouse anti-FLAG monoclonal antibody (M2), the anti-cyclin D2 monoclonal antibody (DCS-3), and the anti-β-actin monoclonal antibody (AC-15) were purchased from Sigma-Aldrich. The mouse anti-Bcl6 monoclonal antibodies (D-8 and C-19) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase-conjugated anti-mouse IgG antibody was obtained from Promega (Madison, WI). Rabbit polyclonal antibodies against synthetic peptides corresponding to the cytoplasmic region of pro-HB-EGF (#H1) and the extracellular region of pro-HB-EGF (#H6) have been described previously (32). Rabbit and mouse normal IgGs were obtained from IBL.

Expression Vectors—The expression vector for the cyan fluorescent protein (CFP)-tagged Bcl6 (CFP-Bcl6) was constructed by inserting human Bcl6 cDNA into the BglII and SalI sites of the pECPF-C1 vector (Clontech). The full-length Bcl6 and a series of their truncated regions were cloned into the BglII and Xhol sites of modified pME18S vector (pME18S-FLAG) (17). Glu-627, Glu-655, and both in the linker region of Bcl6 Znf 4–6 were mutated to Ala to construct three mutants, E627A (m1), E655A (m2), and E627A/E655A (m3). The plasmid for the recombinant expression of glutathione S-transferase (GST)-fused HB-EGF-CTF (pGEX6p1-HB-EGF-CTF) has been described previously (17). All of the cDNA constructs were verified by DNA sequencing using a CEQ 8000 DNA Analysis System (Beckman Coulter).

Cell Culture, cDNA Transfection, and TPA Treatment—Stable transfectants of human fibrosarcoma HT1080 expressing human placental alkaline phosphatase (AP)-tagged pro-HB-EGF (HT1080/AP-HB-EGF) and both AP-HB-EGF and a mutant of ADAM12 lacking the metalloprotease domain (HT1080/AP-HB-EGF/ADAM-12), wild-type pro-HB-EGF (HT1080/wt HB-EGF), an uncleavable form of pro-HB-EGF (HT1080/uc HB-EGF), and a mutant of pro-HB-EGF lacking the cytoplasmic domain (HT1080/ΔC HB-EGF) have been described previously (6, 8, 14, 17). Wild-type and stably transfected HT1080 cells were grown in Eagle’s minimum essential medium (Nikken Biomedical Laboratory) supplemented with 0.1 mM nonessential amino acids (Invitrogen), 10% fetal bovine serum (HyClone, Lot AGN19241), penicillin, and streptomycin sulfate. Human colorectal adenocarcinoma LoVo cells were grown in Dulbecco’s modified Eagle’s medium (Nikken Biomedical Laboratory) containing 10% fetal bovine serum, penicillin, and streptomycin sulfate. Transfections were performed using Lipofectamine® 2000 (Invitrogen) according to the manufacturer’s instructions. The cells were treated with 100 nM TPA in each culture medium for each individual time.

Preparation of Cell Extracts—Cells were washed with ice-cold phosphate-buffered saline (PBS(–)) and lysed in ice-cold lysis buffer (PBS(–) containing 5 mM EDTA, 1% Triton X-100, 0.2 mM p-aminophenyl methanesulfonyl fluoride hydrochloride, and 2 μg/ml aprotinin). Cell lysates were mixed by end-over-end rotation for 30 min at 4 °C and then centrifuged at 9100 × g for 10 min. The resulting supernatants were used as cell extracts.

Immunoprecipitation—Cell extracts of LoVo cells and HT1080/AP-HB-EGF cells transfected with FLAG-tagged full-length Bcl6 (amino acid residues 1–706) were incubated with an antibody to cyclin D2 (DCS-3), the cytoplasmic region of pro-HB-EGF (#H1), or Bcl6 (D-8) for 2 h at 4 °C with end-over-end rotation. Protein G-Sepharose 4 Fast Flow beads (Amersham Biosciences) were then added to the mixture. After a 2-h incubation at 4 °C with mixing, the suspension was centrifuged, and the collected protein G-Sepharose beads were washed three times with lysis buffer. The bound proteins were analyzed by Western blotting using an anti-cyclin D2 antibody (DCS-3), an anti-FLAG monoclonal antibody (M2), or an anti-Bcl6 antibody (D-8), followed by a horseradish peroxidase-conjugated anti-mouse IgG antibody. Immunoreactive proteins were visualized using an AEC substrate kit (Vector Laboratories, Inc.) or the ECL Plus Western blotting Detection system (Amersham Biosciences).

Imaging of CFP Fusion Proteins—Transiently transfected cells expressing CFP-Bcl6 cDNA were cultured for at least 12 h in serum-free medium to decrease endogenous HB-EGF and its shedding. The cells were then used to examine the subcellular localization of CFP-Bcl6 proteins by fluorescence microscopy (IX70, Olympus).

Expression and Purification of Recombinant Proteins—GST and GST-HB-EGF-CTF were produced in and purified from Escherichia coli strain BL21. Protein expression was induced by treatment with 1 mM isopropyl-1-thio-D-galactopyranoside for 4 h at 30 °C. The cells were then transferred to a centrifuge tube, collected by centrifugation, washed once with ice-cold PBS(–), frozen at −80 °C for at least 1 h, resuspended in 10 ml of lysis buffer (50 mM Tris-HCl, pH 8.0, 1 mM MgCl2, and 100 μg/ml lysozyme) by repeated pipetting, and incubated on ice for 10 min. The mixture was then adjusted to 5 mM dithiothreitol, and protease inhibitors (Pefablock SC (Roche Applied Science) and aprotinin) were added. The solution was adjusted to 1% N-laurylsarcosine and then vortexed and sonicated on ice using a UR-20P sonicator (Tomy Seiko Co., Ltd.). After sonication, the lysates were clarified by centrifugation at 10,000 × g for 10 min at 4 °C. The supernatants were transferred to new tubes, and the solution was adjusted to 2% Triton X-100. The lysates were vortexed, and glutathione-Sepharose 4B beads (Amersham Biosciences) were added. After mixing by end-over-end rotation for 30 min at 4 °C, the beads were washed five times with PBS(–) and resuspended in storage buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 5 mM dithiothreitol, and 10% v/v glycerol).
GST Pulldown Assay—Extracts from HT1080 cells expressing various FLAG-tagged Bcl6 mutants were mixed with 2 μg of recombinant GST or GST-HB-EGF-CTF immobilized on glutathione-Sepharose beads for 2 h at 4 °C by end-over-end rotation. After washing the beads, the bound proteins were analyzed by SDS-PAGE, followed by Western blotting using an anti-FLAG antibody (M2), followed by a horseradish peroxidase-conjugated anti-mouse IgG antibody. Immunoreactive proteins were visualized using the ECL Plus Western blotting detection system (Amersham Biosciences). The binding abilities of the Bcl6 ZnF 4–6 E/A mutants (m1–3) were estimated by the ratio of each band intensity (lane 2) in a pulldown assay panel to the corresponding band intensity in a cell lysate panel.

Luciferase Reporter Assay—Plasmids encoding the human cyclin D2 promoter-driven luciferase reporter gene and its mutant (pGL3-wt cyclin D2 promoter and pGL3-mut cyclin D2 promoter) were constructed by inserting the PCR-amplified promoter fragment of cyclin D2 (nucleotides −1560 to −4) or its mutated fragment (three base mutations; see Fig. 4A and Ref. 31) into the pGL3-Control Vector (Promega). Vector pRL-TK (Promega) was used as an internal control. Wild-type and stably transfected HT1080 cells (5 × 10⁴) were transiently transfected with luciferase reporter plasmids using Lipofectamine™ 2000 (Invitrogen). After a 24-h culture, cells were treated with 100 nM siRNA, after which the mRNA and protein levels were examined by RT-PCR and Western blotting, respectively. RT-PCR was performed using the following primer pair: 5′-GGCAGGCUGUAGGCAGGCU (sense) and 5′-AAGGCUUGUGCUUGCUU (antisense). Bcl6 has two characteristic zinc fingers (ZnF), namely ZnF 4–6 and ZnF 7–9. Binding of Bcl6 to the HB-EGF-CTF promoter and the Bcl6 overexpression are both necessary for the interaction between HB-EGF-CTF and Bcl6 in mammalian cells.

Results

HB-EGF-CTF Binds the ZnF Region of Bcl6—To analyze the interaction between HB-EGF-CTF and Bcl6 in mammalian cells, FLAG-tagged Bcl6 (FLAG-Bcl6 as shown in Fig. 1B) were transfected into HT1080 cells stably expressing AP-tagged pro-HB-EGF (HT1080/AP-HB-EGF). Antibodies against the cytoplasmic region of pro-HB-EGF (#H1) immunoprecipitated AP-tagged pro-HB-EGF (Fig. 1A, lower) and an ~90-kDa band from the cell lysates. This corresponds to FLAG-Bcl6, indicating that HB-EGF-CTF physically interacts with Bcl6 (Fig. 1A, upper).

We next performed a GST pulldown assay using GST-tagged HB-EGF-CTF (GST-HB-EGF-CTF) and extracts of HT1080 cells expressing FLAG-Bcl6. Western blotting with an anti-FLAG antibody showed that GST-HB-EGF-CTF, but not GST alone, bound to Bcl6 (Fig. 1C). Bcl6 has two characteristic domains: an amino-terminal BTB domain and a carboxyl-terminal C₂H₂ ZnF domain. We determined which domain is responsible for the interaction with HB-EGF-CTF using two deletion mutants of Bcl6 (BTB+Center and ZnF, Fig. 1B). Extracts from cells expressing these deletion mutants were
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HB-EGF-CTF interacts with Bcl6. A, FLAG-tagged full-length Bcl6 (FLAG-Bcl6) was transiently expressed in HT1080/AP-HB-EGF cells and then immunoprecipitated using an antibody against the cytoplasmic region of HB-EGF (H1) or normal rabbit IgG. Western blotting was carried out using an anti-FLAG monoclonal antibody. B, schematic representation of Bcl6 and its deletion mutants, Bcl6 (amino acids 1–706), BTB+Center (amino acids 1–518), and ZnF (amino acids 519–706). All of the recombinant proteins were expressed as FLAG-tagged fusion proteins. C, recombinant GST-HB-EGF-CTF pulled down Bcl6 and its mutants from crude extracts. Recombinant GST (lane 2) or GST-HB-EGF-CTF (lane 2) was immobilized on glutathione-Sepharose beads. A large fraction of ZnF and a detectable level of BTB+Center were pulled down by GST-HB-EGF-CTF but not by GST alone (Fig. 1C). These data indicate that the ZnF domain mediates the interaction with HB-EGF-CTF.

We have previously shown that some of the PLZF is translocated to the cytoplasm subsequent to the shedding of pro-HB-EGF (17). Here, we investigated whether the localization of Bcl6 is affected by the production of HB-EGF-CTF in HT1080 and HT1080/AP-HB-EGF cells. HT1080 cells, which express a very low level of endogenous pro-HB-EGF (26), were used as a negative control. CFP-Bcl6 showed a diffuse and discrete punctuate pattern in the nucleus, as reported previously for endogenous Bcl6 (28). Unexpectedly, treatment with TPA for up to 60 min did not alter the localization of CFP-Bcl6 in the nucleus in either HT1080 or HT1080/AP-HB-EGF cells (Fig. 1D). The same results were also obtained when HT1080 cells stably expressing CFP-Bcl6 and transiently overexpressing wild-type HB-EGF were used (data not shown). This might be due to the different properties of their center regions with no homology between PLZF and Bcl6. We confirmed that HT1080/AP-HB-EGF cells produce soluble HB-EGF and HB-EGF-CTF in response to TPA treatment (Fig. 1E). These findings indicate that the shedding of pro-HB-EGF and the resulting production of HB-EGF-CTF did not affect the localization of CFP-Bcl6 in the nucleus.

ZnF 4–6 in Bcl6 Is Sufficient for Interaction with HB-EGF-CTF—In initial experiments, we found that HB-EGF-CTF interacts with the ZnF domain of Bcl6. This ZnF domain contains six Krüppel-like C2H2 ZnFs. We therefore determined which of the ZnFs are required for the interaction by a pull-down experiment using GST-HB-EGF-CTF and a series of FLAG-tagged Bcl6 ZnF truncation mutants (Fig. 2A). The results showed that GST-HB-EGF-CTF binds strongly to ZnF 1–6 and ZnF 1–5, weakly to ZnF 1–4, and not at all to ZnF 1–2 or ZnF 1–3 (Fig. 2B). Amino-terminal deletion mutants of the ZnF domain showed that ZnF 4–6 was sufficient for binding HB-EGF-CTF but that ZnF5–6 was not (Fig. 2C). We previously reported that the ZnF5–8 region of PLZF is sufficient for interaction with HB-EGF-CTF (26). To identify the consensus amino acid sequence motif required for the binding of HB-EGF-CTF, we aligned the HB-EGF-CTF binding ZnF region of PLZF and Bcl6. However, no significant homology in the ZnF motifs of PLZF ZnF 5–8 and Bcl6 ZnF 4–6 was found, except for the C2H2 ZnF consensus amino acids and “TGEKP” linker region (Fig. 3A). We further determined whether ZnF 1–4 and ZnF 5–6, which have a linker sequence at their carboxyl and amino termini, respectively (Fig. 3B), can bind HB-EGF-CTF. As shown in Fig. 3C, ZnF 1–4 binds more efficiently than ZnF 1–4 to HB-EGF-CTF. In addition, ZnF5–6 clearly binds to HB-EGF-CTF. These results suggest that the linker region between ZnF 4 and ZnF5 plays a crucial role in the interaction of Bcl6 with HB-EGF-CTF. Because a series of Ala-scan-
ning mutations of this linker region suggest the important role of the Glu, and ZnF 4–6 was sufficient for binding HB-EGF-CTF, we analyzed the binding abilities of E627A (m1), E655A (m2), and E627A/E655A (m3) by a pulldown assay. As shown in Fig. 3D, m1, m2, and m3 mutants showed 57, 76, and 86% reduction of the binding ability, respectively, as compared with wild type. The data presented here suggest that both linker regions in ZnF 4–6 play an important role for the interaction with HB-EGF-CTF.

Production of HB-EGF-CTF Is Essential for Reversing Bcl6-mediated Transcriptional Repression—Because HB-EGF-CTF binds the ZnF region of Bcl6, which is responsible for DNA binding, we hypothesized that the binding of HB-EGF-CTF might affect the ability of Bcl6 to repress transcription. We examined this possibility by using a luciferase reporter with the wild-type cyclin D2 promoter (wt cyclin D2 promoter), which contains a Bcl6 consensus binding site or its mutated promoter (mut cyclin D2 promoter, Fig. 4A) (33). To determine whether the endogenous Bcl6 represses the luciferase expression driven by the cyclin D2 promoter in HT1080 cells, siRNA duplexes targeting human Bcl6 (siBcl6-1 and siBcl6-2) were transfected. We evaluated the Bcl6 knockdown level by RT-PCR and Western blotting. Bcl6 mRNA levels were reduced after 24 h (Fig. 4B), and the amount of Bcl6 protein was specifically reduced (Fig. 4C). The luciferase activity increased in the Bcl6 knockdown cells with statistical significance (Fig. 4D). Because Bcl6 cannot bind the mut cyclin D2 promoter, luciferase expression driven by the mut cyclin D2 promoter was higher than that driven by the wild-type promoter (Fig. 4E). These results indicate that endogenous Bcl6 in HT1080 cells represses the luciferase expression driven by the cyclin D2 promoter.

We next determined whether HB-EGF-CTF signaling affects Bcl6-mediated transcriptional repression. We transfected HT1080 and HT1080/AP-HB-EGF cells with the luciferase reporter gene driven by the wt cyclin D2 promoter or its mutated promoter. The luciferase expression driven by wt cyclin D2 promoter was lower than that mediated by the mut cyclin D2 promoter in both HT1080 and HT1080/AP-HB-EGF cells. Treatment with TPA resulted in the same level of luciferase activity as that induced by the mut cyclin D2 promoter in HT1080/AP-HB-EGF cells but not HT1080 cells (Fig. 4E). This suggests that the production of HB-EGF-CTF reverses Bcl6-mediated transcriptional repression.

To confirm that HB-EGF-CTF signaling is essential for the reversal of the Bcl6-mediated transcriptional repression, we used HT1080 cells stably expressing wild-type pro-HB-EGF (HT1080/wt HB-EGF), an uncleavable form of pro-HB-EGF (HT1080/uc HB-EGF), or pro-HB-EGF lacking the cytoplasmic domain (HT1080/ΔC HB-EGF). The latter construct lacks the 19 carboxyl-terminal amino acids in pro-HB-EGF, and, although it is cleaved in response to TPA, it does not function as

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3 H. Iwabuki and S. Higashiyama, unpublished data.
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HB-EGF-CTF (26). These cell lines express significant levels of HB-EGF and mutant HB-EGFs (Fig. 5A, upper). After TPA treatment, the transcriptional repression by Bcl6 was reversed in HT1080/wt HB-EGF cells but not in HT1080 cells. In addition, the repression was not reversed in HT1080/uc HB-EGF, in which pro-HB-EGF is not shed, or HT1080/ΔC HB-EGF cells, which do not produce functional HB-EGF-CTF (Fig. 5A, lower).

We also examined luciferase activity driven by the cyclin D2 promoter in HT1080/AP-HB-EGF/ΔMP-ADAM12 cells, which express AP-tagged wild-type pro-HB-EGF and ADAM12 lacking the metalloprotease domain (2). Pro-HB-EGF was not shed in these cells, even when treated with TPA (Fig. 5B, upper). In contrast to HT1080/AP-HB-EGF cells, TPA did not induce the recovery of luciferase activity in HT1080/AP-HB-EGF/ΔMP-ADAM12 cells (Fig. 5B, lower). Recovery of luciferase activity was greater in wild-type HB-EGF transfectant (Fig. 5A) than AP-tagged HB-EGF transfectant (Figs. 4E and B), which is consistent with data showing different efficiency of the shedding in wild-type HB-EGF and AP-tagged HB-EGF transfectants (see Western blotting data in Fig. 5, A and B). Taken together, these results reinforce the view that HB-EGF-CTF production as a result of pro-HB-EGF shedding is required for the reversal of cyclin D2 transcriptional repression by Bcl6.

Cyclin D2 Expression in LoVo Cells Is Increased following Pro-HB-EGF Shedding—Because we investigated the HB-EGF-CTF functions in reconstructed systems using some plasmids, we tested whether these phenomena are reproduced in LoVo cells, which express endogenous HB-EGF, Bcl6, and cyclin D2 (data not shown). To analyze the endogenous interaction between HB-EGF-CTF and Bcl6 in LoVo cells, #H1 antibodies were again used. Bcl6 in the precipitate from an extract of LoVo cells by #H1 antibodies was detected by the anti-Bcl6 monoclonal antibody. The results showed that the endogenous Bcl6 was co-immunoprecipitated with HB-EGF-CTF (Fig. 6A). We then determined whether the shedding of pro-HB-EGF could reverse the repression of cyclin D2 expression. Cells were treated with TPA for the indicated periods, after which endogenous cyclin D2 was immunoprecipitated and the expression level analyzed by Western blotting. In the absence of TPA, the level of cyclin D2 protein in LoVo cells was low, but cyclin D2 protein increased significantly after the TPA treatment (Fig. 6B).
To confirm that the up-regulation of cyclin D2 protein depends on Bcl6 and the production of HB-EGF-CTF, siRNA duplexes targeting Bcl6 and HB-EGF (siBcl6-1 and siHB-EGF) were transfected. The protein level of Bcl6 and pro-HB-EGF in LoVo cells was specifically reduced as the result of a 24-h transfection (Fig. 6, C and D). We found that cyclin D2 protein levels were not affected by transfection of the scrambled siRNA (Fig. 6, B and E (left)) and were increased after TPA treatment. In Bcl6 knockdown LoVo cells the level of cyclin D2 protein increased, even in the absence of TPA, compared with untransfected cells (Fig. 6E, middle). TPA treatment increased cyclin D2 protein to some level, which may be due to recruitment of other transcriptional activators. In the HB-EGF knockdown cells, however, the level of cyclin D2 protein increased faintly in the presence of TPA (Fig. 6E, right). This result shows that the production of HB-EGF-CTF is essential for the induction of cyclin D2 expression by reversing Bcl6-mediated transcriptional repression. Furthermore we analyzed the other Bcl6 target genes, MIP-1a and CD69. Unfortunately, the mRNA of MIP-1a and CD69 were not detected in LoVo cells at all before and after production of HB-EGF-CTF (data not shown).

To repress cyclin D2 gene expression, Bcl6 is recruited to the cyclin D2 promoter. HB-EGF-CTF interacts with Bcl6 and reverses Bcl6-mediated transcriptional repression. We therefore investigated the effect of HB-EGF-CTF on Bcl6 binding to the endogenous cyclin D2 promoter by a ChIP assay. Chromatin was immunoprecipitated from LoVo cells with or without TPA treatment by an anti-Bcl6 monoclonal antibody, C-19. PCR primer pairs were designed to selectively detect Bcl6 consensus binding site in the human cyclin D2 promoter. As expected, the cyclin D2 promoter bound to Bcl6 in LoVo cells without TPA treatment, whereas TPA treatment abolished Bcl6 association with the cyclin D2 promoter nearly completely (Fig. 6F, upper). These results were not affected by scrambled siRNA transfection (Fig. 6F, middle). In siHB-EGF-treated LoVo cells, Bcl6 bound to the cyclin D2 promoter regardless of TPA treatment (Fig. 6F, lower). This result explains why HB-EGF-CTF plays a role in the binding of Bcl6 to the cyclin D2 promoter and why the Bcl6-mediated transcriptional repression of cyclin D2 gene was reversed after the production of HB-EGF-CTF.

**DISCUSSION**

Ectodomain shedding of pro-HB-EGF is involved in the activation of two independent signal transduction pathways: signaling from the EGFR after engagement of the shed growth factor and signaling mediated by HB-EGF-CTF (1, 17). Following shedding, HB-EGF-CTF is translocated from the plasma membrane to the nucleus, where it binds and inactivates PLZF (17). We were therefore very interested in determining whether other repressors related to PLZF are also targets of HB-EGF-CTF.

Bcl6 dimerizes and shares a primary sequence and domain structure homology with PLZF. Thus, we examined the interaction between HB-EGF-CTF and Bcl6. In vitro immunoprecipitation and pulldown assays demonstrated that HB-EGF-CTF binds to Bcl6. Using various deletion and truncation mutants, we found that the minimum sequence of Bcl6...
required for HB-EGF-CTF binding is ZnF +5–6 suggesting that a sequence consisting of at least two ZnF motifs with two TGEKP linkers is required for interaction with HB-EGF-CTF. These binding experiments and the alignment of the sequences of the HB-EGF-CTF-binding regions with Bcl6 and PLZF imply that the sequence TGEKP is crucial for binding to HB-EGF-CTF. The linker region is an important structural element that controls the spacing between neighboring ZnF domains along the DNA binding site. The most common linker sequence contains TGEKP between the last histidine of one ZnF and the first histidine of the next ZnF.
conserved aromatic amino acid of the next ZnF (34). Most C_{6}H_{2}
ZnF proteins belong to a class of transcriptional repressors
characterized by amino-terminal POZ, KRAB, or SCAN
domains (35). These repressors have C_{6}H_{2} ZnF repeats con-
ected by the TGXEKP linker sequence, some of which might be
regulated by HB-EGF-CTF.

In addition, we found a weak but reproducible interaction
between the BTB+Center and HB-EGF-CTF (Fig. 1C). BTB+Center binds directly to the cytoplasmic region of pro-
HB-EGF in vitro.\(^4\) The BTB domain binds co-repressors,
including mSin3A, SMRT, NcoR, and histone deacetylases (28,
36). Therefore the binding of HB-EGF-CTF to the
BTB+Center might regulate the interaction between Bcl6 and
corepressors.

We further examined the role of HB-EGF-CTF on Bcl6 func-
tion using a luciferase reporter containing the promoter of
cyclin D2, which is a target of Bcl6 (33). Using this system, we
found that HB-EGF-CTF interferes with Bcl6-mediated tran-
scriptional repression. In LoVo cells, which express endoge-

ous Bcl6, HB-EGF, and cyclin D2, protein levels of cyclin D2
increased after HB-EGF-CTF production and by the knock
down of Bcl6. The result showed that the interaction of HB-
EGF-CTF with Bcl6 down-regulates Bcl6 repressor activity,
at least for the cyclin D2 promoter. Therefore similar to soluble
HB-EGF, HB-EGF-CTF is an important regulator of the cell
cycle via Bcl6 and PLZF.

Bcl6 has been shown to be recruited to the endogenous cyclin
D2 promoter (31). A ChIP assay showed that Bcl6 is dissociated
from the cyclin D2 promoter after TPA treatment (Fig. 6F).
TPA treatment produces soluble HB-EGF and HB-EGF-CTF,
the dissociation of Bcl6 from the cyclin D2 promoter assumes
that HB-EGF-CTF interferes with Bcl6 binding to the cyclin D2
promoter. However, we will study further the possibility of a
direct or an indirect interaction among HB-EGF-CTF, Bcl6,
and cyclin D2 promoter region, because we were not able to
detect the interference of Bcl6-DNA interaction by HB-EGF-
CTF in an electrophoretic mobility shift assay.

We used TPA to stimulate ectodomain shedding. It was
previously reported that TPA treatment induced Bcl6 phos-
horylation and its degradation (37, 38). However, the
amount of Bcl6 protein was not decreased after TPA treat-
ment in LoVo cells (data not shown). This discrepancy might
be dependent on the cell type under study. We attempted to
show that Bcl6-mediated repression was able to be relieved
by HB-EGF-CTF without TPA treatment using expression
vectors of HB-EGF-CTF and the cytoplasmic domain of HB-
EGF. The expressed HB-EGF-CTF was aggregated in the

cytoplasm, and the cytoplasmic region of pro-HB-EGF alone
did not function in reversing Bcl6-mediated transcriptional
(repression (data not shown) suggesting that the transmem-
brane domain of HB-EGF-CTF plays an important role in the
appropriate localization and functioning. We are under the
investigation of developing a functional HB-EGF-CTF deliv-
ering system.

HB-EGF is known to be an effector of protein synthesis in
cardiomyocytes (6). Bcl6 is expressed in cardiomyocytes and
plays a significant role (39). When cardiomyocytes are stimu-
lated by G-protein-coupled receptor agonists, metalloproteases
are activated, causing the shedding of pro-HB-EGF, which
results in the transactivation of EGFR, ultimately leading to
heart hypertrophy. Cardiac hypertrophy is an adaptive
response of the heart that occurs in various cardiovascular dis-
eases (40). A recent report indicated that cyclin D2 induces the
proliferation of cardiac myocytes, and hypertrophic signals up-
regulate cyclin D2 expression in the cardiac myocytes (41, 42).
Furthermore, it has been reported that cyclin D2 is necessary for Myc-induced cardiac hypertrophy (43). Collectively, the
above results suggest that HB-EGF-CTF is able to induce the
proliferation of cardiac myocytes via Bcl6, which reverses cyclin
D2 repression. In summary, our results indicate that pro-HB-
EGF is a bidirectional signaling molecule: soluble HB-EGF activ-
vates EGFR tyrosine kinase, and HB-EGF-CTF reverses tran-
scriptional repression. The function of the latter and its
pathophysiological roles in the regulation of cell growth remain
to be determined.

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