Suppression of the Biological Activities of the Epidermal Growth Factor (EGF)-like Domain by the Heparin-binding Domain of Heparin-binding EGF-like Growth Factor*

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Heparin-binding EGF-like growth factor (HB-EGF) is a member of the EGF family of growth factors that has a high affinity for heparin and heparan sulfate. While interactions with heparin are thought to modulate the biological activity of HB-EGF, the precise role of the heparin-binding domain has remained unclear. We analyzed the activity of wild-type HB-EGF and a mutant form lacking the heparin-binding domain (ΔHB) in the absence of heparin. The activity of the EGF-like domain of HB-EGF was determined by measuring binding to diphtheria toxin (DT) as well as the growth factor activity in EGF receptor-expressing cells. The binding affinity of ΔHB for DT was much higher than that of wild-type HB-EGF in the absence of heparin. The binding affinity of HB-EGF for DT was increased by addition of exogenous heparin and reached the level close to the affinity of ΔHB, whereas that of ΔHB was not affected. Moreover, the growth factor activity of ΔHB was much higher than that of wild-type HB-EGF in the absence of heparin but was not affected by addition of exogenous heparin, whereas HB-EGF had increased growth factor activity with added heparin. These results indicate that the heparin-binding domain suppresses the activity of the EGF-like domain of HB-EGF and that association of heparin with HB-EGF via this domain removes the suppressive effect. Thus, we conclude that the heparin-binding domain serves as a negative regulator of this growth factor.

Cell surface heparan sulfate proteoglycans (HSPGs)1 have been implicated in a variety of cell signaling pathways involving heparin-binding growth factors or cytokines. These growth factors and cytokines form tight complexes with heparin or heparan sulfate are the fibroblast growth factor (FGF) family of growth factors (2), the transforming growth factor-β family of growth factors (3), vascular endothelial growth factor (4), interleukin-3 (5), granulocyte-macrophage colony-stimulating factor (5), interferon-γ (6), Hedgehog (7), and Wnt (7). Many classes of receptors, including receptor serine/threonine kinases and seven pass transmembrane receptors, have now been shown to be modulated by HSPGs (1). Binding of ligand to cell surface HS is thought to result in a high local ligand concentration to activate signaling receptors. Although biochemical and cell culture data suggest that this binding usually facilitates but is not essential for ligand-receptor interactions and signaling, in the case of Wnt and Hedgehog, HSPGs are crucial for proper pathway function during development (8). Studies with FGFs and their receptor tyrosine kinases also documented a coreceptor role for HSPGs and have suggested models in which HS promotes ligand dimerization, leading to receptor dimerization and stimulation of kinase activity (1).

Heparin-binding EGF-like growth factor (HB-EGF), a member of the EGF family growth factors, has a high affinity for heparin and heparan sulfate (HS) (9, 10). HB-EGF is first synthesized as a type I transmembrane protein (pro-HB-EGF) containing heparin-binding and EGF-like domains (9, 11). Pro-HB-EGF is cleaved within the juxtamembrane domain on the cell surface, resulting in the shedding of soluble HB-EGF (sHB-EGF) (12), which acts as a mitogenic signal through the EGF receptor (EGFR) (9). Pro-HB-EGF is biologically active as a juxtacrine growth factor that signals to neighboring cells in a nondiffusible manner (13–15). Pro-HB-EGF forms complexes with CD9 (13, 16–18) and integrin α3β1 (19) on the cell membrane and acts as a receptor for diphtheria toxin (DT), mediating the entry of DT into the cytoplasm (18, 20). sHB-EGF is a potent mitogen and chemoattractant for a number of cell types, including vascular smooth muscle cells, fibroblasts, and keratinocytes (10, 21). HB-EGF has been implicated in a number of physiological and pathological processes, which include wound healing (22, 23), cardiac hypertrophy (24), smooth muscle cell hyperplasia (25), kidney collecting duct morphogenesis (26), blastocyst implantation (27), pulmonary hypertension (28), and oncogenic transformation (29). In addition, we recently demonstrated through analysis of HB-EGF null mice that HB-EGF is an essential factor for normal heart function and valvulogenesis (30).

The modulation of various HB-EGF activities by cell surface HSPGs has been previously described (21). For examples: (i) reduced HS expression on the cell surface decreases the ability of HB-EGF to stimulate the migration of bovine aortic smooth muscle cells (10), (ii) binding of HB-EGF to HSPG-deficient CHO cells expressing EGFR is lower than binding to wild-type

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1 The abbreviations used are: HSPGs, heparan sulfate proteoglycans; HS, heparan sulfate; FGF, fibroblast growth factor; HB-EGF, heparin-binding epidermal growth factor-like growth factor; EGF, epidermal growth factor; R, receptor; DT, diphtheria toxin; CHO, Chinese hamster ovary; CM, conditioned medium; sHB-EGF, soluble HB-EGF; DER, 32D-EGFR.
CHO cells expressing EGF-R, an effect that is rescued by the addition of exogenous hepamin (31), (iii) CHO cells expressing pro-HB-EGF, but deficient in cell surface HS-PGs, were 15-fold less sensitive to DT-toxicity than wild-type CHO cells, but DT sensitivity was restored by addition of either HS or hepamin, which increased the binding affinity of pro-HB-EGF for DT (32). Although previous studies well documented the modulation of the biological activities of HB-EGF with hepamin, the precise role of the hepamin-binding domain remained unclear. Here we analyze the DT binding and growth factor activities of HB-EGF and a mutant form lacking the hepamin-binding domain, in the presence or absence of hepamin. We present evidence that the hepamin-binding domain of HB-EGF suppresses the activity of the EGF-like domain and that binding of hepamin to this domain removes the suppressive effect.

**EXPERIMENTAL PROCEDURES**

**Materials—**DT was produced as previously described (33). Heparin sodium salt (from bovine intestinal mucosa) was purchased from Sigma. Heparitinase (EC 4.2.2.8, catalog no. 100703) was purchased from Seikagaku Kogyo Co. (Tokyo, Japan). Heparin-Sepharose CL-6B was purified from Amersham Biosciences. TALON metal affinity resin was purchased from Clontech Laboratories, Inc. (Palo Alto, CA). Anti-human HB-EGF-neutralizing antibody was purchased from R&D Systems (Minneapolis, MN). Anti-human HB-EGF (H6) antibody was prepared as previously described (14). Anti-human EGF-R antibody (1005) was purchased from Chemicon International (Temecula, CA). Anti-goat IgG antibody was purchased from Invitrogen Corp. (San Diego, CA), generating the pcDNA6/HB-EGF/myc-His and pcDNA6/HB-EGF/myc-His constructs, respectively. The cDNA encoding the hepamin-binding domain deletion mutant of pro-HB-EGF (pro-HB-) was obtained and isolated by PCR using the forward primer (5′-GACCCATGTCTTCGGAAATACAAG-3′) and the reverse primer (5′-CCCCTCTCCGCCTCTGGTGGT-3′), using pH5HBtm, which contains the human HB-EGF deletion mutant (HBtm) missing the transmembrane and cytoplasmic domains (34), as a template. This amplified fragment was self-ligated, generating the pH5HBtm bacteriophage-λ construct. pH5HBtm and pH5BtmHIS were digested with HindIII and XbaI. The digested cDNA fragment containing HBtm or HBtmHIS was then ligated into the HindIII/XbaI sites of pcDNA6/myc-His (Invitrogen Corp., San Diego, CA), generating the pcDNA6/HBtm/myc-His and pcDNA6/HBtmHIS/myc-His constructs, respectively. The cDNA encoding the hepamin-binding domain deletion mutant of pro-HB-EGF (pro-HB-) was obtained and isolated by PCR using the same primer set as above and using pH5HB-EGF, which contains the entire human pro-HB-EGF coding region (18), as a template. This amplified fragment was self-ligated, generating the pH5proHBtm-construct. The integrity of all constructs was confirmed by sequencing.

**Cell Culture and Transfection—**DER cells, murine 32D hematopoietic progenitor cells stably expressing human EGF-R (14), were maintained as previously described (14). Both the CHO mutant cell line pgsD-677 (also known as 677) and the 677H cell line (677 cells stably expressing pro-HB-EGF) (32) were maintained as previously described (32). Stable transfectants of L cells, including LLC cells stably expressing pro-HB-EGF, and LC cells stably expressing CD8 (18), were maintained as previously described (18). To obtain transfectants producing human HB-EGF and sHB, 677 cells were transfected with plasmids encoding HB-EGF cDNA (pcDNA6/myc-His) or sHB cDNA (pcDNA6/HBtmHIS/myc-His) using LipofectAMINE 2000 (Invitrogen) according to the manufacturer’s instructions. 24 h after transfection, the medium was exchanged with fresh RPMI1640 containing 10% fetal calf serum, and cells were cultured for 48 h. Conditioned medium (CM) containing sHB-EGF or sHB was harvested and centrifuged. To obtain stable transfectants of 677 cells and L cells that express pro-HB, cells were transfected with pH5proHBtm by the calcium phosphate method (35). Cells were cultured for 48 h and further cultured for 7 days in the presence of 200 μg/ml G418. Colonies growing in the selection medium were isolated and assayed for DT-binding activity as described above. Positive clones were isolated and subcloned again. Clones with highest expression of human pro-HB were chosen from among 677pro-HB cells derived from 677 cells and Lpro-HB cells derived from L cells.

**Immunoblotting—**Recombinant His-tagged HB-EGF and sHB proteins were collected from conditioned medium of transfected 677 cells using TALON metal affinity resin. Samples were boiled in SDS-PAGE sample buffer with 2-mercaptoethanol, run on an SDS-PAGE gel, and transferred to an Immobilon membrane (Millipore Corp., Bedford, MA). The membrane was blocked with 1% skim milk in TTBS (0.05% Tween 20, 20 mM Tris, pH 7.5, 0.15 M NaCl), incubated with anti-human HB-EGF-neutralizing antibody followed by horseradish peroxidase-conjugated anti-goat IgG antibody. Proteins were visualized using an ECL Western blotting kit (Amersham Biosciences).

To detect pro-HB-EGF and pro-HB protein in transfected 677 cell lysates, cells were lysed with O-glycosylation buffer (60 mM 1-O-acetyl-β-glucopyranoside, 0.15 M NaCl, 20 mM Heps-NaOH, pH 7.2, 10 mM EDTA, 0.5 μM phenylmethylsulfonyl fluoride, 0.15 μM aprotinin, 1 μM leupeptin) and then centrifuged for 20 min at 15,000 × g. The supernatant was boiled in SDS-PAGE sample buffer with 10% 2-mercaptoethanol and then run on an SDS-PAGE gel and transferred to an Immobilon membrane. After blocking with 1% skim milk in TTBS, the membrane was incubated with anti-human HB-EGF-neutralizing antibody and then incubated with horseradish peroxidase-conjugated anti-goat IgG antibody as described above.

**DT Binding Assay—**Purified DT was labeled with Na125I (Amersham Biosciences) by an IODO-GEN Pre-Coated Iodination Tube (Pierce) according to the manufacturer’s instructions. Binding of 125I-DT to HB-EGF secreted into CM was measured as follows: 500 μl of CM containing either sHB-EGF or sHB, both His-tagged, was added to 20 μl of 1 M Heps-NaOH, pH 7.2, and then the mixture was incubated with 20 μl of TALON metal affinity resin at 4 °C for 4 h. After washing the resin with buffer (0.1% bovine serum albumin in phosphate-buffered saline), the gel was suspended with 970 μl of wash buffer. Then the gel was incubated with 10 μl of 125I-DT at the indicated concentrations in the presence or absence of 10 μl of unlabeled DT (final 10 μg/ml) and 10 μl of hepamin at the indicated concentrations. After incubation for 8 h at 4 °C, the gel was washed three times with 1 ml of wash buffer. The radioactivity bound to the gel was counted with a γ-counter. Specific binding of 125I-DT to the recombinant HB-EGF molecules was calculated by subtracting the radioactivity of a sample with excess unlabeled DT from that of the sample without unlabeled DT. Specific binding values were plotted as described by Scatchard (36) to determine the number of DT binding sites and the binding affinity of sHB-EGF or sHB for DT. Binding curves were generated by regression analysis of the data.

**Binding of 125I-DT to pro-HB-EGF at the cell surface was measured as previously described (18), except that 100 ng/ml 125I-DT was used. This concentration (1.7 nm) is close to the concentration required for half-saturation of DT binding to human pro-HB-EGF (18, 37). Non specific binding of 125I-DT was assessed in the presence of 10 μg/ml unlabeled DT. Specific binding was determined by subtracting the nonspecific binding from the total binding obtained using 125I-DT alone. The relative values of 125I-DT binding to pro-HB-EGF and to pro-HB on the cell surface were calculated as the ratio of specific DT binding to the total amount of pro-HB-EGF or pro-HB on the cell surface, measured by the anti-HB-EGF antibody (H-6) binding assay, as previously described (38).

**Determination of the Concentration of sHB-EGF and sHB in the CM Samples—**The concentration of sHB-EGF and sHB in each CM sample was calculated from the Bmax value in the Scatchard plot analysis of DT binding as described above. In each experiment, the actual concentration was determined in which the Bmax value was corrected by a proportion of collected recombinant protein with the metastatic affinity, using by a known concentration of commercial purified recombinant HB-EGF (R&D Systems). CM samples determined for the concentrations of these species were used in the mitogenic assay and in the EGFR autophosphorylation assay as described later.

**Heparitinase Treatments of Cells—**Heparitinase treatments were carried out as described previously (32).

**Mitogenic Assay—**40 μ1 of DER cells (1.0 × 106 cells) in RPMI 1640 containing 10% fetal calf serum were inoculated in each well of a 96-well tissue culture plate. CM samples containing either sHB-EGF or sHB were added to the wells in quadruplicate. The cells were incubated for 48 h at 37 °C. After the incubation period, the cells were further incubated for 2 h without medium change in 1 ml of RPMI 1640 containing 10% fetal calf serum. Cell proliferation was determined using the Cell proliferation assay kit (Roche Molecular Biochemicals, Indianapolis, IN).
sΔHB were diluted with the medium to the indicated concentrations. 50 μl of CM sample, with or without anti-HB-EGF-neutralizing antibody (10 μg/ml), was added to the wells containing the DER cells. 10 μl of heparin at the indicated concentrations was also added to the wells. After 36 h of culture at 37 °C, the cell number in each well was measured by the Cell Count Reagent SF (Nacalai, Kyoto, Japan), according to the manufacturer's instructions. The mitogenic activity of the secreted HB-EGF species was calculated as the difference between the

**Fig. 1.** Characterization of the mutant forms of HB-EGF lacking the heparin-binding domain. **A,** schematic structures of wild-type and mutant forms of HB-EGF, pro-HB-EGF, full-length membrane-anchored HB-EGF; pro-ΔHB, deletion mutant of pro-HB-EGF lacking the heparin-binding domain; sHB-EGF, myc- and His-tagged mutant of pro-HB-EGF with transmembrane and cytoplasmic domain truncation; sΔHB, myc- and His-tagged deletion mutant of sHB-EGF lacking heparin-binding domain; HBD, heparin-binding domain; TMD, transmembrane domain; M, myc tag; H, His tag. **B,** Western blot analysis detecting sHB-EGF and sΔHB in the culture medium (left panel) and pro-HB-EGF and pro-ΔHB in the cell lysates (right panel) of transfected 677 cells. HB-EGF species were detected by immunoblotting using anti-HB-EGF-neutralizing antibody. **C,** heparin-Sepharose chromatography of sHB-EGF (upper) and sΔHB (lower). sHB-EGF was able to bind to the heparin-Sepharose column and was eluted by >0.75 M NaCl, whereas sΔHB did not show any binding to heparin-Sepharose, and almost all sΔHB input was detected in the flow-through fraction.
values obtained with and without anti-HB-EGF-neutralizing antibody. 

**EGFR Autophosphorylation Assay**—DER cells were washed twice and incubated with serum-free RPMI 1640 for 30 min before the experiment. 100 μl of DER cells (4 x 10^5 cells) was treated with 100 μl of CM from mock-transfected cells or cells expressing sHB-EGF or sΔHB, and incubated at 37 °C for 1 min. After incubation, DER cells were lysed with Triton X-100-lysate buffer (1% Triton X-100, 0.15 mM NaCl, 50 mM Tris-HCl, pH 7.5, 1 mM NaVO₃, 0.5 mM phenylmethylsulfonyl fluoride, 0.15 mM aprotinin, 1 μM E-64, 1 μM leupeptin, 0.5 mM EDTA) and then centrifuged for 20 min at 15,000 x g. The supernatant was boiled in SDS-PAGE sample buffer with 10% 2-mercaptoethanol, then run on an SDS-PAGE gel, and transferred to an Immobilon membrane. After blocking with 1% skim milk in TTBS, the membrane was incubated with anti-EGFR or anti-phospho-EGFR antibody and then incubated with horseradish peroxidase-conjugated anti-rabbit IgG antibody. The membrane was finally analyzed using an ECL Western blotting kit.

**RESULTS**

**Mutant Forms of HB-EGF Lacking the Heparin-binding Domain**—To elucidate the role of the heparin-binding domain of HB-EGF, mutant forms of HB-EGF were generated, as shown in Fig. IA. Pro-ΔHB, a membrane-anchored form of HB-EGF lacking the heparin-binding domain (amino acids 93–105), and sΔHB, a myc- and His-tagged soluble form of HB-EGF lacking the heparin-binding domain, were both generated. In addition, sHB-EGF, also a myc- and His-tagged wild-type soluble form, was used as a control. Each construct was expressed in a mutant CHO cell line, 677, which lacks both N-acetylgalcosaminyltransferase and glucuronosyltransferase, enzymes required for the polymerization of HS chains (39).

First, we examined the recombinant proteins from these cell lines by Western blot. sHB-EGF and sΔHB were detected in the conditioned medium with the expected molecular masses ranging from 18 to 28 kDa and from 14 to 24 kDa, respectively (Fig. 1B, left panel). Pro-HB-EGF or pro-ΔHB was detected in the lysate of the transfected 677 cells, with the expected molecular masses ranging from 27 to 52 kDa and 24 to 50 kDa, respectively (Fig. 1B, right panel). Because both sΔHB and pro-ΔHB lack the heparin-binding domain, the molecular masses of these proteins are smaller than wild type, as expected. Immunoblotting of HB-EGF species showed several bands, which represent various post-translational modifications, such as glycosylation and N-terminal processing (11, 40). sHB-EGF and pro-HB-EGF differed among the various lines of the stable transfecants, DT-binding activity was normalized according to the total amount of pro-HB-EGF or pro-ΔHB expressed on the cell surface. In heparin-free conditions, 677pro-ΔHB cells showed much greater binding to 125I-DT than did 677HB cells (Fig. 3A). Addition of heparin restored the DT-binding activity of 677HB cells to levels comparable to that of 677pro-ΔHB cells in heparin-free conditions. No significant effect of heparin on DT binding was observed in 677pro-ΔHB cells. Higher DT binding was also observed for pro-ΔHB than for wild-type pro-HB-EGF in the stable transfecants of L cells (Fig. 3B). In the heparin-free condition, the difference in DT binding between LH and Lpro-ΔHB cells was lower (2-fold difference) than that between 677HB and 677pro-ΔHB cells (5-fold difference), because endogenously expressed HSPG in L cells supports DT binding to pro-HB-EGF (32).

Because CD9 also has been implicated in the up-regulation of the DT-binding activity of pro-HB-EGF (17, 18), we examined DT binding of pro-ΔHB in cells expressing CD9 on the cell surface. Either pro-ΔHB or pro-HB-EGF were transiently introduced into LC cells (18), stably expressing CD9 (LC-pro-ΔHB cells and LC-pro-HB cells, respectively), and we found this was also the case for 677 cells and L cells; LC-pro-ΔHB cells showed much higher binding activity to 125I-DT than did LC-pro-HB cells in the heparin-free conditions (Fig. 3C). Addition of heparin restored DT binding in LC-pro-HB cells, whereas LC-pro-ΔHB cells were unaffected. Thus, up-regulation by CD9 is the mechanism independent of deletion of the heparin-binding domain as well as of interaction of HSPG as described previously (32).

We also examined the effect of cell surface HSPGs on DT binding by pro-ΔHB (Fig. 3D). Treatment of LC-pro-HB cells with heparitinase to diminish HS chains on the surface of LC-pro-HB cells decreased DT binding, whereas subsequent addition of heparin restored DT binding in LC-pro-HB cells to maximal levels. In contrast, neither addition of heparin nor pro-HB-EGF restored DT binding. Therefore, the heparin-binding domain of HB-EGF is essential for heparin-binding property in HB-EGF.

**DT Binding Assay**—HB-EGF has been shown to act as a diphtheria toxin receptor (DTTR) and binds to DT through its EGF-like domain (37). Previously, we reported that pro-HB-EGF associates with cell surface HSPGs, which increases its binding affinity for DT (32). To investigate whether or not the heparin-binding domain influences the biological activity of the EGF-like domain in HB-EGF, we first examined how deletion of the heparin-binding domain affects DT binding activity. We developed a cell-free DT binding assay in which His-tagged sHB-EGF and sΔHB proteins were collected from the conditioned media of transfected 677 cells by a metal affinity resin, and then the binding of 125I-DT to the immobilized sHB-EGF or sΔHB was analyzed in the presence or absence of heparin.

sΔHB showed much higher binding affinity to DT than did sHB-EGF (Fig. 2A), with Scatchard plot analysis yielding \( K_a \) values of \( 1.9 \times 10^9 \) M⁻¹ for sHB-EGF and \( 1.3 \times 10^9 \) M⁻¹ for sΔHB (Fig. 2B). The DT binding activity of immobilized sHB-EGF and sΔHB was also tested at varying concentrations of heparin. The DT-binding activity of sHB-EGF increased with the addition of heparin in a dose-dependent manner, whereas that of sΔHB was not affected by exogenously added heparin (Fig. 2C). Scatchard plot analysis for sHB-EGF binding to DT yielded \( K_a \) values of \( 1.9 \times 10^9 \) M⁻¹ in the absence of heparin and \( 7.2 \times 10^9 \) M⁻¹ in the presence of 100 μg/ml heparin (Fig. 2, D and E), in agreement with our previous observation in DT binding to pro-HB-EGF (32). On the other hand, the \( K_a \) value of sΔHB was unaffected by absence (\( K_a = 1.3 \times 10^9 \) M⁻¹) or presence (\( K_a = 1.4 \times 10^9 \) M⁻¹) of heparin (Fig. 2, F and G). Thus, the binding affinity of sHB-EGF for DT became close to that of sΔHB by addition of exogenous heparin.

Increased DT binding activity resulting from the deletion of the heparin-binding domain was confirmed in the membrane-anchored form of HB-EGF. We analyzed the DT-binding activity of pro-ΔHB and pro-HB-EGF stably expressed on the surface of 677 cells (677pro-ΔHB and 677HB cells, respectively). Because cell surface expression levels of pro-HB-EGF and pro-ΔHB differed among the various lines of the stable transfecants, DT-binding activity was normalized according to the total amount of pro-HB-EGF or pro-ΔHB expressed on the cell surface. In heparin-free conditions, 677pro-ΔHB cells showed much greater binding to 125I-DT than did 677HB cells (Fig. 3A). Addition of heparin restored the DT-binding activity of 677HB cells to levels comparable to that of 677pro-ΔHB cells in heparin-free conditions. No significant effect of heparin on DT binding was observed in 677pro-ΔHB cells. Higher DT binding was also observed for pro-ΔHB than for wild-type pro-HB-EGF in the stable transfecants of L cells (Fig. 3B). In the heparin-free condition, the difference in DT binding between LH and Lpro-ΔHB cells was lower (2-fold difference) than that between 677HB and 677pro-ΔHB cells (5-fold difference), because endogenously expressed HSPG in L cells supports DT binding to pro-HB-EGF (32).
heparitinase treatment affected the DT-binding activity of LC-pro-H9004 HB cells. These results indicate that cell surface HSPGs are not involved in the increased DT binding of pro-H9004 HB.

Growth Factor Assay—We next investigated the effect of deletion of the heparin-binding domain on the growth factor activity of HB-EGF. We compared the mitogenic activities of wild-type sHB-EGF and sΔHB for DER cells, a 32D cell line expressing EGFR that proliferates in an EGFR ligand-dependent manner (14). Like 677 cells, neither parental 32D cells (42) nor DER cells (data not shown) express HSPGs. The mitogenic

![Image](http://www.jbc.org/)
activity of sHB-EGF and sΔHB was analyzed by measuring DER cell growth in the conditioned medium of cells transfected with each construct. As shown in Fig. 4A, mitogenic activity of sΔHB for DER cells was ∼10 times higher than that of wild-type sHB-EGF. In addition, the mitogenic activity of sHB-EGF increased in a dose-dependent manner upon addition of exogenous heparin (10 μg/ml). Figure 4B, effect of heparitinase on DT binding to pro-HB-EGF and pro-ΔHB. LC-pro-HB cells and LC-pro-ΔHB cells were incubated with or without heparitinase (0.02 unit/ml) for 1.5 h at 37 °C. DT binding to these cells was measured in the absence or presence of heparin (10 μg/ml). Open bars, untreated cells; hatched bars, heparitinase-treated cells; dotted bars, heparitinase-treated cells with heparin. All data are expressed as relative values of DT binding normalized to the amount of pro-HB-EGF or pro-ΔHB expressed at the cell surface, which was determined as described under “Experimental Procedures,” and indicated as the ratio of the value against the score of untreated cells without heparin, and shown as mean ± S.E. from three independent experiments. Nonspecific binding was <5% of the total binding.

To investigate whether the effect of heparin on the mitogenic activity of sHB-EGF was mediated through binding of HB-EGF to EGFR, we compared EGFR activation by wild-type sHB-EGF and sΔHB in DER cells, in the presence or absence of heparin. EGFR activation was assayed by Western blot detection of tyrosine-phosphorylated EGFR in DER cells incubated with conditioned medium containing sHB-EGF (324 pM) or sΔHB (240 pM). Conditioned medium from mock-transfected cells did not induce EGFR autophosphorylation (Fig. 5, lanes 1 and 2). In agreement with the results from the mitogenic assay, sΔHB induced autophosphorylation of EGFR at a much higher level than sHB-EGF under heparin-free conditions (Fig. 5, lanes 3 and 5). In the presence of heparin, EGFR autophosphorylation activity of sHB-EGF was greatly increased (Fig. 5, lanes 3 and 4), whereas the activity of sΔHB was unaffected by heparin (Fig. 5, lanes 5 and 6). These results indicate that the difference in mitogenic activity between sHB-EGF and sΔHB, as well as the heparin-induced changes in mitogenic activity of sHB-EGF, occur at the level of interaction between HB-EGF and EGFR.

Taken together, results from both the DT binding assay and growth factor assay indicate that the heparin-binding domain...
is not essential for biological activity of the EGF-like domain in HB-EGF. On the contrary, the heparin-binding domain appears to suppress the function of the EGF-like domain. Restoration of the activity of the EGF-like domain by addition of exogenous heparin indicates that association of heparin with HB-EGF via the heparin-binding domain removes the suppressive effect of this domain.

**DISCUSSION**

As is the case for other heparin-binding factors, HB-EGF activity is modulated by its interactions with heparin-like molecules. Previous data indicated that heparin and HS increase the binding of HB-EGF to EGFR (31). Our previous work has also demonstrated that HSAGs on the cell surface are required for maximal DT binding of pro-HB-EGF (32). Thus, it is conceivable that the heparin-binding domain is required for the full activity of HB-EGF mediated by the interaction of heparin-like molecules. However, our results show that the heparin-binding domain is able to suppress both its DT-binding activity and its EGFR-mediated function as a growth factor and that this domain is not absolutely required for those HB-EGF activities. The mutant form of HB-EGF lacking the heparin-binding domain (ΔHB) showed much higher activity than wild-type HB-EGF both in the DT binding and in the mitogenic signaling for EGFR. The increased activity of ΔHB reached levels comparable to that of wild-type HB-EGF interacting with heparin. These results indicate that heparin interaction with HB-EGF removes the suppressive effect of the heparin-binding domain, with the result being that HB-EGF exhibits maximal activity. This is the first evidence indicating that the heparin-binding domain negatively regulates the activities of the growth factor or cytokine with heparin-binding properties.

FGFs are among the best-studied heparin-binding growth factors. Recent structural studies have clearly demonstrated that heparin or HS directly associates with not only FGF, but also FGFR, in a ternary complex on the cell surface (43). Formation of the ternary complex promotes ligand dimerization, leading to receptor dimerization and stimulation of kinase activity. However, this is not the case for HB-EGF. DT does not appear to bind to heparin (32). In addition, binding of EGF to EGFR does not appear to be affected by the presence of heparin or HSAG on the cell surface (31). These findings suggest that heparin or HS associates only with HB-EGF in interactions between HB-EGF and either DT or EGFR.

The structure of the EGF-like domain of HB-EGF has been solved by crystallographic analysis of the DT-HB-EGF complex (44). As is the case for other EGF family growth factors (45), the three-dimensional structure of the EGF-like domain of HB-EGF is composed of three loops: the A-, B-, and C-loops, which run from the N terminus to the C terminus of the polypeptide. The largest structural differences among the various EGFs occur within the N-terminal A-loop, particularly in the region between the first and second cysteines (44). In HB-EGF, this region contains highly basic charged residues from amino acids 110–113 (RKKY). A mutant form of HB-EGF with 3-amino acid substitutions of Arg110, Lys111, and Lys113 to Leu, Ser, and Asp,

![Figure 4](image1.png) **FIG. 4.** Mitogenic activity of the soluble HB-EGF and the deletion mutant lacking the heparin-binding domain. A, mitogenic activity of sHB-EGF and sΔHB on DER cells. DER cells were cultured in the presence of the indicated concentrations of sHB-EGF (open circles) or sΔHB (closed circles) for 36 h. B, mitogenic activity of sHB-EGF in the presence or absence of heparin. DER cells were cultured with the indicated concentrations of sHB-EGF with varying concentrations of heparin: 0 μg/ml (open circles), 10 μg/ml (open squares), and 100 μg/ml (open triangles). C, mitogenic activity of sΔHB in the presence or absence of heparin. DER cells were cultured with the indicated concentrations of sΔHB with varying concentrations of heparin: 0 μg/ml (closed circles), 10 μg/ml (closed squares), and 100 μg/ml (closed triangles). In all figures, mitogenic activity was calculated as described under the “Experimental Procedures.” Similar results were obtained in three independent experiments.

![Figure 5](image2.png) **FIG. 5.** EGFR autophosphorylation by the soluble HB-EGF and the deletion mutant lacking the heparin-binding domain. DER cells were treated with CM from mock-transfected cells (lanes 1 and 2), sHB-EGF-transfected cells (lanes 3 and 4), or sΔHB-transfected cells (lanes 5 and 6) at 37 °C for 1 min in the presence (lanes 2, 4, and 6) or absence (lanes 1, 3, and 5) of heparin (100 μg/ml). Tyrosine-phosphorylated EGFR (upper panel) and total EGFR (lower panel) in DER cells was detected by Western blot analysis using the anti-human phospho-EGFR antibody and the anti-human EGFR antibody, respectively. The concentration of sHB-EGF and sΔHB in CM was calculated using the DT binding assay as 324 pm and 240 pm, respectively. Similar results were obtained in three independent experiments.
respectively, decreases not only the DT-binding activity, but also the mitogenic signaling activity via EGFR (46), suggesting that the A-loop of the EGF-like domain is critical for the biological activities of HB-EGF. Interestingly, the heparin-binding domain is just adjacent to the N-terminal portion of the A-loop. How does the heparin-binding domain suppress the biological activity of the EGF-like domain of HB-EGF? Although a precise mechanism has not been determined, structural studies point to a hypothetical model. Our data indicate that association of heparin increases the activity of wild-type HB-EGF to levels comparable to that of the mutant form lacking the heparin-binding domain (44). We propose that the heparin-binding domain and its association with heparin-like molecules drastically affect the structure of the A-loop in the EGF-like domain (Fig. 6, A and B). In our model, interaction of heparin with the heparin-binding domain electrostatically neutralizes this domain, resulting in a conformational change such that the A-loop adopts a similar form to that of the mutant HB-EGF form lacking the heparin-binding domain (Fig. 6, B and C).

Upon binding of DT to HB-EGF (Fig. 6D), the A-loop and C-loop face the receptor-binding domain of DT (44). We previously demonstrated that the amino acids Phe115, Leu127, or especially Glu141, within the EGF-like domain are critical for DT-binding activity (38). In particular, Phe115, which is located in the A-loop, and Glu141, which is within the C-loop, play crucial roles in binding between DT and HB-EGF at the interface between these molecules (44). It is possible that the weak binding observed for HB-EGF and DT in the absence of heparin is mediated by the C-loop, which may be distant enough from the heparin-binding domain to be unaffected by structural changes caused by heparin binding.

Based on the crystal structures of the EGF-EGFR ectodomain (47, 48) and the transforming growth factor-α-EGFR ectodomain (49), it is likely that all loop structures in the EGF-like domain participate in the binding of HB-EGF to EGFR (Fig. 6E). The A-loop and C-loop appear to interact with...
domain III of EGFR, whereas B-loop interacts with domain I. We propose that a conformational change in the A-loop induced either by the loss of the heparin-binding domain or by the association of heparin with this domain dramatically enhances the activity of HB-EGF to bind to EGFR. Thus, HB-EGF may weakly interact with EGFR via the B-loop and C-loop in the absence of heparin, but full activation of EGFR may require a heparin-induced conformational change allowing for association of the A-loop with EGFR.

What are the biological implications of the inhibitory regulation of HB-EGF activity by the heparin-binding domain? We suggest that when HB-EGF associates with cell surface HSPGs, both its activity and local concentration are high, whereas when HB-EGF exists as the HSPG-free form, both are low. In this manner, HB-EGF can signal fully only when associated with cell surface HSPGs, both its activity and local concentration are high, whereas when HB-EGF associates with cell surface HSPGs, both its activity and local concentration are high, whereas when HB-EGF exists as the HSPG-free form, both are low. In this manner, HB-EGF can signal fully only when associated with cell surface HSPGs, both its activity and local concentration are high, whereas when HB-EGF exists as the HSPG-free form, both are low.

In conclusion, here we show evidence indicating that the heparin-binding domain of HB-EGF plays an autoregulatory role by suppressing HB-EGF activity, whereas association of heparin with this domain removes this suppression. Although this mechanism needs to be verified by structural studies, our results provide new insights into the mechanisms regulating the activity of the heparin-binding growth factors and cytokines.

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Suppression of the Biological Activities of the Epidermal Growth Factor (EGF)-like Domain by the Heparin-binding Domain of Heparin-binding EGF-like Growth Factor

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