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 × 10⁶ 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-lysis buffer (1% Triton X-100, 0.15 M NaCl, 50 mM Tris-HCl, pH 7.5, 1 mM Na₃VO₄, 0.5 μM phenylmethylsulfonyl fluoride, 0.15 μM aprotinin, 1 μM E-64, 1 μM leupeptin, 0.5 μM EDTA) and then centrifuged for 20 min at 15,000 × 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. 1A. 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*-acetylglucosaminyltransferase 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). sΔHB and pro-ΔHB had similar banding patterns as sHB-EGF and pro-HB-EGF, respectively, indicating that deletion of heparin-binding domain does not affect the post-translational modification of HB-EGF (Fig. 1B).

The heparin-binding activity of sHB-EGF and sΔHB was assessed using heparin-Sepharose chromatography. As shown in the *upper panel* of Fig. 1C, sHB-EGF bound to heparin-Sepharose in 0.05 M NaCl buffer and was eluted in buffer containing more than 0.75 M NaCl, as reported previously (41). On the contrary, almost all of the sΔHB input was detected in the flow-through fraction (Fig. 1C, *lower panel*), indicating that heparin-binding activity of sΔHB was undetectable. A previous study indicated that the heparin-binding domain consisted of a 21-amino acid stretch from amino acid 93 through 113 (41), with the highly basic sequences [93]KRKKK[97], [103]KKR[105], and [110]RKYK[113] contributing the most to the heparin-binding function. However, here we deleted amino acids 93–105, which lie adjacent to but do not overlap the EGF-like domain, and found that the amino acid stretch [93]KRKKKGKGLGKKR[105] is essential for heparin-binding property in HB-EGF.

DT Binding Assay—HB-EGF has been shown to act as a diphtheria toxin receptor (DTR) 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 ^{125}I-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 × 10⁸ M⁻¹ for sHB-EGF and 1.3 × 10⁹ 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 × 10⁸ M⁻¹ in the absence of heparin and 7.2 × 10⁸ 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 ($K_a = 1.3 × 10^9$ M⁻¹) or presence ($K_a = 1.4 × 10^9$ 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 677H cells, respectively). Because cell surface expression levels of pro-HB-EGF and pro-ΔHB differed among the various lines of the stable transfectants, 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 ^{125}I-DT than did 677H cells (Fig. 3A). Addition of heparin restored the DT-binding activity of 677H 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 transfectants 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 677H 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 and L cells; LC-pro-ΔHB cells showed much higher binding activity to ^{125}I-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
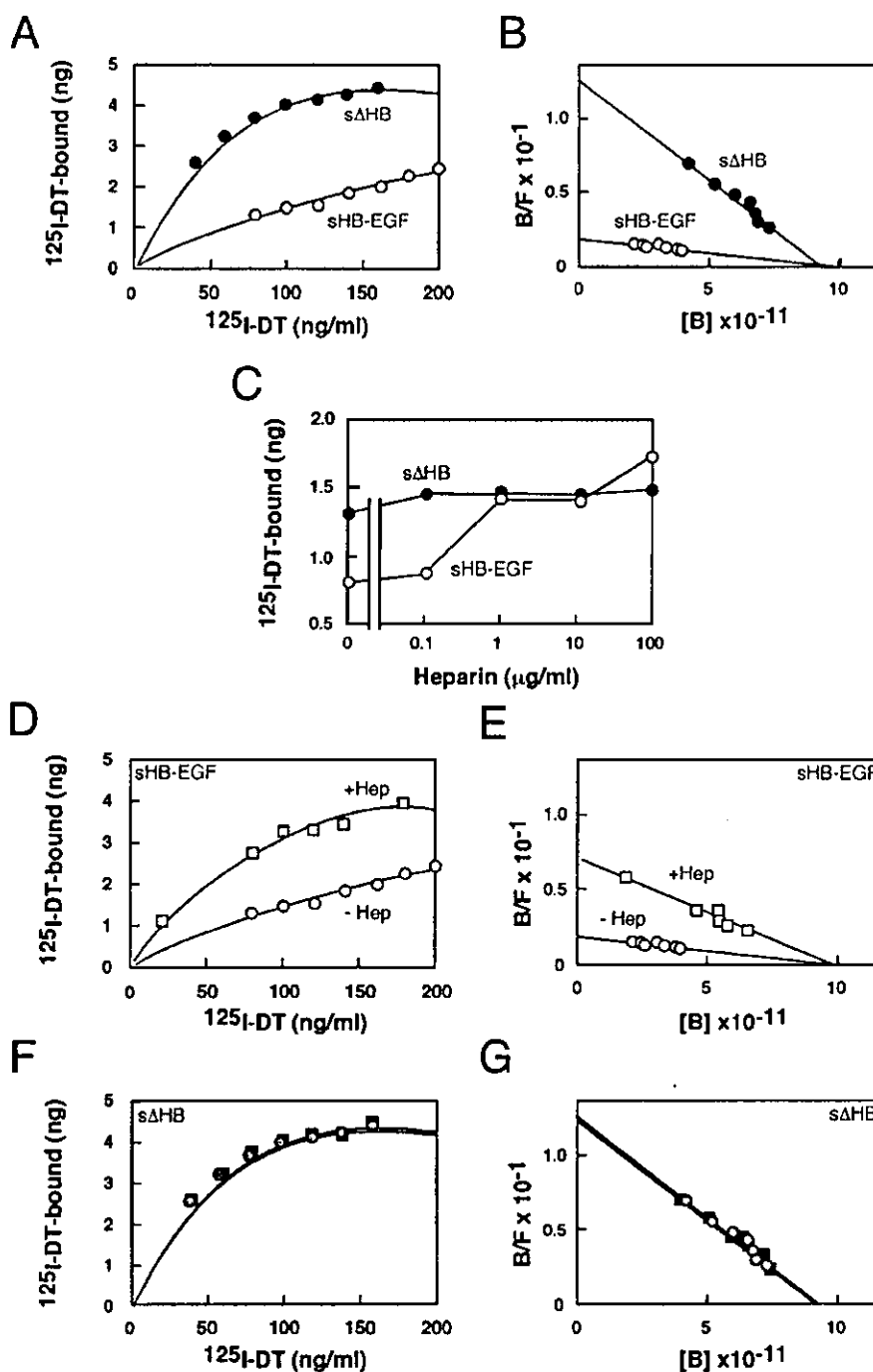
Fig. 2. **DT binding to the soluble HB-EGF and the deletion mutant lacking the heparin-binding domain.** *A*, binding of ^{125}I-DT to sHB-EGF (*open circles*) and sΔHB (*closed circles*). *B*, Scatchard plot analysis of ^{125}I-DT binding to sHB-EGF (*open circles*) and sΔHB (*closed circles*) based on the data shown in *A*. *C*, binding of ^{125}I-DT to sHB-EGF (*open circles*) and sΔHB (*closed circles*) at the indicated concentrations of heparin. The concentration of ^{125}I-DT was fixed at 100 ng/ml. *D*, binding of ^{125}I-DT to sHB-EGF in the presence (*open squares*) or absence (*open circles*) of 100 μg/ml heparin. *E*, Scatchard plot analysis of ^{125}I-DT binding to sHB-EGF in the presence (*open squares*) or absence (*open circles*) of 100 μg/ml heparin based on the data shown in *D*. *F*, binding of ^{125}I-DT to sΔHB in the presence (*closed squares*) or absence (*closed circles*) of 100 μg/ml heparin. *G*, Scatchard plot analysis of the ^{125}I-DT binding to sΔHB in the presence (*closed squares*) or absence (*closed circles*) of 100 μg/ml heparin based on the data shown in *F*. In all figures, data are expressed as specific binding of ^{125}I-DT, which was calculated as described under "Experimental Procedures." In all experiments, nonspecific binding of ^{125}I-DT was <20% of the total binding. Similar results were obtained in three independent experiments.

heparitinase treatment affected the DT-binding activity of LC-pro-ΔHB cells. These results indicate that cell surface HSPGs are not involved in the increased DT binding of pro-ΔHB.

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

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A

B

C

D

FIG. 3. **DT binding to the membrane-anchored HB-EGF and the deletion mutant lacking the heparin-binding domain.** A, binding of ^{125}I-DT (100 ng/ml) to pro-HB-EGF and pro-ΔHB stably expressed in 677 cells (677H cells and 677pro-ΔHB cells, respectively) in the absence (*open bars*) or presence (*closed bars*) of heparin (10 μg/ml). B, binding of ^{125}I-DT (100 ng/ml) to pro-HB-EGF and pro-ΔHB stably expressed in L cells (LH cells and Lpro-ΔHB cells, respectively) in the absence (*open bars*) or presence (*closed bars*) of heparin (10 μg/ml). C, binding of ^{125}I-DT (100 ng/ml) to pro-HB-EGF and pro-ΔHB transiently expressed in LC cells (LC-pro-HB cells and LC-pro-ΔHB cells, respectively) in the absence (*open bars*) or presence (*closed bars*) of heparin (10 μg/ml). D, 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.
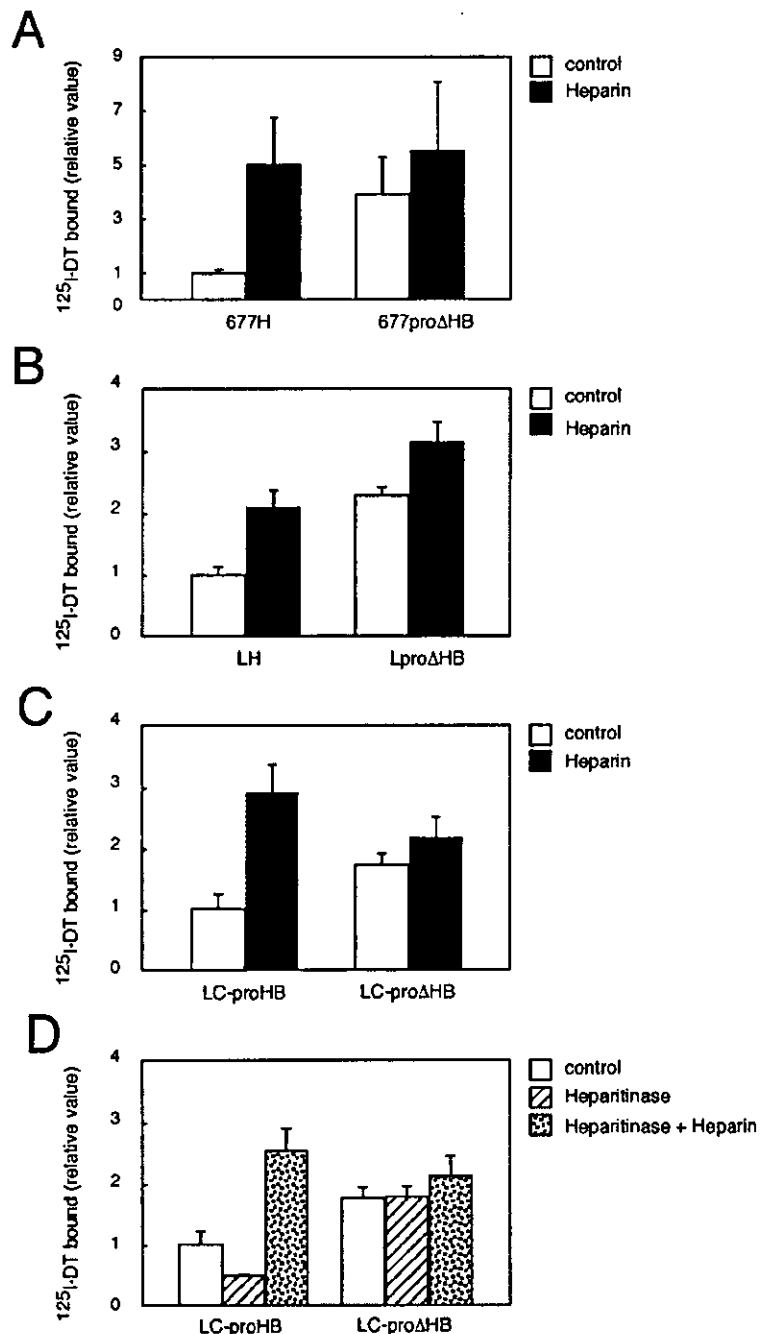
activity of sHB-EGF and sΔHB was analyzed by measuring DER cell growth in the conditioned medium of cells transfected with each construct. As shown in Fig. 4A, mitogenic activity of sΔHB for DER cells was ~10 times higher than that of wild-type sHB-EGF. In addition, the mitogenic activity of sHB-EGF increased in a dose-dependent manner upon addition of exogenous heparin (Fig. 4B), whereas that of sΔHB was unaffected by heparin (Fig. 4C).

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
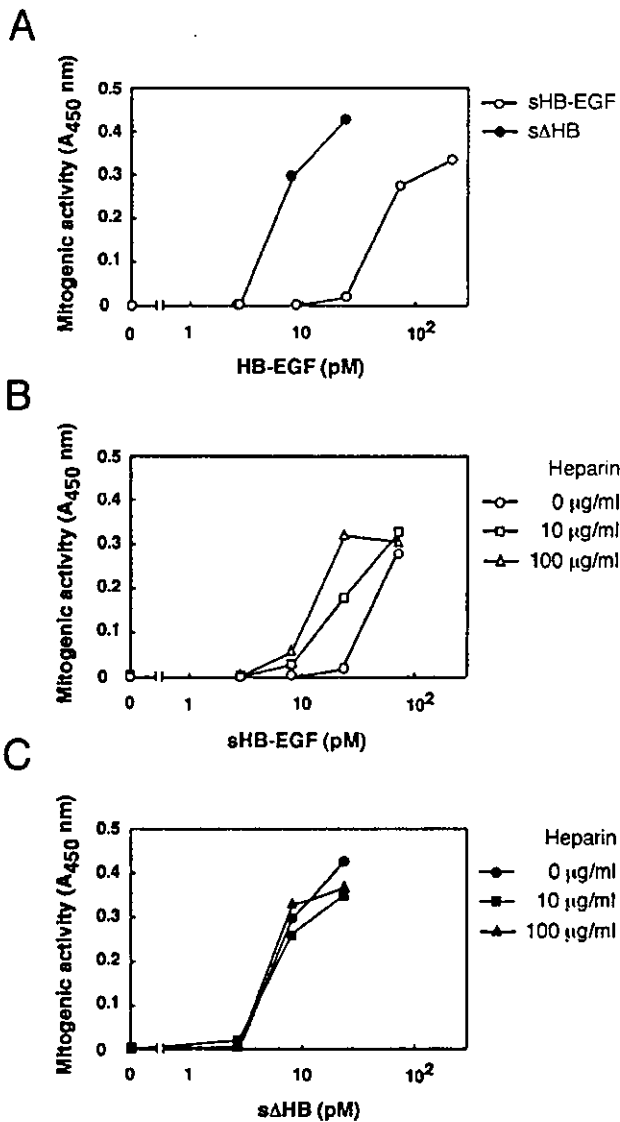
A

B

C

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.

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 mol-
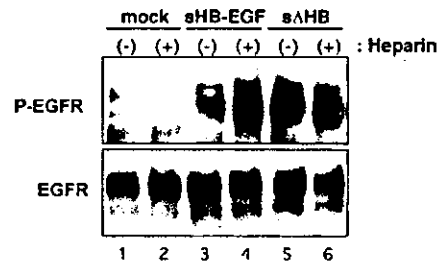

FIG. 5. **EGFR autophosphorylation by the soluble HB-EGF and the deletion mutant lacking the heparin-binding domain.** DER cells were treated with CM from mock-transfected cells (*lanes 1* and *2*), sHB-EGF-transfected cells (*lanes 3* and *4*), or sΔHB-transfected cells (*lanes 5* and *6*) at 37 °C 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.

ecules. Previous data indicated that heparin and HS increase the binding of HB-EGF to EGFR (31). Our previous work has also demonstrated that HSPGs 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 HSPG 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 (RKYK). A mutant form of HB-EGF with 3 amino acid substitutions of Arg[110], Lys[111], and Lys[113] to Leu, Ser, and Asp,

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FIG. 6. **Proposed model for the regulation of HB-EGF biological activities by the heparin-binding domain.** *A*, the structure of the EGF-like domain of HB-EGF is composed of three loops: the A-, B-, and C-loops. In HB-EGF, the A-loop contains highly basic, charged amino acids (represented by a *red line*). Because the heparin-binding domain (*HBD*, represented by a *bold red line*), which is also a highly basic, charged region (+), is juxtaposed with the N-terminal A-loop, the structure of A-loop is affected by the repulsive polar effect between the HBD and the A-loop, converting the EGF-like domain into a conformation that has weak activity. *B*, interaction of heparin, which is highly acidic and charged, with the heparin-binding domain neutralizes polar effect of this domain (±). As a result, the A-loop undergoes a conformational change and the EGF-like domain gains full activity. *C*, deletion of the heparin-binding domain diminishes the polar effect of this domain, mimicking the effect of heparin interaction. This results in a conformational change of the EGF-like domain that confers full activity. *D*, upon binding of HB-EGF with DT, the A-loop and C-loop face to the receptor-binding domain of DT. *E*, during binding of HB-EGF to EGFR, all loop structures in the EGF-like domain participates. The A-loop and C-loop interact with domain III of EGFR, while the B-loop interacts with domain I of EGFR.
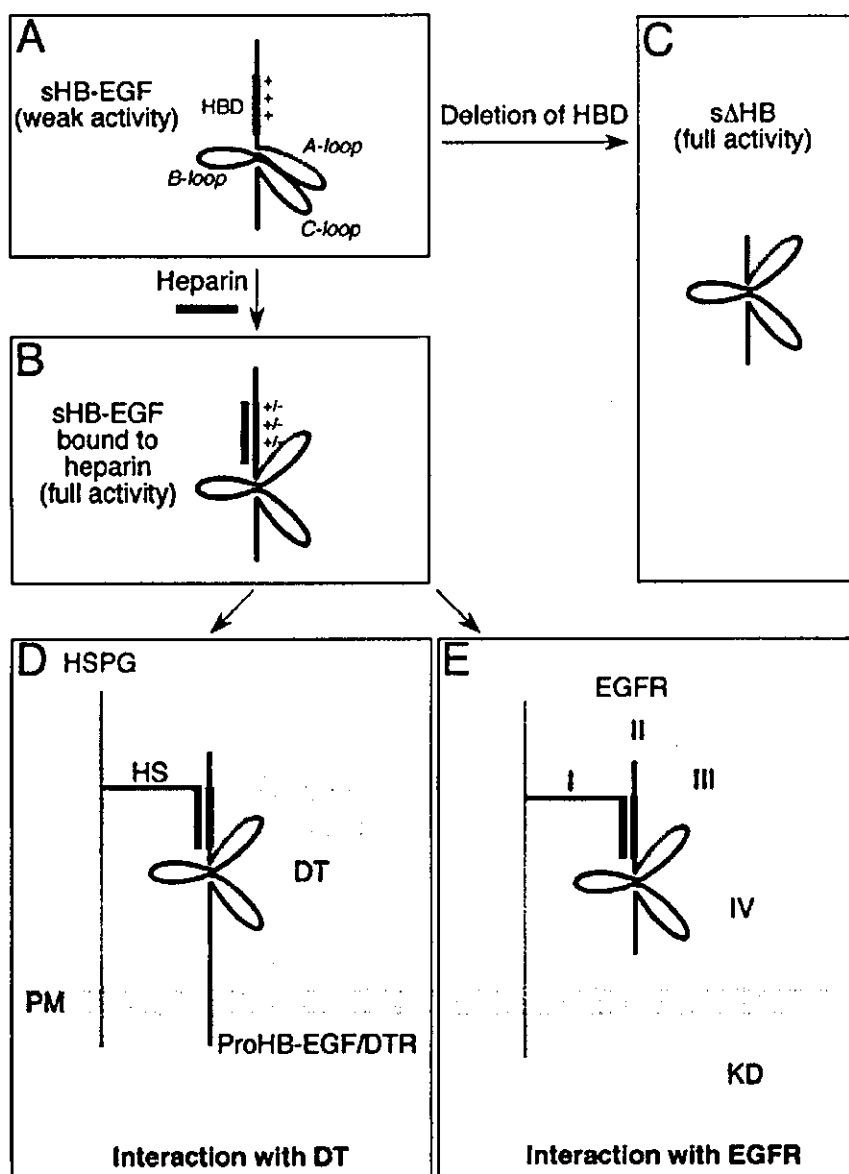
respectively, decreases not only the DT-binding activity,[2] but also the mitogenic signaling activity via EGFR (46), suggesting that the A-loop of the EGF-like domain is critical for the biological activities of HB-EGF. Interestingly, the heparin-binding domain is just adjacent to the N-terminal portion 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. Although the structural relationship between the heparin-binding domain and the EGF-like domain in DT·HB-EGF complex was disordered in the reported crystal structure (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 Phe[115], Leu[127], or especially Glu[141], within the EGF-like domain are critical for DT-binding activity (38). In particular, Phe[115], which is located in the A-loop, and Glu[141], 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 ability 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 both HSPGs and EGFR on the target cell surface.

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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