Heparin-like Molecules on the Cell Surface Potentiate Binding of Diphtheria Toxin to the Diphtheria Toxin Receptor/Membrane-anchored Heparin-binding Epidermal Growth Factor-like Growth Factor*

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Diphtheria toxin receptor (DTR), which is identical to the membrane-anchored form of heparin-binding EGF-like growth factor (proHB-EGF), has a high affinity for heparin. We studied the effect of heparin-like molecules on the binding of diphtheria toxin (DT) to DTR/proHBEGF. Mutant Chinese hamster ovary (CHO) cells deficient in heparan sulfate (HS) proteoglycans were about 15 times less sensitive to DT than wild type CHO-K1 cells. When free heparan sulfate or heparin was added to the culture medium, DT sensitivity of the mutant cells was fully restored. Studies of binding of 125I-labeled DT to HS-deficient CHO cells transfected with human DTR/proHB-EGF cDNA indicated that the increased sensitivity to DT after addition of heparin is due to increased binding of DT to cells. Vero cells display a relatively large amount of heparan sulfate residues compared to CHO-K1 cells or L cells. Enhancement of DT binding by the addition of heparin was also observed with CHO-K1 cells and L cells that had been transfected with human DTR/proHB-EGF cDNA, but the degree of enhancement was less than that observed with the HS-deficient CHO cells. Addition of heparin did not affect DT binding or DT sensitivity of Vero cells. Heparin-dependent binding was observed when intact Vero cells were treated with heparitinase or when the cell membrane was solubilized with a neutral detergent. Scatchard plot analysis for the binding of DT to a recombinant HB-EGF in vitro and to L cells expressing human DTR/proHB-EGF revealed that heparin increases the affinity of DTR/proHB-EGF for DT but does not change the number of binding sites. Although DRAP27/C9 is known to enhance DT binding to DTR/proHB-EGF, the results indicate that heparin and DRAP27/C9 increase DT binding by independent mechanisms. Thus, heparin-like molecules, probably in the form of heparan sulfate proteoglycan on the cell surface, are a third factor required for maximal DT binding activity of cells.

Diphtheria toxin (DT) inhibits protein synthesis of eukaryotic cells by catalyzing the ADP-ribosylation of elongation factor-2, which results in its inactivation (1, 2). The process of DT cytotoxicity includes (i) binding of DT to a specific receptor on the cell surface, (ii) internalization of the toxin by receptor-mediated endocytosis, (iii) translocation of the enzymatically active A fragment from endosome to the cytosol, and (iv) inactivation of elongation factor-2. Although a number of cellular factors are involved in this process, binding of DT to the diphtheria toxin receptor (DTR) is a key step (3).

DTR, the principal protein involved in binding of DT to cells, was first identified and purified from monkey Vero cells (4, 5), one of the cell lines most sensitive to DT (6). A cDNA-encoding DTR was cloned from this cell line (7). DTR is identical to the precursor form of heparin-binding EGF-like growth factor (proHB-EGF) (7, 8) which was originally identified as a heparin-binding growth factor (9). While proHB-EGF is cleaved by an unidentified protease on the cell surface to yield the soluble mature growth factor (HB-EGF) (10), a significant amount of proHB-EGF is left uncleaved on the cell surface, where it acts as a membrane-anchored growth factor (11) and as a DTR. Although proHB-EGF is expressed in species including human, monkey, rat, and mouse with a similar tissue distribution, cells derived from mice and rats are resistant to DT (6, 12, 13). The insensitivity of mouse and rat cells to DT is due to amino acid substitutions in the EGF-like domain of proHB-EGF that reduce binding of DT (14).

DTR/proHB-EGF forms a complex with DRAP27/C9 (8, 15), which belongs to a newly identified tetra membrane-spanning protein family (16). Although direct interaction of DRAP27/C9 with DT has not been observed, DRAP27/C9 greatly up-regulates the binding of DT (8, 16, 17) and the juxtacrine mitogenic activity (11) of DTR/proHB-EGF. Thus, DRAP27/C9 is a second factor determining binding of DT to cells. Although the mechanism of up-regulation by DRAP27/C9 is still not clear, it is neither due to increased expression of the DTR/proHB-EGF gene nor increased DTR/proHB-EGF protein on the cell surface (8). Scatchard plot analysis shows that DRAP27/C9 increases the number of DT-binding sites but does not change their affinity for DT (8).
DTR/proHB-EGF shows strong affinity for heparin and heparan sulfate (HS) (18, 19). As has been shown for other heparin-binding growth factors (20–22), HB-EGF requires heparin or cell surface heparan sulfate proteoglycan (HSPG) for binding to and activation of the EGFR receptor (23, 24). Because the binding site of DTR/proHB-EGF for DT seems to overlap with or be located in close proximity to the binding site for the EGFR receptor (14), we examined whether heparin influences binding of DT to DTR/proHB-EGF. The results indicate that DT requires cell surface heparin-like molecules for maximal binding to DTR/proHB-EGF. Heparin-like molecules on the cell surface, possibly in the form of HSPG, are a third factor influencing binding of DT to cells.

EXPERIMENTAL PROCEDURES

Materials—DT was produced as described previously (25). HS (from bovine intestinal mucosa), dermatan sulfate (from bovine mucosa), sodium salts (heparitinase EC 4.2.2.8, catalog no. 100703), chondroitinase ABC, and mouse anti-HS monoclonal antibody HepSS1 (26) were purchased from Seikagaku Co. (Tokyo, Japan). Heparin (from bovine intestinal mucosa) and N-desulfated heparin (from porcine mucosal heparin) sodium salts were purchased from Sigma. Chondroitin sulfate A and C (from shark cartilage) sodium salts were obtained from Nacalai Tesque, Inc. (Kyoto, Japan). Rabbit anti-human HB-EGF antibody (H6) was obtained from Sepherose gels. The CHO mutant cell line pgsD-677 (677) was kindly provided by Dr. J. D. Esko (Department of Biochemistry, University of Birmingham, AL). CHO-K1 cells and mutant 677 cells were grown in Ham's F-12 medium supplemented with 10% fetal calf serum, 100 units/ml penicillin G, and 100 μg/ml streptomycin as described previously (20). Vero cells and VeroH cells (10) were grown in Eagle's minimum essential medium supplemented with 10% calf serum, nonsolvent amino acids, 100 units/ml penicillin G, and 100 μg/ml streptomycin. LH cells and LCH cells (8) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 units/ml penicillin G, and 100 μg/ml streptomycin.

Flow Cytometric Analysis—Cells were detached by EDTA treatment and suspended in WS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM phosphate buffer, 0.2 mM CaCl2, 0.2 mM MgCl2, 3 mM NaN3, 1 mg/ml bovine serum albumin, pH 7.2). For detection of HS, cell suspension was incubated with 5 μl of FITC-conjugated goat anti-rabbit IgG antibody H6 (diluted 500-fold in WS buffer), washed twice with WS buffer, and then incubated with FITC-conjugated anti-mouse IgG secondary antibody for 1 h at 4 °C. The FITC-conjugated antibody was detected by fluorescence microscope. The cell surface HS content was determined by flow cytometry (Coulter Corp., Hialeah, FL). For each measurement, 10,000 events were collected, and aggregated cells were gated out.

Assay for DT Cytotoxicity—DT cytotoxicity was assayed by measuring the inhibition of protein synthesis as described previously (28). Stable Transfectants of CHO Cells with High Expression of DTR—CHO-K1 cells or mutant 677 cells were transfected with prHBEGF (8) by the calcium phosphate method (29). Cells were cultured for 48 h and further cultured for 7 days in the presence of 200 μg/ml G418. Colonies growing on medium were isolated and assayed for DT binding activity. Positive clones were isolated and subcloned again. K1H cells derived from CHO-K1 cells and 677H cells from 677 cells were the clones with the highest expression of human HB-EGF.

Binding of 125I-labeled DT to Cells—Binding of 125I-labeled DT to cells was measured as described previously (8) except for the binding medium. The same binding assay was used in this study was used for HSPG containing 20 mg HEPES, 1 mg/ml bovine serum albumin, and 3 mM NaN3, pH 7.2. Specific binding was determined by subtracting the counts obtained with cells incubated with 125I-DT and excess unlabeled DT (non-specific binding) from the counts obtained with 125I-DT alone (total binding). In some experiments, values of specific binding were plotted as described by Scatchard (30) to determine the number of DT-binding sites and the binding affinity of DTR/proHB-EGF for DT.

Chlorate and Heparitinase Treatments of Cells—Chlorate and heparitinase treatments were carried out as described previously (23). For treatment with chlorate, CHO cells were incubated for 48 h with Dulbecco's modified Eagle's medium containing 10% dialyzed bovine calf serum (Life Technologies, Inc.) and 30 mM sodium chlorate. For treatment with heparitinase, cells were washed with binding buffer, incubated for 1 h at 37 °C with 0.02 unit/ml heparitinase in the binding buffer. After washing twice with PBS (+) (PBS containing 0.2 mM CaCl2 and 0.2 mM MgCl2), DT binding to the treated cells was measured by 125I-DT-binding assay.

Surface Biotinylation and Immunoprecipitation of DTR—Cells grown in monolayers were labeled with NHS-LC-biotin, and the cell lysate was prepared as described previously (8). The cell lysate was incubated with 650 nmol DT in the presence or absence of 10 μM unlabeled DT for 6 h at 4 °C followed by incubation with anti-DT antibody-conjugated Sepharose as described previously (8). A part of the supernatant was treated with 0.02 unit/ml heparitinase for 1.5 h at 37 °C, then precipitated with DT and immobilized anti-DT antibody. Material recovered from the Sepharose gels was subjected to SDS-PAGE, transferred to an Immobilon membrane (Millipore Ltd., Tokyo, Japan), and blot in cell surface proteins in the precipitate were detected as described previously (8).

Binding of 125I-labeled DT to Recombinant HB-EGF—A 75-amino acid fragment of human recombinant HB-EGF (19) corresponding to residues 73–147 of DTR/proHB-EGF (9, 31) was coupled to CH-activated Sepharose gels. After washing twice with PBS containing 0.2 mM CaCl2 and 0.2 mM MgCl2, DT binding to the treated cells was measured by subtracting the radioactivity of a sample with an excess unlabeled DT from that of the sample without unlabeled DT. All measurements were made in duplicate.

RESULTS

Decreased DT Sensitivity of HS-deficient CHO Cells and Restoration of DT Sensitivity by Addition of HS or Heparin—A mutant of CHO cells, pgsD-677, lacks both N-acetylgalactosaminyltransferase and glucuronyltransferase, enzymes required for the polymerization of HS chain, and does not produce HSPG on the cell surface (32, 33). Flow cytometric analysis of 677 cells and K1 cells stained with anti-HS antibody verified that there are no detectable HS residues on the cell surface of 677 cells while a significant amount of HS was observed on the cell surface of K1 cells (Fig. 1). As shown in Fig. 2, the ED50 for inhibition of protein synthesis by DT for 677 cells, 230 ng/ml, was 15 times greater than for wild type cells, which had an ED50 of 15 ng/ml.

Binding of basic fibroblast growth factor (bFGF) to 677 cells is restored by addition of exogenous HS or heparin (20). As shown in Fig. 3A, DT sensitivity of the 677 cells was increased in a dose-dependent manner by addition of HS to the medium. At 100 μg/ml HS, the ED50 of 677 cells was about 10 ng/ml, which is similar to the value for wild type cells in the absence of HS. DT sensitivity of wild type cells was not increased at low concentrations of HS (0.1–1.0 μg/ml), but was slightly increased at high concentrations (10–100 μg/ml) (Fig. 3B). The sensitivity of 677 cells to DT was also increased by addition of heparin (Fig. 3C). The ED50 for 677 cells at 0.1, 1, and 10 μg/ml heparin was 60, 28, and 24 ng/ml, respectively, while ED50 in the absence of heparin was 180 ng/ml. Heparin in high concentration (10–100 μg/ml) increased DT sensitivity of the wild type cells as well, but the effect of heparin was less in K1 cells than in 677 cells (Fig. 3D). Heparin was more effective than HS for both cell lines, as was reported for bFGF (34, 35). The fact that the addition of HS or heparin restores DT sensitivity in 677 cells indicates that the decreased sensitivity of 677 cells is due to the absence of HSPG in this mutant cells and that heparin-like molecules are involved in determining DT sensitivity.
Other related glycosaminoglycans (GAG) were tested. N-Desulfated heparin, chondroitin sulfate A, chondroitin sulfate C, and hyaluronic acid did not increase DT sensitivity, but dermatan sulfate was as effective as heparin (data not shown), as was previously observed for FGF (35, 36). These results suggest that both the saccharide core structure and the level of sulfation influence the enhancing effect.

Heparin Potentiation of DT Binding to 677 Cells—To examine whether the effect of heparin and HS on DT sensitivity is due to an increase of DT binding or some other mechanism, binding of 125I-labeled DT to K1 cells and 677 cells was studied in the presence or absence of heparin. Because specific binding of DT to CHO-K1 cells is too low to detect (37), we transfected wild type CHO-K1 cells and mutant 677 cells with human DTR/proHB-EGF cDNA. K1H cells and 677H cells, stable transfectants expressing human HB-EGF, were used for DT-binding experiments. Specific binding of 125I-DT to 677H cells was clearly observed without heparin. Fig. 4A shows that binding of 125I-DT to 677H cells is much higher in the presence of 0.1 μg/ml heparin, whereas binding to K1H cells was increased 2.3-fold. The effect of heparin on DT binding is well correlated with the effect on DT sensitivity, indicating that the decreased DT sensitivity of 677 cells is due to reduced binding of DT to DTR/HB-EGF in the absence of heparin or HS molecules.

Effect of Chlorate Treatment on DT Binding—Chlorate is an inhibitor of sulfation. The requirement for heparin-like molecules for binding of DT was further studied by treating cells with chlorate to reduce the amount of cell surface HS residues. K1H cells and 677H cells were incubated with chlorate for 48 h, then the specific binding of 125I-DT to the cells was measured. In K1H cells, the chlorate treatment diminished the binding of 125I-DT to 40% of the value for untreated controls (Fig. 5). When heparin was added to the medium, binding of 125I-DT to chlorate-treated cells was restored. In the presence of heparin binding of DT to both chlorate-treated cells and untreated cells was about 1.5 times higher than that observed with untreated cells.
cells without heparin. Binding of similar amounts of DT to both chlorate-treated cells and untreated cells in the presence of chlorate rules out the possibility that chlorate treatment diminished the amount of DTR/proHB-EGF. In the case of 677H cells, binding of DT was not affected by chlorate treatment, which is consistent with the HSPG deficiency of 677 cells. We concluded that heparin-like molecules are involved in DT binding at least in CHO cells, and that DT sensitivity of 677 cells is reduced due to the deficiency in HSPG.

Requirement for Heparin-like Molecules for DT Binding to Vero Cells—To examine whether heparin-dependent DT binding is a general property of DTR/proHB-EGF or specific to CHO cells, the requirement of heparin-like molecules for binding of DT to Vero cells was examined. Addition of heparin up to 50 mg/ml did not enhance DT sensitivity (data not shown) or DT binding (Fig. 6A). Flow cytometric analysis of cells stained with anti-HS antibody showed that the amount of HS-GAG on Vero cells was about 80 times higher than the amount on CHO-K1 cells (Fig. 1).

When cell surface HS-GAG was diminished by treatment of Vero cells with heparitinase, heparin-dependent binding was observed. Heparitinase treatment greatly diminished cell surface HS contents, but did not affect the amount of DTR/proHB-EGF (data not shown). Under these conditions, binding of 125I-DT to heparitinase-treated Vero cells was about 50% of the

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**Fig. 4.** Heparin enhancement of binding of DT to DTR/proHB-EGF. A, binding of DT to 677H cells in the presence or absence of heparin. 677H cells were incubated with various concentrations of 125I-DT in the presence (closed squares) or absence (closed circles) of heparin (10 μg/ml) for 15 h at 4 °C. Data are expressed as specific binding. Similar results were obtained in three separate experiments. B, comparison of heparin dependence on DT binding of 677H cell to K1H cells. 677H (closed circles) and K1H cells (open circles) were incubated with 125I-DT (100 ng/ml) in the presence of various concentrations of heparin for 15 h at 4 °C. Then cells were washed, and the radioactivity associated with the cells was determined. Data are expressed as relative values compared to controls without heparin. In these experiments nonspecific binding of 125I-DT was <20% of the total binding.

**Fig. 5.** Effect of chlorate on DT binding to CHO cells. K1H cells and 677H cells were incubated with or without 30 mM chlorate for 48 h as described under "Experimental Procedures." Then DT binding was determined by incubation with 125I-DT (100 ng/ml) in the presence or absence of heparin (10 μg/ml) for 15 h at 4 °C. Data are expressed as specific binding and shown as means ± S.E. of three independent experiments. Open bar, untreated cells; closed bar, heparitinase-treated cells; hatched bar, untreated cells with heparin; dotted bar, heparitinase-treated cells with heparin. Nonspecific binding was <5% of the total binding.

**Fig. 6.** Involvement of heparin-like molecules in DT binding to Vero cells. A, effect of heparitinase on DT binding to intact Vero cells. Vero cells were incubated with or without heparitinase (0.02 unit/ml) for 1.5 h at 37 °C. Then DT binding to Vero cells was measured by incubation with 125I-DT (100 ng/ml) for 15 h at 4 °C in the presence or absence of heparin (10 μg/ml). Data are expressed as specific binding and shown as means ± S.E. of three independent experiments. Open bar, untreated cells; closed bar, heparitinase-treated cells; hatched bar, untreated cells with heparin; dotted bar, heparitinase-treated cells with heparin. Nonspecific binding was <5% of the total binding. B, precipitation of DTR/proHB-EGF with DT and anti-DT antibody from VeroH cell lysates. Surface-biotinylated VeroH cells were solubilized and incubated with or without heparitinase (0.02 unit/ml) for 1.5 h at 37 °C. Then DTR/proHB-EGF was precipitated with DT and immobilized anti-DT antibody in the presence or absence of heparin (10 μg/ml). Precipitated material was subjected to SDS-PAGE, followed by Western blotting with streptavidin-horseradish peroxidase.
binding to untreated cells. Binding was fully restored by addition of exogenous heparin (Fig. 6A). Heparin-dependent binding of DT was also observed in Vero cells lysates. VeroH cells, Vero cells overexpressing DTR/proHB-EGF, were surface-biotinylated and lysed. The lysate was precipitated with DT and immobilized anti-DT antibody in the presence or absence of exogenous heparin. The precipitated materials were analyzed by SDS-PAGE. Similar species of HB-EGF/DTR with molecular masses of 21–28 kDa were precipitated from the cell lysate in the presence or absence of heparin, but much more DTR/proHB-EGF was precipitated in the presence of heparin (Fig. 6B). Furthermore, when cell lysate was first treated with heparitinase and then precipitated with DT and anti-DT antibody, the amount of DTR/proHB-EGF precipitated was greatly diminished. Addition of heparin to heparitinase-treated cell lysate restored the precipitation efficiency. These results indicate that heparin-like molecules are involved in DT binding by Vero cells.

Heparin and DRAP27/CD9 Potentiate DT Binding by Independent Mechanism—DRAP27/CD9 is known to enhance DT binding activity in the presence of DTR/proHB-EGF (8, 16), and we showed in this study that heparin-like molecules are required for maximal DT binding. To determine whether heparin and DRAP27/CD9 potentiate DT binding by independent mechanisms, we examined the effect of heparin on the binding of DT to LH cells and LCH cells. LH cells are stable transfectants of mouse L cells expressing mouse L cells expressing human DTR/proHB-EGF alone, and LCH cells are stable transfectants of mouse L cells expressing both DTR/proHB-EGF and DRAP27/CD9 (8). LH cells and LCH cells express similar amounts of DTR/proHB-EGF and DRAP27/CD9 on the cell surface, but LCH cells bind DT 15–30 times more than LH cells due to the enhancing effect of DRAP27/CD9. The gel was incubated with 125I-DT (25 ng/ml) in the presence or absence of heparin (Fig. 7B). Scatchard plot analysis showed that the K_d values in presence of heparin was 7.7 × 10^8 M⁻¹, compared to 2.6 × 10^8 M⁻¹ in the absence of heparin. B_max values were similar with or without heparin, indicating that heparin potentiates DT binding activity of HB-EGF by increasing the affinity and of DTR/proHB-EGF for DT rather than by increasing the number of binding sites.

Heparin Enhances Binding of Diphtheria Toxin—A human recombinant HB-EGF corresponding to residues 73–147 of the DTR/proHB-EGF precursor was immobilized on CH-activated Sepharose 4B gels, and binding of 125I-DT to the Sepharose gels was measured in the presence of increasing concentrations of heparin. Fig. 7A shows that heparin-dependent binding was observed with the immobilized HB-EGF as well. Saturation binding experiments were also performed with increasing concentrations of 125I-DT in the presence or absence of heparin (Fig. 7B). Scatchard plot analysis showed that the K_d values for LH cell in presence of heparin was 7.7 × 10^8 M⁻¹, compared to 2.6 × 10^8 M⁻¹ in the absence of heparin. B_max values for LH cell were 1.5 and 0.56 × 10^9 M⁻¹, respectively, while the K_d values for LH cells in the presence or absence of heparin were 1.5 × 10^9 M⁻¹ and 0.56 × 10^9 M⁻¹. DRAP27/CD9 increased DT-binding site numbers rather than changing the affinity (8, 17). The difference in the affinities for DT of the LH cells and LCH cells used here is probably due to clonal variations. We conclude that DRAP27/CD9 and heparin potentiate DT binding to DTR/proHB-EGF by independent mechanisms.

Heparin Does Not Directly Interact with DT—Heparin has a strong affinity for heparin and HS (18, 19), suggesting that the effect of heparin on the binding of DT is due to the interaction of heparin with DTR/proHB-EGF. Although there is no reason to expect direct interaction between DT and heparin, the possibility that heparin also interacts with DT and
modifies directly the receptor-binding property of DT was re-
mained. To examine whether DT itself binds heparin, 125I-DT
was incubated with heparin-Sepharose and binding of 125I-DT
to heparin-Sepharose gels was measured. As shown in Table I,
only 1.1% of the radioactivity of 125I-DT added remained to
heparin-Sepharose gels in normal saline, and this value was
almost similar to that obtained by using control Sepharose gels.
When 125I-DT was incubated with heparin-Sepharose gels in
the presence of the recombinant HB-EGF, more than 34% of DT
added was adsorbed to heparin-Sepharose gels. These results
dearly indicate that DT binds to heparin-Sepharose in the
presence of HB-EGF but does not directly interact with
heparin.

**DISCUSSION**

DT specifically binds to the diphtheria toxin receptor, the
DTR/proHB-EGF molecule. Binding of DT to the receptor is one
of the key steps in the intoxication process, and the sensitivity
of cells to DT is primarily determined by the number of recep-
tors (3, 37, 38). Our recent studies revealed, however, that a
second factor, in addition to DTR/proHB-EGF itself, is required
for maximal DT binding activity. The membrane protein
DRAP27/CD9 up-regulates DT binding and DT sensitivity as a
co-factor of DTR/proHB-EGF (8, 15, 16). We showed here that
heparin-like molecules, probably in the form of HSPG on the
cell surface, are a third factor required for maximal DT binding
d and DT sensitivity. The requirement for heparin-like molecules
for full DT binding activity is indicated by the following evi-
dence: (i) HS-deficient CHO mutant 677 cells are less sensitive
to DT than the wild type CHO-K1 cells, and the decreased DT
sensitivity is restored by the addition of free HS or heparin; (ii)
677H cells bind 125I-DT in a heparin-dependent manner; (iii)
treatment with chlorate or heparitinase to diminish cell sur-
f ace HS decreases the binding of DT; (iv) binding of 125I-DT to
a recombinant HB-EGF in vitro is heparin-dependent. We pro-
pose that the heparin-like molecules responsible for enhancing
DT binding probably exist as HSPG rather than as proteogly-
cans with dermatan sulfate or free heparin, because DT bind-
ing activity of Vero cells and Vero cell lysates was greatly
diminished by treatment with heparitinase, which hydrolyses
HS-GAG, but not dermatan sulfate or heparin.

Increased binding of DT after addition of free heparin or HS
was most striking in HS-deficient 677 cells, but was also ob-
served to a lesser extent in CHO-K1 cells and L cells expressing
human HB-EGF (LH and LCH cells). No increase of DT binding
was observed when heparin was added to Vero cells. The dif-
ference in heparin dependence among cell lines can be ex-
plained by differences in the amount of cell surface HS-GAG.
The amount of HS on the surface of Vero cells is about 70 times
higher than on CHO-K1 cells and about 35 times higher than
on L cells. The amount of HS on CHO-K1 cells and L cells does
not appear to be sufficient for interaction with all of the DTR/
proHB-EGF, thus exogenous heparin or HS increases the bind-
ing of DT to these cells, although the effect is less than that
seen with 677 cells. Since DTR/proHB-EGF molecules are over-
expressed in LH and LCH cells, much more HSPG may be
required for maximal DT binding by these cells, making the

**FIG. 8.** Binding of 125I-DT to LH cells and LCH cells in the
presence or absence of heparin. A, LH (open symbols) and LCH cells
(closed symbols) were incubated for 15 h at 4 °C with binding medium
containing various concentrations of 125I-DT in the presence (squares)
or absence (circles) of heparin (10 μg/ml). Cell-associated radioactivity
was determined. Data are expressed as specific binding. B, Scatchard
plot of the specific binding of 125I-DT to LH cells in the presence
(squares) or absence (circles) of heparin. C, Scatchard plot of the specific
binding of 125I-DT to LCH cells in the presence (closed squares)
or absence (closed circles) of heparin. Nonspecific binding of 125I-DT was
<10% of the total binding.

**Table I**

| Gel          | Recombinant HB-EGF | 125I-DT bound* |
|--------------|--------------------|----------------|
|              |                    |cpm            | %             |
| Sepharose    | –                  | 100 ± 15       | 0.2 ± 0.2     |
|              | +                  | 517 ± 201      | 1.0 ± 0.4     |
| Heparin-Sepharose | –          | 582 ± 153      | 1.1 ± 0.3     |
|              | +                  | 17,069 ± 2,685 | 33.6 ± 5.3    |

*a Means ± SD of triplicate samples.*
heparin dependence more apparent. Vero cells contain abundant HS on the cell surface, and exogenous heparin is not required for maximal binding. However, when cell surface HS is removed by the treatment with heparitinase, or when the cell membrane is solubilized, Vero cells show heparin-dependent binding of DT.

How is the binding of DT to the cells increased by heparin-like molecules? DTR/proHB-EGF, like mature HB-EGF, binds heparin tightly, while DT itself is not bound to heparin-Sepharose (Table I). Therefore, the effect of heparin on the binding of DT must result from the interaction of heparin with DTR/proHB-EGF molecules. Highly cationic amino acids clusters located within and upstream of the EGF-like domain of HB-EGF form the putative heparin-binding domain (19). We have previously shown that DT binds to the EGF-like domain of DTR/proHB-EGF molecule. Because cells expressing a deletion mutant of DTR/proHB-EGF, HΔ (63–105) that lacks most of the heparin-binding domain still bind DT (14), it is likely that the heparin-binding domain modulates the binding activity for DT rather than forming the binding site itself. Therefore, we propose that binding of heparin, or HSPG, to the heparin-binding domain brings about a conformational change in the DTR/proHB-EGF molecule, resulting in increased affinity for DT by an allosteric effect, similar to the induced-fit model shown originally in the interaction of bFGF with heparin (20). Heparin and DRAP27/CD9 potentiate the binding of DT by different mechanisms. Heparin increases the affinity of DTR/proHB-EGF for DT but does not change the number of binding sites, while DRAP27/CD9 increases the number of DT-binding sites but does not change the Ks value (8, 17). The Ks for DT of L cells expressing both DTR/proHB-EGF and DRAP27/CD9 for DT is still lower than that of Vero cells (8, 17). We showed here that Ks values of LH cells and LCH cells for DT in the presence of heparin are similar to that of Vero cells. Therefore, the lower abundance of heparin-like molecules on the cell surface is likely to be responsible for the lower affinity of L cells.

We propose a model for the role of HSPG and DRAP27/CD9 in the binding of DTR/proHB-EGF molecule with DT (Fig. 9). Although the precise mechanism for the enhancing effect of DRAP27/CD9 is not clear, we speculate that DTR/proHB-EGF alone may not be stably retained on the cell surface. Association of DRAP27/CD9 with DTR/proHB-EGF may stabilize DTR/proHB-EGF, and thus DRAP27/CD9 would up-regulate DT binding and mitogenic activity. Cell surface HSPG, or exogenous heparin, binds to the heparin-binding domain of DTR/proHB-EGF, and changes the conformation of the EGF-like domain, resulting in increased affinity for DT.

The present studies demonstrate that full DT binding activity is achieved in the presence of heparin-like molecules. DT sensitivity is higher at low cell densities than under confluent conditions (39), indicating that close contacts between cells are not required for the maximal DT sensitivity. This implies that DTR/proHB-EGF on the cell surface can interact with its HSPG on the same cell. We showed recently that the complex comprising DTR/proHB-EGF and DRAP27/CD9 associates with integrin α3β1 as well (40). DTR/proHB-EGF may form a large functional complex including DRAP27/CD9, integrin α3β1 and HSPG. Although present study does not define whether a specific class of HSPG is involved in the interaction with DTR/proHB-EGF, studies of specificity of HSPG interacting with DTR/proHB-EGF may provide further understanding of the physiological role of DTR/proHB-EGF as a membrane-anchored growth factor, as well as the DT receptor.

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FIG. 9. Proposed model for the roles of DRAP27/CD9 and heparin-like molecules in binding of DT to DTR/proHB-EGF. a, DTR/proHB-EGF alone in the plasma membrane is not available for DT-binding. b, DRAP27/CD9 binds to orients DTR/proHB-EGF, making it accessible to DT. c, cell surface HSPGs or free heparin bind to DTR/proHB-EGF at the heparin-binding domain, and induce a conformational change that results in increased affinity of DTR/proHB-EGF molecule for DT. d, the DTR/proHB-EGF/DRAP27/CD9/HSPG-DT complex.
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