Diphtheria toxin (DT) binding to the epidermal growth factor (EGF)-like domain of human membrane-anchored heparin-binding EGF-like growth factor (proHB-EGF), the human DT receptor (DTR). DT does not bind to mouse proHB-EGF because of amino acid substitutions within the EGF-like domain. We made 10 independent mutants, replacing a single amino acid within the EGF-like domain of human DTR/proHB-EGF with the corresponding amino acid residue in mouse proHB-EGF. The mutant proteins were transiently expressed in mouse L cells either expressing or not expressing DRAP27/CD9, and DT binding was measured. DT binding activity of GST fusion proteins containing the mutated EGF-like domain was also determined by a cell-free binding assay. The largest effect was seen with E141H, and second largest effects were seen with F115Y and L127F in all of the assay systems. We conclude that Phe115, Leu127, and Glu141 are critical amino acid residues for DT binding activity, Phe115, Leu127, and Glu141, are all located on the same face of the EGF-like domain, suggesting that this face of DTR/proHB-EGF interacts with the receptor-binding domain of DT.

Diphtheria toxin (DT) (1) \((M_r = 58,342)\) is secreted by toxigenic strains of Corynebacterium diphtheriae (for reviews, see Refs. 1 and 2). DT inhibits cellular protein synthesis in eukaryotes by ADP-ribosylating elongation factor 2 through ADP-ribosylation. DT is composed of two fragments, A and B, which are covalently linked by a disulfide bond. The crystal structure of DT reveals that DT is actually composed of three distinct domains, the catalytic domain (C-domain), which is A-fragment itself, the transmembrane domain (T-domain), which is essential for the enzymatically active C-domain is translocated to the cytosol (15). The DTR is the principal protein involved in binding DT to cells, and the DT sensitivity of cells is determined primarily by the presence or absence of DTR (16). DTR was purified from monkey Vero cells (17, 18), one of the cell lines most sensitive to DT (19). A cDNA encoding DTR was cloned from this cell line (20). DTR is identical to the precursor form of heparin-binding EGF-like growth factor (proHB-EGF) (20, 21) that was originally identified as the heparin-binding member of the epidermal growth factor family (22, 23). Although proHB-EGF is cleaved by an unidentified protease on the cell surface to yield the soluble mature growth factor (HB-EGF) (24), a significant amount of proHB-EGF is left uncleaved on the cell surface of many cell types, where it acts as a membrane-anchored growth factor (25) and as the DTR (21). It has also been shown that heparin and heparan sulfate proteoglycan binds to DTR/proHB-EGF and influences its DT binding activity (26).

DTR/proHB-EGF exists on the cell surface as a complex with a tetramembrane-spanning protein DRAP27/CD9 (21, 27, 28). DRAP27/CD9 itself does not bind DT, but it enhances the DT binding activity of DTR/proHB-EGF (21, 27, 29). DRAP27/CD9 also greatly up-regulates the juxtacrine mitogenic activity of DTR/proHB-EGF (25).

Although proHB-EGF is ubiquitously expressed in species including human, monkey, rat, and mouse (30), cells from mice and rats are resistant to DT (19, 31, 32). Transfection of human or mouse proHB-EGF cDNAs into mouse L cells revealed that mouse cells are insensitive to DT because mouse proHB-EGF does not bind the toxin (33). Use of human/mouse proHB-EGF chimeras demonstrated that DT binds to the EGF-like domain of human proHB-EGF but not to the EGF-like domain of mouse proHB-EGF (33). There are 10 amino acid differences between the EGF-like domain of human DTR/proHB-EGF and that of mouse proHB-EGF. Thus, mouse proHB-EGF is a natural mutant form of DTR. These nonconserved amino acid residues are good candidates for site-directed mutagenesis to study the relationship of proHB-EGF primary structure to its function as the DTR.

We introduced several mutations within the coding region for the EGF-like domain of human DTR/proHB-EGF and identified amino acid residues required for DT binding activity. A computer model of the tertiary structure of the human EGF-
like domain of HB-EGF, based on the NMR structure of TGF-α (34), was made to examine the spatial arrangement of residues involved in DT binding.

EXPERIMENTAL PROCEDURES

Materials—DT was prepared as described previously (6). Rabbit anti-human proHB-EGF antibody (H-6) was produced as described previously (21) and affinity-purified using a synthetic peptide corresponding to amino acid residues 54–73 of human DTR/proHB-EGF conjugated to Sepharose CL-6B (Pharmacia Biotech Inc., Tokyo, Japan). Affinity-purified goat anti-rabbit IgG antibody was purchased from Organon Teknika Corp., Durham, NC.

Plasmid Constructions—All point mutations to alter amino acid residues were introduced into pTHG-1, which contains the entire human DTR/proHB-EGF coding region (33), by site-directed mutagenesis using an in vitro mutagenesis kit (Amersham, Ltd., Tokyo, Japan). Four chimeric plasmids containing multiple substitutions were constructed as follows. Three plasmids, pTMHG-1, pTMHGDS-1, and pTMHGDK-1, which encode mouse proHB-EGF, human/mouse HB-EGF chimera H(106–136), and human/mouse HB-EGF chimera H(106–186), respectively (33), were digested with PvuII and BalI, and the PvuII-BalI fragment of human DTR/proHB-EGF cDNA was inserted. This segment encodes the recognition site for anti-human proHB-EGF antibody (H-6) (21), which can be used as an immunological tag to measure the surface expression of proHB-EGF. The resulting plasmids were named pTMHG-1tag, pTMHGDS-1tag, and pTMHGDK-1tag, respectively. Two plasmids, pTMHGDS-1tag and pTMHGDK-1tag, were digested either with DraII and SaeI or with SaeI and SalI then ligated with synthetic DNAs encoding the corresponding regions of mouse proHB-EGF, except for Phe127. Compared with human proHB-EGF, the resulting plasmids have four types of multiple mutations within the EGF-like domain (Fig. 1). For each of the mutant proteins constructed for this study, the amino acid substitutions are indicated. Restriction enzyme sites used for constructing the chimeric plasmids are also indicated. pre, signal sequence; pro, pro domain; HBD, heparin-binding domain; TM, transmembrane domain; CM, cytoplasmic domain.

FIG. 1. Structures of human DTR/proHB-EGF (hHB-EGF), mouse proHB-EGF (mHB-EGF), and the human DTR/proHB-EGF mutants constructed for the study. A schematic structure of proHB-EGF and the human DTR/proHB-EGF mutants is shown at the top of the figure. Alignment of the amino acid sequences of the EGF-like domains of human and mouse proHB-EGF are also shown with only the residues that differ in mouse HB-EGF indicated. For each of the different mutant proteins constructed for this study, the amino acid substitutions are indicated. Restriction enzyme sites used for constructing the chimeric plasmids are also indicated. pre, signal sequence; pro, pro domain; HBD, heparin-binding domain; TM, transmembrane domain; CM, cytoplasmic domain.

FIG. 2. Linearity of the assay for cell surface proHB-EGF. Human DTR/proHB-EGF cDNA was transfected into LC cells. After incubation for 1 day, transfected cells were detached from dishes and mixed with untransfected LC cells at various ratios, and samples of 1 x 10^5 cells were replated on new dishes. After further incubation for 1 day, the amount of proHB-EGF molecules on the cell surface was determined using anti-HB-EGF antibody and 125I-labeled secondary antibody as described under “Experimental Procedures.” The cell-associated radioactivity of the secondary antibody was plotted against the number of transfected cells (•). The amount of DT bound to the cells was concurrently determined (○).

as follows. DNA fragments encoding the EGF-like domain, Asp^{106–Pro}^{149}, were amplified from plasmids for wild-type human DTR/proHB-EGF and single mutants using the polymerase chain reaction. The
The amounts of proHB-EGF expressed on the cell surface and the amounts of DT bound to the cells were determined as described under “Experimental Procedures” by using 1 × 10⁶ transfected cells and 1 × 10⁵ transfected cells, respectively. The amounts of DT bound to the cells were calculated from the values of specific binding of [¹²⁵I]-DT. The DT binding activity is expressed as the calculated value B/A. Average DT binding activity was calculated from data of three experiments. Standard deviations of the DT binding activity are shown in parentheses.

### Table I

| Protein | proHB-EGF on the cell surface | [¹²⁵I]-DT bound to the cells | DT binding activity B/A | Average DT binding activity | Relative DT binding activity % WT |
|---------|-------------------------------|-----------------------------|--------------------------|-------------------------------|----------------------------------|
| Human proHB-EGF (WT) | 48.1 | 4.12 | 0.086 | 0.089 (0.0042) | 100 |
| | 50.0 | 4.28 | 0.086 | 42.2 | 3.99 | 0.095 |
| | 50.5 | 0.58 | 0.012 | 50.0 | 0.57 | 0.011 |
| | 48.3 | 0.57 | 0.012 | 33.7 | 2.07 | 0.061 |
| | 35.3 | 2.17 | 0.062 | 36.3 | 2.13 | 0.059 |
| | 51.5 | 3.92 | 0.076 | 45.7 | 3.85 | 0.084 |
| | | 45.5 | 3.63 | 0.080 | 40.2 | 3.52 | 0.088 |
| | | 44.5 | 3.60 | 0.079 | 42.3 | 3.47 | 0.082 |
| | | 39.9 | 0.69 | 0.017 | 40.6 | 0.70 | 0.017 |
| | | 38.3 | 0.65 | 0.017 | 42.9 | 2.72 | 0.097 |
| | | | 25.6 | 2.66 | 0.104 |
| | | | 28.6 | 2.68 | 0.094 |
| | | 41.8 | 1.29 | 0.031 | 39.1 | 1.27 | 0.032 |
| | | 36.0 | 1.21 | 0.034 | 33.9 | 1.50 | 0.044 |
| | | 33.6 | 1.52 | 0.045 | 36.5 | 1.57 | 0.043 |
| | | | 33.1 | 2.53 | 0.076 |
| | | | 33.1 | 2.55 | 0.077 |
| | | 28.0 | 0 | 0 | 0 |
| | | 29.3 | 0 | 0 | 0 |
| | | 27.8 | 0 | 0 | 0 |

The purities of all the fusion proteins were estimated to be about 90% by SDS-polyacrylamide gel electrophoresis. For binding assays, glutathione-Sepharose 4B (Pharmacia) according to the manufacturer’s instruction. The purities of all the fusion proteins were estimated to be about 90% by SDS-polyacrylamide gel electrophoresis. For binding assays, glutathione-Sepharose 4B (Pharmacia) according to the manufacturer’s instruction. The purities of all the fusion proteins were estimated to be about 90% by SDS-polyacrylamide gel electrophoresis. For binding assays, glutathione-Sepharose 4B (Pharmacia) according to the manufacturer’s instruction. The purities of all the fusion proteins were estimated to be about 90% by SDS-polyacrylamide gel electrophoresis. For binding assays, glutathione-Sepharose 4B (Pharmacia) according to the manufacturer’s instruction. The purities of all the fusion proteins were estimated to be about 90% by SDS-polyacrylamide gel electrophoresis. For binding assays, glutathione-Sepharose 4B (Pharmacia) according to the manufacturer’s instruction. The purities of all the fusion proteins were estimated to be about 90% by SDS-polyacrylamide gel electrophoresis. For binding assays, glutathione-Sepharose 4B (Pharmacia) according to the manufacturer’s instruction. The purities of all the fusion proteins were estimated to be about 90% by SDS-polyacrylamide gel electrophoresis. For binding assays, glutathione-Sepharose 4B (Pharmacia) according to the manufacturer’s instruction. The purities of all the fusion proteins were estimated to be about 90% by SDS-polyacrylamide gel electrophoresis. For binding assays, glutathione-Sepharose 4B (Pharmacia) according to the manufacturer’s instruction. The purities of all the fusion proteins were estimated to be about 90% by SDS-polyacrylamide gel electrophoresis. For binding assays, glutathione-Sepharose 4B (Pharmacia) according to the manufacturer’s instruction. The purities of all the fusion proteins were estimated to be about 90% by SDS-polyacrylamide gel electrophoresis. For binding assays, glutathione-Sepharose 4B (Pharmacia) according to the manufacturer’s instruction. The purities of all the fusion proteins were estimated to be about 90% by SDS-polyacrylamide gel electrophoresis. For binding assays, glutathione-Sepharose 4B (Pharmacia) according to the manufacturer’s instruction.
Surface—L cells or LC cells transiently transfected with various mutant plasmids were incubated at 4 °C for 2 h with 5 μg/ml H-6 antibody in binding medium (Dulbecco's modified Eagle's medium containing nonessential amino acids, 20 mM HEPES-NaOH (pH 7.2) supplemented with 10% calf serum). Cells were washed with chilled washing buffer (phosphate-buffered saline supplemented with 0.5 mM CaCl₂, 0.5 mM MgCl₂, 5 mM NaN₃, and 1% calf serum) three times and then incubated at 4 °C for 2 h with 1 μg/ml ¹²⁵I-goat anti-rabbit IgG antibody in binding medium. Finally, cells were washed with washing buffer three times and the cell-associated radioactivity was counted.

Computer Modeling of HB-EGF—Computer modeling was interactively performed using the Homology program (Biosym Technologies, San Diego, CA). Side chains of the minimized average structure of TGF-α (34) were replaced with those of corresponding residues in human HB-EGF. Conformations of side chains were selected from a rotamer library (36, 37), avoiding severe steric repulsion with other parts of the molecule. Amino acid residues that were identical in the two amino acid sequences were not changed. The model was bathed in 1723 TIP-3P water (38). The whole system was fully energy-minimized by program AMBER 4.1 (39) with dielectric constant = 1, cut-off of nonbonded interaction = 12 Å, and a truncated octahedral periodic boundary condition applied to generate the final model of human HB-EGF.

### RESULTS AND DISCUSSION

The 10 independent mutants of human DTR/proHB-EGF with substitutions of single amino acid residues from the sequence of mouse proHB-EGF and three chimeric proteins with multiple substitutions studied here are shown in Fig. 1. The mutant proteins, wild-type human DTR/proHB-EGF, and mouse proHB-EGF were transiently expressed in recipient cells, and the DT binding activity of the cells was measured. Both mouse L cells and LC cells were used as the recipient cells. L cells do not express DRAP27/CD9 (25); thus effects of the amino acid alternations on DT binding could be observed independently of effects on the interaction of DTR/proHB-EGF with DRAP27/CD9. The LC cell line is a stable transfectant of mouse L cells that express DRAP27/CD9. Using this cell line allows the effects of the amino acid alternations to be observed under conditions in which DTR can interact with DRAP27/CD9.

### Table II

**DT binding activity of mutant diphtheria toxin receptor proteins expressed in LC cells**

| Protein | proHB-EGF on the cell surface A | ¹²⁵I-DT bound to the cells B | DT binding activity B/A | Average DT binding activity | Relative DT binding activity | Enhancement of DT binding |
|---------|-------------------------------|-----------------------------|-------------------------|-----------------------------|-----------------------------|-----------------------------|
| Human proHB-EGF (WT) | 4.02 | 1.37 | 0.34 | 0.340 (0.000) | 100 | 3.8 |
| | 4.38 | | 1.49 | 0.34 | | |
| F115Y | 3.76 | 0.79 | 0.21 | 0.220 (0.010) | 65 | 18 |
| | 3.05 | 0.70 | 0.23 | | | |
| K122R | 4.04 | 1.19 | 0.29 | 0.320 (0.030) | 94 | 5.2 |
| | 2.55 | 0.88 | 0.35 | | | |
| V124L | 3.62 | 1.03 | 0.28 | 0.310 (0.010) | 91 | 3.9 |
| | 2.34 | 0.79 | 0.34 | | | |
| K125Q | 4.06 | 1.38 | 0.34 | 0.355 (0.015) | 104 | 4.3 |
| | 2.95 | 1.09 | 0.37 | | | |
| L127F | 3.48 | 0.78 | 0.22 | 0.230 (0.010) | 68 | 14 |
| | 2.75 | 0.67 | 0.24 | | | |
| A129T | 2.94 | 1.08 | 0.37 | 0.335 (0.035) | 99 | 3.4 |
| | 1.77 | 0.53 | 0.30 | | | |
| I133K | 4.04 | 1.17 | 0.29 | 0.315 (0.025) | 93 | 9.8 |
| | 2.97 | 1.00 | 0.34 | | | |
| H135L | 1.80 | 0.59 | 0.33 | 0.350 (0.020) | 103 | 8.0 |
| | 3.20 | 1.19 | 0.37 | | | |
| E141H | 2.18 | 0.06 | 0.03 | 0.030 (0.000) | 8.8 | — |
| | 3.09 | 0.08 | 0.03 | | | |
| S147T | 2.00 | 0.63 | 0.32 | 0.325 (0.005) | 96 | 4.3 |
| | 3.79 | 1.24 | 0.33 | | | |
| A129T/I133K/ H135L | 1.81 | 0.42 | 0.23 | 0.210 (0.020) | 62 | ND |
| | 1.95 | 0.37 | 0.19 | | | |
| F115Y/K122R/ V124L/K125Q/ E141H/S147T | 1.87 | 0.02 | 0.01 | 0.010 (0.000) | 2.9 | ND |
| | 3.08 | 0.03 | 0.01 | | | |
| A129T/I133K/ H135L/E141H/ S147T | 1.91 | 0.01 | 0.005 | 0.0065 | 1.9 | ND |
| | 2.64 | 0.02 | 0.008 | (0.0015) | | |
| F115Y/K122R/ V124L/K125Q/ L127F/A129T/ I133K/H135L/ E141H/S147T (mouse proHB-EGF) | 2.24 | <0.01 | <0.004 | <0.0045 | <1.5 | — |
| | 2.18 | <0.01 | <0.005 | | | |

Draper 27/CD9 (25); thus effects of the amino acid alternations on DT binding could be observed independently of effects on the interaction of DTR/proHB-EGF with DRAP27/CD9. The LC cell line is a stable transfectant of mouse L cells that express DRAP27/CD9. Using this cell line allows the effects of the amino acid alternations to be observed under conditions in which DTR can interact with DRAP27/CD9.

Although the transfection efficiency for the recipient cells
used in this study was usually 30–50%, the efficiency varied from experiment to experiment. Furthermore, the efficiency of DTR/proHB-EGF expression on the cell surface may differ among mutants. Therefore, to compare DT binding activities of the mutant proteins with that of wild type we normalized DT binding to the amount of proHB-EGF expressed on the cell surface. ProHB-EGF on the cell surface was measured for each set of transfected cells using an anti-human proHB-EGF antibody and $^{125}$I-labeled secondary antibody as described under “Experimental Procedures.” The anti-human proHB-EGF antibody used here was obtained by immunizing rabbits with a synthetic peptide corresponding to amino acids 54–73 of human DTR/proHB-EGF. This antibody reacts with all of the mutant proteins used.

Although it would be difficult to determine the absolute amounts of proHB-EGF by such an indirect method, for the present purpose only, the relative amounts of proHB-EGF on the cell surface must be determined. To verify that this method allows accurate measurement of the relative amount of proHB-EGF on the cell surface, we performed a model experiment. LC cells transfected with human DTR/proHB-EGF cDNA were mixed with untransfected LC cells at various ratios, and the cells were treated with anti-human proHB-EGF antibody and $^{125}$I-labeled secondary antibody. As shown in Fig. 2, the amount of $^{125}$I-labeled secondary antibody bound is proportional to the number of transfected cells added. Similar results were obtained using L cells as recipients (data not shown).

Table I shows the amount of proHB-EGF expressed on the cell surface and the amount of $^{125}$I-DT bound to the L cells transfected with each mutant plasmid. The DT binding activities, obtained by dividing the specific binding of DT by the amount of proHB-EGF expressed on the cell surface, are also shown. Among the 10 single mutants, the largest effect was seen with E141H, for which no DT binding activity was detected using this binding assay. The second largest effect was seen with F115Y and L127F, for which DT binding activity decreased by about 86 and 81%, respectively. Smaller effects were seen with K122R, I133K, H135L, and S147T, for which DT binding activity decreased by about 31, 64, 51, and 16%, respectively. The other three single mutants, V124L, K125Q, and A129T, did not show significant effects on DT binding activity.

DT binding activities of the mutant DTR/proHB-EGF proteins expressed in LC cells are shown in Table II. The E141H substitution also showed the largest effect on the DT binding in these cells, with DT binding activity decreased by about 91%. F115Y and L127F also showed reduced binding activity, but the other seven single mutants, K122R, V124L, K125Q, A129T, I133K, H135L, and S147T, did not have significant effects. Effects of combinations of substitutions were also studied. The multiple mutants F115Y/K122R/V124L/K125Q/E141H/S147T and A129K/I133K/H135L/E141H showed DT binding activity about 3 and 2% that of wild type, respectively. These values are lower than that of the single mutant E141H but higher than that of mouse proHB-EGF. The triple mutant, A129T/I133K/H135L, showed DT binding activity about 62% that of wild type despite the fact that single amino acid alternations in these three residues (Ala$^{129}$, Ile$^{133}$, and His$^{135}$) did not show negative effects. This indicates that amino acid substitutions whose influence on DT binding was not apparent by testing of individual substitutions may collectively have significant effects.

To assess the effect of each single amino acid alternation introduced in the EGF-like domain by a different system, GST fusion proteins containing wild-type and mutant forms of the EGF-like domain of human DTR/proHB-EGF were made, and DT binding activities were determined in a cell-free system (Table III). As in the transfection assays, DT binding activity was greatly reduced with E141H, and F115Y and L127F showed reductions of 51 and 68%. Smaller effects were seen with K122R, A129T, I133K, and H135L. The other single mutants, V124L, K125Q, and S147T, did not show significant effect on DT binding activity in this assay.

Both human and mouse HB-EGF bind to EGF-receptor and have mitogenic activity. Substitution of amino acid residues in the EGF-like domain of human DTR/proHB-EGF by the corresponding amino acid residues in the mouse protein is therefore not expected to result in an undesirable change in the overall structure of the EGF-like domain. However, this approach has a limitation for evaluating the effects of substitutions at different sites. In the case of F115Y, K122R, V124L, L127F, and S147T, the amino acid substitutions are relatively homologous amino acid residues, whereas the substitutions in K125Q, I133K, H135L, and E141H involve nonhomologous or oppositely charged amino acid residues. Generally, substitution with a nonhomologous or oppositely charged amino acid would influence the activity more strongly than substitution with a homologous amino acid. Thus the failure to observe an effect of the substitutions used in this study does not necessarily indicate that the site does not play a role in DT binding, and further analysis is necessary to clarify the contributions of such sites.

Nevertheless, the reduced binding activities of F115Y, L127F, and E141H are prominent among the mutants studied here. In all three DT binding assays, transfection into L cells or LC cells or binding of recombinant GST fusion proteins, the E141H substitution showed the largest effect, as indicated in earlier studies (33, 40). Reduced DT binding activity of F115Y and L127F was also observed in all three binding assays, even though both mutants involve substitution of a nonpolar amino acid residue by another residue of the same type. We conclude that these three substitutions are most critical for the loss of DT binding activity in mouse proHB-EGF.

DRAP27/CD9 associates with DTR/proHB-EGF and enhances its DT binding activity. When DT binding activities of the mutant DTR proteins expressed in L cells were compared with their binding activities in LC cells, enhancement of DT binding was observed for all of the single mutants in the presence of DRAP27/CD9 (compare Table I with Table II).
Thus, the single amino acid alternations introduced in this study do not block the enhancement DT binding by human DTR/proHB-EGF.

Although the tertiary structure of the EGF-like domain of HB-EGF has not been determined, the solution structures of the EGF-like domains of EGF and TGF-α were determined by NMR studies (34, 41, 42). All members of the EGF family of growth factors, including HB-EGF, have six conserved cysteine residues and several other conserved amino acids in their EGF-like domains. Furthermore, HB-EGF, EGF, and TGF-α all bind to the EGF receptor. Therefore, we expect the main chain folds of the EGF-like domain of HB-EGF to be similar to those of EGF and TGF-α. Because HB-EGF and TGF-α have the same number of the amino acid residues inside the EGF-like domain and EGF has one additional amino acid residue between the second and third cysteine residues, we chose TGF-α as the template structure for a computer model of the tertiary structure of the EGF-like domain of HB-EGF. Human HB-EGF and mouse HB-EGF bind to same EGF receptor, and mouse HB-EGF is as mitogenic for cells as human HB-EGF. Thus, the main chain structures of the EGF-like domain are not likely to be greatly affected by substitution of amino acid residues in the human DTR/proHB-EGF sequence with corresponding amino acid residues in mouse proHB-EGF.

Fig. 3 shows two views of the model in different orientations. In the model, the three amino acid residues critical for DT binding activity, Phe115, Leu127, and Glu141, are all located on the same face. We propose that the face containing Phe 115, Leu127, and Glu 141 is important for DT recognition by the EGF-like domain of DTR/proHB-EGF and that these residues play roles in DT binding. The other seven amino acid residues in the EGF-like domain that differ between human and mouse, except for Ala129, are located on other faces. Although Ala129 is on the same face as Phe115, Leu127, and Glu141, A129T did not show significantly decreased DT binding activity. Ala129 may not be involved in DT recognition or Ala129 may be involved in DT recognition, but the homologous substitution Thr for Ala may not have a significant effect.

Reduced DT binding activity was observed with K122R, I133K, and H135L in the absence of DRAP27/CD9 (Table I and III) but not in the presence of DRAP27/CD9 (Table II). The presence of DRAP27/CD9 may compensate for the negative effects of these mutants by interacting with DTR/proHB-EGF. The tertiary structure model predicts that amino acid residues Lys122, Ile133, and His135 are located on the face opposite the one containing Phe115, Leu127, and Glu141. Therefore, it is intriguing to speculate that DRAP27/CD9 interacts with the face on which Lys122, Ile133, and His135 are located and helps stabilize the structure of DTR/proHB-EGF, reducing the effects of the substitutions.

In human amphiregulin, amino acid residues Phe115, Leu127, and Glu141 are conserved, but DT does not bind to human amphiregulin (33). Whereas amino acid residues Phe115, Leu127, and Glu141 are important for the interaction of HB-EGF with DT, other residues within the EGF-like domain also affect DT recognition. Amino acid residues conserved in both human and mouse HB-EGF probably participate in DT recognition, especially amino acid residues located in the face including Phe115, Leu127, and Glu141.

Alanine replacements in DT indicated that residues Lys516 and Phe530, located within a loop of the R-domain of DT, are involved in DTR recognition (5). All three amino acid residues that we identified as critical for DT binding activity are located in a loop in the EGF-like domain of DTR/proHB-EGF and that these residues play roles in DT binding. It would be interesting to examine whether Lys516 and Phe530 of DT interact directly with Phe115, Leu127, or Glu141 of DTR/proHB-EGF. Further site-directed mutagenesis studies of DT and DTR/proHB-EGF can provide more precise understanding of DT-DTR interactions.

Another approach to the study of DT-DTR interaction would be structural analysis of the DT-DTR complex. An analysis of the crystal structure of DT-HB-EGF complex is under way by S. Choe and associates in the Salk Institute. Their results also...
suggest that Phe^{115}, Leu^{127}, and Glu^{141} of the DTR/proHB-EGF are located in the face interacting with DT.²

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