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A Diphtheria Toxin Receptor Deficient in Epidermal Growth Factor–Like Biological Activity

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Targeted cell ablation in animals is a powerful method for analyzing the physiological function of cell populations and generating various animal models of organ dysfunction. To achieve more specific and conditional ablation of target cells, we have developed a method termed Toxin Receptor mediated Cell Knockout (TRECK). A potential shortcoming of this method, however, is that overexpression of human heparin-binding epidermal growth factor–like growth factor (hHB-EGF) as a diphtheria toxin (DT) receptor in target cells or tissues may cause abnormalities in transgenic mice, since hHB-EGF is a member of the EGF growth factor family. To create novel DT receptors that are defective in growth factor activity and resistant to metalloprotease-cleavage, we mutated five amino acids in the extracellular EGF-like domain of hHB-EGF, which contains both DT-binding and protease-cleavage sites. Two of the resultant hHB-EGF mutants, I117A/L148V and I117V/L148V, possessed little growth factor activity but retained DT receptor activity. Furthermore, these mutants were resistant to metalloprotease-cleavage by 12-*O*-tetradecanoylphorbol-13-acetate stimulation, which is expected to enhance DT receptor activity. These novel DT receptors should be useful for the generation of transgenic mice by TRECK.

Key words: cell ablation, diphtheria toxin receptor, growth factor, HB-EGF, site-directed mutagenesis.

Abbreviations: ADAM, a disintegrin and metalloprotease; DT, diphtheria toxin; h, human; HA, haemagglutinin; HB-EGF, heparin-binding epidermal growth factor–like growth factor; IL-3, interleukin-3; IRES, internal ribosomal entry site; m, mouse; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; TRECK, toxin receptor–mediated cell knockout.

Ablation of specific cells or tissues in an animal model is a powerful method for analyzing the physiological function of cells or tissues. Moreover, targeted cell ablation is useful for generating animal models of specific organ dysfunction, which could be utilized in clinical investigations, such as stem cells transplantation. To accomplish the ablation of specific cells or tissues in animals, several methods have been established. One strategy is surgical disruption of the target cells. Another is based on genetic approaches, for example, expression of a cytotoxic gene such as diphtheria toxin fragment A (DT-A) under the control of a tissue-specific promoter in transgenic mice (*1*).

To achieve more specific and conditional ablation of target cells, we have developed a method termed Toxin Receptor mediated Cell Knockout (TRECK). In this method, we have used the human heparin-binding epidermal growth factor–like growth factor (hHB-EGF) as a diphtheria toxin (DT) receptor in the target cells of transgenic mice (*2*). DT binds to the extracellular EGF-like domain (*3*) of the membrane-anchored precursor form of HB-EGF on the cell surface of toxin-sensitive cells through the DT-B fragment, and is incorporated into the cells by receptor-mediated endocytosis. In the acidic conditions of the late endosome, DT-A is translocated into the cytosol and inactivates eukaryotic elongation factor 2 (eEF-2) through ADP-ribosylation. This induces the inhibition of protein synthesis leading to cell death. Though even a single molecule of DT-A introduced into the cytosol is capable of killing all eukaryotic cells (*4*), mouse and rat cells are less sensitive to DT (*5*). Because amino acid residues in the EGF-like domain of mouse HB-EGF (mHB-EGF) are different from those in hHB-EGF, DT can not bind the mHB-EGF (*3*). Therefore, intraperitoneal injection of even high doses of DT (50 μg/kg weight) causes no abnormalities in wild type mice. However, administration of a small amount of DT was observed to cause fulminant hepatitis in transgenic mice expressing the gene for the hHB-EGF under the control of the liver cell–specific albumin promoter (*2*).

However, HB-EGF is a member of the EGF family growth factor (*6*), and similar to EGF and other EGF family proteins, HB-EGF binds to and phosphorylates the EGF receptor (EGFR). HB-EGF is first synthesized as a membrane-anchored protein containing heparin-binding, EGF-like, transmembrane, and cytosolic domains.

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A membrane-anchored form of HB-EGF, which also acts as a DT receptor in toxin-sensitive cells, is cleaved on the cell surface by proteases of a disintegrin and metalloprotease (ADAM) family (*7*, *8*), resulting in the secretion of a soluble form of HB-EGF. The secreted soluble form of hHB-EGF has mitogenic activity against several cells, including mouse cells such as BALB-3T3 (*6*). The membrane-anchored form of HB-EGF is not only a precursor form of soluble HB-EGF, but is thought to possess biological activity and transduce the signals to neighboring cells in a membrane-associated manner (*9*). Thus, it is possible that the overexpression of hHB-EGF in specific cells or tissues might cause abnormalities in transgenic mice. Moreover, soluble hHB-EGF cannot serve as a DT receptor but might act as a neutralizing antibody against DT injected into transgenic mice. Therefore, we attempted to inactivate the EGF-like activity of hHB-EGF without abolishing DT receptor function.

Here, we show that the hHB-EGF mutant (I117A/L148V) possessed little growth factor activity, but still functioned as a DT receptor, although the DT sensitivity of retrovirally infected cells decreased about 10-fold compared to wild type. In addition, a second hHB-EGF mutant, (I117V/L148V), exhibited greatly reduced growth factor activity, and the DT sensitivity was almost similar to wild type. Furthermore these mutants showed resistance to 12-*O*-tetradecanoylphorbol-13-acetate (TPA) stimuli, which accelerates the cleavage of membrane-anchored HB-EGF (*10*). Therefore, these hHB-EGF mutants could be useful for making transgenic mice by TRECK.

MATERIALS AND METHODS

Plasmid Constructions—All point mutations to alter amino acid residues of hHB-EGF were introduced by site-directed mutagenesis using polymerase chain reaction (PCR) amplification as follows. The sequence of all PCR primers is summarized in the Table 1. In the first PCR steps, the plasmid pRcHBEGF (*11*) was amplified in two separate reactions using the following oligonucleotide primers, P1-*Bam*HI and M2, M1 and P2-*Eco*RI, respectively. Next, these two amplified fragments were mixed in the same tubes and amplified in a second PCR reaction using primers, P1-*Bam*HI and P2-*Eco*RI. The *Bam*HI–*Eco*RI full-length fragments generated by the second PCR steps were introduced into the pMX-IRES-GFP retroviral plasmid (*12*) kindly provided by T. Kitamura (The University of Tokyo). Double amino acids mutations were introduced by similar sequential PCR reactions. Enforced secreted-hHB-EGF constructs (1–148) were obtained by PCR amplification using P1-*Eco*RI and P2-148t-*Eco*RI or P2-L148Vt-*Eco*RI, and amplified fragments were inserted into *Eco*RI sites of a mammalian expression vector pCAGGS (*13*). HA-tagged hHB-EGF constructs in Fig. 5 were obtained by PCR amplification using P1-*Bam*HI and P2-HA-*Eco*RI, and the amplified fragments were inserted into the *Bam*HI–*Eco*RI sites of pMX-IRES-GFP. Construction of all mutant plasmid was confirmed by DNA sequencing.

Cell Cultures—NIH 3T3 cells used as the recipient cells for infection experiments and HeLa cells (HeLa tet off cells, Clontech) were maintained in Dulbecco's modified Eagle's medium (DMEM) (Sigma) supplemented with 100 μg/ml streptomycin, 100 units/ml penicillin G, and 10% foetal bovine serum (FBS) (Thermo Trace Ltd., Melbourne, Australia). PLAT-E cells (*14*) are ecotropic retroviral packaging cell lines kindly provided by T. Kitamura (The University of Tokyo) and maintained in DMEM supplemented with 1 μg/ml puromycin, 10 μg/ml blastcidine, 100 μg/ml streptomycin, 100 units/ml penicillin G, and

Table 1. **The sequences of all PCR primers used in this study**.

Primer name		Sequence
P1-*Bam*HI		5′- ACG GGATCC TCGAAAGTGACTG-GTGCCTCGCCGCCT -3′
P1-*Eco*RI		5′- GAGCTC GAATTC TCGAAAGTGA-CTGGTGCCTCGCCG -3′
P2-*Eco*RI		5′- AGCGGC GAATTC TCAGTGGGA-ATTAGTCATGCCCAA -3′
P2-148t-*Eco*RI		5′- CAGCTG GAATTC TCAGAGGCT-CAGCCCATGACA -3′
P2-L148Vt-*Eco*RI		5′- CAGCTG GAATTC TCACACGCT-CAGCCCATGACACCT -3′
P2-HA-*Eco*RI		5′- CAG GAATTC TCA **AGATCCAGC-GTAATCTGGAACATCGTATGGGTA** -GTGGGAATTAGTCATGCCCAACTT -3′
M1	I117A	5′- TACAAGGACTTCTGCGCCCATGG-AGAATGCAAA -3′
	I117V	5′- TACAAGGACTTCTGCGTGCATGG-AGAATGCAAA-3′
	V124A	5′- GGAGAATGCAAATATGCCAAGG-AGCTCCGGGCT -3′
	V124F	5′- GGAGAATGCAAATATTTCAAGGA-GCTCCGGGCT -3′
	V124L	5′- GGAGAATGCAAATATCTGAAGG-AGCTCCGGGCT -3′
	R128H	5′- TATGTGAAGGAGCTCCACGCTC-CCTCCTGCATC -3′
	R128K	5′- TATGTGAAGGAGCTCAAGGCTC-CCTCCTGCATC -3′
	Y138D	5′- ATCTGCCACCCGGGTGACCATG-GAGAGAGGTGT -3′
	Y138L	5′- ATCTGCCACCCGGGTCTGCATG-GAGAGAGGTGT -3′
	L148V	5′- TGTCATGGGCTGAGCGTGCCA-GTGGAAAATCGC -3′
M2	I117A	5′- TTTGCATTCTCCATGGGCGCAG-AAGTCCTTGTA -3′
	I117V	5′- TTTGCATTCTCCATGCACGCA-GAAGTCCTTGTA -3′
	V124A	5′- AGCCCGGAGCTCCTTGGCATA-TTTGCATTCTCC -3′
	V124F	5′- AGCCCGGAGCTCCTTGAAATAT-TTGCATTCTCC -3′
	V124L	5′- AGCCCGGAGCTCCTTCAGATATT-TGCATTCTCC -3′
	R128H	5′- GATGCAGGAGGGAGCGTGGAGCT-CCTTCACATA -3′
	R128K	5′- GATGCAGGAGGGAGCCTTGAGCT-CCTTCACATA -3′
	Y138D	5′- ACACCTCTCTCCATGGTCACCCGG-GTGGCAGAT -3′
	Y138L	5′- ACACCTCTCTCCATGCAGACCCG-GGTGGCAGAT -3′
	L148V	5′- GCGATTTTCCACTGGCACGCTC-AGCCCATGACA -3′

Bold sequence encodes HA tag and underlined sequences encode mutated amino acid residues.

10% FBS. DER cells, the stable transformants of 32D cells expressing human EGFR (*15*), kindly provided by E. Mekada (Osaka University) were used as target cells for the growth factor activity assay and maintained in RPMI medium (Sigma) supplemented with 100 μg/ml streptomycin, 100 units/ml penicillin G, 10% FBS, and 5% WEHI-3 cell conditioned medium. WEHI-3 conditioned medium was used as a source of interleukin-3 (IL-3) and were prepared as described (*16*).

Retroviral Infection—PLAT-E cells (2×10^6) were seeded in 60-mm dishes and cultured for 12–18 h in the absence of antibiotics. Three micrograms of retroviral expression plasmid containing the insert encoding various hHB-EGF mutants were mixed with 9 μl FuGENE6 (Roche) and added into the culture medium according to the manufacturer's instructions. After 24 h, the medium was replaced with 4 ml of fresh DMEM/10% FBS and the cells were incubated for another 24 h. Then, the supernatants were collected for infection of recipient cells. For infection, NIH 3T3 cells (2×10^5) were seeded in 100-mm dishes 12–18 h prior to infection and incubated with 5 ml of virus stock and 5 ml of fresh DMEM/10% FBS for 24 h in the presence of Polybrene (8 μg/ml) (Sigma), then medium was changed to fresh DMEM/10% FBS. For further infection, cells were seeded in new 100-mm dishes and incubated with the same virus stock. Cells were used for experiments after a few passages from the second infection.

Measurement of Growth Factor Activity of Retrovirally Infected Cells—Cells were seeded into 12-well dishes in 1 ml DMEM/10% FBS and cultured for 24 h. Then, the cells were washed with RPMI 1640 medium twice and 1×10^5 DER cells were co-cultured with the cells for 36 h in 2 ml RPMI 1640/10% FBS. Next, DER cells were harvested into 96-well dishes by gentle pipetting, and growth of DER cells was evaluated by measuring the cell number using a cell count reagent (Nacalai Tesque), according to the manufacturer's instructions.

Measurement of DT Sensitivity—Cells (2×10^4) were seeded into 24-well dishes in 0.5 ml DMEM/10% FBS. After incubation for about 24 h, various concentrations of DT purified as described previously (*2*) were added and the cells were cultured for further 12 h. Cells were incubated for 1 h in methionine/cysteine-free DMEM (Sigma) containing 5 μCi [^{35}S]methionine/cysteine (Daiichi Pure Chemicals, Japan) and then were lysed in 0.1 N NaOH. Proteins in this lysate were precipitated by 10% trichloroacetic acid. The radioactivity of acid-insoluble materials trapped by glass filter was measured in a liquid scintillation counter.

Measurement of Soluble Growth Factor Activity—For production of soluble form of hHB-EGF, HeLa cells (5×10^5) were seeded in 60-mm dishes and cultured for 12–18 h. Ten micrograms of plasmid containing hHB-EGF (1–148 amino acid residues) were transfected by the calcium phosphate method. After 24 h, the medium was replaced with 4 ml of fresh DMEM/10% FBS and the cells were incubated for another 24 h. Then, the supernatants were collected and the amount of soluble form of HB-EGF in the supernatants was estimated by western blot analysis. For measurement of growth factor activity, the amounts of soluble form of hHB-EGF in the samples were equalized by diluting with medium and DER cells (1×10^4) were seeded in 96-well dishes. The samples were added to each well and the DER cells were incubated for 36 h. Growth of DER cells was evaluated by measuring the cell number using a cell count reagent (Nacalai Tesque).

Measurement of Membrane-Anchored Growth Factor Activity—Cells were seeded into 12-well dishes in 1 ml DMEM/10% FBS and cultured for 24 h. Then, the cells were washed with 2 M NaCl–containing RPMI 1640 medium to remove soluble HB-EGF trapped by cell surface heparan sulfate proteoglycans (*9*) and fixed with 10% formaldehyde. The formalin-fixed cells were washed for five times with RPMI 1640 medium, and 1×10^5 DER cells were co-cultured on the fixed cells in 2 ml RPMI 1640/10% FBS for 36 h. Next, DER cells were harvested into 96-well dishes by gentle pipetting, and growth of DER cells was evaluated by quantitation of cell number using a cell count reagent (Nakarai Tesque), according to the manufacturer's instructions.

Detection of Membrane-Anchored Form of HB-EGF—Cells were collected and suspended in lysis buffer [10 mM Tris-HCl (pH 7.4), 1% NP-40, 1 mM EDTA, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 1 μg/ml pepstatin A, and 100 μg/ml PMSF in PBS]. After 10–20 min of incubation on ice, the lysates were centrifuged at 12,000 × *g* for 2 min and the supernatants were collected. For detection of hHB-EGF in retrovirally infected cell lysates, western blot analysis was performed with goat anti–human HB-EGF antibody (R&D) and the antibodies were visualized with horseradish peroxidase–conjugated anti–goat IgG (Jackson) using ECL Western Blotting Detection Reagents (Amersham Biosciences).

Detection of Soluble Form of HB-EGF—Fifteen microliters of conditioned medium of transfected HeLa cells was mixed with SDS sample buffer and loaded in 12% acrylamide gel. After transfer to nitrocellulose membrane, the membrane was incubated with goat anti–human HB-EGF antibody (R&D). The antibodies were visualized with horseradish peroxidase–conjugated anti–goat IgG (Jackson) using ECL Western Blotting Detection Reagents (Amersham Biosciences).

Detection of HB-EGF Cleavage—Cells were cultured for 18 h in serum-free DMEM medium and treated with 64 nM TPA (Nacalai Tesque, Japan) for 30 min. Then, cell lysates were collected as described above and western blot analysis was performed with mouse anti-hemagglutinin (HA) monoclonal antibody (12CA5) (Roche) and rabbit anti–green fluorescent protein (GFP) antibody (Clontech). The antibodies were visualized with horseradish peroxidase–conjugated anti–mouse IgG (DAKO) or anti–rabbit IgG (DAKO) using ECL Western Blotting Detection Reagents (Amersham Biosciences).

RESULTS

Determination of the Critical Residues in Mutational Analysis of hHB-EGF—To search the critical residues to diminish the growth factor activity of hHB-EGF without losing the activity as a DT receptor, we used computer simulation analysis of the crystal structure of the complex of DT with hHB-EGF (*17*), based on a previous reports concerned with the affinity of hEGF for EGFR (*18*). The results are summarized in Fig. 1. First, the amino acid residues involved in growth factor activity may be

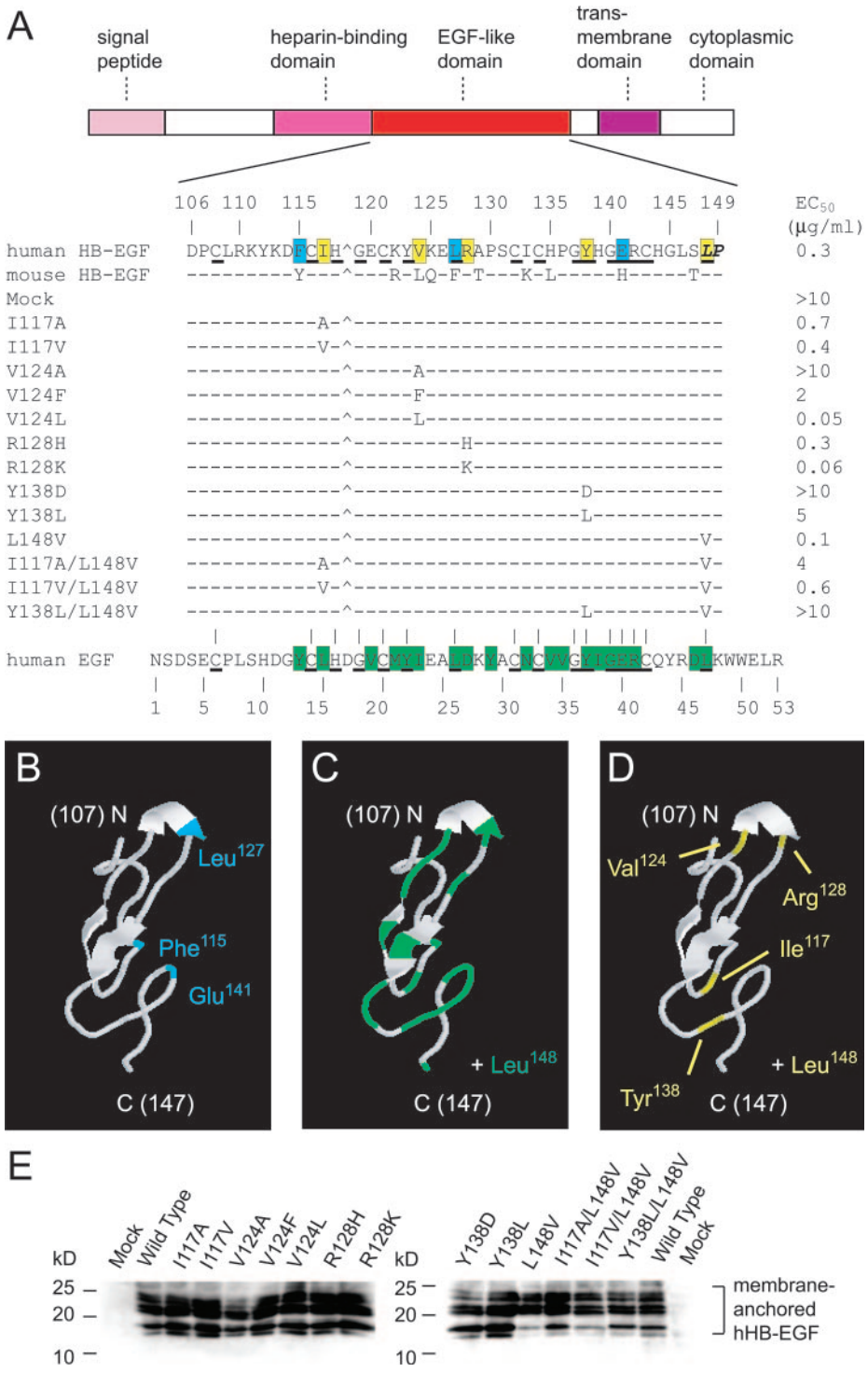

Fig. 1. **A schematic representation of determining the critical residues in mutational analysis of hHB-EGF.** (A) A schematic structure of hHB-EGF, alignment of the amino acid sequences of the EGF-like domain of hHB-EGF and hEGF. The cyan boxes in the EGF-like domain of hHB-EGF indicate three crucial amino acid residues for DT binding (*19*). Amino acids marked in bold italic affected the cleavage of membrane-anchored form of hHB-EGF accelerated by TPA stimuli (*31*). The green boxes in hEGF indicate the amino acid residues that substantially reduced affinity to EGFR (*18*). Ten amino acid residues shown in the EGF-like domain of mHB-EGF differ from those in hHB-EGF. Amino acids marked by heavy underlines indicate amino acid residues conserved between the EGF-like domains of hHB-EGF and hEGF. The amino acid residues substituted in this study are shown in yellow boxes. EC_{50} were determined from the data shown in Fig. 3. (B–D) Computer models of the tertiary structure of the EGF-like domain of hHB-EGF. The tertiary structure of the EGF-like domain of hHB-EGF (Phe^{107}–Ser^{147}) was referred to the complex with DT (*17*) and added with Leu^{148}. The important amino acid residues are shown in the same colors with in A; three crucial amino acid residues for DT binding (B), the corresponding amino acid residues to those of hEGF that substantially reduced affinity to EGFR (C), and the amino acid residues substituted in this study (D). (E) The amount of hHB-EGF protein in retrovirally infected cells was confirmed by western blot analysis using goat anti–human HB-EGF antibody. The multiple bands of membrane-anchored hHB-EGF represent various N-terminal truncations and glycosylations.

surmised from reports on hEGF, as HB-EGF binds to the EGFR as well as EGF. Mutation of the amino acid residues of hEGF shown in green boxes in Fig. 1, A and C, substantially decreased its affinity for EGFR (*18*). Second, a site-directed mutagenesis study of hHB-EGF suggested that three amino acid residues, Phe^{115}, Leu^{127}, and Glu^{141} are crucial for DT binding in the EGF-like domain of hHB-EGF (Fig. 1, A and B) (*19*). Thus we could not substitute these three amino acids whereas they were candidates with reduced affinity to EGFR (Fig. 1, B and C). Third, a study of the crystal structure of the complex of DT with the EGF-like domain of hHB-EGF (*17*) showed that amino acid residues, Ile^{117}, Arg^{128}, and Tyr^{138} do not interact with DT, but that amino acid residues, Leu^{15}, Asp^{27}, and Tyr^{37} in hEGF, which correspond to Ile^{117}, Arg^{128}, and Tyr^{138} in hHB-EGF, respectively, bind to EGFR with substantially reduced affinity (Fig. 1, A and D) (*18*). Although the amino acid residue, Val^{124} in

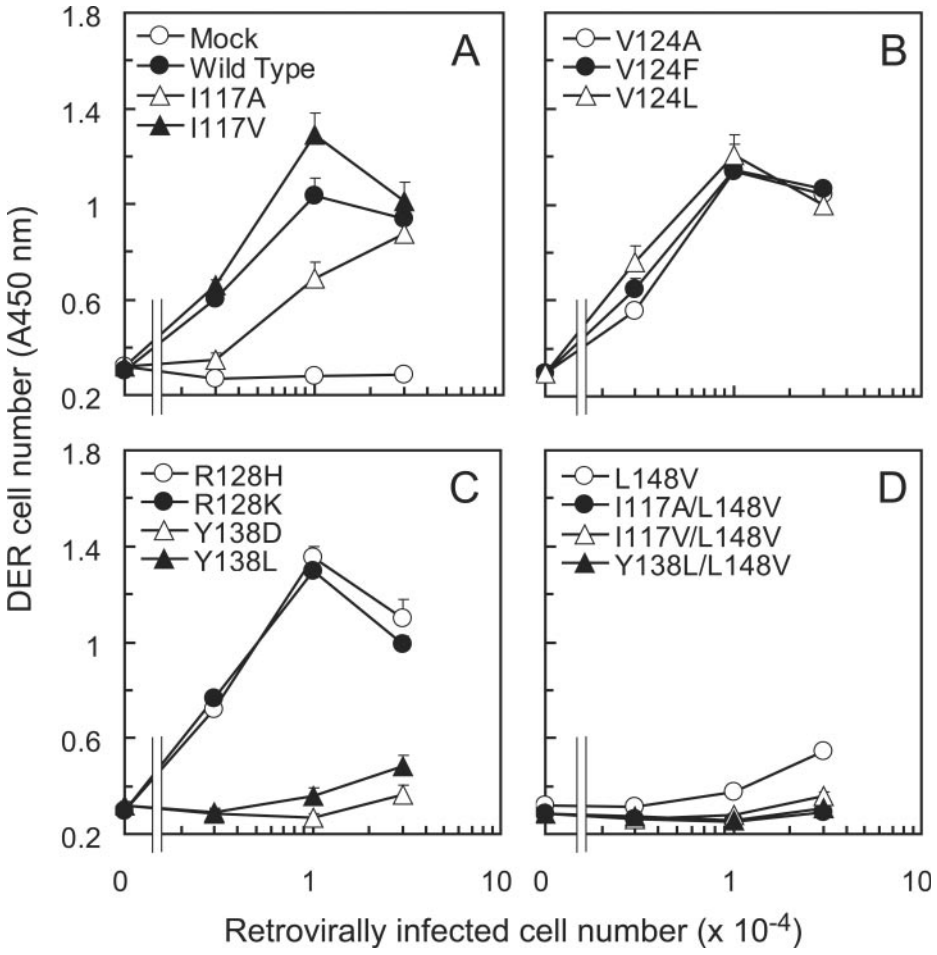

Fig. 2. **Growth factor activity of hHB-EGF mutants in mouse cells.** (A–D) DER cells, which grow well in the presence of hHB-EGF, were co-cultured for 36 h with mouse NIH 3T3 cells expressing hHB-EGF mutants by retroviral infection, then the cell number was measured by using a cell count reagent. The values represent the average of at least two experiments performed on different days and the bars indicate the standard errors from the means.

hHB-EGF interacts with DT (*17*), Ile^{23} in hEGF, which corresponds to Val^{124} in hHB-EGF, binds with low affinity to EGFR (*18*). Mutation of Val^{124} to hydrophobic residues would not be expected to have a significant effect on DT binding since mutation to V124L was shown to retain 90% of the DT binding activity relative to the wild type hHB-EGF (*19*). Although the amino acid Leu^{148} was not included in the complex of hHB-EGF crystal structure study (*17*), it was shown that EGFR affinity was considerably affected by amino acid substitution of Leu^{47} in hEGF (*18*), which corresponds to Leu^{148} in hHB-EGF. From the crystal structure study of the complex of hEGF and its receptor extracellular domains, Leu^{15}, Ile^{23}, and Leu^{47} in hEGF, which correspond to Ile^{117}, Val^{124}, and Leu^{148} in hHB-EGF, respectively, interact hydrophobically with EGFR (*20*). Based on these reports, we substituted five amino acid residues, Ile^{117}, Val^{124}, Arg^{128}, Tyr^{138}, and Leu^{148} in order to inactivate the growth factor activity of hHB-EGF without eliminating DT receptor function. All hHB-EGF mutants were expressed in mouse NIH 3T3 cells using retroviral infection as described in Materials and Methods, because retroviral infection enabled us to express hHB-EGF mutants similarly in almost all cells, which is essential for determining their biological activities. Indeed, the amount of hHB-EGF protein in retrovirally infected cells was almost similarly in all cells used in this study as confirmed by western blot analysis (Fig. 1E).

Growth Factor Activity in Retrovirally Infeced Cells—We first examined the growth factor activity of hHB-EGF mutants using DER cells, which are stable transformants of IL-3–dependent 32D cells expressing human EGFR and which can proliferate in the presence of hHB-EGF without IL-3 (*15*). DER cells were co-cultured with hHB-EGF mutants–expressing 3T3 cells by retroviral infection in a medium containing 10% FBS. After incubation for 36 h, DER cells were separated to 96-well dishes by gentle pipetting and cell count reagent was added to the medium. After incubation for 3 h, DER cell number was determined. As shown in Fig. 2A, DER cells proliferated when co-cultured with the cells expressing wild type hHB-EGF and this growth was dependent on the number of co-cultured cells. In contrast, no growth of DER cells was observed with mock-infected cells. NIH 3T3 cells expressing the hHB-EGF mutant (I117A) exhibited less stimulation of DER cell growth (Fig. 2A), while 3T3 cells expressing the hHB-EGF mutants (Y138D, Y138L, and L148V) exhibited only weak growth-stimulatory activity (Fig. 2, C and D). Amino acid substitutions in Val^{124} and Arg^{128} had no effect on DER stimulatory activity (Fig. 2, B and C), whereas the double amino acid mutations (I117A/L148V, I117V/L148V and Y138L/L148V) resulted in almost complete loss in DER growth stimulatory activity (Fig. 2D).

DT Sensitivity in Retrovirally Infected Cells—Next, we examined the DT-sensitivity of retrovirally infected

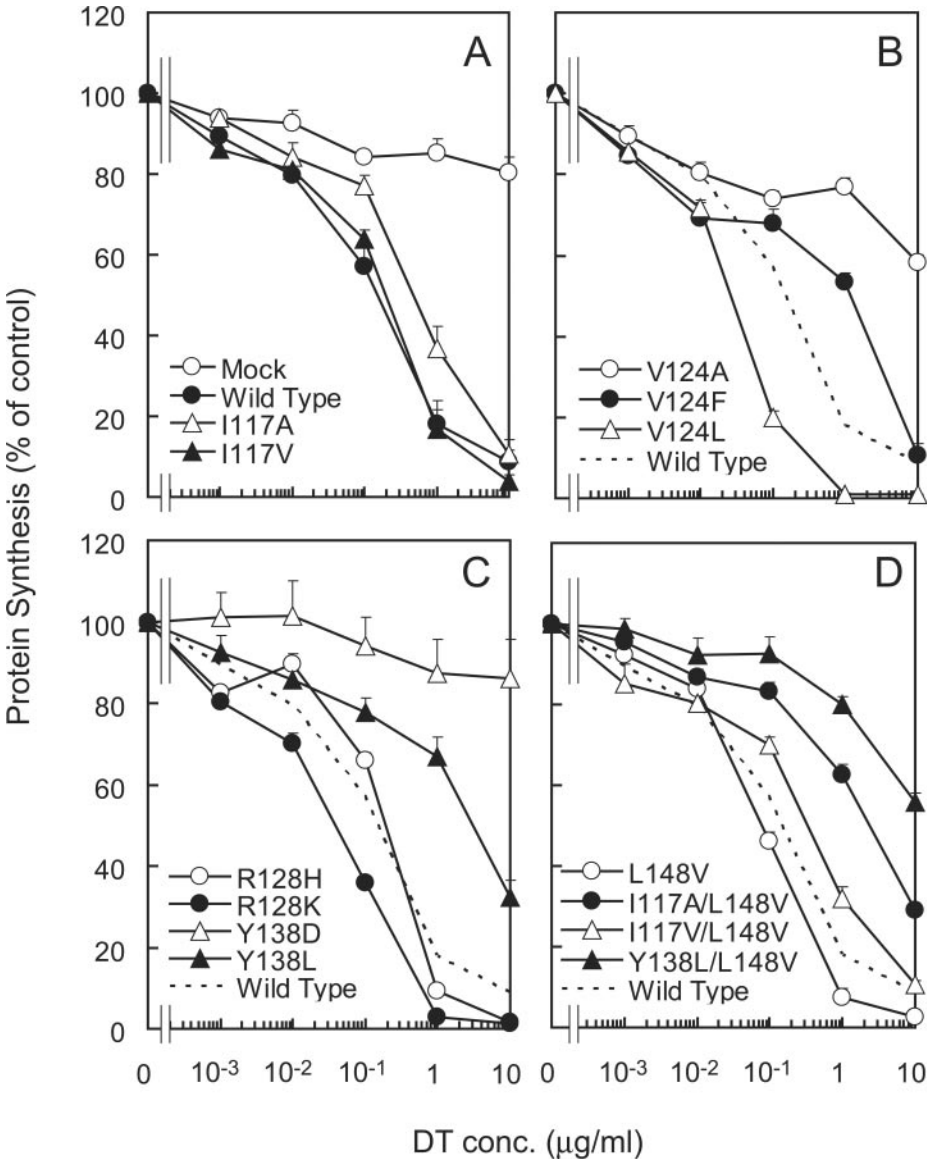

Fig. 3. **DT sensitivity of hHB-EGF mutants in mouse cells.** (A–D) NIH 3T3 cells expressing hHB-EGF mutants were incubated with various concentrations of DT for 12 h and then incubated with [^{35}S]methionine/cysteine for 1 h. Radioactivity incorporated into the protein was measured. EC_{50} was defined as the effective concentration of DT which reduced protein synthesis to 50% of the control values and these are shown in Fig. 1A. The values represent the average of at least two experiments performed on different days and the bars indicate the standard errors from the means.

cells, which expressed hHB-EGF mutants. Normally, DT can bind to hHB-EGF and is taken into the cell by receptor-mediated endocytosis, where it inhibits protein synthesis. We therefore incubated cells expressing hHB-EGF mutants with various concentrations of DT and measured protein synthesis by [^{35}S]methionine/cysteine incorporation. As shown in Fig. 3, a decrease in the rate of protein synthesis was observed in cells expressing wild type hHB-EGF, but little in mock-infected cells. In this study, EC_{50} was defined as the effective concentration of DT that reduces protein synthesis to 50% of the control, and these EC_{50} values are listed in Fig. 1A. Cells expressing the hHB-EGF mutants, I117A, I117V, R128H, R128K, and L148V exhibited DT sensitivity (Fig. 3, A, C, and D), although this sensitivity was slightly lower compared with the I117A mutant (Fig. 3A). DT sensitivity was higher in cells expressing V124L (Fig. 3B), and lower in cells expressing V124F or Y138L (Fig. 3, B and C). Cells expressing V124A showed little DT sensitivity (Fig. 3B) and DT sensitivity was abolished in cells expressing Y138D (Fig. 3C). DT sensitivity was slightly low for the double amino acid mutant I117V/L148V and lower still with the I117A/L148V and Y138L/L148V mutants. Although DT sensitivity is reduced, the I117A/L148V still clearly could function as a DT receptor. Together, these results suggest that the hHB-EGF mutants L148V, I117A/L148V, and I117V/L148V possessed little growth factor activity but maintained their DT receptor function.

Growth Factor Activity of Soluble hHB-EGF—We also examined the growth factor activity mediated solely by soluble form of hHB-EGF. To obtain soluble forms of hHB-EGF mutants, enforced secreted constructs were generated by insertion of a stop codon after Leu^{148}, which is the major cleavage site of membrane-anchored form of hHB-EGF (*21*). The secreted constructs were transfected into HeLa cells and the conditioned medium was collected. After estimation of the amount of soluble form of hHB-EGF in the conditioned medium of transfected cells by western blot analysis, the amount of soluble hHB-EGF mutants in the samples was equalized (Fig. 4D).

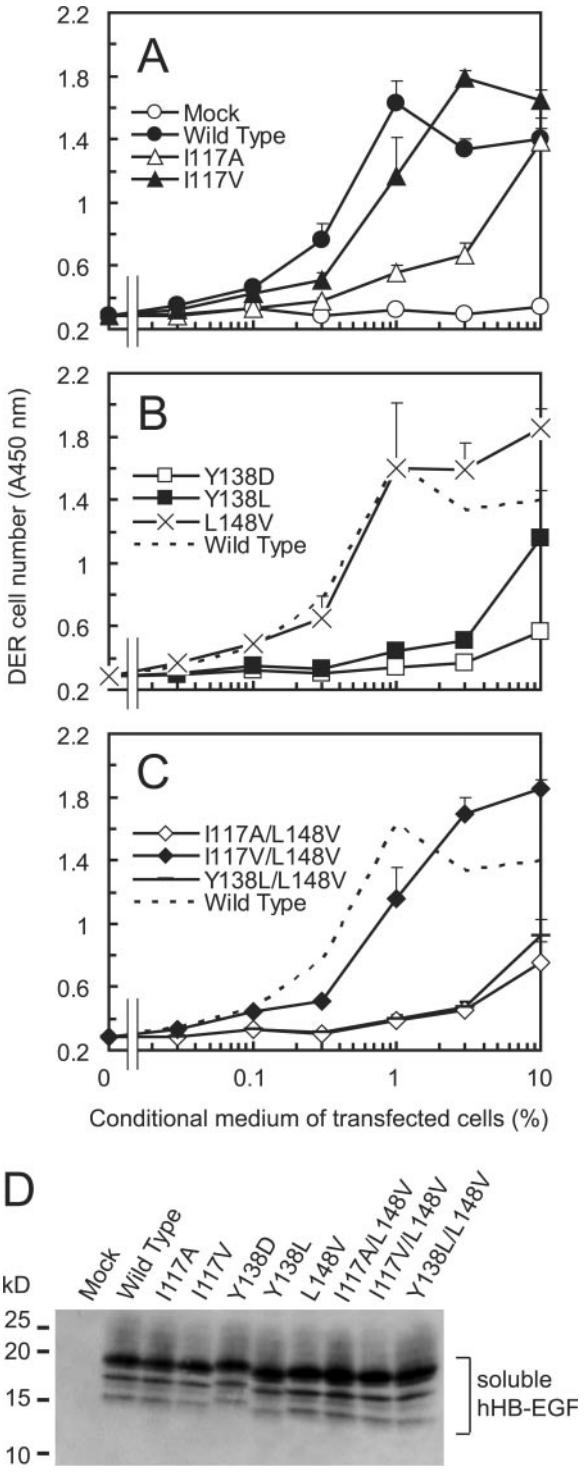

Fig. 4. **Growth factor activity of soluble form of hHB-EGF mutants.** (A–C) DER cells were incubated with the conditioned medium containing secreted hHB-EGF mutants for 36 h. Then, growth of DER cells was evaluated by measuring the cell number using a cell count reagent. The conditioned medium was used in the dilution as shown in D. The values represent the average of three independent experiments and the bars indicate the standard errors from the means. (D) The amounts of soluble form of hHB-EGF mutants in samples after equalization were confirmed by western blot analysis using anti–HB-EGF antibody. The multiple bands of hHB-EGF represent various N-terminal truncations and glycosylations.

The samples were added to DER cells in 96-well dishes. After incubation for 36 h, DER cell number was determined. As well as Fig. 2A, DER cells proliferated when added the conditioned medium of cells transfected with soluble form of wild type hHB-EGF and this growth was dependent on the amount of the conditioned medium. No growth of DER cells was observed in the medium of mock-transfected cells. Growth stimulation was slightly reduced in hHB-EGF mutant (I117V) (Fig. 4B). Great reduction on growth stimulatory activity was observed in hHB-EGF mutants (I117A, Y138D, Y138L, I117A/L148V, and Y138L/L148V) (Fig. 4, A, B, and C). These results were largely correlated with the observations in Fig. 2. No reduction on growth stimulatory activity was observed in hHB-EGF mutant (L148V) (Fig. 4B) and slight reduction in hHB-EGF (I117V/L148V) (Fig. 4C), thought these hHB-EGF mutants exhibited weak or almost no growth stimulatory activity (Fig. 2D). These results suggest that amino acid substitution in Leu^{148} may have different effect on growth stimulatory activity between soluble form and membrane-anchored form of hHB-EGF.

Cleavage of Membrane-Anchored hHB-EGF—The membrane-anchored form of hHB-EGF functions as a DT receptor but is cleaved at the cell surface by proteases and becomes soluble and cannot serve as a DT receptor. This cleavage of membrane-anchored hHB-EGF is accelerated by TPA stimulation (*10*). We examined TPA-induced cleavage of membrane-anchored form of hHB-EGF mutants by western blot analysis. The hHB-EGF mutant–expressing cells were incubated with or without TPA for 30 min and the HA-tagged cytosolic domains of hHB-EGF were detected with anti-HA antibodies in the cell lysate (Fig. 5, A and B). The multiple bands of approximate 25 kDa molecular marker are the membrane-anchored form of hHB-EGF and the smallest band near 15 kDa molecular marker labeled as the tail fragment is the proteolytically cleaved fragment comprising the cytosolic and transmembrane domains. The addition of TPA induced cleavage and generated a large amount of the tail fragment in cells expressing wild type hHB-EGF, but few tail fragments were detected in cells expressing the hHB-EGF mutants (L148V, I117A/L148V, and I117V/L148V) (Fig. 5B). This result suggests that the hHB-EGF mutants (L148V, I117A/L148V, and I117V/L148V) were resistant to TPA-induced cleavage.

Growth Factor Activity of Membrane-Anchored hHB-EGF—The membrane-anchored form of HB-EGF transduces biological signals to neighboring cells in a membrane-associated manner (*9*). The growth factor activity shown in Fig. 2 represents both the soluble and membrane-anchored growth factor activities of hHB-EGF. However, several of the mutants exhibiting reduced growth factor activity (L148V, I117A/L148V, and I117V/L148V) were not cleaved in the presence of TPA (Fig. 5B) and soluble growth stimulatory activity observed in Fig. 4 was different from that in Fig. 2. We therefore examined growth factor activity mediated solely by the membrane-anchored form of hHB-EGF to examine the possibility that the reduced growth factor activity exhibited by some of the mutants may be due to an inability to produce soluble forms of hHB-EGF. NIH 3T3 Cells expressing the hHB-EGF mutants by retroviral infection were fixed with 10% formaldehyde before co-culture with DER cells,

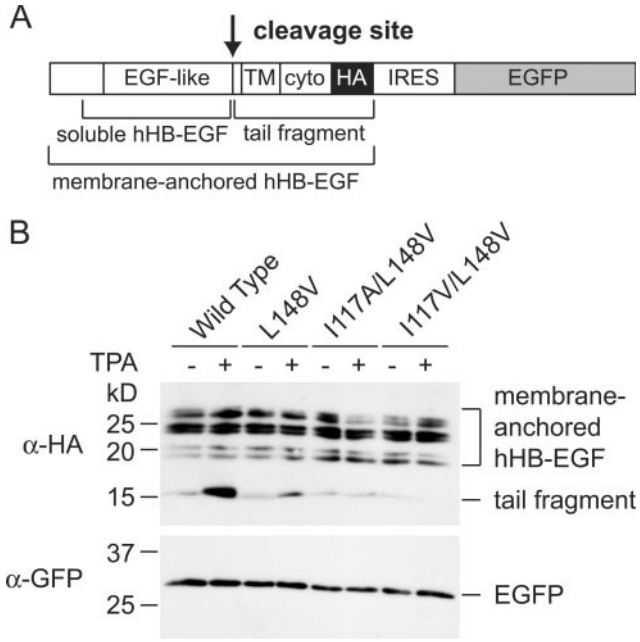

Fig. 5. **Cleavage of membrane-anchored hHB-EGF.** (A) A schematic structure of the hHB-EGF constructs used here. The tail fragment represents a proteolytically cleaved fragment consisting of the transmembrane (TM) and cytosolic (cyto) domains. (B) Cells were incubated in serum-free medium for 18 h and incubated with or without 64 nM TPA for 30 min. Then the cells were harvested and the cleavage of membrane-anchored hHB-EGF was examined by western blot analysis using anti-HA antibody and anti-GFP antibody. EGFP expressed in the same construct linked by an IRES sequence is shown in the lower panel as a loading control. Similar results were obtained from the experiments performed on different days. The multiple bands of membrane-anchored hHB-EGF represent various N-terminal truncations and glycosylations.

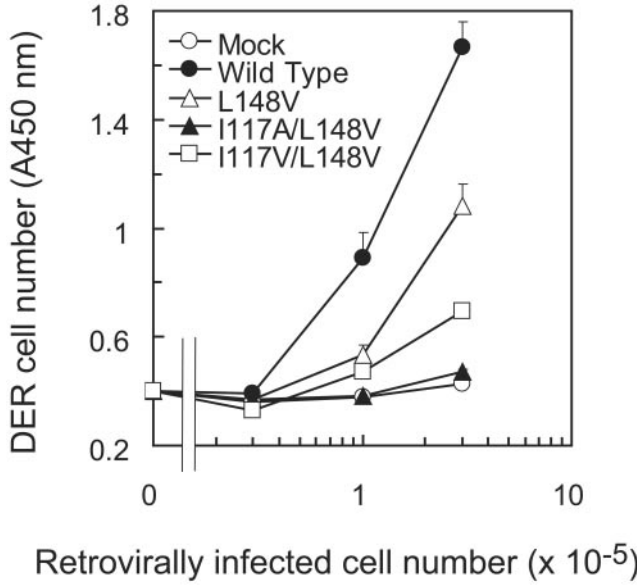

Fig. 6. **Growth factor activity of the membrane-anchored form of hHB-EGF mutants.** DER cells, which grow well in the presence of hHB-EGF, were used as targeted cells and co-cultured on hHB-EGF expressing effector cells fixed with 10% formaldehyde for 36 h, after which DER cell number was measured using a cell count reagent. The values represent the average of at least two experiments and the bars indicate the standard errors from the means.

thus eliminating the possibility that they could produce any soluble form of hHB-EGF. After incubation for 36 h, the number of DER cells was determined as described for Fig. 2. As shown in Fig. 6, DER cells proliferated when co-cultured on formalin-fixed cells expressing wild type hHB-EGF but not on mock-infected cells. The growth of DER cells was reduced, although not abolished when co-cultured with cells expressing L148V mutant. This suggests that the significant reduction of the growth factor activity of this mutant observed in Fig. 2 was due to the inability of this mutant to produce a soluble form. However, the I117A/L148V and I117V/L148V mutants showed little and greatly reduced DER cell stimulatory activity, respectively, suggesting that amino acid substitutions in these mutants disrupted its physical interaction with EGFR.

DISCUSSION

To accomplish the conditional ablation of specific cells or tissues in animals, we used hHB-EGF as a DT receptor in the liver cells of transgenic mice (*2*). It was reported that HB-EGF caused increased DNA synthesis in rat hepatocytes (*22*) and mouse fetal liver epithelial cells (*23*) in primary cultures. In addition, HB-EGF mRNA expression was observed to increase during rat liver regeneration (*24*), and hepatocyte DNA replication was increased in HB-EGF–injected mice but delayed in HB-EGF null mice after partial hepatectomy (*25*). These observations suggest that HB-EGF plays an important role in liver regeneration. Although transgenic mice expressing hHB-EGF in liver cells showed no abnormalities under normal conditions (*2*), hepatocyte proliferation was accelerated after partial hepatectomy in mice transgenic for hepatocyte-specific hHB-EGF expression (*26*). Moreover, it was also reported that HB-EGF plays an important role *in vivo*, such as in cardiac hypertrophy (*8*), pancreatic fibrosis (*27*), heart development and valvulogenesis (*28*, *29*). This shows that overexpression of hHB-EGF in specific cells or tissues could cause abnormalities in transgenic mice. Therefore in order to use hHB-EGF as a DT receptor to conditionally ablate cells in transgenic mice it is necessary to reduce the EGF-like activity of hHB-EGF without destroying DT receptor function.

Substitution of the amino acid residue, Leu^{15} in hEGF, which corresponds to Ile^{117} in hHB-EGF, results in substantially decreased EGFR affinity; for example, Val substitution results in a reduction in affinity to 18%, while Ala substitution results in a reduction to 2.7% compared to wild type (*18*). The I117V mutation scarcely affected hHB-EGF growth factor activity, whereas I117A caused a reduction in activity (Figs. 2A and 4A). The crystal structure of hHB-EGF indicates that amino acid residue Ile^{117} does not interact with DT (*17*), however sensitivity to DT was decreased by the I117A or I117A/L148V mutations (Fig. 3, A and D), suggesting the possibility that the larger overall structure of hHB-EGF was

slightly affected by the point mutation of I117A. It had been expected that mutation of Leu^{148} in hHB-EGF would cause a significant reduction in growth factor activity because the corresponding mutation of Leu^{47} in hEGF has a pronounced effect on EGFR affinity (*18*). The crystal structure of hEGF suggests that Leu^{47} hydrophobically interacts with EGFR (*20*), suggesting that this residue plays an important role in binding to EGFR. However, mutation of the corresponding Leu^{148} residue in hHB-EGF to Val (L148V) had no or only a weak effect on growth factor activity (Figs. 4B and 6). This may be due to the fact that the EGF-like domain of hHB-EGF terminates with Leu^{148}, but the corresponding hEGF domain consisting of 53 amino acid residues includes 6 amino acid residues C-terminal to Leu^{47} (Fig. 1A). Double point mutations such as I117A/L148V caused a further reduction in hHB-EGF growth factor activity (Fig. 6), as observed in mutagenesis studies on hEGF, which showed that combinations of point mutations in hEGF caused a cumulative reduction in EGFR affinity (*18*). Amino acid substitutions in Val^{124} and Arg^{128} did not reduce growth factor activity (Fig. 2, B and C) and mutations in Tyr^{138} affected DT sensitivity (Fig. 3C). These results indicated that amino acid substitutions in Ile^{117} and Leu^{148} may be effective in inactivating hHB-EGF growth factor activity of without eliminating DT receptor function. In the another study examining the creation of a soluble hHB-EGF for use as a diphtheria toxin antidote it was found that recombinant truncated hHB-EGF with the mutations I117A/L148A had low mitogenic activity but showed inhibition of DT binding similar to that of wild type (*30*).

It was reported that the hHB-EGF mutants L148S and L148S/P149T were resistant to cleavage accelerated by the stimuli such as TPA or lysophosphatidic acid (*31*, *32*). This suggested that the point mutation introduced into Leu^{148} would affect cleavage of the membrane-anchored form of hHB-EGF, and indeed we observed reduced TPA-induced cleavage of the hHB-EGF mutant L148V. It is expected that the suppression of the cleavage of the membrane-anchored form of hHB-EGF would prevent the reduction of DT sensitivity in the hHB-EGF expressing mouse cells, since soluble hHB-EGF cannot serve as a DT receptor. However, we found that DT sensitivity in the mouse cells expressing hHB-EGF mutant (L148V) was not significantly higher than those expressing wild type hHB-EGF. This may be because there is still an excess of membrane-anchored hHB-EGF present even after TPA stimulation, as shown in Fig. 5B. On the other hand, while the membrane-anchored form of HB-EGF is assumed to have biological activity in cultured cells (*9*), a study examining mice deficient in the cleavage of the membrane-anchored form of HB-EGF showed phenotypes similar to the HB-EGF knock-out mice (*32*). Thus, the physiological function of the membrane-anchored form of HB-EGF is unclear. Furthermore, the mutant mice producing an excess of soluble form of HB-EGF showed severe abnormalities in the skin and heart (*32*), suggesting that the soluble form plays a crucial role in HB-EGF physiological functions. Moreover, cardiac hypertrophy was blocked by an inhibitor of the ADAM12 metalloproteinase, which cleaves membrane-anchored HB-EGF (*8*). In this study, hHB-EGF mutants containing a mutation of L148V, which renders them resistant to TPA stimuli, showed significantly reduced growth factor activity under conditions where both soluble and membrane-anchored forms are measured, as in our Fig. 2, compared to the membrane-anchored form only as in our Fig. 6. Therefore, we might expect that the side effects of hHB-EGF overexpression in transgenic mice might be prevented by suppressing the cleavage of the membrane-anchored form of HB-EGF.

In the present study, the hHB-EGF mutant I117A/L148V showed reduced soluble and membrane-anchored growth factor activity (Figs. 2D and 6), but retained its function as a DT receptor, although the DT sensitivity, *i.e.* EC_{50} of infected cells, decreased about 10-fold relative to wild type (Figs. 1A and 3D). The cytotoxic effects observed when using TRECK depends on the dose of DT and the expression levels of hHB-EGF in the transgenic mice (*2*). However, the dose of DT injected into transgenic mice is generally low, for example 5–500 ng/kg weight in the liver injury model, compared to the maximum tolerated dose of 50 μg/kg weight in wild type mice (*2*). Also, the expression level of hHB-EGF depends on its promoter activity in different tissues. Thus, a 10-fold decrease in DT sensitivity may be inconsequential for most applications using TRECK. In cases where the expression of hHB-EGF is low or in tissues that require a large amount of DT to be destroyed, the hHB-EGF mutant (I117V/L148V) may be preferable. This may be demonstrated by the fact that the DT sensitivity of the cells infected with hHB-EGF mutant (I117V/L148V) was almost similar level to wild type, and little growth factor activity was detected in an assay that included the soluble forms (Fig. 2D). Although hHB-EGF mutant (I117V/L148V) resulted in only a slight reduction of secreted growth factor activity (Fig. 4), this mutant was resistant to cleavage of the membrane-anchored form (Fig. 5). As mentioned above, the actual physiological conditions are probably close to the conditions employed for the assay in Fig. 2, and the growth factor activity shown in Fig. 4 and Fig. 6 may not need to be considered in most cases.

We conclude that the hHB-EGF mutants I117A/L148V or I117V/L148V could be more useful than wild type hHB-EGF for conditional cell ablation by TRECK without the side effects of EGF-like growth factor activity.

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