Heparin-binding epidermal growth factor (EGF)-like growth factor (HB-EGF) is a member of the EGF family of growth factors. The membrane-anchored form of HB-EGF (proHB-EGF) is mitogenically active to neighboring cells as well as being a precursor of the soluble form. In addition to its mitogenic activity, proHB-EGF has the property of binding to diphtheria toxin (DT), serving as the specific receptor for DT. Tetramembrane-spanning protein CD9, a member of the TM4 superfamily, is physically associated with proHB-EGF at the cell surface and up-regulates both mitogenic and DT binding activities of proHB-EGF. To understand this up-regulation mechanism, we studied essential regions of both CD9 and proHB-EGF for up-regulation. Immunoprecipitation experiments revealed that not only CD9 but also other TM4 proteins including CD63, CD81, and CD82 associate with proHB-EGF on the cell surface. However, these TM4 proteins did not up-regulate DT binding activity of proHB-EGF. Transfection of a series of chimeric constructs comprising CD9 and CD81 showed that the major extracellular domain of CD9 is essential for up-regulation. Assays of DT binding activity and juxtacrine mitogenic activity of the deletion mutants of proHB-EGF and chimeric molecules, derived from proHB-EGF and TGF-α, showed that the essential domain of proHB-EGF for up-regulation is the EGF-like domain. These results indicate that the interaction of the extracellular domains of both molecules is important for up-regulation.

Heparin-binding epidermal growth factor-like growth factor (HB-EGF) is a member of the EGF family (1, 2) that encompasses a number of structurally homologous mitogens including EGF, TGF-α, vaccinia virus growth factor, amphiregulin, β-cellulin, and epiregulin. Similar to other EGF family growth factors, HB-EGF binds to and stimulates EGF receptors, and thus soluble mature HB-EGF is a potent mitogen for a number of cells including NIH3T3 cells, bovine aortic smooth muscle cells, rat hepatocytes, and human keratinocytes (3). HB-EGF is synthesized as a trans-membrane protein of 208 amino acids composed of signal peptide, pro, heparin-binding, EGF-like, juxtamembrane, transmembrane, and cytoplasmic domains. Although the membrane-anchored form of HB-EGF (proHB-EGF) is cleaved on plasma membrane to yield a soluble HB-EGF (4, 5), a considerable amount of proHB-EGF remains uncleaved on the cell surface. Indeed, proHB-EGF is not only a precursor protein for soluble HB-EGF but also a membrane-anchored growth factor itself. As shown in other membrane-anchored growth factors and lymphokines, proHB-EGF transduces mitogenic signals to neighboring cells in a nondiffusible manner, the so-called “juxtacrine mechanism” (6).

proHB-EGF serves as the specific receptor for diphtheria toxin (DT) (7, 8) and mediates endocytosis of the receptor-bound toxin. In the endocytic vesicle, DT or its fragment A penetrates the endosome membrane and reaches to the cytosol (9) where it inhibits the eukaryotic system for protein synthesis by inactivating elongation factor 2 through ADP-ribosylation (10). proHB-EGF binds DT through the EGF-like domain (11, 12), as it binds to the EGF receptor. CRM197, a nontoxic mutant of DT, inhibits mitogenic activity of HB-EGF by preventing binding of HB-EGF to the EGF receptor (11), thus the binding site of proHB-EGF for DT has been suggested as being located in close proximity to, or overlapping with, that for the EGF receptor. The proHB-EGF gene has been identified in mammals and chicken, and thus most mammalian cells are sensitive to DT. However, cells derived from mouse or rat are resistant to DT, because proHB-EGF of these animals has amino acid substitutions in the EGF-like domain and therefore does not bind DT.

proHB-EGF forms a complex with CD9 (7, 13) and integrin α5β1 (14) on the cell surface. CD9, originally identified as a DT receptor-associated protein (DRAP27) (15), has four membrane spanning domains, belongs to the transmembrane 4 superfamily (TM4SF) that includes CD37, CD53, CD63, CD81, CD82, and CD151 (16). CD9 up-regulates both DT binding and juxtacrine mitogenic activities of proHB-EGF when CD9 is co-expressed with proHB-EGF, although CD9 itself does not have those properties (6, 7). CD9 does not enhance the proHB-EGF transcription, and neither does it increase the number of proHB-EGF molecules on the cell surface (6, 7), indicating that this up-regulation of proHB-EGF activities by CD9 is due to protein-protein interaction between proHB-EGF and CD9.
Scatchard plot analysis of DT binding to proHB-EGF indicated that increased DT binding with CD9 is due to an increase in the number of effective binding sites for DT (7). However, the precise mechanism of up-regulation remains to be elucidated.

In this report we have studied the regions of proHB-EGF and CD9 responsible for up-regulation. Results showed that the second extracellular domain of CD9 and the EGF-like domain of proHB-EGF are essential for the up-regulation of proHB-EGF, indicating that interaction of these molecules at the extracellular domains is important.

**EXPERIMENTAL PROCEDURES**

**Reagents and Antibodies—** DT was produced as described previously (17). Rabbit anti-HB-EGF antisera H6 were obtained as described previously (7). Goat anti-human proHB-EGF were purchased from R & D Systems (Minneapolis, MN). Mouse anti-CD9, -CD63, and -CD9 antibodies were from MBL Co., Ltd (Nagoya, Japan). Mouse anti-CD9 monoclonal antibodies were obtained from Serotec Ltd. (Oxford, UK). Rabbit anti-goat IgG and goat anti-rabbit IgG were from Cappel Laboratories (Durham, NC).

**Plasmid Constructions—** Plasmids encoding monkey CD9 (pRCt1843) and human proHB-EGF (pRBHECKG) were used as described previously (13). cDNA encoding human CD9 and CD81 were kindly provided by Dr. H. Hotta (Kobe University) and Dr. S. Levy (Stanford University), respectively. cDNA of human CD82 was obtained by polymerase chain reaction from the human B cell cDNA library. These cDNAs were inserted into the HindIII/Kit site in the expression vector, pRC/CMV.

CD9/CD81 chimeras were constructed as follows. NheI site and NarI sites were introduced in CD9 cDNA by substituting 172Ct to A and 335A to C, respectively. The mutations introduced were synonymous; thus no amino acid substitutions occurred. Then CD9/CD81 chimeras were constructed by substituting to the corresponding polymerase chain reaction fragments of CD81 cDNA with corresponding restriction enzyme sites as illustrated in Fig. 3A.

The deletion mutants of proHB-EGF were derived from pTHG-1 (11). XhoI and BamHI sites were introduced in pTHG-1 by site-directed mutagenesis from CAC to GAG and from ACAAA to GGAT, respectively, as illustrated in Fig. 4A. These alterations resulted in amino acid substitutions of P149E, T160G, and T161S. The deletions were made by digesting proHB-EGF cDNA with corresponding restriction enzyme sites and linking them with oligonucleotide. In the case of the FRM construct, synthetic nucleotide of the corresponding region of transferrin receptor was inserted. ΔCyto was made by introducing stop codon at the 530 base pair by site-directed mutagenesis.

**HB-EGF/TGF-α chimeras** were based on the mutant proHB-EGF, which has additional Bal1, XhoI, and BamHI sites (see Ref. 11 and Fig. 4A). proHB-EGF has DraII and KpnI sites in its sequence as shown in Fig. 4. Each domain of TGF-α was generated by polymerase chain reaction using corresponding primer and inserted into HB-EGF cDNA digested by corresponding restriction enzymes. To construct TGJ, synthetic nucleotide corresponding to the juxtamembrane domain of TGF-α was inserted into HB-EGF cDNA digested by XhoI and BamHI (Fig. 5A).

**Cell Culture and Transfection—** Monkey Vero cells, human HT1080D cells, mouse L cells, and their derivatives were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 units/ml of penicillin G, and 100 μg/ml of streptomycin. EP170.7 cells, obtained from Dr. J. Pierce (National Institutes of Health, Bethesda, MD) were grown in RPMI 1640 medium supplemented with 10% fetal calf serum, 5% WEHI-3 cell conditioned medium, 100 units/ml of penicillin G, and 100 μg/ml of streptomycin. Transfection of plasmids into recipient cells was done as described previously (15). Transfected cells were cultured for 48 h and then used for further studies. LC, LH, and LCH cells are stable transfectants of L cells expressing monkey CD9 alone, human proHB-EGF alone and both monkey CD9 and human HB-EGF, respectively (7). Stable transfectants expressing TG-E alone (LTG-E cell) or both TG-E and CD9 (LTG/E/D cell) were isolated from L cells in a selection medium containing 40 μg/ml of G418 after transfection with each of the plasmids. HT1080D cells were cloned in the selection medium containing 40 μg/ml of G418 after transfection with monkey CD9 into HT1080 cells. VeroHKa cells were Vero cells stably expressing proHB-EGF and CD82.

**Cell Surface Biotinylation, Immunoprecipitation, and SDS-PAGE—** Cell surface biotinylation was carried out as described previously (14). Briefly, cells were washed and incubated in a biotinylating solution containing 0.2 mg/ml NHS-LC-Biotin (Pierce) for 30 min at 4 °C. The reaction was stopped by the addition of 40 μl of glycine. For immunoprecipitation, cells were lysed with HBS (10 mM Hepes, 150 mM NaCl, pH 7.0) containing 10 μg/ml CHAPS, 10 μg/ml chymostatin, and 20 μg/ml antipain. The lysate was cleared of insoluble material by centrifugation at 40,000 × g for 30 min. The supernatant was precipitated with primary antibodies at a concentration of 5 μg/ml followed by the addition of Sepharose 4B-conjugated goat anti-mouse antibodies. The gel was washed with washing buffer (HBS containing 10 μg/ml CHAPS) then boiled with SDS-PAGE sample buffer. Material recovered from the gel was analyzed by SDS-PAGE. Samples subjected to SDS-PAGE were electrophoresed to an Immobilon membrane. The membrane was blocked with TBS containing 3% bovine serum albumin (Sigma) at room temperature for 1 h, and proteins were detected by incubation with 100 ng/ml horseradish peroxidase-streptavidin (Pierce), then analyzed with an ECL-Western blotting kit (Amersham Pharmacia Biotech).

**DT Binding and Antibody Binding Assay—** Binding of 125I-labeled DT to cells was measured as described previously (12). Nonspecific binding of 125I-DT was assessed in the presence of a 100-fold excess of unlabeled DT. Specific binding was determined by subtracting the nonspecific binding from the total binding obtained with 125I-DT alone. The amount of DT bound to the cells was calculated from the value of the specific binding of DT. The amounts of proHB-EGF expressed on the cell surface were determined as described previously (12). Briefly, cells were cultured in HB-EGF antibody medium at 4 °C for 2 h. After washing three times, cells were incubated with 1 μg/ml 125I-secondary antibody in binding medium. Finally cells were washed with washing buffer three times, and the cell-associated radioactivity was counted. DT binding activity was expressed as the calculated value B/A, in which A is the amount of proHB-EGF expressed on the cell surface, whereas B is the amount of DT bound to the cells.

**Juxtacrine Assay—** Juxtacrine mitogenic activity of proHB-EGF was monitored by measuring the incorporation of [3H]thymidine into DNA of EP170.7 cells as described previously (6). Stable transfectants were plated in 24-well plates and incubated for 1 day. The cells were washed twice with Dulbecco's modified Eagle's medium containing 10% fetal calf serum and 2 mM NaCl and fixed with 4% paraformaldehyde for 5 min. The fixed cells were washed twice with 10% fetal calf serum/RPMI 1640, and EP170.7 cells were added in co-culture. After incubation for 36 h, [3H]thymidine (37 kBq/ml) was added to the well, and the culture cells were incubated for 4 h. The EP170.7 cells were harvested and analyzed for incorporation of [3H]thymidine into DNA.

**RESULTS**

Association between proHB-EGF and TM4SF Proteins—We have shown that proHB-EGF forms a complex with CD9 and integrin α5β1 (14). Recent studies also show that integrins α5β1 and α6β1 associate with not only CD9 but also other TM4SF proteins including CD63 and CD81 (19). Furthermore, TM4SF proteins including CD9, CD63, CD81, and CD82 have a tendency to associate each other (20). We examined whether other members of the TM4SF associate with proHB-EGF by co-precipitation experiments using a lysate of VeroHKa cells. VeroHKa cells are stable transfectants of Vero cells overexpressing proHB-EGF and CD82 and also endogenously express CD9, CD63, and CD81 of TM4SF. Cell lysates of surface-biotinylated VeroHKa cells were immunoprecipitated with specific antibody against CD9, CD63, CD81, or CD82, and the precipitated material was subjected to SDS-PAGE and Western blotting followed by staining of the biotinylated proteins. Anti-CD9 antibody precipitated integrin α5β1, proHB-EGF, and unidentified proteins including those at 95 and 48 kDa and other minor bands, as well as CD9 itself, as previously shown (14) (Fig. 1, A and B, lane 2). Antibodies against CD63, CD81, and CD82 also co-precipitated integrin α5β1 and proHB-EGF (Fig. 1, A and B, lanes 3 and 4). Western blotting analysis of co-precipitated material labeled with anti-integrin α5 antibody confirmed that the band at 150 kDa was integrin α5 (Fig. 1C). Similarly, anti-proHB-EGF antibody confirmed that the bands at 20–27 kDa were proHB-EGF (data not shown). However, the bands of CD63 and CD82, which generally migrate over a broad range because of their extensive glycosylation, were difficult to see in precipitates of the biotinylated cell lysate, as previously men-
tioned (19). Associations among TM4SF proteins were also detected. Anti-CD63, -CD81, and -CD82 antibodies co-precipitated the 27-kDa band of CD9 (Fig. 1A, lanes 3–5), as confirmed by Western blotting using anti-CD9 antibody (data not shown). Western blotting analysis also revealed the co-precipitation of CD63 and CD81 with anti-CD9 antibody (data not shown). These results indicated that CD63, CD81, and CD82 also associate with proHB-EGF and that these TM4SF proteins are included in the integrin α3β1, CD9, and proHB-EGF complex.

CD9 associates with proHB-EGF, and it up-regulates both DT binding activity and the mitogenic activity of proHB-EGF. We tested whether other TM4SF molecules up-regulate DT binding activity of proHB-EGF, as with CD9. LH cells, stable transformants of L cells expressing human proHB-EGF, were transfected with plasmids encoding CD9, CD63, CD81, or CD82. Expression of transfected cDNA at the cell surface was confirmed by indirect immunofluorescence. proHB-EGF molecules expressed on cell surfaces were determined by the binding of the anti-HB-EGF antibody followed by the 125I-labeled secondary antibody. DT binding activity was normalized from the amount of proHB-EGF molecules expressed on the cell surface as described previously (12). Although CD63, CD81, and CD82 are able to associate with proHB-EGF, these TM4SF molecules did not enhance the DT binding activity of proHB-EGF at all (Fig. 2A). Similar results were obtained using stable transfectants of LH cells expressing each of the TM4SF molecules (data not shown).

CD63, CD81, and CD82 associate with proHB-EGF but do not up-regulate DT binding activity of proHB-EGF. These results raise the possibility that CD63, CD81, or CD82 may inhibit the effect of CD9 in a competitive manner. We examined this possibility using HT1080D cells that are stable transformants of HT1080 cells expressing CD9. HT1080D cells express low amounts of human proHB-EGF endogenously. After transfection with CD63, CD81, or CD82 into HT1080D cells, the DT binding activity of proHB-EGF was measured. As shown in Fig. 2B, the ectopic expression of these TM4SF proteins diminished the up-regulation effect of CD9. These results indicate that CD63, CD81, and CD82 have the ability to reduce

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**Fig. 1. Co-precipitation of integrin and HB-EGF with TM4SF proteins.**

A, VeroHKa cells were surface biotinylated and then lysed with 10 mM CHAPS solution. The lysate was precipitated with anti-CD9, -CD63, -CD81, and -CD82 antibodies. The precipitates were analyzed by SDS-PAGE. Cell lysate used for immunoprecipitation of CD9 was 20% of that of the others used. Bars on the left margin show molecular mass markers in kDa. B, the long time exposure of A. proHB-EGF (20–27 kDa) were coprecipitated with anti-CD9, -CD63, -CD81, and -CD82 antibodies. C, co-precipitation of integrin α3. Immunoprecipitates from VeroHKa cells with anti-CD9, -CD63, -CD81, and -CD82 antibodies were subjected to Western blotting using anti-integrin α3 subunit antibody. Integrin α3 (150 kDa) was coprecipitated with anti-CD9, -CD63, -CD81, and -CD82 antibodies.

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**Fig. 2. Effect of TM4SF expression on DT binding to proHB-EGF.** LH cells (A) or HT1080D cells (B) were transfected with control plasmids (RcCMV), or plasmids encoding CD9, CD63, CD81, or CD82. 48 h after transfection DT binding activity was determined as described under “Experimental Procedures.” Data represent the means ± S.D. of the results obtained from triplicate samples. DT binding activity of LH cells was up-regulated by CD9 but not by other TM4SFs. In HT1080D cells, expressing both proHB-EGF and CD9, transfection of CD63, CD81, or CD82 reduced DT binding activity.
molecules were expressed on the cell surface in the expected sizes and associated with proHB-EGF as shown in co-immunoprecipitation experiments with anti-CD9 antibody or anti-CD81 antibody (data not shown). Among the chimeric constructs, DR11 and TA2 enhanced DT binding activity, whereas DR8 did not enhance it (Fig. 3D). These results indicated that the second extracellular loop of CD9 is important for up-regulation.

**Domain of proHB-EGF Necessary for Up-regulation by CD9**—We also studied the domain(s) within proHB-EGF necessary for up-regulation by CD9. To examine this we made deletion mutants of proHB-EGF. proHB-EGF can be structurally divided into seven domains: pre, pro, heparin-binding, EGF-like, juxtamembrane, transmembrane, and cytoplasmic domains. Of these domains, the heparin-binding, EGF-like, juxtamembrane, or cytoplasmic domain was deleted, and the constructs were termed ΔHBD, ΔEGF, ΔJxM, and ΔCyto, respectively. FRM is a chimeric construct in which the transmembrane domain of proHB-EGF was substituted to that of the transferrin receptor. Because the transmembrane domain is essential for anchoring of membrane proteins, a mutant form lacking transmembrane domain was not constructed. Schematic structures of these constructs are shown in Fig. 4A. It should be noted that deletion mutants shown here were made from the pseudo-wild type of proHB-EGF, which has amino acid substitutions of P149E, T160G, and T161G. The pseudo-wild type had DT binding activity, which was as up-regulated by CD9 as that of wild type proHB-EGF.

Plasmids encoding each proHB-EGF mutant and plasmids encoding CD9 or vectors only were introduced into L cells. These deletion mutants were expressed on the cell surface of the L cells in expected sizes and coprecipitated with CD9 (Fig. 4B). The DT binding activity of the transfected cells was next determined. The results are shown in Fig. 4C. Under these conditions DT binding activity of wild type proHB-EGF was up-regulated about 15-fold by the transfection with CD9. ΔHBD, ΔCyto, and FRM were significantly up-regulated by CD9, although the up-regulation for FRM seemed to be lower than wild type. These results indicated that the heparin-binding, transmembrane, and cytoplasmic domains were not essential for up-regulation and that the remaining domains must be responsible for the up-regulation. Because DT binding activity was not observed in the cells transfected with ΔEGF or ΔJxM, regardless of the presence or absence of CD9, the effect of CD9 on the DT binding activity of ΔEGF or ΔJxM could not be determined. ΔEGF and ΔJxM were expressed on the cell surface in amounts similar to wild type proHB-EGF (data not shown); therefore deletion of these domains must cause a loss of DT binding activity. The loss of DT binding activity in ΔEGF is reasonable because DT binds to the EGF-like domain of proHB-EGF (11). The failure of ΔJxM DT binding is surprising and is probably due to the impaired access of DT to the EGF-like domain of proHB-EGF without the juxtamembrane domain.²

**HB-EGF/TGF-α Chimeric Analysis**—Deletion mutant analysis still retained the possibility that either the EGF-like or the juxtamembrane domain was responsible for the up-regulation mechanism. To determine the domain necessary for up-regulation, we made a series of chimeric constructs between proHB-EGF and TGF-α. TGF-α does not bind DT (11), and the juxtamitogenic activity of TGF-α is not up-regulated by CD9 (6). N-terminal regions containing pre, pro, and heparin-binding, EGF-like, and juxtamembrane domains or both transmembrane and cytoplasmic domains of proHB-EGF were replaced

² T. Takahashi and E. Mekada, manuscript in preparation.
receptor. The whose transmembrane domain is substituted with that of transferrin receptor. The striped box represents the region of the transferrin receptor. The asterisk shows bands of CD9. A, up-regulation of DT binding activity of proHB-EGF deletion mutants by CD9. These chimeras were transfected into L cells with or without CD9 cDNA, and DT binding activity was determined. Data represent the means ± S.D. of the results obtained from triplicate samples.

with the corresponding domains of TGF-α, termed TGN, TGE, TGJ, or TGMC, respectively, as illustrated in Fig. 5A. TGN-JMC, the chimera in which the EGF-like domain of proTGF-α was replaced with that of proHB-EGF was also constructed. These chimeras were transfected into L cells with or without CD9 cDNA, and DT binding activity was determined. As shown in Fig. 5B, DT binding of TGN, TGJ, and TGMC were up-regulated 5–10 fold by the expression of CD9, whereas that of wild type proHB-EGF was 10-fold. Consistent with the deletion analysis, the substitution of the N-terminal, juxtamembrane, transmembrane, or cytoplasmic domain with the corresponding domain of TGF-α did not affect the up-regulation properties.

TGE had no DT binding activity, as expected, and thus the effect of CD9 could not be determined. It is also noteworthy that the DT binding of TGJMC was also up-regulated by the expression of CD9. These results indicate that the pre, pro, heparin-binding, juxtamembrane, and cytoplasmic domains are not essential and that these domains are dispensable to the corresponding domains of TGF-α for up-regulation by CD9, whereas the EGF-like domain of proHB-EGF is responsible for up-regulation.

Although the above studies indicated that the EGF-like domain of proHB-EGF would be essential for up-regulation by CD9, DT does not bind to the EGF-like domain of TGF-α and thus up-regulation of DT binding activity in TGE by CD9 could not be directly determined. To examine whether the replacement of the EGF-like domain of proHB-EGF by that of TGF-α results in the loss of up-regulation, juxtacrine growth factor assay was performed as described previously (6). To do this assay we used stable transfecants expressing proHB-EGF alone (LH), TGE alone (L/TGE), both proHB-EGF and CD9 (LCH), or both TGE and CD9 (L/TGE/D). As shown in Fig. 6A, LCH cells display much higher juxtacrine activity than LH cells, despite LCH cells expressing lower numbers of proHB-EGF molecules on the cell surface (Fig. 6B). In the case of TGE, two independently isolated clones of L/TGE4/D (L/TGE4/D2 and L/TGE4/D7) cells had rather lower juxtacrine activity than L/TGE4 cells (Fig. 6A), although these three cell lines expressed similar numbers of TGE molecules on the cell surface (Fig. 6B). Similar results were also obtained using other stable transfecant cell lines, L/TGE15 and L/TGE15/D2 (data not

**FIG. 4.** Analysis of proHB-EGF deletion mutants. A, schematic representation of proHB-EGF deletion mutants. ΔHBD, ΔEGF, ΔJxM, and ΔCyto are deletion mutants of the heparin-binding, EGF, juxtamembrane, and cytoplasmic domains, respectively. FRM is a mutant whose transmembrane domain is substituted with that of transferrin receptor. The striped box represents the region of the transferrin receptor. Bl, Bst11; D, DraI; X, XhoI; Bm, BamHI; K, KpnI. B, association of CD9 with proHB-EGF mutants. LC cells were transfected with control vector or proHB-EGF mutants. After incubation for 48 h, cells were surface biotinylated and lysed with 10 mM CHAPS solution. The lysates were immunoprecipitated with anti-CD9 antibody, and the precipitates were analyzed by SDS-PAGE. The asterisk shows bands of CD9.

**FIG. 5.** Analysis of proHB-EGF/TGF-α chimera. A, schematic representation of proHB-EGF/TGF-α chimera. Open and shaded boxes indicate the regions of proHB-EGF and TGF-α, respectively. B, up-regulation of DT binding activity of proHB-EGF/TGF-α chimeras by CD9. L cells were co-transfected with plasmids encoding one of the proHB-EGF/TGF-α chimeras (wild type HB-EGF, TGN, TGE, TGJ, TGMC, and TGJMC) and either control Rc/CMV vector (open boxes) or plasmids encoding CD9 (shaded boxes). After incubation for 48 h, DT binding activity was determined. DT binding activities are expressed as relative values, which were obtained by comparing with those of wild type proHB-EGF without CD9. Data represent the means ± S.D. of the results obtained from triplicate samples.
Previous studies indicated that the number of proHB-EGF molecules at the cell surface was not changed in the presence or absence of CD9 (7). Furthermore, direct binding of DT with CD9 has not been detected. Scatchard plot analysis of DT binding to proHB-EGF indicated that increased DT binding with CD9 is due to an increase in the number of effective binding sites for DT rather than any increased binding affinity for DT (7). Therefore, an increase in the number of effective binding sites must be attributable to protein-protein interaction between proHB-EGF and CD9. Co-precipitation studies showed that not only CD9 but also CD63, CD81, and CD82 are associated with proHB-EGF and integrin αβ1. However, among these TM4SF proteins only CD9 up-regulated the DT binding activity of proHB-EGF. We took advantage of the inability of CD81 to up-regulate to analyze the region necessary for up-regulation. A series of chimeric molecules was made to analyze the region of CD9 essential for up-regulation. All the chimeric molecules expressed on the cell surface, but only constructs that had a second extracellular domain of CD9 had up-regulation activity. Hence, we concluded that the second loop of CD9 is sufficient for up-regulation and that all of the transmembrane domains and the first loop between the first and the second transmembrane domains are exchangeable with CD81. Consistent with our present results, a recent report has suggested that the latter half of CD9 is necessary for up-regulation of DT sensitivity (35).

We also studied the domain of proHB-EGF necessary for up-regulation by using a series of deletion mutants of proHB-EGF and chimeric molecules between proHB-EGF and TGF-α. DT binding assay revealed that none of the domains of proHB-EGF, except for the EGF-like domain, were essential for up-regulation, whereas the chimeras that have the EGF-like domain of proHB-EGF were all up-regulated by CD9, suggesting the involvement of the EGF-like domain in up-regulation. The chimeric molecule TGE, which has the EGF-like domain of TGF-α, does not bind to DT; thus up-regulation of DT binding was not determined by this chimera. To circumvent this difficulty, juxtacrine mitogenic assay was performed, and the results showed that juxtacrine activity of TGE is not up-regulated by CD9. Thus, the domain of proHB-EGF necessary for up-regulation is the EGF-like domain. From these results, together with data obtained from CD9/CD81 chimera, we concluded that the extracellular domains of CD9 and the EGF-like domain of proHB-EGF are important for up-regulation.

Our studies demonstrated that proHB-EGF associated with CD63, CD81, and CD82 as well as with CD9, but these TM4SF proteins and some CD9/CD81 chimera failed to up-regulate DT binding activity. Moreover, it was shown that TGE was associated with CD9 (Fig. 4B) but that the juxtacrine activity was not up-regulated by CD9. Therefore, association of CD9 with proHB-EGF is probably essential but is not enough for up-regulation. We attempted to define the domains responsible for the association of proHB-EGF and CD9 in this study. However, because all of the deletion mutants of proHB-EGF, including ΔEGF and FRM, were co-precipitated with CD9, at more or less the same efficiency compared with wild type proHB-EGF, these studies did not allow us to define a particular domain of proHB-EGF necessary for association. These results may suggest that multiple domains, e.g., the EGF-like domain and the transmembrane domain, are involved in association, but the possibility of the artifact being due to the overexpression of CD9 and proHB-EGF cannot be ruled out.

The present study showed that not only CD9 but also other TM4SF members are associated with proHB-EGF. The role of the TM4SF members in this complex is unclear, but they would have different functions at cell-cell contact sites, because only
CD9 of these TM4SF members up-regulates proHB-EGF. Subcellular localization of each TM4SF member seems to be different. Immunofluorescence studies showed that CD9 and CD81 localized mainly at the cell surface, whereas CD63 mainly localizes at lysosomes and secreted vesicles. Thus CD63 may have functions in the transport of proHB-EGF or integrin α3β1 to or from cell surfaces. Consistent with this notion, association of CD63 with PI4 kinase has been reported, and its role for integrin internalization has been suggested (36). Diphtheria toxin is internalized with proHB-EGF, but the domains responsible for internalization have not been found. It would be intriguing to speculate that internalization of DT-bound proHB-EGF is achieved by assistance from a TM4SF member. Further study is needed to clarify the role of each TM4SF member in the complex.

In conclusion, the present domain analysis of CD9 and proHB-EGF suggests the importance of the interactions of both molecules at their extracellular domains. The precise molecular mechanism for up-regulation still remains to be clarified. Further studies, especially of the extracellular domains, would help with understanding the molecular mechanism of up-regulation and also help to create dominant negative forms of these proteins.

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