

The Membrane Protein CD9/DRAP 27 Potentiates the Juxtacrine Growth Factor Activity of the Membrane-anchored Heparin-binding EGF-like Growth Factor

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Abstract. The membrane-anchored heparin-binding EGF-like growth factor precursor (proHB-EGF)/diphtheria toxin receptor (DTR) belongs to a class of transmembrane growth factors and physically associates with CD9/DRAP27 which is also a transmembrane protein. To evaluate the biological activities of proHB-EGF/DTR as a juxtacrine growth factor and the biological significance of its association with CD9/DRAP27, the mitogenic activity of proHB-EGF/DTR was analyzed using stable transfectants of mouse L cells expressing both human proHB-EGF/DTR and monkey CD9/DRAP27, or either one alone. Juxtacrine activity was assayed by measuring the ability of cells in co-culture to stimulate DNA synthesis in an EGF receptor ligand dependent cell line, EP170.7. LH-2 cells expressing human proHB-EGF/DTR stimulated EP170.7 cell growth moderately. However, LCH-1 cells, a stable co-transfectant expressing both human

proHB-EGF/DTR and monkey CD9/DRAP27 cDNAs, dramatically unregulated the juxtacrine growth factor activity of proHB-EGF/DTR approximately 25 times over that of LH-2 cells even though both cell types expressed similar levels of proHB-EGF/DTR on the cell surface. Anti-CD9/DRAP27 antibodies which were not able to neutralize the mitogenic activity of soluble HB-EGF suppressed LCH-1 cell juxtacrine growth activity to the same extent as did anti-HB-EGF neutralizing antibodies and CRM 197, specific inhibitors of human HG-EGF. These findings suggest that optimal expression of the juxtacrine growth activity of proHB-EGF/DTR requires co-expression of CD9/DRAP27. These studies also indicate that growth factor potentiation effects which have been observed previously for soluble growth factors also occurs at the level of cell surface associated growth factors.

HEPARIN-binding EGF-like growth factor (HB-EGF)¹ was first identified as a 20–22-kD glycoprotein by macrophages and macrophage-like cells (3, 18). It is structurally a member of the EGF family which encompasses a number of structurally homologous mitogens including EGF, transforming growth factor- α (TGF- α), vaccinia virus growth factor (VGF) (9), amphiregulin (AR)

(43), and β -cellulin (42). Like EGF, TGF- α , and AR, HB-EGF binds to and stimulates phosphorylation of the EGF receptor. Secreted mature HB-EGF is a potent mitogen for NIH3T3 cells, bovine aortic smooth muscle cells (BASMC) (18, 20), rat hepatocytes (23), and human keratinocytes (16, 30).

Analysis of the nucleotide sequence of human HB-EGF cDNA predicts a precursor protein of 208 amino acids composed of putative signal peptide, heparin-binding, EGF-like, transmembrane and cytoplasmic domains (18, 19, 47). The HB-EGF precursor can be cleaved to yield a mature biologically active protein containing 75–86 amino acids (19). While mature HB-EGF is a potent mitogen, there is also the possibility that proHB-EGF is biologically active as well. For example, it has been shown that several growth factors and lymphokines are synthesized as membrane-anchored proteins, including the EGF family of growth factors, tumor necrosis factor- α (TNF- α), colony-stimulating factor-1

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1. *Abbreviations used in this paper:* AR, amphiregulin; BASMC, bovine aortic smooth muscle cells; CSF-1, colony-stimulating factor-1; DT, diphtheria toxin; DTR, DT receptor; EGFR, EGF receptor; HB-EGF, heparin-binding-EGF; hHB-EGF, human HB-EGF; HSPG, heparan sulfate proteoglycans; KL-1-2, c-kit ligand-1,2; TNF- α , tumor necrosis factor- α ; VGF, vaccinia virus growth factor.

(CSF-1), and c-kit ligand-1,2 (KL-1,2) (reviewed in reference 31). These transmembrane forms are biologically active. For example, the TGF- α precursor in co-culture stimulate EGF receptor (EGFR) phosphorylation, mitogenesis, and Ca²⁺ uptake in which has been termed "juxtacrine stimulation" (1, 4, 6, 51). Transmembrane KL is required for the development of melanocytes, germ cells, and hematopoietic stem cells. Soluble KL can not substitute for the transmembrane form in vivo (7, 14, 21).

An important feature of proHB-EGF is that it is not only a growth factor precursor but that it acts as the specific receptor for diphtheria toxin (DT) and mediates endocytosis of the receptor-bound toxin resulting in its entry into the cytoplasm (25, 35). Interestingly, proHB-EGF/DTR receptor (DTR) forms a complex with another membrane protein known as CD9 (25, 32). CD9 was first identified as a cell surface antigen on lymphohemopoietic cells (26, 27). It is identical to the 27-kD diphtheria toxin receptor associated protein (DRAP27) (24). Anti-DRAP27 antibodies inhibit the binding of DT to proHB-EGF/DTR and co-precipitate proHB-EGF/DTR and CD9/DRAP27 suggesting that these two proteins are closely associated in the cell membrane (24, 25). Furthermore, co-expression of CD9/DRAP27 with proHB-EGF/DTR markedly enhances DT binding and DT sensitivity of cells (8, 25, 32). The ability of CD9/DRAP27 to upregulate DT sensitivity prompted us to investigate the role of CD9/DRAP27 in regulating HB-EGF growth factor activity, especially as a co-factor in juxtacrine stimulation.

Using a co-culture system of donor cells expressing proHB-EGF/DTR in contact with acceptor cells expressing EGF receptor, we demonstrate: (a) that proHB-EGF/DTR stimulates cell growth in a juxtacrine manner; and (b) that co-expression of CD9/DRAP27 upregulates proHB-EGF/DTR juxtacrine growth factor activity to a great extent.

Materials and Methods

Materials

Sulfo-NHS-Biotin and immobilized protein A were purchased from Pierce Chemical Co. (Rockford, IL). ECL-Western blotting kits were purchased from Amersham (Buckinghamshire, England). CRM197 was prepared as described previously (49). Anti-human HB-EGF antibodies, H6 (25), and M6 were produced by immunizing rabbits with synthetic peptides GGGRRDRKVRDLQEQADLDLLR and ENEEKVKGMTNSH, corresponding to amino acids 54-73 (extracellular domain) and 195-208 (cytoplasmic domain) of the human HB-EGF precursor, respectively (18). Anti-human HB-EGF neutralizing antibodies were directed against a 75-amino acid form of recombinant human HB-EGF and prepared in goats by Deborah Damm (Scios Nova Inc., Mountain View, CA) (16). Anti-CD9 monoclonal antibody, ALB-6, was purchased from MBL (Nagoya, Japan).

Transfections

Both transient and stable transfections were carried out using human HB-EGF cDNA (19) for the expression of proHB-EGF/DTR, and monkey DRAP27 cDNA (32) for the expression of CD9/DRAP27 as described previously (25). Stable transfectants expressing proHB-EGF/DTR alone (LH-1, 2, 3, and 4) were derived from parental L cells and transfectants expressing both proHB-EGF/DTR and CD9/DRAP27 (LCH-1) were derived from LC14 cells, a CD9/DRAP27 transfected cell line previously described (32). L cells and LC14 cells (5×10^5 cells) were transfected with 20 μ g of pRcHB-EGF by the calcium-phosphate method (10), cultured for 48 h and further cultured for 7 d in selection medium DME containing 10% FCS and 1 mg/ml G418. Colonies growing in the selection medium were isolated and analyzed for ¹²⁵I-DT binding as described previously (25). LH-1,

LH-2, LH-3, and LH-4 cells which express proHB-EGF/DTR at different levels, and LCH-1 cells which express both proHB-EGF/DTR and CD9/DRAP27 were passaged in culture and used for further experiments.

Human TGF- α cDNA was a kind gift from Dr. Rik Derynck (University of California, San Francisco, CA). A 720-bp HindIII-EcoRI fragment of TGF- α subcloned into pRK7 α (11) was inserted into a HindIII-EcoRI site of pBluescript II SK(-). The resulting plasmid containing TGF- α cDNA was digested at Apal-XbaI sites. An Apal-XbaI fragment was inserted into the corresponding restriction enzyme sites in the expression vector, pRc/CMV (Invitrogen Corp., San Diego, CA). The resulting plasmid was transfected transiently as described previously (25) by the calcium phosphate method (10).

Cell Culture and Growth Factor Activity Measurements

L, LC, 3279-10, and F-10 melanoma cell lines were grown in DME supplemented with 10% FCS, penicillin (100 U/ml) streptomycin (100 μ g/ml). HB-EGF-transfected cell lines were cultured in the same media with the addition of G418 (0.5 mg/ml). EPI70.7 cells, obtained from Dr. Jackie Pierce (NIH, Bethesda, MD) (37), were grown in RPMI 1640 medium supplemented with 10% FCS, 5% WEHI-3 cell conditioned medium, penicillin (100 U/ml), and streptomycin (100 μ g/ml) (5). Human-mouse hybrid 3279-10 cells and its transfectants expressing CD9/DRAP27 were grown in DME supplemented with 10% FCS, penicillin (100 U/ml), streptomycin (100 μ g/ml), and 0.3 mg/ml G418 (32).

Growth factor activity was monitored by measuring the incorporation of [³H]thymidine into the DNA of exponentially growing EPI70.7 cells. EPI70.7 cells were washed twice with RPMI 1640 lacking WEHI-3 conditioned medium, and then plated at 2×10^4 cells/well in a volume of 200 μ l in 96-well plates. Samples of recombinant HB-EGF with and without blocking reagents were added and 36 h later, [³H]thymidine (1 μ Ci/well; 1 mCi = 37MBq) was added to a well. After a 4-h incubation, the EPI70.7 cells were harvested and [³H]thymidine incorporated into DNA was measured with the Betaplate system (Pharmacia LKB, Uppsala, Sweden).

The juxtacrine growth factor assays were carried out in several ways as follows: (a) Co-culture of formalin fixed transfectants and EPI70.7 cells: Parental cells and transfectants of L cells, and human-mouse hybrid cells were plated in DME/10% FCS (500 μ l/well) in 24-well plates and incubated for 12 h prior to washing and fixation. The cells were washed twice with DME/10% FCS/2 M NaCl to remove soluble HB-EGF trapped by cell surface heparan sulfate proteoglycans (34), and fixed with 5% buffered formalin for 5 min. The formalin fixed cells were washed twice with RPMI 1640/10% FCS and EPI70.7 cells (1×10^5 cells/500 μ l/well) were added in co-culture. After 48 h, [³H]thymidine (1 μ Ci/well; 1 mCi = 37MBq) was added to the wells and the co-cultured cells were incubated for 4 h. The EPI70.7 cells were harvested and analyzed for incorporation of [³H]thymidine into DNA. (b) Co-culture of live transfectants and EPI70.7 cells: transiently transfected L cells were plated in DME/10% FCS (500 μ l/well) in 24-well plates, incubated for 12 h and washed twice with RPMI 1640/10% FCS. Incubation with EPI70.7 cells and [³H]thymidine labeling EPI70.7 cells was carried out as described in a. (c) Transwell plates: transient transfectants of L cells were plated in DME/10% FCS (500 μ l/well) in 24-well plates. After washing the cells either with or without fixation as described above, the cells were covered with 250 μ l of RPMI 1640/10% FCS. Transwells (12-mm diam, 0.4- μ m pore size; Costar, Cambridge, MA) were introduced into the wells of the 24-well plate containing L cell transfectants and EPI70.7 cells (1×10^5 cells/250 μ l/well) were added to the Transwells. [³H]Thymidine labeling of EPI70.7 cells were carried out as described in a.

Flow Cytometry

Stably transfected cell lines were grown in 10 cm culture dishes at a density of 5×10^5 cells/dish for 12 h. To remove any soluble HB-EGF which might bind to heparan sulfate proteoglycans (HSPG) on the cell surface, cells were washed twice with medium containing 2 M NaCl, and twice with PBS (34). The cells were detached from dishes with PBS containing 0.5 mM EDTA, and centrifuged at 500 g for 3 min. After washing the cell pellets twice with ice-cold PBS, the pellets were incubated with anti-HB-EGF antibody, H6, or anti-CD9 monoclonal antibody ALB-6 for 30 min on ice. The cells were washed twice with ice-cold PBS and then incubated with FITC-conjugated goat anti-rabbit IgG (Cappel, Oregon Teknika, Durham, NC) or FITC-conjugated goat anti-mouse IgG (Becton Dickinson, San Jose, CA) on ice for 30 min. The cells were washed twice with

ice-cold PBS and analyzed using a FACScan® (Becton Dickinson). A scatter window was set to eliminate dead cells and cell debris. 1×10^4 cells were acquired by list mode, and measurements were performed on a single cell basis and displayed as frequency distribution histograms.

Northern Blot Analysis

Total RNA was isolated and analyzed by Northern blot from L and F-10 cells as described previously (12). Briefly an aliquot of total RNA (2.3 μ g) was denatured by formaldehyde, electrophoresed on a 1.2% agarose gel, and transferred to a Hybond-N filter (Amersham Intl., Amersham, UK). Hybridization was performed with single-stranded, uniformly 32 P-labeled cDNA probes corresponding to an EcoRI-XhoI fragment of the mouse CD9 gene.

Cell Surface Biotinylation, Immunoprecipitation, and Western Blotting

LH and LCH cells (3×10^6 cells) were grown overnight in 10 cm dishes. Cells were washed three times with ice-cold Hanks buffer, and biotinylated by 30-min incubation on ice with 0.1 mg/ml of sulfo-NHS-biotin (Pierce Chemical Co., Rockford, IL) in 100 mM Hepes, pH 8.0, 0.15 M NaCl. Excess reagent was quenched and removed by washing with DME/10% FCS. Cells were lysed with a lysis buffer (500 μ l of 1% Triton X-100, 3 mM EDTA, 1 mM benzamidine, 1 mM p-amidino phenylmethylsulfonyl fluoride, 5 μ M 3,4-dichloroisocoumarin, 10 μ g/ml Aprotinin, and 0.4 M NaCl in 20 mM Hepes, pH 7.2). Supernatants of the cell lysates were incubated for 2 h at 4°C with 10 μ g of H6 IgG followed by the incubation for 2 h at 4°C with 10 μ l of protein A trisacryl (50% suspension) (Pierce Chemical Co.). Samples were dissolved in SDS-PAGE sample buffer and fractionated by 15% SDS-PAGE. Proteins in the gels were transferred to a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany) in 150 mM 3-[cyclohexylamino]-1-propane-sulfonic acid (CAPS) buffer, pH 11, containing 20% methanol at 180 mA for 3 h. The nitrocellulose membrane was blocked with 5% skimmed milk in PBS and incubated for 30 min at room temperature with avidin-HRP (Vectastatin, Burlingame, CA). HRP was detected by ECL kit (Amersham) and autoradiography. Intensity of bands exposed to a X-OMAT film (Kodak, Rochester, NY) was measured with the Scanning Imager (Molecular Dynamics, Sunny Vale, CA).

Results

proHB-EGF/DTR Mediates Mitogenic Activity in a Juxtacrine Manner

To test the potential juxtacrine activity of proHB-EGF/DTR, we developed a co-culture system in which donor cells expressing proHB-EGF/DTR and acceptor cells expressing EGFR but not HB-EGF were incubated together. For the latter, EP170.7 cells were chosen, since they are uniquely IL-3 dependent but have been transfected with EGFR and respond only to EGFR ligands in the absence of IL-3. Mouse L cells transfected with human HB-EGF (hHB-EGF) cDNA were used as the source of membrane-anchored growth factor. Since previous studies showed that L cells transfected with hHB-EGF cDNA produce substantial proHB-EGF/DTR on the cell surface but also spontaneously release some soluble HB-EGF into the conditioned media (25), these cells were fixed with 5% buffered formalin prior to co-culture to prevent processing, a method previously used to analyze cell associated CSF-1 (46). The EP170.7 cells were overlaid on to a fixed monolayer of L cells and co-cultured for 48 h. The contact-dependent growth stimulation of EP170.7 cells by L cells was then assayed by measuring the incorporation of [3 H]thymidine into EP170.7 cell DNA. Mock L cell transfectant did not stimulate EP170.7 cell growth under these conditions (Fig. 1 A). However, hHB-EGF-transfected L cells stimulated the growth of EP170.7 cells in proportion

to the number of L cells plated. At 20×10^4 L cells, a 4,500-fold increase in EP170.7 DNA synthesis was seen compared to control mock transfectants (465,000 cpm vs. 103 cpm) (Fig. 1 A). As a control, the hHB-EGF-transfected L cells did not stimulate parental 32D cells lacking EGFR (data not shown). To determine whether a juxtacrine cell-to-cell contact mechanism was involved in stimulating EP170.7 cell growth in the co-culture system, the two cell types were grown in Transwells so that EP170.7 cells and L cells were separated physically but diffusion of soluble HB-EGF could occur. As shown in Fig. 1 A, this procedure abrogated more than 90% of EP170.7 cell growth induced by hHB-EGF transfectants at 20×10^4 cells/well. Moreover, conditioned media prepared from fixed hHB-EGF transfectants at 20×10^4 cells/ml co-cultured with and without EP170.7 cells were tested for EP170.7 cell DNA synthesis in order to estimate the level of released HB-EGF. Mitogenic activities in both the conditioned media were undetectable level. These results suggested that growth stimulation of EP170.7 cells by co-cultured hHB-EGF transfectants is mediated mainly by cell-to-cell contact via proHB-EGF/DTR-EGFR interactions in a juxtacrine manner.

To ensure that no artifact of co-culture occurred with fixed cells, the juxtacrine activity of proHB-EGF/DTR was also analyzed in a co-culture of live non-fixed cells. Live HB-EGF transfectants greatly enhanced EP170.7 cell proliferation, about 16-fold over mock transfectants at 5×10^4 cells/well (Fig. 1 B). Physical blocking of cell-to-cell contact in Transwells abrogated EP170.7 cell growth by about 40% (Fig. 1 B), suggesting that while live L cells stimulate EP170.7 cell proliferation in a juxtacrine manner, a substantial amount of paracrine activity mediated by a soluble HB-EGF occurred in the live cell co-culture as well. In fact both conditioned media prepared from hHB-EGF transfectants at 5×10^4 cells/ml with and without EP170.7 cells induced DNA synthesis of EP170.7 cells corresponding to 20-30% activity of the total activity shown in the co-culture at 5×10^4 cells of hHB-EGF transfectants. Given the undetectable level of HB-EGF paracrine activity in the fixed L cell co-culture unlike that in live cells, this method was chosen as the best way to measure proHB-EGF/DTR juxtacrine activity.

A series of four LH cell lines were cloned and analyzed quantitatively for both juxtacrine activity and for proHB-EGF/DTR protein levels by flow cytometry using an antibody (H6) directed against the extracellular domain of proHB-EGF/DTR. These cell lines were named LH-1, LH-2, LH-3, and LH-4 in order of increased cell surface proHB-EGF/DTR expression as measured by flow cytometry (data not shown). In addition, the expression of cell surface proHB-EGF/DTR was analyzed quantitatively by a combination of cell surface biotinylation and immunoprecipitation using an antibody (M6) that recognizes the cytoplasmic domain of proHB-EGF/DTR, and which as a consequence immunoprecipitates proHB-EGF/DTR specifically, but can not precipitate any soluble forms of mature HB-EGF potentially trapped by cell surface HSPG. As shown in Fig. 2 A, proHB-EGF/DTR migrated as heterogeneous molecules with molecular masses of about 20, 24, and 27 kD on SDS-PAGE, consistent with our previous report (25). The expression level of proHB-EGF/DTR in the four L cell transfectants was estimated by measuring the intensities of the

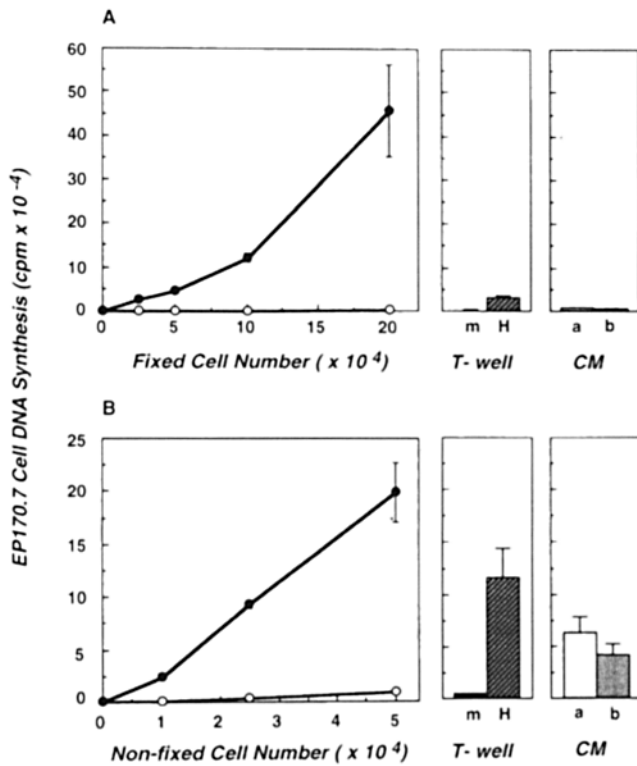


Figure 1. Juxtacrine growth factor activity of L cells expressing proHB-EGF/DTR. Formalin fixed (A) or non-fixed (B) L cells transiently transfected with HB-EGF cDNA and plated at various number of cells/well were co-cultured with 1×10^5 EP170.7 cells and after 48 h, the incorporation of [3 H]thymidine into EP170.7 cell DNA was measured. \circ , Mock-transfected L cells; \bullet , transiently transfected L cells. (T-well) Co-culture was also carried out in Transwells (T-well) using 20×10^4 fixed HB-EGF transfected L cells/well (bar graph in A; m, mock; H, HB-EGF) or 5×10^4 unfixed HB-EGF-transfected L cells/well (bar graph in B; m, mock; H, HB-EGF). (CM) EP170.7 cell DNA synthesis assay was carried out to estimate the mitogenic activities in the media conditioned by 20×10^4 fixed HB-EGF-transfected L cells alone (bar graph in A; a), 20×10^4 fixed HB-EGF-transfected L cells with 1×10^5 EP170.7 cells (bar graph in A; b), 5×10^4 unfixed HB-EGF-transfected L cells alone (bar graph in B; a) and 5×10^4 unfixed HB-EGF-transfected L cells with EP170.7 cells (bar graph in B; b).

bands using a densitometer (Fig. 2 B). LH-2, LH-3, and LH-4 cells expressed 2.8, 14, and 73 times more immunoprecipitable proHB-EGF/DTR than LH-1 cells, respectively. The L cells and the four stable transfectants were fixed and co-cultured with EP170.7 cells and their growth stimulatory activities were measured (Fig. 2 C). Unlike L cells, four LH cell transfectants promoted EP170.7 cell growth. The growth factor activities of LH-2, LH-3, and LH-4 cells were 2.6, 13, and 61 times greater than that of LH-1 cells, respectively, at 10×10^4 cells/well. Thus the juxtacrine activity of the LH transfectants correlated positively with proHB-EGF/DTR cell surface levels in a very quantitative manner.

Coexpression of CD9/DRAP27 with proHB-EGF/DTR Greatly Enhances Juxtacrine Growth Stimulation Activity

Previous studies on the diphtheria toxin receptor indicated that proHB-EGF/DTR forms a complex with CD9/DRAP27

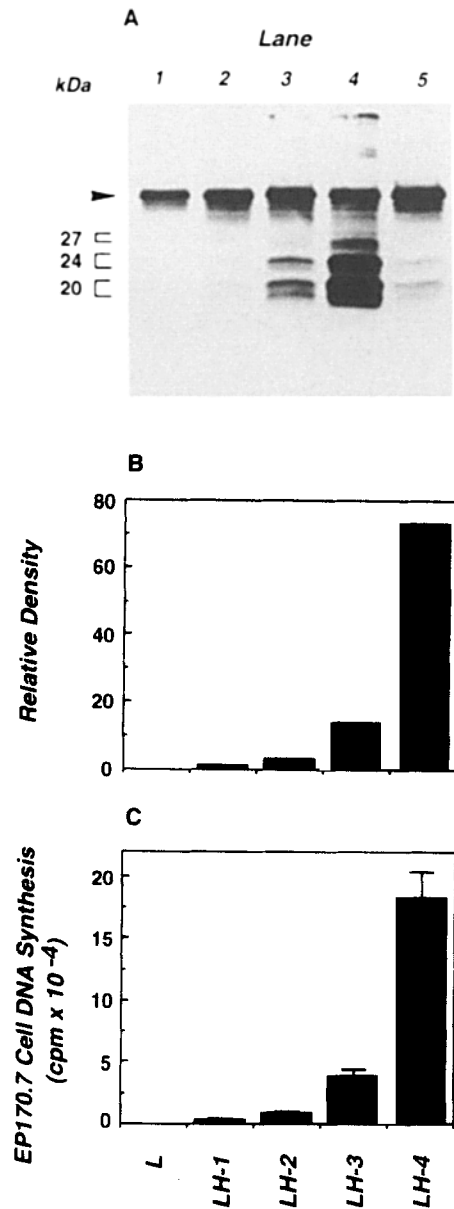


Figure 2. proHB-EGF/DTR protein levels on the cell surface and juxtacrine growth factor activities on HB-EGF-transfected L cells. (A) Immunoprecipitation of proHB-EGF/DTR. Cell surface proteins were biotinylated and the proHB-EGF/DTR was immunoprecipitated with anti-HB-EGF antibodies (M6) from L cells (lane 1) and four HB-EGF-transfected cell lines (lane 2, LH-1; lane 3, LH-2; lane 4, LH-3; lane 5, LH-4). The immunoprecipitated protein was fractionated by SDS-PAGE and after transfer to a nitrocellulose membrane, the biotinylated proteins were probed with avidin-HRP and detected by autoradiography. Bands pointed out by an arrowhead were nonspecifically detected. (B) Densitometric analysis of the immunoprecipitated proHB-EGF/DTR shown in A. For each cell type, the intensities of the immunoreactive bands ranging from 20–27 kD were measured together. (C) Juxtacrine growth factor activities of L cells and the four stable HB-EGF transfectants. Cells were plated at 10×10^4 cells/well and juxtacrine activity was measured as in Fig. 1 A.

in the cell membrane and that CD9/DRAP27 enhances the number of functional diphtheria toxin receptor without an increase in the number of cell surface proHB-EGF/DTR molecules (25). Accordingly, we analyzed whether CD9/

DRAP27 enhances the juxtacrine activity of proHB-EGF/DTR as well. Northern blot analysis showed that transcripts of the CD9 gene were undetectable in L cells but that the probe could detect CD9 mRNA in a control melanoma F-10 cell line (Fig. 3 A). Thus, monkey CD9/DRAP27 cDNA was co-transfected with HB-EGF cDNA into L cells, and a stable transfectant cell line (LCH-1) was cloned. A stable transfectant expressing CD9/DRAP27 alone (LC) was also cloned. CD9/DRAP27 protein expression in both LCH-1 and LC cells was analyzed by flow cytometry and shown to be about equivalent (Fig. 3 B). Quantitative immunoprecipitation demonstrated that LCH-1 cells (Fig. 4 A, lane 4) produced 1.3 times more proHB-EGF/DTR than did the LH-2 cells

(Fig. 4 A, lane 2), and about 20 times less than LH-4 cells (Fig. 4 A, lane 3). The juxtacrine growth factor activities of LH-2 and LCH-1 cells were compared (Fig. 4 B), and despite a comparable production of proHB-EGF/DTR, LCH-1 cells displayed about 33 times more juxtacrine activity than did LH-2 cells at 5×10^4 cells/well. Actually the LCH-1 cells displayed even more juxtacrine activity, 2.8-fold at 5×10^4 cells/well, than did LH-4 cells, which are high expressors of proHB-EGF/DTR (Fig. 4 A, lane 3). LC cells showed very little, if any, juxtacrine activity. These results suggested that CD9/DRAP27 upregulate proHB-EGF/DTR juxtacrine activity to a large extent.

Neutralization of proHB-EGF/DTR Activity

The anti-HB-EGF neutralizing antibody which specifically neutralizes the mitogenic activity of human HB-EGF but not other EGF family members such as EGF, TGF- α , AR, and β -cellulin (16), inhibited approximately 90% of EP170.7 jux-

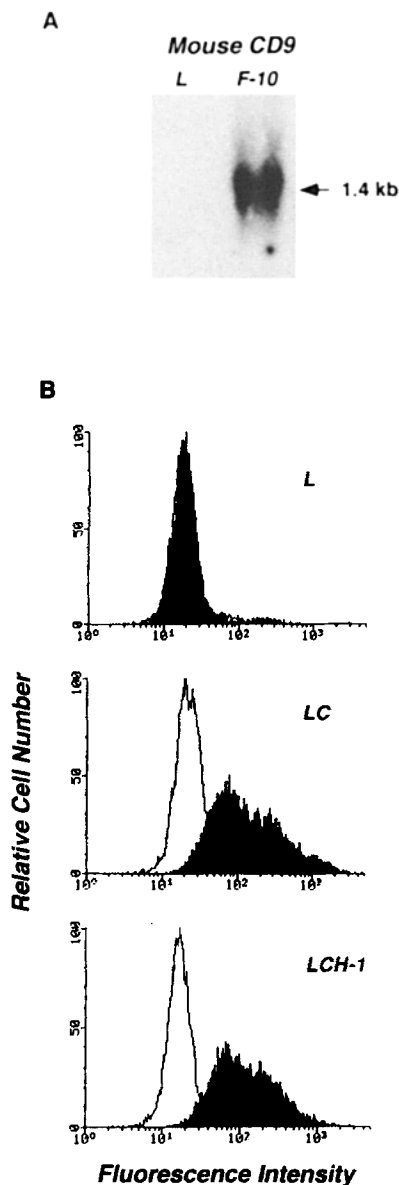


Figure 3. Expression of CD9/DRAP27. (A) Expression of CD9 mRNA in L cells. L cell and control mouse melanoma F-10 cell RNAs were subjected to Northern blotting using a mouse CD9 cDNA probe. (B) CD9/DRAP27 protein expressed on L, LC, and LCH-1 cells. The cells were incubated with anti-CD9 antibody ALB6, then with FITC-conjugated goat anti-mouse IgG, and fluorescence intensity was measured by flow cytometry.

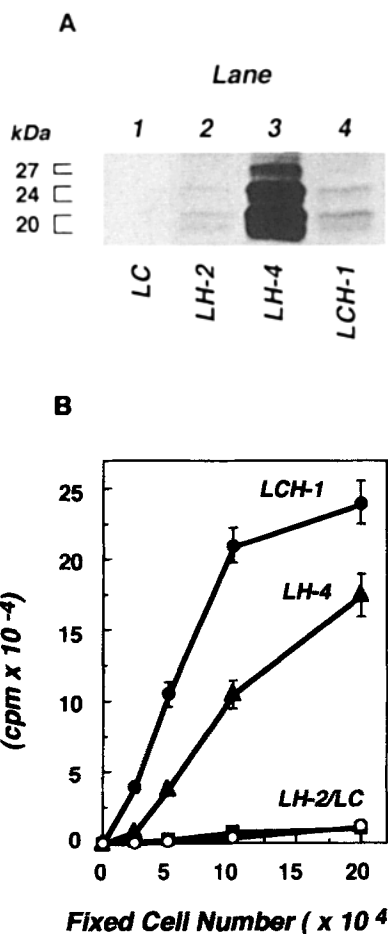


Figure 4. Upregulation of proHB-EGF/DTR juxtacrine growth factor activity of CD9/DRAP27 co-expression. (A) Immunoprecipitation of pro-HB-EGF/DTR. proHB-EGF/DTR produced by LC (lane 1) and L cell HB-EGF transfectants, LH-2 (lane 2), LH-4 (lane 3), and LCH-1 (lane 4) was biotinylated, immunoprecipitated with anti-HB-EGF antibody M6, and after SDS-PAGE analyzed with avidin-HRPO. (B) Juxtacrine growth factor activity. The four cell lines described in A were co-incubated with EP170.7 cells and juxtacrine growth factor activity measured as in Fig. 1 A. LC (\circ), LH-2 (\bullet), LH-4 (\blacktriangle) LCH-1 (\bullet).

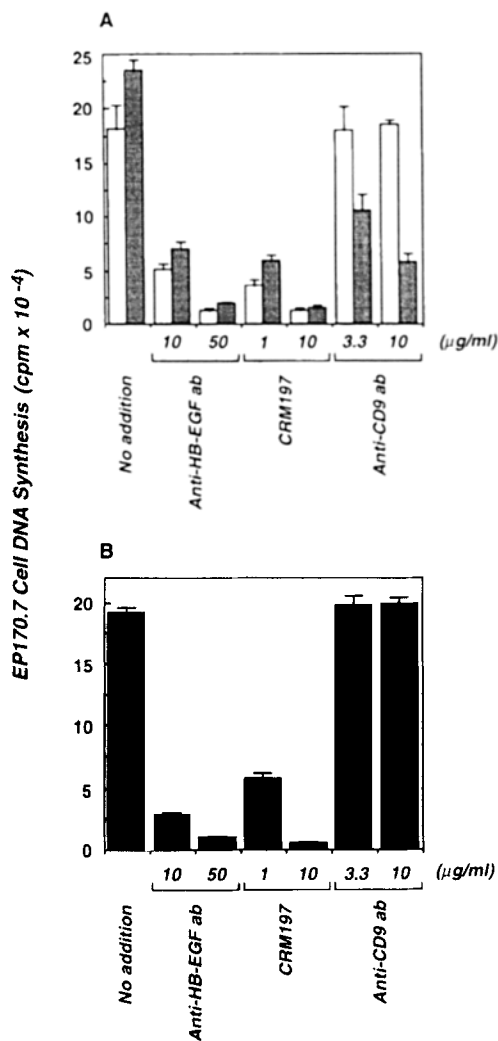


Figure 5. Neutralization of proHB-EGF/DTR juxtacrine growth factor activity. (A) Inhibition of EP170.7 cell growth induced by proHB-EGF/DTR in a juxtacrine manner. Anti-HB-EGF blocking antibodies, CRM197, or anti-CD9 antibodies, at the indicated concentrations, were added to EP170.7 cells (2×10^4 cells/well) being co-cultured with L cell HB-EGF transfectants (1×10^5 cells/well). LH-4 cells, white bars; LCH-1 cells, gray bars. (B) Effect of anti-CD9 antibodies on a secreted form of HB-EGF. Anti-CD9 antibodies, anti-HB-EGF blocking antibodies or CRM-197, at the indicated concentrations, were added to 2 ng/20 μ l of 75 amino acid recombinant mature human HB-EGF (46), the mixtures were added to a 96-well plate of EP170.7 cells and the ability to stimulate DNA synthesis in EP170.7 cells was measured.

tacrine cell growth stimulated by LH-4 or LCH-1 cells (Fig. 5 A). CRM 197, a non-toxic mutant of DT which binds to proHB-EGF/DTR and inhibits soluble HB-EGF specifically but not other EGF receptor ligands, such as EGF, TGF- α , AR, and β -cellulin (33) also inhibited over 90% of the juxtacrine growth activities of these two cell lines. These results confirmed that the growth stimulation of EP170.7 cells by L cell transfectants was due to proHB-EGF/DTR and not to other EGFR ligands. Since CD9/DRAP27 upregulated the juxtacrine growth activity of proHB-EGF markedly, and a tight physical association between proHB-EGF and CD9/

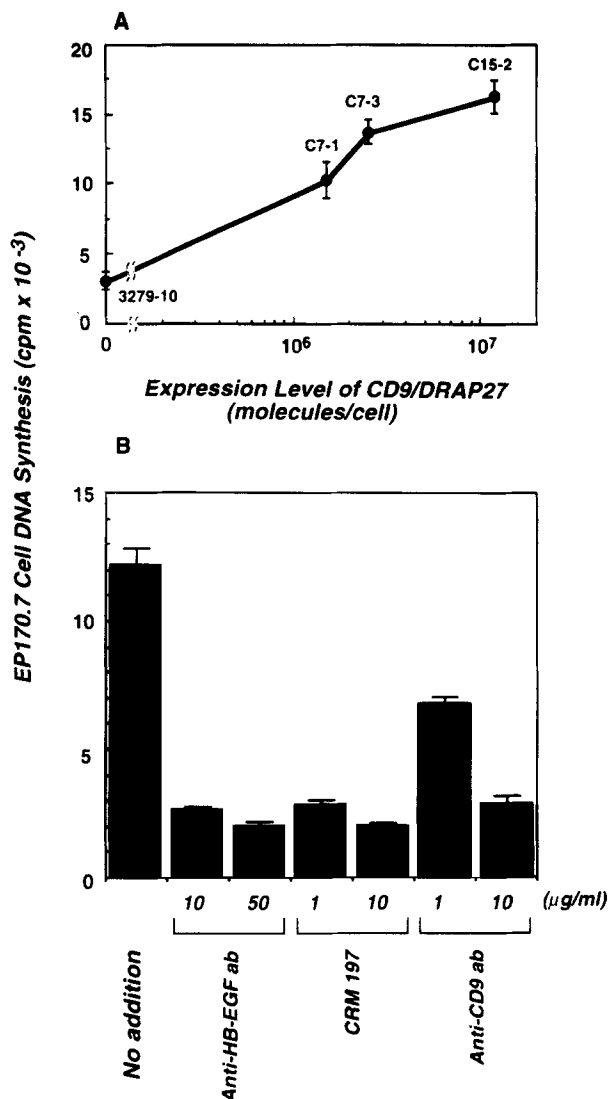


Figure 6. Effect of CD9/DRAP27 co-expression on the juxtacrine growth factor activity of human-mouse hybrid cells. (A) Human-mouse hybrid 3279-10 cells and three stable transfectants expressing variable amount of CD9/DRAP27 were plated at 2×10^5 cells/well and juxtacrine growth factor activity for EP170.7 cells was measured as in Fig. 1 A. The numbers of CD9/DRAP27 molecules on the cell surface were determined by binding experiments with a saturating amount (100 μ g/ml) of ¹²⁵I-labeled anti-DRAP27 ab (007) at 4°C for 6 h (32). (B) Neutralization of juxtacrine activity. Anti-HB-EGF blocking antibodies, anti-CD9 antibodies (ALB6), and CRM 197 were added to co-cultures of C15-2 cells (the highest expressor of CD9/DRAP27) and EP170.7 cells at 2×10^5 cells/well and juxtacrine activity measured as in A.

DRAP27 on the cell membrane has been shown (25), the effect of anti-CD9 antibodies on the juxtacrine growth factor activity of cell surface proHB-EGF was also tested. The anti-CD9 antibodies at 10 μ g/ml suppressed the juxtacrine growth factor activity of LCH-1 cells by 75%, but had no effect on the juxtacrine growth factor activity of LH-4 cells (Fig. 5 A). These results demonstrated that anti-CD9 antibodies did not directly inhibit proHB-EGF juxtacrine activity, but inhibited indirectly probably due to the steric

hindrance. The lack of direct inhibition was further demonstrated by showing that anti-CD9 antibodies did not inhibit the mitogenic activity of soluble mature HB-EGF whereas anti-HB-EGF neutralizing antibodies and CRM197 inhibited this activity totally (Fig. 5 B).

Dose-dependent Up-regulation of proHB-EGF/DTR Juxtacrine Activity by CD9/DRAP27 in Cells Expressing Endogenous Human proHB-EGF/DTR

As described above, L cells transfected with hHB-EGF showed significant growth stimulation of EP170.7 cells in a juxtacrine manner and co-expression of CD9/DRAP27 markedly enhanced its activity. However, these cells are transfectants that over-express proHB-EGF/DTR and may not reflect a situation in which proHB-EGF/DTR is expressed naturally. Therefore, the potentiating effect of CD9/DRAP27 on the juxtacrine activity of proHB-EGF/DTR in 3279-10 cells which express proHB-EGF/DTR was examined naturally. The cell line 3279-10 is a hybrid cell line of human skin fibroblasts and mouse L cells, which carried only two human chromosomes 5 and 22 (17). Human HB-EGF/DTR and CD9/DRAP27 are located in chromosome 5 (13) and chromosome 12 (2), respectively. As expected the cell line expresses human HB-EGF/DTR but not human CD9/DRAP27 (32). CD9/DRAP27 cDNA was transfected into 3279-10 cells and three stable transfectants C7-1, C7-3, and C15-2 were isolated which expressed different levels of CD9/DRAP27 (32). The juxtacrine activities of these cell lines were measured as a function of CD9/DRAP27 expression (Fig. 6 A). The levels of juxtacrine growth factor activity correlated positively with the level of CD9/DRAP27 antigen on the cell surface (Fig. 6 A). In addition, the juxtacrine activity expressed by the highest CD9/DRAP27 expressor, C15-2, was neutralized by anti-HB-EGF neutralizing antibodies, by CRM 197 and by anti-CD9 antibodies to about 20% of control, which is equivalent to the juxtacrine growth

factor activity of 3279-10 cells not expressing CD9/DRAP27 (Fig. 6 B). Thus, the potentiating activity of CD9/DRAP27 could be demonstrated for both transfected and endogenous proHB-EGF/DTR.

Coexpression of CD9/DRAP27 Does Not Upregulate proTGF- α Activity

To evaluate the specificity of CD9/DRAP27 on the juxtacrine activity of proHB-EGF, we tested the effect of CD9/DRAP27 on proTGF- α juxtacrine activity transiently expressed on L cells. Transient transfectants of proTGF- α expressed significant juxtacrine activity as compared with mock transfectants as well as did proHB-EGF transient transfectants (Fig. 7). While cotransfection of CD9/DRAP27 cDNA upregulated the juxtacrine growth factor activity of proHB-EGF for a great extent, it did not alter the juxtacrine activity of proTGF- α at all. These results suggest that CD9/DRAP27 might be a unique upregulator for proHB-EGF, and that an NH₂-terminal extended sequence, heparin-binding domain, existed in proHB-EGF, but not in proTGF- α might be required for the upregulation of its juxtacrine activity.

Discussion

We have demonstrated in these studies that: (a) HB-EGF exists in a cell-associated form, proHB-EGF/DTR, which is a juxtacrine growth factor for cells expressing EGFR; and (b) proHB-EGF/DTR juxtacrine growth factor activity is up-regulated markedly by another transmembrane protein known as CD9/DRAP27. Previously, most studies on HB-EGF have involved the secreted form, a 20–22-kD protein mitogenic in a paracrine manner for cells such as fibroblasts, SMC and keratinocytes. However, the open reading frame of HB-EGF cDNA predicts an HB-EGF precursor form which could be processed to a mature secreted form. When mouse L cells are transfected with human HB-EGF cDNA encoding the whole precursor molecule, they express cell surface proHB-EGF/DTR that is found in 20-, 24-, and 27-kD forms as determined by flow cytometry and immunoprecipitation with highly specific anti-HB-EGF antibodies. These multiple forms might represent different glycosylation forms of HB-EGF which has been shown to be a glycoprotein or multiple forms produced by processing as shown for mature HB-EGF (19).

Juxtacrine activity can be demonstrated by the ability of L cells transfected with HB-EGF cDNA to stimulate in coculture, DNA synthesis in EP170.7 cells, a myeloid cell line engineered to express EGFR (37). The parental cell line of EP170.7, 32D, is not stimulated by HB-EGF-transfected cells in a juxtacrine manner nor are parental L cells capable of stimulating juxtacrine activity. The level of juxtacrine stimulation correlates positively with the number of transfected L cells used and the amount of proHB-EGF/DTR being produced as monitored by flow cytometry and immunoprecipitation. To show that these proHB-EGF/DTR-EGFR juxtacrine interactions were mediated by cell-cell contact, cells were plated in a Transwell dish in which the proHB-EGF/DTR-transfected L cells were physically separated from the EP170.7 cells. Under these conditions, virtually no juxtacrine growth factor activity occurred. Further, neither of the

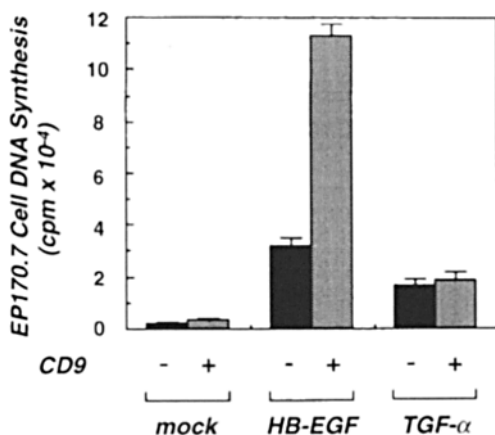


Figure 7. Effect of CD9 co-expression on the juxtacrine growth factor activity of proTGF- α . Formalin fixed L cells transiently transfected with HB-EGF cDNA or TGF- α cDNA, or cotransfected with CD9/DRAP27 cDNA, and plated at 2×10^5 cells/well in 24-well plates were co-cultured with 1×10^5 EP170.7 cells. After a 48-h incubation, the incorporation of [³H]thymidine into EP170.7 cell DNA was measured.

conditioned media obtained from the fixed cells alone nor the fixed cells co-cultured with EPI70.7 cells stimulated EPI70.7 cell DNA synthesis. In our experiments, juxtacrine activity was measured using formalin-fixed L cell HB-EGF transfectants so as to eliminate the possible processing of precursor to mature HB-EGF during the co-culture. Previous studies have shown that proHB-EGF/DTR can be processed to mature HB-EGF, for example, by phorbol esters (38). When living, non-fixed, L cell transfectants rather than fixed one were used in Transwell experiments and the conditioned media from the non-fixed transfectants co-cultured with and without EPI70.7 cells were examined, about 20–60% of the total mitogenic activity persisted suggesting that in living cells, a degree of processing occurs giving rise to substantial paracrine activity. As a consequence, fixed cell co-cultures were used throughout the study in order to measure strictly juxtacrine activity. In another study, it was found that proHB-EGF/DTR purified from baculovirus-infected insect cells was mitogenic consistent with the ability of this form of HB-EGF to be mitogenic in a juxtacrine manner (36). Taken together, all of the evidence demonstrates that proHB-EGF/DTR is a juxtacrine growth factor capable of stimulating DNA synthesis in adjacent cells expressing the EGFR. Therefore, HB-EGF is similar in this activity to TGF- α , TNF- α , CSF-1, and the c-kit ligand, all of which are capable of juxtacrine activity (31).

Recently, it was demonstrated that the receptor for diphtheria toxin is the same molecule as the HB-EGF precursor (25, 35). In the course of analyzing DT sensitivity of cells, it was found that a 27-kD transmembrane protein identical to the CD9 antigen increased cell sensitivity to DT toxicity (32). This protein was found to associate physically with DTR and was named diphtheria toxin receptor associated protein 27, or DRAP27 (32). CD9/DRAP27 up-regulates DT sensitivity without altering the number of cell surface proHB-EGF/DTR molecules (25). Although the mechanism of CD9/DRAP27 action is unknown, a tight interaction of proHB-EGF/DTR and CD9/DRAP27 is implied by the ability of anti-CD9/DRAP27 antibodies to co-precipitate these two molecules. The tight association with CD9/DRAP27 might somehow activate proHB-EGF/DTR and increase its functionality.

We have found that CD9/DRAP27 also up-regulates proHB-EGF/DTR juxtacrine activity quite markedly. When two transfected L cell lines express similar levels of cell surface proHB-EGF/DTR but one of them expresses, in addition, CD9/DRAP27, the juxtacrine activity of the proHB-EGF/DTR and CD9/DRAP27 co-expressants is increased about 25-fold over the L cells expressing proHB-EGF/DTR only. CD9/DRAP27 expression increases proHB-EGF/DTR juxtacrine activity but does not increase proHB-EGF/DTR levels suggesting a potentiation activity rather than the induction of de novo proHB-EGF/DTR synthesis. The potentiating activity of CD9/DRAP27 for proHB-EGF/DTR juxtacrine activity can also be demonstrated for cells producing endogenous proHB-EGF/DTR. The human-mouse hybrid cell line, 3279-10, has human chromosome 5, which is where the HB-EGF/DTR gene maps (13), but lacks chromosome 12, which is where CD9/DRAP27 maps (2). Typically, 3279-10 cells show only a moderate amount of DT sensitivity (32) and of proHB-EGF/DTR juxtacrine activity. However, when these cell lines are transfected with CD9/

DRAP27, juxtacrine activity is increased in direct proportion to the amount of CD9/DRAP27 expressed. The role of CD9/DRAP27 in potentiating proHB-EGF/DTR juxtacrine growth factor activity is further demonstrated in that anti-CD9 antibodies, which do not inhibit HB-EGF mitogenic activity directly, will inhibit juxtacrine activity in cells co-expressing both transmembrane proteins to base-line levels found in the absence of CD9/DRAP27 expression. Taken together, it is quite evident that CD9/DRAP27 upregulates proHB-EGF/DTR juxtacrine growth factor activity dramatically. The mechanism for the CD9/DRAP27 effect on proHB-EGF/DTR activity is unknown but probably reflects the tight association of the two transmembrane proteins, for example: (a) ProHB-EGF/DTR is a relatively small molecule on the cell surface, and might be covered with other membrane proteins. Association of CD9/DRAP27 with proHB-EGF/DTR might open appropriate space to give access to EGFR. (b) ProHB-EGF/DTR and CD9/DRAP27 may associate with other molecules to form a functional juxtacrine device, or CD9/DRAP27 may mediate the association of proHB-EGF/DTR and the third molecule resulting in the formation of an active juxtacrine complex.

A novel finding of this study is that CD9/DRAP27, a protein previously shown to be involved in platelet aggregation (22, 45), association with small GTP-binding proteins (41) and sensitivity to DT (8, 25, 32) also has an unexpected functional role in regulating cell growth. Indeed, this study may be the first demonstration that insoluble cell surface-associated growth factors can be potentiated in their growth factor activity, in the case of proHB-EGF/DTR, by another transmembrane protein, CD9/DRAP27. Previously, soluble paracrine growth factors have been shown to be potentiated by other molecules. For example, heparin potentiates the mitogenic activity of FGF-1 (40, 48), FGF-2 (39, 52), and VEGF (15). However, unlike proHB-EGF/DTR, there is no evidence as yet that soluble HB-EGF, a potent mitogen for SMC, fibroblasts (18), and keratinocytes (16, 30), needs to be potentiated by any factors to be optimally bioactive. Thus, it may be that the soluble and insoluble forms of HB-EGF are regulated differently. If one imagines cell-associated HB-EGF, which has a cytoplasmic domain, to be a receptor with the ectodomain of EGFR as the ligand, then an analogy can be made to other receptors whose binding of ligand is enhanced by the presence of cell surface transmembrane proteins. For example TGF- β binding to its high affinity receptor is dependent on interaction of type I and II receptors with the type III receptor, betaglycan (29, 50).

It is still unclear whether CD9/DRAP27 could associate with other membrane-anchored growth factors and upregulate their mitogenic activities. Although upregulation of the mitogenic activity of proTGF- α was not observed in L cells coexpressed with CD9/DRAP27, proTGF- α may form a complex with CD9/DRAP27. Since it has been reported that proTGF- α associates with a kinase complex, suggesting the existence of two directional signaling between proTGF- α and EGFR (44), CD9/DRAP27 might be involved in a reverse mode of the signaling. It is under way to investigate the regulation of other membrane-anchored growth factor activities such as proAR, pro β -cellulin, and proheparin.

These studies indicate that juxtacrine mechanisms of regulating growth and differentiation, first shown for TGF- α (1, 4, 6, 51) may be more complex than previously envi-

sioned, and may depend on the interaction of multiple factors reported recently (44), as seems to be the case for growth factor receptors (28). Since transmembrane growth factors may play a key role in regulating growth and differentiation, further investigation is needed to elucidate the cofactors that modulate juxtacrine activity and the mechanisms by which they act.

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References

- Anklesaria, P., J. Teixido, M. Laiho, J. H. Pierce, J. S. Greenberger, and J. Massague. 1990. Cell-cell adhesion mediated by binding of membrane-anchored transforming growth factor α to epidermal growth factor receptors promotes cell proliferation. *Proc. Natl. Acad. Sci. USA*. 87: 3289-3293.
- Benoit, P., M. S. Gross, P. Frachet, J. Frezal, G. Uzan, C. Boucheix, and N. V. Cong. 1991. Assignment of the human CD9 gene to chromosome 12 (region P13) by use of human specific DNA probes. *Hum. Genet.* 86:268-272.
- Besner, G., S. Higashiyama, and M. Klagsbrun. 1990. Isolation and characterization of macrophage-derived heparin binding growth factor. *Cell Regul.* 1:811-819.
- Blasband, A. J., D. M. Gilligan, L. F. Winchell, S. T. Wong, N. C. Luetjete, K. T. Rogers, and D. C. Lee. 1990. Expression of the TGF α integral membrane precursor induces transformation of NRK cells. *Oncogene*. 5:1213-1221.
- Blotnick, S. B., G. E. Peoples, M. R. Freeman, T. J. Eberlein, and M. Klagsbrun. 1994. T lymphocytes synthesize and export heparin-binding epidermal growth factor-like growth factor and basic fibroblast growth factor, mitogens for vascular cells and fibroblasts: differential production and release by CD4⁺ and CD8⁺ T cells. *Proc. Natl. Acad. Sci. USA*. 91:2890-2894.
- Brachmann, R., P. B. Lindquist, M. Nagashima, W. Kohr, T. Lipari, M. Napier, and R. Derynck. 1989. Transmembrane TGF- α precursors activate EGF/TGF- α receptors. *Cell*. 56:691-700.
- Brannan, C. I., S. D. Lyman, D. E. Williams, J. Eisenman, D. M. Anderson, D. Cosman, M. Bedell, N. A. Jenkins, and N. G. Copeland. 1991. Steel-Dickie mutation encodes a c-Kit ligand lacking transmembrane and cytoplasmic domains. *Proc. Natl. Acad. Sci. USA*. 88:4671-4674.
- Brown, J. G., B. D. Almond, J. G. Naglich, and L. Eidels. 1993. Hypersensitivity to diphtheria toxin by mouse cells expressing both diphtheria toxin receptor and CD9 antigen. *Proc. Natl. Acad. Sci. USA*. 90: 8184-8188.
- Carpenter, G., and M. I. Wahl. 1990. The epidermal growth factor family. In *Peptide Growth Factors and Their Receptors* I. M. B. Sporn and A. B. Roberts, editors. Springer-Verlag/New York, New York. 69-171.
- Chen, C. A. and H. Okayama. 1988. Calcium phosphate-mediated gene transfer: a highly efficient transfection system for stably transforming cells with plasmid DNA. *Biotechniques*. 6:632-638.
- Derynck, R., A. B. Roberts, M. E. Winkler, E. Y. Chen, and D. V. Goeddel. 1984. Human transforming growth factor- α : precursor structure and expression in *E. coli*. *Cell*. 38:287-297.
- Dluz, S. M., S. Higashiyama, D. Damm, J. A. Abraham, and M. Klagsbrun. 1993. Heparin-binding EGF-like growth factor expression in cultured fetal human vascular smooth muscle cells. *J. Biol. Chem.* 268: 18330-18334.
- Fen, Z., M. S. Dhady, M. Yoshizumi, R. J. Hilkert, T. Quertermous, R. L. Eddy, T. B. Shows, and M.-E. Lee. 1993. Structural organization and chromosomal assignment of the gene encoding the human heparin-binding epidermal growth factor-like growth factor/diphtheria toxin receptor. *Biochemistry*. 32:7932-7938.
- Flanagan, J. G., D. C. Chan, and P. Leder. 1991. Transmembrane form of the kit ligand growth factor is determined by alternative splicing and is missing in the *S^H* mutant. *Cell*. 64:1025-1035.
- Gitay, G. H., S. Soker, I. Vlodayvsky, and G. Neufeld. 1992. The binding of vascular endothelial growth factor to its receptors is dependent on cell surface-associated heparin-like molecules. *J. Biol. Chem.* 267:6093-6098.
- Hashimoto, K., S. Higashiyama, H. Asada, E. Hashimura, T. Kobayashi, K. Sudo, T. Nakagawa, D. Damm, K. Yoshikawa, and N. Taniguchi. 1994. Heparin-binding EGF-like growth factor is an autocrine growth factor for human keratinocytes. *J. Biol. Chem.* 269:20060-20066.
- Hayes, T., Y. Kaneda, T. Uchida, and Y. Okada. 1987. Regional assignment of the gene diphtheria toxin sensitivity using subchromosomal fragment in microcell hybrids. *Chromosoma*. 96:26-32.
- Higashiyama, S., J. A. Abraham, J. Miller, J. C. Fiddes, and M. Klagsbrun. 1991. A heparin-binding growth factor secreted by macrophage-like cells that is related to EGF. *Science (Wash. DC)*. 251:936-939.
- Higashiyama, S., K. Lau, G. E. Besner, J. A. Abraham, and M. Klagsbrun. 1992. Structure of Heparin-binding EGF-like growth factor: multiple forms, primary structure, and glycosylation of the mature protein. *J. Biol. Chem.* 267:6205-6212.
- Higashiyama, S., J. A. Abraham, and M. Klagsbrun. 1993. Heparin-binding EGF-like growth factor stimulation of smooth muscle cell migration: dependence on interactions with cell surface heparan sulfate. *J. Cell Biol.* 122:933-940.
- Huang, E. J., K. H. Nocka, J. Buck, and P. Besmer. 1991. Differential expression and processing of two cell associated form of the kit-ligand: KL-1 and KL-2. *Mol. Biol. Cell*. 3:349-362.
- Huang, M. M., Z. Indik, L. F. Brass, J. A. Hoxie, A. D. Schreiber, and J. S. Brugge. 1992. Activation of Fc γ RII induces tyrosine phosphorylation of multiple proteins including Fc γ RII. *J. Biol. Chem.* 267:5467-5473.
- Ito, N., S. Kawata, S. Tamura, S. Kiso, H. Tsushima, D. Damm, J. A. Abraham, S. Higashiyama, N. Taniguchi, and Y. Matsuzawa. 1994. Heparin-binding EGF-like growth factor is a potent mitogen for rat hepatocytes. *Biochem. Biophys. Res. Commun.* 198:25-31.
- Iwamoto, R., H. Senoh, Y. Okada, T. Uchida, and E. Mekada. 1991. An antibody that inhibits the binding of diphtheria toxin to cells revealed the association of a 27-kDa membrane protein with the diphtheria toxin receptor. *J. Biol. Chem.* 266:20463-20469.
- Iwamoto, R., S. Higashiyama, T. Mitamura, N. Taniguchi, M. Klagsbrun, and E. Mekada. 1994. Heparin-binding EGF-like growth factor, which acts as the diphtheria toxin receptor, forms a complex with membrane protein DRAP27/CD9, which up-regulates functional receptors and diphtheria toxin sensitivity. *EMBO (Eur. Mol. Biol. Organ.) J.* 13:2322-2330.
- Jones, N. H., M. J. Borowitz, and R. S. Metzgar. 1982. Characterization and distribution of a 24,000-molecular weight antigen defined by a monoclonal antibody (DU-ALL-1) elicited to common acute lymphoblastic leukemia (cALL) cells. *Leuk. Res.* 6:449-464.
- Kersey, J. H., T. W. Lebien, C. S. Abramson, R. Newman, R. Sutherland, and M. Greaves. 1981. p24: a human leukemia-associated and lymphohemopoietic progenitor cell surface structure identified with monoclonal antibody. *J. Exp. Med.* 153:726-731.
- Klagsbrun, M., and A. Baird. 1991. A dual receptor system is required for basic fibroblast growth factor activity. *Cell*. 67:1-3.
- Lopez-Casillas, F., J. L. Wrana, and J. Massague. 1993. Betaglycan presents ligand to the TGF β signaling receptor. *Cell*. 73:1435-1444.
- Marikovsky, M., K. Breuing, P. Y. Liu, E. Eriksson, S. Higashiyama, P. Farber, J. Abraham, and M. Klagsbrun. 1993. Appearance of heparin-binding EGF-like growth factor in wound fluid as a response to injury. *Proc. Natl. Acad. Sci. USA*. 90:3889-3893.
- Massague, J., and A. Pandiella. 1993. Membrane-anchored growth factors. *Annu. Rev. Biochem.* 62:515-541.
- Mitamura, T., R. Iwamoto, T. Umata, T. Yomo, I. Urabe, M. Tsuneoka, and E. Mekada. 1992. The 27-kD diphtheria toxin receptor-associated protein (DRAP27) from Vero cells is the monkey homologue to human CD9 antigen: expression of DRAP27 elevates the number of diphtheria toxin receptors on toxin-sensitive cells. *J. Cell Biol.* 118:1389-1399.
- Mitamura, T., S. Higashiyama, N. Taniguchi, M. Klagsbrun, and E. Mekada. 1995. Diphtheria toxin binds to the EGF-like domain of human heparin-binding EGF-like growth factor/diphtheria toxin receptor and inhibits specifically its mitogenic activity. *J. Biol. Chem.* In press.
- Moscattelli, D. 1987. High and low affinity binding sites for basic fibroblast growth factor on cultured cells: absence of a role for low affinity binding in the stimulation of plasminogen activator production by bovine capillary endothelial cells. *J. Cell Physiol.* 131:123-130.
- Naglich, J. G., J. E. Metherall, D. W. Russell, and L. Eidels. 1992. Expression cloning of a diphtheria toxin receptor: identity with a heparin-binding EGF-like growth factor precursor. *Cell*. 69:1051-1061.
- Ono, M., G. Raab, K. Leu, J. A. Abraham, and M. Klagsbrun. 1994. Purification and characterization of transmembrane forms of heparin-binding epidermal growth factor-like growth factor. *J. Biol. Chem.* 269: 31315-31321.
- Pierce, J. H., M. Ruggiero, T. P. Fleming, P. P. D. Fiore, J. S. Greenberger, L. Varticovski, J. Schlessinger, G. Rovera, and S. A. Aaronson. 1988. Signal transduction through the EGF receptor transfected in IL-3-dependent hematopoietic cells. *Science (Wash. DC)*. 239:628-631.
- Raab, G., S. Higashiyama, S. Hetelekidis, J. A. Abraham, D. Damm, M.

- Ono, and M. Klagsbrun. 1994. Biosynthesis and processing by phorbol ester of the cell surface-associated precursor form of heparin-binding EGF-like growth factor. *Biochem. Biophys. Res. Commun.* 204:592-597.
39. Rappraeger, A. C., A. Krufka, and B. Olwin. 1991. Requirement of heparan sulfate for bFGF-mediated fibroblast growth and myoblast differentiation. *Science (Wash. DC)*. 252:1705-1708.
 40. Sakaguchi, K., M. Yanagishita, Y. Takeuchi, and G. D. Aurbach. 1991. Identification of heparan sulfate proteoglycan as a high affinity receptor for acidic fibroblast growth factor (aFGF) in a parathyroid cell line. *J. Biol. Chem.* 266:7270-7278.
 41. Seehafer, J. G., and A. R. E. Sahw. 1991. Evidence that the signal-initiating membrane protein CD9 is associated with small GTP-binding proteins. *Biochem. Biophys. Res. Commun.* 179:401-406.
 42. Shing, Y., G. Christofori, D. Hanahan, Y. Ono, R. Sasada, K. Igarashi, and J. Folkman. 1993. Betacellulin: a mitogen from pancreatic β cell tumors. *Science (Wash. DC)*. 259:1604-1607.
 43. Shoyab, M., G. D. Plowman, V. L. McDonald, J. G. Bradley, and G. J. Todaro. 1989. Structure and function of human amphiregulin: a member of the epidermal growth factor family. *Science (Wash. DC)*. 243:1074-1076.
 44. Shum, L., S. A. Reeves, A. C. Kuo, E. S. Fromer, and R. Derynck. 1994. Association of the transmembrane TGF- α precursor with a protein kinase complex. *J. Cell Biol.* 125:903-916.
 45. Slupsky, J. R., J. C. Cawley, L. S. Griffith, A. R. E. Shaw, and M. Zuzel. 1992. Role of Fc γ RII in platelet activation by monoclonal antibodies. *J. Immunol.* 148:3189-3194.
 46. Stein, J., G. V. Borzillo, and C. W. Rettenmier. 1990. Direct stimulation of cells expressing receptors for macrophage colony-stimulating factor (CSF-1) by a plasma membrane-bound precursor of human CSF-1. *Blood*. 76:1308-1314.
 47. Thompson, S. A., S. Higashiyama, K. Wood, N. S. Pollitt, D. Damm, G. McEnroe, B. Garrick, N. Ashton, K. Lau, N. Hancock, M. Klagsbrun, and J. A. Abraham. 1994. Characterization of sequences within heparin-binding EGF-like growth factor that mediate interaction with heparin. *J. Biol. Chem.* 269:2541-2549.
 48. Thornton, S. C., S. N. Mueller, and E. M. Levine. 1983. Human endothelial cells: use of heparin in cloning and long term serial cultivation. *Science (Wash. DC)*. 222:623-625.
 49. Uchida, T., A. M. Pappenheimer Jr., and R. Greany. 1973. Diphtheria toxin and related proteins; isolation and properties of mutant proteins serologically related to diphtheria toxin. *J. Biol. Chem.* 248:3838-3844.
 50. Wang, X.-F., H. Y. Lin, E. Ng-Eaton, J. Downward, H. F. Lodish, and R. A. Weinberg. 1991. Expression cloning and characterization of the TGF- β type III receptor. *Cell*. 67:797-805.
 51. Wong, S. T., L. F. Winchell, B. K. McCune, H. S. Earp, J. Teixido, J. Massague, B. Herman, and D. C. Lee. 1989. The TGF- α precursor expressed on the cell surface binds to the EGF receptor on adjacent cells, leading to signal transduction. *Cell*. 56:495-506.
 52. Yayon, A., M. Klagsbrun, J. D. Esko, P. Leder, and D. M. Ornitz. 1991. Cell surface, heparin-like molecules are required for binding of basic fibroblast growth factor to its high affinity receptor. *Cell*. 64:841-848.