Amino-terminal Processing of Cell Surface Heparin-binding Epidermal Growth Factor-like Growth Factor Up-regulates Its Juxtacrine but Not Its Paracrine Growth Factor Activity*

(Received for publication, May 24, 1996, and in revised form, August 22, 1996)

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Human heparin-binding epidermal growth factor (EGF)-like growth factor (HB-EGF) expressed on Chinese hamster ovary (CHO) cells is synthesized as a 19-kDa major, and 22- and 27-kDa minor, membrane-anchored precursors (proHB-EGF). In contrast, the 27-kDa species is major and the 19- and 22-kDa ones are minor in mouse proHB-EGF. The juxtacrine growth factor activities of human and mouse proHB-EGFs on CHO cells toward EP170.7 cells in co-culture are significantly different. To investigate the relationship between the juxtacrine growth factor activities and the molecular species, we prepared human-mouse chimeras. Chimeras that have the human amino-terminal sequence and a mouse EGF-like domain showed approximately 5-fold up-regulation of the juxtacrine growth factor activity and the predominance of a 19-22-kDa major species. In contrast, chimeras that have the mouse amino-terminal sequence with a human EGF-like domain showed approximately 5-fold down-regulation of the juxtacrine activity and the predominance of the 27-kDa major species.

A Gly32-HB-EGF (117-amino acid form), which is amino-terminally truncated, induced the same mitogenic activity as that of Arg73-HB-EGF (75-amino acid form), which is amino-terminally truncated. These results strongly suggested that amino-terminal processing of human proHB-EGF would be required for up-regulation of its juxtacrine growth factor activity, but not for its paracrine activity.

Heparin-binding EGF1-like growth factor (HB-EGF) was first identified as a 20–22-kDa soluble glycoprotein produced by macrophages and macrophage-like cells (1–3). It is structurally a member of the epidermal growth factor family, which encompasses a number of structurally homologous mitogens including EGF, transforming growth factor-α, vaccinia virus growth factor (4), amphiregulin (5), β-cellulin (6), and epiregulin (7). HB-EGF binds to and stimulates phosphorylation of the EGF receptor (EGFR) as well as all members of EGF family. Secreted HB-EGF is a potent mitogen for NIH 3T3 cells, bovine aortic smooth muscle cells (1, 8, 9), rat hepatocytes (10), and human keratinocytes (11).

The analysis of the nucleotide sequence of human HB-EGF cDNA predicts a precursor protein of 208 amino acids composed of a putative signal peptide and heparin-binding, EGF-like, transmembrane, and cytoplasmic domains (1–2, 12). The HB-EGF precursor can be cleaved on the plasma membrane to yield a biologically active protein comprising 75–87 amino acids (2, 13, 14). While soluble HB-EGF is a potent mitogen, proHB-EGF is also biologically active in two ways, one as a juxtacrine growth factor (15), and the other as a diphtheria toxin receptor (DTR) (16, 17). It has also 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, colony-stimulating factor-1, and e-Kit ligands 1 and 2 (18). These transmembrane forms are biologically active. For example, the transforming growth factor-α precursor in co-culture stimulates EGFR phosphorylation, mitogenesis, and Ca2+ uptake (19–22). Transmembrane e-Kit ligand is required for the development of melanocytes, germ cells, and hematopoietic stem cells. Soluble e-Kit ligand cannot substitute for the transmembrane form in vivo (23–25).

An important feature of proHB-EGF is the formation of a complex with another transmembrane protein known as CD9 (17, 26) and/or heparan sulfate proteoglycan (8) to express the maximal activities of both the juxtacrine growth factor and diphtheria toxin receptor (15, 17, 27). It has also been reported that proHB-EGF and CD9 form a complex with integrin αβ1 on the cell surface (28); thus, integrin may affect the juxtacrine activity of proHB-EGF. Moreover, the carboxyl-terminal processing of proHB-EGF, which yields the soluble form, is regulated through a protein kinase C-induced mechanism, suggesting that an unidentified processing system is involved in the regulation of the juxtacrine growth factor activity (14). Therefore, the juxtacrine mechanism is a complex process and the mechanism is different from that of the case in the soluble HB-EGF.

Using a co-culture system of donor cells expressing proHB-EGF in contact with acceptor cells expressing EGFR, we demonstrate here that amino-terminal processing of proHB-EGF is a requisite for full stimulation of cell growth in a juxtacrine manner. We also present the evidence that the amino-terminal...
processing does not affect the full expression of the paracrine growth factor activity of HB-EGF.

**EXPERIMENTAL PROCEDURES**

**Materials**

Sulfo-NHS (N-hydroxy succinimide)-biotin was purchased from Pierce. Rabbit anti-human HB-EGF antibody H1 was raised against a synthetic peptide corresponding to amino acids 185–208 of the HB-EGF precursor, which are in the cytoplasmic domain, as described previously (29). An enhanced chemiluminescence (ECL) kit was purchased from Amersham (Buckinghamshire, United Kingdom). Recombinant human HB-EGF, produced in an *Escherichia coli* system (12), was a kind gift from Dr. Judith A. Abraham (Scios Inc., Mountain View, CA).

**Cell Culture**

Chinese hamster ovary cells (CHO K1) were grown in Ham’s F-12 supplemented with 10% fetal calf serum (FCS), penicillin (100 units/ml), and streptomycin sulfate (100 μg/ml). EP170.7 cells (30) were grown in RPMI 1640 medium supplemented with 10% FCS, 5% WEHI-3 cell conditioned medium (CM) (31). EP170.7 cells (100 μl/ml), and streptomycin sulfate (100 μg/ml). Stable transfectants expressing human proHB-EGF (h-proHB-EGF), mouse proHB-EGF (m-proHB-EGF), and human/mouse chimeric proHB-EGF were prepared by transfecting parental CHO cells with 20 μg of each plasmid construct DNA (26) by the calcium-phosphate method (32). *Spodoptera frugiperda* SE21 cells were maintained at 28 °C in Grace’s insect medium (Life Technologies, Inc.) supplemented with 10% FCS, 3.3 g/liter yeastolate, 3.3 g/liter lactate, 100 units/ml penicillin, 10 μM antipain, 5 μM 3,4-dichloroisocoumarin, and 0.4 μM aprotinin, 10 mM NaCl, 0.67 mM KCl, 8 mM Na₂HPO₄, 1.4 mM KH₂PO₄) overnight at 4°C. The densities of bands on the exposed films were determined with a Pharmacia model 610A phenylthiohydantoin-derivative analyzer (Perkin Elmer). The samples were dissolved in the SDS-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and then fractionated by 15% SDS-PAGE (33). Proteins in the gels were transferred to nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany) in 150 mM CAPS buffer, pH 11, containing 20% methanol at 180 mA for 3 h. The nitrocellulose membranes were blocked with 5% skim milk in PBS (137 mM NaCl, 0.67 mM RCl, 5 mM Na₂HPO₄, 1.4 mM KH₂PO₄) overnight at 4°C. The membranes were then incubated for 30 min at room temperature with avidin-conjugated horseradish peroxidase (HRP) (Vector Laboratories Inc., Burlingame, CA). After being washed five times at intervals of 10 min with 0.05% Tween 20 in PBS, the membranes were treated with enhanced chemiluminescence (ECL) Western blotting detection reagents for 1 min (Amersham) at room temperature. The membranes were exposed to Kodak scientific imaging films (Eastman Kodak Co.). The densities of bands on the exposed films were determined with a Scanning Imager (Molecular Dynamics).

**Northern Blot Hybridization**

Total RNA was prepared from the cells according to the method of Chomczynski and Sacchi (34). RNA was electrophoresed on 1% agarose gel and was transferred onto a Zeta-probe membrane (Bio-Rad) by capillary action (35). The membrane filter was hybridized with 32P-labeled monkey CD9 cDNA (36), a 0.6-kilobase pair Xba1-HindIII fragment at 4°C in hybridization buffer (35). The filter was washed with 2 × standard saline citrate at 55°C, and exposed to a Fuji Film imaging plate in the BAS2000 bioimage analyzer (Fuji Photo Film) overnight.

**Expression of Soluble HB-EGF in a Baculovirus-Insect Cell System**

Expression of soluble HB-EGF in a baculovirus-insect cell system was carried out essentially by the method described previously (37).

**Expression of Recombinant Proteins**

In expression experiments, SE21 cells were infected with the recombinant viruses at a multiplicity of infection of 1–2 CM was harvested 72–96 h post-infection and used for HB-EGF purification.

**Purification of Recombinant Soluble HB-EGF**

Secreto hHB-EGF produced by insect cells infected with recombinant baculovirus was purified from the insect cell CM by the method described previously with modifications (3). The insect cell CM (0.5 liters) was applied to an A-Heparin Toyopearl 650 x column (2.5 × 4 cm; Tosoh, Osaka, Japan) equilibrated with 0.5 M NaCl and 0.01 M Tris-HCl, pH 7.2. After extensive washing with the equilibration buffer, the bound proteins were eluted batchwise with 1.2 M NaCl and 0.01 M Tris-HCl, pH 7.2. The column was washed with the equilibration buffer, and the bound proteins were eluted with a 40-mllinear gradient of 0.2–2 M NaCl in 0.01 M Tris-HCl, pH 7.2. The bio-active fractions were collected and applied to a C₄ reversed phase column (4.6 × 250 mm; Vydac, Hesperia, CA) equilibrated with 5% acetonitrile in 0.05% trifluoroacetic acid, in a Shimadzu LC-10A HPLC system. The column was washed extensively with the equilibration buffer, and bound protein fractions were then eluted with a 60-mllinear gradient of 25–60% acetonitrile in 0.05% trifluoroacetic acid at the flow rate of 1 ml/min.

**Amino Acid Composition and Amino-terminal Sequence Analyses of Recombinant Human HB-EGF**

The purified hHB-EGF (approximately 10 pmol) was applied to a Hitachi L-8500 amino acid analyzer (Hitachi, Tokyo, Japan) for amino acid composition analysis, and an Applied Biosystems model 492 micromass analyzer-analyzer to determine the on-line Applied Biosystems model 610A phenylthiohydantoin-derivative analyzer (Perkin Elmer) for amino-terminal sequence analysis.

**Growth Factor Assay**

**Soluble Growth Factor Activity Measurements—**EP170.7 cells were washed with RPMI 1640 medium supplemented with 10% FCS, penicillin (100 units/ml), and streptomycin sulfate (100 μg/ml). The cells (2 × 10⁵) were plated on 96-well plates in a total volume of 200 μl. Appropriate volumes of samples were added to each well, and the EP170.7 cells were then incubated for 36 h. Ten μl of [3H]thymidine solution (1 μC/10 μl PBS, ICN Biomedicals Inc., Costa Mesa, CA) were added and after 4 h of incubation, the incorporation of [3H]thymidine into DNA was measured using a 1205 Betaplate system (Pharmacia Biotech Inc.). Soluble recombinant HB-EGF (HB-EGFₙ, or Arg₂–HB-EGF) (12) was used as a standard.

**Juxtacrine Growth Factor Activity Measurements—**The juxtacrine growth factor assay was carried out as described previously (15). Briefly, CHO transfectants (1 × 10⁵ cells/well) were plated in Ham’s F-12, 10% FCS (500 μmol/l) for 12 h, and then incubated for 12 h prior to washing, fixation, and incubation in Ham’s F-12, 10% FCS, 2 mM NaCl to remove soluble HB-EGF trapped by cell surface heparan sulfate proteoglycan (38), and then fixed with 5% buffered formalin for 5 min. The formalin-fixed cells were washed twice with RPMI 1640, 10% FCS, and then EP170.7 cells (1 × 10⁵ cells/500 μmol/l) were added to the fixed cells. After 48 h, 10 μl of [3H]thymidine solution (1 μC/10 μl PBS, ICN Biomedicals Inc.) (1 μC/well; 1 μCi =
Juxtacrine growth factor activities of human and mouse proHB-EGF—

**RESULTS**

**Juxtacrine Growth Factor Activities of Human and Mouse HB-EGFs**—It has been reported that cell surface proHB-EGF is able to stimulate adjacent cell growth through cell to cell contact (15). Since CHO cells did not show juxtacrine growth factor activity in our assay system, stable transfectants of CHO cells with hHB-EGF or mHB-EGF cDNA were cloned and subjected to the juxtacrine growth factor assay. One of the stable transfectants with hHB-EGF cDNA, CHO<sub>proHB-EGF</sub>, showed significantly high juxtacrine growth factor activity as compared with that of a mock transfectant (Fig. 1A). In contrast, one of the cloned stable transfectants with mHB-EGF cDNA, CHO<sub>mproHB-EGF</sub>, showed extremely low juxtacrine growth factor activity (Fig. 1A). The expression levels of h- and mproHB-EGFs on both transfectants were estimated by the combination of biotinylation and immunoprecipitation methods. Although, as shown in Fig. 1B, the proHB-EGF protein levels were almost equal to each other, mproHB-EGF appeared on an SDS gel as 22–27-kDa heterogeneous bands, which were much higher than the 19–22-kDa heterogeneous bands of hproHB-EGF. The same results were obtained when mouse L929 cells were used as host cells.

**Endogenous CD9 Expression Is Enough for the Juxtacrine Growth Factor Activity in CHO Cells—Modulation of HB-EGF juxtacrine growth factor activity by CD9 has been reported (15). The difference of CD9 expression level in the two transfectants described in Fig. 1 might affect their juxtacrine growth factor activities. To investigate the implication of the difference between their CD9 expression levels, first CD9 mRNA expression was examined by Northern blot analysis, and second CD9 cDNA was transiently introduced into CHO<sub>proHB-EGF</sub> and CHO<sub>mproHB-EGF</sub> cells. Total RNA extracted from each cell was electrophoresed. Hybridization was performed with a key CD9 cDNA according to the method described under “Experimental Procedures.” B, effects of transient expression of CD9 on the juxtacrine growth factor activities of CHO<sub>proHB-EGF</sub> and CHO<sub>mproHB-EGF</sub> cells. Mock or CD9 cDNA was transiently transfected into CHO<sub>proHB-EGF</sub> and CHO<sub>mproHB-EGF</sub> cells. Their juxtacrine growth factor activities were measured according to the method described under “Experimental Procedures.” The juxtacrine growth factor activity of CHO<sub>proHB-EGF</sub> was increased by the additional expression of hproHB-EGF, suggesting that the proHB-EGF protein level produced by CHO<sub>mproHB-EGF</sub> cells is appropriate for the estimation of CD9 as a juxtacrine up-regulator.

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**NH<sub>2</sub>-terminal Processing of ProHB-EGF**

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2. T. Nakagawa, S. Higashiyama, T. Mitamura, E. Mekada, and N. Taniguchi, unpublished observation.
same as those of h-proHB-EGF and m-proHB-EGF-H1, respectively (Fig. 3B). These results indicate that the molecular mass specificities of the human and mouse species are characterized by the amino-terminal 68-amino acid sequence, which would have different substrate specificities for some unidentified processing proteases.

Juxtacrine Growth Factor Activities of Human/Mouse Chimeric ProHB-EGFs—Since the juxtacrine growth factor activity of m-proHB-EGF was much lower than that of h-proHB-EGF, even though the m-proHB-EGF protein was produced in amounts equal to that of the h-proHB-EGF (Fig. 1, A and B), the human/mouse chimeric proHB-EGFs expressed on CHO cells were examined for juxtacrine growth factor activity. Each chimeric construct was transfected and stable clones with different expression levels were isolated to quantitatively analyze their juxtacrine growth activities. The juxtacrine growth factor activity and produced protein level of each clone were measured, as shown in Fig. 4 (A, B, D, and E). Quantitative comparison of their juxtacrine growth factor activities was carried out by plotting their activities against their protein expression levels. While m-proHB-EGF-H1 proteins weakly induced the growth of EP170.7 cells in a juxtacrine manner, wild type h-proHB-EGF showed significantly increasing juxtacrine growth promoting activity in parallel with the increase in the protein level on the cell surface (Fig. 4, B, C, E, and F), as also shown in Fig. 1A. Human/mouse chimeras, h(68–208), comprising h-proHB-EGF with the mouse amino-terminal sequence, showed approximately 5 times less specific activity in comparison with that of the wild type h-proHB-EGF at the initial linear phase (Fig. 4C). In contrast, human/mouse chimeras, h(1–68)-H1, comprising m-proHB-EGF with the human amino-terminal and cytoplasmic sequence, showed approximately 8 times greater specific activity as compared with that of the wild type mouse proHB-EGF at the initial linear phase (Fig. 4F).

Mutual replacement of the amino-terminal portions of human and mouse proHB-EGFs caused drastic changes in their molecular species produced by CHO cells. h(68–208) chimeras were apparently less processed and composed of a major 27-kDa species, while the controls, h-proHB-EGFs, were composed of a major 19-kDa species (Fig. 4A). Furthermore, h(1–68) chimeras were well processed and composed of a major 19-kDa species, while the controls, m-proHB-EGF-H1 proteins, were composed of a major 27-kDa species (Fig. 4D). Inhibition and acceleration of the processing of h(68–208) and h(1–68)-H1 proteins were well correlated with up- and down-regulation of their juxtacrine growth factor activities, respectively (Fig. 4, A, B, D, and E). Based on these results, the processing at the amino-terminal portion would be a requisite for full activity of the juxtacrine growth factor.

Paracrine Growth Factor Activity of Amino-terminally Extended HB-EGF—The paracrine growth factor activities of soluble hHB-EGFs were estimated toward EP170.7 cells. The soluble hHB-EGF produced by recombinant baculovirus-infected Sf21 cells existed in the CM as heterogeneous molecular forms (data not shown). The largest soluble form of hHB-EGF was purified from the CM and migrated as a 19-kDa band on an SDS-PAGE gel. Amino-terminal sequencing analyses revealed that hHB-EGF started at G32LAA... of its primary translation form named Gly32-hHB-EGF (Fig. 5A). The specific activity of Gly32-hHB-EGF produced in the baculovirus system was identical to that of a 75-amino acid form of hHB-EGF (Arg15-hHB-EGF) produced in an E. coli system with an Ed50 of 200 pm (12) (Fig. 5B). The Arg73-hHB-EGF has been reported to have the same specific activity as U-937-derived HB-EGF (12). These results indicate that the 41-amino acid extension at the amino-terminal end has no effect on the specific activity of hHB-EGF as to the paracrine growth factor activity. Based on these results, amino-terminal truncation might have no effect on the paracrine growth factor activity of HB-EGF.

DISCUSSION

HB-EGF has been reported to associate with heparan sulfate proteoglycan and/or CD9 to express optimal mitogenic activity in juxtacrine and paracrine manners (8, 15). In particular, on juxtacrine stimulation, complex formation of proHB-EGF with CD9 has been reported to be a requisite for the activation of EGFR on adjacent cells (15). This time, juxtacrine growth factor analysis of m-proHB-EGF provided us with a great opportunity to investigate the enzymatic activation mechanism of HB-EGF. Using a CHO cell system that was not affected by CD9 expression level, we demonstrated that amino-terminal truncation of proHB-EGF up-regulates its juxtacrine growth factor activity to a great extent, while it has no effect on its optimal paracrine growth factor activity.

Two kinds of human/mouse chimeric proHB-EGF with sub-
stituted amino-terminal 67-amino acid sequences have been produced, and their protein production level and juxtacrine growth factor activities have been quantitatively analyzed. A human/mouse chimera with the mouse amino-terminal sequence appeared predominantly as a 27-kDa species on the CHO cell surface, and its juxtacrine activity was suppressed about 5-fold in comparison with that of the wild-type proHB-EGF. In contrast, a human/mouse chimera with the human amino-terminal sequence comprised a major 22-kDa species, and its juxtacrine growth factor activity was up-regulated about 8-fold as compared with that of the wild type proHB-EGF. These results lead us to the conclusion that the amino-terminal extension of HB-EGF suppresses its juxtacrine growth factor activity, and the amino-terminal processing is required for the optimal expression of its juxtacrine activity. However, it is intriguing that this conclusion is not applicable to the expression of HB-EGF. The precise molecular mechanism has not been elucidated yet, the amino-terminal extension sequence might interfere with the association with CD9 or the interaction of the complex with EGFR.

The existence of the R57DRKVR sequence in human HB-EGF strongly indicates that the endoprotease, furin (39), could be involved in the amino-terminal processing. This would
be also supported by the facts that the 24-kDa species (Asp\textsuperscript{63,65}h-HB-EGF shown in Fig. 5A) of the purified h-HB-EGF from U-937 cell CM was cleaved amino-terminally at its post-R\textsuperscript{57}DRKVR (3, 37) and that h-HB-EGF was not processed in LoVo/Fur 1 cells (40), which have an active furin.\textsuperscript{2} In contrast, LoVo cells, which lack a functional furin, but was processed in their processing sites, which might suggest the physiological importance of their processing.

The physiological meaning of the amino-terminal processing of EGF superfamily members remains unknown. Not only HB-EGF but also transforming growth factor-\(\alpha\), amphiregulin, \(\beta\)-cellulin, and neuregulins have multiple amino-terminal processing sites, which might suggest the physiological importance of their processing.

Acknowledgments—We are very grateful to Y. Sakamoto (Osaka University Medical School) for amino acid sequence and composition analyses. We also thank Dr. K. Goishi (Asahikawa Medical School) for helpful discussions.

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