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The Chemical Synthesis and Binding Affinity to the EGF Receptor of the EGF-like Domain of Heparin-binding EGF-like Growth Factor (HB-EGF)

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Abstract: Heparin-binding EGF-like growth factor (HB-EGF), which belongs to the EGF-family of growth factors, was isolated from the conditioned medium of macrophage-like cells. To investigate the effect of *N*- and *C*-terminal residues of the EGF-like domain of HB-EGF in the binding affinity to the EGF receptor on A431 cell. We synthesized HB-EGF(44–86) corresponding to the EGF-like domain of HB-EGF and its *N*- or *C*-terminal truncated peptides. Thermolytic digestion demonstrated three disulfide bond pairings of the EGF-like domain in HB-EGF is consistent with that of human-EGF and human-TGF-α. HB-EGF(44–86) showed high binding affinity to EGF-receptor, like human-EGF. The truncation of the *C*-terminal Leu^{86} residue from HB-EGF(44–86), HB-EGF(45–86) or HB-EGF(46–86) caused a drastic reduction in the binding affinity to the EGF receptor. These results suggest that the EGF-like domain of HB-EGF plays an important role in the binding to the EGF receptor, and its *C*-terminal Leu^{86} residue is necessary for binding with the EGF-receptor. In addition, the deletion of the two *N*-terminal residues (Asp^{44}-Pro^{45}) from HB-EGF(44–86) caused a 10-fold decrease in relative binding affinity to the EGF receptor. This indicates that the two *N*-terminal residues of the EGF-like domain of HB-EGF are necessary for its optimal binding affinity to the EGF receptor.

Keywords: binding affinity; EGF-like domain; EGF receptor; HB-EGF

INTRODUCTION

Heparin-binding EGF-like growth factor (HB-EGF) was initially identified from the conditioned medium of the macrophage-like U-937 cell lines [1]. HB-EGF is a member of the epidermal growth factor (EGF) family, which encompasses a number of structurally homologous mitogens including EGF [2], transforming growth factor (TGF-α) [3], amphiregulin (AR) [4], vaccinia growth factor (VGF) [5], β-cellulin (BTC) [6] and epiregulin [7]. HB-EGF was known to bind and stimulate the phosphorylation of the EGF receptors on A431 human epidermoid carcinoma cells [8]. More recently, HB-EGF has been shown to bind and stimulate HER4 as well as EGF receptor [9]. Like other members of the EGF family, HB-EGF is synthesized as a transmembrane protein (proHB-EGF) and can be cleaved at the plasma membrane to yield soluble HB-EGF [10,11]. HB-EGF is a potent mitogen for a number of cells including NIH-3T3 cells, smooth muscle cells (SMC), epithelial

Abbreviations: EGF, epidermal growth factor; TGF, transforming growth factor.

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cells and keratinocytes [8]. Unlike other EGF family peptides, HB-EGF possesses the heparin-binding domain with a stretch of a 21 cationic amino acid residues in the *N*-terminal region [1,12–14]. The interaction of the heparin-binding domain of HB-EGF with cell surface heparin sulfate proteoglycan was known to be important for its optimal biological activity [8].

HB-EGF has in common an amino acid motif containing six cysteine-residues in the *C*-terminal region known as the EGF-like domain [1] (Figure 1). The highly conserved six cysteine-residues of the EGF-like domain of HB-EGF form the consensus $CX_7CX_4CX_{10}CXCX_8C$. This spacing pattern of six cysteine-residues in the EGF-like domain of HB-EGF is almost similar to that of six cysteine-residues in the other EGF family peptides (Figure 1). Also, the several specific amino acid residues involved in EGF receptor binding and biological activity in EGF and TGF-α are conserved in the EGF-like domain of HB-EGF. In our previous study, the synthetic HB-EGF(44–86) corresponding to the EGF-like domain of HB-EGF was reported to have a similar mitogenic activity in NIH-3T3 fibroblast cell lines as compared with human-EGF [15].

In this study, in order to examine the importance of the *N*- and *C*-terminal residues of the EGF-like domain of HB-EGF in the interaction with the EGF receptor on A431 cells, HB-EGF(44–86) and its *N*-terminal truncated peptides [HB-EGF(45–86) and HB-EGF(46–86)] were synthesized by a solid phase method [16]. The *C*-terminal Leu^{86} residue truncated peptides [HB-EGF(44–85), HB-EGF(45–85) and HB-EGF(46–85)] were prepared by the enzymatic digestion of HB-EGF(44–86), HB-EGF(45–86) and HB-EGF(44–86) with carboxypeptidase A, respectively. The S-S pairing of the oxidized form of HB-EGF(44–86), HB-EGF(45–86) and HB-EGF(44–86) was determined by thermolytic digestion. The binding affinity to the EGF receptor on A431 cells of the synthetic peptides was investigated.

MATERIALS AND METHODS

Chemicals

Fmoc-amino acids and reagents were obtained from commercial sources.

Cell Lines

Human epidermoid carcinoma A431 cell lines were obtained from Riken Cell Bank (Tsukuba, Japan). The cells were routinely grown in DMEM (Dulbecco modified Eagles medium) with 10% heat inactivated fetal calf serum (FCS), 50 unit/ml penicillin G and 100 mg/ml streptomycin sulfate.

Peptide Synthesis

Peptides were synthesized by a solid phase method [16] using HMP-resin as a starting material. The Fmoc-group was removed with 20% piperidine in NMP. The functional side chains of amino acids were protected by the tBu group for Asp, Glu, Ser, and Tyr; Trt for Cys, His, Asn and Gln; Boc for Lys; Mtr for Arg. Fmoc-Leu-OH was loaded onto the HMP-resin using DCC/DMAP [Fmoc-Leu-OH : DCC : DMAP = 1.5 : 1.2 : 0.1 equiv]. Fmoc-amino acids in each step were coupled with either Bop/HOBt or HBTU/HOBt in NMP. Coupling completion was checked by means of Kaiser's ninhydrin test. Incomplete coupling reactions were either re-coupled until the negative result in the Kaiser's ninhydrin test was obtained or capped with benzoic anhydride in DMF: DCM (1 : 3, vol/vol). After completion of the peptide chain elongation, the protected-peptide resin (100 mg) was treated with TFA-based deprotecting reagent (Reagent K) (TFA: 8.25 ml, thioanisole: 0.5 ml, phenol: 750 mg, EDT: 0.25 ml, H_2O: 0.5 ml) for 3 h at room temperature. After removing volatile materials under a vacuum, the crude products were washed with cold ethyl ether to eliminate trace amounts of scavengers, and then extracted with 30 ml of CH_3CN containing 0.1% β-mercaptoethanol. The extracts were eluted with H_2O and lyophilized to give the

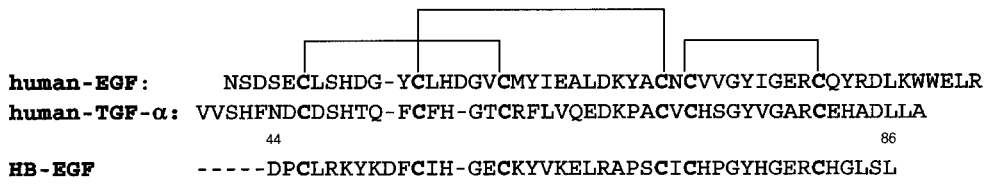

Figure 1 Amino acid sequences of human-EGF, human-TGF-α and HB-EGF(44–86).

reduced (non-disulfude bridged) crude peptide. In order to form the three disulfide bridges of the peptides, the reduced peptides were oxidized in 0.1 M Tris-HCl buffer (pH 8.2) containing 0.1 mM EDTA, 30 mM oxidized glutathione and 15 mM reduced glutathione overnight at room temperature. The oxidation progress of the reduced peptides was checked by the elution profile of analytical reversed-phase HPLC (Beckman, Ultrasphere C_{18}, 5 μm, 4.5 × 250 mm). The amino composition of the oxidized (disulfide bridged) peptides was determined by amino acid analysis after 6 N HCl hydrolysis containing 2% phenol at 110 °C for 22 h. The molecular weight of the oxidized peptides was determined by a fast atom bombardment mass spectrometer (FAB-MS) (JEOL, JMS-HX 110 double-focusing mass spectrometer).

Thermolytic Digestion of the Oxidized Form of HB-EGF(44–86), HB-EGF(45–86) and HB-EGF(46–86)

The oxidized form (500 μg) of HB-EGF(44–86), HB-EGF(45–86) and HB-EGF(46–86) was dissolved in 0.5 ml of 0.1 M pyridine–acetic acid buffer (pH 6.5), respectively. Themolysin (50 μg, Boehringer Mannheim) was added, and then the solution was maintained for 24 h at 45 °C. The digestion mixtures were fractionated by analytical reversed-phase HPLC (Beckman, Ultrasphere C_{18}, 5 μm, 4.5 × 250 mm). The fragments containing Cys residues were determined by amino acid analysis. The amino acid sequence of the fragments containing Cys residues was determined by amino acid sequencing.

Carboxypeptidase A Digestion of the Oxidized Form of HB-EGF(44–86), HB-EGF(45–86) and HB-EGF(46–86)

The oxidized form (300 μg) of HB-EGF(44–86), HB-EGF(45–86) and HB-EGF(46–86) was dissolved in 0.5 ml of 100 mM sodium phosphate buffer (pH 6.5), respectively. Carboxypeptidase A (50 μg, Boehringer Mannheim) was added, and then the solution was maintained for 24 h at 35 °C. The digestion mixtures were fractionated by analytical reversed-phase HPLC (Beckman, Ultrasphere C_{18}, 5 μm, 4.5 × 250 mm).

Radioreceptor Competition Assay

A431 cells were plated on 24-well plates at a density of 5.0×10^4 cells/well and incubated at 37 °C in a 5% CO_2 atmosphere for 2 days, at which time the cells were about 80% confluent. The medium was decanted and replaced with 500 μl of serum-free DMEM medium containing 1 mg/ml bovine serum albumin (BSA), and incubated for 2 h, and then cooled by incubation for 1 h in ice-cooled Hanks balanced salt solution (HBSS). Binding media (0.5 ml of bicarbonate-free DMEM supplemented with 40 mM HEPES, pH 7.2, and 1 mg/ml BSA) containing [^{125}I] human-EGF (20 pM) and the peptides were added to each well. The plates were incubated for 3 h at 4 °C. The medium was aspirated and the monolayers were washed three times with 0.5 ml of HBSS. The cells were lysed with 0.5 ml of 0.4 M NaOH. The lysate was transferred to the vials and ^{125}I levels were counted in an automatic well Gamma system (Aloka, ARC-1000M). Nonspecific binding was determined in triplicate wells containing 160 nM unlabelled human-EGF and normally represented less than 60 cpm. This value was subtracted from the total binding to obtain specific cell-bound [^{125}I]-human EGF.

RESULTS AND DISCUSSION

The EGF-like domain of HB-EGF contains six Cys, three Tyr, three Arg and four His residues susceptible in the side reactions which occur during TFA deprotection. Thus, in order to minimize the undesirable side reaction during the deprotection step, the protected peptide-resin was treated with Reagent K [17, 18] known as an effective deprotection reagent in the synthesis of complex peptides. After reducing with β-mercaptoethanol, the crude reduced (non-disulfide linked) peptides were revealed as a main peak in analytical reversed-phase HPLC (data not shown). The six Cys residues of the reduced peptides of HB-EGF(44–86), HB-EGF(45–86) and HB-EGF(46–86) were oxidatively refolded by air oxidation in a highly diluted aqueous buffer in the presence of reduced and oxidized glutathione to form three intermolecular disulfide linkages. The amino acid composition and molecular weight of the oxidized form of HB-EGF(44–86), HB-EGF(45–86) and HB-EGF(46–86), determined by amino acid analysis and FAB-MS, were in good agreement with the theoretical values, respectively (Tables 1 and 2).

The disulfide pairing of 1–3, 2–4 and 5–6 in the EGF family peptides including human-EGF and human-TGF-α has been characterized. The correct disulfide pairing in human-EGF and human-TGF-α was reported to be essential for binding with the EGF

Table 1 Amino Acid Composition of the Oxidized Form of the Peptides

Amino acids	HB-EGF(44–86)	HB-EGF(45–86)	HB-EGF(46–86)	HB-EGF(44–85)	HB-EGF(45–85)	HB-EGF(46–85)
Asp, Asn	1.9 (2)	1.0 (1)	0.8 (1)	2.0 (2)	1.1 (1)	1.1 (1)
Ser	1.9 (2)	1.8 (2)	1.8 (2)	1.8 (2)	2.3 (2)	1.7 (2)
Glu, Gln	3.3 (3)	3.4 (3)	3.2 (3)	3.0 (3)	3.4 (3)	3.2 (3)
Pro	3.1 (3)	2.7 (3)	1.9 (2)	2.7 (3)	3.2 (3)	2.2 (2)
Gly	4.4 (4)	4.3 (4)	3.9 (4)	4.1 (4)	4.2 (4)	3.7 (4)
Ala	1.1 (1)	1.2 (1)	1.0 (1)	0.9 (1)	1.1 (1)	1.0 (1)
Val	1.0 (1)	1.0 (1)	1.1 (1)	0.9 (1)	1.1 (1)	1.0 (1)
Ile	1.8 (2)	1.8 (2)	1.7 (2)	1.7 (2)	1.7 (2)	1.6 (2)
Leu	4.0 (4)	4.0 (4)	4.0 (4)	3.0 (3)	3.0 (3)	3.0 (3)
Tyr	2.9 (3)	2.7 (3)	3.1 (3)	2.9 (3)	2.8 (3)	2.9 (3)
Phe	1.1 (1)	0.9 (1)	1.0 (1)	1.1 (1)	0.9 (1)	1.1 (1)
His	4.4 (4)	3.7 (4)	4.0 (4)	3.9 (4)	3.7 (4)	3.8 (4)
Lys	3.6 (4)	3.6 (4)	3.7 (4)	3.9 (4)	4.1 (4)	3.8 (4)
Arg	3.1 (3)	2.7 (3)	2.9 (3)	2.9 (3)	3.0 (3)	2.8 (3)

Table 2 Molecular Weights of the Synthetic Peptides Determined by FAB-MS

Peptide	Calculated value	Observed value
HB-EGF(44–86)	4858.8	4958.7
HB-EGF(45–86)	4843.7	4842.7
HB-EGF(46–86)	4746.6	4745.7
HB-EGF(44–85)	4845.6	4846.2
HB-EGF(45–85)	4730.6	4829.7
HB-EGF(46–85)	4632.7	4633.4

receptor and mitogenic activity. For this reason, to determine the three disulfide pairings in the oxidized form of HB-EGF(44–86), HB-EGF(45–86) and HB-EGF(46–86), the peptides were digested with thermolysin. The digested fragments were fractionated by analytical reversed-phase HPLC (Figure 2). Peaks containing Cys residues were analysed by amino acid sequencing. Similar to our previous study [15], peak 1 contained two sequences, Ile^{71}-Cys-His-Pro-Gly^{75} and Cys^{81}-Arg-Glu-Gly-His-Tyr^{86}. Peak 3 consisted of two sequences, Phe^{53}-Cys^{54} and Leu^{65}-Arg-Ala-Pro-Ser-Cys^{70} (data not shown). Peaks 2, 2′ and 2″ involved the two sequences paired by Cys^{46} and Cys^{59} (Table 3). The results indicated the oxidized form of HB-EGF(44–86), HB-EGF(45–86) and HB-EGF(46–86) has the three disulfide linkages between Cys^{46}-Cys^{59} (first Cys–third Cys), Cys^{54}-Cys^{70} (second Cys–fourth Cys) and Cys^{72}-Cys^{81} (fifth Cys–sixth Cys), respectively. Thus, the pattern of three disulfide linkages in these peptides was

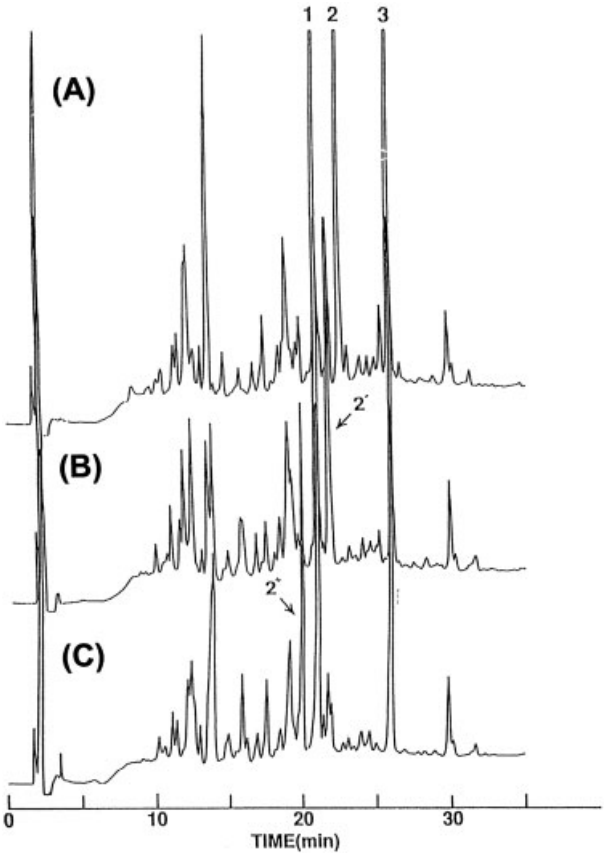

Figure 2 Fractionation of thermolytic digested fragments of HB-EGF(44–86) **(A)**, HB-EGF(45–86) **(B)** and HB-EGF(46–86) **(C)** by analytical reversed-phase HPLC using Beckman ODS (4.6 × 250 min) with linear gradient of 0–80% CH_3CN/H_2O/0.1% TFA over 35 min at a flow rate of 1 ml/min. UV absorption was monitored at 214 nm.

Table 3 Sequence Analysis of Peak 2, 2′ and 2″ after Thermolytic Digestion of HB-EGF(44–86), HB-EGF(45–86) and HB-EGF(46–86)

Cycle no.	Peak 2[a]		Peak 2′		Peak 2″	
	Sequence I (pmol)	Sequence II (pmol)	Sequence I (pmol)	Sequence II (pmol)	Sequence I (pmol)	Sequence II (pmol)
1	Ile 42.5	Arg 19.4	Ile 164.67	Pro 164.67	Ile 224.57	Cys ND
2	His 3.6	Pro 23.0	His 143.99	Cys ND	His 118.57	
3	Gly 14.0	Cys ND	Gly 244.17		Gly 150.80	
4	Glu 13.1		Glu 260.80		Glu 108.26	
5	Cys ND		Cys ND		Cys ND	
6	Lys 14.6		Lys 120.80		Lys 98.83	
7	Tyr 11.0		Tyr 63.26		Tyr 58.59	

ND: not determined.
[a] Data were taken from reference [15].

Peak 2
Asp^{44}-Pro-Cys^{46}
|
Ile^{55}-His-Gly-Glu-Cys^{59}-Lys-Tyr_{61}

Peak 2′
Pro^{45}-Cys^{46}
|
Ile-His-Gly-Glu-Cys^{59}-Lys-Tyr

Peak 2″
Cys^{46}
|
Ile-His-Gly-Glu-Cys^{59}-Lys-Tyr

consistent with that of human-EGF and human-TGF-α. The elimination of the *C*-terminal Leu^{86} residue from HB-EGF(44–86), HB-EGF(45–86) and HB-EGF(46–86) was performed by carboxypeptidase A digestion. The resulting peptides, HB-EGF(44–85), HB-EGF(45–85) and HB-EGF(46–85) were confirmed by amino acid analysis and FAB-MS (Tables 1 and 2).

Specific binding of each peptide was assessed with respect to its ability to complete with ^{125}I-labelled wild-type human-EGF bound to the EGF receptors in membrane fractions of A431 cells. The competition curves, presented in Figure 3, permit the estimation of IC_{50} values which indicate the concentration of competing species required for inhibition of binding by 50%. The ratio of wild-type human-EGF and HB-EGF peptide IC_{50} values was used as an index of the relative affinity of the EGF receptor for each peptide (Table 4). HB-EGF(44–86) showed a similar high binding affinity to the EGF receptor as human-EGF. The truncation of *C*-terminal Leu^{86} residue from HB-EGF(44–86), HB-EGF(45–86) and HB-EGF(46–86) caused a drastic reduction in the binding affinity to the EGF receptor (Figure 3 and Table 4). These results suggest that the EGF-like domain of HB-EGF plays an important role in the binding to the EGF receptor and its *C*-terminal Leu^{86} residue is necessary for the binding to the EGF receptor.

It has been reported that deletion or substitution of the Leu^{47} residue (EGF numbering system) corresponding to this position in the human- or mouse-EGF induced a drastic loss in binding with the EGF receptor on A431 cells and mitogenic activity in mouse 3T3 fibroblast cells [19–23]. Also, the synthetic amphiregulin (44–84) corresponding to the EGF-like domain of amphiregulin showed no affinity for the EGF receptor and no mitogenic activity [24]. This is thought to be due to deletion of the highly conserved Leu residue at this position of the EGF-like domain of amphiregulin. However, the EGF-like domain of β-cellulin conserved with the Leu residue at this position displayed equipotent activity in mitogenesis in NIH-3T3 fibroblast cells, when compared with human-EGF (data not shown). Therefore, these results suggest that the Leu residue located at the fifth position from the *C*-terminal Cys residue (sixth Cys) of the EGF family growth factors is essential for the EGF receptor binding on A431 cells and mitogenic activity in fibroblast cells.

In addition, the *N*-terminal truncated peptides, HB-EGF(45–86) and HB-EGF(46–86), displayed 5- and 10-times less binding affinity to the EGF receptor than HB-EGF, respectively. This fact suggests that the two *N*-terminal residues (Asp^{44}-Pro^{45}) of the EGF-like domain of HB-EGF play a critical role in showing optimal binding affinity to the EGF receptor.

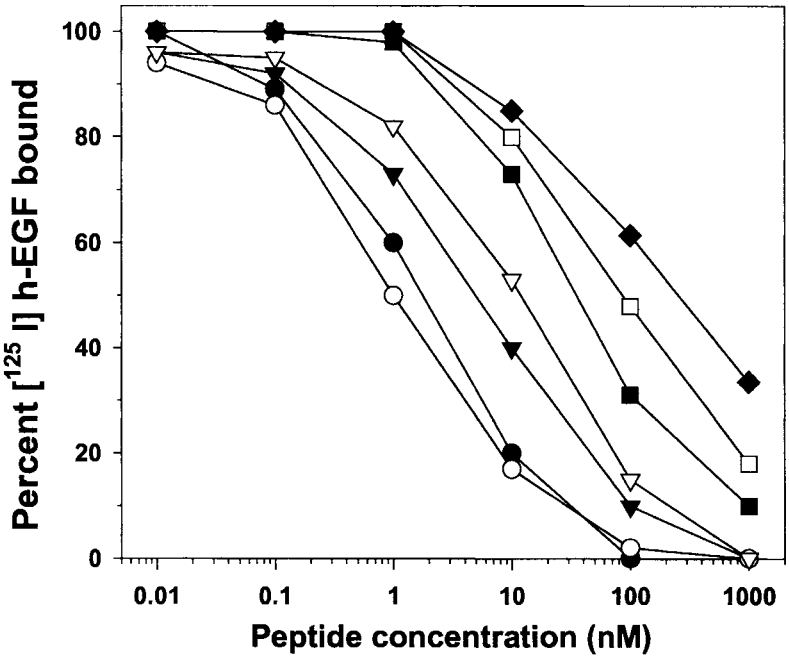

Figure 3 Inhibition of binding of ^{125}I-human-EGF to A431 cells by human-EGF, HB-EGF(44–86) and its truncated peptides. Human-EGF (●), HB-EGF(44–86) (○), HB-EGF(45–86) (▼), HB-EGF(46–86) (▽), HB-EGF(44–85) (■), HB-EGF(45–85) (□) and HB-EGF(46–85) (♦).

Table 4 Binding Affinities of h-EGF, HB-EGF(44–86) and Truncated Peptides

Peptide	IC_{50} (nM)[a]	Relative binding affinity (%)[b]
Human-EGF	2	100
HB-EGF(44–86)	1	200
HB-EGF(45–86)	5	40
HB-EGF(46–86)	9	22.2
HB-EGF(44–85)	35	5.7
HB-EGF(45–85)	83	2.4
HB-EGF(46–85)	234	0.9

[a] Data are representative of average values from at least two dependent determinations.
[b] Relative binding affinity = IC_{50} [human-EGF] / IC_{50} (peptide) × 100 %.

In conclusion, the EGF-like domain of HB-EGF plays an important role in the binding to the EGF-receptor and its *C*-terminal Leu^{86} residue is necessary for the binding with the EGF receptor. In addition, the two *N*-terminal residues of the EGF-like domain of HB-EGF are necessary for its optimal binding affinity to the EGF-receptor.

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