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The significance of disulfide bonding in biological activity of HB-EGF, a mutagenesis approach

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Abstract

A site-directed mutagenesis approach was taken to disrupt each of 3 disulfide bonds within human HB-EGF by substituting serine for both cysteine residues that contribute to disulfide bonding. Each HB-EGF disulfide analogue (HB-EGF-Cys/Ser_{108/121}, HB-EGF-Cys/Ser_{116/132}, and HB-EGF-Cys/Ser_{134/143}) was cloned under the regulation of the mouse metallothionein (MT) promoter and stably expressed in mouse fibroblasts. HB-EGF immunoreactive proteins with M_r of 6.5, 21 and 24kDa were observed from lysates of HB-EGF and each HB-EGF disulfide analogue. HB-EGF immunohistochemical analyses of each HB-EGF stable cell line demonstrated ubiquitous protein expression except HB-EGF-Cys/Ser_{108/121} and HB-EGF-Cys/Ser_{116/132} stable cell lines which exhibited accumulated expression immediately outside the nucleus. rHB-EGF, HB-EGF, and HB-EGF_{134/143} proteins competed with ¹²⁵I-EGF in an A431 competitive binding assay, whereas HB-EGF-Cys/Ser_{108/121} and HB-EGF-Cys/Ser_{116/132} failed to compete. Each HB-EGF disulfide analogue lacked the ability to stimulate tyrosine phosphorylation of the 170kDa EGFR. These results suggest that HB-EGF-Cys/Ser_{134/143} antagonizes EGFRs.

Keywords

HB-EGF; disulfide bond; cysteine; tyrosine phosphorylation; EGF receptors

Introduction

Heparin-binding EGF-like growth factor (HB-EGF), a member of the EGF superfamily, is initially synthesized as a membrane-bound protein that undergoes extensive post-translational modification. Initially, the extracellular domain of HB-EGF is processed by a furin-like enzyme, which cleaves the 208 amino acid proHB-EGF precursor at Arg₆₂-Asp₆₃ [1–3], and a disintegrinase and metalloprotease (ADAM) between Pro₁₄₈-Val₁₄₉ or Glu₁₅₁-Asn₁₅₂, resulting in the release of soluble, mature HB-EGF that is collectively referred to as ectodomain shedding [4–7]. Following ectodomain shedding, the HB-EGF intracellular domain is processed by an unidentified protease resulting in a carboxy-terminal HB-EGF domain, termed HB-EGF C [8,9]. Soluble, mature and carboxy-terminal domains of HB-EGF are potent stimulators of cellular proliferation whose action is mediated in an EGF receptor (EGFR)-dependent and EGFR-independent mechanisms, respectively [1,8,9].

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A 36–40 amino acid extracellular motif containing 6 conserved cysteines with the consensus sequence CX₇CX₄CX₁₀CXCX₈C contributing to the formation of 3 disulfide bonds (Cys₁–Cys₃, Cys₂–Cys₄, and Cys₅–Cys₆), referred to as the EGF-like domain, is thought to be required for EGF family members to bind and activate EGFRs [10,11]. The three dimensional structure of HB-EGF [12] and EGF [13,14] specify an amino-terminal domain containing the first two disulfide bonds forming a two-stranded anti-parallel β -sheet and a carboxy-terminal domain containing the third disulfide bond contributing to two short β -sheets for the EGF-like domain of HB-EGF or EGF itself. This domain within EGF is responsible for binding EGFRs, Her1/erbB1 and Her4/erbB4, in a 2 ligand: 2 receptor dimeric complex resulting in activation of endogenous receptor tyrosine kinase (RTK) autophosphorylation [1,15,16,17].

The present study exploits mouse fibroblasts that stably express HB-EGF or each of three individually disrupted HB-EGF disulfide bonds, termed HB-EGF-Cys/Ser_{108/121}, HB-EGF-Cys/Ser_{116/132}, and HB-EGF-Cys/Ser_{134/143}, to examine whether the disulfide bonds within HB-EGF are required for HB-EGF to undergo proteolytic processing, maintain its high affinity binding to EGFRs, and stimulate tyrosine phosphorylation of EGFRs. These data provide insight into the structural requirements of the extracellular domain within HB-EGF necessary in maintaining bioactivity.

Materials and Methods

Cloning and DNA sequencing

HB-EGF- Cys/Ser_{108/121}, HB-EGF- Cys/Ser_{116/132}, and HB-EGF- Cys/Ser_{134/143} were cloned by overlapping PCR. The first of two cysteine residues involved in each disulfide bond was produced by two separate overlapping PCR reactions using an external forward non-mutagenic primer and internal reverse mutagenic primer or an external reverse non-mutagenic primer and internal forward mutagenic primer. The overlapping DNA products were diluted 1:10, combined and subjected to a second round of PCR using only the external non-mutagenic primer set. These individual single cysteine mutations (amino acid residues 116, 121, or 143) were first generated and used as the template for substitution of serine for the second encoded cysteine of the disulfide bond for HB-EGF- Cys/Ser_{108/121}, HB-EGF-Cys/Ser_{116/132}, and HB-EGF- Cys/Ser_{134/143}. PCR reactions contained ~4 ng/ μ l of HB-EGF cDNA, 0.5 μ l forward and reverse primers (10 μ M), 2.0 μ l of 10X reaction buffer, 0.5 μ l dNTPs, 0.5 μ l Taq polymerase (New England Biolabs: Beverly, MA), and dH₂O to bring the volume to 20 μ l. Each reaction was subjected to 94°C (5 min), 35 cycles of 94°C (30 sec), 58°C (1 min), and 72°C (40 sec), followed by an extension at 72°C (10 min). Reaction products were separated by 0.8% agarose gel electrophoresis, purified with the Wizard Gel & PCR DNA clean up kit (Promega: Madison, WI), cloned into TOPO® (Invitrogen: Carlsbad, CA) cloning vector, and sequenced to verify each cysteine to serine substitution using an ABI 310. The HB-EGF- Cys/Ser_{108/121}, HB-EGF- Cys/Ser_{116/132}, and HB-EGF- Cys/Ser_{134/143} were excised from TOPO by EcoRV/EcoRI digests and fused downstream of the MT promoter.

Cell Culture and Transfection

Mouse fibroblasts (MLC) were maintained in DMEM (Cellgro, Herndon, VA) supplemented with 10% fetal bovine serum, penicillin (100U/ml) and streptomycin (100 μ g/ml) (BioWhittaker, Walkersville, MD). 4.0 μ g of either pMT-HB-EGF, HB-EGF- Cys/Ser_{108/121}, HB-EGF- Cys/Ser_{116/132}, or HB-EGF- Cys/Ser_{134/143} and 400ng pSV-neo, were co-transfected into MLCs using Lipofectamine (Invitrogen, Carlsbad, CA) according to the manufacturer's recommendations. pSV-neo alone was transfected into MLC as a control cell line. 48 hours post-transfection the cells were placed on neomycin (G418) selection (1mg/ml). Single colonies were picked and propagated generating neomycin resistant pMT-HB-EGF, HB-EGF- Cys/Ser_{108/121}, HB-EGF- Cys/Ser_{116/132}, and HB-EGF- Cys/Ser_{134/143} stable cell

lines. Two independent stable cell lines were examined for native HB-EGF and each HB-EGF analogue in all experiments.

Northern blotting

Total cellular RNA was extracted from HB-EGF, HB-EGF- Cys/Ser_{108/121}, HB-EGF- Cys/Ser_{116/132}, HB-EGF- Cys/Ser_{134/143} and pSV-neo MLC stable cell lines using TriReagent (Molecular Research Center, Cincinnati, OH), DNase-digested with DNA-free (Ambion, Austin, TX), and quantified by absorbance at 260 nm according to manufacturer's recommendations. Northern blotting was performed as previously described [9,19].

Western Blotting

Cell extracts from HB-EGF, HB-EGF- Cys/Ser_{108/121}, HB-EGF- Cys/Ser_{116/132}, HB-EGF- Cys/Ser_{134/143} and pSV-neo MLC stable cell lines were collected and analyzed using an HB-EGF-C antisera, as described [9].

HB-EGF Immunohistochemistry

HB-EGF, HB-EGF- Cys/Ser_{108/121}, HB-EGF- Cys/Ser_{116/132}, HB-EGF- Cys/Ser_{134/143} and pSV-neo MLC stable cell lines were plated at a density of 5×10^4 cells/well in an 8-well chamber slide and incubated at 37°C (16h) in 5% CO₂ and fixed overnight in HistoPrep™-buffered 10% formalin at 4°C. Fixed cells were washed in PBS/0.05% Tween-20, incubated with rabbit anti-human HB-EGF-C peptide antibody [9] at 1:200 at 4°C overnight. Cells were then incubated with [FITC]-conjugated AffiniPure goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) at room temperature 1 hr, washed 3 times 5 min each and stained with 4,6-diamino-2-phenylindole (DAPI) for visualization of nuclei.

Immunofluorescence was detected using a fluorescence BX51 light microscope (Olympus America, Inc., Melville, NY). Negative controls consisted of each cell line in which the primary antibody was omitted (data not shown).

Competitive Binding Assay

To assess whether proteins derived from HB-EGF, HB-EGF-Cys/Ser_{108/121}, HB-EGF- Cys/Ser_{116/132}, HB-EGF-Cys/Ser_{134/143} and pSV-neo MLC stable cell lines were able to bind EGFRs, competitive EGF radioreceptor assays using ¹²⁵I-EGF (Perkin Elmer, Boston, MA) were performed (two times in triplicate using 2 independent stable cell lines each) using human squamous epithelial carcinoma cells (A431), as previously described [9]. To assure accurate amounts of HB-EGF protein from each HB-EGF stable cell line was analyzed in the receptor binding assays, western blotting was performed using an antibody that recognizes the amino-terminal EGFR binding domain of HB-EGF, 2μg/ml, (R&D Systems) with 50ng of recombinant HB-EGF (R&D Systems) and 50ng of HB-EGF protein from each stable cell line, corresponding to 10μl, 20μl, 17μl, and 3μl of cellular lysate from HB-EGF, HB-EGF-Cys/Ser_{108/121}, HB-EGF- Cys/Ser_{116/132} and HB-EGF- Cys/Ser_{134/143} stable cell lines, respectively (Fig. 4A, inset). The largest volume of cellular lysate applied at each HB-EGF concentration in the receptor assay was the amount of lysate added for the pSV-neo MLC stable cell line.

Tyrosine phosphorylation of EGFR in response to HB-EGF and HB-EGF disulfide analogues

Cellular lysates (15μg) from each of HB-EGF, HB-EGF-Cys/Ser_{108/121}, HB-EGF-Cys/Ser_{116/132}, HB-EGF-Cys/Ser_{134/143} and pSV-neo MLC stable cell lines were obtained by scraping cells in lysis solution (1% Triton-X 100, 100mM Tris pH 7.4), quantified, and analyzed by western blot analysis as described above. In order to discern possible differences in HB-EGF, HB-EGF-Cys/Ser_{108/121}, HB-EGF-Cys/Ser_{116/132}, HB-EGF-Cys/Ser_{134/143} and pSV-neo MLC stable cell lines signaling through tyrosine phosphorylation of EGFRs, A431

cells were treated with ~50ng–100ng of HB-EGF of each for 15 min., solubilized in lysis buffer and quantified. Western blots of each A431 treated lysates (15µg) were placed in blocking solution [5% non-fat dry milk in TBS/0.1% Tween (TBS/T)], incubated with either anti-phosphotyrosine-EGFR antibody (1:1000) or anti-EGFR antibody (1:1000) overnight at 4°C with gentle agitation, washed 3X 5 min each in TBS/T, incubated with a HRP-conjugated goat anti-rabbit IgG (1:2000) secondary antibody in blocking solution and immunoreactive proteins were determined by application of SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) and developed.

Results

HB-EGF and HB-EGF disulfide analogue mRNA expression in mouse fibroblasts

To demonstrate that human HB-EGF, HB-EGF-Cys/Ser_{108/121}, HB-EGF-Cys/Ser_{116/132}, or HB-EGF-Cys/Ser_{134/143} mRNA is expressed in stably transfected MLCs, total cellular RNA was analyzed by northern blot analyses using a ³²P-labeled human HB-EGF cDNA resulting in similar levels of expression of a 0.7kb HB-EGF transcript (Fig. 1A, lanes 2–5). pSV-neo MLC lacked the 0.7kb HB-EGF transcript and served as a negative control (Fig. 1A, lane 1). To assure equivalent amounts of RNA were analyzed, the blot was stripped and incubated with a ³²P-labeled GAPDH DNA fragment resulting in a 1.4kb transcript present in all cell lines (Fig. 1B).

HB-EGF protein heterogeneity

Cellular lysates from HB-EGF, HB-EGF-Cys/Ser_{108/121}, HB-EGF-Cys/Ser_{116/132}, HB-EGF-Cys/Ser_{134/143} and pSV-neo MLCs were examined by western blotting using HB-EGF C antisera resulting in immunoreactive proteins of 6.5, 21, and 24kDa for HB-EGF and each HB-EGF disulfide analogue (Fig. 2A, lanes 2–5). The 21 and 24kDa proteins exhibit a doublet due to amino-terminal heterogeneity in ectodomain shedding [18]. The 6.5kDa protein is the intracellular processed form of HB-EGF (HB-EGF-C). Lysates from HB-EGF-Cys/Ser_{134/143} resulted in a greater amount of immunoreactive proteins as compared to stable cell lines expressing native HB-EGF, HB-EGF-Cys/Ser_{108/121}, or HB-EGF-Cys/Ser_{116/132} (Fig. 2, lane 5A). These results are not due to analysis of a greater amount of protein for HB-EGF-Cys/Ser_{134/143} because probing the same blot with an actin antibody resulted in equivalent amount of immunoreactive 43kDa actin protein (Fig. 2B). This blot is representative of 2 independent experiments from 2 stable cell lines each.

Subcellular localization of HB-EGF and HB-EGF disulfide analogues

HB-EGF immunohistochemistry was performed to address possible differences in post-translational processing by each HB-EGF disulfide analogue as compared to HB-EGF and pSV-neo cell lines. Immunofluorescence results suggest that HB-EGF-Cys/Ser_{108/121} and HB-EGF-Cys/Ser_{116/132} are processed differently than HB-EGF and HB-EGF-Cys/Ser_{134/143}. HB-EGF and HB-EGF-Cys/Ser_{134/143} demonstrated a ubiquitous immunofluorescent expression pattern throughout the cells. However, HB-EGF-Cys/Ser_{108/121} and HB-EGF-Cys/Ser_{116/132} appeared localized immediately outside the nucleus possibly in the rough endoplasmic reticulum, the site for disulfide bond formation and protein folding (Fig. 3).

Biological properties of HB-EGF and HB-EGF disulfide analogues

HB-EGF and each HB-EGF disulfide analogue were examined for their ability to bind EGFRs. Lysates from HB-EGF, HB-EGF-Cys/Ser_{108/121}, HB-EGF-Cys/Ser_{116/132}, and HB-EGF-Cys/Ser_{134/143}, and pSV-neo stably transfected MLCs were assayed for their ability to compete with ¹²⁵I-EGF in a competitive receptor binding assay using A431 cells. Results indicate that HB-EGF, HB-EGF-Cys/Ser_{134/143}, and recombinant HB-EGF (R&D Systems)

displaced ^{125}I -EGF with a similar EC_{50} (65–85ng), although lysates from pSV-neo MLC, HB-EGF-Cys/Ser_{108/121}, and HB-EGF-Cys/Ser_{116/132}, failed to displace ^{125}I -EGF (Fig. 4A). In sum, these results indicate that the first and second disulfide bonds are necessary to maintain high affinity binding of HB-EGF to EGFR; however, the third disulfide bond is not required to maintain EGFR binding activity.

Since HB-EGF and HB-EGF-Cys/Ser_{134/143} were able to compete with ^{125}I -EGF for EGFRs, we addressed the biological activity of HB-EGF as well as each HB-EGF disulfide analogue by their ability to induce tyrosine phosphorylation of EGFRs in A431 cells. Recombinant HB-EGF and protein from the HB-EGF stable cell line induced tyrosine phosphorylation of the 170kDa EGFR (Fig. 4B, lanes 1 and 3, top panel). However, protein from each disulfide analogue and pSV-neo failed to induce tyrosine phosphorylation EGFRs (Fig. 4B, lanes 2, 4–6, top panel). To assure equivalent amounts of A431 protein was analyzed, an equivalent blot was incubated with an EGFR antibody demonstrating equivalent amounts of EGFR in each sample (Fig. 4B, bottom panel).

Discussion

Recent studies have provided evidence suggesting that the conserved six cysteine motif contributing to the 3 disulfide bonds present in all EGF family members may not be necessary for binding and bioactivity. For example, a combinatorial approach to replacing cysteine residues of EGF peptides with α -amino-butyric acid (Abu) demonstrated the first disulfide bond may be removed (mEGF_{4–48} Abu 6,20) and retain native structure and significant mitogenic activity [20,21]. In contrast, removal of the other disulfide bonds singly or in pairs within the EGF peptides altered their tertiary structure and bioactivity [21]. Secondly, an alternatively spliced transcript of HB-EGF encoding the signal peptide, propeptide, heparin-binding, and the first two disulfide bonds of the EGF-like domain was identified from a cDNA library of green monkey Vero cells and termed the short form of HB-EGF (SF-HB-EGF) [22]. SF HB-EGF exhibited high affinity for heparin, mitogenic activity, but failed to bind EGFRs, suggesting that SF HB-EGF may utilize an alternative EGFR-independent signaling pathway. Furthermore, disulfide plasticity has been demonstrated in mEGF_{1–53} capable of accommodating seven different disulfide bonding patterns while maintaining structural requirements [23].

We determined the importance of the three disulfide bonds independently using the entire full-length HB-EGF cDNA using a site-directed mutagenesis approach in which the two cysteine residues that contribute to each disulfide bond were substituted with serine. Our results demonstrate that the first and second disulfide bonds of HB-EGF are necessary for binding EGFRs while its third disulfide bond is not required to maintain high affinity to EGFRs. This result is surprising in that a comparable EGF peptide mutation (mEGF_{4–48}, Abu 33,42) significantly altered structure and reduced mitogenic activity [21]. It is possible that the amino acids surrounding these disulfide bonds may contribute to the overall folding of HB-EGF as evidenced by data in which modification of Leu₄₇ in EGF and Leu₄₈ in TGF α reduces biological activity [25]. We hypothesize that the entire proHB-EGF molecule, that is *o*-glycosylated at two sites in the ectodomain [26], may contribute to the overall structure of HB-EGF and likely a more representative *in vivo* approach to studying the biological significance of disulfide bonds.

HB-EGF bioactivity was measured by the ability of lysates from HB-EGF, HB-EGF- Cys/Ser_{108/121}, HB-EGF- Cys/Ser_{116/132}, HB-EGF- Cys/Ser_{134/143}, and pSV-neo stable cell lines to induce tyrosine phosphorylation of EGFRs in A431 cells [1,3]. Recombinant HB-EGF and protein from HB-EGF stable cell line stimulated tyrosine phosphorylation of the EGFR, whereas protein derived from each HB-EGF disulfide analogue cell line failed to induce EGFR

tyrosine phosphorylation. It was unexpected that HB-EGF- Cys/Ser_{134/143} was able to compete with ¹²⁵I-EGF for EGFRs but not stimulate EGFR phosphorylation. These results suggest that HB-EGF- Cys/Ser_{134/143} may antagonize EGFRs. Previous reports identified an HB-EGF antagonist, CRM 197, an analogue of diphtheria toxin (DT). Membrane bound forms of HB-EGF act as receptors for DT and CRM197 specifically inhibits HB-EGF binding to EGFRs [27].

Disulfide bond formation is a complex process that occurs in the endoplasmic reticulum catalyzed by protein disulfide isomerase [28]. In general, there are two stages of quality control in the ER, primary and secondary. Primary quality control is undergone by virtually all proteins regulating transport of proteins from the ER to the golgi apparatus regulated by a number of molecular chaperones [29]. Deviations from a protein's native conformation due to incomplete folding or mis-folding often leads retention in the ER. HB-EGF immunohistochemical staining of HB-EGF-Cys/Ser_{108/121}, and HB-EGF-Cys/Ser_{116/132} exhibited specific staining immediately outside the nucleus, suggesting disruption of the first and second disulfide bonds within HB-EGF alter processing resulting in increased degradation or ER retention. To address this, stable cell lines were chosen that expressed similar HB-EGF mRNA levels. Western blot analysis using an HB-EGF C antibody resulted in various levels of HB-EGF protein expressed, although the same amount of total protein was analyzed. Combined with the results from immunohistochemistry HB-EGF and HB-EGF- Cys/Ser_{134/143} appeared to be processed through the ER with no problem, unlike HB-EGF-Cys/Ser_{108/121} and HB-EGF- Cys/Ser_{116/132}. This is likely due to processing within the ER, but increased HB-EGF degradation for these HB-EGF disulfide analogues cannot be ruled out.

Results from this study demonstrated that disruption of the first and second disulfide bond influenced processing rates, was required to maintain high affinity binding to EGFRs, and lacked the ability to stimulate EGFR phosphorylation. In contrast, disruption of the third disulfide bond of HB-EGF was processed similar to native HB-EGF, not required to maintain high affinity binding to EGFRs and lacked the ability to stimulate tyrosine phosphorylation of EGFR.

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Fig. 1. Northern blot analysis of total cellular RNA from HB-EGF, HB-EGF- Cys/Ser_{108/121}, HB-EGF- Cys/Ser_{116/132}, HB-EGF- Cys/Ser_{134/143}, and pSV-neo stably transfected mouse fibroblasts. (A) A 0.7kb recombinant HB-EGF transcript is present in all HB-EGF and HB-EGF analogue stable cell lines (lanes 2–5) when incubated with a full-length ³²P-labeled HB-EGF cDNA as probe. No HB-EGF mRNA was detected in pSV-neo mouse fibroblasts (MLC) (lane 1). (B) A GAPDH probe was used to assure equivalent amounts of RNA were analyzed for all RNA samples from each stable cell line examined.

Fig. 2. HB-EGF western blot analyses of cellular extracts from HB-EGF, HB-EGF- Cys/Ser_{108/121}, HB-EGF- Cys/Ser_{116/132}, HB-EGF- Cys/Ser_{134/143}, and pSV-neo stably transfected mouse fibroblasts. (A) Cell extracts were examined using an HB-EGF C antibody resulting in immunoreactive proteins of 6.5, 20 and 24 kDa (HB-EGF C, TM2 and TM1, respectively) for HB-EGF and each HB-EGF disulfide analogue (lanes 2–5) and pSV-neo mouse fibroblasts (lane 1). (B) A mouse actin antibody, resulting in a 43kDa immunoreactive protein present in all cell lysates (lanes 1–5).

Fig. 3.

Immunohistochemical analysis of HB-EGF, HB-EGF- Cys/Ser_{108/121}, HB-EGF- Cys/Ser_{116/132}, HB-EGF- Cys/Ser_{134/143}, and pSV-neo stably transfected mouse fibroblasts. Fixed cells were incubated with an HB-EGF C antibody and then with a FITC-conjugated secondary antibody (FITC). DNA staining with DAPI was used to localize the nuclei for each stable cell line. No HB-EGF immunofluorescence was observed in pSV-neo mouse fibroblasts (MLC).

Fig. 4.

Competitive EGFR binding assay and induction of EGFR tyrosine phosphorylation by HB-EGF, HB-EGF- Cys/Ser_{108/121}, HB-EGF- Cys/Ser_{116/132}, HB-EGF- Cys/Ser_{134/143}, and pSV-neo proteins derived from stably transfected mouse fibroblasts. (A) The ¹²⁵I-EGF bound versus unbound ratio in the presence of increasing amounts of unlabeled recombinant proteins from each stable cell line was examined using A431 cells. rHB-EGF, HB-EGF, and HB-EGF- Cys/Ser_{134/143} competed with ¹²⁵I-EGF with similar EC₅₀ (65–85ng). HB-EGF- Cys/Ser_{108/121}, HB-EGF- Cys/Ser_{116/132}, and pSV-neo failed to compete with ¹²⁵I-EGF. Each point represents the mean for two experiments (each in triplicate). HB-EGF western blot of 50ng each of rHB-EGF, HB-EGF, HB-EGF- Cys/Ser_{108/121}, HB-EGF- Cys/Ser_{116/132}, HB-EGF- Cys/Ser_{134/143}, (A inset, lanes 1 and 3–6) and lysate from pSV-neo (A inset, lane 2) (B) Western blot analysis of EGFR and tyrosine phosphorylated EGFR (P-EGFR) was analyzed by treatment of A431 cells with 50ng rHB-EGF or proteins from each stable cell line. rHB-EGF and HB-EGF stimulated tyrosine phosphorylation of the 170kDa EGFR, whereas HB-EGF-

Cys/Ser_{108/121}, HB-EGF- Cys/Ser_{116/132}, HB-EGF- Cys/Ser_{134/143}, and pSV-neo proteins did not (B, upper panel). Equivalent levels of immunoreactive 170kDa EGFR were observed for each experiment (B, lower panel).