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Biochemical and Biophysical Research Communications 342 (2006) 19–27
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Sequestering ErbB2 in endoplasmic reticulum by its autoinhibitor from translocation to cell surface: An autoinhibition mechanism of ErbB2 expression

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Received 8 January 2006
Available online 2 February 2006

Abstract

ErbB2 is differentially overexpressed in tumor versus host tissues, suggesting that an autoregulation mechanism may modulate the expression of ErbB2 and control cell growth. A truncated ErbB2 extracellular domain, herstatin has been shown to bind to ErbB2 and inhibit the growth of tumor cells expressing ErbB2. In the present study, the interaction of herstatin and ErbB2 in vivo was observed by confocal microscopy. The aggregation of ErbB2 and herstatin was found in endoplasmic reticulum (ER). The decrease of ErbB2 on the cell surface was accompanied with the increased colocalization of ErbB2 and herstatin in the cytoplasm, suggesting that the formation of ErbB2/herstatin complex may prevent transit from ER to cell surface of ErbB2. The formation of ErbB2 and herstatin complex was further confirmed by immunoprecipitation. The results demonstrate that sequestering ErbB2 molecules intracellularly by herstatin may be a possible mechanism of the cell growth inhibition.

Keywords: erbB2; Ectodomain; Herstatin; Autoinhibitor; Receptor internalization; Cell proliferation; Autoregulation; Endoplasmic reticulum; Growth factor receptor; Confocal microscopy

Receptor tyrosine kinases of the ErbB family play an important role in cell growth, differentiation, tissue development, and tumor generation and/or progression [1–3]. This family consists of four structurally related transmembrane receptors, the epidermal growth factor (EGF) receptor (EGFR or ErbB1), ErbB2, ErbB3, and ErbB4. EGF family ligands bind to the extracellular domain (ECD) of ErbB receptors, resulting in autophosphorylation of specific tyrosine residues within the cytoplasmic domain [3–5]. Ligand-induced activation of ErbB receptors often involves multiple receptor members. Ligand recognition by a specific ErbB receptor not only results in homodimerization of the same receptor, but also in recruitment of related receptors forming heterodimers via combinatorial protein interactions. The signaling network of ErbB proteins is based on receptor homo- and heterodimers [6–8].

Over a dozen different EGF-like ErbB ligands have been identified. EGF, transforming growth factor-α (TGF-α) and other EGF-related ligands bind to ErbB1. ErbB3 and ErbB4 interact with a large group of structurally related EGF-like peptides [3,9]. ErbB2 does not bind any EGF family ligand with high affinity and its homodimerization is thought to occur in a ligand-independent manner [1,2,10]. However, ErbB2 functions as a coreceptor and is a preferred heteroassociation partner of all other ErbB proteins [6,11,12]. ErbB2 can be activated by constitutive autophosphorylation when overexpressed or via heterodimerization and transphosphorylation with ligand-bound EGFR, ErbB3, and ErbB4 [6,13–15]. ErbB2-containing heterodimeric receptor combinations have been found to be more

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doi:10.1016/j.bbrc.2006.01.115

mitogenic and transforming [16–18]. The receptor complexes have a higher ligand affinity and signaling potency by virtue of the potent latent kinase activity of ErbB2 [3,5,12]. The expression levels of the various receptors and the concentrations of respective ligands determine the composition of homo- and heterooligomers [6,18].

Activation-induced endocytosis and downregulation are important in limiting the duration of receptor activation. ErbB2 is differentially overexpressed in tumor versus normal tissues [19], suggesting that under normal physiological conditions there must be an autoregulation mechanism that may modulate the expression of ErbB2 and control cell growth. A crucial physiological mechanism of ErbB receptor signal attenuation involves the downregulation of receptors from the cell surface. For example, upon EGF stimulation, EGFR undergoes ligand-induced endocytosis, ubiquitinylation, and subsequent sorting to the lysosome for degradation [20]. In contrast, ErbB2 avoids delivery to lysosomes and subsequent proteolysis and has a long half-life at the plasma membrane [21–23]. The oncogenic potential of ErbB2 may in part be explained by its capability to avoid downregulation. The reason why ErbB2 escapes the lysosomal pathway and remains at the cytoplasmic membrane has remained elusive.

Recently, an autoinhibitor of ErbB2, herstatin has been identified [24]. It is a secreted product of ErbB2 gene containing ErbB2 ECD subdomain I and II followed by an intron-encoded 79 amino acid sequence. This truncated ErbB2 ECD has been shown to bind to EGFR and ErbB2, block homomeric and heteromeric receptor interactions, and inhibit cell growth [25,26]. However, the mechanism of proliferation inhibition of herstatin has not been well understood.

To investigate the mechanisms of autoregulation of ErbB2 expression, in the present study, the mammalian expression vectors containing cDNAs of ErbB2, ErbB2 ECD mutant, herstatin and herstatin mutant were constructed, and the interaction between herstatin and ErbB2 in the transfected cells was studied by confocal microscopy and immunoprecipitation.

Materials and methods

cDNA constructs, cell culture, and DNA transfections. The herstatin sequence was cloned as previously described by Doherty et al. [24]. A mammalian expression vector was constructed by inserting *Hin*dIII–*Eco*RI fragment of herstatin into pCDNATM3.1/myc-His(−) (InvitrogenTM, Life Technologies) designated pCDNA3.1/hers. The plasmids pEGFP-N1 and pDsRed-Express-N1 were from Clontech. pEGFP-N1 encodes a GFP variant (EGFP) and pDsRed-Express-N1 a variant of *Discosoma* sp. red fluorescent protein (DsRed). The full-length herstatin cDNA cut from pCDNA3.1/hers by *Xho*I and *Hin*dIII sites was fused to the N-terminus of DsRed (designated pDsRed/hers) by the same sites. The 79 aa region deleted herstatin mutant was also fused to the N-terminus of DsRed designated pDsRed/mhers. The plasmid pCDNA3 containing full-length ErbB2 cDNA (pCDNA3/ErbB2) was kindly provided by Dr. Yanjun Jia (Institute of Transfusion Medicine, Beijing). The ErbB2 ECD cDNA was fused to the N-terminus of EGFP by *Xho*I and *Hin*dIII sites (pEGFP-N1/ErbB2) as previously described [27]. All standard molecular cloning techniques were carried out according to published procedures. All expression constructs were verified by sequence analysis.

SKBR3, MCF7, COS7, and CHO cells were obtained from American Type Culture Collection. The cells were cultured at 37 °C, 5% CO_2 in DMEM supplemented with 10% FCS. The cells were transfected with pCDNA3.1/hers or with empty vector by using the Lipofectamine (Life Technologies) reagent according to the manufacturer's protocol. Transfection efficiencies were compared by using plasmid pEGFP-N1. Stable pools of transfectants were selected in medium containing 800 μg/ml G418 (Sigma). For the studies by confocal microscopy, plasmid pEGFP-N1/ErbB2 was cotransfected with pDsRed/hers or pDsRed/mhers into CHO cells. For the studies by immunofluorescence staining and immunoprecipitation, plasmids pCDNA3.1/hers and pCDNA3/ErbB2 were cotransfected into CHO, COS7, and SKBR3 cells.

Cell lysate preparation, Western blotting, and immunoprecipitation. The cells transfected with pCDNA3.1/hers were grown on six-well plates. After transfection for 48 h, the cells were washed with PBS and lysed in ice-cold lysis buffer (100 mM DTT, 2% SDS, 10% glycerol, 0.1% bromophenol blue, and 50 mM Tris, pH 6.8). After the cells had been scraped from the plates, samples were centrifuged at 10,000*g* at 4 °C for 10 min and supernatants transferred to new tubes. Samples were then boiled in electrophoresis sample buffer and loaded onto SDS–PAGE gels. After transfer to nitrocellulose membranes, filters were blocked for 1 h in blocking buffer [50 mM Tris–Cl, pH 7.5, 100 mM NaCl (Tris-buffered saline, TBS) containing 5% dried milk and 0.2% Tween 20] and then incubated for 1 h with the monoclonal antibody against 79 aa region of herstatin (Upstate Biotechnology) diluted in blocking buffer. After being washed with TBS (50 mM Tris–Cl, pH 7.5, 100 mM NaCl, and 0.2% Tween 20), filters were incubated with horseradish peroxidase-conjugated secondary antibodies (Beijing Zhongshan Golden Bridge Biotechnology) for 30 min and bands visualized by the Enhanced Chemiluminescence system (Amersham Pharmacia Biotech).

For immunoprecipitation, cells cotransfected with pCDNA3.1/hers and pCDNA3/ErbB2 were washed with phosphate-buffered saline (PBS) and lysed in ice-cold lysis buffer [(50 mM Tris–Cl, pH 7.4, 100 mM NaCl, 10% glycerol, 1% Nonidet P-40, and 1× cocktail (Roche)]. Samples were centrifuged at 10,000*g* at 4 °C for 10 min and supernatants transferred to new tubes with the anti-herstatin antibody or the antibody against c-ErbB2, Ab-20 (NeoMarker), which recognizes ErbB2 ECD, and protein A/G PLUS-Agarose (Santa Cruz Biotechnology). Immunoprecipitated samples were then blotted on nitrocellulose and detected with Ab-20 or anti-herstatin antibody and horseradish peroxidase-conjugated secondary antibody (Beijing Zhongshan). The bands were visualized by the Enhanced Chemiluminescence system.

Cell proliferation measurements. Subconfluent monolayer cultures were trypsinized and the cells transfected with either pCDNA3.1/hers or empty vector were plated in quadruplicate in 96-well plates to a density of 5000 per well. Cell proliferation was analyzed by an MTT-based assay as follows. The medium in each well was replaced with 100 μl of medium containing MTT at 0.5 μg/μl and plates were returned to the incubator for 4 h. The medium-MTT was then removed, 100 μl of dimethylsulfoxide was added to each well and the plate kept in agitation for 10 min in the dark to dissolve the MTT-formazan crystals. The absorbance of the samples was then recorded at 570 nm. The results are presented as means ± the standard deviation (SD). The experiment was repeated for three times.

Confocal microscopy. CHO cells grown in 35-mm glass bottom dishes (MatTek) were cotransfected with pDsRed/hers or pDsRed/mhers and pEGFP-N1/ErbB2. After transfection for 48 h, the cells were washed with cold PBS and observed under a laser scanning confocal microscope (Radiance 2100, Bio-Rad).

For endoplasmic reticulum (ER) localization, the cotransfected cells were stained with ER-Tracker™ Blue-White DPX (E-12353) following the manufacturer's instructions.

To exclude the interferences of GFP and DsRed in the fusion proteins and to confirm the intracellular retention of ErbB2 caused by the overexpression of herstatin, COS-7 and SKBR3 cells were cotransfected with the plasmids pCDNA3.1/hers and pCDNA3/ErbB2, and then stained with the monoclonal antibody against herstatin, polyclonal antibody against ErbB2 and corresponding secondary antibodies (FITC-labeled goat-anti-mouse IgG and Rhodamine-labeled goat anti-rabbit IgG).

Statistical analysis. Data were expressed as means ± SD. Analysis of data was performed using the Student's *t* test.

Results

Proliferation inhibition of the cells expressing herstatin

The plasmids containing ErbB2, ErbB2 ECD mutant, herstatin, and herstatin mutant were illustrated in Fig. 1. The plasmid pCDNA3.1/hers was transfected into SKBR3, MCF7, and CHO cells. MCF7 and CHO cells express low level of ErbB2, whereas SKBR3 cells express high level of ErbB2. After transfection for 48 h, the cell lysates were prepared and herstatin expression was examined. Fig. 2A shows a Western blot analysis of herstatin expression in CHO, SKBR3, and MCF7 cells. An about 60 kDa protein was detected in each transfected cell line with the antibody against 79 aa region of herstatin. A much larger protein,

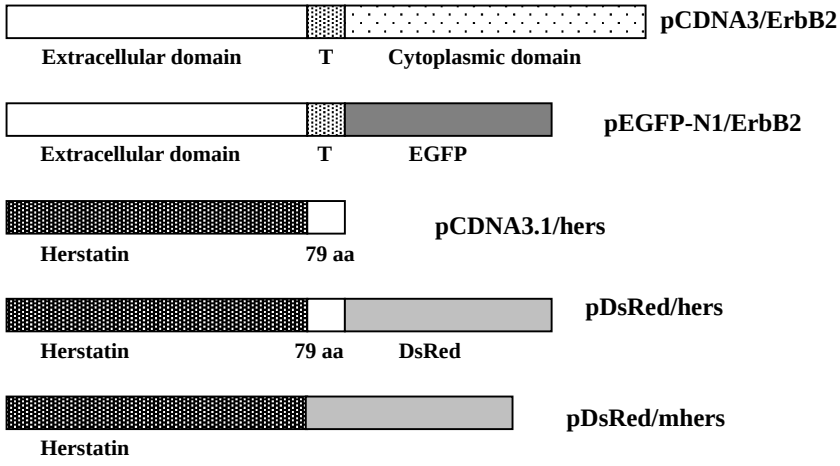

Fig. 1. Schematic diagram of the mammalian expression vectors.

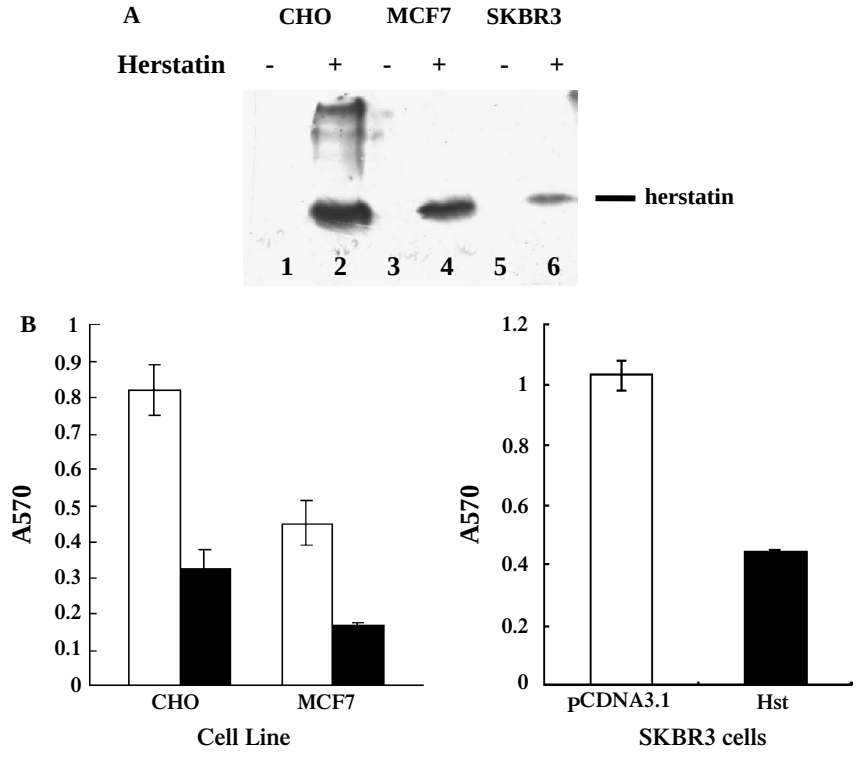

Fig. 2. The expression of herstatin in the transfected cells (A). The cells were transfected with plasmid pCDNA3.1/hers. After transfection for 48 h, the lysates were prepared and analyzed by Western blotting with the monoclonal antibody against herstatin. Lanes 1, 3, and 5, CHO, MCF7, and SKBR3 cells transfected with empty plasmid pCDNA3.1; lanes 2, 4, and 6, CHO, MCF7, and SKBR3 cells transfected with pCDNA3.1/hers. The inhibition of proliferation activity of the cells expressing herstatin (B). Subconfluent monolayer cultures were trypsinized, and the cells transfected with either pCDNA3.1/hers or empty vector were plated in quadruplicate in 96-well plates. Cell proliferation was analyzed by an MTT-based assay. Empty square, the cells transfected with the empty plasmid pCDNA3.1; solid square, the cells transfected with pCDNA3.1/hers.

possibly an aggregate of herstatin, was also found in CHO cells, but not in MCF7 and SKBR3 cells, which may be due to the higher transfection efficiency of CHO cells leading to the higher expression of herstatin in CHO cells than MCF7 and SKBR3 cells. The efforts to select the clones highly expressing herstatin from SKBR3 cells were hindered because of an inhibitory effect on cell survival. Since the transfection efficiency of CHO cells is much higher than any other cell lines and CHO cells express herstatin at a higher level, they were mainly used as the model cells in this study.

To determine the effects of herstatin expression on the cell growth, the proliferation activity of the CHO and MCF7 cells was analyzed. As shown in Fig. 2B, the growth of the cells transfected with pCDNA3.1/hers was considerably slower than the cells transfected with the empty plasmid. To ensure that the growth inhibition was not an artifact of the transfection, this experiment was repeated for three times and the results were very consistent.

Intracellular colocalization and complex formation of ErbB2 and herstatin

The expression profile of ErbB2 ECD and herstatin was observed under a laser scanning confocal microscope. The expression of herstatin-DsRed fusion protein (red) was distributed in the cytoplasm when the cells were transfected with pDsRed/hers only (Fig. 3A). Because of the existence of the membrane anchoring region, ErbB2/GFP fusion

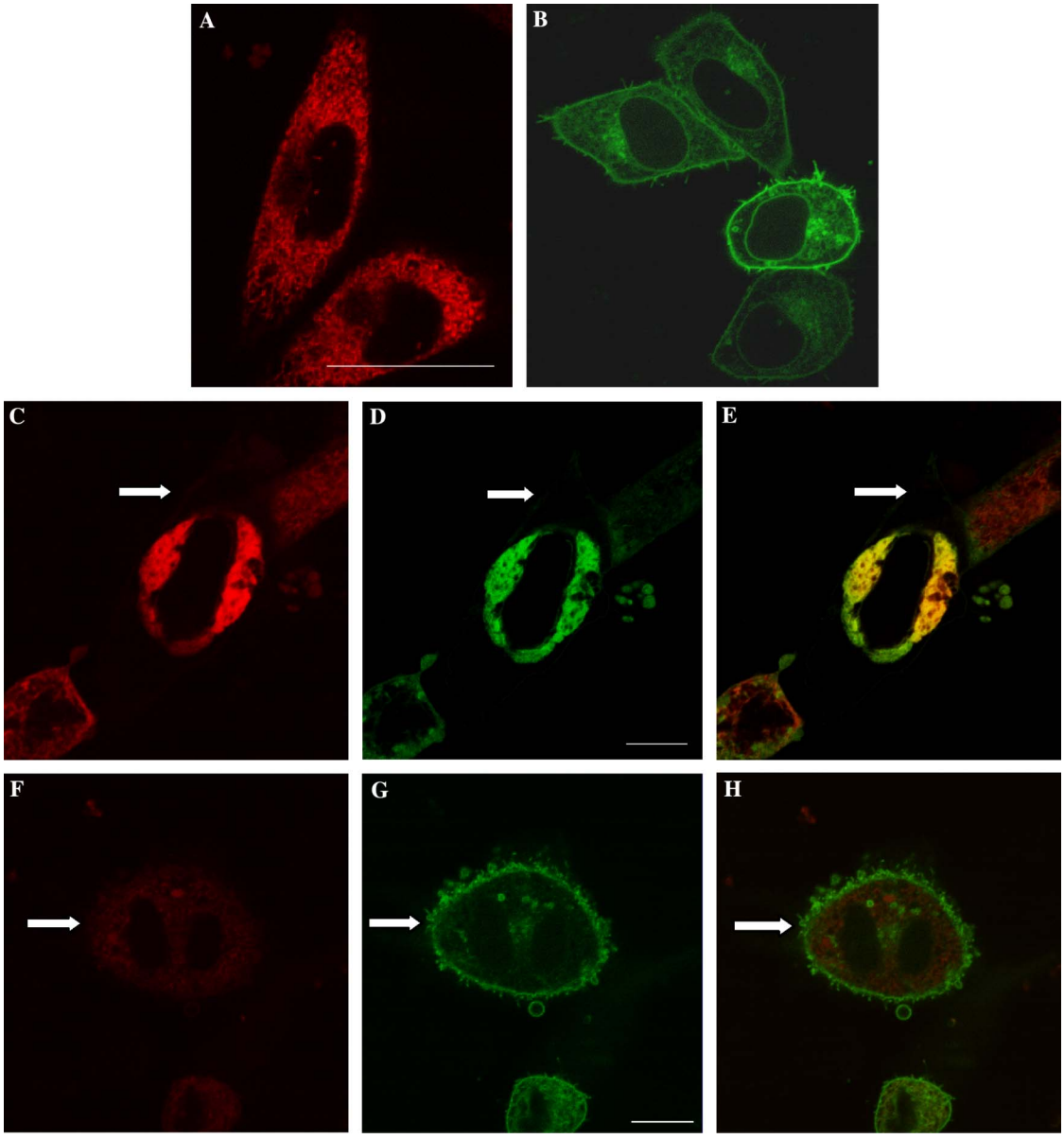

Fig. 3. Colocalization of herstatin and ErbB2 in CHO cells. CHO cells grown in 35-mm dishes were cotransfected with pDsRed/hers and pEGFP-N1/ErbB2. After washing with cold PBS, cells were observed under a laser scanning confocal microscope. (A) The cells transfected with pDsRed/hers only; bar = 50 μM. (B) pEGFP-N1/ErbB2 only; (C–H) the cells cotransfected with pDsRed/hers and pEGFP-N1/ErbB2. (C,F) Herstatin-DsRed fusion protein; (D,G) ErbB2-EGFP fusion protein; (E,H) merged image from (C,D or F,G), respectively. Bar = 10 μM. ⇨ Cell membrane.

protein (green) was mainly located on the cytoplasmic membrane surface when the cells were transfected with pEGFP-N1/ErbB2 only (Fig. 3B). While both plasmids were cotransfected into the cells, ErbB2 proteins on the cell surface were strikingly decreased in some cells, but an intense colocalization was observed as shown in Figs. 3C–E that two types of fluorescences overlapped well around the nucleus, yielding a yellowish or an orange color in merged images (Fig. 3E). However, the expression of either ErbB2 or herstatin varied in the individual cell significantly because transient transfection allows variance in gene expression. Interestingly, the stronger the expression of herstatin was, the weaker the expression of ErbB2 was on the cell surface. On the other hand, when the herstatin expression was not evident, the distribution of ErbB2 and herstatin was separated with the red color slightly seen in the cytoplasm and the green one clearly on the cell surface (Figs. 3F–H).

To confirm that the accumulation of herstatin and ErbB2 in the cytoplasm was not due to the aggregation of DsRed and EGFP in the fusion proteins, the COS7 cells were cotransfected with the plasmids pCDNA3.1/hers and pCDNA/ErbB2 that do not contain DsRed and EGFP genes, stained with anti-herstatin and anti-ErbB2 antibodies and then detected with corresponding fluorescence labeled secondary antibodies. Herstatin was stained as green with FITC-conjugated secondary antibody and ErbB2 stained as red with Rhodamine-labeled secondary antibody (Figs. 4A and B). Similar expression patterns were seen in cotransfected COS7 cells (Figs. 4C–E), showing that herstatin and ErbB2 were colocalized in the cytoplasm when the expression of herstatin was high. Furthermore, ErbB2 molecules on the cell membrane were decreased in herstatin expressing cells. In contrast, the location of ErbB2 at the membrane surface was not affected in the cells expressing low level of herstatin or not expressing at all (Figs. 4F–H).

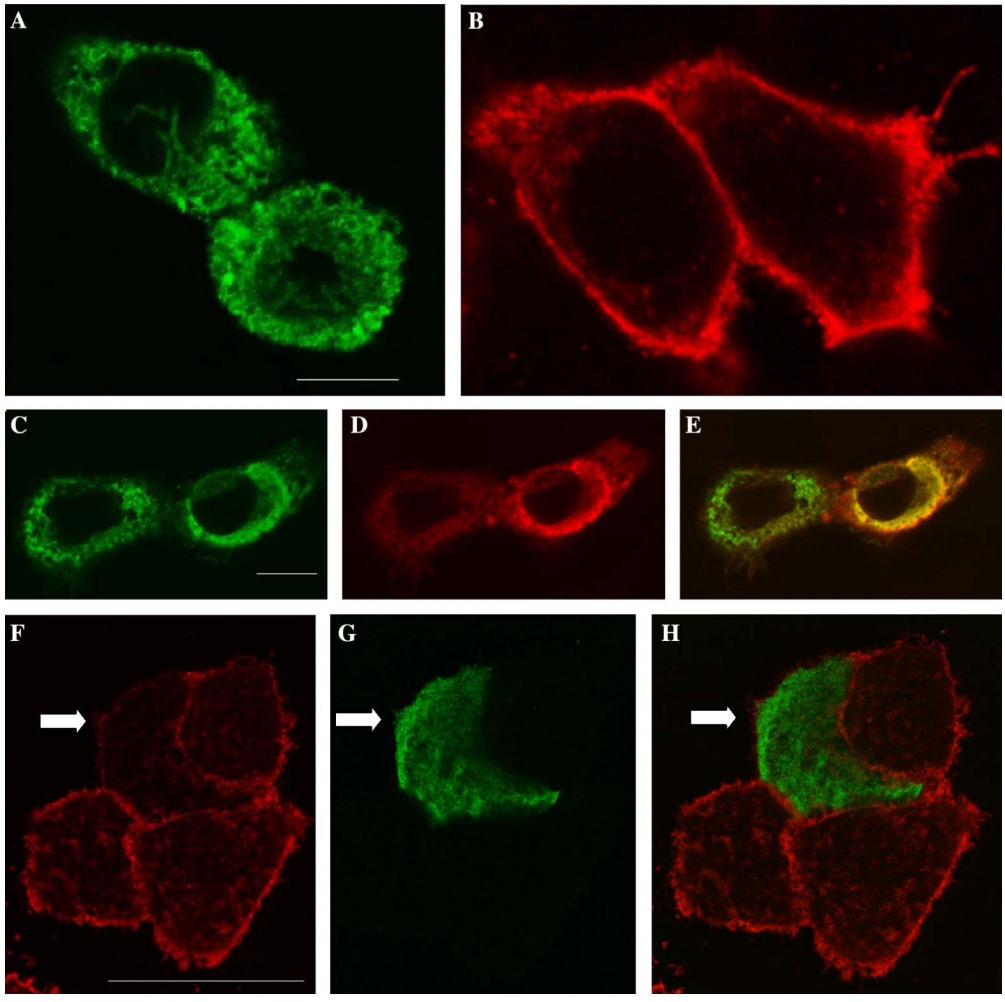

Fig. 4. Colocalization of herstatin and ErbB2 in COS7 cells. COS7 cells were cotransfected with the plasmids pCDNA3.1/hers and pCDNA/ErbB2, stained with anti-herstatin, anti-ErbB2 antibodies and the corresponding fluorescence labeled secondary antibodies. Herstatin was stained with FITC-conjugated secondary antibody and ErbB2 stained with Rhodamine-labeled secondary antibody. (A) The cells transfected with pCDNA3.1/hers; (B) pCDNA/ErbB2; (C–E) the cells cotransfected with pCDNA3.1/hers and pCDNA/ErbB2; (C) herstatin; (D) ErbB2; (E) merged image from (C,D). Bar = 10 μM. (F–H) ErbB2 molecules on the cell membrane was decreased in a herstatin expressing cell. The location of ErbB2 at the membrane surface was not affected in the cells expressing low level of herstatin or no expressing at all. Bar = 50 μM. ⇨ Cell membrane.

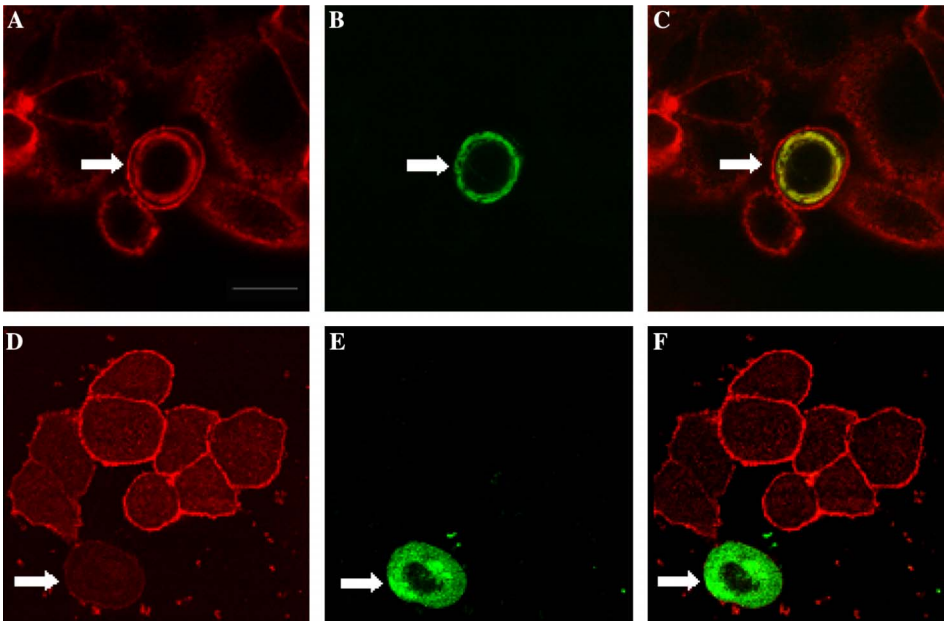

Fig. 5. Colocalization of herstatin and ErbB2 in SKBR3 cells. SKBR3 cells were transfected with the plasmid pCDNA3.1/hers and stained with anti-herstatin, anti-ErbB2 antibodies, and the corresponding fluorescence labeled secondary antibodies. Herstatin was stained with FITC-conjugated secondary antibody and ErbB2 stained with Rhodamine-labeled secondary antibody. (A,D) ErbB2; (B,E) herstatin; (C,F) colocalization of ErbB2 and herstatin. Bar = 50 μM. ⇨ Cell membrane.

The transfected CHO cells expressed higher level of herstatin than MCF7 and SKBR3 cells did as shown in Fig. 2A that the aggregates of herstatin were detected in the lysates of CHO cells but rarely in MCF7 and SKBR3 cells. To rule out the possibility that ErbB2 may associate with aggregated herstatin, SKBR3 cells were transfected with pCDNA3.1/hers. Fig. 5 shows that the intense colocalization of herstatin and ErbB2 was clearly seen in transfected SKBR3 cells (Figs. 5A–C). All the cells highly expressing herstatin did not survive. The results were very consistent with the previous studies [25]. In some SKBR3 cells transfected with herstatin, ErbB2 molecules on the cell membrane were largely decreased (Figs. 5D–F). The results demonstrate that the overexpression of herstatin can cause retention of ErbB2 in ER and inhibit cell growth.

It has been reported that herstatin binds to ErbB2 by its C-terminal 79 aa region. To confirm that the aggregation of ErbB2 in the cytoplasm is due to the interaction with herstatin, the plasmids pDsRed/mhers containing a herstatin mutant gene, in which the sequence encoding C-terminal 79 aa region was deleted, and pEGFP-N1/ErbB2 were cotransfected into the cells. After 48 h, the cotransfected cells were stained with ER-Tracker Blue-White DPX dye to determine the intracellular compartment localization of ErbB2 and herstatin. ER-Tracker Blue-White DPX dye is a photostable probe that is selective for ER in live cells. The cotransfected CHO cells were stained for 30 min, fixed with 3.7% formaldehyde for 10 min, and visualized under a confocal microscope. Fig. 6 shows that both ErbB2 and herstain aggregated in ER (blue). The localization of ErbB2-GFP fusion protein on the cell surface was remarkably decreased when herstatin was expressed highly (Figs. 6A–D). Whereas, when 79 aa region was deleted from herstatin, colocalization of herstatin and ErbB2 did not occur (Figs. 6E–H). Although herstatin mutant was expressed highly in the cytoplasm, ErbB2 appeared strikingly on the cell membrane surface, but only herstatin was localized within ER.

To further confirm that colocalization of ErbB2 and herstatin reflected protein–protein interaction, the immunoprecipitation was performed. From the lysate of CHO cells cotransfected with pCDNA3/ErbB2 and pCDNA3.1/hers or SKBR3 cells transfected with pCDNA3.1/hers, p185 ErbB2 protein was coprecipitated with the antibody against 79 aa region of herstatin (Fig. 7A). An about 60 kDa protein corresponding to herstatin and 185 kDa protein corresponding to ErbB2 were detected by the antibody against ErbB2 ECD. By using anti-ErbB2 antibody, herstatin was also coimmunoprecipitated from the cotransfected cells (Fig. 7B), demonstrating the formation of herstatin and ErbB2 complexes in the cells.

Discussion

ErbB receptors engage in an extensive network of homo- and heteroassociations resulting in signal transduction [3–5]. Ligand-mediated activation of ErbB receptors occurs by interaction of the ECDs of these receptors with specific members of the EGF family [7]. ErbB activity is also modulated by cell-surface ErbB proteins and other modulators. These modulators may augment or suppress signaling efficiency by serving as retention proteins and holding receptors at or away from sites of signaling or influence the specificity of ErbB signaling through differential receptor

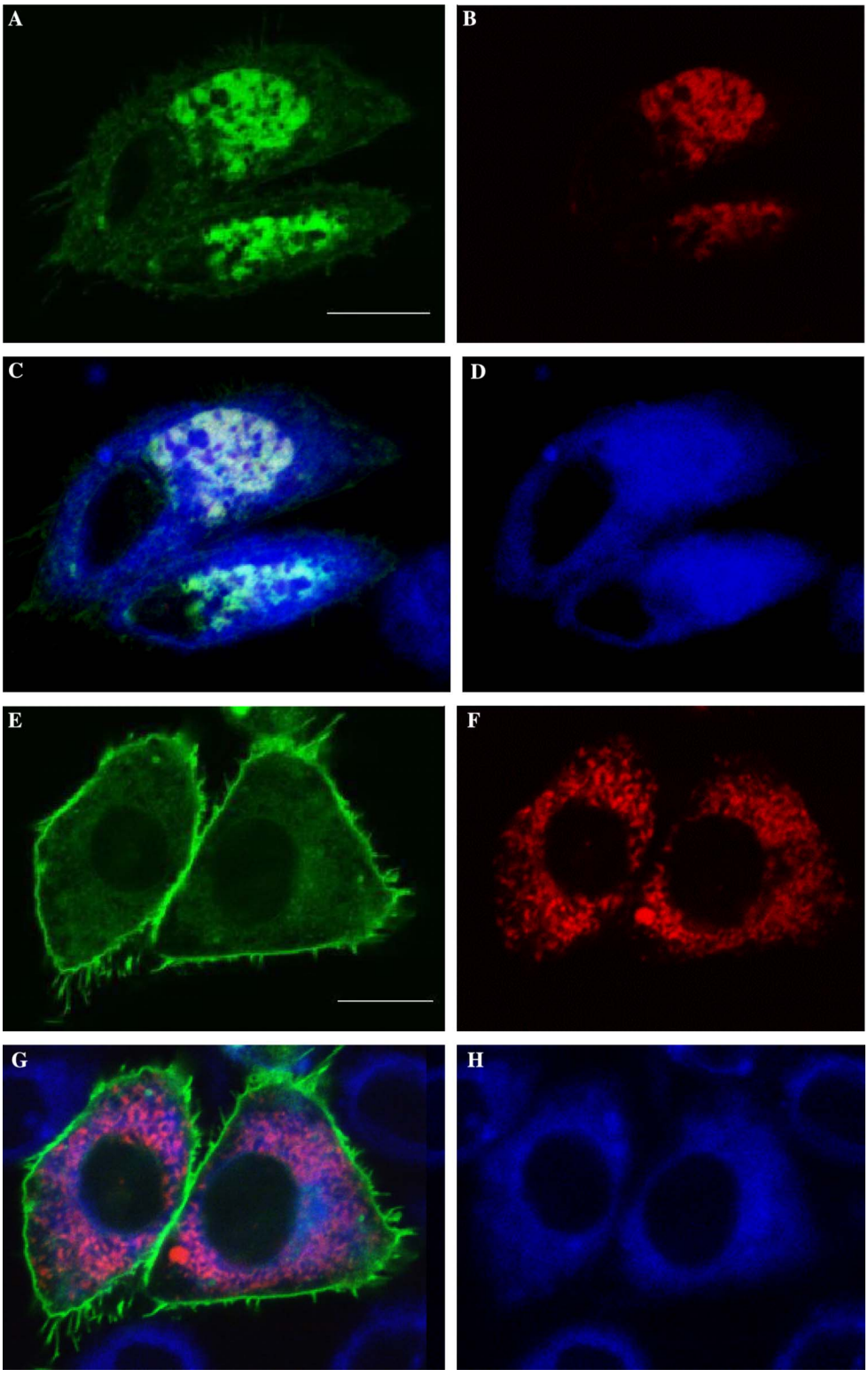

Fig. 6. Identification of localization of herstatin and ErbB2 in ER. The cotransfected CHO cells were stained with ER-Tracker Blue-White DPX dye for 30 min, fixed with 3.7% formaldehyde for 10 min and visualized under a confocal microscope. (A–D) The cells cotransfected with pDsRed/hers and pEGFP-N1/ErbB2. (A) ErbB2; (B) herstatin; (C) merged image from (A,B); (D) ER. (E–H) The cells cotransfected with pDsRed/mhers, in which the sequence encoding 79 aa region was deleted, and pEGFP-N1/ErbB2. (E) ErbB2; (F) herstatin mutant; (G) merged image from (E,F); (H) ER. Bar = 10 μM.

localization [28]. A hypothesis on the major fate of erbB2 retained in the ER is intracytosolic degradation by the proteasome [20].

Receptor localization plays an important role in the reception of paracrine signals from adjacent cells. One function of erbB2 receptor localization is to send different

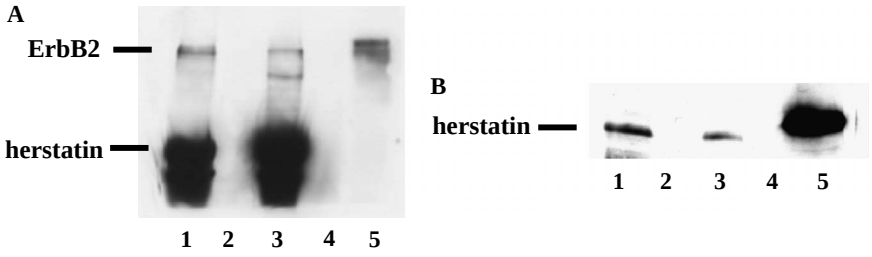

Fig. 7. Formation of complex of ErbB2 and herstatin in the cells. The cells cotransfected with pCDNA3.1/hers and pCDNA3/ErbB2 were lysed. Samples were immunoprecipitated with the antibody against herstatin and protein A/G PLUS-Agarose and then blotted on nitrocellulose. Immunoprecipitated samples were detected with the antibody against c-ErbB2, Ab-20 and horseradish peroxidase-conjugated secondary antibodies. The bands were visualized by the Enhanced Chemiluminescence system (A). Lane 1, the CHO cells cotransfected with pCDNA3/ErbB2 and pCDNA3.1/hers; lane 2, the CHO cells transfected with pCDNA3.1; lane 3, the SKBR3 cells transfected with pCDNA3.1/hers; lane 4, the SKBR3 cells transfected with pCDNA3.1; lane 5, SKBR3 cell lysate. Samples were immunoprecipitated with the anti-ErbB2 antibody and protein A/G PLUS-Agarose. Immunoprecipitated samples were detected with the anti-herstatin antibody and horseradish peroxidase-conjugated secondary antibodies (B). Lane 1, the CHO cells cotransfected with pCDNA3/ ErbB2 and pCDNA3.1/hers; lane 2, pCDNA3.1 empty plasmid; lane 3, the SKBR3 cells transfected with pCDNA3.1/hers; lane 4, pCDNA3.1 empty plasmid; lane 5, total lysate from CHO cells transfected with pCDNA3.1/hers.

signals to the cells depending on the growth factor ligand. The gathering of excess ErbB2 at the cell surface results in constitutive activation of signaling cascades that drive tumor cell growth.

An anti-ErbB2 monoclonal antibody, Herceptin or trastuzumab, which is widely used in treatment of breast cancer, is generally believed to exert its antitumor effect by driving internalized ErbB2 away from the recycling pathway and toward the lysosomal degradative pathway [29–31]. It has been also reported that ErbB2 could be functionally inactivated by intracellular expression of a single-chain antibody, which retains the receptor in ER and prevents its translocation to the plasma membrane [32–34]. ER retention of ErbB2 has no effect on proliferation of cells with low levels of ErbB2; however, it blocks growth of ErbB2-overexpressing tumor cells [31].

It is important to understand the normal process of receptor downregulation. Autoregulation may be a common feature among oncogenes. Recently, a natural autoinhibitor of ErbB2, herstatin has been reported. It is not detected by Northern analysis of carcinoma cell lines [24]. It has been shown that herstatin binds to ErbB2 receptor ECD, blocks receptor activation, and inhibits the proliferation activity of ErbB2 expressing cells and EGF-stimulated Akt phosphorylation [25,26].

Binding of herstatin to ErbB2 receptor in vitro has been investigated by pull-down assay [24]. The binding site of ErbB2 extracellular domain with Herstatin has also been identified in vivo [35]. In the present study, the interaction of herstatin and ErbB2 in vivo was observed by confocal microscopy. Colocalization of ErbB2 and herstatin was found in ER. It is noteworthy that the decrease of ErbB2 on the cell surface was accompanied with the increased colocalization of ErbB2 and herstatin in ER, suggesting that the formation of ErbB2/herstatin complex may prevent transit through ER and cell surface localization of ErbB2 and affect the association of ErbB2 with other members of the ErbB family. However, low expression of herstatin may not be able to sequester ErbB2 within ER as seen in Fig. 3 that herstatin and ErbB2 were present separately in the cytoplasm and on the cytoplasmic membrane surface, indicating that retention of ErbB2 in ER was dependent on the expression level of herstatin. It has been demonstrated that moderate overexpression of ErbB2 fails to cause transformation in NIH3T3 cells, whereas high overexpression is transforming in cell culture models. This result is consistent with the studies by Azios et al. [25], showing that tyrosine phosphorylation of ErbB2 is inhibited by expression of herstatin and the extent of inhibition depends on the ratio of herstatin to erbB2 introduced into the cells. The formation of ErbB2 and herstatin complex was further confirmed by using immunoprecipitation and Western blotting analysis. The results indicate herstatin can bind to ErbB2 in the cells and cause the retention of ErbB2 in ER. Herstatin is a secreted alternative product of the ErbB2 gene. The activity of herstatin to associate with ErbB2 in vitro and the inhibition of cell growth by exogenous herstatin have been reported, suggesting that the complex formation of ErbB2 and herstatin could occur either during their biosynthesis and/or through binding of secreted herstatin [25,36]. It is possible that both intracellular herstatin and/or secreted herstatin were responsible for receptor inhibition.

Taken together, our results demonstrate that sequestering ErbB2 molecules intracellularly by herstatin may be a possible mechanism of the cell growth inhibition. By blocking transportation of ErbB2 from ER to the plasma membrane surface, the interaction between ErbB2 and growth factors and subsequent signal transduction can be inhibited. In this way, herstain may be involved in the maintenance of the balance of ErbB2 expression under physiological conditions.

Acknowledgments

We thank Dr. Yanjun Jia for providing us plasmid pCDNA3/ErbB2. This work was supported by 863 Program (2003AA215101) and Beijing Natural Science Foundation (No. 7051006).

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