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The cytoplasmic tail of heparin-binding EGF-like growth factor regulates bidirectional intracellular trafficking between the plasma membrane and ER

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ABSTRACT

Heparin-binding epidermal growth factor (EGF)- like growth factor (HB-EGF) is synthesized in the ER, transported along the exocytic pathway, and expressed on the plasma membrane as a type I transmembrane protein. Upon extracellular stimulation, HB-EGF, either proHB-EGF or the shed form HB-EGF-CTF, undergoes endocytosis and is then transported retrogradely to the ER. In this study, we showed the essential contribution of the short cytoplasmic tail of HB-EGF (HB-EGF-cyto) to the bidirectional intracellular trafficking between the ER and plasma membrane and revealed several critical amino acids residues that are responsible for internalization from the plasma membrane and ER targeting. We suggest that these anterograde and retrograde sorting signals within HB-EGF-cyto are strictly regulated by protein modification and conformation.

1. Introduction

Heparin-binding epidermal growth factor (EGF)-like growth factor (HB-EGF) is a member of the EGF family. HB-EGF is expressed in multiple tissues, and participates in a variety of normal and aberrant processes including blastocyst implantation, wound healing, smooth muscle cells hyperplasia, and tumor growth [reviewed in 1]. The membrane-anchored form of HB-EGF is the receptor for diphtheria toxin [2]. HB-EGF is synthesized by ER-bound ribosomes and expressed at the cell surface as a type I transmembrane precursor (proHB-EGF). The extracellular portion of proHB-EGF contains a heparin-binding domain and a bioactive EGF-like sequence; the intracellular portion (HB-EGF-cyto) consists of 24 amino acids [3,4]. The EGF-like growth factor domain alone is sufficient to elicit EGF receptor (EGFR) phosphorylation via autocrine, juxtacrine, and paracrine signaling pathways. ProHB-EGF is cleaved at the juxtamembrane site by activated metalloproteases, yielding a soluble growth factor and a carboxyl-terminal fragment containing the transmembrane and cytoplasmic segments (HB-EGF-CTF). This process, termed ectodomain shedding, can be stimulated by various physiological and pharmacological agonists, including 12-*O*-tetradecanoylphorbol-13-acetate (TPA) [5], calcium ionophores [6], and ligands of G protein coupled receptors [7]. We previously showed that in addition to these well-known signaling pathways mediated by the extracellular EGF-like growth factor domain, the membrane-anchored HB-EGF-cyto (maHB-EGF-cyto), which indicates both unshed proHB-EGF and HB-EGF-CTF (see Fig. 1(A)), migrates from the plasma membrane to the ER and nuclear envelope (NE) [8]; there, maHB-EGF-cyto faces to the nucleoplasm and directly regulates gene expression [9,10]. However, the molecular mechanism underlying the bidirectional intracellular trafficking of this protein between the plasma membrane and ER remains unknown. How is the newly synthesized proHB-EGF, which possesses ER retrieval activity, delivered to the plasma membrane? How is the ER retrieval activity activated in response to shedding stimuli? A major goal of this article is thus to reveal the molecular mechanism that regulates the intracellular sorting of maHB-EGF-cyto in opposite directions. In this study, to address these questions, we used various mutants of HB-EGF and a monoclonal antibody against HB-EGF-cyto.

Abbreviations: TPA, phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate; EGF, epidermal growth factor; HB-EGF, heparin-binding EGF-like growth factor; EGFR, EGF receptor; HB-EGF-cyto, cytoplasmic tail of HB-EGF; maHB-EGF-cyto, membrane anchored HB-EGF-cyto; CTF, carboxy-terminal fragment; NE, nuclear envelope; TCA, trichloroacetic acid

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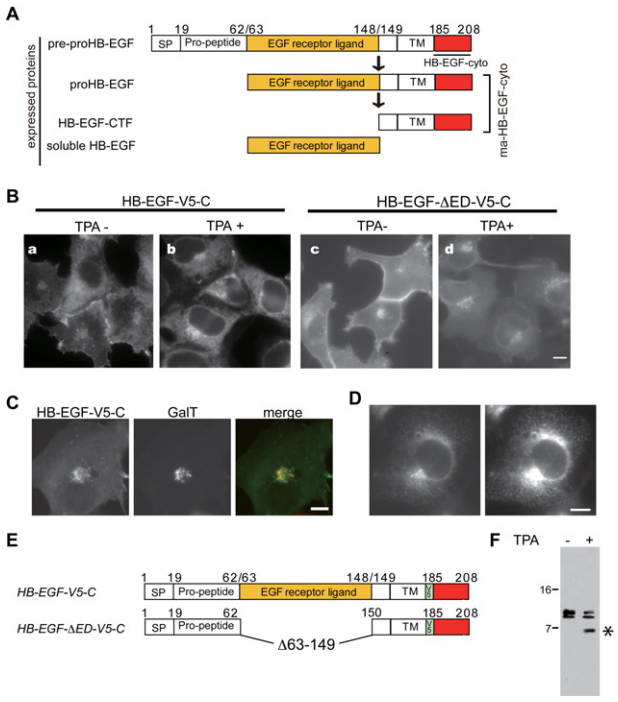

Fig. 1. The extracellular domain is not essential for the intracellular trafficking of maHB-EGF-cyto. (A) Schematic presentation of HB-EGF processing. HB-EGF is synthesized as pre-proHB-EGF by ER-bound ribosomes, and expressed at the plasma membrane as proHB-EGF. ProHB-EGF is cleaved at the juxtamembrane domain, yielding soluble HB-EGF and HB-EGF-CTF. Here we collectively refer to proteins containing the membrane-anchored cytoplasmic tail (proHB-EGF and HB-EGF-CTF) as 'maHB-EGF-cyto'. SP: signal peptide and TM: transmembrane domain. (B) HT1080 cells were transiently transfected with HB-EGF-V5-C (left) or HB-EGF-ΔED-V5-C (right). Cells were treated with TPA, and then fixed; intracellular localization was visualized with anti-V5 mAb. (C) HB-EGF-V5-C was transfected into the GalT stably expressing COS1 cells. Then cells were stained with anti-V5 mAb. (D) HB-EGF-V5-C transfected HT1080 cells were fixed with PFA and permeabilized with Tween 20. Then cells were immunostained with anti-V5 mAb. Right panel is an over exposed photo. (E) Schematic presentation of an HB-EGF mutant lacking the extracellular domain (HB-EGF-ΔED-V5-C). V5-epitope tag (indicated as V5) was inserted just downstream of the transmembrane domain, because tagging at the C terminus interferes with the function of HB-EGF [9] and stabilizes phosphorylation of maHB-EGF-cyto [14]. (F) HB-EGF-ΔED-V5-C was transiently transfected into HT1080 cells. After TPA treatment, total cell lysate were analyzed by immunoblotting using anti-V5 mAb. * indicates the carboxyl-terminal fragment of HB-EGF-ΔED-V5-C. Bar, 10 μm.

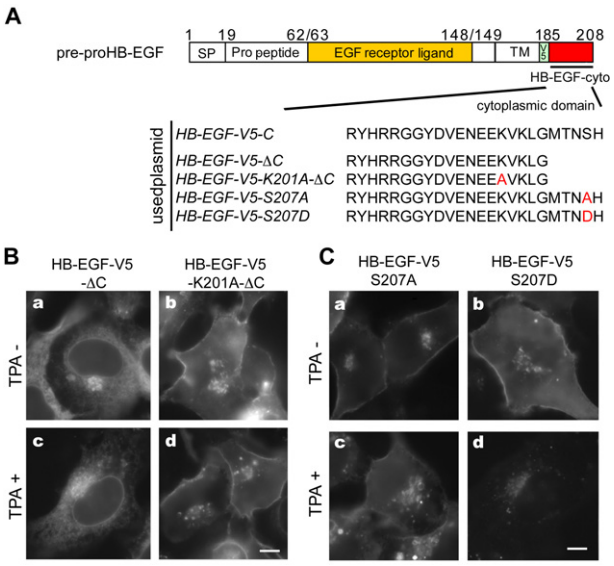

Fig. 2. The cytoplasmic tail of HB-EGF contributes to bidirectional sorting to the plasma membrane and ER. (A) Schematic presentation of HB-EGF constructs used in this study. The mutated amino acids are indicated in red. The sizes of expressed proteins were verified by Western blotting using anti-V5 mAb (data not shown). (B, C) HT1080 cells were transfected with indicated plasmids and treated with TPA, followed by fixation. Cells were stained with anti-V5 mAb. Bar, 10 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2. Materials and methods

2.1. Construction of plasmids

The plasmids encoding YFP-fused HB-EGF (pEYFP-N1-HB-EGF) and HB-EGF with the carboxyl-terminal 10 amino acids deleted were described previously [9]. Internally V5-tagged HB-EGF (pME18S-HB-EGF-V5-C), HB-EGF-V5-ΔC, HB-EGF-V5-K201A-ΔC, HB-EGF-V5-K201A, and constructs encoding GFP fused to the C-terminal 10- or 15-amino acid regions of HB-EGF-cyto (199–208 and 194–208, respectively) were described previously [8]. HB-EGF-V5-S207D and HB-EGF-V5-S207A were constructed by oligonucleotide-directed mutagenesis using HB-EGF-V5-C as a template. HB-EGF-ΔED-V5-C was constructed by overlapping PCR. All cDNA constructs were verified by DNA sequencing.

2.2. Antibodies

Rat monoclonal antibodies against HB-EGF-cyto (mAbC and mAbD) were raised against a synthetic peptide corresponding to HB-EGF-cyto (185–208). The details were described in [8]. Mouse anti-V5 mAb was purchased from Invitrogen. Rabbit anti-GFP polyclonal antibodies were from Medical & Biological Laboratories. All fluorescently labeled secondary antibodies were purchased from Jackson ImmunoResearch. Anti-HB-EGF polyclonal antibodies (#H1) were described in [11].

2.3. Cell culture, transfection and TPA treatment

HT1080 cells were grown in Eagle's minimum essential medium supplemented with 10% fetal calf serum and 0.1 mM non-essential amino acids. COS cells stably expressing human β-1,4-galactosyltransferase-GFP (GalT-GFP) [12] were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Transfections were performed using Lipofectamine™ 2000 (Invitrogen). 12-*O*-tetradecanoylphorbol-13-acetate (TPA) was purchased from Sigma, dissolved in DMSO as a 100 μM stock, and stored at −30 °C. For TPA treatment, TPA was added to the culture medium at a final concentration of 100 nM and incubated for 30 min at 37 °C.

2.4. Immunofluorescence

For Figs. 1(B,C) and 2(B,C), cells were fixed with 4% formaldehyde at RT for 20 min and permeabilized with 0.4% Triton X-100. For Fig. 1(D), cells were fixed with 4% formaldehyde at RT for 20 min and permeabilized with 0.1% Tween 20. For Fig. 3(B), cells were fixed with ice-cold 10% trichloroacetic acid (TCA) at RT for 20 min and permeabilized with 0.4% Triton X-100 for 5 min. Cells were then blocked with 2% BSA, incubated with primary antibodies overnight at 4 °C, and subsequently incubated with Cy3- or FITC-conjugated secondary antibodies. Image acquisition was performed using a microscope (IX70; Olympus) equipped with a camera (ORCA-ER; Hamamatsu).

2.5. Western blotting

Indicated plasmids were transfected into HT1080 cells. After 16 h, cells were treated with TPA and collected in SDS–PAGE sample buffer. Total cell lysates were separated by SDS–PAGE and transferred to nitrocellulose membranes. Blots were blocked with 3% skim

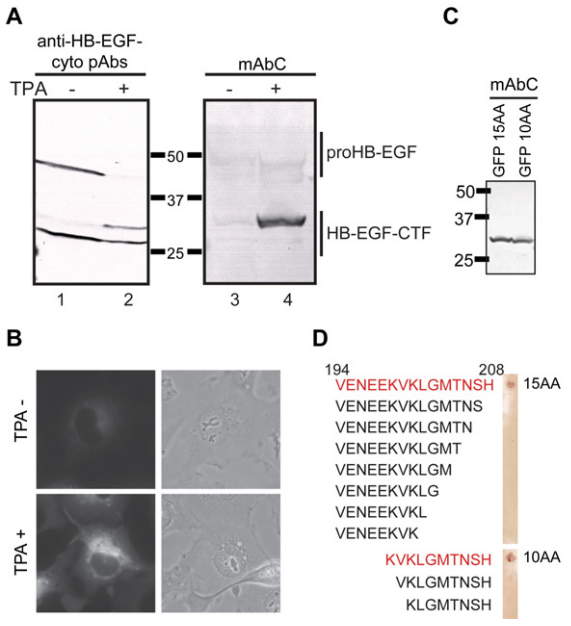

Fig. 3. A monoclonal antibody against the cytoplasmic tail of HB-EGF, mAbC, recognizes maHB-EGF-cyto only after addition of shedding stimuli. (A) Cells were transiently transfected with carboxyl-terminally YFP-tagged HB-EGF. Cells were then treated with TPA and analyzed by Western blotting using anti-HB-EGF-cyto pAbs (left) or mAbC (right). To clearly separate the two HB-EGF-CTF bands, YFP-tagged HB-EGF was used because the HB-EGF-CTFs are too small to separate clearly (calculated molecular weight is around 6.5 kDa). (B) HB-EGF-V5-C transiently expressing cells were treated with TPA and fixed with ice-cold 10% TCA; cells were then permeabilized with 0.4% Triton X-100 and stained with mAbC. (C) Total cell lysate expressing GFP fused to the 15 or 10 carboxyl-terminal amino acids of proHB-EGF (194–208 or 199–208, respectively) were analyzed by immunoblotting using mAbC (right). (D) Identical quantities of the indicated peptides were blotted onto nitrocellulose membrane and detected with mAbC.

milk, and then incubated with the primary antibody followed by HRP-conjugated secondary antibodies.

2.6. Dot blotting

Synthetic peptide was obtained from Sigma Aldrich and dissolved according to the manufacturer's instructions. Each peptide solution was applied to the nitrocellulose and dried. The nitrocellulose sheets were washed, blocked with 3% skim milk/PBS-T, and incubated with primary antibody followed by HRP-conjugated secondary antibody. Detection was performed using an AEC kit (Vector Laboratories).

3. Results

3.1. The extracellular domain of HB-EGF is not essential for intracellular trafficking

In the absence of ectodomain-shedding stimuli, proHB-EGF was predominantly localized at the plasma membrane and in the perinuclear regions [8] (also see Fig. 1(B,a)). The perinuclear signal is colocalized with a medial-*trans*-Golgi marker, GalT (Fig. 1(C)). This signal probably represents newly synthesized (pre)proHB-EGF because it disappeared after translation block by cycloheximide (data not shown). Here we use the term 'maHB-EGF-cyto' to refer collectively to proHB-EGF and HB-EGF-CTF (please see Fig. 1(A)), because proHB-EGF as well as HB-EGF-CTF are targeted to the ER and NE (ER/NE). In response to the shedding stimuli, maHB-EGF-cyto was relocalized to the ER (Fig. 1(B,b) and (D)) as shown previously [8]. Here in Fig. 1(D), we used Tween 20 to permeabilize the plasma membrane and to retain the ER meshwork structure. In order to identify the domain responsible for the intracellular trafficking of HB-EGF, we deleted the extracellular EGF-like growth factor domain (HB-EGF-ΔED-V5-C, Fig. 1(E)). The truncated mutant produced a protein of the predicted size, and shed after exposure to shedding stimuli (Fig. 1(F)). This mutant was localized at the plasma membrane and Golgi apparatus in the absence of shedding stimuli (Fig. 1(B,c)) and relocalized to the ER/NE in response to shedding stimuli (Fig. 1(B,d)), in a manner similar to the wild type; however, the level of accumulation at the Golgi complex was slightly higher than in the wild type, possibly because of differences in glycosylation efficiency. This result indicates that the extracellular domain of proHB-EGF is not essential for intracellular trafficking in either direction, i.e., to the plasma membrane or to the ER.

3.2. The cytoplasmic tail of proHB-EGF contributes to retrograde trafficking from the plasma membrane to the ER

Next, to investigate the roles of the cytoplasmic tail of proHB-EGF (HB-EGF-cyto) in intracellular trafficking between the plasma membrane and ER, we introduced several mutations (Fig. 2(A)). HB-EGF lacking the 5 C-terminal amino acids (HB-EGF-V5-ΔC) was retained at the ER (Fig. 2(B,a)) because of an unintentionally generated K(X)KXX-type ER retrieval signal [13]. This localization was not affected in the presence of shedding stimuli (Fig. 2(B,c)). On the other hand, HB-EGF-V5-K201A-ΔC was observed at the plasma membrane and the Golgi apparatus, similar to the wild type (Fig. 2(B,b)), but was not relocalized to the ER/NE after exposure to shedding stimuli (Fig. 2(B,d)). Previously, we showed that K201A does not affect endocytosis [8]. Thus, these results indicate that the carboxyl-terminal 5 amino acids are not required for plasma membrane targeting, but are indispensable for internalization. The carboxyl-terminal 5 amino acids contain a conserved serine residue (S207) that is phosphorylated in response to shedding stimuli [14]. To examine whether phosphorylation of this residue plays a role in the regulation of maHB-EGF-cyto localization, we replaced S207 with A or D (termed HB-EGF-V5-S207A or HB-EGF-V5-S207D, respectively; Fig. 2(A)). Western blotting analysis showed that neither mutant had an effect on ectodomain shedding efficiency (data not shown). S207A was localized at the plasma membrane in the absence of shedding stimuli (Fig. 2(C,a)). However, the efficiency of internalization of this mutant is less than wild type (Fig. 2(C,c)), implying that either the presence of serine at position 207 or phosphorylation of this residue is required for the regulation of internalization but not for plasma membrane localization. In contrast, the phosphorylation-mimic mutant, S207D was expressed at the plasma membrane (Fig. 2(C,b)) and disappeared from the plasma membrane after shedding stimuli (Fig. 2(C,d)) indicating the internalization in response to shedding stimuli; however, this protein did not migrate to the ER/NE, but rather accumulated at the intracellular vesicles in addition to the Golgi apparatus (Figs. 2(C,d) and S1). Thus, S207 phosphorylation is not sufficient for the induction of internalization in the absence of shedding stimuli, suggesting that dephosphorylation or further modification is required at the intracellular vesicles prior to targeting to the ER/NE. Taken together, these results indicate that specific residues in the cytoplasmic tail of HB-EGF contribute to retrograde trafficking from the plasma membrane to the ER.

3.3. A monoclonal antibody recognizes HB-EGF-cyto only after exposure to shedding stimuli

The cytoplasmic tail of HB-EGF has critical functions in its intracellular trafficking. We thus raised monoclonal antibodies against a synthetic peptide corresponding to HB-EGF-cyto (185–208), obtaining two clones, designated mAbC and mAbD. Details of mAbD were described previously [8]. In this paper, we analyzed the regulation of maHB-EGF-cyto intracellular localization using mAbC. In Western blotting analysis, anti-HB-EGF-cyto polyclonal antibodies recognized proHB-EGF in the absence of shedding stimuli; in the presence of

shedding stimuli, the antibody detected additional two bands corresponding to HB-EGF-CTF. The upper HB-EGF-CTF band, but not the lower band, was increased in the presence of shedding stimuli (Fig. 3(A), lanes 1 and 2); this observation could be explained by S207 phosphorylation [14]. HB-EGF-CTF could be observed even in the absence of shedding stimuli, possibly due to a basal level of shedding activity, as the serum in the culture medium contains growth factors that induce ectodomain shedding [15].

On the other hand, mAbC unexpectedly recognized only the TPA-induced upper band of HB-EGF-CTF, but not full length HB-EGF, in Western blotting (Fig. 3(A), lanes 3 and 4), even though mAbC was raised against a synthetic peptide containing no modification. This observation implied the presence of an epitope-masking posttranslational protein modification(s) of proHB-EGF under steady-state conditions, and the removal of this modification after exposure to the shedding stimuli.

In immunofluorescence microscopy, anti-V5 mAb stained the plasma membrane and the Golgi complex at steady state; after exposure to shedding stimuli, the antibody stained the ER/NE (Fig. 1(B) and (D)). In contrast, mAbC did not yield any significant signals when cells were fixed with paraformaldehyde in the presence or absence of shedding stimuli (data not shown). Since mAbC recognizes the slow-migrating HB-EGF-CTF on Western blots of samples exposed to shedding stimuli (Fig. 3(A)), we attempted to preserve the antigenicity for mAbC during fixation using multiple fixation methods, including methanol, ethanol, and TCA. We found that mAbC could stain maHB-EGF-cyto when cells were fixed with TCA, after exposure to shedding stimuli but not at steady state (Fig. 3(B)). TCA effectively inactivates soluble phosphatases, thus preserving the phosphorylation level during sample processing for immunofluorescence staining [16]. This result suggest that phosphorylation in the HB-EGF-cyto might induce a conformational change and expose the epitope for mAbC; as mentioned above, no phosphorylation or protein modifications is not contained in the antigen peptide.

3.4. Amino acids 199–208 are required for recognition by mAbC

To identify the epitope of mAbC, we fused GFP with the carboxyl-terminal 10 or 15 amino acids of HB-EGF-cyto (residues 199–208 or 194–208, respectively), expressed these constructs in HT1080 cells, and subjected cell lysates to Western blotting (Fig. 3(C)). mAbC recognized both constructs, indicating that the epitope of mAbC resides in the amino acids 199–208. Next, to determine the exact epitope recognized by mAbC, we subjected a series of peptides derived from amino acids 194–208 to dot blotting. However, removal of even a single residue (H208 or K199) from either end of the carboxyl-terminal 10 amino acids almost completely abolished reactivity with mAbC (Fig. 3(D)). Therefore, we concluded that the entire carboxyl-terminal 10-amino acid region (residues 199–208) is required for recognition by mAbC.

4. Discussion

HB-EGF shows bidirectional intracellular trafficking, anterograde and retrograde transport between the plasma membrane and ER, and is able to localized to five different subcellular localizations, the plasma membrane, Golgi apparatus, ER, nuclear envelope, and perinuclear vesicles; the latter are not further specified. This study revealed two main findings; essential contribution of cytoplasmic tail to the bidirectional intracellular trafficking of HB-EGF and critical amino acids residues that are responsible for retrograde sorting from the plasma membrane to the ER.

Retrograde sorting acts on cargo molecules targeted to endosomes from the plasma membrane. These cargo molecules are then transported to the trans-Golgi network (TGN), Golgi membrane, or in some cases ER via retrograde route [17–19]. However retrograde sorting route from the plasma membrane to the ER has to date only been well characterized for some of the toxin [20]. Therefore it is to be addressed that we first revealed several critical amino acid residues that are responsible for internalization from the plasma membrane and ER.

4.1. A model for anterograde transport from the ER to plasma membrane

Based on our results described here and our previous observations (summarized in Fig. 4(A)), we propose a model for the intracellular trafficking of maHB-EGF-cyto (Fig. 4(B)). Amino acids 185–198 of maHB-EGF-cyto are capable of directing a reporter protein to the ER and NE [8]; nevertheless, the newly synthesized proHB-EGF is sorted to the plasma membrane. This indicates that ER retrieval/retention activity is inactivated when proHB-EGF is synthesized in the ER, probably by protein modification, steric masking by a partner subunit [21,22], or a conformational change. The exact molecular mechanism for the inactivation of HB-EGF-cyto ER targeting activity (185–198) remains unknown; however, our Western blotting experiment (Fig. 3(A)), in which mAbC could not detect proHB-EGF, implies that epitope-masking posttranslational protein modification(s) are present in the epitope domain (199–208) of newly synthesized proHB-EGF. The epitope domain contains amino acids residues such as K199, K201, and T205 which could have posttranslational modifications such as methylation, acetylation, or phosphorylation. Shedding stimuli increase mAbC accessibility to the epitope region (Fig. 3(A) and (B)) and simultaneously activate ER retrieval activity. Thus one possible model that fits the existing data is that during or just after protein translation, a protein modification occurs in mAbC epitope region (199–208) that suppresses ER retention/retrieval activity (185–198) via conformational change. With ER targeting blocked, the maHB-EGF-cyto protein is instead targeted to the plasma membrane (Fig. 4(B,①,②)).

4.2. A model for the internalization from the plasma membrane and retrograde transport to the ER

Our mutagenesis analysis showed that the carboxyl-terminal 5 amino acids (203–208), and especially phosphorylation of S207, are indispensable for internalization after exposure to shedding stimuli (Fig. 2). In addition, the S207D and K201A mutant accumulated at a perinuclear organelle after exposure to shedding stimuli (Figs. 2(C) and S1 and [8], respectively). Moreover, the phosphorylation at S207 [14] may be essential for the recognition of HB-EGF-cyto by mAbC in immunofluorescence microscopy. Based on these observations, we speculate that in response to shedding stimuli, two events may occur at the same time, resulting in the internalization and activation of ER targeting (Fig. 4(B,④,⑤)): first, an epitope-exposing conformation change is induced by S207 phosphorylation (④); second, epitope masking posttranslational modification (①) is concomitantly removed. Neither the precise timing nor the location of these reactions is known. The first event could explain the unstable conformation of HB-EGF, as observed in immunofluorescence microscopy (Fig. 2(B)); the second event may explain the recognition of the HB-EGF-cyto epitope by mAbC only after shedding stimuli exposure, as observed in Western blotting (Fig. 2(A)). The results obtained with the S207D mutant indicate that S207 phosphorylation is not sufficient for the induction of internalization in the absence of shedding stimuli (Fig. 2(C)), supporting the idea that two events are required and suggesting some function(s) for S207 in the ultimate localization of the protein (ER, plasma membrane, or perinuclear vesicles). In this regard, it is noteworthy that a chimeric protein containing the carboxyl-terminal 10 amino acids (199–208) accumulates at the perinuclear structure [8], whereas perinuclear targeting ability is also

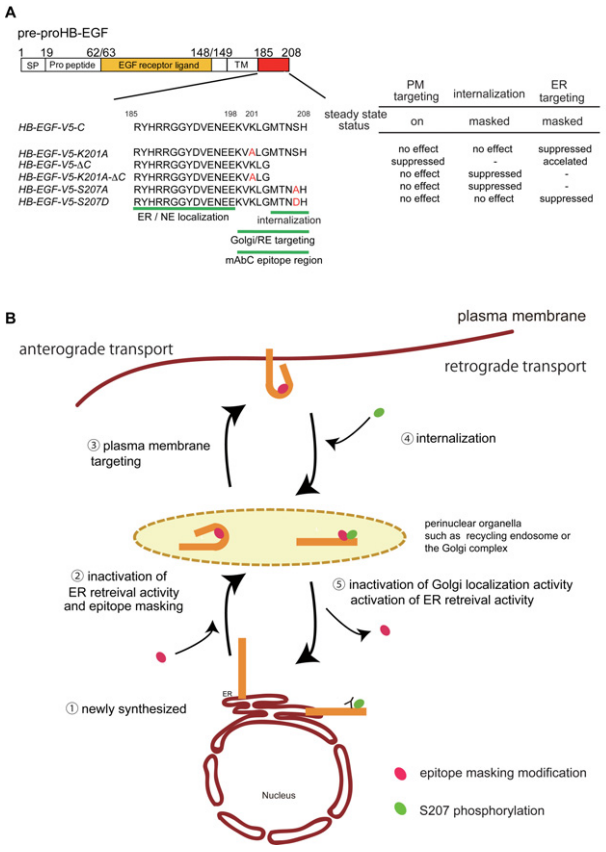

Fig. 4. A working model for the intracellular trafficking of maHB-EGF-cyto and its localization. (A) Summary of constructs used in mutagenesis analysis and their behavior with respect to plasma membrane targeting, internalization, and ER targeting. Functional domains within the cytoplasmic tail involved in intracellular trafficking are in green; the region required for mAbC detection is in red. (B) A model in which post-translational modification regulates intracellular trafficking of HB-EGF. Since the extracellular domain of HB-EGF is not essential for intracellular trafficking (Fig. 1), only HB-EGF-cyto is shown (orange bar). ① Pre-proHB-EGF is synthesized by ER-bound ribosomes. ② Epitope masking (199–208) occurs during or just after protein synthesis Fig. 3(A), and suppresses the ER retrieval activity (185–198) via a conformation change. ③ In this situation, at steady state, proHB-EGF targets to the plasma membrane. ④ Internalization requires S207 phosphorylation Fig. 2(C). ⑤ Accessibility of the epitope domain to mAbC in immunofluorescence microscopy Fig. 3(B) suggests that unstable protein modification(s) such as phosphorylation occur after exposure to shedding stimuli, concomitant with the removal of the epitope-masking protein modification Fig. 3(A), resulting in activation of ER retrieval activity. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

inactivated in proHB-EGF at steady state, consistent with the epitope-masking protein modification invoked in our model (Fig. 4(B,②)). S207D accumulated intracellular vesicles might be recycling endosomes, since the NE targeting of maHB-EGF-cyto utilizes the Rab11 dependent retrograde membrane trafficking pathway [8]. Here we used TPA as a shedding stimulus. Other shedding stimuli, such as bEGF or EGF also induces NE localization of maHB-EGF-cyto [8], thus the response of maHB-EGF-cyto to each shedding stimulus might have its own mechanism. Further studies will be necessary to determine the exact protein modifications and conformation of this protein. The results reported here, however, show that the cytoplasmic tail of HB-EGF contains sorting signals involved in trafficking a single protein in opposite directions; these signals are likely to be strictly regulated by protein modification and conformation.

Acknowledgments

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Supplementary Material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.fob.2012.09.002.

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