Constitutive Lysosomal Targeting and Degradation of Bovine Endothelin-converting Enzyme-1a Mediated by Novel Signals in Its Alternatively Spliced Cytoplasmic Tail*

(Received for publication, June 22, 1998, and in revised form, October 7, 1998)

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Endothelin-converting enzyme-1 (ECE-1) is a type II membrane protein that catalyzes the proteolytic activation of big endothelin-1 to endothelin-1 (ET-1). The subcellular distribution of ECE-1, and hence the exact site of physiological activation of big ET-1, remains controversial. Here, we demonstrate with several complementary methods that the two alternatively spliced bovine ECE-1 isoforms, ECE-1a and ECE-1b, differing only in the first 30 amino acids of their N-terminal cytoplasmic tails, exhibit strikingly distinct intracellular sorting patterns. Bovine ECE-1a, which is responsible for the intracellular cleavage of big ET-1 in endothelial cells, is constitutively recruited into the lysosome, whereas it is rapidly degraded. In contrast, bovine ECE-1b, the isoform found in cultured smooth muscle cells, is transported to the plasma membrane by a default pathway and functions as an ectoenzyme. Mutational analyses reveal that the N-terminal tip of the cytoplasmic domain of bovine ECE-1a contains novel proline-containing signals that mediate constitutive lysosomal targeting. Analyses of chimeric ECE-1/transferrin receptors demonstrate that the cytoplasmic tail of bovine ECE-1a is sufficient for the lysosomal delivery and rapid degradation. Our results suggest that the distinct intracellular targeting of bovine ECE-1 isoforms may provide new insights into functional aspect of the endothelin system and that the cell permeability of ECE inhibitor compounds should be carefully considered during their pharmacological development.

* This work was supported by grants from the Ministry of Education, Science and Culture of Japan and a Japan Heart Foundation Research Grant. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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§ The abbreviations used are: ET, endothelin; ECE, endothelin-converting enzyme; CHO, Chinese hamster ovary; EC, endothelial cells; EIA, enzyme immunoassay; PCR, polymerase chain reaction; TR, human transferrin receptor; NGS, normal goat serum; PBS, phosphate-buffered saline.

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the cell surface. In contrast, in vascular endothelial cells and Chinese hamster ovary (CHO) cells transfected with the ECE-1 cDNA, cleavage of endogenously synthesized big ET-1 occurred intracellularly during transit through the Golgi apparatus (14, 27). Thus, there appear to be different conversion sites of big ET-1. However, it is unclear whether the same ECE molecule is responsible for these different conversions. Clarifying the exact site at which ECE-1 functions is of importance to understand its physiological roles and to design ECE inhibitors for therapeutic purpose.

Recently, two subisoforms of ECE-1, termed ECE-1a and ECE-1b, which differ from each other only in the N-terminal tip of their cytoplasmic tail, were identified (14, 24, 28, 29). Structural studies of the human ECE-1 gene indicate that these two isoforms are generated by alternative splicing (29). To investigate the significance of these two isoforms of ECE-1, we have studied the possibility that the N-terminal cytoplasmic tails of bovine ECE-1a and ECE-1b determine subcellular localization of the protein and hence the site at which big ET-1 is converted to the mature peptide. We provide morphological, biochemical, and pharmacological evidence showing that these subisoforms exhibit distinct intracellular sorting patterns, both in native vascular cells and in transfected CHO cells. Furthermore, we have uncovered signals in the alternatively spliced cytoplasmic tail of bovine ECE-1a that are responsible for constitutive lyssosomal targeting of this integral membrane protein.

**EXPERIMENTAL PROCEDURES**

**Reagents—**Synthetic human big ET-1(1–38) and ET-1 were obtained from Sigma. FR901533 (WS97098B) and FR139317 (WS97098B) were gifts from Fujisawa Pharmaceutical Co., Ltd. Fura-2-AM was from Molecular Probes, Inc. (Eugene, OR).

**Enzyme Immunoassay of Endothelin Peptides—**The supernatant of transfected cells was directly applied to a sandwich-type enzyme immunoassay (EIA) that showed no cross-reactivity between big ET-1 and ET-1 (14).

**Cell Culture—**CHO cells were cultured as described (14). The coding region of bovine ECE-1a or ECE-1b was subcloned into the pME18S vector (14).

Supernatants from transfected clones (CHO/ECE-1a and CHO/ECE-1b) were performed as described (14). Transfected colonies were isolated by alternative splicing of the transfectant clones (CHO/ECE-1a and CHO/ECE-1b) were performed as described (14). Clones that showed a similar level of expression by Northern blotting were chosen for further analysis. Endothelial cells were isolated from bovine coronary arteries by collagenase treatment and were cultured in Dulbecco's modified Eagle's medium containing 10% (v/v) fetal bovine serum. Smooth muscle cells were isolated from bovine trachea by collagenase treatment and were cultured in Dulbecco's modified Eagle's medium in the presence or absence of FR901533 at the designated concentrations and were incubated at 37 °C. The supernatant was directly used for reverse transcriptase PCR.

**Reverse Transcription-PCR—**RNA was extracted from cells using RNA STAT-60 (TEL-TEST "B", Inc.) as recommended by the manufacturer.

First-strand cDNA synthesis was carried out with 1 μg of total RNA and oligo(dT)12-18 primers by using SuperScript reverse transcriptase II (Life Technologies, Inc.) as recommended by the manufacturer. The PCR contained 20 mM Tris-HCl (pH 8.5), 50 mM KCl, 1.5 mM MgCl2, a 0.2 mM concentration of each dNTP, a 100 mM concentration of each amplification primer, 10 ng of first strand cDNA, and 2.5 units of Taq DNA polymerase. The primers 5′-ATGTGCTCCCCGGGGCA-3′ and 5′-TTGGTGGACGTCCACTTGAAGG-3′ were used for bovine ECE-1b-specific amplification (93 base pairs expected). Thirty cycles of PCR were performed at an annealing temperature of 60 °C, and the PCR products were separated on a 2% agarose gel. The PCR products were verified by DNA sequencing.

**Quantitative PCR—**Quantitative PCR was performed using real-time PCR detection technology that was analyzed on a model 7700 Sequence Detector (Applied Biosystems) (30, 31). The assay uses the 5′-nucleotide activity of Taq polymerase to cleave a nonextendible hybridization probe during the extension phase of PCR. The fluorescent reporter located on the 5′-end of the probe is released from a quenching dye present on the 5′-end, and fluorescent emission is measured in real time. The amount of a specific transcript was calculated by determining the point at which the fluorescence exceeds a threshold limit (usually 10 times the S.D. values of the base line). The primers 5′-ATGTCTACCTA-CAAGCGGGCCA-3′ and 5′-TTGGTGAGCCTCACTTGAAGG-3′ were used for bovine ECE-1b-specific amplification, and the primers 5′-GAAGGCGTTGAAGAGGAC-3′ and 5′-TGGCGCATTCCGATATGAT-3′ were used for amplification of the common region of bovine ECE-1. The hybridization probes were 5′-TACACGGTCCTTCCGGACAGCGAGT-3′ (ECE-1b specific) and 5′-TCATCCATCCACTTCGGGTTGGCTCA-3′ (common region). These oligonucleotide probes, which bind to the amplified PCR products, were labeled with a reporter dye, FAM (6-carboxyfluorescein), on the 5′-nucleotide and a quenching dye, TAMRA (6-carboxytetramethylrhodamine), on the 5′-nucleotide. The PCR products were quantitated with an Applied Biosystems 7700 PCR Thermal Cycler. The fluorescence of the probe during the extension phase of PCR was monitored by subtracting the amount of ECE-1b from the total amount of ECE-1.

**Fluorescent Immunocytochemistry—**Cells were seeded on coverslips and cultured for 2 days.

Fluorescent immunocytochemistry was performed for intracellular staining, during which cells were fixed and permeabilized in methanol for 5 min at −20 °C. After washing in phosphate-buffered saline (PBS), PBS containing 10% (v/v) normal goat serum (NGS/PBS) was added. Following a 1-h incubation at 37 °C, the NGS/PBS was replaced with buffer containing polyclonal antibody (1:100) directed against bovine ECE-1 C-terminal peptides. After incubation for 90 min at 37 °C, the cells were washed six times with PBS for 10 min each and then incubated in NGS/PBS containing 7.5 μg/ml of fluorescein isothiocyanate-goat anti-rabbit IgG (Zymed Laboratories Inc.) for 2 h. Competitive inhibitors were included at 10 μM, and the cells were washed nine times with PBS for 10 min each. The coverslips were mounted on microscope slides with 90% (v/v) glycerol, 50 mM Tris-HCl (pH 9.0), and 2.5% (w/v) 1,4-diazobicyclo[2.2.2]octane. The rhodamine-lentil lectin (Vector Laboratories), used to counterstain the Golgi apparatus, was incubated at 2.5 μg/ml. Monoclonal antibody UHS, which recognizes hamster lysosomal membrane glycoprotein B, was obtained from The Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA).

For cell surface staining, cells were fixed in PBS containing 4% paraformaldehyde for 15 min at room temperature. Following two washes in PBS, cells were incubated in PBS containing 10% (w/v) nonfat dry milk (milk/PBS) for 1 h at 37 °C. Cells were incubated in milk/PBS containing a polyclonal antibody (1:100) directed against ECE-1 C-terminal peptides for 90 min at 37 °C. The cells were washed six times with PBS for 10 min each and then incubated in milk/PBS containing 7.5 μg/ml of fluorescein isothiocyanate-labeled goat anti-rabbit IgG. After 45 min at 37 °C, the cells were washed nine times with PBS for 10 min each. The coverslips were mounted on microscope slides as described above. Three negative control conditions were examined: staining with preimmune serum, antibody after preabsorption, and omission of primary antibody. None of these conditions resulted in cell staining.

**Ca2+ Transient Bioassay—**The stable transfected CHO cell lines, CHO/ECE-1a and ECE-1b, were transiently transfected with the ETα endothelin receptor expression construct and loaded with Fura 2-AM. Synthetic human ET-1 or big ET-1 was applied to these cells, and intracellular calcium changes were monitored by a Jasco CAM-110 digital ion analyzer as described (14). In some experiments, cells were pretreated with FR139317, an ETα receptor antagonist, or phosphoramidon.

**Molecular Labeling and Immunoprecipitation—**Cells were plated onto a 60-mm dish to obtain 70–80% confluency and grown overnight. Cells were washed twice with starvation medium (methionine and cysteine-free Dulbecco's modified Eagle's medium supplemented with...
% (v/v) fetal bovine serum), preincubated in starvation medium for 1 h, and incubated for 1 h in 1.5 ml of the same medium containing 100 μCi/ml Trans²S-labeled (ICN Biomedical). Pulse-labeled cells were chased for the designated times in complete medium. In some experiments, cells were preincubated for 2–6 h in the medium containing 100 μm phosphoramidon, 30 μm ammonium chloride, or 100 μM chloroquine and chased for 0, 1, 2, and 4 h in the same medium. At each time point, labeled cells were placed on ice and solubilized with lysis buffer (50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 1% “Zwittergent”). ECE-1a and ECE-1b were immunoprecipitated from postnuclear supernatants using the polyclonal antibody directed against the C-terminal 16 amino acids of bovine ECE-1. Immunoprecipitates were analyzed on 7.0% polyacrylamide gels. Quantitation of radioactivity was performed on a Phosphorimager (Molecular Dynamics, Inc., Sunnyvale, CA).

**Expression of the Two Alternative Spliceforms of Bovine ECE-1**—Recent studies have shown that the ECE-1 gene contains two alternative promoters and first exons, generating ECE-1a and ECE-1b polypeptides that differ from each other in the N-terminal half of their cytoplasmic tails (24, 29) (see Fig. 7). We found that bovine arterial and bronchial smooth muscle cells, both possessing endothelin receptors (3), exclusively express ECE-1b mRNA (Fig. 1A). In contrast, both ECE-1a and ECE-1b mRNAs were detectable in cultured vascular endothelial cells by nonquantitative reverse transcription-PCR (Fig. 1A). Therefore, we determined the relative quantity of the two isoforms in both cell types by real-time quantitative PCR using a fluorogenic 5′ nuclease assay (30, 31). As shown in Fig. 1B, 95% of ECE-1 mRNA expressed in endothelial cells was ECE-1a mRNA, indicating that endothelial cells predominantly express ECE-1a.

**Distinct Subcellular Localization of ECE-1a and ECE-1b**—Since the only sequence difference between bovine ECE-1a and ECE-1b was found in the tip of the cytoplasmic tails, we investigated the possibility that these isoenzymes exhibit different subcellular localizations. We immunostained both endothelial cells and smooth muscle cells with antibodies that recognize the common C-terminal ectodomain of bovine ECE-1. Without prior permeabilization, endothelial cells stained only faintly (Fig. 1C). After permeabilization, endothelial cells showed strong staining in intracellular vesicles, the majority of which overlapped with Golgi staining, visualized using lentil lectin (Fig. 1C). In contrast, smooth muscle cells exhibited a robust cell surface staining without permeabilization (Fig. 1C). With permeabilization, smooth muscle cells exhibited both intracellular and cell surface staining (data not shown). These findings indicate that the endothelial cells predominantly express intracellular ECE-1, whereas the smooth muscle cells express ECE-1 on the cell surface. To examine where the actual cleavage of endogenously produced big ET-1 occurs in these endothelial cells, we cultured the cells in the presence of phosphoramidon, a cell-permeable ECE-1 inhibitor (27), or FR901533, a non-cell-permeable ECE-1 inhibitor (14). We then assayed the levels of big and mature ET-1 peptides secreted into the medium. Secretion of mature ET-1 from these cells was significantly inhibited by phosphoramidon, with a concomitant increase in big ET-1 secretion (Fig. 1D). In contrast, FR901533, at concentrations sufficient to inhibit extracellular cleavage of big ET-1 (see Fig. 3A), could not inhibit the processing of big ET-1 in endothelial cells, presumably because the compound did not have access to the site of big ET-1 cleavage. These findings support the notion that cleavage of endogenously produced big ET-1 occurs intracellularly in endothelial cells.

To examine whether the distinct subcellular localization of bovine ECE-1a and ECE-1b could be reproduced in heterologous cell lines, we stably transfected CHO cells (which do not express detectable levels of ECE-1) with either bovine ECE-1a or ECE-1b cDNAs and immunostained multiple transfected clones with an anti-ECE-1 antibody. Most cells from monoclonal CHO/ECE-1a transfected cell lines were not stained without prior permeabilization (Fig. 2A). With permeabilization, a robust staining of CHO/ECE-1a cells was observed in Golgi-like areas, in which we also observed strong lectin staining (Fig. 2B). In contrast, CHO/ECE-1b cells exhibited strong cell surface staining without prior permeabilization (Fig. 2C).

To functionally confirm the intracellular versus cell surface localization of bovine ECE-1a and ECE-1b proteins in transfected cells, we co-cultured CHO/prepro-ET-1 cells, which secrete big ET-1, with the same number of either CHO/ECE-1a or CHO/ECE-1b cells and assessed the generation of mature ET-1.
in the medium. CHO/ECE-1b cells generated large amounts of mature ET-1 when cocultured with CHO/prepro-ET-1 cells (Fig. 3A). The production of mature ET-1 was efficiently inhibited by FR901533, with a reciprocal increase in big ET-1 levels, indicating that cleavage occurred in the extracellular space where FR901533 had access. In contrast, co-culture of CHO/ECE-1a and CHO/prepro-ET-1 cells yielded only low levels of mature peptide, indicating that CHO/ECE-1a cells express little functional ECE-1 on the cell surface (Fig. 3A). This is not due to an absence of functional ECE-1 in CHO/ECE-1a cells, since we previously found that these same cell lines, when further transfected with prepro-ET-1 cDNA, could cleave 50–90% of the endogenously synthesized big ET-1 intracellularly (14). Furthermore, we assessed the functional localization using CHO cell lines that express both ECE-1 and ET-A receptor as reporter cells. When CHO/ECE-1b cells were transiently transfected with an ETA receptor cDNA, these cells became responsive to exogenous big ET-1 as assessed by intracellular 

$$\text{Ca}^{2+}$$ transients (Fig. 3B). The action of big ET-1 was completely abolished by an ECE inhibitor (Fig. 3B), indicating that exogenous big ET-1 could be efficiently cleaved into active peptide by ECE-1b present on the cell surface. In contrast, co-expression of ECE-1a and the ETA receptor did not render CHO cells responsive to exogenous big ET-1, while ET-1 produced a response of similar amplitude to that observed in CHO/ECE-1b cells, indicating that little functional ECE-1 is expressed on the cell surface in CHO/ECE-1a cells (Fig. 3B).

**Selective Lysosomal Sorting and Rapid Degradation of ECE-**
**Lysosomal Targeting of Bovine Endothelin-converting Enzyme-1a**

CHO/ECE-1a cells—In the process of further dissecting the cellular mechanisms that may lead to the distinct steady-state subcellular localization of bovine ECE-1a and ECE-1b, we found that the turnover rate of ECE-1a protein was much higher than that of ECE-1b. CHO/ECE-1a and CHO/ECE-1b cells were metabolically labeled with \(^{35}\)S–amino acids for 60 min and then rinsed with medium. ECE-1a protein was then chased with unlabeled amino acids for various time intervals, cell extracts were prepared and immunoprecipitated with an anti-ECE-1 antibody. These pulse-chase experiments demonstrated that, although ECE-1a and ECE-1b mRNAs are translated at similar rates (compare the initial lanes in Fig. 4), the half-life of ECE-1a protein (~1.5 h) is much shorter than that of ECE-1b (~20 h).

A previous study reported that treatment of vascular endothelial cells with phosphoramidon caused a marked increase in the cellular amount of ECE-1 protein, although the mechanism responsible for this was unknown (18, 28). We were able to reproduce these observations: when the endothelial cells were cultured in the presence of phosphoramidon for 24 h, they contained significantly larger amounts of ECE-1 protein as compared with untreated CHO/ECE-1a cells (Fig. 5A). The level of ECE-1 mRNA was unaffected by phosphoramidon treatment (data not shown). Importantly, however, this phenomenon was not observed in the smooth muscle cells (Fig. 5A). Similar experiments in transfected CHO cells confirmed that this “pseudoinduction” of the ECE-1 protein occurs only in CHO/ECE-1a cells and not in CHO/ECE-1b cells (Fig. 5A). Taken together, these findings led us to hypothesize that phosphoramidon may somehow protect bovine ECE-1a from its rapid degradation, thereby causing an accumulation of the isoenzyme. Indeed, we found that, in both endothelial and CHO/ECE-1a cells, phosphoramidon treatment markedly prolonged the half-life of ECE-1a protein as judged by pulse-chase immunoprecipitation (Figs. 4 and 5B).

These findings prompted us to speculate that ECE-1a protein is constitutively recruited into a lysosomal compartment, where it is rapidly degraded. To test this hypothesis, we treated CHO/ECE-1a cells with inhibitors of lysosomal function. Both NH\(_4\)Cl and chloroquine markedly prolonged the half-life of ECE-1a protein in these cells (Fig. 5C). Furthermore, after the NH\(_4\)Cl or chloroquine treatment, strong ECE-1 immunoreactivity was detected in coarse granular compartments more distal to the nuclei, which we did not previously see in untreated CHO/ECE-1a cells (compare Figs. 2 and 6). Double staining of CHO/ECE-1a cells in the presence of lysosomal inhibitors with an anti-ECE-1 antibody and a monoclonal antibody that recognizes lysosomal membrane glycoprotein B confirmed that ECE-1–immunoreactive granular structures were also lysosomal membrane glycoprotein B–positive (Figs. 6, G and H). These results indicate that bovine ECE-1a is constitutively targeted to lysosomes. We observed similar ECE-1–immunoreactive granular compartments in phosphoramidon–treated CHO/ECE-1a cells and endothelial cells (Fig. 6, B and F). In contrast, the addition of FR901533 up to 100 \(\mu\)M did not cause the accumulation of bovine ECE-1 protein in either CHO/ECE-1a cells or endothelial cells (data not shown).

**The Cytoplasmic Tail of Bovine ECE-1a Contains Signals That Mediate the Constitutive Lysosomal Targeting**—The cDNA-predicted bovine ECE-1a and ECE-1b polypeptides have a N-terminal putative cytoplasmic tail of 56 residues and 51 residues, respectively (Fig. 7). The last (C-terminal) 24 amino acids of these cytoplasmic tails as well as the entire transmembrane domains and ectodomains are identical between the two isoenzymes. Therefore, the structural determinants that cause the striking difference in subcellular localization and trafficking of bovine ECE-1a and ECE-1b proteins must be embedded within the first ~30 amino acid residues of the cytoplasmic tails. To further elucidate the nature of these presumptive signal(s), we performed a series of mutagenesis studies within the cytoplasmic tails. The mutant constructs were stably transfected into CHO cells, and multiple clones from each transfection were assessed by immunofluorescence staining as well as by pulse-chase immunoprecipitations. A deletion of all of the alternatively spliced portion of the tail resulted in a robust cell surface expression of the mutant protein, which was indistinguishable from the distribution of wild-type bovine ECE-1b.
This indicates that, in the absence of the putative signal(s), the enzyme goes to the cell surface by default. In fact, a deletion of the first 5 amino acids at the N-terminal tip of the bovine ECE-1a tail is sufficient to cause a full cell surface expression (Fig. 7, ECE-1aΔ2–6), indicating that this portion of bovine ECE-1a tail contains at least a part of the essential signal that prevents cell surface expression. Alanine scan mutagenesis studies in the critical N-terminal portion of bovine ECE-1a tail revealed that there are two appreciable clusters of indispensable amino acid residues: one at Pro3-Arg4 and the other at Pro12-Leu13-Leu14 (Fig. 7). In all cases, the results from pulse-chase immunoprecipitation experiments were in accordance with the immunofluorescence assessments. We observed a rapid turnover of the mutant protein whenever the protein exhibited intracellular localization; in contrast, mutant proteins that exhibited cell surface localization were all long lived (data not shown).

Next, we examined whether the cytoplasmic tail of bovine ECE-1a is sufficient to mediate the lysosomal targeting of a heterologous membrane protein. Chimeric constructs were created by attaching the cytoplasmic tail of bovine ECE-1a or ECE-1b to the transmembrane and ectodomain of the TfR, another type II membrane protein (Fig. 8A). Pulse-chase experiments with stable transfectant CHO cells constitutively expressing these constructs demonstrated that the half-life of the ECE-1a–TfR chimeric molecule (1 h) was similar to that of ECE-1a and was much shorter than that of ECE-1b–TfR (7 h) and wild-type TfR (10 h, data not shown) (Fig. 8B). These findings indicate that ECE-1a–TfR protein is constitutively recruited to the lysosomal compartment and rapidly degraded. Immunostaining of these cells using antibodies that recognize the extracellular domain of the human transferrin receptor revealed that the ECE-1a–TfR chimeric protein exhibited juxtanuclear staining, which is similar to the pattern observed for wild type bovine ECE-1a protein (data not shown). Taken together, these results indicate that the cytoplasmic tail of bovine ECE-1a is sufficient to mediate the lysosomal targeting of a heterologous membrane protein.
were pulse-labeled with 35S label for 1 h and then chased for the designated time in the complete medium. ECE-1 protein was immunoprecipitated and analyzed on SDS-polyacrylamide gels as described under “Experimental Procedures.” The relative amount of immunoprecipitated at each time point was calculated as a percentage of the amount labeled at 0 h. An autoradiograph from a representative experiment is shown, and the quantitative data shown represent the mean of results from three independent experiments in which three different clones of CHO cells were analyzed for ECE-1a and ECE-1b.

ECE-1a contains signals necessary and sufficient for lysosomal targeting of this membrane protein.

**DISCUSSION**

We have shown that the alternative splicing of the ECE-1 gene first exon generates two distinct integral membrane isoenzymes, which exhibit strikingly different subcellular localizations. ECE-1a, the predominant isoform found in cultured vascular endothelial cells, resides in an intracellular compartment that largely overlaps with the Golgi apparatus. Our experiments have shown that this isoenzyme is responsible for the intracellular, co-secretory cleavage of endogenously produced big ET-1 in endothelial cells. In contrast, ECE-1b is expressed on the cell surface as an ectoenzyme in the endothelin receptor-containing smooth muscle cells. We have shown that this isoenzyme can catalyze the cell surface activation of extracellularly supplied big ET-1. Obviously, a more careful examination on the expression pattern of ECE-1a versus ECE-1b will be required to establish these relationships. Nevertheless, based on our present findings, we propose a model of endothelin-mediated cell-cell communications depicted in Fig. 9. This scheme dictates that cleavage of big ET-1 by the “generator” cells takes place primarily inside the cell, provided that those cells express sufficient levels of ECE-1, as in the case of vascular endothelial cells. ET-1 secreted as mature peptide probably works locally in a paracrine or autocrine manner. In addition to the intracellular cleavage of big ET-1 at the level of the generator cells, our model dictates that extracellular big ET-1 will be further cleaved by cell surface ECE-1 expressed by the “target” cells, with the resultant mature peptide readily acting on ET receptors. Extracellular big ET-1 may not only come from generator cells that do not express high levels of ECE-1; it may also be able to travel a considerable distance. In this context, it is interesting to note that the circulating plasma half-life of big ET-1 is significantly longer than that of mature ET-1 (33). The absolute concentration of circulating big ET-1 is also higher than that of mature peptide (34).

Our model is also supported by the observation obtained from a recent series of endothelin-related gene disruption studies in mice. ECE-1−/− mice (19) reproduced the phenotype observed in ET-1−/− deficient embryos (10): craniofacial and cardiovascular abnormalities. However, a large amount of mature ET-1 peptide was still present in near term ECE-1−/− embryos. This suggests that mature ET-1 must be produced at the exact sites where it functions, since the mature peptide present could not rescue the developmental phenotype of ECE-1−/− mice. This demonstrates that endothelin secreted as mature peptide acts in a highly local fashion. On the other hand, ET-1−/− embryos showed an incomplete penetrance of cardiovascular abnormalities.
abnormalities, which are observed in all ECE-1\(^{−/−}\) and ET\(^{−/−}\) embryos (13, 19). This suggests that the cardiovascular phenotype in ET\(^{−/−}\) embryos is partially rescued by maternally or placentally produced big ET-1, but not mature ET-1, which is delivered to the embryo and locally processed to ET-1 by ECE-1. This establishes that big ET-1 can work as a long distance carrier of the biological signals of ET-1. Taken together, these genetic studies strongly suggest that ET-1 functions only locally, whereas big ET-1 can act as a distant carrier of ET-1 signals.

In the results presented in this paper, it does not appear that ECE-1a is actively retained within Golgi in the same fashion as glycosyltransferases and other Golgi-resident membrane proteins (35). Instead, the Golgi-like localization of ECE-1a protein in the steady state appears to be due to its constitutive targeting to a lysosomal compartment, where the protein is rapidly degraded by an acidification-dependent mechanism. This scheme is highly analogous to the one for the constitutive lysosomal sorting and rapid degradation of P-selectin in endothelial cells (36, 37) (see below). Interestingly, treatment of ECE-1a–expressing cells with the cell-permeable ECE-1 inhibitor phosphoramidon prevents the rapid turnover of the isoenzyme, resulting in a direct accumulation of the protein in the lysosomal compartments. Phosphoramidon, a competitive inhibitor of ECE-1, presumably causes a conformational change in ECE-1a by binding its active pocket, rendering the isoenzyme immune to the lysosomal degradation machinery. Alternatively, phosphoramidon may inhibit other lysosomal metalloprotease(s) that are essential for initiating the rapid degradation of ECE-1a. In this case, we expect that lysosomal degradation of a number of other proteins unrelated to ECE-1 may possibly be prevented by phosphoramidon.

Alanine scan mutagenesis studies demonstrated that the cytoplasmic tail of ECE-1a contains two clusters of indispensable amino acid residues: Pro\(^{3}\)-Arg\(^{4}\) and Pro\(^{12}\)-Leu\(^{13}\)-Leu\(^{14}\).
ble amino acid residues, Pro\textsuperscript{3}-Arg\textsuperscript{4} and Pro\textsuperscript{12}-Leu\textsuperscript{13}-Leu\textsuperscript{14}, both of which are essential for specifying the steady-state intracellular localization and rapid lysosomal turnover of ECE-1a protein. To specifically determine the influence of these residues on the intracellular targeting of ECE-1a, it will be necessary to assess the relative amount of ECE-1a localized on the cell surface of these mutants by quantitative assay methods including cell surface biotinylation or iodination. Nevertheless, these findings suggest that the sorting determinant mediating lysosomal targeting of bovine ECE-1a is located within the two proline-containing signals. It has been previously demonstrated that constitutive, direct lysosomal targeting of P-selectin at the trans-Golgi network can be abrogated by deleting a 10-amino acid stretch, DGKCPLNPHS, from the C-terminal cytoplasmic tail (36). Interestingly, this stretch of P-selectin sequence contains two proline residues, in the contexts PLN and PH. More recently, the proline residue within the sequence KCPL was shown to make a major contribution to the efficiency of lysosomal targeting of P-selectin without affecting internalization (37). It is tempting to speculate that ECE-1a and P-selectin are constitutively delivered to lysosomal compartments through proline-containing signals by similar molecular mechanisms. In this regard, it has also been shown that P-selectin reaches lysosomes in CHO cells via the plasma membrane (38). We cannot establish from the results presented in this paper whether ECE-1a is delivered to lysosomes directly from the trans-Golgi network, or indirectly via the plasma membrane. However, our live cell-based assay using ECE inhibitors as pharmacological probes, coupled to our preliminary internalization experiments using ECE-1a–TfR chimera\textsuperscript{2} suggests that ECE-1a is delivered to lysosomes without appearing on the plasma membrane.

Studies on cultured cells transfected with ECE cDNA have demonstrated that ECE-1a is localized to the plasma membrane by immunofluorescence microscopy analysis (17, 21, 24). In fact, we observed cell surface expression of ECE-1 in a small subset of CHO/ECE-1a cells by immunocytochemistry, although the majority of cells showed intracellular localization. However, our biochemical and pharmacological studies demonstrated that all CHO/ECE-1a stable transfectant cell lines exhibit lysosomal targeting. In this regard, we observed that ECE-1a protein does “leak” to the cell surface when it is mas-

\textsuperscript{2} N. Emoto and M. Matsuo, unpublished observations.
sively overexpressed, for example, under a strong promoter after transient transfection into the episomal replication-competent COS cells (data not shown; see Ref. 17). We feel that cell surface localization of ECE-1 observed in a few CHO/ECE-1a cells is largely due to massive overexpression. This suggests that the lysosomal targeting of ECE-1a occurs through a saturable mechanism. It is tempting to hypothesize that proline-containing sequences unique to the ECE-1a tail may interact with other regulatory proteins involved in lysosomal trafficking at the trans-Golgi network. A differential protein interaction screen using cytoplasmic tails of ECE-1a and ECE-1b as positive and negative “baits” may prove a feasible strategy to identify such proteins.

Subcellular localization of ECE-1 has remained controversial. A number of apparently contradicting observations have been reported in recent years using a variety of cultured cells (14, 18, 21, 23, 39). Our present study suggests that some of the controversies may have arisen due to the presence of two distinct spliceforms of ECE-1 that exhibit completely different subcellular localizations. ECE-1 provides an attractive target for pharmacological intervention to reduce the formation of active endo-
terin production. However, the results presented here indicate that a careful consideration on cell permeability of inhibitor compounds is essential in the future development of ECE-1 inhibitors.

Acknowledgments—We thank Sumio Kyoto for a sample of FR901533, Nobshiro Suzuki and Hirokazu Matsumoto for the EIA antibodies, and Damiane deWit for technical assistance.

REFERENCES

1. Yanagisawa, M., Kurihara, H., Kimura, S., Tomobe, Y., Kobayashi, M., Mitsui, Y., Yazaki, Y., Goto, K., and Masaki, T. (1988) Nature 332, 411–415
2. Inoue, A., Yanagisawa, M., Kimura, S., Kasuya, Y., Miyauuchi, T., Goto, K., and Masaki, T. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 2663–2667
3. Araki, H., Horii, S., Aramori, I., Ohkubo, H., and Nakanishi, S. (1990) Nature 348, 730–732
4. Sakurai, T., Yanagisawa, M., Takawa, Y., Miyazaki, H., Kimura, S., Goto, K., and Masaki, T. (1990) Nature 348, 732–735
5. Clozel, M., Breu, V., Burri, K., Cassal, J. M., Fischli, W., Gray, G. A., Hirth, G., Loffler, B. M., Muller, M., Neidhart, W., and Ramuz, H. (1993) Nature 365, 759–761
6. Kowalski, W., Sutsch, G., Hunziker, P., Muller, P., Kim, J., Oechslin, E., Schmitt, R., Jones, R., and Bertel, O. (1995) Lancet 346, 732–736
7. Sakai, S., Mitsuhashi, T., Kobayashi, M., Yamaguchi, I., Goto, K., and Sugishita, Y. (1996) Nature 384, 355–355
8. Minamino, T., Kurihara, H., Takahashi, M., Shimada, K., Maemura, K., Oda, H., Ishikawa, T., Uchiyama, T., Tanzawa, K., and Yazaki, Y. (1997) Circulation 95, 221–230
9. Krum, H., Viskoper, R. J., Lacourciere, Y., Budde, M., and Charlon, V. (1998) N. Engl. J. Med. 338, 784–790
10. Kurihara, Y., Kurihara, H., Suzuki, H., Kodama, T., Maemura, K., Nagai, R., Oda, H., Kuwaki, T., Cao, W. H., Kamada, N., Jishage, K., Ouchi, Y., Azuma, S., Toyoda, Y., Ishikawa, T., Kumada, M., and Yazaki, Y. (1994) Nature 368, 703–710
11. Baynash, A. G., Hosoda, K., Giard, A., Richardson, J. A., Emoto, N., Hammer, R. E., and Yanagisawa, M. (1994) Cell 77, 1277–1285
12. Hosoda, K., Hammer, R. E., Richardson, J. A., Baynash, A. G., Cheung, J. C., Giard, A., and Yanagisawa, M. (1994) Cell 79, 1267–1276
13. Clouthier, D. E., Hosoda, K., Richardson, J. A., Williams, S. C., Yanagisawa, H., Kuwaki, T., Kumada, M., Hammer, R. E., and Yanagisawa, M. (1998) Development 125, 813–824
14. Xu, D., Emoto, N., Giard, A., Slaughter, C., Kaw, S., deWit, D., and Yanagisawa, M. (1994) Cell 78, 473–485
15. Shimada, K., Takahashi, M., and Tanzawa, K. (1994) J. Biol. Chem. 269, 18275–18278
16. Emoto, N., and Yanagisawa, M. (1995) J. Biol. Chem. 270, 15262–15268
17. Takahashi, M., Fukuda, K., Shimada, K., Barnes, K., Turner, A. J., Ikeda, M., Knie, H., Yamamoto, Y., and Tanzawa, K. (1995) Biochem. J. 311, 657–665
18. Barnes, K., Shimada, K., Takahashi, M., Tanzawa, K., and Turner, A. J. (1996) J. Cell Sci. 109, 919–928
19. Yanagisawa, H., Yanagisawa, M., Kapur, R. P., Richardson, J. A., Williams, S. C., Clouthier, D. E., deWit, D., Emoto, N., and Hammer, R. E. (1998) Development 125, 825–836
20. Gui, G., Xu, D., Emoto, N., and Yanagisawa, M. (1993) J. Cardiac. Pharmacol. 22, 853–856
21. Schweizer, A., Valdenaire, O., Nelbock, P., Deuschle, U., Dumas Milne Edwards, J. B., Stumpf, J. G., and Loffler, B. M. (1997) Biochem. J. 326, 871–877
22. Harrison, V. J., Barnes, K., Turner, A. J., Wood, E., Corder, R., and Vane, J. R. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 6344–6348
23. Barnes, K., Brown, C., and Turner, A. J. (1998) Hypertension 31, 3–9
24. Shimada, K., Takahashi, M., Ikeda, M., and Tanzawa, K. (1995) FEBS Lett. 371, 140–144
25. Barnes, K., Walden, B. J., Wilkinson, T. C., and Turner, A. J. (1997) J. Neurochem. 68, 570–577
26. Matsumura, Y., Hisaki, K., Takaoka, M., and Morimoto, S. (1990) Eur. J. Pharmacol. 185, 163–166
27. Begawa, R., Matsamura, Y., Tsuchiya, Y., Takaoka, M., and Morimoto, S. (1990) Biochem. Biophys. Res. Commun. 171, 669–675
28. Schmidt, M., Kroger, B., Jacob, E., Seulberger, H., Subkowski, T., Otter, R., Meyers, C., Schmalzing, G., and Hillen, H. (1994) FEBS Lett. 356, 238–243
29. Valdenaire, O., Rohrbacher, E., and Mattei, M. G. (1995) J. Biol. Chem. 270, 29794–29798
30. Lush, S. M., Di Marco, F., Levin, N., Armanini, M., Xie, M. H., Nelson, C., Bennett, G. L., Williams, S. C., Spaen, S. A., Gurney, A., and de Sauvage, F. J. (1997) J. Mol. Endocrinol. 18, 75–88
31. Casteels, K. M., Mathieu, C., Waer, M., Valckx, D., Overbergh, L., Laureys, J. M., and Rouillon, R. (1998) Endocrinology 139, 95–102
32. Sakamoto, A., Yanagisawa, M., Sawamura, T., Enoki, T., Ohtani, T., Sakurai, T., Nakao, K., Toyo-oKA, T., and Masaki, T. (1993) J. Biol. Chem. 268, 8547–8553
33. Henssen, A., Ahlborg, G., Ostosson-Seeberger, A., and Lundberg, J. M. (1995) Regul. Pept. 55, 287–297
34. Suzuki, N., Matsumoto, H., Kitada, C., Kimura, S., Miyauuchi, T., and Fujino, M. (1990) J. Immunol. Methods 137, 165–170
35. Pelham, H. R., and Munro, S. (1993) Cell 75, 603–605
36. Green, S. A., Setiadi, H., McEver, R. P., and Kelly, R. B. (1994) J. Cell Biol. 126, 435–448
37. Blagoveshchenskaya, A. D., Norcott, J. P., and Cutler, D. F. (1998) J. Biol. Chem. 273, 2729–2737
38. Setiadi, H., Dusl, M., Green, S. A., Canfield, W. M., and McEver, R. P. (1995) J. Biol. Chem. 270, 26818–26826
39. Turner, A. J., and Tanzawa, K. (1997) FASEB J. 11, 355–364