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Molecular isolation and characterization of novel four subisoforms of ECE-2

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Abstract

Endothelin-converting enzymes (ECEs) are the key enzymes in the endothelin (ET) biosynthesis that catalyze the conversion of big ET, the biologically inactive precursor of mature ET. Two enzymes, termed ECE-1 and ECE-2, have been molecularly identified. Here, we report novel four subisoforms of ECE-2 that differ in their N-terminal cytoplasmic tails, termed ECE-2a-1, ECE-2a-2, ECE-2b-1, and ECE-2b-2. RT-PCR analysis of these subisoforms in bovine tissues demonstrated that their tissue distribution was strikingly different. ECE-2a-1 and ECE-2a-2 are expressed in a variety of tissues including liver, kidney, adrenal gland, testis, and endothelial cells, while ECE-2b-1 and ECE-2b-2 are expressed abundantly in brain and adrenal gland. Furthermore, ECE-2a-1 and ECE-2b-2 were revealed to be predominant forms as compared to ECE-2a-2 and ECE-2b-1, respectively. Immunohistochemical analyses of CHO cells, stably expressing ECE-2a-1 or ECE-2b-2, revealed that both ECE-2a-1 and ECE-2b-2 were localized in intracellular compartments but not on the cell surface. Detailed analysis of ECE-2 subisoforms will provide crucial information to clarify the physiological function of ECE-2.

Keywords: Endothelin; Endothelin-converting enzyme; Alternative splicing; Metalloprotease; Sorting signal

Endothelin-converting enzymes (ECEs) are the rate-limiting enzymes to produce mature ET-1. Two isoforms of ECE (ECE-1 and ECE-2) have been molecularly identified [1–3]. Both ECE-1 and ECE-2 belong to the type II membrane-bound zinc metalloprotease family that contains a zinc-binding consensus motif (HEXXH) [4]. At present, five members of this family other than ECEs have been identified, neutral endopeptidase 24.11 (NEP), Kell blood group protein, the product of PEX gene, soluble secreted endopeptidase (SEP), and ECEL-1 [5–9]. Their identical structural features are the short cytoplasmic tail in the N-terminal, single transmembrane domain, and a large extracellular domain containing the zinc-binding motif that catalyzes their enzymatic activities in the C-terminal. Endopeptidases of this family play crucial roles in the processing and metabolizing peptides including hormones, immunoregulatory peptides, vasoactive peptides, and neuropeptides.

ECE-2 has been cloned from bovine adrenal cortex and reported to possess 59% homology with ECE-1. Although ECE-2 shows specific activity to produce mature ET-1 from big ET-1 like ECE-1, the striking difference of ECE-2 from ECE-1 is its acidic pH optimum of 5.5, suggesting that ECE-2 is involved in the ET synthesis at the intracellular compartments in which the pH is acidic [3]. Recently, ECE-2 has been reported to be expressed in various tissues including cultured human vascular endothelial cells, uterus, ovary, heart, lung, and liver, suggesting its various functions [10,11]. However, physiological function of ECE-2 is still unclear.

Previously, ECE-1, NEP, and SEP have been reported to have several subisoforms that are produced by an alternative splicing. Human ECE-1 consists of four subisoforms which differ from each other only in their N-terminal cytoplasmic domains [12–15]. NEP

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comprises three subisoforms which resulted from an alternative splicing in the 5′ untranslated region [16,17]. SEP has two isoforms that differ by only 23 amino acids following the transmembrane domain, one of which is secreted extracellularly whereas the other is anchored at the endoplasmic reticulum (ER) [8,18]. Since the presence of subisoforms contributes to their functional diversity, analysis of subisoforms is important to clarify their physiological functions.

Considering that ECE-2 also belongs to the type II membrane-bound zinc metalloprotease family allowed us to hypothesize that ECE-2 might have splicing variants. Here, we report the identification and characterization of novel four subisoforms of bovine and mouse ECE-2. Detailed analysis of these subisoforms will provide precious information to clarify the physiological function of ECE-2.

Materials and methods

Cloning of bovine and mouse ECE-2. Bovine ECE-2b cDNA was obtained by 5′ rapid amplification of cDNA ends (5′ RACE) using bovine brain total RNA. The first strand cDNA was synthesized with SuperScript II reverse transcriptase (Life Technologies) by using a gene specific primer, 5′-GGCAGTAAGCACTTTCTCATTGGC-3′ (corresponding to the amino acids 986–1009 of bovine ECE-2). An oligo(dC) anchor was added to the 3′ end of first strand cDNA using terminal deoxynucleotidyltransferase. The first polymerase chain reaction (PCR) was performed as recommended by the manufacturer with a gene specific 3′ primer, 5′-TGGTCCACCTGGATAATATTGCTG-3′ (corresponding to the amino acids 916–939 of bovine ECE-2) and a 5′ anchor primer. The product was then subjected to the second PCR by using a nested specific 3′ primer, 5′-CGTTCCAGCCACCAATCT TGTC-3′ (corresponding to amino acids 779–800 of bovine ECE-2). The product of the second PCR was subcloned into pT7Blue T-vector (Novagen) and sequenced. Both strands of cDNA were sequenced at least twice by Amersham Alfred DNA sequencer.

Reverse-transcription PCR. First strand cDNA synthesis was carried out with 1 μg of total RNA from bovine tissues and oligo$(dT)_{12-18}$ primers by using SuperScript II reverse transcriptase (Life Technologies). The PCR contained 20 mM Tris–HCl (pH 8.5), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 100 nM of each amplification primer, 10 ng of first strand cDNA, and 2.5 U of Taq DNA polymerase. The sense primers, 5′-ACGCTGCGATGGGAGACCATG GATG-3′ (amino acids 143–167 of bovine ECE-2a) for ECE-2a and 5′-GCCCCGACTCCAGCATGAGGGT-3′ (amino acids 170–191 of bovine ECE-2b) for ECE-2b, and the common antisense primer, 5′-GGCAAGTGCTATGGGATGGGTCTCT-3′ were used. Thirty-two PCR cycles were performed at an annealing temperature of 62.1 °C and then the PCR products were separated on a 1% agarose gel. These PCR products were verified by sequencing.

Cell culture and transfection. The Chinese hamster ovary (CHO) cells were cultured in HamF12 and Dulbecco's modified Eagle's medium (1:1 mixture) supplemented with 10% fetal bovine serum. Cells were grown in a humidified incubator at 37 °C in an atmosphere of 5% CO_2 and 95% air. The coding region of the bovine ECE-2a-1 or ECE-2b-2 was subcloned into pME18Sf(−) expression vector (2). Stable transfection of CHO cells and isolation of transfectant clones were performed as described [2].

Antibody and Western blot. Antibody against ECE-2 was synthesized by immunizing rabbits with a synthetic peptide corresponding to the C-terminal 16 amino acids of bovine ECE-2 as previously described [3]. Western blot analysis was performed with horseradish peroxidase conjugated anti-rabbit IgG as second antibody and then signals were detected using an ECL detection kit (Amersham Pharmacia).

Immunohistochemistry. CHO/ECE-2a-1 or CHO/ECE-2b-2 cells were seeded onto coverslip glasses and cultured for two days. For intracellular staining, cells were fixed and permeabilized in cold methanol for 5 min at 4 °C. After washing three times in phosphate-buffered saline (PBS), PBS containing normal goat serum (1:10 v/v) (NGS/PBS) was added. Following 30 min of incubation at 37 °C, the NGS/PBS was replaced with the NGS/PBS containing 1% (v/v) polyclonal ECE-2 antibody. After incubation for 2 h at 37 °C, the cells were washed four times with PBS for 10 min each. Following a 1 h incubation in the NGS/PBS containing 7.5 μg/ml of fluorescein isothiocyanate-goat anti-rabbit IgG (Zymed Laboratories) at 37 °C, the cells were washed six times with PBS for 15 min each. The coverslips were mounted on microscope slides with 90% (v/v) glycerol–50 mM Tris–HCl and 2.5% (w/v) 1,4-diazabicyclooctane. For cell surface staining, cells were fixed in PBS containing 4% paraformaldehyde for 15 min at room temperature. After fixation, cells were stained with the same methods as those used for intracellular staining described above.

Results

Cloning of ECE-2 isoform

To compare the nucleotide sequence of ECE-2 among the different species, we performed screening of human, mouse, and rat cDNA libraries and isolated full-length ECE-2 cDNAs. Sequencing of these ECE-2 cDNAs revealed that only bovine ECE-2 showed no similarity to the others in the N-terminal cytoplasmic domain (Fig. 1A). Therefore, we further investigated the possibility that ECE-2 may have spliced variants.

To isolate a bovine ECE-2 counterpart corresponding to human and mouse ECE-2, we performed 5′ RACE using total RNA of bovine brain. We successfully isolated a novel bovine ECE-2 subisoform, termed ECE-2b, that showed high similarity to human and mouse ECE-2 in the cytoplasmic tail (Figs. 1B and C). We named the original bovine ECE-2, ECE-2a and a novel subisoform, ECE-2b. The similarity of bovine ECE-2b to human and mouse ECE-2 in the cytoplasmic tail is 82% and 65%, respectively. ECE-2a contains two di-leucine based motifs (LL) in its cytoplasmic domain, while ECE-2b includes one tyrosine based motif (YKRA) and one LL motif in the same portion (Fig. 1B).

To examine their expressions, we designed gene specific primers for bovine ECE-2a and ECE-2b and performed RT-PCR using total RNA from bovine brain and kidney. We obtained the PCR products of the expected sizes and, unexpectedly, several additional bands. Sequencing of these PCR products revealed that both ECE-2a and ECE-2b consist of two subisoforms, termed ECE-2a-1, -2a-2 and ECE-2b-1, -2b-2 (Fig. 2A). Both ECE-2a-2 and -2b-2 contain the same 29-amino acid insertion including the tyrosine-containing motif.

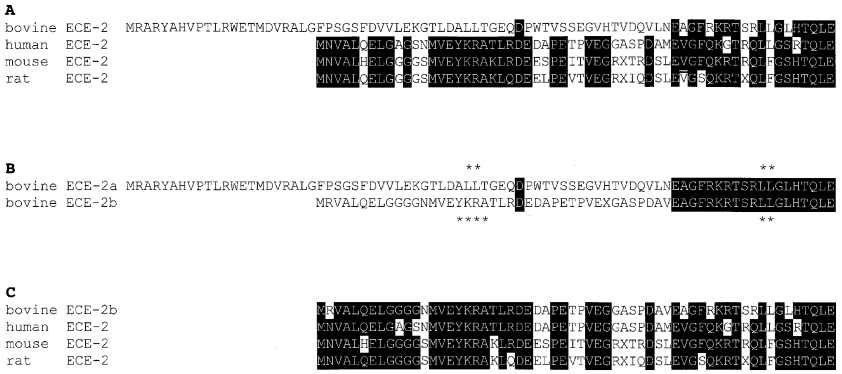

Fig. 1. Bovine ECE-2. (A) Amino acid sequence alignment of bovine, human, mouse, and rat ECE-2 in the N-terminal cytoplasmic domain. (B) Amino acid sequences of bovine ECE-2a and ECE-2b in the N-terminal cytoplasmic domain. Di-leucine based (LL) and tyrosine-containing motifs (YKRA) are highlighted by asterisks. (C) Amino acid sequence of bovine ECE-2b shows high similarity to human, mouse, and rat ECE-2.

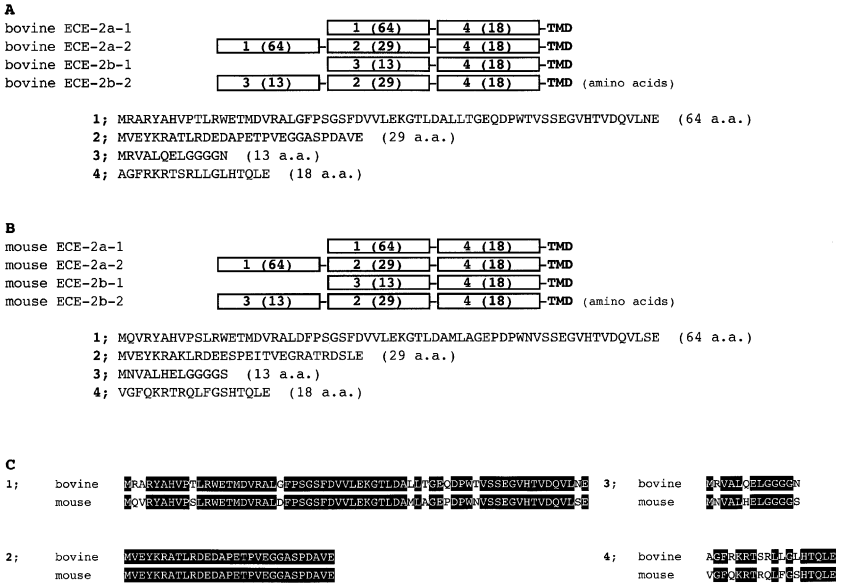

Fig. 2. ECE-2 subisoforms of bovine and mouse amino acid sequence of bovine (A) and mouse (B) ECE-2a-1, -2a-2, and ECE-2b-1, -2b-2. (C) Amino acid sequence of bovine and mouse ECE-2 subisoforms showed high similarity.

We searched the EST database of GenBank to identify four subisoforms of ECE-2 in other species. One EST sequence (accessory number 1598057) of mouse showed high similarity to bovine ECE-2a. We designed gene specific primers for the putative mouse ECE-2a and ECE-2b and performed RT-PCR using total RNA from mouse brain and kidney. As we expected, we obtained the PCR products of several different sizes. Sequencing of these PCR products revealed that mouse ECE-2 also consists of four subisoforms (Fig. 2B). These four subisoforms were highly conserved between bovine ECE-2a and mouse (Fig. 2C).

Tissue distribution of ECE-2 subisoforms

To examine the tissue distribution of the four ECE-2 subisoforms and to assess their relative expression levels, RT-PCR analysis of bovine ECE-2a and ECE-2b was performed in various bovine tissues (Fig. 3). ECE-2a-1 was abundantly expressed in liver, kidney, adrenal gland, testis, and endothelial cells whereas ECE-2b-2 was highly expressed in brain and adrenal gland. Tissue distributions of ECE-2a-2 and ECE-2b-1 were similar to those of ECE-2a-1 and -2b-2, respectively, while their expressions were very less similar.

Immunohistochemistry of CHO/ECE-2 cells

To analyze the subcellular localization of bovine ECE-2 subisoforms, we performed the immunohistochemistry of CHO cells that stably express bovine ECE-2a-1 or ECE-2b-2. We selected these two subisoforms because of their predominant expressions assessed by RT-PCR and of containing different targeting

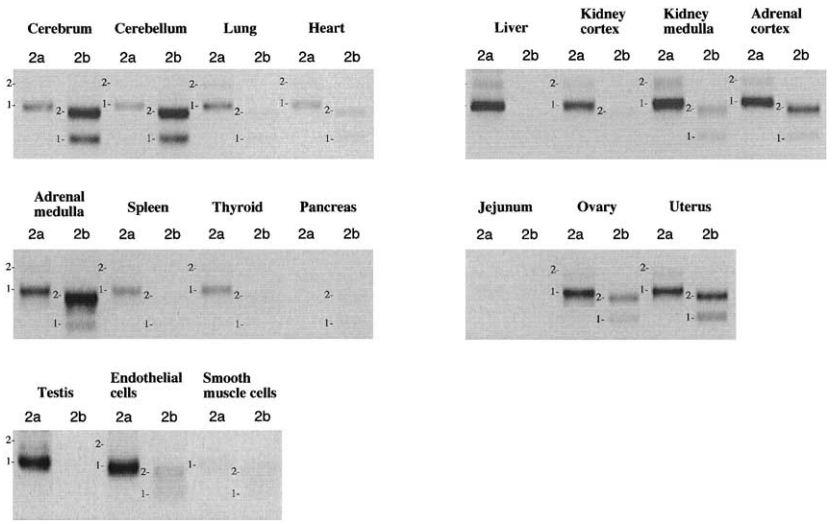

Fig. 3. RT-PCR analysis of ECE-2 subisoforms in bovine tissues. One μg of total RNA from 18 bovine tissues was examined. PCR products were separated on a 1% agarose gel. The size of PCR product is 320 (for ECE-2a-1), 397 (for ECE-2a-2), 208 (for ECE-2b-1), and 295 (for ECE-2b-2) base pairs.

motifs. For intracellular staining, cells were permeabilized by methanol, fixed, and stained with ECE-2 polyclonal antibody. MOCK-transfected CHO cells showed no immunoreactivity against ECE-2 antibody (data not shown). Both CHO/ECE-2a-1 cells and CHO/ECE-2b-2 cells showed a strong immunoreactivity in intracellular compartments, appeared in the Golgi apparatus and secretory granules (Figs. 4A and B). For cell surface staining, cells were fixed without permeabilization and both CHO/ECE-2a-1 and CHO/ECE-2b-2 cells did not show any immunoreactivity (Figs. 4C and D). These results indicate that both ECE-2a-1 and ECE-2b-2 are located in intracellular compartments but not on the cell surface.

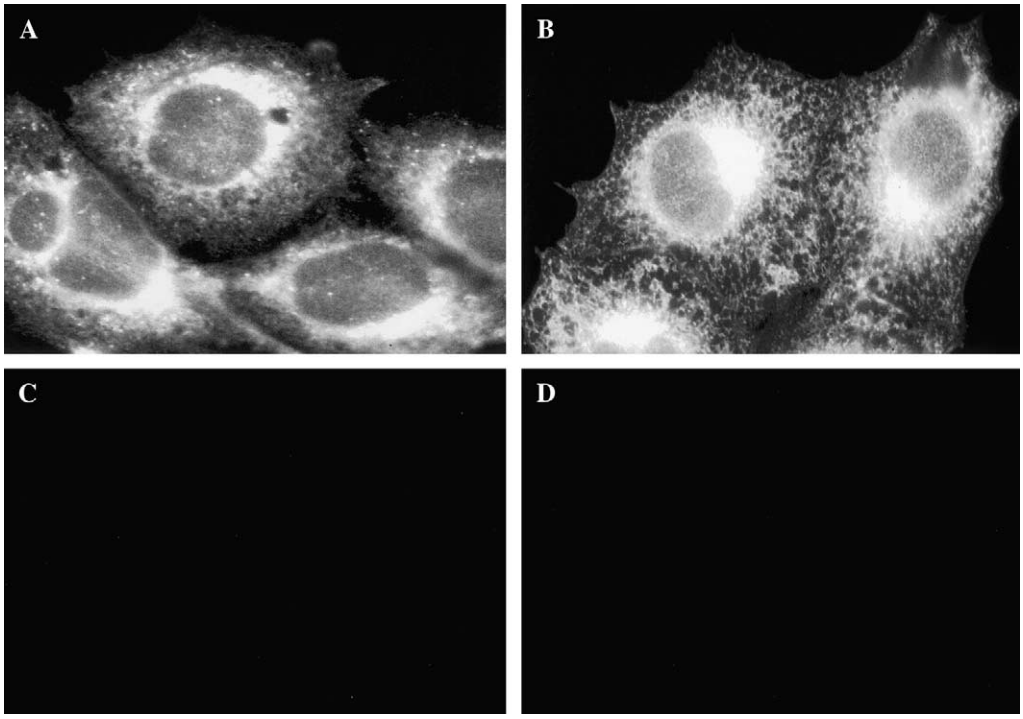

Fig. 4. Subcellular localization of ECE-2a-1 and ECE-2b-2. Subcellular localization of ECE-2 isoforms was examined using CHO cells stably expressing bovine ECE-2a-1 (A and C) or ECE-2b-2 (B and D). For intracellular staining, cells were fixed and permeabilized with cold methanol (A and B). For cell surface staining, cells were fixed by 4% PFA (C and D). Both ECE-2a-1 and ECE-2b-2 are localized intracellularly.

Discussion

In the present study, we described the identification and characterization of novel subisoforms of ECE-2, which differ only in their N-terminal cytoplasmic tails. During the preparation of this manuscript, the existence of three human ECE-2 subisoforms (ECE-2A, ECE-2B, and a splicing variant of ECE-2B) has been reported [19]. Here, we demonstrated the existence of three ECE-2 subisoforms, ECE-2a-1, ECE-2b-1, and ECE2b-2, in bovine and mouse that are homologous to human ECE-2 mRNAs, ECE-2A, a splicing variant of ECE-2B, and ECE-2B, respectively. Furthermore, we were able to identify an additional ECE-2 transcript (ECE-2a-2) for the first time. Although we have not confirmed the presence of ECE-2a-2 transcript in human, the highly conserved structures among different species in this metalloprotease family suggest that ECE-2a-2 may exist in human.

ECE-2 belongs to the type II membrane-bound zinc metalloprotease family, some members of which have been reported to produce several subisoforms by alternative splicing mechanisms. ECE-1 exists as four distinct subisoforms (ECE-1a, ECE-1b, ECE-1c, and ECE1d) which differ only in their N-terminal regions. Several lines of evidence have shown that these subisoforms exhibit the distinct subcellular localizations and intracellular sorting patterns (see below) [14]. Although a number of apparently contradicting observations regarding the subcellular localization of ECE-1 polypeptides have been reported, the presence of these subisoforms appears to account for some of these controversies [4,14,15,20]. SEP, the most recently identified member of this family, has two mRNAs, SEP^{Δ} and SEP, generated by alternative splicing of a single gene [8]. These two isoforms differ from each other in the presence of a 23-amino acid insertion flanking the transmembrane domain of SEP. The unique feature of SEP is that alternative splicing regulates the ER localization or secretion of SEP polypeptides [8]. After translation, both isoforms are inserted into the ER as type II membrane proteins. SEP^{Δ} then becomes an ER resident, whereas SEP is proteolytically cleaved and transported into the extracellular compartment [18]. Three distinct NEP mRNAs have been isolated, differing only in their N-terminal portion [16,17]. However, the differences in the sequences reside only in 5′-noncoding regions, suggesting that these spliceoforms exhibit no functional differences. Thus, the presence of alternative spliced variants appears to be the unique feature among the members of this family.

The tissue distribution patterns of the four ECE-2 subisoforms examined by RT-PCR revealed that the relative expression ratios of the four mRNA species differ in various bovine tissues. ECE-2a-1 and ECE-2a-2 were expressed ubiquitously in a wide variety of tissues including liver, kidney, adrenal gland, gonadal organs, and vascular endothelial cells. In contrast, ECE-2b-1 and ECE-2b-2 expression showed a highly restricted pattern of distribution. ECE-2b-1 and ECE-2b-2 were found to be major isoforms in neuronal tissues including cerebrum, cerebellum, and adrenal medulla. Thus, the expressions of ECE-2 subisoforms are regulated in a tissue- and cell-specific manner.

Recent gene targeting studies with mouse ECE-1 and ECE-2 genes revealed that ECE-2 plays crucial roles in the formation of cardiac outflow structures [11]. Based on our RT-PCR results that ECE-2a-1 is a predominant subisoform in the heart, ECE-2a-1 contributes to the proper formation of cardiac outflow structures. ECE-2 polypeptide was shown to be located in secretory vesicles of cultured vascular endothelial cells [10]. Since ECE-2a-1 is the major transcript among ECE-2 mRNAs, ECE-2a-1 may be responsible for the conversion of big ET-1 in secretory vesicles in endothelial cells. In contrast, ECE-2b appears to function in neuronal tissues judging from its unique expression pattern of distribution. ECE-2 mRNA was reported to be enriched in the catecholaminergic nuclei in brain including substantia nigra compacta, raphe nuclei, and locus coeruleus [21,22]. The regional distribution of ECE-2b transcript implies a crucial role for ECE-2 mRNA in neuronal function.

Our immunohistochemical studies demonstrated that both ECE-2a-1 and ECE-2b-2 proteins expressed in CHO cells are localized in intracellular compartments. Since ECE-1 subisoforms have been reported to show diverse subcellular localization patterns, we initially expected that ECE-2 subisoforms may show distinct subcellular localization. However, we failed to detect any differences in their localization, indicating that alternative spliced cytoplasmic tails have no effect on the subcellular localization. These findings raised at least two possibilities. First, CHO cells may not be the suitable cell type for ECE-2 subisoforms to investigate their subcellular localization. It is possible that other cultured cells derived from neuronal tissues may give a different observation. Second, similar intracellular localization of ECE-2a and ECE-2b proteins in the steady state is mediated by the different sorting patterns that are analogous to ECE-1 subisoforms. For example, human ECE-1a is strongly expressed at the cell surface while human ECE-1b is expressed in the intracellular compartments [14,23]. ECE-1a contains one di-leucine based motif (LL) while ECE-1b has both a tyrosine containing motif (YKRA) and di-leucine motifs (LL, LV). The tyrosine containing and di-leucine based motifs have been reported to function as sorting signals [24]. Mutation of di-leucine motifs induces the expression of ECE-1b proteins at the cell surface [23]. These results indicate that sorting or targeting motifs play a critical role in the intracellular trafficking of ECE-1 subisoforms. ECE-2a-2 and ECE2b-2 contain tyrosine containing motifs (YKRA)

and all four subisoforms have one or two di-leucine based motifs (LL), suggesting that they may show distinct intracellular trafficking. Further studies with several complementary methods are required to clarify their distinct protein targeting and internalization.

In conclusion, we have identified four ECE-2 subisoforms of bovine and mouse. They showed different tissue distributions but similar subcellular localizations. Since ECE inhibitor is a fascinating target to treat many diseases, a detailed analysis of ECE-2 isoforms will provide precious information to design the rational ECE inhibitor.

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