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Identification and characterization of two isoforms of an endothelin-converting enzyme-1

Kohei Shimada[a], Masaaki Takahashi[a], Masahiro Ikeda[b], Kazuhiko Tanzawa[a,*]

[a]*Biological Research Laboratories, Sankyo Co. Ltd., 1-2-58 Hiromachi, Shinagawa-ku, Tokyo 140, Japan*
[b]*Pharmacology and Molecular Biology Research Laboratories, Sankyo Co. Ltd., 1-2-58 Hiromachi, Shinagawa-ku, Tokyo 140, Japan*

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Abstract **We report the cloning and sequencing of 5′-terminal region of a β form of rat ECE-1 cDNA which is different only in its N-terminal amino-acid sequence to the cDNA we have cloned previously (α form [K. Shimada et al. (1994) J. Biol. Chem. 269, 18275–18278]). No significant difference was found in the specific activity and substrate specificity between the two isoforms. The expression level of ECE-1α mRNA was higher than that of ECE-1β in various rat cells and tissues, suggesting that the physiologically important isoform is ECE-1α. The present findings verified the presence of two forms of ECE-1 over many species, which are created probably through alternative splicing.**

Key words: Endothelin-converting enzyme; Endothelin; Alternative splicing; Rat ECE-1β

1. Introduction

Endothelins (ETs) are potent vasoconstrictive 21 amino-acid peptides originally isolated from the supernatant of cultured porcine aortic endothelial cells [1]. ETs are generated by a unique proteolytic cleavage of big endothelins (big ETs) at the Trp^{21}–Val^{22} or Trp^{21}–Ile^{22} bond catalyzed by an endothelin-converting enzyme (ECE) [2].

Purification and cDNA cloning studies of rat ECE-1 revealed that it is an unique, highly glycosylated neutral metalloprotease with a single transmembrane domain near the N-terminus [3,4]. In the light of the subsequent studies being carried out both on bovine ECE-1, the results, however, were somehow in contradiction to each other on the basis of their N-terminus alone; i.e. Xu et al. [5] and Ikura et al. [6] have cloned and sequenced one form of bovine ECE-1 cDNA, while Schmidt et al. have cloned another form of bovine ECE-1 cDNA. The latter was almost the same as the former except for its N-terminal 28 amino-acid residues [7]. Schmidt et al. went on to discuss that the sequence reported by Xu et al. resulted from a misinterpretation of incorporated intronic sequences and does not reflect the authentic amino-acid sequence [7]. This speculation was further supported by the fact that the sequence of bovine ECE-1 cDNA cloned by Schmidt et al. was more homologous to rat ECE-1 sequence than the bovine sequences that others have cloned [4–7]. In addition, the sequence of human ECE-1 cDNAs, cloned by Schmidt et al. and Yorimitsu et al., were also homologous to that of rat ECE-1 cDNA [4,7,8]. Interestingly, the sequence of human ECE-1 cDNA which we cloned was different from the one of Schmidt et al. had reported at the 5′-terminus [9]. From these observations, therefore, it is pertinent to assume that there are two types of ECE-1 mRNAs in each animal species which differ by their N-termini. Yorimitsu et al. previously suggested the possible presence of alternatively spliced forms [8]. We tentatively designated each of these two sequences as α and β form, as guided by the suggestion of Turner and Murphy [10]. The rat cDNA which we cloned [4], and bovine and human cDNAs cloned by Schmidt et al. and Yorimitsu et al. [7,8] are recognized as α forms while bovine cDNAs cloned by Xu et al. [5] and by Ikura et al. [6], and human cDNA which we cloned [9] are all collectively termed β forms.

Here we report the cloning and sequencing of the 5′-terminal region of the β form of rat ECE-1 cDNA, and reveal that the α form is dominant in various cells and organs. In addition, enzymatic properties of the two forms of ECE-1 are also extensively compared.

2. Materials and methods

2.1. Cell culture

SV40-transformed rat lung vascular endothelial cells (TRLEC-03 cells) were kindly supplied by Dr. S. Tsurufuji, Institute of Cytosignal Research, Inc., and were cultured on a collagen-coated flask (Corning) in a RPMI-1640 medium containing 10% FCS. COS-1 cells were cultured in DMEM containing 10% FCS. Human umbilical vein endothelial cells (HUVEC) were purchased from Kurabo, Japan and cultured as described [11]. All cells were grown in a humidified incubator in a 5% CO_2/95% air atmosphere at 37°C. All media were purchased from Gibco BRL and supplemented with penicillin/streptomycin.

2.2. Cloning of the 5′-terminal region of rat ECE-1β cDNA and human ECE-1α cDNA

Rat lung total RNA was prepared and used for cloning of the 5′-terminal region of rat ECE-1β cDNA. A sense primer (GTGGGAACCAGACCACCC) was designed from the homology between the 5′-noncoding region of bovine and human ECE-1β cDNA sequences [5,6,9], and an antisense primer (GGGGTCCATGGAGTTTAGGATGGAGCTGGT) was based on rat ECE-1α cDNA [4]. The rat ECE-1β cDNA was amplified from single-stranded cDNA from rat lung total RNA using the sense primer and the antisense primer. Reverse transcription-PCR was performed using RNA-PCR kit (Takara Shuzo, Japan) according to the manufacturer's instruction. The 354 bp PCR product was subcloned into a pCR II TA vector (Invitrogen) and sequenced automatically on both strands. The *Eco*RI (vector)–*Bln*I (211) region of rat ECE-1α cDNA [4] was replaced by the *Eco*RI (vector)–*Bln*I (235) fragment of the product to construct rat ECE-1β cDNA. The resultant full length rat ECE-1β cDNA was inserted into a pcDL-SRα296 vector [12] for expression.

The total RNA of HUVEC was prepared and used for cloning of the 5′-terminal region of human ECE-1α cDNA. The cDNA was amplified from single stranded HUVEC cDNA using a sense primer (atgatgaattcatgcatGTGTCCGCCCCTGCTGTCGGCGCTG) and an antisense primer (GCAGGGGTCCACTGTGGGGTCC) which were designed

*Corresponding author. Fax: (81) (3) 5436 8565.

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based on human ECE-1α cDNA [7]. The 360 bp PCR product was subcloned and sequenced as described above. Since human ECE-1α and -1β cDNA sequences are identical except for their 5′-terminal region, the *Eco*RI (vector)–*Hin*dIII (331) region of human ECE-1β cDNA cloned previously [9] was replaced by the *Eco*RI(primer)–*Hin*dIII (292) fragment of the product to construct human ECE-1α cDNA. The resultant full-length human ECE-1α cDNA was inserted into the pcDL-SRα296 vector for expression.

2.3. Northern blot analysis of human ECE-1α and -1β mRNA

Four μg of poly(A)$^+$ RNA prepared from HUVEC was separated by formaldehyde/1.0% agarose gel electrophoresis and transferred to a Hybond N$^+$ membrane (Amersham Corp.). 5′-endlabelled oligonucleotide probe for human ECE-1α mRNA (GTCCACCAGGTCCTCCTCGTCCAGCGTGGCCCGCTTGTACGTCGA) or for human ECE-1β mRNA (CCCTTGGAGGAAGGGGTTCCGCTGCAGGCCCAGGCCCTGGAGAGG) was used as a probe, respectively. Membranes were washed in 2×SSC, 0.1% SDS at 60°C and analyzed by BAS 2000 system (FUJIX).

2.4. Competitive PCR of rat ECE-1α and -1β transcripts

The levels of rat ECE-1α and -1β transcripts were measured by competitive PCR [13] using their respective cDNAs, which were reverse-transcribed from RNA samples. The 5′-terminal regions of rat ECE-1α and -1β cDNAs were synthesized by PCR using a sense primer (ATGATGTCATCCTACAAGCGGGCC) for ECE-1α cDNA, a sense primer (ATGGGCAGCCTGAGGCCTCCCCAG) for ECE-1β cDNA, and an antisense primer (GGGTCCATGGAGTTTAGGATGGAGCTGGT) for both cDNAs, and the PCR products (303 bp for ECE-1α and 327 bp for ECE-1β) were subcloned into a pCR II TA vector to construct pCRrECE-1α and pCRrECE-1β, respectively. For construction of competitor DNA, a vector region of each plasmid was amplified using an antisense primer (CCGGGGGCTGCGAAGGTTCAC) and a sense primer (CTGGTGGTTCTGGTGACGCTTCTGGCAGCAGGG), and blunt-ended PCR products were self-ligated. The resultant plasmids, pCRrECE-1α-d and pCRrECE-1β-d, carry a 43 bp deletion within each cDNA. These plasmids were subjected to PCR after their linearization with restriction enzyme digestion.

From 100 ng of poly (A)$^+$RNA prepared from TRLEC-03 cells or several rat tissues, cDNAs were synthesized using random 9mer primer in a total volume of 20 μl and diluted 10-fold with H_2O. Aliquots of 2 μl of these dilutions were subjected to the quantitative PCR analysis in the presence of linearized competitor DNA using sense primer for ECE-1α or sense primer for ECE-1β, and antisense primer for both cDNAs as described above. As a control, glyceraldehyde 3-phosphate dehydrogenase (G3PDH) cDNA was amplified using rat G3PDH control amplimer set (Clontech).

2.5. Expression of ECE-1α and -1β in COS-1 cells

Expression vector for rat or human ECE-1α or -1β was transfected into COS-1 cells. The solubilized membrane fraction was prepared from each cell as described previously [4]. The solubilized membrane fractions were separated on 4–20% SDS-PAGE under reducing condition, and transferred to polyvinylidene difluoride membrane (Millipore). Immunoblot analysis probed with anti-ECE-1 monoclonal antibody, AEC32-236, was carried out as described previously [9]. The amount of immunoreactive ECE-1 in each sample was determined by measurement of chemiluminescence using ARGUS-100 (Hamamatsu photoelectronics). Purified rat lung ECE-1 [3] was used as a standard. The enzyme reaction was carried out at 37°C for 2 h in 100 μl of reaction mixture consisting of 100 mM Tris-HCl (pH 7.0), 0.1% (w/v) BSA, 0.1 mM PMSF, 20 μM pepstatin A, 20 μM leupeptin, 4 μg of the solubilized membrane protein, and 0.1 μM of human big ETs. Produced ETs were determined by a sandwich enzyme immunoassay [14].

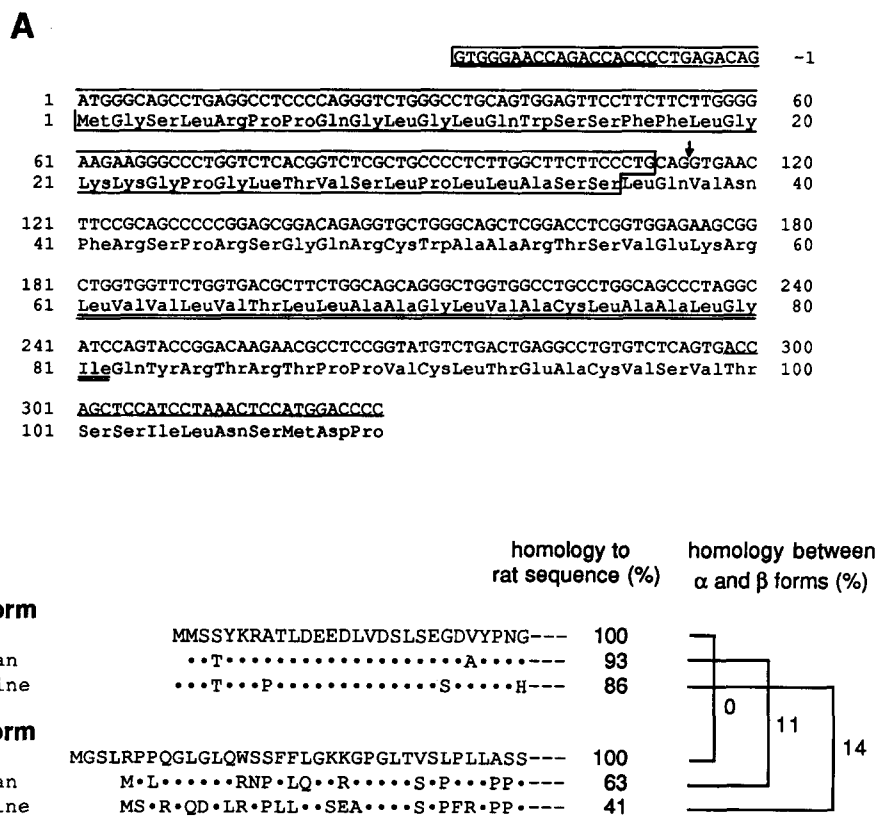

Fig. 1. (A) Nucleotide and deduced amino-acid sequences of cloned region of rat ECE-1β cDNA. Primer regions used for the cloning by reverse transcription-PCR are underlined. Nucleotide and amino-acid sequences differ from those of rat ECE-1α and are boxed. Putative transmembrane domain is doubly underlined. Putative splicing site is indicated by an arrow. (B) Alignment of N-terminal amino-acid sequences of ECE-1α and -1β. Amino-acid sequences of α-form of rat [4], human and bovine [7] or β-form of rat [this paper], human [9] and bovine [6] are aligned. Homology to rat sequence in each form and homology between α and β forms in each species are indicated. Dots indicate amino-acids identical to the respective rat sequences. Dashes represent amino-acid sequences identical to that of the another form of each species.

2.6. Indirect immunofluorescence

COS-1 cells transfected with either human ECE-1α or -1β cDNA were grown on glass coverslips for 48 h. All subsequent processing was performed at room temperature. The cells were fixed with 3% formaldehyde in PBS for 15 min, quenched with 0.1 M glycine in PBS, and incubated in 0.1% saponin in PBS for 10 min for permeabilized samples. The samples were blocked with normal 10% goat serum in PBS for 1 h, incubated with AEC 32-236 for 1 h, washed in PBS, and then incubated with FITC-conjugated goat anti-mouse IgG (Jackson) for 1 h. In all cases, the antibodies were diluted in 10% goat serum in PBS. The coverslips were mounted in a 1:1 mixture of PBS: glycerol containing 2% 1,4-diazabicyclo[2.2.2.]octane to prevent photobleaching. Samples were viewed under a Bio-Rad MRC-600 confocal fluorescence microscope (Bio-Rad) and photographed using a HC1500 film processor (Toshiba, Japan).

3. Results

3.1. Structure of ECE-1α and -1β

5′-terminal region of rat ECE-1β cDNA was amplified by reverse-transcription PCR from rat lung RNA as described in section 2. A sense primer was designed from homology between bovine and human ECE-1β cDNA sequences [5,6,9] and an antisense primer was designed based on rat ECE-1α cDNA [4]. Fig. 1A shows cloned region of rat ECE-1β cDNA sequence and its deduced amino-acid sequence. The cDNA sequence obtained was different from that of rat ECE-1α cDNA by its 5′-terminal 138 bp sequence, resulting in complete differentiation of the N-terminal amino-acid sequence from that of rat ECE-1α, although the cDNA sequence in other regions was completely identical to that of rat ECE-1α. Fig. 1B shows the alignment of N-terminal amino-acid sequences of rat, human and bovine ECE-1α and -1β. ECE-1α was highly conserved among 3 species, whereas the homology of ECE-1β among 3 species was much lower than that of ECE-1α. Complete identity of human ECE-1α and -1β cDNAs except for their 5′-terminal regions [7–9] strongly suggests that these isoforms are produced by alternative splicing from the same gene. Genomic PCR was next carried out using either rat ECE-1α or -1β specific primers. The primers used were the same as those used for quantitation of each mRNA described above (see [2.4]). When rat ECE-1α specific primers were used, we obtained PCR product having the same size as respective cDNA, whereas no PCR product was synthesized when rat ECE-1β specific primers were used

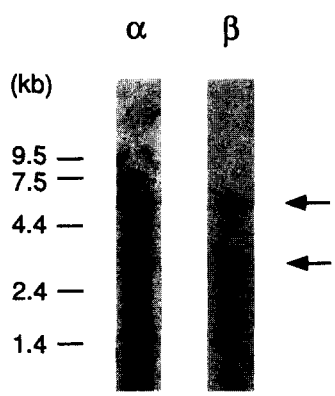

Fig. 2. Northern blot analysis of ECE-1α and -1β transcripts in HUVEC. The 5.0-kb and 3.0-kb ECE-1 mRNAs are indicated by arrows. RNA blot of 4 μg of HUVEC poly(A)$^+$ RNA was hybridized with ECE-1α specific probe (α) or ECE-1β specific probe (β). Positions of M_r markers are shown.

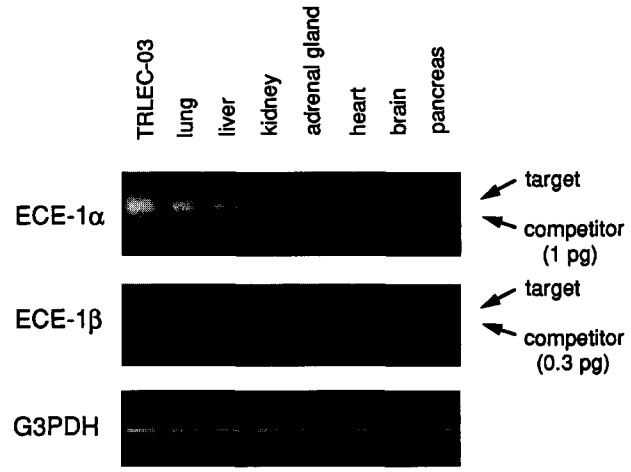

Fig. 3. Competitive PCR analyses of ECE-1α and -1β transcripts in various cells and tissues. Single-stranded cDNAs were reverse-transcribed from the poly (A)$^+$ RNA of various cells and tissues indicated. Each cDNA was subjected to ECE-1α specific PCR (ECE-1α) or ECE-1β specific PCR (ECE-1β) in the presence of a 1 pg of competitor DNA for ECE-1α or 0.3 pg of a competitor DNA for ECE-1β, respectively. cDNAs were also subjected to G3PDH specific PCR (G3PDH). The amplified products were subjected to electrophoresis on agarose gels and were visualized by staining with ethidium bromide.

(data not shown). Human genomic PCR was also carried out using either human ECE-1α or -1β specific primers corresponding to the sequence used for rat genomic PCR, and the results were identical with those of rat genomic PCR (data not shown). These results suggest the absence of an intronic sequence in this region of ECE-1α pre-mRNA, and inversely, the presence of a relatively large intronic sequence in that of ECE-1β pre-mRNA. Indeed, the consensus sequence for a splice acceptor signal, $(Py)_nNCAG/G$ [15], is found in rat ECE-1α cDNA (CCCTACAG/G) [4]. Putative splicing site in ECE-1β cDNA is indicated in Fig. 1A. The splice acceptor signal sequence in the same location is also found in the bovine and human ECE-1α cDNAs [7,8]. Comparing the human ECE-1β cDNA, which we have reported previously [9] and the rat ECE-1β cDNA cloned in this study, together with bovine cDNAs [5,6] indicates the existence of a β form of transcript in all these species tested. Northern blot analysis also elucidated the presence of both forms of ECE-1 mRNA in HUVEC (Fig. 2). The two different sizes of the mRNA are presumably generated by alternative poly(A) addition in the 3′-noncoding region as observed in bovine ECE-1 mRNA [5].

3.2. Determination of expression levels of ECE-1α and -1β mRNAs in various cells and tissues

We next examined the expression levels of ECE-1α and -1β mRNAs in several rat tissues and cells by competitive PCR (Fig. 3). ECE-1α mRNA was more abundant than ECE-1β mRNA in all the tissues examined. As previously observed, ECE-1 mRNAs were expressed at a high level in lung and adrenal gland compared with other organs [4], but the ratio between α and β in these organs was similar to that in other organs. In the case of TRLEC-03 cells, in particular, no ECE-1β mRNA was detected. Northern blot analyses of human ECE-1α and -1β also clarified that ECE-1α mRNA was more abundant than ECE-1β mRNA in all the tissues investigated

(data not shown). Reverse-transcription PCR analyses on HUVEC RNA revealed that ECE-1 mRNA of the α form is also more abundant than that of the β form (data not shown).

3.3. Comparison of enzymatic properties of ECE-1α and -1β

For comparison of enzymatic properties of ECE-1α and -1β, either ECE-1α or -1β cDNA were expressed in COS-1 cells. As an antibody which recognizes both ECE-1α and -1β, we here employed monoclonal antibody AEC32-236 [9], because it reacts with truncated rat ECE-1α which lacks the N-terminal 98 amino-acid sequence [16] as well as intact human, rabbit and bovine ECE-1 [9]. Immunoblot analysis showed the presence of immunoreactive proteins in solubilized membrane fractions prepared from these cells (Fig. 4). As previously reported, ECE-1 is a highly glycosylated protein, but no difference was found in glycosylation levels of the two forms.

Enzyme activities of these recombinant proteins were shown in Table 1. Both forms of ECE-1 showed almost the same specific activities and substrate specificities, and similar results were obtained when the concentration of substrates was varied from 0.1 μM to 2 μM (data not shown). Specific activities of human ECE-1α and -1β were, however, significantly lower than those of rat ECE-1α and -1β probably due to differences in their glycosylation levels (Fig. 4) as described previously [9]. Subcellular localizations of human ECE-1α and -1β expressed in COS-1 cells were studied using AEC32-236 by confocal laser immunofluorecescent micrography. Both human ECE-1α and -1β were found to be mainly localized on the plasma membrane with the clustering structure (Fig. 5) as observed with rat ECE-1α [16] without any further difference in their morphological feature. The result indicates that the differences in N-terminal amino-acid sequences between α and β do not affect their subcellular localization.

4. Discussion

In the present study, we have accomplished the cloning of 5′-terminal region of a new rat ECE-1β cDNA by reverse-transcription PCR derived from rat lung RNA. This result together with previous observations indicates the presence of two isoforms of ECE-1, whose sequences are different only in the N-termini over many species. Both isoforms were also detected by Northern blotting of HUVEC RNA. These isoforms are probably produced by alternative splicing and/or alternative promoter. Sequence analysis of the ECE-1 genomic DNA will reveal the mechanism of the production of ECE-1 isoforms.

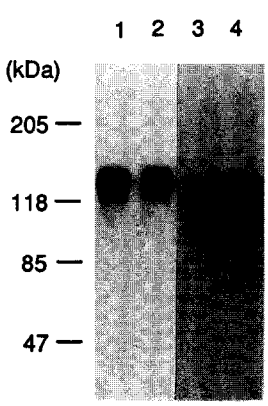

Fig. 4. Immunoblot analysis of ECE-1α and ECE-1β. Immunoblot analysis was carried out using indicated amounts of membrane fractions prepared from COS-1 cells transfected with rat ECE-1α cDNA, 2.5 μg (lane 1), rat ECE-1β cDNA, 2.5 μg (lane 2), human ECE-1α cDNA, 2.0 μg (lane 3) and human ECE-1β cDNA, 2.0 μg (lane 4). Positions of M_r markers are shown.

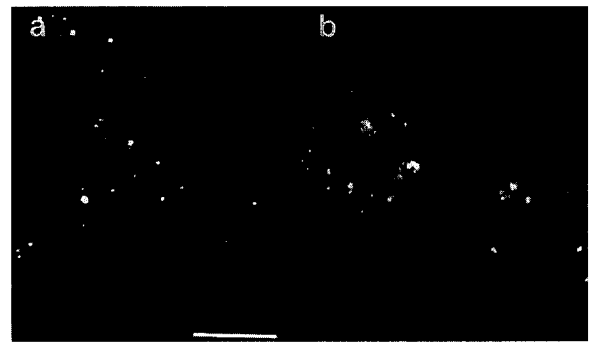

Fig. 5. Subcellular localization of ECE-1 by confocal laser immunofluorescent analysis. COS-1 cells transfected with human ECE-1α cDNA (a) or ECE-1β cDNA (b) were fixed and permeabilized for both cell-surface and intracellular staining. Bar, 10 μm.

Neutral endopeptidase 24.11 (NEP), which is also a membrane-bound neutral metalloprotease and has sequence and structural homology to ECE-1 [4,5], exhibits a broad tissue distribution [17]. Li et al. demonstrated three alternatively spliced forms of rat NEP mRNA. In this case, each contains unique 5′-untranslated sequences including a promoter region but having a common coding sequence. The expressions of these transcripts are regulated in a cell- and tissue-specific manner [18]. In the case of ECE-1, the sequences of two forms of mRNA are different in the 5′-untranslated sequence which probably includes both a promoter region and a small coding region, but mRNA of α form is always 5- to 10-fold more abundant than mRNA of β form in any cells and organs examined, and little tissue-specificity was observed. The observation that α is a dominant form probably reflects the fact that two forms exist in the one cell, yet a possibility remains that the cells expressing only the α form are much more abundant than the cells expressing the β form only. In TRLEC-03 cells, no ECE-1β mRNA was detected. It is possible that the transformation of the cells by SV-40 DNA resulted in down-regulation of ECE-1β mRNA. Schmidt et al. cloned 5′-terminal cDNAs of both human and bovine ECE-1α by rapid amplification of the cDNA ends (RACE) method [7]. This method would result in amplification of only the dominant form of cDNA when several alternatively spliced mRNAs with similar sizes are present in the sample. Our results presented here well explain why they cloned only α-type of ECE-1 cDNAs of both bovine and human.

Table 1
Specific activities and substrate specificities of rat and human ECE-1α and -1β

Substrate	Rat		Human	
	α	β	α	β
Big ET-1	42* (100%)	35 (100%)	3.6 (100%)	3.4 (100%)
Big ET-2	15 (36%)	13 (36%)	1.9 (52%)	1.7 (50%)
Big ET-3	21 (50%)	17 (47%)	1.7 (48%)	1.5 (45%)

Specific activities for big ET-1 are set to 100%.
*Specific activity (nmol/h·mg).

The present results indicate that ECE-1α plays a major role for the conversion of big ETs to ETs compared to ECE-1β. The significance of ECE-1β's presence as a minor form is unclear so far, as there is no difference between the two forms in specific activity, substrate specificity and subcellular localization. ECE-1 forms a disulfide-linked dimeric structure as revealed by our recent observation in SDS-PAGE analyses and cross-linking experiment [16]. It is possible that ECE-1 exists as either homodimer ($\alpha\alpha$ or $\beta\beta$) or heterodimer ($\alpha\beta$) in native cells. Recombinant ECE-1α and -1β, which is present only as respective homodimer, showed both almost same specific activities and substrate specificities. These results coincide with the structural feature of ECE-1, that the large C-terminal region, probably externally facing the plasma membrane, contains a catalytic domain, whereas the small cytoplasmic N-terminal region may not contribute to its catalytic activity [4,16]. In some type II membrane-anchored proteins, the N-terminal amino-acid sequence plays an important role for subcellular localization of the protein [19]. No difference was observed, however, in subcellular localization of ECE-1α and -1β as far as homodimers $\alpha\alpha$ and $\beta\beta$ were concerned. The role of N-terminus in heterodimer, if any, remains to be elucidated.

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