Human endothelin-converting enzyme (ECE-1): three isoforms with distinct subcellular localizations

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Endothelin-converting enzyme 1 (ECE-1) is a membrane-bound metalloprotease that catalyses the conversion of inactive big endothelins into active endothelins. Two different isoforms (ECE-1a and ECE-1b) have previously been identified for human ECE-1. In the present study we have cloned a novel human ECE-1 isoform, termed ECE-1c, and have thus shown for the first time the existence of three distinct ECE-1 isoforms. The three isoforms differ only in their N-terminal regions and are derived from a single gene through the use of alternative promoters. Ribonuclease protection experiments revealed that, although the relative levels of the three isoform mRNA species vary between

human tissues, ECE-1c mRNA is generally the predominant isoform messenger. Immunofluorescence microscopy analysis showed distinct subcellular localizations for the three isoforms: whereas ECE-1a and ECE-1c are localized at the cell surface, ECE-1b was found to be intracellular and showed significant colocalization with a marker protein for the *trans*-Golgi network. We determined that the three isoforms have similar kinetic rate constants (K_m , k_{cat} and V_{max}) for the processing of big endothelin 1 and that the big endothelin isoforms 1, 2 and 3 are cleaved with similar relative velocities of 1.0:0.1:0.1 by the three isoenzymes.

INTRODUCTION

Endothelin, first isolated and sequenced from cultured pig endothelial cells [1], has opened a new field of research activities in recent years. The endothelin family consists of three distinct isopeptides, ET-1, ET-2 and ET-3 which derive from three distinct genes [2]. To form active endothelins, big endothelins have to be cleaved exactly at the Trp²¹-Val/Ile²² peptide bond. A putative endopeptidase specific for this cleavage has been claimed [1] and referred to as endothelin-converting enzyme (ECE). Complementary DNA species coding for two ECE forms, termed ECE-1 and ECE-2, have been isolated [3–7]. Both enzymes are zinc metalloproteases, are inhibited by phosphoramidon, and show structural similarities with a short N-terminal domain, a single transmembrane segment and a large C-terminal part including the active site of the enzyme. The neutral pH optimum as well as the more abundant tissue distribution of ECE-1 suggests, however, that ECE-1 is the enzyme mainly responsible for the conversion of big ET-1 into ET-1.

We and others have previously reported the existence of two human ECE-1 isoforms [8,9] that differ only in their N-terminal parts. These isoforms, ECE-1a and ECE-1b, were further shown to be derived from the same gene by the use of two alternative promoters and by complex splicing events [8]. Here we report the identification of an additional novel human ECE-1 mRNA. This new mRNA encodes a 754-residue isoform that we termed ECE-1c. We studied the relative abundance of the three different mRNA species in several human tissues and compared the biochemical activity and subcellular localization of the corresponding ECE-1 isoforms.

MATERIALS AND METHODS

Cloning of human ECE-1c cDNA

The 5' regions of ECE-1 mRNA species were determined with the SLIC methodology [10]. In brief, first-strand cDNA species were synthesized with an ECE-1-specific primer (5'-TGATGATTGCTTGGTTGTG-3') and total RNA (5 µg) was extracted from human umbilical vein endothelial cells (HUVEC and ECV304) [11]. The purified single-stranded cDNA species were ligated at their 3' ends to a 50-residue oligonucleotide and used as a template for nested polymerase chain reaction with two successive sets of primers. Each set was composed of an ECE-1 primer (5'-CGCCAGAAGTACCACCAACACCACC-3' and 5'-AGCCGCTTCTCCACCTGGGTCCGTG-3') and of a primer complementary to the 50-residue oligonucleotide. The amplification products were probed by Southern blotting with a fourth ECE-1 primer (5'-CGGAGAGCGAGTCCACCAGGTC-3'), subcloned into the pCR2 plasmid vector (Invitrogen), and sequenced.

Ribonuclease protection

Total RNA was prepared from HUVEC and ECV304 cells, human pheochromocytoma and adrenal glands by the Chomczynski methodology [12]; human tissue (liver, placenta, testis, small intestine, lung, heart and kidney) poly(A)⁺ RNA species were purchased from Clontech. RNA samples (10 µg of total RNA or 1 µg of poly(A)⁺ RNA) were protected as previously described [13] with a ³²P-labelled anti-sense RNA, transcribed *in*

Abbreviations used: CHO, Chinese hamster ovary; DHFR, dihydrofolate reductase; ECE, endothelin-converting enzyme; HUVEC, human umbilical vein endothelial cells; MEM, minimal essential medium; TGN, *trans*-Golgi network.

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The nucleotide sequence results reported here will appear in DDBJ, EMBL and GenBank Nucleotide Sequence Databases under the accession number X98272.

Figure 1 ECE-1 isoforms

(**A**) Amino acid sequences of the human ECE-1 isoform N-termini. Dashes represent residues identical with the line above. (**B**) Nucleotide sequences. Entries 1, 2 and 3 show the sequences specific for ECE-1a, ECE-1b and ECE-1c, respectively; entry 4 displays the sequence common to both ECE-1b and ECE-1c, and entry 5 shows the first nucleotides of the rest of the sequence, common to all isoforms. The corresponding residues (aa) and exons are also indicated. (**C**) Organization of the 5' region of the ECE-1 gene. Exons 4-19 (common to all isoforms), which encode the major part of ECE-1 cDNA [8], are not represented. Four exons (numbered 1c, 1b, 2 and 3) and their corresponding parts in the 5' regions of ECE-1a, ECE-1b and ECE-1c mRNA species are shown. The start codons of the three isoforms are indicated. The 3' part (black box) of exon 3, including 142 nt, is common to the three isoforms, whereas its 5' part (empty rectangle) is specific for ECE-1a. Exon 2 is common to ECE-1b and ECE-1c. ECE-1b- and ECE-1c-specific sequences correspond to exons 1b (exon 1 in [8]) and 1c respectively. Promoters (P) located upstream of exons 1c, 1b and 3 drive the transcription of ECE-1c, ECE-1b and ECE-1a mRNA species respectively. The size of the intron between exons 1c and 1b could not be assessed.

vitro from a 313 bp cloned ECE-1b cDNA fragment ranging from the ECE-1b start codon to the ECE-1 unique *HindIII* site. Protected RNA species were resolved on sequencing gels with known DNA sequences for size determination.

Expression of ECE-1 isoforms

A dihydrofolate reductase (DHFR)-deficient Chinese hamster ovary cell line (CHO DHFR⁻) was cultured in minimal essential medium α (MEM α) supplemented with 10% (v/v) heat-inactivated fetal calf serum, 100 i.u./ml penicillin and 100 μ g/ml streptomycin at 37 °C in a humidified air/CO₂ (19:1) atmosphere. The three isoform cDNA species were inserted into the eukaryotic expression vector pN346, which contains the DFHR gene. CHO DHFR⁻ cells were transfected with the three constructs by using Lipofectamine (Gibco BRL). Selection was performed with MEM α lacking ribonucleosides and deoxyribonucleosides (MEM α ⁻; Gibco BRL). Resistant colonies were screened by the measurement of endothelin-converting activity and immunofluorescence microscopy. Selected clones were expanded for

further study and maintained in selective MEM α ⁻ containing 10% (v/v) dialysed heat-inactivated fetal calf serum, 100 i.u./ml penicillin and 100 μ g/ml streptomycin.

Antibodies

A peptide common to all three isoforms (NH₂-LQVNFHSPR-SGQR-CO₂H) and N-terminally elongated by one cysteine residue was synthesized, conjugated to bovine thyroglobulin by using *N*-maleoyl- β -alanine-*N'*-hydroxysuccinimide as previously described [14], then purified. MORO IBM mice were immunized and boosted at 4-week intervals, yielding antiserum P6. The polyclonal antibody against murine γ -adaplin [15] was a gift from Dr. L. Traub (Washington University Medical School, St. Louis, MO, U.S.A.).

Immunofluorescence microscopy

Cells were grown in eight-well multichamber Permanox slides (CHO and ECV304 cells) or eight-well multichamber glass slides

Figure 2 Ribonuclease protection analysis

(A) The anti-sense RNA probe was transcribed from a cloned ECE-1b cDNA fragment. RNA fragments protected by the 32 P-labelled probe are indicated (solid line) for each ECE-1 isoform. Abbreviation: b, bases. (B) Relative levels of human ECE-1 mRNA species determined by ribonuclease protection analysis. Signals corresponding to the three isoforms (a, b and c, for ECE-1a, ECE-1b and ECE-1c respectively) are indicated. Lane 1, ECV304 cells; lane 2, HUVEC; lane 3, liver; lane 4, placenta; lane 5, testis; lane 6, small intestine; lane 7, lung; lane 8, heart; lane 9, kidney; lane 10, adrenal gland; lane 11, pheochromocytoma. Poly(A)⁺ RNA (1 μ g) (lanes 3–9) or total RNA (10 μ g) (lanes 1, 2, 10 and 11) were used.

coated with 1 % (w/v) gelatin in PBS (HUVEC). For the single immunofluorescence the procedure described by Schweizer et al. [16] was used. In brief, formaldehyde-fixed and saponin-permeabilized cells were incubated with mouse polyclonal anti-(ECE-1) antibody, P6, followed by FITC-conjugated goat anti-mouse antibody (Cappel). For double immunofluorescence, cells were first washed once with PBS and then fixed and permeabilized for 2 min at room temperature with methanol/acetone (1:1) precooled to -20°C . After two washes each with PBS and 0.2 % (v/v) pig skin gelatin in PBS, the cells were sequentially incubated with the rabbit anti-(γ -adapin) polyclonal antibody, Texas Red-labelled goat anti-rabbit antibody (Cappel), the mouse polyclonal antibody P6, and FITC-conjugated goat anti-mouse antibody. The antibodies were diluted in PBS containing 0.2 % (v/v) pig skin gelatin, and each antibody incubation was followed by four washes with the same solution. The specimens were examined with a Zeiss Axioscope microscope connected to a video imaging system.

Measurement of ECE-1 isoform activity

For each ECE-1 isoform, transfected CHO cells were cultured in 60 mm plates to confluency in MEM α ⁺ supplemented with 10 % (v/v) dialysed fetal calf serum. Before ECE activity measurement the cells were washed with fresh medium and subsequently incubated for 30 min at 37°C with 1 ml of medium containing various concentrations of big ET-1. After incubation the medium was collected, shock-frozen and stored at -80°C until ET-1 determination. Cells were scraped off, washed with PBS, resuspended in 1 ml of buffer A [10 mM Tris/5 mM

MgCl₂/100 μ M PMSF/20 μ M *trans*-epoxysuccinyl-L-leucyl-amido-(4-guanidino)butane (E-64)/20 μ M leupeptin/20 μ M pepstatin (pH 7.0)] and sonicated for 10 s at 0°C . The cell homogenate was subjected to either protein and ECE-1 isoform quantification or membrane preparation.

For membrane preparation, cell homogenates were centrifuged twice at 20000 *g* for 20 min each and resuspended in 500 μ l of buffer A. Membrane protein (1–20 μ g) was incubated in buffer B [50 mM Tris/HCl (pH 7.0)/0.1 % BSA/0.1 % NaN₃] with various concentrations of big ET-1 in a total volume of 160 μ l for 30 min at 37°C . The enzymic reaction was stopped by the addition of 10 μ l of 150 mM EDTA, pH 7.0.

To measure the enzyme reaction constants $V_{\max}$ and K_m , whole cells and cell membrane preparations were incubated in the presence of 0.075–1.5 μ M big ET-1. The substrate hydrolysis rate (v) per mg of ECE-1 (μ mol of ET/min per mg) was calculated by using the amount of ECE-1 per mg of membrane or total cell protein determined by quantitative immunoblotting (see below). The maximal substrate conversion under the conditions used was at most 4 %. K_m values were calculated with the Hanes plot ($[S]$ against $[S]/v$, where $[S]$ is substrate concentration). $V_{\max}$ values were calculated from an Eadie–Hofstee plot (v against $v/[S]$). The Hill coefficient, h , was determined as the slope of the curve of $\log[v/(V_{\max} - v)]$ against $\log[S]$.

To determine the K_i values of phosphoramidon for the inhibition of the ECE-1 isoforms, CHO membrane preparations were incubated in the presence of 0, 0.2, 0.5, 1.0 or 5.0 μ M phosphoramidon as described above. Results were plotted on Lineweaver–Burk diagrams. The mode of inhibition of the ECE-1 isoforms was competitive. The K_i values were obtained from secondary plots of the slope of the respective Lineweaver–Burk curves against the concentration of phosphoramidon.

The relative conversions of human big ET-1, ET-2 and ET-3 were determined at a substrate concentration of 0.5 μ M under the conditions described above. The production of ET-1 (ET-2, ET-3) in culture medium or buffer B was measured with a specific radioimmunoassay as previously described [17].

All kinetic studies on whole cells and cell membrane preparations were performed as three independent experiments. Mock-transfected CHO cells and their membrane preparations showed no cleavage of human big ET-1 within the incubation period used in these experiments. The hydrolysis of big endothelin was linear with time (up to 90 min) and protein (up to 20 μ g) under the assay conditions used. Each determination of ET-1 produced was performed in triplicate.

Overall ECE-1 isoform expression in transfected CHO cells was determined by quantitative immunoblotting (B.-M. Löffler and M. Mangold, unpublished work). In brief, cell homogenates (10 and 20 μ g), cell membrane preparations (5 and 10 μ g), purified mouse IgG (0.4–20 fmol) and recombinant human ECE-1 purified from HEK-293 cells (0.4–20 fmol) were heated to 55°C for 30 min in non-reducing sample buffer (Novex) and subjected to SDS/PAGE [4–20 % (w/v) gradient gels; Novex] as described by Laemmli [18]. Electrophoresis was performed at 4°C with a constant current of 25 mA per gel for 1.5 h. Proteins were transferred to Immobilon-P membranes (Millipore) at 4°C for 2 h at a constant 75 V with 25 mM Tris/192 mM glycine/15 % (v/v) methanol as a buffer system. See-Blue prestained standards (Novex) were used as molecular mass markers. The membranes were blocked for 18 h at 4°C , washed with PBS supplemented with 0.2 % (v/v) Tween-20 (PBS-T), incubated for 3 h at 20°C with antiserum P6 (1:500, v/v) and washed with PBS-T. ECE-1 and mouse IgG were detected with the enhanced chemiluminescence system (Amersham) in accordance with the manufacturer's instructions. Enhanced chemiluminescence hyperfilms

were scanned and the ECE-1 signals were quantified with Scan Analysis version 2.50 (Biosoft). Only the ECE-1 signal at 250 kDa was used for quantification. The ECE-1 content of samples was calculated by using purified recombinant ECE-1 and purified IgG as standards. Samples and standards were always run on the same gel. Determinations were performed in duplicate. Protein was determined by the method of Lowry, with BSA as a standard protein [19].

RESULTS

Cloning of an additional ECE-1 isoform

A PCR-based methodology named SLIC [10] was employed to clone the 5' extremities of ECE-1 mRNA species from HUVEC and ECV304 cells. Three populations of clones could thus be distinguished. Two groups of clones corresponded to the previously known ECE-1a and ECE-1b mRNA [8], whereas the third group suggested the existence of a new type of ECE-1 mRNA. This new messenger displays 81 bases of ECE-1b specific sequence, but diverges from ECE-1b mRNA upstream from these 81 bases (Figure 1B), encoding a novel ECE-1 isoform of 754 amino acid residues, termed ECE-1c.

Distribution of ECE-1 isoform mRNA species in human tissues

To confirm the existence of the three ECE-1 isoform mRNA species and to assess their relative abundances, ribonuclease

protection assays were performed on various human tissues. The RNA probe was synthesized from a cloned ECE-1b cDNA fragment and was devised to detect three signals corresponding to protected ECE-1 mRNA species of 179 (ECE-1a), 260 (ECE-1c) and 313 bases (ECE-1b) (Figure 2A). The results clearly show that the relative levels of expression of the three ECE-1 isoform mRNA species differed between various tissues (Figure 2B). ECE-1c mRNA was in general more abundant than the two other isoform mRNA species, except in HUVEC, where ECE-1a mRNA was predominant.

Subcellular localization of the three human ECE-1 isoforms

To examine the subcellular distribution of the human ECE-1 isoforms, indirect immunofluorescence microscopy was performed. Stable cell lines expressing ECE-1a, ECE-1b or ECE-1c were fixed with paraformaldehyde and permeabilized with saponin to reveal both cell-surface and intracellular staining. The polyclonal antibody P6, which recognizes a peptide common to all three isoforms, was then used for detection. Non-transfected control cells showed no staining with the P6 antibody (Figure 3A), consistent with previous evidence that CHO cells have little or no endogenous ECE-1 activity. As seen in Figure 3(B), cells transfected with ECE-1a displayed bright cell-surface staining, indicating that this isoform is predominantly localized at the plasma membrane. A similar distribution was obtained for the ECE-1c isoform (Figure 3D). However, in addition to strong

Figure 3 Immunofluorescence localization of the three ECE-1 isoforms in CHO cells

Non-transfected CHO DHFR⁻ cells (A), or CHO DHFR⁻ cells stably expressing ECE-1a (B), ECE-1b (C) or ECE-1c (D) were fixed with paraformaldehyde and permeabilized with saponin. For detection, polyclonal antibody P6 followed by FITC-conjugated goat anti-mouse antibody was used. In contrast with ECE-1a and ECE-1c, the ECE-1b isoform is predominantly localized intracellularly.

Figure 4 Double-immunofluorescence localization of ECE-1b (A) and γ -adaptin (B) in CHO cells

CHO DHFR⁻ cells stably expressing the ECE-1b isoform were fixed and permeabilized with methanol/acetone followed by sequential incubation with rabbit anti- γ -adaptin, Texas Red-labelled goat anti-rabbit antibody, mouse polyclonal antibody P6 and FITC-conjugated goat anti-mouse antibody.

expression at the plasma membrane, cells positive for ECE-1c also showed some intracellular, Golgi-like staining. In contrast with ECE-1a and ECE-1c, the staining pattern for ECE-1b was not characteristic for a cell-surface protein but was found to be predominantly intracellular (Figure 3C). When cells expressing the ECE-1b isoform were stained with the P6 antibody, a juxtanuclear Golgi-like pattern could be seen. In addition, there was some labelling of more peripheral punctate structures, which might represent coated vesicles or endosomal structures.

To study the relationship of the ECE-1b-positive structures and the Golgi apparatus, double immunofluorescence was performed. The stable cell line expressing ECE-1b was double-stained with the mouse polyclonal antibody P6 against ECE-1 and a rabbit antibody against γ -adaptin, a well-established marker protein for the *trans*-Golgi network (TGN) [20,21]. As shown in Figure 4, the overall distributions of ECE-1b and γ -adaptin were very similar, indicating that the ECE-1b isoform is present in the TGN.

We next examined the localization of endogenous ECE-1 isoforms in the endothelial cell lines HUVEC and ECV304. As shown above, ECE-1a mRNA is the predominant isoform mRNA expressed in HUVEC (Figure 2), whereas ECV304 cells contain exclusively ECE-1c and ECE-1b mRNA species (Figure 2). Immunofluorescence analysis with the polyclonal antibody P6 revealed ECE-1 at the cell surface in both endothelial cell lines

Figure 5 Immunofluorescence localization of ECE-1 in HUVEC (A) and ECV304 (B) cells

Cells were fixed with paraformaldehyde, permeabilized with saponin and then labelled with the polyclonal antibody against ECE-1 followed by FITC-conjugated goat anti-mouse antibody.

(Figure 5). In HUVEC (Figure 5A), plasma membrane staining was present as patches or clusters as described previously [22], whereas ECV304 cells (Figure 5B) showed a more homogeneous cell-surface labelling of ECE-1. Some ECE-1 in ECV304 cells was also present in intracellular Golgi-like structures (Figure 5B). These results with the endothelial cell lines clearly confirm in an endogenous system the distributions of ECE-1a, b and c found in transfected CHO cells.

Taken together, the morphological analysis of human ECE-1a, b and c revealed that the isoforms differ in their subcellular localizations.

Cleavage of big endothelins by the ECE-1 isoforms

To compare their enzymic properties, the three ECE-1 isoforms were expressed in CHO cells and their endothelin-converting activity was analysed either on intact cells or on cell membrane preparations with a specific radioimmunoassay [17]. All three isoforms cleaved big ET-1 with comparable efficiencies (Table 1) and showed similar K_m values ($0.5 \pm 0.2 \mu\text{M}$, means $\pm$ S.D.) on both whole cells and membrane preparations. The k_{cat} values ($2.9 \pm 0.3 \text{ s}^{-1}$, means $\pm$ S.D.) measured on membrane preparations were also similar for the three isoforms. The ECE-1a isoform showed a similar maximal specific activity of $0.65\text{--}0.80 \mu\text{mol}$

Table 1 Kinetic parameters of the ECE-1 isoforms

The catalytic activity of the ECE-1 isoforms was determined in assay buffer (membrane preparations) or culture medium (cells) with human big ET-1 as a substrate for 30 min at 37 °C as described in the Materials and methods section. Data are means $\pm$ S.D. for three independent measurements. $V_{\max}$, maximal initial specific velocity of ET-1 production/min per mg of ECE-1. The $V_{\max}$ value of ECE-1b on whole cells was calculated on the basis of the total amount of enzyme present in these cells. It does not take into account the inaccessibility of the intracellularly localized ECE-1b to the substrate.

Enzyme preparation	$V_{\max}$ ($\mu\text{mol}/\text{min per mg}$)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\text{s}^{-1} \cdot \mu\text{M}^{-1}$)	h	K_i (μM)
ECE-1a						
Cells	0.8 ± 0.2	0.75 ± 0.07	3.3 ± 0.9	4.4		
Membranes	0.65 ± 0.05	0.45 ± 0.06	2.7 ± 0.2	6.0	1.03 ± 0.06	0.41 ± 0.09
ECE-1b						
Cells	$0.03 \pm 0.02^*$	0.41 ± 0.20				
Membranes	0.63 ± 0.03	0.52 ± 0.05	2.6 ± 0.1	5.2	1.08 ± 0.11	0.37 ± 0.11
ECE-1c						
Membranes	0.55 ± 0.05	0.43 ± 0.08	2.3 ± 0.2	5.8	0.98 ± 0.07	0.33 ± 0.07

* Apparent maximal specific velocity.

of endothelin/min per mg at 37 °C, regardless of whether it was measured on whole cells or on membrane preparations. Comparable maximal specific activities were measured for ECE-1b and ECE-1c on membrane preparations (Table 1). In contrast, the apparent maximal specific activity observed for ECE-1b on whole cells was much lower ($0.03 \mu\text{mol}$ of endothelin/min per mg), in line with the predominantly intracellular localization of ECE-1b that we have observed by immunofluorescence microscopy (Figure 3C). By using the enzymic activity ratio of ECE-1b-expressing cells and their respective membrane preparations we calculated that only approx. 5% of ECE-1b is expressed on the plasma membrane. The Hill coefficient, h , was close to 1.0 for all three isoforms. Phosphoramidon inhibited all three isoforms competitively with similar K_i values of approx. $0.4 \mu\text{M}$ (Table 1). In addition, all three isoforms displayed a clear preference for big ET-1 over big ET-2 and big ET-3. The relative cleavage ratios (big ET-1 to big ET-2 to big ET-3) were calculated to be 1:0.01:0.14 for ECE-1a, 1:0.03:0.13 for ECE-1b and 1:0.03:0.10 for ECE-1c.

DISCUSSION

The present study shows that ECE-1 exists as three distinct isoforms encoded by the same gene. These three isoforms show clear differences both in tissue distribution and subcellular localization.

We had previously established the existence of two human ECE-1 isoforms, termed ECE-1a (758 residues) and ECE-1b (770 residues) [8]. These isoforms differ in their N-termini and originate from the same gene through the use of two different promoters [8]. Thus ECE-1 gene exons 1b and 2 are specific for ECE-1b mRNA, whereas a 5' portion of exon 3 is specific for ECE-1a (Figure 1C). This paper describes the isolation of an additional, previously unknown human ECE-1 mRNA. The newly discovered transcript contains the second ECE-1b specific exon (exon 2 in Figure 1C), but displays a different sequence upstream of this exon. This new mRNA thus encodes an additional ECE-1 isoform, ECE-1c, consisting of 754 residues. The three human ECE-1 isoform sequences are presented in Figure 1. The existence of two human ECE-1 isoforms has been reported by Shimada et al. [9]. ECE-1 β is identical with our ECE-1a, whereas ECE-1 α is in fact a truncated form of ECE-1b (lacking the first 17 residues) because it was deduced from an incomplete cDNA [6]. Two ECE-1 isoforms each have so far also been identified for rat [3,23] and bovine [4,6].

The existence of a third isoform sheds new light on the organization of the ECE-1 gene, which we have described recently [8]. We had previously shown that two alternative promoters drive the transcription of ECE-1a and ECE-1b RNA species [8]. Sequence analysis of the 200 bp between exons 1b and 2 and of a 1.5 kb region upstream of exon 1b did not reveal an ECE-1c-specific sequence. Therefore the generation of ECE-1c mRNA can be explained only by the presence of (at least) an additional exon (1c) and of a third promoter upstream of exon 1b. The complex organization of the 5' region of the ECE-1 gene is shown in Figure 1(C): three distinct promoters drive the transcription of the different RNA species. Exons 4–19 and the 3' half of exon 3 are common to all isoform messengers. ECE-1a mRNA begins with the whole exon 3. Exon 2 is common to ECE-1b and ECE-1c mRNA species, and part of exon 3 is spliced out so that these two mRNA species contain only the last 142 nt of this exon. ECE-1b mRNA additionally contains exon 1b, and ECE-1c at least one specific upstream exon (exon 1c).

The tissue distribution patterns of the three ECE-1 isoforms were assessed by a ribonuclease protection assay. The relative expression ratios of the three isoform mRNA species differ significantly between various human tissues (Figure 2). In particular, ECE-1c mRNA was found to be the major isoform mRNA in most of the tested human tissues, whereas ECE-1a mRNA was predominant in HUVEC. ECE-1b signals were clearly weaker than both ECE-1a and ECE-1c signals in all tissues except in ECV304 cells and pheochromocytoma, where ECE-1a could hardly be detected, or not at all. These differences in the tissue distributions of the three isoform mRNA species are in line with the existence of isoform-specific promoters.

The three isoforms were expressed in CHO cells and their functions characterized. They were all found to cleave the three big endothelins, but with a clear preference for big ET-1, which is in agreement with previous results [3,4,6,23]. No significant difference between the isoforms could be detected with respect to their kinetically characteristic parameters K_m , k_{cat} , $V_{\max}$ and h if measured on membrane preparations of CHO cells. This was expected because the active site of the enzyme is located in its C-terminal part, which is common to all three isoforms. The kinetic parameters of the three isoforms were determined for the membrane-bound dimeric form of ECE-1 and not for the pure solubilized enzyme. Thus the precision of the ECE-1 quantification as well as the crude membrane environment might influence the values of the kinetic parameters determined in this study. However, solubilization and enzyme purification as has

been used by Ohnaka [24] might also influence kinetic parameters owing to the use of detergents and the partial inactivation of the enzyme during purification. It is important to note that ECE-1 activity in our membrane preparations did not alter over several freezing and thawing cycles, indicating a high enzyme stability (results not shown). In agreement with earlier reports, we could not detect any unspecific cleavage of big endothelin by the CHO cells used to express the ECE-1 isoforms [4,7]. Furthermore the K_m values reported in the present study for the ECE-1 isoforms are comparable to those reported for rat ECE-1 [25]. The average maximal specific activity of the three isoforms of $0.75 \pm 0.14 \mu\text{mol}$ of endothelin/min per mg of ECE-1 is 1.8-fold that reported for ECE-1 purified from pig aortic endothelium [24]. This difference might be due to the intact membrane environment under the present assay conditions, to losses of enzyme activity during purification, or to the different enzyme assays used.

In contrast with the enzymic data, immunofluorescence analysis of ECE-1a, ECE-1b and ECE-1c revealed a clear difference in their subcellular localizations. ECE-1b was found to be intracellular, significantly co-localizing with the TGN marker γ -adaptin. From our activity data we could calculate that approx. 95% of the totally expressed ECE-1b remained intracellular. Most ECE-1a and ECE-1c, in contrast, was present at the cell surface, with some additional intracellular staining for ECE-1c. Recently, human ECE-1 was localized to the plasma membrane in HUVEC by using immunomagnetic separation techniques and immunofluorescence microscopy [22]. We also found ECE-1 at the cell surfaces of HUVEC and an additional endothelial cell line ECV304. In ECV304 cells some ECE-1 was also detected intracellular. These distributions are in line with the localization of ECE-1a, b and c in transfected CHO cells because ribonuclease protection experiments clearly showed that ECE-1a mRNA is the predominant isoform mRNA in HUVEC, whereas ECV304 cells contain exclusively ECE-1b and ECE-1c mRNA species. The distributions of ECE-1 in the endothelial cell lines further demonstrate that the plasma membrane localization of ECE-1a and c in transfected CHO cells is not due to an overexpression of the isoforms in this system. Consistent with our results, a plasma membrane localization was also found for the above-mentioned ECE-1 β isoform when transiently expressed in COS cells [9].

The most striking finding of our immunofluorescence analysis is the intracellular distribution of ECE-1b. A comparison of the amino acid sequences of ECE-1 isoforms reveals that the Golgi localization of ECE-1b is most likely conferred by its 17 N-terminal residues (Figure 1A). The correct localization of ECE-1b could be mediated by a retrieval mechanism via the plasma membrane. Retrieval has, for example, been shown to be the mechanism by which the integral membrane protein TGN38 is targeted to the TGN [26,27]. Interestingly, ECE-1 was observed to redistribute to an intracellular wheatgerm agglutinin-positive phosphoramidon [22], indicating that it can cycle between the plasma membrane and intracellular sites. Alternatively, ECE-1b localization could be achieved by retention, as shown for a number of Golgi proteins [28].

In conclusion, the identification of three ECE-1 isoforms reveals a new level of complexity of the endothelin system. Because the respective mRNA species, which are under the control of alternative promoters, are differentially expressed in a

cell-specific manner, the ECE-1 isoenzyme expression patterns might be of regulatory importance and provide a fine modulation of local endothelin levels. Furthermore the different subcellular localizations of these isoforms, in particular the intracellular localization of ECE-1b, might provide new insights into functional aspects of the endothelin system, and could be of importance for the development and evaluation of ECE-1-selective inhibitors.

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