Construction, expression and characterization of a soluble form of human endothelin-converting-enzyme-1

Petra Korth[a], Giorgia Egidy[a], Charles Parnot[a], Jean-Michel LeMoullec[b], Pierre Corvol[a], Florence Pinet[a],*

[a]INSERM U36, Collège de France, 3 rue d'Ulm, 75005 Paris, France
[b]Roussel Uclaf, 102 route de Noisy, 93235 Romainville, France

Abstract Endothelin-converting-enzyme-1 (ECE-1) belongs to the family of zinc metallopeptidases and is responsible for generating endothelin (ET) peptides from their inactive precursors the big endothelins (bigET). The enzyme is a type II integral membrane protein consisting of a short amino-terminal cytosolic domain of 56 amino acids, a single transmembrane domain and a large putative extracellular domain containing the catalytic site. Recombinant and native ECE-1 are expressed as a dimer. We have constructed a soluble form of ECE, named sECE*, by fusing the cleavable signal peptide of pro-opiomelanocortin in frame to the complete extracellular domain of human ECE-1. Stable expression of this construct in CHO cells resulted in the secretion of a fully active enzyme. In contrast to membrane-bound ECE, sECE* was expressed as a monomer, highly glycosylated, as assessed by gel filtration and Western blot. However, recombinant sECE* converted bigET-1 with similar specific activity as ECE-1a. This activity was completely inhibited by phosphoramidon, but not by thiorphan and captopril. sECE* was active in a broad range of pH, showing an optimum of 6.6–6.8 for bigET-1. Thus, the extracellular domain alone is sufficient for conferring full ECE-1 activity, inhibitors recognition and substrate specificity.
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Key words: Human; ECE-1; Endothelin; Soluble enzyme; Immunoprecipitation; Glycosylation

1. Introduction

The endothelins are a recently discovered family of three 21-amino acid peptides that regulate vascular tone [1,2] and play an important role in cardio-vascular and enteric nervous system development [3,4]. Endothelin-2 (ET-2) and endothelin-3 (ET-3) differ from endothelin-1 (ET-1) by 2 and 6 amino acids respectively, and are encoded by separate genes [5]. ETs are initially synthesized as large inactive precursor proteins called preproETs (212, 178 and 238 amino acids, respectively) which are first cleaved at two pairs of basic amino acids [6] to generate bigET-1, bigET-2 and bigET-3 (38–41 amino acids). They are then cleaved by endothelin-converting enzyme (ECE) (EC 3.4.24.71) at a Trp-Val (for bigET-1 and bigET-2) or Trp-Ile (for bigET-3) site to produce the 21-residue active peptides [7].

*Corresponding author. Fax: +33 1 44 27 16 91.
E-mail: pinet@infobiogen.fr

ECE is a key enzyme in the biosynthesis of the endothelins as the biological activities of bigETs are negligible [8,9]. ECE is a new membrane-bound metalloprotease [7,10,11] with structural homology to neutral endopeptidase (NEP) [12] and Kell blood group protein [13], displaying 39% similarity to NEP and 32% to Kell protein. ECE is inhibited by phosphoramidon but not by thiorphan, a specific inhibitor of NEP [14]. Two human ECE-1 isoforms, ECE-1a and ECE-1b [15–17] have recently been cloned. They differ only by their N-terminal extremities and are encoded by a single gene [18].

ECE-1 is assumed to be a type II integral membrane protein with a short N-terminal cytoplasmic tail, a single transmembrane hydrophobic helix and a large putative extracellular domain containing the catalytic site [19]. Membrane preparations of Cos and CHO cells transfected with the cloned ECE-1 efficiently convert bigET-1 in vitro but are less efficient for the conversion of bigET-2 and bigET-3, suggesting that this isoform is specifically implicated in bigET-1 processing [20,21]. Recently, using a live-cell assay, Parnot et al. [22] showed that ECE-1a is able to process bigET-1 into ET-1 both intracellularly (probably within vesicles) and extracellularly at the plasma membrane [22].

ECE-1 is a member of the zinc metalloprotease family, harbouring the HEXXH zinc-binding motif. Recent studies using site-directed mutagenesis have shown that two glutamic acids (residues 592 and 651) and one histidine (residue 716) are responsible for catalytic activity and zinc binding in rat ECE-1a [23]. ECE exists as a dimer and this process is due to the sole Cys412 as shown by site-directed mutagenesis of rat ECE-1a. It was also shown that the equivalent residue (Cys416) in human ECE-1a is involved in dimerization [24]. The contribution of other amino acids to the catalytic activity is still unknown as well as the role of the cytosolic and membrane-spanning domains in the transport of the protein to the cell surface and in the protein conformation necessary for catalytic activity and substrate specificity.

The purpose of the present study was to determine the role of the intracellular and the transmembrane domains and to evaluate whether a soluble form of ECE-1 could be transported to the cell surface and secreted as an active form. For this purpose, the intracytoplasmic and membrane-spanning domains of ECE-1 were substituted by the signal peptide of a secretory protein (pro-opiomelanocortin) (POMC) as described previously for NEP [25]. The resulting truncated enzyme is not inserted in the plasma membrane but can be recovered as a soluble form of ECE-1 in the culture medium of stably transfected CHO cells and exhibits the same enzymatic characteristics as the membrane-bound enzyme. Finally, the soluble enzyme appears predominantly as a monomer

compared to the membrane-bound enzyme which is expressed mainly as a dimer. This monomer of ECE will be a useful tool in structural and functional studies.

2. Materials and methods

2.1. Plasmid construction

The pcDNA3 expression vector (Invitrogen) in which ECE-1a was subcloned [22], named pcDNA3-ECE-1a, was digested by *Hin*dIII which removed the first 287 bp of the ECE cDNA corresponding to the N-terminal cytoplasmatic tail and the transmembrane domain (amino acids 1 to 78). To create a secreted ECE (sECE*), the following sequence was added 5'-AGCTTACTAGTATGCCGAGATCGT-GCTGCAGCCGCTCTCGGGGGCCCTGTTGCTGGCCTTGCTG-CTTCAGGCCTCCATGGAAGTGCGTGG<u>CATGC</u>AGTACCAGA-CAAGATCCCCCTCTGTGTGTGCCTGAGCGA-3': The nucleotide sequence corresponds to the human POMC signal peptide followed by a *Sph*I site (underlined nucleotides) and the ECE sequence corresponding to the amino acids 78 to 90 (bold nucleotides) with a *Hin*dIII site added at both ends. This double stranded oligonucleotide was subcloned in the expression vector described above. This plasmid was named pcDNA3-sECE*.

2.2. Expression in CHO cells

CHO-K1 cells were maintained in Ham's F-12 medium supplemented with 10% fetal calf serum, 0.5 mM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. To establish the CHO/ECE-1a and CHO/sECE* clones, CHO cells were transfected with 10 µg of the corresponding plasmids using the calcium phosphate precipitation method [26]. Single colonies of primary G418-resistant (750 µg/ml) transformants were isolated and first screened by RNA dot blot. Cell colonies expressing ECE mRNA were selected and subcloned using the limiting dilution technique. Pure cell lines were further assayed for their ECE activity.

2.3. Membrane preparation

CHO cells expressing the human ECE-1a were grown to confluence, washed twice with ice-cold PBS and collected. All subsequent steps were performed at 4°C. The cells were homogenized in 20 mM Tris-HCl, pH 7.0, 5 mM $MgCl_2$, 0.1 mM phenylmethylsulfonyl-fluoride, 20 µM pepstatin A and 20 µM leupeptin using a polytron homogenizer. The homogenate was centrifuged at $1300 \times g$ for 10 min, and the resulting supernatant was further centrifuged at $100000 \times g$ for 60 min. The crude membrane pellet thus obtained was solubilized for 60 min in the homogenization buffer containing 0.5% (w/v) Triton X-100 to give a final protein concentration of 2–3 µg/ml. The sample was centrifuged at $100000 \times g$ for 60 min and the resulting supernatant was used as solubilized membrane.

2.4. ECE activity assay

Selected CHO clones expressing sECE* (CHO/sECE*) were grown to confluence and washed twice with PBS. 3×10^6 cells were incubated for a 24 h period with 3 ml serum-free medium (Ultra-CHO, Bio-Whittaker or DMEM, Boehringer) supplemented with 0.5 mM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. The medium was recovered, centrifuged and concentrated by ultra-filtration (Millipore, Ultra-free centrifugal filter unit with a 50 000 NMWL Biomax high-flux polysulfone membrane). To test the stability of sECE* activity in the two culture mediums used, bigET-1 conversion rate was determined under the same conditions in freshly harvested medium and after various storage periods at 4°C.

The enzymatic conversion of bigET into ET peptides was carried out in 100 µl buffer A (50 mM Tris-HCl (pH 7.0), 150 mM NaCl, 0.1 µM $ZnCl_2$ and 0.1% BSA) by incubating the solubilized membrane protein or concentrated medium with 0.1 µM bigET-1, bigET-2 or bigET-3 for 15 min at 37°C, unless otherwise stated. For some experiments, samples were preincubated at 37°C with various protease inhibitors added to buffer A, prior to the addition of bigET substrates. To determine IC_{50} of phosphoramidon various concentrations from 0.5 µM to 50 µM were used. The reaction was stopped by adding 100 µl of 5 mM EDTA and then subjected to a commercial EIA kit (Cayman Chemical Co., Ann Arbor, USA) to quantitate ET-1, ET-2, or ET-3 generation. For the determination of K_m values, human bigET-1 was used at concentrations of 50, 100, 330, 500, 667 nM, 1, 2

and 5 µM at pH 6.8 for 30 and 60 min to keep substrate conversion below 10%.

2.5. Immunological characterization of recombinant ECE-1a and sECE*

Antiserum 473-17-A was raised in rabbits against a synthetic peptide corresponding to amino acid sequence 473–489 of the extracellular part of human ECE-1a. The antisera obtained were screened by RIA using the ^{125}I-labelled corresponding peptide. Preimmune serum of each rabbit was used for negative control. Using immunoblot analysis the crossreactivity of antiserum 473-17-A to purified NEP (a generous gift of Dr. G. Boileau) was examined, since comparison of the corresponding cDNA sequences showed high level of homology. The antiserum did not recognize human NEP up to a concentration of 2 µg of pure protein when analyzed by immunoblot (data not shown).

To further characterize this antiserum, immunoprecipitation was carried out after metabolic labelling of CHO/ECE-1a, CHO/sECE* and untransfected CHO cells. Cells grown to confluence were starved of methionine and cysteine for 60 min and pulse-labelled for 30 min with 50 µCi of [^{35}S]Pro-mix (Amersham, UK) in methionine and cysteine-free Ham's F-12 medium. After a 3 h chase in complete medium, cells were solubilized by overnight incubation in buffer B (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1%Triton X-100, 1 mM EDTA). The samples were centrifuged $12000 \times g$ for 10 min and the resulting supernatant was incubated with 5 µl of polyclonal antiserum 473-17-A and 60 µl of protein A/Sepharose (100 mg/ml, Pharmacia LKB) overnight at 4°C. The beads were washed once with buffer B, once with 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% Triton X-100, 1 mM EDTA, and once in 20 mM Tris-HCl pH 6.8. The antigen eluted in boiling loading buffer was resolved by 7.5% SDS/PAGE, and the gel was dried and subjected to autoradiography.

Secreted and membrane-bound ECE were analyzed by SDS-PAGE followed by Western blot. Solubilized membrane proteins (ECE-1a) or concentrated medium (sECE*) were resolved on a 7.5% polyacrylamide gel [27] and transferred to a polyvinylidene fluoride microporous membrane (Immobilon P, Millipore). Immunoelectrophoretic blot analysis was carried out as described [28]. The primary antiserum was used in 1:300 dilution. In some experiments sECE* (25–30 µg) and ECE-1a (10–15 µg) were deglycosylated using *N*-Glycosidase F deglycosilation kit (Boehringer Mannheim), essentially as recommended by the manufacturer and subjected to Western analysis.

2.6. Gel filtration

2 ml Ultra-CHO medium containing sECE* was dialyzed against 3 l of 50 mM Tris-HCl (pH 7.0) containing 100 mM NaCl and 1 µM $ZnCl_2$. A 0.5 ml aliquot was applied to a Superose 12 (HR 10/30) column equilibrated with the same buffer used for dialysis. The column was eluted with the same buffer at a flow rate of 0.4 ml/min, and 0.2 ml fractions were collected. All fractions were concentrated to 50 µl using speed-vac centrifugation. The ECE activity was estimated after in vitro incubation of 10 µl of each fraction with 0.1 µM big-ET-1 for 60 min as described above. Subsequently the ET-1 generation was measured by EIA.

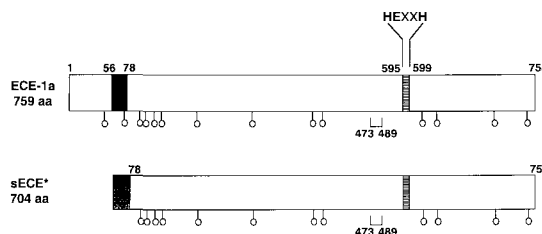

Fig. 1. Schematic representation of the soluble form of ECE (sECE*) compared to ECE-1a. Black block: membrane-spanning domain, dashed block: consensus zinc binding site, O: cysteine residue, grey block: POMC signal peptide (24 amino acids). Numbers correspond to amino acid residues of human ECE-1a. The polyclonal antibody used in this study was made against a peptide corresponding to the sequence 473–489 of human ECE-1a.

3. Results

3.1. Construction and expression of sECE* in CHO cells

In order to obtain a secreted ECE, a truncated form of ECE was constructed by deleting the coding region of the cytoplasmic and membrane-spanning domains followed by fusing in frame the sequence of the cleavable signal peptide from human POMC to the coding region of the complete extracellular domain (amino acids 78–758) of ECE-1a (Fig. 1). Preliminary studies indicated that CHO cells showed no endogenous bigET-1 conversion activity and were therefore a suitable expression system. Stable cell lines expressing wild-type ECE-1a and sECE* were established. The recombinant clones expressing the highest ECE activity were chosen for further studies. For the detection of ECE protein an antiserum was raised against a synthetic peptide corresponding to the amino acids 473–489 of ECE-1a. Specificity of the antiserum was tested by immunoprecipitation using the CHO/ECE-1a cells or the untransfected cells, as control. As shown in Fig. 2, in CHO/ECE-1a cell lysates, the antiserum immunoprecipitated an antigen which migrated, in non-reducing conditions, as a major protein of an apparent molecular mass of 250–300 kDa, and a minor protein of 120 kDa. In presence of β-mercaptoethanol, only a broad and thick band around 120 kDa was observed. This pattern of bands is consistent with the described homodimeric membrane-bound ECE-1a. No immunoprecipitated radiolabelled proteins were detected in non-transfected CHO cells (Fig. 2).

In CHO/sECE* conditioned media, the antiserum 473-17-A recognized a broad band of approximately 115 kDa indicating that these cells released soluble ECE (Fig. 3). No immunoreactive proteins were present in CHO/ECE-1a and CHO conditioned media (Fig. 3). Solubilized membrane proteins with reducing agent, revealed a broad band of 120 kDa in CHO/ECE-1a cell membranes, but none in non-transfected CHO control cells (Fig. 3). Interestingly, in CHO/sECE* membranes the 120 kDa band was absent, instead, a discrete band of approximately 90 kDa was present. Pulse-chase experiments in CHO/sECE* cells followed by immunoprecipitation confirmed the time-dependent secretion of sECE* (data not shown).

3.2. Characterization of enzymatic activity of sECE*

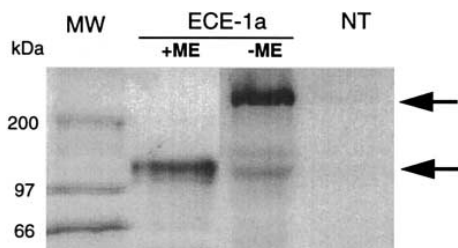In preliminary experiments two different culture media were

Fig. 3. Efficient secretion of sECE* in transfected CHO cells. The concentrated conditioned medium (25 μg protein) and solubilized membranes (15 μg protein) from non-transfected (NT), CHO/ECE-1 and CHO/sECE* cells were analysed by Western blot using the ECE polyclonal antiserum 473-17A. The amount of medium from CHO/sECE* and membranes of CHO/ECE-1a loaded onto the gel had the same enzymatic activity (270 pmol/ml/min). Numbers on the right show the position of molecular weight markers.

Fig. 2. Characterization of the polyclonal antiserum 473-17-A. Non-transfected (NT) and CHO/ECE-1a cells were pulse-labelled with 50 μCi of [35S]methionine and cysteine for 30 min and chased for 3 h. Cells were solubilized and immunoprecipitated with the polyclonal antiserum 473-17A as described in Section 2. Immunoprecipitated samples were analysed by SDS-PAGE after boiling in the absence (−ME) or presence (+ME) of β-mercaptoethanol. The gel was exposed for autoradiography. Arrows indicate the dimeric and the monomeric forms of the enzyme. The left lane contained 14C-labelled markers.
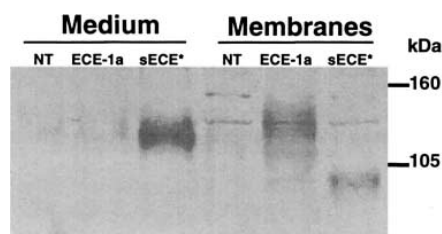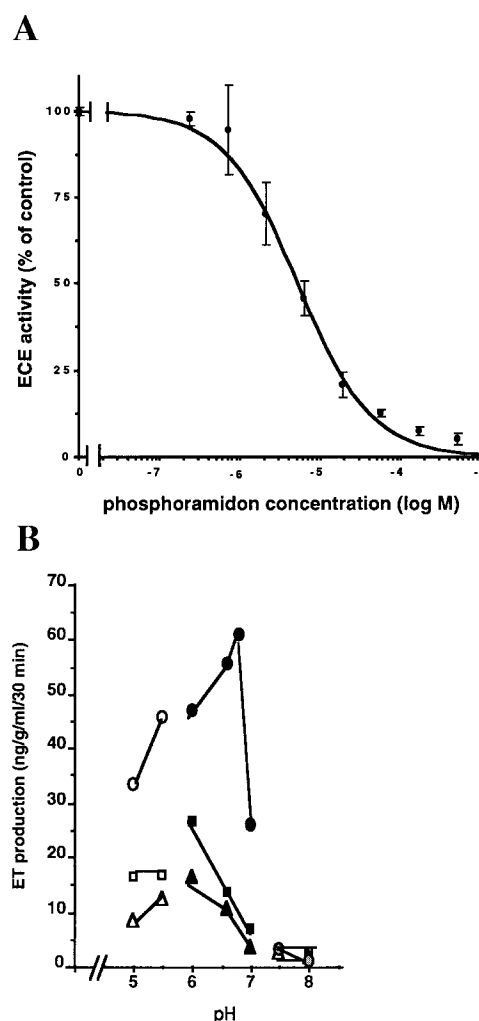

Fig. 4. In vitro characterization of sECE* from CHO/sECE* conditioned medium. A: Concentration-dependent inhibition of sECE* activity by phosphoramidon. 50 μl of CHO/sECE* conditioned medium were used. B: pH profile of sECE* activity using 10 μl of 10× concentrated conditioned medium on isopeptide substrates. BigET-1 (○, ●, grey circle), bigET-2 (□, ■, grey box) or bigET-3 (△, ▲, grey triangle) at 100 nM were used as substrates and generation of corresponding mature peptides was measured by EIA. The pH was determined by using 50 mM buffers MES (open symbols), HEPES (black symbols) and Tris HCl (grey symbols). Specificity of the reaction was tested by preincubation with 100 μM phosphoramidon (not shown).

tested for sECE* production from CHO/sECE* cells. The bigET-1 conversion activity was slightly higher with Ultra-CHO compared to serum-free DMEM medium indicating a larger amount of sECE* secretion or a better stability. Indeed, full stability of sECE* in Ultra-CHO medium lasted at least three months, therefore Ultra-CHO medium was used in all subsequent experiments. Cell culture medium recovered from confluent CHO/sECE* cells efficiently converted bigET-1 into ET-1 whereas medium recovered from non-transfected CHO or CHO/ECE-1a cells showed no detectable cleavage of big-ET-1 (data not shown). Conversely, membrane fraction of CHO/ECE-1a cells exhibited ECE activity whereas membrane fractions from CHO/sECE* or non-transfected CHO cells did not convert bigET-1 into ET-1 (data not shown). The soluble ECE activity was inhibited by phosphoramidon in a dose-dependent manner with an apparent IC_{50} value of 6 μM (Fig. 4A). The enzyme was not inhibited in vitro by captopril, thiorphan, pepstatin A, leupeptin, PMSF, E-64 or chymostatin, however the chelating agents EDTA and 1,10-phenanthroline completely inhibited ECE activity secreted from CHO/sECE* cells (Table 1). Recombinant sECE* converted bigET-1 much more efficiently than bigET-2 and bigET-3 (Fig. 4B). Soluble ECE activity was detectable in a relative broad range of pH 5.0–7.0 with different maximums for the three bigETs (Fig. 4B). BigET-1 was most efficiently cleaved at pH 6.6–6.8, whereas conversion of bigET-2 and bigET-3 showed a maximum at pH 6.0. The bigET-converting activity was completely blocked by 100 μM phosphoramidon at each pH tested indicating that any non-specific degradation occurred at low pH values.

The enzymatic properties of sECE* were very similar to ECE-1a. Further kinetic analyses revealed that K_m for bigET1 of the recombinant soluble enzyme was 1.98 $μM \pm 0.87$ (mean $\pm$ S.E.M., $n=4$), very similar to the membrane anchored enzyme (8.03 $μM \pm 2.05$ ($n=2$) determined in the same experiments). Moreover, it is interesting to notice that when the same activity was loaded into a gel, an immunoreactive band with comparable intensity was revealed (Figs. 3 and 6).

3.3. Estimation of molecular mass of sECE*

Size-exclusion chromatography was performed to estimate the apparent molecular mass of native soluble ECE. Using a FPLC superose 12 column, sECE* activity eluted as a single peak, corresponding to a molecular mass of 110–140 kDa
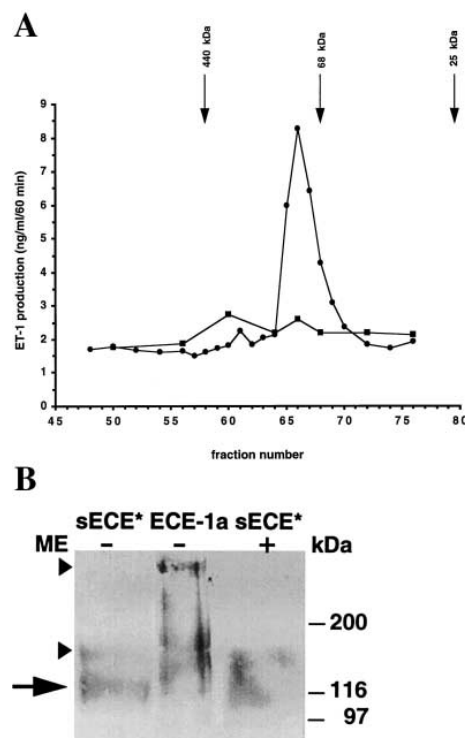

Fig. 5. Determination of molecular mass of native sECE*. A: FPLC analysis of sECE*. 0.5 ml of CHO/sECE* medium was applied to a Superose 12 HR10/30 column. 0.2 ml fractions were collected and analyzed. ECE activity was estimated after incubation of 1/5 of each fraction with 0.1 μM bigET-1 for 60 min in absence (●) or presence of 100 μM phosphoramidon (■). Arrows indicate the elution positions of ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), bovine serum albumin (67 kDa), and chymotrypsine (25 kDa). B: Western blot of 10 μl of 30× concentrated CHO/sECE* medium and 15 μg of CHO/ECE-1a membranes. Samples boiled in SDS gel sample buffer in the presence (+) and absence (−) of β-mercaptoethanol (ME) were subjected to SDS-PAGE and analyzed by immunoblotting using antiserum 473-17A. Arrow indicates the monomeric form of sECE*, arrow heads the monomeric and dimeric forms of full length ECE-1a for comparison. Lines on the right show the position of molecular weight markers.

(Fig. 5A). sECE* activity was totally inhibited by preincubating the fractions in the presence of 100 μM phosphoramidon confirming the specificity of the assay (Fig. 5A).

In addition, in contrast to ECE-1a, sECE* appeared as a

Table 1
Inhibition profile of sECE* activity

Compound	Concentration (μM)	ECE activity (% of control)
EDTA	1	47
	10	8
1,10-Phenanthroline	10	10
	100	0
Thiorphan	10	96
	50	79
Captopril	10	99
	50	90
PMSF	100	103
E-64	50	101
Chymostatin	100	106
Pepstatin A	20	111
Leupeptin	20	93

The indicated amount of inhibitor were preincubated for 15 min at 37°C in 100 μl of ECE assay buffer with 5 μl of 10× concentrated medium from CHO/sECE* before the addition of 0.1 μM 5 mM EDTA. 2 μl of reaction mixture was tested for ET-1 concentration in EIA. Control activity (100%) was determined in the absence of inhibitors. Data are the means of two independent experiments.
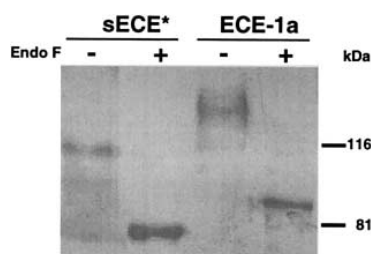
Fig. 6. Deglycosylation of sECE* and ECE-1a. Recombinant sECE* and ECE-1a enzymes were mock treated (−) or treated with 2 units of endoglycosidase F (+), under reducing conditions and analysed by Western blot. The amount of medium from CHO/sECE* cells and membranes of CHO/ECE-1a cells loaded onto the gel had the same enzymatic activity (430 pmol/ml/min). Lines on the right show the position of molecular weight markers.

broad band with an apparent molecular mass of 115 kDa regardless of the presence of β-mercaptoethanol (Fig. 5B). Deglycosylation of ECE-1a and sECE* with endoglycosidase F resulted in both cases in single sharp bands of 80 and 70 kDa respectively (Fig. 6).

4. Discussion

The two human ECE-1 isoforms, which differ only by their N-terminal intracellular regions, are encoded by a single gene [18]. The two isoforms seem to have the same specific activity of cleavage of bigET-1 to generate ET-1 [16]. Until now, no biological soluble form of ECE has been characterized, although Takada et al. [29] reported the presence of a phosphoramidon-sensitive ECE in the cytosolic fraction of bovine endothelial cells. Therefore, it was interesting to create a soluble form of ECE-1 to investigate its biochemical characteristics. Like NEP, ECE-1 is a class II integral membrane protein with a short N-terminal cytoplasmic tail, a transmembrane domain that represents the uncleaved signal peptide, and a large putative extracellular domain.

The aim of this work was to determine if a recombinant soluble form of ECE-1 containing only the extracellular domain, retains enzymatic activity. To construct a soluble form of ECE-1, the same strategy as Lemay et al. [25] for solubilizing NEP was used by substituting the cytoplasmic and membrane-spanning domains of ECE for the signal peptide of POMC, a secretory protein. It was expected that the in frame fusion of the POMC signal peptide would direct the routing of ECE devoid of its membrane domain through the secretory apparatus and allow its secretion in the extracellular medium.

The polyclonal antibody raised against a peptide corresponding to amino acids 473–489 of the extracellular part of ECE recognizes specifically both forms (the membrane-bound and soluble) of ECE-1 without background staining of untransfected cells. By comparison to the CHO/ECE-1a cell membranes, CHO/sECE* cell membranes had no immunoreactive 120 kDa band, indicating that ECE devoid of its membrane domain is efficiently secreted into the culture medium.

The catalytic and biochemical properties of the soluble enzyme recovered in the culture medium of CHO/sECE* cells were determined using an in vitro enzymatic assay. The observed high stability of sECE* in Ultra-CHO culture medium demonstrated that the CHO/sECE* cell line was a useful tool to produce large amounts of sECE* without the need to pu-

rify it for studying its enzymatic properties. We show that, in our expression system, sECE* containing medium and CHO/ECE-1a membrane fractions exhibit the same enzymatic properties. sECE* activity was inhibited dose dependently by phosphoramidon but not by thiorphan, a specific NEP inhibitor [14] nor by captopril, a specific ACE inhibitor [30]. Moreover, sECE* was not blocked by inhibitors of serine, aspartic or cysteine peptidases but completely inhibited by the chelating agents EDTA and 1,10-phenanthroline, showing that the soluble ECE keeps all the characteristics of zinc metalloproteases. These results demonstrate that the soluble form of ECE-1 exhibits the same properties versus the inhibitors as the membrane form, ECE-1a. Therefore, we conclude that neither the intracellular nor the transmembrane domain play a role in inhibitor binding.

sECE* also kept the properties of substrate specificity as it cleaved preferentially bigET-1 versus bigET-2 and bigET-3. The similar K_m for bigET-1 and the comparable amount of protein revealed on Western blot for the same activity demonstrates that the specific activity of both, soluble and full length enzymes, appear to be identical. Also, pH activity range differed not much between the two enzymes. sECE* was active in a broad range of pH showing still 50% of activity at pH 5.0 and a pH optimum between 6.6 and 6.8 for cleaving bigET-1. For the first time, the pH optimum for bigET-2 and bigET-3 conversion was examined which was 6.0 for both substrates. However, at each pH tested, sECE* cleaved preferentially bigET-1. These results therefore demonstrate that anchoring of the ectodomain into the plasma membrane is neither important for the catalytic activity of ECE nor for its substrate recognition.

The purified rat lung ECE-1 and the recombinant ECE-1a expressed in Cos cells are both composed of two disulfide-linked subunits [31]. Recent analysis using site-directed mutagenesis revealed that Cys416 in human ECE-1 is responsible for dimer formation, and that this dimerisation favours conversion of bigET-1 into ET-1 [23,24]. In the present study, in contrast to the membrane-bound ECE-1a, soluble ECE appeared as a monomer on gel filtration and Western blot. Nevertheless, sECE* kinetic parameters did not differ significantly from the wild-type enzyme indicating that dimerization does not appear to be essential nor preferential for full ECE activity.

Faster electrophoretic mobility of recombinant sECE*, indicating an apparent molecular mass lower than ECE-1a is in accordance with the theoretical size of the truncated protein (78 amino acids less) which seems to be indeed cleaved at the site of the signal peptide. The difference was still visible after deglycosylation, suggesting that both enzymes are quantitatively similarly glycosylated, even though slightly altered glycosylation pattern cannot be ruled out with the present data. The apparent molecular mass after deglycosylation for both enzymes is consistent with the expected size according to the sequence. The maintenance of the same glycosylation level of the soluble ECE compared to the membrane-bound enzyme may be important for activity since it has been demonstrated for NEP, that transport to the cell surface and enzymatic activity are both dependent on sugar residues of NEP probably through conformational constraints [32].

Purification of large amounts of membrane-bound ECE has been particularly difficult due to the detergent requirement in all separation steps, as well as the risk of contamination of the

preparation with unspecific ET degrading activities and the impaired stability of enzyme activity. This recombinant soluble ECE will be easily purified from the incubation medium of cultured cells without the use of detergent. This enzyme kept the same enzymatic properties as the membrane-bound ECE-1. A well-characterized soluble form of endothelin-converting enzyme is of interest for further structural studies by site-directed mutagenesis, development and easy screening of inhibitors and eventually crystallization.

References

[1] Kennedy, R., Haynes, W. and Webb, D. (1993) Clin. Endocrinol. 39, 259–265.

[2] Masaki, T. (1995) Annu. Rev. Pharmacol. Toxicol. 35, 235–255.

[3] Gershon, M.D. (1995) Curr. Biol. 5, 601–604.

[4] Kurihara, Y., Kurihara, H., Oda, H., Maemura, K., Nagai, R., Ishikawa, T. and Yazaki, Y. (1995) J. Clin. Invest. 96, 293–300.

[5] Inoue, A., Yanagisawa, M., Takuwa, Y., Mitsui, Y., Kobayashi, M. and Masaki, T. (1989) J. Biol. Chem. 264, 14954–14959.

[6] Seidah, N., Day, R., Marcinkiewicz, M. and Chretien, M. (1993) Ann. New York Acad. Sci. 680, 135–146.

[7] Opgenorth, T., Wu-Wong, J. and Shiosaki, K. (1992) FASEB J. 6, 2653–2659.

[8] Kashiwabara, T., Inagaki, Y., Ohta, H., Iwamatsu, A., Nomizu, M. and Nishikori, K. (1989) FEBS Lett. 247, 73–76.

[9] Kimura, S., Kaysuya, Y., Sawamura, T., Shinmi, O., Sugita, Y., Yanagisawa, M., Goto, K. and Masaki, T. (1989) J. Cardiovasc. Pharmacol. 13, S5–S7.

[10] Ikegawa, R., Matsumura, Y., Tsukahara, Y., Takaoka, M. and Morimoto, S. (1990) Biochem. Biophys. Res. Commun. 171, 669–675.

[11] McMahon, E., Palomo, M., Moore, W., McDonald, J. and Stern, M. (1991) Proc. Natl. Acad. Sci. USA 88, 703–707.

[12] Malfroy, B., Kuang, W., Seedburg, P., Mason, A. and Schofield, P. (1988) FEBS Lett. 229, 206–210.

[13] Lee, S., Zambas, E., Marsh, W. and Redman, C. (1991) Proc. Natl. Acad. Sci. USA 88, 6353–6357.

[14] Roques, B., Noble, F., Dauge, V., Fournie-Zaluski, M. and Beaumont, A. (1993) Pharmacol. Rev. 45, 87–146.

[15] Schmidt, M., Kroger, B., Jacob, E., Seulberger, H., Subkowski, T., Ottep, R., Meyer, T., Schmalzing, G. and Hillen, H. (1994) FEBS Lett. 356, 238–243.

[16] Shimada, K., Takahashi, M., Ikeda, M. and Tanzawa, K. (1995) FEBS Lett. 371, 140–144.

[17] Yorimitsu, K., Moroi, K., Inagaki, N., Saito, T., Masuda, Y., Masaki, T., Seino, S. and Kimura, S. (1995) Biochem. Biophys. Res. Commun. 208, 721–727.

[18] Valdenaire, O., Rohrbacher, E. and Mattei, M.G. (1995) J. Biol. Chem. 270, 29794–29798.

[19] Turner, A.J. and Murphy, L.J. (1995) Biochem. Pharmacol. 5, 91–102.

[20] Shimada, K., Takahashi, M. and Tanzawa, K. (1994) J. Biol. Chem. 269, 18275–18278.

[21] Xu, D., Emoto, N., Giad, A., Slaughter, C., Kaw, S., deWit, D. and Yanagisawa, M. (1994) Cell 78, 473–485.

[22] Parnot, C., LeMoullec, J.-M., Cousin, M.-A., Guedin, D., Corvol, P. and Pinet, F. (1997) Hypertension 30, 837–844.

[23] Shimada, K., Takahashi, M., Turner, A.J. and Tanzawa, K. (1996) Biochem. J. 3154, 863–867.

[24] Hoang, V.M., Sansom, C.E. and Turner, A.J. (1996) Biochem. Soc. Trans. 24, 471S.

[25] Lemay, G., Waksman, G., Roques, B.P., Crine, P. and Boileau, G. (1989) J. Biol. Chem. 264, 15620–15623.

[26] Graham, F. and VanDerEb, A. (1975) Virology 52, 456–476.

[27] Laemmli, U.K. (1970) Nature 227, 680–685.

[28] Wei, L., Alhenc-Gelas, F., Soubrier, F., Michaud, A., Corvol, P. and Caluser, E. (1991) J. Biol. Chem. 266, 5540–5546.

[29] Takada, J., Okada, K., Ikenaga, T., Matsuyama, K. and Yano, M. (1991) Biochem. Biophys. Res. Commun. 176, 860–865.

[30] Cushman, D.W., Cheung, H.S., Sabo, E.F. and Ondetti, M.A. (1977) Biochemistry 16, 5484–5491.

[31] Takahashi, M., Fukuda, K., Shimada, K., Barnes, K., Turner, A.J., Ikeda, M., Koike, H., Yamamoto, Y. and Tanzawa, K. (1995) Biochem. J. 311, 657–665.

[32] Lafrance, M.-H., Vezina, C., Wang, Q., Boileau, G., Crine, P. and Lemay, G. (1994) Biochem. J. 302, 451–454.