Endothelin-converting enzyme-1 (ECE-1) cleaves big-endothelins, as well as bradykinin and beta-amylloid peptide. Several isoforms of ECE-1 (a-d) have been identified to date, they differ only in their N-terminus but share the catalytic domain located in the C-terminal end. Using quantitative PCR, we found ECE-1d to be the most abundant type in several endothelial cells (EC) types. In addition to full-length ECE-1 forms we have identified novel, alternatively spliced mRNAs of ECE-1 b-d. These splice variants (SVs) lack exon 3', which codes for the transmembrane (TM) region and is present in full-length forms. SVs mRNA were highly expressed in EC derived from macro and microvascular beds but much less so in other, non-endothelial cells expressing ECE-1, which suggests that the splicing mechanism is cell-specific. Analyses of ECE-1d and its SV form in stably transfected HEK293 cells revealed that both proteins were recognized by anti C-terminal ECE-1 antibodies, but anti N-terminal antibodies only bound ECE-1d. The novel protein, designated ECE-1sv, has an apparent MW of 75 kDa; by using site directed mutagenesis its start site was identified in a region common to all ECE-1 forms suggesting that ECE-1 b-d SV mRNAs are translated into the same protein. In agreement with the findings demonstrating common C-terminus for ECE-1sv and ECE-1d, both exhibited a similar catalytic activity. However, immunofluorescence staining and differential centrifugation revealed a distinct intracellular localization for these two proteins. Presence of ECE-1sv in different cellular compartments than full-length forms of the enzyme may suggest a distinct physiological role for these proteins.

Endothelin-converting enzyme-1 (ECE-1) is a type II membrane protease that belongs to the nephrilysin (NEP) family of zinc metallopeptidases (1,2). ECE-1 is abundantly expressed in the vascular endothelial cells (EC) of all tissues but is also found in nonvascular cells (3-6). This enzyme is characterized by a single transmembrane region, a short N-terminal cytosolic tail and a large C-terminal extracellular domain that contains the enzymatic active site (7). ECE-1 is a glycosylated protein with 10 putative N-linked glycosylation sites (8). The best characterized substrates are the ETs family consisting of three isopeptides, termed ET-1, ET-2 and ET-3, which are derived from distinct genes (9,10). ET-1, the most abundant of the three, is a pleiotropic peptide; although best known for its vasoconstricting activity it has diverse biological functions. These include roles in processes such as embryonic development, cardiovascular homeostasis, vascular permeability and angiogenesis (11-13). The three ETs mediate their various effects via two G protein-coupled receptors: ETA and ETB (14,15). ETs are synthesized from ~ 200 aa precursor -prepro ET (ppET). After removal of their signal peptide ETs are processed by dibasic pair-specific enzymatic activity to form the respective inactive big-ETs (38-41 residues long; 1,4). ECE-1 then specifically hydrolyzes the Trp21-Val/Ile22 bonds of big-ETs to produce biologically active ETs (1,16). ECE-1 null mice exhibit a phenotype similar to that of ET-1 or ETA deficient mice thus demonstrating the physiological relevance of ECE-1 in generating bioavailable ET-1 (17).

Four isoforms of human ECE-1 (1a, 1b, 1c, and 1d) have been identified to date (8,18-20). The four proteins are encoded by one gene but each is expressed from a distinct promoter that regulates the expression of the four unique amino termini (8,18-20). Although the ectodomain containing the active site is identical in each of the isoforms, the amino-terminal sequences appear to be responsible for
MATERIALS AND METHODS

**Materials**

Dulbecco’s Minimum Essential Medium (DMEM) low glucose, DMEM with Ham’s F12 1:1 (v/v) nutrient mixture, SuperScriptII RNase H– reverse transcriptase, calf serum (CS) and Ultra pure electrophoresis agarose gel were obtained from Gibco BRL Life Technologies (Gaithersburg, MD, USA). Vitrogen, type I collagen from Cohesion Technologies (Palo Alto, CA, USA). penicillin, streptomycin, and fetal calf serum (FCS) were from Biological Industries (Beit Haemek, Israel). TRI Reagent from MRC (Cincinnati, OH). Deoxynucleotide triphosphates, random hexamer oligodeoxynucleotides, and Taq DNA polymerase were from Fermentas (Vilnius, Lithuania). Oligo-dT and oligonucleotide primers were synthesized by MWG Biotech AG (Ebersberg, Germany). The real-time PCR SYBR Green master-mix kit was from Eurogentec (Seraing, Belgium). Protease inhibitor cocktail for mammalian cell extracts and horseradish peroxidase-conjugated goat anti-rabbit IgG were from Sigma (St Louis, MO, USA). Protein quantification kit was from Bio-Rad Laboratories (Hercules, CA, USA). Hifidelity Taq polymerase was from Takara (Otsu, Shiga, Japan). Restriction enzymes from Fermentas (Hanover MD, USA). DpnI was from New England Biolabs (Beverly, MA, USA). FuGENE 6 Transfection Reagent from Roche (Indianapolis, IN). pGEM-T vector, pcDNA6/V5-His version C and Blasticidin are from Invitrogen (Carlsbad, CA, USA). N-octyl glucoside and phosphoramidon were from Sigma Aldrich (St Louis MO, USA). BK-2 was synthesized by Sigma-Genosys (The Woodlands, TX).

**Cell cultures**

Bovine aortic EC (BAEC) were kindly provided by I. Vlodavsky of the Hadassah-Hebrew University Hospital, Jerusalem, Israel, and the cells were grown in complete DMEM containing 10% calf serum and 2 mM glutamine. Microvascular EC derived from the bovine corpus luteum (29-31), termed luteal EC were grown in complete DMEM Ham’s F12 containing 10% FCS and 2 mM glutamine on plates pre-coated with 2% Vitrogen. Experiments were carried out on cells from passages 5-12, with 70-80% confluence. Human embryonic kidney cell cultures (HEK-293) and Chinese hamster ovary cell cultures (CHO) were cultured in complete DMEM Ham’s F12 containing 10% FCS and 2 mM glutamine.

**Enrichment of luteal steroidogenic and endothelial cells**

For enrichment of luteal cell subpopulations, mid cycle corpora lutea were dispersed by using collagenase IV as previously described (32). Briefly, corpora lutea were sliced and dispersed in M-199 containing 0.5% BSA and collagenase (420 U/ml). Dispersed luteal cells were mixed with epoxy magnetic beads pre-coated with Bandeiraea simplicifolia lectin-1 (BS-1), a lectin specific for bovine EC. Both BS-1 positive cells (EC) and non-adherent cells (enriched steroidogenic cells) were collected and further processed for RNA extraction.

**Production of bECE-1 constructs**

The cDNA sequences of full length bovine ECE-1d and ECE-1d splice variant (SV) were amplified with 1d and ECE-1–end as primers (Table 1). The amplification products were separated on agarose gels and the corresponding single bands were extracted and cloned onto pGEM-TEasy vector. Inserts were subsequently subcloned into pcDNA vector (pcDNA6/V5) and sequenced. ECE-1d and SV plasmids were mutated (mut) at the putative start site of the latter (ATG located between bases 207-209) as follows: 26 bp complementary sense and antisense oligos, containing the desired mutation (ATG to TTT), were used in a PCR reaction with the original SV plasmid as a template. Template plasmid was then digested with DpnI. A shorter SVcut construct lacking the first 169 bp of SV was generated by digesting SV with Eco91I (BstEII). HEK-293 cells were transfected by FuGENE 6 Transfection Reagent. Stably transfected cells lines (containing bECE-1d, SV and

differences in subcellular localization (19,21-23). ECE-1 isoforms were mainly studied in cell lines overexpressing each isoform separately. This may explain why it is still unclear how abundant each of the ECE-1 isoforms is in naturally expressing cells.

Several studies have shown that ECE-1 efficiently hydrolyzes a number of peptide hormones other than ETs, these include bradykinin, substance P, and neurotensin (24). An exciting novel splice variant for ECE-1 is the β-amyloid peptide that is implicated in the pathogenesis of Alzheimer’s disease (25,26).

Inhibitors of ECE-1 are considered to be valuable therapeutic agents and were developed for the treatment of various disorders linked to elevated ET-1 levels (27,28). Numerous peptides or non-peptidyl ECE-1 inhibitors have already been produced, but, contrary to initial expectations none is currently used for therapeutic purposes, perhaps because of insufficient knowledge of the ECE-1 family of proteins in naturally expressing cells.

In this paper we report the prevalence of ECE-1 isoforms a-d in EC and the initial characterization of a novel splice variant of ECE-1 that lacks the transmembrane domain.
SVcut) were established using Blasticidin (1ug/ml) as a selective antibiotic.

**Cell fractionation and Western blot analysis**

The procedure for total cell extracts was carried out as we have previously described (32,33). Briefly, cells were homogenized in lysis buffer (25 mM Tris HCl, 100 mM NaCl, 0.5% deoxycholate, 0.5% NP40, 5mM EDTA, at pH 7.5 and 10% protease inhibitor cocktail). Cell extracts were sonicated on ice for 10 sec at low speed. For sub-cellular localization of ECE-1 forms, HEK-293 cells were homogenized in lysis buffer without detergents and then centrifuged for 15 min at 15000xg. The resulting pellet (containing particulate fraction) was dissolved in lysis buffer containing 0.5% deoxycholate and 0.5% NP40. The supernatant was centrifuged at 44000xg for 1hr. The supernatant obtained after ultracentrifugation was defined as cytosolic fraction. The cytosol and cell particulate fractions were separated by 7.5% SDS/PAGE, under reducing conditions. Protein concentrations were determined by using Bio-Rad DC reagents. All steps were performed on ice and samples were kept at -80C until use. Proteins were electrically transferred to nitrocellulose membranes. After 2 h blocking in TBST + 5% low fat milk, membranes were incubated with the appropriate ECE-1 antibodies. Anti total ECE-1 antiserum (anti C-terminal antibody; 4788) was raised against a synthetic peptide comprising the last 16 amino acids of ECE-1 and was affinity purified by means of the immunizing peptide immobilized on a Sepharose 4B column. Two antibodies raised against N-terminal sequences of ECE-1 were also used: ds-90 which recognizes the cytosolic sequence of ECE-1d and 1207 which recognizes ECE-1bcd (34). The membranes were washed three times and then incubated with HRP-conjugated goat anti rabbit IgG for 1h at room temperature. A chemiluminescent signal was generated with SuperSignal and the membranes were exposed to X-ray film.

**Cell free transcription / translation system – (TNT)**

The T7 transcription / translation system with S\textsuperscript{35} methionine was used to probe translated products of the various plasmids (bECE-1d, bECE-1d-mut SV, SVmut and SVCut). Briefly, 1mg plasmid DNA was incubated with TNT master mix (Promega) and S\textsuperscript{35} methionine, 90 min at 30°C. The resulting proteins were then run on an SDS/PAGE gel under reducing conditions and the gel was dried (Bio-Rad gel dryer) and exposed to X-rays film.

**Immunofluorescence**

CHO or HEK-293 cells were seeded on 14-mm coverslips and transfected with plasmids coding for either ECE-1d or SV. They were cultivated for 48 h before fixation with cold methanol for 5 min. Nonspecific binding was saturated with 10% normal goat serum in PBS. Cells were then incubated with the primary antibodies directed against the C-terminus of ECE-1 in 1% normal goat serum in PBS. Secondary goat antibodies directed against rabbit IgG were coupled to AlexaFluor-555 (Molecular Probes). Nuclei were labeled using To-Pro-3 (Molecular Probes). Coverslips were mounted with Mowiol and observed with a TCS SP2 confocal microscope (Leica Microsystems).

**Biological activity**

ECE-1 activity was measured using BK2 peptide (aminomethylcoumarin-RPPGFSAFR-dinitrophenyl) as a substrate. The proteolysis of this quenched peptide at the Ala7-Phe8 bond by ECE-1 has already been characterized (23, 35). ECE-1 activity of HEK–293 cells stably expressing ECE-1d, SV, and SV cut was assayed as described by Luciani et al (36). Non-transfected cells served as a negative control and BAEC as a positive control (endogenous activity). Cells were grown to 80% confluence, washed twice with PBS and harvested. The cells were pelleted at 300xg, re-suspended in 200 ul ice-cold 50 mM Tris/maleate, pH 6.8 containing 1% (w/v) N-octyl glucoside (as permeabilization agent) and protease inhibitor cocktail and sonicated. Following 1 h of incubation on ice, the extracts were centrifuged (15000xg, 15min, 4C) and the protein content of the supernatants was measured with Bio-Rad DC reagents. ECE-1 activity was assayed in white 96-well microplates in a final volume of 100 ul. Substrate (BK-2, final concentration 30 uM) and 20 ug of cellular protein extracts were incubated in Tris-maleate with or without phosphoramidon (100 uM). Fluorescence was measured ($\lambda_{ex} = 330$ nm; $\lambda_{em} = 420$ nm) in a multiwell plate reader fluorimeter (Varian/Cary Eclipse™ Fluorimeter, Melbourne Australia). Blanks consisting of all reagents except either cell extract or substrate gave only negligible conversion. Fluorescence was measured at several time points for each cell type until complete hydrolysis was achieved. An incubation time of 90 min was chosen as it gave the maximal activity. Relative BK-2 breakdown was calculated by subtracting the values obtained in the presence of phosphoramidon from the total fluorescence at the corresponding time point.

**RNA Extraction and RT-PCR**

Total RNA was extracted from the cells using Tri-reagent. One microgram of total RNA was reverse transcribed and semi-quantitative PCR was performed as described previously (37). The sequence of the primers used in PCR reactions is shown in Table 1.
Real time PCR: the PCR reaction was performed as we previously described (33), using the SYBR green I PCR kit as recommended by the manufacturer with ROX passive reference. The fold change of target gene normalized to an endogenous reference, G3PDH and was calculated by the following equation = 2 ^ - ΔCt where ΔCt = [(Ct target - Ct G3PDH)]. To compare the amplification efficiency of the primers for different ECE-1 isoforms, plasmids containing inserts of each ECE isoform: ECE-1a, ECE-1b, ECE-1c, and ECE-1d were generated by PCR with the corresponding 5' primers and ECE-1 common reverse primer (Table 1). These constructs were electroporated into CHO cells as follows: 10x10^6 cells were electroporated by using a Gene-Pulser (370V, capacitance 960 µf) with 10 µg plasmid DNA. Cells were then transferred into complete DMEM/F12 medium and cultured for 16 and 48 hours. RNA was extracted from the cells and cDNA was analyzed by real time PCR with two sets of primers. The first primer set included isoform specific primers (Table 1), the second set comprised primers amplifying the common sequence of ECE-1 and was used to normalize for the total amount of ECE-1 expressed in the transfected cells. The ratios of the expression of ECE-1a, b, c, and d to that of total ECE-1 was 2.95, 0.72, 1.24, and 0.47, respectively. The results obtained were corrected for primer efficiency. Statistical analysis
The differences between groups were analyzed by one-way ANOVA, employing the post hoc multiple comparisons Dunnett’s test. Analysis was performed using SPSS (SPSS Inc., Chicago, IL, USA, v. 10.05 for Windows). Differences were considered significant if P < 0.05.

RESULTS

Identification and occurrence of the spliced ECE-1 forms of mRNA

PCR of cDNA derived from several EC types: BAEC and luteal EC with a 5’ primer specific for each ECE-1 isoform and a common reverse primer located ~500bp downstream (Table 1) generated two products for isoforms b, c and d (Fig. 2). The upper bands were of the expected size and the lower ones appeared approximately 140 bp shorter. The existence of these two PCR products was also observed when other 3’ primers of ECE-1 were used (data not shown). The upper and lower bands were excised, cloned and sequenced. The upper bands were the expected PCR products based on the known sequences of bECE-1 b-d (38) and the lower bands were splice variants (SVs) lacking the same 142 bp sequence, corresponding to exon 3’ found in all known ECE-1 isoforms (18; Fig. 3A). This region in isoform ECE-1d is highlighted (in Fig. 3B). No such splice variant (lacking exon 3’) was observed for ECE-1a isoform whose transcription begins further downstream, in exon 3. Similar shorter spliced variants were also detected in human umbilical vein EC (data not shown).

We next sought to determine the total ECE-1 expression and the ratio between the expression of splice variant (SV) mRNAs (of ECE-1b, c, d) and the full-length forms of ECE-1. For that we designed primers which spanned a unique sequence of SV forms (produced by the end of exon 2’ merged with exon 4 ;Fig. 3A). This primer (with a reverse primer spanning the region between 333-352 in the SV sequence of ECE-1d; Table 1) should amplify all three SV forms. Full-length ECE-1 forms were amplified with a primer that resides in the sequence of exon 3’ which is absent from SV forms (Table 1). Figure 4 depicts the ratio of SV to full length ECE-1 in endothelial (BAEC and luteal EC) and non-endothelial (luteal steroidogenic) cell types. In all EC types the expression of SV forms was high and ranged between 21 to 37% of that of the full-length forms of the enzyme. Interestingly, regardless of total ECE-1 levels, in luteal EC- whether cultured or freshly isolated - the proportion of SV was higher than in BAEC or luteal steroidogenic cells. For instance, in luteal steroidogenic cells expressing similar total ECE-1 levels as freshly isolated luteal EC, the SV forms were only 6% of full length mRNA levels as compared to 22.5 % in luteal EC (Fig. 4). Characterization of translated forms of ECE-1

As ECE-1d was found to be the most abundant form (Fig. 1) we cloned and stably expressed its...
full-length and SV forms. An additional cDNA, in which part of the 5' end of SV was deleted (SVcut) was stably expressed in HEK-293 cells as well. An antibody that recognizes the common C-terminal end of the enzyme identified a protein product in all cells expressing ECE-1d, SV and SVcut (Fig. 5A). Cells transfected with full-length ECE-1d expressed as expected a protein of approximately 120 kDa, whereas SV and SVcut transfected cells both expressed a protein of approximately 75 kDa (Fig. 5A). Two different antisera directed against N-terminal parts of the molecule (one specific for ECE-1d; Fig. 5a and the second for forms b, c and d; Fig. 5B&C) were then examined in western blot analysis. These two antibodies were readily bound to full-length ECE-1d, but did not recognize the protein products of either SV or SVcut. These findings therefore suggest that SV forms share only the C-terminal end with ECE-1d. As N-terminal antibodies did not recognize the translated products of SV and SVcut (Fig. 5) it appeared that these forms have a different start codon from that used in ECE-1d. This was in fact expected since splicing out of 142 bp segment would modify the reading frame. Because cDNAs of both SV and the shorter form -SVcut cDNA were translated into proteins with the same apparent MW, it suggested that another start codon was located further downstream. ATG located between 207-209 bp in the cDNA sequence of the SV form of ECE-1d could drive translation in-frame with that of conventional ECE-1. Additionally, this ATG is a good candidate to be the translation initiator since it lies within a kozak sequence. To test this assumption, an SV construct mutated at ATG207 was produced. As shown in Fig. 6 mutating the ATG to TTT eliminated the SV protein which suggests that ATG207 is indeed the putative start codon for the SV form. This was confirmed by western blot analysis of cells expressing SV plasmid as well as in a cell-free translation system (TNT; Fig. 6). Mutating the same ATG in the ECE-1d sequence did not affect its translation (Fig. 6a).

Since the catalytic domain of the enzyme is found in its far C-terminal end, the translation product of SV and SVcut, designated from here on – ECE-1sv is expected to retain its bioactivity. Therefore we next examined the catalytic activity of extracts of HEK-293 that stably expressed ECE-1d, SV and SVcut by using the quenched BK2 peptide (aminomethylcoumarin-RPPGFSAFR-dinitrophenyl) as a substrate (Fig. 7). While non-transfected cells had only marginal activity, SV- and SVcut- overexpressing cells exhibited enzymatic activity that was similar to that exhibited by full-length ECE-1d (Fig. 7). This enzymatic activity was inhibited by phosphoramidon, the common NEP/ECE-1 inhibitor. The catalytic activity of ECE-1sv was comparable on a total protein basis with that exhibited by cells expressing ECE-1d. These data further support those shown in Fig 5b, suggesting that full-length ECE-1 and ECE-1sv share their C-terminal parts.

**Localization of ECE-1sv and ECE-1d in overexpressing cells**

The coding sequences of ECE-1d and its splice variant were transiently transfected in HEK 293 and CHO cells and their intracellular localization was probed by indirect immunofluorescence using anti C-terminal antibody and western blots pf extracts after differential centrifugation (Fig. 8 A, B). As expected, using immunofluorescence, ECE-1d was present in plasma membrane and vesicles (23; Fig. 8A) and in the particulate cell fraction (15,000xg pellet; Fig. 8B). ECE-1sv, on the other hand exhibited a diffused cytosolic labeling by immunofluorescence (Fig. 8A), in agreement, western blot data (Fig. 8 B) demonstrated that most of ECE-1sv forms were detected in the 44,000xg supernatant fraction, cytosol. The sequence of these two proteins predicts their different sub-cellular localization; while ECE-1d, as other full-length forms of ECE-1, contain TM domain and is therefore a membrane-anchored protein, ECE-1sv lacks TM sequence (or signal peptide) and therefore is expected to remain cytosolic.

**Native expression of ECE-1sv protein in normal cells**

Having demonstrated that EC abundantly expressed SV mRNA we next examined whether ECE-1sv, as characterized in cells overexpressing SV cDNA, was endogeneously present in normal cells. Data presented in Fig. 9 show a western blot obtained by using an anti ECE-1 antibody. A 75 KDa protein was identified in BAEC, luteal EC and also in corpus luteum, a highly vascular tissue. As expected these samples expressed full-length ECE-1 proteins which appeared as two bands (Fig. 9), that most probably corresponded to different glycosylation forms of the enzyme.

**DISCUSSION**

This study documented the prevalence of the various ECE-1 isoforms, and found that ECE-1d was the most abundant and ECE-1a the least expressed. The mRNA of isoforms ECE-1b and c were also rather scarce, with levels 6 -10 folds lower than that of ECE-1d. However, these were not the only ECE-1
forms found in EC, we have also identified novel splice variants of ECE-1b, c and d mRNA which lacked exon 3' that codes for the TM domain of the enzyme. These mRNA species are abundantly expressed in EC, in microvascular EC they comprise up to 40% of full length ECE-1 mRNA. We used plasmid containing the coding sequence of the SV form of ECE-1d overexpressed in HEK cells and in a cell free translation system to demonstrate that it is translated into a protein of apparent MW of 75 kDa. This protein was recognized by anti ECE-1 antibody raised against the C-terminal end of the enzyme. The start site of this protein was further downstream in a region common to all ECE-1 forms, which suggests that the three mRNA species were translated into one protein -ECE-1sv. Since the catalytic domain of the enzyme is found in its far C-terminal end, the translation product is expected to retain its bioactivity. Indeed, the ECE-1sv protein was biologically active, cleaved a synthetic ECE-1 substrate as efficiently as the full-length enzyme. Lastly, we have identified a protein with an apparent MW as ECE-1sv in normal EC and within a highly vascular endocrine gland such as the corpus luteum.

The hitherto known isoforms of ECE-1 arise from the use of alternative promoters upstream of exon 3' (18,19). ECE-1sv is the first identified ECE-1 isoform that arises from an internal exon splicing. Alternative splicing is widespread in mammalian gene expression and is a major contributor to the functional complexity of mammalian genomes (39,40). Variant splice patterns are often characteristic of specific stages of development, particular tissues or a disease state (41). Whether the ratio of ECE-1 to its splice variant form also differs between different physiological or pathological conditions is as yet unknown. However it is noteworthy that ECE-1sv was highly expressed in EC derived from macro and microvascular beds but much less so in steroidogenic cells that also express ECE-1, and this could imply that there is a cell-specific splicing mechanism. Since ECE-1sv is not routed to the secretory pathway it is expected to remain non-glycosylated as indeed occurred (TNT compared with westerns), but nonetheless the protein retained its catalytic activity. Therefore these findings suggest that glycosylation may not be necessary for the enzyme to be bioactive. In agreement with this conclusion, deglycosylation of purified ECE-1 did not significantly alter its enzyme activity (42). Mutating two of ECE-1 glycosylation sites is however enough to render the enzyme inactive (43), which is expected considering the importance of glycosylation for proper protein folding in the secretory pathway (for review see (44). Our data thus suggest that molecular chaperones can efficiently compensate for the lack of glycosylation, and promote proper folding of ECE-1sv in the cytosol.

Catalytic activity was demonstrated here by using BK-2 as a substrate but its physiological substrates still remain to be determined. The catalytic site of full-length ECE-1 faces the extracellular milieu or the luminal side of vesicles, whereas the active site of ECE-1sv would resides within the cells cytoplasm. What are the putative substrates of this unique isoform? It is tempting to speculate that cytosolic ECE-1sv may degrade small peptides such as angiotensin I, bradykinin, neurotensin, and substance P, internalized via their receptors or it could also cleave β-amyloid peptide. All of these peptides (24,25) were formerly shown to be very efficiently degraded by soluble ECE-1 engineered by truncating its TM domain. Breaking down these small peptides by ECE-1sv could act to terminate their signaling in a manner analogous to the action of insulin degrading enzyme (IDE). IDE is a neutral thiol metalloprotease, ubiquitously expressed, which is present mainly in cytosol and peroxisomes. The active site of IDE, HEXXXH is very similar to that of ECE-1 – HELTH, which could explain the overlapping specificity of these enzymes – i.e. cleavage of insulin and amyloid peptides.

Interestingly, the presence of a soluble intracellular form of ECE-1 in endothelial and vascular smooth muscle cells was postulated in the past (38,45) however no molecular identification was provided before.

The mechanism responsible for ECE-1sv translation most likely involves re-initiation of translation. This was shown to occur with mRNAs that have small ORFs near the 5' end (46). These short ORFs are initiated at the respective start codons of isoforms ECE-1b,c and d and terminated by a stop codon produced by splicing out exon 3' (~50 codons). It is also noteworthy that the start codon that initiates the translation of ECE-1sv - ccATGg - has a strong Kozak's motif (46).

The present findings concerning the prevalence of ECE-1 isoforms in EC may shed new light on these isoforms within their physiological context. Using quantitative real time PCR we demonstrated here that ECE-1d is the dominant isoform. This was true for all EC types examined regardless of their origin. Currently, there is ambiguity regarding the abundance of ECE-1 isoforms in EC. Both ECE-1a and 1c were claimed to be the major isoforms (8,47-50) and although these were based on either observations made prior to the discovery of ECE-1d sequence (19) or on the use methods that are quantitatively less reliable, it persisted in the literature.

The physiological significance of the different
ECE-1 isoforms emanates from their distinct cellular and subcellular distribution. For example ECE-1a is only expressed in vascular EC and not in other cell types expressing the enzyme such as smooth muscle cells or steroidogenic luteal cells (32,50). Examination of the subcellular distribution of ECE-1 isoforms overexpressed in AtT-20 neuroendocrine cells (23), showed that ECE-1a and 1c were present at the plasma membrane whereas ECE-1b and ECE-1d were retained inside the cells. However in stably transfected CHO-K1 cells, ECE-1d was present at the plasma membrane yet at a lower level than ECE1a (19). Whether ECE-1d subcellular localization is cell dependent or not, being the dominant ECE-1 isoform in ECs, it seems imperative to gain a better understanding of its transcriptional regulation, sorting and routing in naturally expressing EC.

The findings presented here which demonstrate the plethora of different ECE-1 forms, full-length and spliced variants, that co-exist within EC offer a novel perspective on the physiological activation of ET-1 and of other substrates of ECE-1 family of proteins.

**FOOTNOTES**

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**REFERENCES**

FIGURE LEGENDS

**Fig. 1.** Abundance of mRNA expression of each ECE-1 isoform (ECE-1a, -1b, -1c and -1d) in different endothelial cell types. Total RNA was extracted from BAEC, freshly isolated and cultured luteal EC; (LEC) and reverse transcribed. Following real-time PCR, relative mean mRNA levels for each cell type were calculated using the ΔCt method (see Methods). Data are the means ± SEM from four different experiments. Different letters indicate significant differences among isoforms within each cell type (*P* < 0.05).

**Fig. 2.** Amplification of full length ECE-1 isoforms and alternatively spliced forms in BAEC, freshly isolated and cultured luteal EC (LEC). Total RNA was extracted from the various endothelial cell types and reverse transcribed. The PCR reaction was conducted with a 5’ primer specific for each ECE-1 isoform and a common reverse primer (see Table 1). Inverse images of ethidium bromide stained agarose gel show amplification of the various products.

**Fig. 3.** A schematic representation of ECE-1 gene structure and its mRNA. A - ECE-1 gene structure showing the first alternative (1c, 1b, 2, 3) exons and their promoters (p). Exons 4–19, common to all isoforms and encoding the major part of ECE-1 cDNA, are not represented at the same scale. Exons are numbered according to Valdenaire et al., 1999 (20). Exons of the four different full length ECE-1 mRNAs together with those of the spliced variants mRNAs are depicted below. B- The sequence of the first ~300 nucleotides of ECE-1d showing the spliced region (gray) corresponding to exon 3’. The putative ATG start codon of the spliced variant is underlined.

**Fig. 4.** The mRNA expression of total ECE-1 levels and the ratio (%) of SV to full length ECE-1 isoforms in BAEC, luteal EC (LEC; freshly isolated and cultured) and in luteal steroidogenic cells (LSC). Data are the means ± SEM from six different separate experiments. Following real-time PCR, relative mean mRNA levels for each cell type were calculated using the ΔCt method (see Methods). Different letters indicate significant differences among cell types (*P* < 0.05).

**Fig. 5.** Detection of ECE-1 proteins in HEK-293 cells stably expressing bECE-1d, SV and SVcut plasmids. Total proteins were extracted in lysis buffer and processed as detailed in Methods. Twenty
five mg of each cell extracts were separated by SDS-PAGE under reducing conditions. Proteins were detected by Western blots using C-terminal specific antibody (A) and two specific N-terminal antibodies (B and C). NT- non transfected cells.

Fig. 6. Mutation of the putative bECE-1sv start codon. The following plasmids: bECE-1d, bECE-1d mut, SV, SVmut and SVcut were examined, either directly in a cell-free transcription / translation system – (TNT) reaction (A) or after transfection into HEK-293 cells (B). Protein extracts of HEK-293 cells were detected by Western Blot with an anti C-terminal ECE-1 antibody. NT- non transfected cells.

Fig. 7. Biological activity of proteins extracted from HEK-293 cells overexpressing ECE-1d, SV and SVcut plasmids and BAEC. Total protein extracts (20 ug) were incubated in Tris/maleate buffer containing the substrate (BK-2, 30 uM) with or without phosphoramidon (100 uM). Fluorescence was measured (lex = 330 nm; lem = 420 nm) in a multiwell plate reader fluorimeter. Data (means ± SEM ;n=3) are the percentages of specific BK-2 breakdown. For each cell type (HEK-293 cells overexpressing ECE-1d, SV and SVcut plasmids and BAEC) fluorescence was measured at several time points and data presented are from the time point that gave maximal specific fluorescence. NT- non transfected cells. Different letters indicate significant differences among samples (P < 0.05).

Fig. 8. Subcellular localization of ECE-1d and SV. A. immmunoflourescence The subcellular distribution of ECE-1d (a and c) and of SV (b and d) was investigated in transiently transfected CHO (a and b) and HEK-293 (c and d) cells using the antibodies directed the C-terminus of ECE-1. Immunoreactivity was detected with an antibody coupled to AlexaFluor-555 (red). Nuclei were stained with To-Pro-3 (blue). ECE-1d is present in intracellular vesicles concentrated in the perinuclear region. SV is detected as a diffuse staining of the cytosol. (bar = 10 mm). B. Western blot analysis. Upper panel- cytosolic fraction (supernatant of 44000xg); lower panel – particulate fraction (15000xg pellet). For details refer to M&M. Protein were detected by western blot analysis using an antibody recognizing the common C-terminal end of ECE-1.

Fig. 9. Native expression of ECE-1sv protein in corpus luteum (CL) tissue, cultured LEC and BAEC. Total proteins were extracted in lysis buffer and detected by western blot analysis with an anti C-
terminal antibody. Doubled headed arrows mark full-length ECE-1 forms; Double-headed arrows indicate putative ECE-1sv.
Table 1. PCR Primer list

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Sequence</th>
<th>Product Length</th>
</tr>
</thead>
<tbody>
<tr>
<td>G3PDH</td>
<td>Forward Reverse</td>
<td>5’ – GGCGTGAACCACGAGAAGTAT- 3’ 5’ – CGTGGACAGTGGTCAAAAGT – 3’</td>
<td>141</td>
</tr>
<tr>
<td>ECE1-total</td>
<td>Forward Reverse</td>
<td>5’ – TGTGGCGGCTGGATCAAAGC – 3’ 5’ – AGGTGCTTGATGATGGATG – 3’</td>
<td>103</td>
</tr>
<tr>
<td>ECE-1a</td>
<td>Forward Reverse</td>
<td>5’ - GTTCTCTCCTGGATTAG – 3’ 5’ – CTTGTCTCTGTATTTGGATGC – 3’</td>
<td>178</td>
</tr>
<tr>
<td>ECE-1b</td>
<td>Forward</td>
<td>5’ – TGTCGGGCGCTGGGGATGTC– 3’</td>
<td>113</td>
</tr>
<tr>
<td>ECE-1c</td>
<td>Forward</td>
<td>5’ - CGGAGC CGCGAGCGAGCGAT – 3’</td>
<td>109</td>
</tr>
<tr>
<td>ECE-1d</td>
<td>Forward</td>
<td>5’ – CCATGGAGGCCTAAGAGAGT – 3’</td>
<td>142</td>
</tr>
<tr>
<td>ECE1-b,c,d</td>
<td>Reverse</td>
<td>5’– GAAGTTCACCTGCAAGTGGT – 3’</td>
<td></td>
</tr>
<tr>
<td>ECE-sv</td>
<td>Forward Reverse</td>
<td>5’ – TACCCCAACCCACCTGCAGGAACG-3’ 5’ – AGGTGCTTGATGATGGCTTGG – 3’</td>
<td>226</td>
</tr>
<tr>
<td>ECE-1</td>
<td>Forward Reverse</td>
<td>5’- GCAGGTGAACCTCCAGAGG – 3’ 5’ - CTTGTCTCTGTATTTGGATGC – 3’</td>
<td>150</td>
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<tr>
<td>Full length</td>
<td>Reverse</td>
<td>5’- GAAGGGGGAGGTTGAGTAGT – 3’</td>
<td>1a 571 1b 503 1c 507 1d 533</td>
</tr>
<tr>
<td>ECE-1 common</td>
<td>Reverse</td>
<td>5’ - CCGTCACCAGACTTCCACACT - 3’</td>
<td>1d - 2312 SV - 2170</td>
</tr>
</tbody>
</table>
Fig. 1

mRNA expression of ECE-1 isoforms (arbitrary Units)

LEC

BAEC

Freshly dispersed EC

ECE-1a
ECE-1b
ECE-1c
ECE-1d
Fig. 2
Fig. 3
Fig. 4

Relative mRNA expression of total ECE-1 (arbitrary units)

% SV mRNA of Full-length ECE-1

LEC  BAEC  Freshly dispersed LEC  LSC
Fig. 5
A. TNT

Fig. 6

B. Western Blot
Specific cleavage of BK-2
(% over cell treated with enzyme inhibitor)

Fig. 7
Fig. 8
Fig. 9
Supplemental figure

Low SV expression

High SV expression
Endothelin converting enzyme-1: Abundance of isoforms a-d and identification of a novel alternatively spliced variant lacking a transmembrane domain
Rina Meidan, Eyal Klipper, Tamar Gilboa, Laurent Muller and Nitzan Levy

J. Biol. Chem. published online September 26, 2005

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