Palmitoylation of the three isoforms of human endothelin-converting enzyme-1

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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. Here we have examined whether the three isoforms of human ECE-1 (ECE-1a, ECE-1b and ECE-1c) are modified by the covalent attachment of the fatty acid palmitate and have evaluated a potential functional role of this modification. To do this, wild-type and mutant enzymes were expressed and analysed by metabolic labelling with [³H]palmitate, immunoprecipitation and SDS/PAGE. All three ECE-1 isoforms were found to be palmitoylated via hydroxylamine-sensitive thioester bonds. In addition, the isoforms showed similar levels of acylation. Cys⁴⁶ in ECE-1a, Cys⁵⁸ in ECE-1b and Cys⁴² in ECE-1c were identified as sites of palmitoyl-

ation and each of these cysteines accounted for all the palmitoylation that occurred in the corresponding isoform. Immunofluorescence analysis demonstrated further that palmitoylated and non-palmitoylated ECE-1 isoforms had the same subcellular localizations. Moreover, complete solubility of the three isoforms in Triton X-100 revealed that palmitoylation does not target ECE-1 to cholesterol and sphingolipid-rich membrane domains or caveolae. The enzymic activities of ECE-1a, ECE-1b and ECE-1c were also not significantly affected by the absence of palmitoylation.

Key words: metalloprotease, post-translational modification.

INTRODUCTION

The family of endothelins consists of the three related vasoactive peptides, ET-1, ET-2 and ET-3 [1,2], and is thought to play an important role in cardiovascular pathophysiology. The conversion of the inactive precursor intermediates, big endothelins, to the biologically active peptides is catalysed by a neutral zinc metalloprotease, called endothelin-converting enzyme (ECE)-1 [3,4]. A second endothelin-converting enzyme (ECE-2) can also convert big endothelins into endothelins, but only under acidic conditions (pH 5.5) [5]. A third enzyme, named ECE-3, which selectively cleaves big ET-3, was recently purified from bovine iris [6].

ECE-1 is a type-II integral membrane protein with a single transmembrane domain, a short N-terminal cytosolic tail and a large extracellular domain that contains the HEXXH zinc-binding motif (HEALTH) [3,4]. The enzyme is a highly glycosylated protein with 10 putative N-linked glycosylation sites and exists as a disulphide-linked dimer of 250 kDa [7–9]. It has high structural and sequence homology with NEP (neutral endopeptidase or enkephalinase), Kell blood-group antigen (KELL), PEX and ECE-2 (for a review see [10]). Three distinct isoforms of human ECE-1 have recently been identified and termed ECE-1a, ECE-1b and ECE-1c [11]. They are derived from a single gene through the use of alternative promoters. The three isoforms have cytoplasmic tails of 56 (ECE-1a), 68 (ECE-1b) and 52 (ECE-1c) amino acids, respectively. These cytoplasmic tails differ only in their N-terminal regions, whereas the 22 cytoplasmic residues adjacent to the transmembrane domain as well as the transmembrane and extracytoplasmic domains are common to all three isoforms. Whereas the three isoforms have similar enzymic properties, they show distinct subcellular localizations when

expressed in Chinese hamster ovary (CHO) or Madin–Darby canine kidney (MDCK) cells ([11,12] and A. Schweizer and J. Rohrer, unpublished work). ECE-1a and ECE-1c are localized at the plasma membrane whereas ECE-1b was found to be predominantly intracellular. Two ECE-1 isoforms, ECE-1 α and ECE-1 β , have so far been identified in rat [13]. They correspond to human ECE-1c and ECE-1a, respectively, and show a predominantly plasma-membrane localization [14].

The common part of the cytoplasmic tails of ECE-1a, ECE-1b and ECE-1c includes a conserved cysteine residue located 10 amino acids from the transmembrane domain (Figure 1). Since cysteine residues close to the membrane bilayer have often been found to undergo palmitoylation via a thioester linkage [15], we were interested to determine whether ECE-1 is modified by this fatty acid and to investigate a possible functional role. A wide variety of viral and cellular proteins are covalently modified by the post-translational addition of the 16-carbon saturated fatty acid palmitate. Palmitoylation occurs in membrane proteins with hydrophobic transmembrane segments as well as in hydrophilic proteins. Examples include the glycoproteins of vesicular stomatitis and Sindbis virus [16,17], the nerve-terminal proteins growth-associated protein-43 (GAP-43), synaptosomal-associated protein of 25 kDa (SNAP-25) and SCG10 [18–20], G-protein α subunits (for reviews see [21,22]) and the human transferrin receptor [23,24], as well as members of the G-protein-coupled-receptor family, such as the α_{2A} -adrenergic receptor [25] and the β_2 -adrenergic receptor [26]. In particular, the two receptors for the endothelin peptides, endothelin receptor A (ET_A) and endothelin receptor B (ET_B), have also been shown to be modified by palmitate [27,28]. For some of the palmitoylated proteins the covalent attachment of fatty acid was shown to be of significant functional importance. Palmitoylation has been

Abbreviations used: ECE, endothelin-converting enzyme; NEP, neutral endopeptidase; KELL, Kell blood-group antigen; CHO, Chinese hamster ovary; MDCK, Madin–Darby canine kidney; FCS, fetal calf serum; mAb, monoclonal antibody.

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Figure 1 Schematic illustration of the cytoplasmic tails of the three human ECE-1 isoforms

Selected amino acids (aa) of the ECE-1a, ECE-1b and ECE-1c cytoplasmic tails are shown; the common cysteine residues are highlighted; the half boxes represent the termini of the single transmembrane domain of each isoform of ECE-1. The cytosolic tails of the three isoforms differ only in their N-terminal sequences; the 22 cytosolic amino acids adjacent to the transmembrane domains as well as the transmembrane and luminal domains are identical in all three isoforms.

implicated in membrane association [20–22,29–32], can affect intracellular trafficking [33–37] and has been shown to be involved in several aspects of cellular signalling, including G-protein coupling [26–28,38].

In the present study, we demonstrate that the three human ECE-1 isoforms are palmitoylated via hydroxylamine-sensitive thioester bonds. Cys⁴⁶ in ECE-1a, Cys⁵⁸ in ECE-1b and Cys⁴² in ECE-1c were identified as the only sites of acylation. In addition, the extent of the palmitoylation was shown to be comparable in all isoforms. Modification with palmitate is not required for the enzymic activity or subcellular localization of the ECE-1 isoforms.

EXPERIMENTAL PROCEDURES

Materials

Enzymes used in molecular cloning were obtained from Boehringer Mannheim (Mannheim, Germany); Lipofectamine and Lipofectamine Plus were from Gibco-BRL (Gaithersburg, MD, U.S.A.); Redivue Pro-mix [³⁵S] cell-labelling mix was from Amersham Life Science (Cleveland, OH, U.S.A.); [³H]palmitate was from NEN Life Science Products (Boston, MA, U.S.A.); Protein A-Sepharose beads were from Repligen Corp. (Cambridge, MA, U.S.A.); rabbit anti-mouse antibody was from Zymed Laboratories (San Francisco, CA, U.S.A.); and protease inhibitors and hydroxylamine were from Sigma (St. Louis, MO, U.S.A.). Oligonucleotides were synthesized by Microsynth (Balgach, Switzerland).

Recombinant DNA procedures

The three ECE-1 isoform cDNA species that contained the Kozak sequence GCCGCCACC upstream of their corresponding initiator, ATG [11], were inserted into the eukaryotic expression vectors pcDNA3 (InVitrogen, Leek, The Netherlands) and pSFFVneo [36].

All mutant forms of ECE-1 were created by standard PCR protocols using the overlap-extension technique. For the construction of the mutants ECE-1a C46A, ECE-1b C58A and ECE-1c C42A, the corresponding ECE-1 isoform cDNAs in pcDNA3 were used as templates together with bp 310–330 of the ECE-1c coding sequence as upstream primer and a downstream primer consisting of an *Nhe*I restriction site, an *Eco*RI restriction site and the Kozak sequence GCCGCCACC followed by the first 21 bp of the corresponding isoform coding sequence. Appropriate pairs of complementary oligonucleotides, which encoded the desired mutation, were chosen as internal primers. The final PCR

products were digested with *Nhe*I and *Hind*III, and the purified fragments were subcloned into the plasmid pcDNA3.1(+)-ECElum. This plasmid was generated by subcloning an ECE-1 cDNA fragment corresponding to the ECE-1 coding sequence (common to all three isoforms) from downstream of the unique *Hind*III site (bp 257 in ECE-1c) to the first 96 bp of the 3' non-coding region, into the *Hind*III and *Eco*RV sites of pcDNA3.1(+). All mutants were verified by sequencing the final plasmid.

Cell culture and transfection

An MDCK cell line was maintained in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal calf serum (FCS), 100 units/ml penicillin and 100 µg/ml streptomycin at 37 °C in a 5% humidified CO₂ atmosphere. The cells were transfected with 20 µg of *Xba*I-linearized DNA using lipofectamine (Gibco-BRL) according to the manufacturer's directions. Selection was performed with neomycin (G418) and resistant colonies were screened for expression by immunoblotting and immunofluorescence microscopy. CHO cells were cultured in minimal essential medium-α supplemented with 5% heat-inactivated FCS, 100 units/ml penicillin and 100 µg/ml streptomycin. Transfections were performed in 100-mm plates with 8 µg of DNA using Lipofectamine Plus (Gibco-BRL) according to the manufacturer's directions.

Antibodies

For the detection of ECE-1, monoclonal antibody (mAb) ECE-6, which recognizes all three isoforms, was used. Affinity-purified rabbit polyclonal antisera against caveolin-1 was generously provided by Dr. K. Fiedler (Biozentrum, Basel, Switzerland).

Metabolic labelling with [³⁵S]methionine/cysteine and [³H]palmitate

Cells were grown in 100-mm dishes. For labelling with [³H]palmitate the cells were washed twice with PBS and labelled in growth medium containing 5% FCS, 20 mM Hepes (pH 7.4) and 600 µCi of [³H]palmitate for 90 min at 37 °C. For labelling with [³⁵S]methionine/cysteine, the cells were rinsed twice with PBS, preincubated in 5 ml of methionine- and cysteine-free growth medium, containing 10% dialysed FCS and 20 mM Hepes (pH 7.4) for 20 min, and then pulsed for 6 h with 100 µCi of Redivue Pro-mix [³⁵S] cell-labelling mix in 5 ml of pre-incubation medium.

Immunoprecipitation, SDS/PAGE and fluorography

Proteins were immunoprecipitated from Triton X-100-solubilized cells as described in [36]. The immunocomplexes were released from the beads by boiling for 3 min in electrophoresis sample buffer containing 62.5 mM Tris/HCl (pH 6.8), 2% SDS, 10% glycerol and 0.001% Bromophenol Blue. Immunoprecipitated proteins were separated on SDS/polyacrylamide minigels (6% gel; Bio-Rad, Hercules, CA, U.S.A.) using the Laemmli system and visualized by fluorography using salicylate and Kodak X-Omat AR films.

Hydroxylamine treatment

ECE-1 protein labelled with [35 S]methionine/cysteine or [3 H]palmitate was electrophoresed in duplicate lanes on SDS/polyacrylamide gels (6% gel). The gels were fixed with 25% methanol/10% acetic acid for 90 min, and subsequently rinsed in water three times for 10 min to remove the acetic acid. One gel was treated overnight with 1 M Tris (pH 7.0) as a control, while the other gel was soaked for the same time in 1 M hydroxylamine (pH 7.0). The gels were then rinsed in water and prepared for fluorography.

Immunofluorescence microscopy

Transfected cells were plated in eight-well multichamber glass slides. The immunofluorescence procedure was that of Schweizer et al. [11]. In brief, formaldehyde-fixed and saponin-permeabilized cells were incubated with mAb ECE-6 followed by FITC-conjugated goat anti-mouse antibody (Cappel, Durham, NC, U.S.A.). To probe for surface expression of proteins, cells were cooled to 4 °C for 20 min and kept on ice for the subsequent steps. The cells were washed once with PBS/0.2% BSA and incubated with mAb ECE-6 for 45 min. After six wash steps with PBS/0.2% (w/v) BSA, the cells were fixed with 3% (w/v) para-formaldehyde; first for 30 min on ice and subsequently for 30 min at room temperature followed by staining with FITC-conjugated goat anti-mouse antibody (Cappel). An inverted Leica microscope equipped with the TCS confocal system and an Ar/Kr laser was used to obtain images.

Measurement of ECE-1 activity

The activity of wild-type and mutant ECE-1 isoforms was measured on membrane preparations of transfected CHO cells as described in Schweizer et al. [11]. Briefly, for membrane preparations, cell homogenates were centrifuged twice at 20000 *g* for 20 min each and resuspended in buffer A [10 mM Tris/HCl (pH 7.5)/100 μ M PMSF/40 μ M leupeptin/40 μ M trans-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E-64)]. Cell-membrane preparations were incubated in buffer B [50 mM Tris/HCl (pH 7.0)/0.1% BSA/0.1% Na $_2$ S $_2$ O $_3$] with 0.3 μ M big ET-1 for 30 min at 37 °C. The enzymic reaction was stopped by the addition of 150 mM EDTA (pH 7.0). The production of ET-1 was measured with a specific radioimmunoassay as described previously [39]. The values obtained for the enzymic activities were then corrected for the differences in expression levels.

The ECE-1 expression was determined by immunoblotting as follows. Equal amounts of cell-membrane preparations were heated to 50 °C for 30 min in reducing sample buffer and subjected to SDS/PAGE. Proteins were transferred on to Immobilon-P membranes (Millipore, Bedford, MA, U.S.A.) with 25 mM Tris/192 mM glycine/15% (v/v) methanol as a buffer system. The membranes were blocked for 2 h with Tris-buffered saline/0.05% (v/v) Tween 20/20% FCS [where

Tris-buffered saline is 137 mM NaCl/3 mM KCl/25 mM Tris (pH 8.0)]. The blots were incubated subsequently for 3 h with mAb ECE-6 in Tris-buffered saline/0.05% (v/v) Tween 20/5% sheep serum followed by a horseradish peroxidase-conjugated anti-mouse secondary antibody. ECE-1 was visualized using the enhanced chemiluminescence (ECL) system (Amersham) according to the manufacturer's directions. The autoradiographs were quantified using a personal densitometer (Molecular Dynamics, Sunnyvale, CA, U.S.A.). The levels of ECE-1 expression among individual transfections differed at the most by 4-fold and the differences were not specific for a particular isoform species or for palmitoylated versus non-palmitoylated isoforms.

To measure the enzyme reaction constants K_m and V_{max} , cell-membrane preparations were incubated in the presence of 0.075–1.5 μ M big ET-1. K_m and V_{max} values were calculated as described previously [11] except that V_{max} values are given per mg of total membrane protein and were corrected for differences in expression levels as described above.

Protein was determined by the method of Lowry with BSA as a standard protein.

Triton X-100 solubility

After two washes with PBS, transfected MDCK cells grown in 100-mm plates were scraped in PBS and centrifuged at 4 °C. Cells were homogenized in 1.5 ml of TNET buffer [50 mM Tris/HCl (pH 8.0)/150 mM NaCl/2 mM EDTA/1% Triton X-100] containing a 1:500 dilution of a protease-inhibitor cocktail (5 mg/ml benzamide/1 mg/ml each of pepstatin A, leupeptin, antipain and chymostatin). Following a 30-min solubilization step on ice, the cells were centrifuged for 30 min at 175000 *g*_{av} in a Ti100.3 rotor (Beckman Instruments, Fullerton, CA, U.S.A.). The resulting supernatants (Triton X-100-soluble fractions) were harvested carefully and proteins were precipitated by the method of Wessel and Flügge [40]. The resulting precipitates (Triton X-100-soluble fractions) and the pellets in the Ti100.3 tubes following centrifugation (Triton X-100-insoluble fractions) were resuspended in PBS containing the above-described protease-inhibitor cocktail. Equal volumes of both fractions were heated in reducing sample buffer to either 50 °C for 30 min (for analysis of ECE-1 isoforms) or to 95 °C for 3 min (for analysis of caveolin-1). Proteins were separated on SDS/polyacrylamide gels [6% (ECE-1) or 13% (caveolin-1) gels] using the Laemmli system and transferred on to nitrocellulose membranes. The immunoreaction for the detection of ECE-1 was as described above. For the detection of caveolin-1, the nitrocellulose sheet was blocked with 3% non-fat dry milk powder in PBS, incubated with an affinity-purified rabbit polyclonal antibody against caveolin-1 (diluted 1:500 in PBS/3% powdered milk) followed by a horseradish peroxidase-conjugated anti-rabbit secondary antibody (Amersham). For development, the ECL detection system (Amersham) was used according to the manufacturer's directions. The blotting of ECE-1 isoforms and caveolin-1 within the same samples allowed a direct comparison of their distributions.

RESULTS

Palmitoylation of ECE-1 isoforms

To determine whether ECE-1 is post-translationally modified by the covalent attachment of the 16-carbon fatty acid palmitate, MDCK cell lines expressing the three human ECE-1 isoforms, ECE-1a, ECE-1b and ECE-1c (Figure 1), were labelled with either [3 H]palmitate or [35 S]methionine/cysteine. The expressed isoforms were then immunoprecipitated and subjected to SDS/

Figure 2 Palmitoylation of ECE-1a, ECE-1b and ECE-1c

MDCK control cells (lanes 1, 5, 9 and 13) and MDCK cells stably expressing ECE-1a (lanes 2, 6, 10 and 14), ECE-1b (lanes 3, 7, 11 and 15) or ECE-1c (lanes 4, 8, 12 and 16) were labelled with [³H]palmitate (**A**) or [³⁵S]methionine/cysteine (**B**). Duplicate lanes were separated on SDS/polyacrylamide gels (6% gels). One gel was soaked overnight in 1 M Tris (pH 7.0) as a control, whereas the other gel was treated overnight with 1 M hydroxylamine (NH₂OH; pH 7.0), followed by fluorography. The numbers on the right indicate the positions of molecular-mass standards in kDa.

PAGE under non-reducing conditions followed by fluorography. As shown in Figure 2, all three ECE-1 isoforms incorporated efficiently both [³⁵S]methionine/cysteine and [³H]palmitate (Figure 2B, lanes 10–12 and Figure 2A, lanes 2–4, respectively), whereas in non-transfected control cells, ECE-1 was not labelled (Figure 2A, lane 1 and Figure 2B, lane 9). The same result was obtained when the isoforms were expressed in CHO cells (results not shown). It is also apparent from Figure 2 that ECE-1a, ECE-1b and ECE-1c showed similar levels of palmitoylation. Quantification of several experiments revealed that the relative

Figure 3 Cys⁴⁶, Cys⁵⁸ and Cys⁴² are the sites of palmitoylation in ECE-1a, ECE-1b and ECE-1c, respectively

CHO cells expressing mutant ECE-1 isoforms were labelled with [³H]palmitate or [³⁵S]methionine/cysteine followed by immunoprecipitation and SDS/PAGE (6% gels). The numbers on the right indicate known molecular mass in kDa.

palmitoylation levels were 1 (ECE-1a), 1.12 (ECE-1b) and 0.85 (ECE-1c). As expected for a thioester linkage [41,42], the ³H label bound to the ECE-1 isoforms was removed by treatment with hydroxylamine at neutral pH (Figure 2A, lanes 6–8). This treatment had no effect on the amount of [³⁵S]methionine/cysteine incorporated into the isoforms (Figure 2B). Taken together, these data establish that ECE-1a, ECE-1b and ECE-1c are modified by thioester-linked palmitate.

Cys⁴⁶, Cys⁵⁸ and Cys⁴² are the sites of palmitoylation in ECE-1a, ECE-1b and ECE-1c, respectively

Cys⁴⁶ in ECE-1a, Cys⁵⁸ in ECE-1b and Cys⁴² in ECE-1c are the only cysteines in the cytoplasmic tails of the isoforms and none are present in the parts of the transmembrane domains adjacent to the cytoplasmic sequences. Thus, Cys⁴⁶, Cys⁵⁸ and Cys⁴² were the likely candidates to undergo palmitoylation [15]. To test this directly, site-directed mutagenesis was used to replace these cysteine residues with alanines (creating mutants ECE-1a C46A, ECE-1b C58A and ECE-1c C42A). CHO cells expressing the three mutant ECE-1 isoforms were labelled with [³H]palmitate or [³⁵S]methionine/cysteine and then analysed as before. As seen in Figure 3, all three mutants showed normal incorporation of [³⁵S]methionine/cysteine (Figure 3, lanes 4–6) whereas no bands were visible with [³H]palmitate (Figure 3, lanes 1–3). Thus the specific replacement of Cys⁴⁶, Cys⁵⁸ or Cys⁴² in the corresponding ECE-1 isoform by alanine abolished palmitoylation completely.

These data demonstrate that Cys⁴⁶, Cys⁵⁸ and Cys⁴² are the sites of acylation in ECE-1a, ECE-1b and ECE-1c, respectively, and that these cysteines account for all the palmitoylation that occurs in the human ECE-1 isoforms.

Palmitoylation is not essential for the subcellular localization of the ECE-1 isoforms

Previous work has established the subcellular localization of the human ECE-1 isoforms expressed in CHO cells [11]: whereas ECE-1b was found to be predominantly intracellular, ECE-1a

Figure 4 Immunofluorescence localization of ECE-1a, ECE-1b and ECE-1c in non-permeabilized CHO cells

Non-permeabilized CHO cells expressing ECE-1a (**A**), ECE-1b (**B**) and ECE-1c (**C**) were stained with an anti-ECE-1 mAb followed by FITC-conjugated goat anti-mouse antibody. In contrast with ECE-1a and ECE-1c, the ECE-1b isoform was predominantly localized intracellularly.

Figure 5 Palmitoylation is not essential for the subcellular localization of the ECE-1 isoforms

CHO cells expressing ECE-1a (**A**), ECE-1b (**B**), ECE-1c (**C**), ECE-1a C46A (**D**), ECE-1b C58A (**E**) and ECE-1c C42A (**F**) were fixed with paraformaldehyde and permeabilized with saponin. For detection, an anti-ECE-1 mAb followed by FITC-conjugated goat anti-mouse antibody were used.

and ECE-1c were present at the cell surface with some additional intracellular staining for ECE-1c. Here, the differences in cell-surface expression between the isoforms was confirmed by labelling of non-permeabilized CHO cells expressing ECE-1a, ECE-1b and ECE-1c with anti-ECE mAbs (Figure 4). To investigate whether acylation is required for the distinct subcellular localization of the ECE-1 isoforms, the intracellular trafficking of the non-palmitoylated mutant isoforms was assessed by indirect immunofluorescence. For this purpose, CHO cells expressing ECE-1a C46A, ECE-1b C58A and ECE-1c C42A as well as ECE-1a, ECE-1b and ECE-1c as controls were fixed with paraformaldehyde and permeabilized with saponin to

visualize both cell-surface and intracellular staining. Detection with an anti-ECE-1 mAb revealed that the subcellular distribution of the non-palmitoylated ECE-1 mutants (Figures 5D–5F) was the same as the localization of the corresponding wild-type isoforms (Figures 5A–5C and [11]). Both ECE-1a C46A and ECE-1c C42A displayed bright cell-surface staining. In addition, ECE-1c C42A showed some intracellular Golgi-like staining. In contrast with ECE-1a C46A and ECE-1c C42A, ECE-1b C58A was found to be predominantly intracellular. As for the wild-type, a Golgi-like pattern and some labelling of more peripheral structures that might represent coated vesicles or endosomal structures were seen.

Figure 6 Triton X-100 solubility of ECE-1 isoforms

MDCK cells expressing ECE-1a, ECE-1b and ECE-1c were solubilized with Triton X-100 at 4 °C and then separated by centrifugation into Triton X-100-soluble (S) and -insoluble (I) fractions. Proteins of the two fractions were subjected to SDS/PAGE and immunoblotting with anti-ECE-1 mAbs (**A**) or anti-caveolin-1 polyclonal antibodies (**B**). The numbers on the right indicate the positions of molecular-mass standards in kDa.

The palmitoylation of several proteins is known to affect their association with cholesterol and sphingolipid-rich membrane domains or caveolae ([30–33]; for a review see [22]). These are microdomains of the plasma membrane that are characterized by their non-ionic detergent-insolubility and have been implicated in transcytosis and signal transduction. To examine whether palmitoylated ECE-1 isoforms reside in Triton X-100-insoluble membranes, MDCK cells expressing ECE-1a, ECE-1b and ECE-1c were extracted with Triton X-100 at 4 °C. Soluble and insoluble fractions were prepared and assayed for the presence of ECE-1 by SDS/PAGE and immunoblotting with an ECE-1 mAb. As shown in Figure 6, all three ECE-1 isoforms were found exclusively in the Triton X-100-soluble fraction. In contrast, caveolin-1, a marker protein of caveolae [43], was predominantly ($\geq 93\%$) recovered in the Triton X-100-insoluble fraction, consistent with previous results [44]. These data demonstrate that palmitoylation does not target ECE-1 to cholesterol and sphingolipid-rich membrane domains or caveolae.

Taken together, the immunofluorescence and biochemical analyses indicate that palmitoylation is not crucial for the subcellular localization of the ECE-1 isoforms.

Figure 7 Enzymic activities of palmitoylated and non-palmitoylated ECE-1 isoforms

The catalytic activities of palmitoylated (ECE-1a, ECE-1b and ECE-1c) and non-palmitoylated (ECE-1a C46A, ECE-1b C58A and ECE-1c C42A) isoforms were determined on membrane preparations with human big ET-1 as a substrate. For all measurements, the values obtained for the enzymic activities were corrected for the differences in expression levels. The data are means $\pm$ S.E.M. for three independent experiments. In each experiment the enzymic activity of a wild-type or mutant isoform was calculated from 2–4 separate samples and the value obtained for each palmitoylated wild-type isoform was set to 100%.

Table 1 Comparison of kinetic parameters of palmitoylated and non-palmitoylated ECE-1 isoforms

The results are for three independent experiments, and are expressed as means $\pm$ S.D. Values indicating mg of protein refer to total membrane protein.

	K_m (μ M)	V_{max} (nmol/min per mg)
ECE-1a	0.29 ± 0.09	0.78 ± 0.06
ECE-1a C46A	0.39 ± 0.07	0.88 ± 0.19
ECE-1b	0.48 ± 0.11	0.89 ± 0.23
ECE-1b C58A	0.33 ± 0.08	0.86 ± 0.17
ECE-1c	0.73 ± 0.25	0.86 ± 0.16
ECE-1c C42A	0.50 ± 0.10	0.68 ± 0.10

Effect of palmitoylation on the enzymic activity of the ECE-1 isoforms

To further elucidate a potential functional role of the palmitoylation of ECE-1, we next investigated the impact of the absence of palmitoylation on the ability of the isoforms to cleave big endothelins. To compare their enzymic properties, ECE-1a, ECE-1b and ECE-1c, as well as ECE-1a C46A, ECE-1b C58A and ECE-1c C42A, were expressed in CHO cells and their endothelin-converting activity was analysed on cell-membrane preparations with a specific radioimmunoassay [11,39]. When the enzymic activities were expressed as a function of ECE-1 content, as determined by quantitative Western blotting, it could be calculated that the non-palmitoylated ECE-1b C58A cleaved big ET-1 with the same efficiency as the corresponding wild-type ECE-1b isoform ($100 \pm 9\%$; Figure 7). Comparable results were obtained for ECE-1a C46A and ECE-1c C42A, which were 88 ± 8 and $92 \pm 9\%$ as active as the wild-type ECE-1a and ECE-1c, respectively (Figure 7). The K_m and V_{max} values were also

similar for palmitoylated and non-palmitoylated ECE-1 isoforms (Table 1). These data show that palmitoylation is not critical for the enzymic properties of the three ECE-1 isoforms.

DISCUSSION

The present study demonstrates that the three isoforms of human ECE-1 are covalently modified by thioesterification of palmitic acid at a common cytoplasmic cysteine residue located 10 amino acids from the transmembrane domain. Non-palmitoylated ECE-1a, ECE-1b and ECE-1c isoforms showed normal enzymic activities and correct subcellular localizations.

Most of the cysteines identified as acylation sites in integral membrane proteins are found in the part of the cytoplasmic domain of the protein adjacent to the lipid bilayer or in the part of the transmembrane domain that is near the cytoplasmic sequence [15,45]. However, comparison of the amino acid sequences surrounding the acylation sites fails to reveal a general consensus sequence [45], and the exact position of the cysteine varies considerably. For instance, the transferrin receptor contains a palmitoylated cysteine in its transmembrane domain [23,24]. The HLA-D-associated invariant chain [46], on the other hand, has a palmitoylation site located two amino acids from the transmembrane domain, whereas this distance is six amino acids in the vesicular stomatitis virus glycoprotein [47], between 11 and 15 amino acids in the β_2 -adrenergic receptor [26], endothelin receptor B [28], bovine opsin [48] and bovine rhodopsin [49], and 29 and 33 amino acids in the cation-dependent mannose 6-phosphate receptor [36]. The 10-amino acid spacing of Cys⁴², Cys⁵⁸ and Cys⁴⁶ in the ECE-1 isoforms further adds to the diversity found for the sites of acylation in integral membrane proteins and thus supports the notion of multiple palmitoyltransferases with diverse structural requirements.

An interesting property of the palmitoylation of the ECE-1 isoforms is that all three isoforms are acylated and show similar levels of modification despite differences in their subcellular localization. This raises the question of where the isoforms are acylated. Palmitoylation has been reported to occur at different subcellular sites. Attachment of fatty acid to the viral proteins vesicular stomatitis virus glycoprotein and the influenza virus haemagglutinin, for example, occurs in a pre-/early Golgi compartment [50,51]. Palmitoylation of many cellular proteins including the transferrin receptor [52] and the immunoglobulin E receptor [53], on the other hand, have been shown to occur at the plasma membrane. Accordingly, the characterization of a G-protein palmitoyltransferase activity that is strongly enriched in plasma membranes has been reported [54]. In addition, Das et al. [55] have recently purified and characterized a plasma membrane-associated enzyme with protein palmitoyl acyltransferase activity using spectrin as substrate. In contrast, Gutierrez and Magee [56] have identified a protein palmitoyltransferase activity that co-fractionated with Golgi markers, and Liu et al. [57] isolated a protein palmitoyltransferase from rat liver microsomes. Taken together, these findings point to the existence of multiple palmitoyltransferases with various subcellular locations. The three ECE-1 isoforms could therefore be palmitoylated by more than one transferase at their different localizations. Alternatively, a single common enzyme that is most probably present in the Golgi apparatus or in a pre-Golgi compartment could be responsible for the acylation of the isoforms. Yet another possibility, which cannot be excluded, is that palmitoylation of ECE-1 may occur non-enzymically. *In vitro*, non-enzymic or autocatalytic acylation has been reported for several proteins, including rhodopsin and certain G-protein α subunits ([58,59] and references therein). For the G α subunits, many of the

characteristics of the autoacylation are similar to those observed for their palmitoylation *in vivo*, raising the possibility that the palmitoylation could proceed non-enzymically in the cell [60].

ECE-1 isoforms carrying Cys $\rightarrow$ Ala mutations did not have any apparent altered biological properties. For instance, palmitoylated and non-palmitoylated ECE-1 isoforms had the same subcellular localization, as judged by immunofluorescence microscopy. As for the corresponding wild-type isoforms, ECE-1b C58A was found to be predominantly intracellular, whereas ECE-1a C46A and ECE-1c C42A were present at the cell surface with some additional intracellular staining for ECE-1c C42A. The finding that the different subcellular distributions of the ECE-1 isoforms were maintained in the absence of acylation indicates that palmitoylation is most probably not involved in the regulation of the intracellular trafficking of the isoforms. Likewise, acylation of the human cell-surface glycoprotein CD4 and the vesicular stomatitis virus glycoprotein was not critical for their transport and subcellular localization [47,61]. In contrast, palmitoylation of Cys³⁴ in the cytoplasmic tail of the cation-dependent mannose 6-phosphate receptor is essential for the normal trafficking of the receptor [36]. In addition, reduced cell-surface expression was reported for the unpalmitoylated forms of the luteinizing hormone/human chorionadotropin and vasopressin V2 receptors [34,35,37]. Another example of how palmitoylation can affect localization is by targeting proteins such as endothelial nitric oxide synthase, influenza haemagglutinin and the protein tyrosine kinase Lck to Triton X-100-insoluble cholesterol and sphingolipid-rich membrane domains or caveolae ([30–33], for a review see [22]). When this possibility was tested for ECE-1, all three isoforms were found to be excluded completely from these structures. As for the localization, we could not detect any significant effect of palmitoylation on the enzymic activity of ECE-1. Both palmitoylated and non-palmitoylated ECE-1 isoforms cleaved big ET-1 with comparable efficiency and had similar K_m and V_{max} values. Thus the function of the covalent attachment of palmitate to ECE-1 remains elusive. Similarly, palmitoylation of the HIV envelope protein [62] and the human dopamine D₁ receptor [63] did not have any obvious biological effects. For some of the palmitoylated transmembrane proteins, on the other hand, acylation was found to be of significant functional relevance. Palmitoylation of the cation-dependent mannose 6-phosphate receptor, for example, is essential for its lysosomal enzyme-sorting function [36]. Palmitoylation also plays a role in the signal-transduction activities of several G-protein-coupled receptors such as the β_2 -adrenergic receptor [26,38] and the two endothelin receptors ET_A and ET_B [27,28]. Prevention of palmitoylation of the β_2 -adrenergic receptor, for instance, markedly impairs its ability to mediate agonist stimulation of adenylate cyclase [26,38]. Finally, it has been suggested that the palmitoylation of some proteins may be a method of blocking otherwise active cysteines. Given the lack of apparent consequences of preventing the palmitoylation of ECE-1, this is a possibility that also cannot be excluded for ECE-1 as a potential role for its acylation.

ECE-1 belongs to a family of zinc metallopeptidases that are defined by a HEXXH zinc-binding motif [64]. Despite significant sequence homology of ECE-1 with the other family members, NEP, KELL, PEX and ECE-2 (for a review see [10]), none of these proteins has a cysteine residue localized in either its cytosolic tail or the adjacent part of the transmembrane domain. They are therefore most likely not palmitoylated. Whereas PEX has a putative site for prenylation [65], no lipid modifications at all are known for NEP, ECE-2 or KELL. In contrast, both rat and bovine ECE-1 have a cysteine residue at the same position relative to the transmembrane domain as the human ECE-1

isoforms. Not only is the exact position of the cysteine conserved between the three species but so too are the amino acids surrounding the cysteine. It will be of interest to confirm that rat and bovine ECE-1 are also palmitoylated.

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