Endothelin-converting enzyme-like 1 (ECEL1) is present both in the plasma membrane and in the endoplasmic reticulum

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Enzymes of the M13 family of zinc-containing endopeptidases are recognized as important regulators of neuropeptide and peptide hormone activity. Peptidases of this family are type II integral-membrane proteins characterized by short cytosolic domains and large extracellular domains containing the active site. The M13 family has, at present, seven members, including ECEL1 (endothelin-converting enzyme-like 1), one of the newest members. ECEL1 is expressed predominantly in the central nervous system. It has been proposed that the enzyme has a role in the nervous regulation of the respiratory system. No physiological substrate has been identified yet. To better understand the function(s) of this enzyme, we have expressed human ECEL1 in cultured cells and monitored its biosynthesis and subcellular localization. Immunoblot and cell-surface biotinylation analysis of transfected cells expressing ECEL1 showed that only a fraction of the protein travelled to the cell surface, while most of the enzyme was present in an intracellular compartment identified by confocal immunofluorescence microscopy and cell fractionation as the ER (endoplasmic reticulum). Pulse–chase experiments showed that ER-localized ECEL1 was stable, with a half-life of more than 3 h. Endogenous ECEL1 from mouse pituitary gland had a similar distribution between the cell surface and the ER. Finally, using domain-swapping experiments with neprilysin, another member of the M13 family, we showed that localization of ECEL1 to the ER requires both the transmembrane and cytoplasmic domains. It thus appears that ECEL1 may have functions both at the cell surface and in the ER.

Key words: biosynthesis, cellular localization, endoplasmic reticulum, endothelin-converting enzyme-like 1 (ECEL1), heterologous expression, monoclonal antibody.

INTRODUCTION

Endopeptidases of the M13 family are important regulators of neuropeptide and peptide hormone activity, and have been identified as putative targets for therapeutic intervention using inhibitors (reviewed in [1]). Seven members have been characterized so far. These are: NEP (neprilysin; also known as neutral endopeptidase-24.11 and CALLA) (reviewed in [2]); ECE-1 and ECE-2 (endothelin-converting enzymes 1 and 2) (reviewed in [3]); PHEX (phosphate-regulating gene with homologies to endopeptidases on the X chromosome; formerly known as PEX) (reviewed in [4]); the Kell blood group protein (reviewed in [5]); SEP/NL1/NEP2 (designated NEP2 in the present paper) [6–8]; and finally, ECEL1/DINE (ECE-Like 1/damage-induced neuronal endopeptidase; designated ECEL1 in the present paper) [9,10]. The hydropathy profiles of sequences deduced from the cDNAs of the M13 family members predict integral-membrane proteins of type II topology, with short N-terminal cytosolic domains, followed by a transmembrane segment and a large extracellular domain or ectodomain of approx. 700 amino acid residues containing the active site of the enzymes.

ECEL1 is one of the newest members of the M13 family. It was first identified by homology cloning [9], and was subsequently cloned as a transcript whose expression was induced in response to nerve injury [10]. It has been reported to be expressed specifically in the nervous system [9], and gene disruption experiments in the mouse suggest a critical role of the enzyme in the nervous regulation of the respiratory system [11]. More recently, high levels of ECEL1 expression were associated with neuroblastoma tumours presenting a favourable prognosis of spontaneous regression [12]. ECEL1 is a 775-amino-acid residue glycoprotein, and its ectodomain harbours the H^{612}EXXH (where 'X' denotes any amino acid) and G^{671}ENIAD motifs, which are hallmarks of the Zn^{2+}-metallopeptidases of the M13 family. Several other amino acid residues that have been shown in NEP to be important for enzymic activity are also present in ECEL1. Overall, ECEL1 has 48 % identity with NEP in the last 500 amino acid residues, where most of the active site residues are located [9]. The physiological substrate(s) of ECEL1 is still unknown, but the enzyme was shown to have activity with a synthetic tripeptide substrate [10].

Little is known about ECEL1 cellular localization. Immunofluorescence analysis of transfected cells suggested that the enzyme is essentially localized in the ER (endoplasmic reticulum) [13]. However, the fate of the enzyme in the ER, and the mechanism for its ER localization, have not been addressed. In the present study, we have examined the biosynthesis and subcellular distribution of ECEL1 in transfected HEK-293 (human embryonic kidney-293) cells expressing this enzyme. We show that a small fraction of ECEL1 is transported to the cell surface, but most of the protein is present in the ER, where it is stable. A similar distribution was found for endogenous ECEL1 from mouse pituitary gland. Finally, we show that both the transmembrane and cytoplasmic domains are necessary for ER localization of ECEL1.

MATERIALS AND METHODS

Cloning of human ECEL1 and construction of expression vectors

All DNA manipulations were performed according to standard protocols [14,15]. Human ECEL1 full-length cDNA was obtained

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; ECE, endothelin-converting enzyme; ECEL1, ECE-like 1; endo H, endoglycosidase H; ER, endoplasmic reticulum; HEK-293, human embryonic kidney-293; NEP, neprilysin; NHS-SS-biotin, biotin disulphide *N*-hydroxysuccinimide ester; PBS-Ca^{2+}-Mg^{2+}, PBS containing 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$; PHEX, phosphate-regulating gene with homologies to endopeptidases on the X chromosome; PNGase F, peptide N-glycosidase F; TGN, *trans*-Golgi network.

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by screening a λgt10 human brain cDNA library (ClonTech) using as probe a partial human ECEL1 cDNA obtained by homology cloning, as described previously [7]. A 2800 bp clone was isolated, and sequencing revealed a nucleotide sequence essentially identical with that reported by Valdenaire et al. [9], and originally designated XCE (results not shown).

For expression of human ECEL1 protein in mammalian cultured cells, a *Hin*dIII/*Xba*I DNA fragment containing 200 nt of the 5′untranslated region, the entire coding sequence and 339 nt of 3′ untranslated region of ECEL1 mRNA was cloned into vector pCDNA3/RSV [16], resulting in plasmid pwtECEL1. This vector also contains the aminoglycoside phosphotransferase 3′ gene, which confers resistance to the antibiotic Geneticin (G418).

Construction of ECEL1–NEP chimaeras

To construct the E/N/N chimaera (where E represents ECEL1 and N represents NEP; cytosolic/transmembrane/ectodomain respectively), the cytosolic domain of ECEL1 was fused to the NEP transmembrane domain and ectodomain by PCR-based techniques [17] using oligonucleotides 5′-GGTCTGACATGGATTGG-3′ and 5′-CAGGACAAGGACCGAGAGGCTGATCTCCTCGCGCCGGTTCCAGCGCGGCAG-3′, and 5′-GATTTAGGTGACACTATAG-3′ and 5′-CTGCCGCGCTGGAACCGGCGCGAGGAGATCAGCCTCTCGGTCCTTGTCCTG-3′ as primers, resulting in pCDNA3/RSV-E/N/N.

To construct the E/E/N chimaera, the cytosolic and transmembrane domains of ECEL1 were fused to the NEP ectodomain by PCR as described above, using oligonucleotides 5′-GGTCTGACATGGATTGG-3′ and 5′-AGTCTGATGACTTGCAAATACCATCATCGAGGGCCAGCATAGCCGCCAGAATGGC-3′, and 5′-GATTTAGGTGACACTATAG-3′ and 5′-GCCATTCTGGCGGCTATGCTGGCCCTCGATGATGGTATTTGCAAGTCATCAGACT-3′ as primers, resulting in pCDNA3/RSV-E/E/N.

To construct the N/E/N chimaera, the NEP transmembrane domain was replaced with that of ECEL1 in the vector pRC/CMV-NEP [18] by performing an all-around PCR, using oligonucleotides containing the ECEL1 transmembrane domain nucleotide sequence: 5′-GAAGAAACAGCGATGGACTCGACTGGTGTGCCTGCTGTCGGGGCTGGTGTTCGCCGCCG-3′ and 5′-ATGCAGTCTGATGACTTGCAAATACCATCATCGAGGGCCAGCATAGCCGCCAGAATGGCGCAGAGGC-3′. Purification of PCR-amplified DNA fragments was performed using QIAquick PCR purification kit (Qiagen). PCR reactions were allowed to proceed using the *Pfu* DNA polymerase (Stratagene). The resulting pRC/CMV-N/E/N vector was verified by nucleotide sequencing.

Monoclonal antibody production

A plasmid for the production in *Escherichia coli* of a GST (glutathione S-transferase)–ECEL1 fusion protein was constructed using the pGEX-2T expression vector (Amersham Pharmacia Biotech Inc., Baie d'Urfé, Canada). Vent polymerase (New England Biolabs, Mississauga, ON, Canada) and ECEL1 cDNA were used to amplify by PCR a 969 bp fragment using, as the sense primer, 5′-CGGATCCGACAAGCTCACCTATGGCACCATC-3′ (starts at Asp^{142} of the published sequence; [9]) and, as the antisense primer, 5′-CGAATTCTCAGGCAGCTGAGAAGTGCTCATGTAC-3′ (ends at Ala^{465} of the published sequence; [9]) containing *Bam*HI and *Eco*I restriction sites respectively (shown underlined in the sequences). Both pGEX-2T and the PCR product were digested with *Bam*HI and *Eco*I, and ligated. Production of the recombinant fusion protein in *E. coli* AP 401 strain and purification of the ECEL1 moiety were performed according to the manufacturer's instructions (Amersham Pharmacia Biotech Inc.). Immunization of mice, cell fusion and hybridoma screening were performed as described previously [19]. One cloned hybridoma was expanded and injected into the peritoneal cavity of pristane-treated mice. The ascitic fluid was then collected and used in subsequent experiments. This monoclonal antibody was designated 7B6.

Transfections, cell culture and immunoblotting

The HEK-293 cell line (A.T.C.C. no. CRL-1573) was cultured and transfected by the calcium phosphate method, as described previously [20]. Stable transfectants were selected in 4 % G418 (BRL Life Technologies, Burlington, ON, Canada), and maintained in DMEM (Dulbecco's modified Eagle's medium; BRL Life Technologies, Burlington, ON, Canada) containing 10 % (v/v) fetal bovine serum at 37 °C in 5 % CO_2/95 % air. At 24 h before analysis of protein expression, sodium butyrate was added to the culture medium to a final concentration of 10 mM.

Expression of ECEL1, PHEX, NEP and NEP2 proteins was monitored by immunoblotting analysis of cell extracts and culture media, as described previously [7], using the specific monoclonal antibody 7B6 (described in the present study), 13B12 [21], 18B5 [22] or mouse polyclonal antibodies [7] respectively. The glycosylation state of the proteins was determined as described previously [7].

Biotinylation of cell-surface proteins

Stably transfected HEK-293 cells were plated at a density of 2×10^5 cells/well in a 24-well plate for 3 days, and the medium was replaced with supplemented medium containing 10 mM sodium butyrate 24 h before the experiment. All subsequent steps were performed at 4 °C, essentially as described previously [23]. Cells were washed twice with PBS containing 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$ (PBS-Ca^{2+}-Mg^{2+}). Cells were then incubated with NHS-SS-biotin (biotin disulphide *N*-hydroxysuccinimide ester; 2 mg/ml; Pierce, Rockford, IL, U.S.A.) in biotinylation buffer [2 mM $CaCl_2$/150 mM NaCl/10 mM triethanolamine (pH 8.0)] for 25 min with gentle agitation. Cells were then washed with biotinylation buffer, and the labelling procedure was repeated under the same conditions. Unreacted NHS-SS-biotin was quenched by the addition of quench buffer (100 mM glycine in PBS-Ca^{2+}-Mg^{2+}). The cells were rinsed twice with quench buffer, and then incubated in the same buffer for 20 min with gentle agitation and rinsed twice with PBS-Ca^{2+}-Mg^{2+}. The cells were then scraped and solubilized by incubation in lysis buffer [1 % (v/v) Triton X-100/150 mM NaCl/5 mM EDTA/50 mM Tris/HCl (pH 7.5)] for 60 min. The lysates were clarified by centrifugation at 14 000 ***g*** for 30 min. Biotin-labelled proteins were separated from unlabelled proteins by adding 0.2 ml of NeutrAvidin resin (Pierce) to 0.9 ml of clarified lysate and incubating at 4 °C overnight with end-over-end rotation. The resin was collected by centrifugation at 5000 ***g*** for 15 min at 4 °C. The supernatant was removed and saved for further analysis. The pellet was then washed three times with lysis buffer, twice with high-salt wash buffer [0.1 % Triton X-100/500 mM NaCl/5 mM EDTA/50 mM Tris/HCl (pH 7.5)], once with no-salt wash buffer [50 mM Tris/HCl (pH 7.5)], and finally resuspended in 0.15 ml of Laemmli [24] sample buffer containing 143 mM 2-mercaptoethanol. Protein samples were subjected to SDS/PAGE (7.5 % gels) and analysed by immunoblotting. First, the blot was probed with the monoclonal antibody 7B6. Then, to rule out the possibility that intracellular proteins could be biotinylated, the blot was stripped and re-probed with rabbit anti-actin antibody.

Solubilization of ECEL1 from mouse pituitary

All steps were performed at 4 °C. Pituitaries from female BALB/c mice (3–4 weeks old) were homogenized (10 % w/v) in homogenizing buffer [25 mM Tris/HCl (pH 7.5)/3 M NaCl/50 mM EDTA/1 mM pepstatin A/2 μg/ml leupeptin/2 μg/ml aprotonin/0.6 mM PMSF/2 mM 2-mercaptoethanol] using a Polytron homogenizer. The homogenate was centrifuged at 1000 ***g*** for 15 min and the supernatant was kept at 4 °C. The pellet was washed with homogenizing buffer and centrifuged as above. The supernatants were combined and centrifuged at 100 000 ***g*** for 60 min. The pellet was resuspended in homogenization buffer containing 1 % Triton X-100, incubated for 1 h and centrifuged at 100 000 ***g*** for 60 min. The supernatant was analysed by SDS/PAGE and immunoblotting, as described above.

Confocal microscopy

Labelling of stably transfected HEK-293 cells expressing ECEL1 was performed on fixed and permeabilized cells using methanol/acetone (4:1, v/v). Cells were incubated with mouse anti-ECEL1 (7B6) antibody, and either rabbit anti-calnexin (a gift from Dr John Bergeron, Department of Anatomy and Cell Biology, McGill University, Montreal, Canada) or rabbit anti-giantin (Covance, Princeton, NJ, U.S.A.). Proteins were revealed with Alexa-488 goat anti-mouse or Alexa-567 goat anti-rabbit antibodies (Molecular Probes, Eugene, OR, U.S.A.). Confocal microscopy was performed with the 100× Nikon Plan Apochromat objective of a dual-channel Bio-Rad 600 laser-scanning confocal microscope, equipped with a krypton/argon laser and the corresponding dichroic reflectors to distinguish between Alexa-488 and Alexa-567 labelling.

Subcellular fractionation

Five confluent 100 mm Petri dishes of transfected HEK-293 cells were washed twice with PBS, before scraping the cells from the dish. The cells were then spun down at 1500 rev./min (200 ***g***) for 5 min and resuspended in 2 ml of homogenization buffer [10 mM Tris/HCl (pH 7.5)/12.5 % sucrose/50 mM EDTA/1 mM pepstatin A/2 μg/ml leupeptin/2 μg/ml aprotonin/0.6 mM PMSF/2 mM 2-mercaptoethanol]. The cells were homogenized with a Potter–Elvehjem homogenizer at 4 °C, and centrifuged at 1000 ***g*** at 4 °C for 15 min. The supernatant was layered on to a sucrose gradient consisting of nine steps from 22–60 % (w/v), and centrifuged at 135 000 ***g*** for 2.5 h at 4 °C. The gradient was fractionated from the top into 14 aliquots of 0.77 ml. Fractions were analysed as described above by SDS/PAGE (7.5 % gels) and immunoblotting, using the following antibodies for the indicated cellular compartments: calnexin for ER (Sigma), and β-COP protein for Golgi–ER transport vesicles (Sigma).

Pulse–chase studies

ECEL1-transfected HEK-293 cells were grown in 60 mm dishes, washed in DMEM lacking methionine and incubated in the same medium for a period of 20 min at 37 °C. The cells were then labelled for 30 min at 37 °C by the addition of [^{35}S]methionine (200 μCi/dish; ICN, Irvine, CA, U.S.A.). After the radioactive pulse, the cells were rinsed twice in DMEM containing an excess of non-radioactive methionine (0.15 mg/ml), and were chased for various periods of time in this medium ($t = 0$, 15, 30, 60, 90, 120 and 180 min). The cells were then washed and scraped into cold PBS. Cells were collected by centrifugation (200 ***g***), solubilized with 200 μl of 1 % *N*-octylglucoside, and centrifuged (200 ***g***) once again. The supernatant was collected and incubated with monoclonal antibody 7B6 for 16 h at 4 °C with end-over-end rotation. A secondary mouse antibody was added for 2 h at 4 °C, and mixed by end-over-end rotation. Protein A–Sepharose beads (Amersham Pharmacia Biotech Inc.; 25 μl of a 10 % suspension) were added, and the incubation was allowed to proceed for 2 h at 4 °C. Beads were collected by centrifugation (15 000 ***g***), washed three times and resuspended directly in Laemmli buffer [24]. The mixture was boiled for 3 min, centrifuged (15 000 ***g***), and the supernatant was analysed both by SDS/PAGE on a 7.5 % (w/v) gel and by fluorography. Some samples were treated with endo H (endoglycosidase H), as described previously [19], before SDS/PAGE.

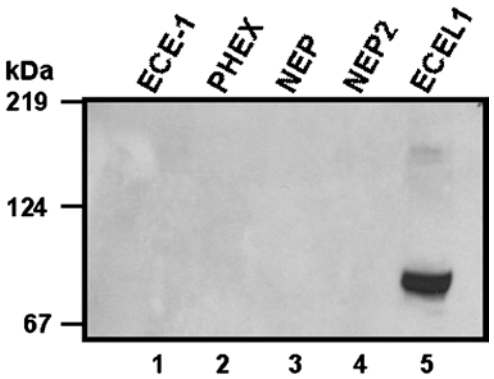

Figure 1 Specificity of ECEL1 monoclonal antibody

Extracts of HEK-293 cells transfected with ECEL-1, PHEX, NEP or ECEL1 cDNA (lanes 1, 2, 3 and 5 respectively) or culture medium from HEK-293 cells transfected with NEP2 cDNA (lane 4) were subjected to SDS/PAGE on 7.5 % gels under reducing conditions, and detected by immunoblotting using the monoclonal antibody 7B6. The positions of molecular-mass markers (in kDa) are shown on the left.

RESULTS

Monoclonal antibody 7B6 specifically recognizes ECEL1

A monoclonal antibody designated 7B6, directed against the N-terminal portion of human ECEL1 ectodomain (Asp^{142}–Ala^{465}), was developed. The specificity of this antibody was assessed using protein extracts from cells transfected with human ECEL1, human PHEX, rabbit NEP, mouse NEP2 or human ECE-1 cDNAs. Immunoblot analysis showed that the antibody recognizes an ECEL1 band migrating at approx. 95 kDa (Figure 1, lane 5). This antibody did not recognize recombinant ECE-1, PHEX, NEP or NEP2 proteins (Figure 1, lanes 1–4 respectively). However, these proteins were detected when the blot was re-probed with the corresponding antibodies (results not shown).

Two ECEL1 proteins, different in their glycosylation states, are present in transfected cells

HEK-293 cells were transfected with expression vector pwt-ECEL1, and pools of stable transfectants were established. Immunoblotting of extracts of control and transfected HEK-293 cells with the ECEL1-specific monoclonal antibody 7B6 led to the detection of a 95 kDa band in the extract of transfected cells (Figure 2A, lane 3), but not in the extract of control cells (Figure 2A, lane 1). No protein was detected in the culture medium (Figure 2A, lanes 2 and 4, for control and transfected cells respectively).

To characterize the glycosylation state of ECEL1, we next subjected the recombinant proteins to deglycosylation by PNGase F

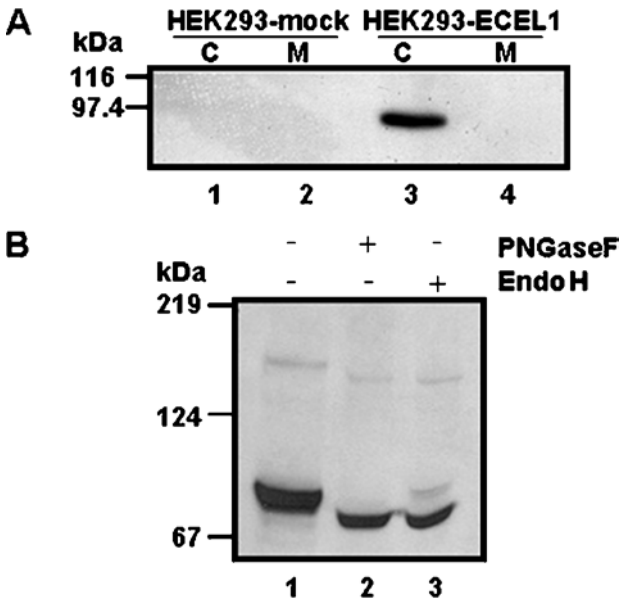

Figure 2 Immunoblot analysis of ECEL1-transfected HEK-293 cells

(**A**) Cell extracts (C) or culture media (M) of mock-transfected or ECEL1-transfected HEK-293 cells were separated by SDS/PAGE on 7.5 % gels under reducing conditions and detected by immunoblotting using the monoclonal antibody 7B6. (**B**) Cell extract of ECEL1-transfected HEK-293 cells was analysed as in (**A**), either without treatment (lane 1) or after digestion with PNGase F (lane 2) or endo H (lane 3). The position of molecular-mass markers (in kDa) is shown on the left.

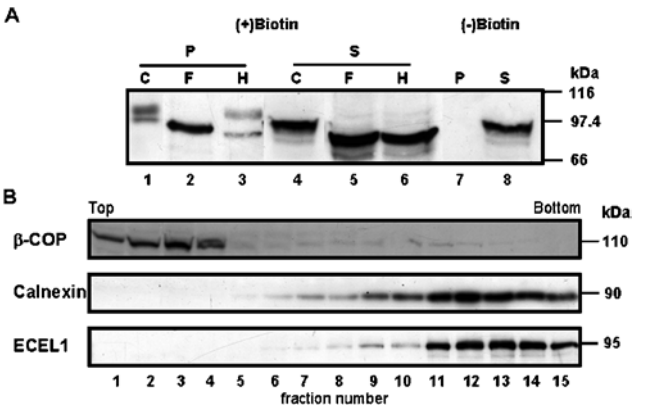

Figure 3 Biotinylation of cell-surface ECEL1

(**A**) Transfected HEK-293 cells were incubated with NHS-SS-biotin and solubilized. Biotin-labelled proteins were separated from unlabelled proteins by adding NeutrAvidin resin. ECEL1 present in the pellet or supernatant was detected by immunoblotting using monoclonal antibody 7B6 after separation of the proteins by SDS/PAGE on 7.5 % gels. P, pellet; S, supernatant; C, control; F, treated with PNGase F; H, treated with endo H. The position of molecular-mass markers (in kDa) is shown on the right. (**B**) Subcellular fractionation of ECEL1-transfected HEK-293 cells. Extracts from ECEL1-transfected HEK-293 cells were centrifuged on a 22–60 % (w/v) sucrose gradient. The gradient was fractionated, and fractions were analysed by SDS/PAGE on 7.5 % gels and immunoblotting using antibodies directed against calnexin, β-COP or ECEL1. The position of molecular-mass markers (in kDa) is shown on the right.

(peptide N-glycosidase F) and endo H. PNGase F removes high mannose, as well as most complex N-linked, oligosaccharides added in the Golgi complex. In contrast, endo H removes N-linked oligosaccharide side chains of the high-mannose type found on proteins in the ER that have not yet transited through the Golgi complex. Thus resistance to endo H can be used as an indication that the protein has travelled through the Golgi complex. PNGase F treatment revealed that ECEL1 is N-glycosylated, since its molecular mass decreased from 95 kDa to 85 kDa following digestion (Figure 2B; compare lanes 1 and 2). This decrease in molecular mass is consistent with the presence of three putative N-glycosylation sites in the ECEL1 primary structure [9]. Digestion of ECEL1-transfected cell extracts with endo H also resulted in an increased electrophoretic mobility of most of the ECEL1 protein (Figure 2B, lane 3). However, a small portion of ECEL1, migrating with a molecular mass of approx. 105 kDa, was resistant to digestion by endo H (Figure 2B, lane 3). This species was not observed in the untreated sample (Figure 2B, lane 1), probably due to the high intensity of the 95 kDa band. As a control for endo H digestion, NEP, a member of the M13 family previously reported to be transported to the cell surface [25], was expressed in HEK-293 cells, and was shown to be resistant to endo H digestion (results not shown). Our results indicate that at least two ECEL1 proteins, different in their glycosylation states, are present in HEK-293-transfected cells. The major 95 kDa species has not acquired complex oligosaccharides, suggesting that it has not travelled through the Golgi apparatus. In contrast, the minor 105 kDa species has acquired complex sugars, suggesting that it travelled through the Golgi, and possibly to the cell surface.

The ECEL1 105 kDa species is present at the cell surface, whereas the 95 kDa protein is localized in the ER

To test the hypothesis that the minor 105 kDa ECEL1 species is localized at the cell surface, transfected HEK-293 cells were subjected to surface biotinylation. The avidin-bound material contained two ECEL1 bands, with respective masses of 100 and 105 kDa (Figure 3A, lane 1). Both species were sensitive to PNGase F treatment (Figure 3A, lane 2), but only the 100 kDa protein was sensitive to endo H digestion (Figure 3A, lane 3). In contrast, the supernatant containing the non-biotinylated material not bound to the avidin beads showed only the 95 kDa protein (Figure 3A, lane 4), and this species was sensitive to digestion by both PNGase F and endo H (Figure 3A, lanes 5 and 6 respectively). No biotinylated actin could be detected when the blot was re-probed with the anti-actin antibodies (results not shown), and in the absence of biotinylation, no ECEL1 protein was found associated with the avidin beads (Figure 3A, lane 7), but was present in the supernatant (lane 8).

The absence of complex oligosaccharides on ECEL1 95 kDa species suggests that it is present in a pre-Golgi compartment, possibly the ER. To confirm this subcellular localization of the 95 kDa protein, transfected HEK-293 cells were fixed, permeabilized and labelled with anti-ECEL1 antibody and either anti-calnexin or anti-giantin antibodies as markers for the ER [26] and Golgi apparatus [27] respectively, and examined by confocal immunofluorescence microscopy. Both anti-ECEL1 and anti-calnexin antibodies displayed a similar punctate pattern, and the merge of both images shows almost perfect co-localization (Figure 4, top panels). In contrast, ECEL1 did not localize with the Golgi marker giantin (Figure 4, bottom panels). Transfected HEK-293 cells were also fractionated on sucrose gradients, and the distribution of ECEL1 in the gradient was compared with that of β-COP (a marker for Golgi-to-ER transport vesicles) and calnexin. The distribution of the ECEL1 95 kDa protein was closely matched with that of calnexin (Figure 3B), thus confirming its presence in the ER.

Endogenous ECEL1 also distributes between plasma membrane and ER in mouse pituitary cells

To determine whether endogenous ECEL1 is also mostly localized in the ER, extracts of mouse pituitary, a tissue reported to express

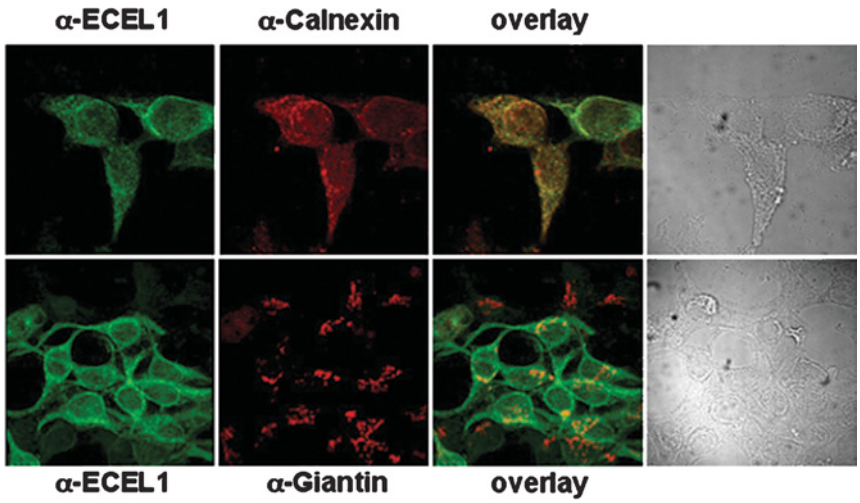

Figure 4 Co-localization of ECEL1 with calnexin

Stable transfectants of HEK-293 cells expressing ECEL1 were permeabilized and labelled with the anti-ECEL1 (7B6) antibody and goat anti-mouse Alexa-488 (panels labelled 'α-ECEL1'). The same field was then labelled with anti-calnexin antibodies (ER marker) and goat anti-rabbit Alexa-567 (panels labelled 'α-calnexin') or anti-giantin antibodies (*cis*- and median-Golgi marker) and goat anti-rabbit Alexa 567 (panel α-giantin). The centre-right panels show the merged images of ECEL1 and ER or Golgi apparatus labelling (panels labelled 'overlay'). The right panels show phase images of the fields.

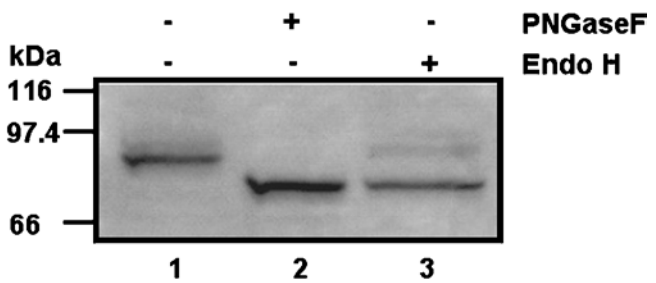

Figure 5 Immunoblot analysis of endogenous ECEL1 from mouse pituitary

Mouse pituitary homogenates were separated by SDS/PAGE on 7.5 % gels under reducing conditions and ECEL1 was revealed by immunoblotting using monoclonal antibody 7B6 either without treatment (lane 1) or following treatment with PNGase F (lane 2) or endo H (lane 3). The position of molecular-mass markers (in kDa) is shown on the left.

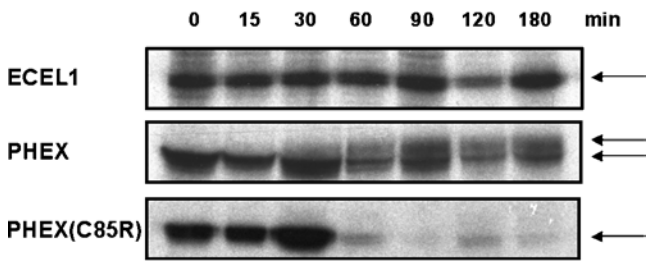

Figure 6 Biosynthesis of ECEL1, PHEX and mutant PHEX proteins

HEK-293 cells transfected with ECEL1, PHEX or mutant PHEX (C85R) cDNAs were pulsed for 30 min with [^{35}S]methionine and chased with unlabelled methionine for the indicated periods of time. Cell lysates were then subjected to immunoprecipitation with ECEL1 or PHEX antibody, and analysed by SDS/PAGE on 7.5 % gels. Arrows indicate the core-glycosylated and mature forms of the proteins.

ECEL1 [9], were analysed by immunoblotting with antibody 7B6. The 95 kDa ECEL1 protein was observed in the pituitary extract (Figure 5, lane 1). This protein was sensitive to PNGase F digestion (Figure 5, lane 2), indicating that it is glycosylated. As in transfected cells, digestion with endo H revealed the presence of two species (Figure 5, lane 3). The major species had its molecular mass decreased to 85 kDa by the treatment with endo H, indicating that it had not acquired complex sugars, whereas the minor 105 kDa species was resistant to digestion. If present, the 100 kDa species could not be detected, since it is expected to be sensitive to endo H digestion.

ECEL1 95 kDa protein is stable in the ER

The biosynthesis and processing of ECEL1 was studied by pulse–chase experiments. HEK-293 cells expressing ECEL1 were pulse-labelled for 30 min with [^{35}S]methionine and cysteine, and chased for the periods of time indicated in Figure 6. For comparison purposes, PHEX, a member of the M13 family known to be transported to the cell surface, was also expressed in HEK-293 cells and studied by pulse–chase. As observed previously [20], a 95 kDa PHEX species corresponding to the core-glycosylated protein was initially synthesized (Figure 6). Within 60 min, a more slowly migrating form of approx. 105 kDa was evident, consistent with the maturation of the protein in the Golgi compartment, where terminal glycosylation occurs. ECEL1 was also synthesized as a 95 kDa species (Figure 6) that was endo H-sensitive (results not shown), but unlike PHEX, this 95 kDa ECEL1 form was never chased into a slower migrating species and showed no significant decrease with time. Even after 180 min and endo H digestion, we could not detect the 105 kDa form observed at steady state by immunoblotting in either transfected cells or the mouse pituitary (results not shown).

It is generally accepted that slow folding or misfolded proteins present in the ER are degraded rapidly. To compare the half-life of ECEL1 with that of a misfolded protein, we also expressed in HEK-293 cells PHEX mutant $Cys^{85} \rightarrow Arg$ (C85R). This mutation was found to result in retention of the protein in the ER [20], causing X-linked hypophosphataemia (XLH) in humans. Only the 95 kDa core-glycosylated form of the C85R mutant

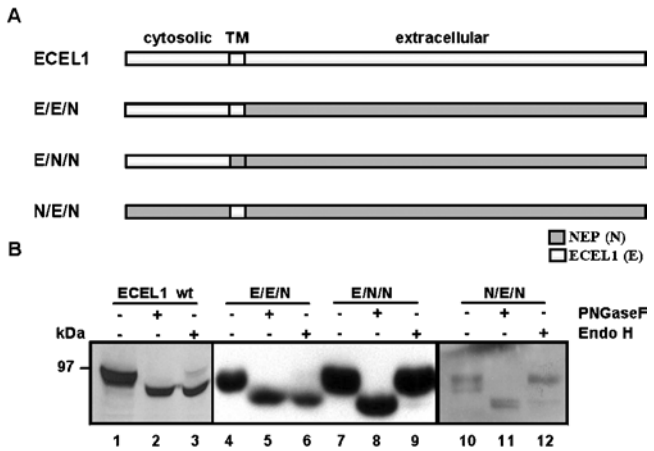

Figure 7 Schematic representation of ECEL1/NEP chimaeric protein (A) and immunoblot analysis of ECEL1/NEP chimaera (B)

(**A**) A Schematic representation of the various ECEL1/NEP chimaeric proteins is shown. White boxes represent ECEL1 (E); grey boxes show NEP (N). The box labelled 'cytosolic' shows the cytosolic domain; boxes labelled 'TM' are the transmembrane domain; and those labelled 'extracellular' are the extracellular domain, or ectodomain. (**B**) HEK-293 cells were transiently transfected with ECEL1 or ECEL1/NEP cDNAs. Cell extracts were digested (+) or not (−) with PNGase F or endo H before electrophoresis on 6 % gels under reducing conditions, and proteins were revealed by immunoblotting with anti-NEP antibody. The position of molecular-mass markers (in kDa) is shown on the left.

PHEX protein was synthesized (Figure 6). In contrast with ECEL1, a rapid decrease in the abundance of the C85R PHEX protein was clearly apparent with increasing time, indicating that the mutant protein was rapidly degraded.

The transmembrane and cytoplasmic domains of ECEL1 are involved in ER retention/localization

ER-localized membrane proteins usually have retention/localization signals in their structure [28]. To determine whether ECEL1 harbours such retention/localization signals, we constructed chimaeric type II membrane proteins, consisting of parts of ECEL1 fused to NEP (Figure 7A). Chimaeric protein E/E/N (see the Materials and methods section for explanatory details) was constructed by joining ECEL1 cytosolic and transmembrane domains to NEP ectodomain, whereas chimaeric protein E/N/N was obtained by fusing ECEL1 cytosolic domain to NEP transmembrane and ectodomain, and chimaeric protein N/E/N was obtained by replacing NEP transmembrane domain with that of ECEL1 (Figure 7A). The chimaeric proteins were expressed in HEK-293 cells, and cell extracts were analysed by immunoblotting with or without digestion by PNGase F and endo H. As observed for ECEL1 (Figure 7B, lanes 1–3), chimaeric protein E/E/N was sensitive to digestion by both PNGase F and endo H (Figure 7B, lanes 4–6), indicating that the protein had not acquired complex sugars. In contrast, proteins E/N/N and N/E/N were resistant to digestion with endo H (Figure 7B, lanes 7–9 and 10–12 respectively), suggesting that they reached the Golgi apparatus. This result indicates that both ECEL1 transmembrane and cytoplasmic domains are necessary for ER localization of the enzyme.

DISCUSSION

ECEL1 is one of the newest members of the M13 family of peptidases. It is found primarily in the central nervous system [9], and the phenotype of homozygote knock-out mice indicates that the enzyme plays an important role in the central control of breathing [11]. However, the mechanism by which ECEL1 controls this vital function and the natural substrate(s) of the enzyme are still unknown. To gain an insight into the physiological function of ECEL1, we have raised a specific monoclonal antibody and used it to study the biosynthesis of the enzyme.

Consistent with previous immunofluorescence results [13], we found that ECEL1 is mostly located in the cellular ER. However, our work clearly shows that a small portion of ECEL1 (probably less than 10 %) reaches the cell surface. This is most evident in experiments of cell-surface biotinylation of transfected HEK-293 cells, where two ECEL1 forms of 105 and 100 kDa respectively were detected in the plasma membrane. These two species differed in their glycosylation pattern, since the 100 kDa protein was sensitive to endo H digestion whereas the 105 kDa ECEL1 was resistant. It is unusual to find an endo H-sensitive protein at the cell surface, but the lack of biotin labelling of the ER-localized 95 kDa ECEL1 species and the absence of biotinylated actin suggest a specific cell-surface labelling of the 100 kDa ECEL1. A similar distribution of ECEL1 between the ER and cell surface was obtained when pituitary extracts were analysed by immunoblotting, indicating that this dual distribution is not due to overexpression of the protein in cultured transfected cells. The presence of ECEL1 at the cell surface suggests that one function of the enzyme consists in regulating the activity of extracellular peptides. NEP and ECE-1, two enzymes of the M13 family, are known to regulate the activity of extracellular peptides. NEP participates in the breakdown of peptides such as enkephalins and substance P, which are involved in pain control, and atrial natriuretic peptide, which regulates water and sodium reabsorption at the kidney (for a review, see [1]), whereas ECE-1 activates endothelin precursors giving rise to endothelins, which are powerful vasoconstrictors (for a review see [3]). More work is needed to identify ECEL1 extracellular substrates.

The presence of large amounts of ECEL1 in the ER can be due to defective intracellular transport to the plasma membrane, to slow folding of the protein and subsequent degradation, or to specific localization of the protein in the ER. To distinguish between these possibilities, we performed pulse–chase experiments. Two important observations were made: (1) core-glycosylated ECEL1 proved to be very stable in the ER; and (2) exit of ECEL1 from the ER after biosynthesis is slow, since after 3 h of chase we could not detect an endo H-resistant form (105 kDa species) of the enzyme. This last observation may explain the low amounts of ECEL1 at the cell surface. The stability of ECEL1 in the ER contrasts remarkably with the stability of PHEX mutant C85R, used as a control in this study, which was almost totally degraded after 60 min of chase. Similarly, misfolded proteins, such as CFTR (cystic fibrosis transmembrane conductance regulator) mutants [29] or unassembled subunits of multisubunit protein complexes (e.g. of the nicotinic acetylcholine receptor) that are trapped in the ER, present very short half-lives [30]. The stability of ECEL1 in the ER suggests that this enzyme is specifically retained, and has a physiological role in this cellular compartment.

Consistent with the hypothesis that ECEL1 is specifically retained in the ER are the results of domain-swapping experiments in which ECEL1 domains were exchanged for equivalent regions of NEP. Our findings indicated that both ECEL1 transmembrane and cytoplasmic domains are required for ER localization. Transmembrane domains have been identified as primary determinants mediating ER localization of several proteins [28]. However, in many cases, a contribution to localization was observed from the flanking cytosolic or luminal domains. This is the case for the yeast Sec12p [31] and the mammalian p63 [32] and cytochrome b_5

proteins [33], and all type II integral membrane proteins, as is ECEL1. As for ECEL1, the yeast protein Sec12p was shown to require signals in both its transmembrane and cytosolic domains to achieve ER residency, whereas the mammalian p63 protein has determinants in its three domains (cytosolic, transmembrane and luminal), and cytochrome b_5 possesses determinants in its luminal domain in addition to the transmembrane domain. More studies are needed to identify precisely the determinants responsible for ER localization present in ECEL1 cytosolic and transmembrane domains.

Although other enzymes of the M13 family, such as the ECEs and NEP2, have also been found in intracellular locations, ECEL1 is the first member of this family shown to possess a localization determinant in its transmembrane domain. ECE-1 is found as three isoforms, generated by the use of three distinct promoters on the same gene [34]. These isoforms, designated ECE-1a, ECE-1b and ECE-1c, differ only in their N-terminal cytosolic domain. Whereas ECE-1a and ECE-1b have completely different N-terminal amino acid sequences, ECE-1c corresponds to a truncated form of ECE1-b, lacking its first 17 N-terminal residues. Immunofluorescence microscopy has indicated that ECE-1a and ECE-1c are present at the cell surface [34], whereas ECE-1b localizes to intracellular compartments, identified as the TGN (*trans*-Golgi network) [34] or early and late endosomes [35]. Clearly, ECE-1b is localized to intracellular structures by its cytosolic domain.

The absence of activity at the cell surface indicates that ECE-2 is also present in intracellular compartments. Substrate localization and a requirement for an acidic pH for full activity suggest that this compartment is the TGN [36]. The structural determinant responsible for this TGN localization has not been identified. In the case of NEP2, both a membrane-bound and a soluble secreted enzyme have been reported [6–8]. Whereas the soluble secreted form of NEP2 is transported to the cell surface, immunofluorescence microscopy analysis of transfected cells and sensitivity to endo H have suggested that the membrane-bound protein is localized in early compartments of the secretory pathway, possibly the ER [6,12,37]. Here again, the structural determinant responsible for the intracellular localization of the membrane-bound NEP2 has not been identified.

In conclusion, our results clearly indicate that ECEL1 is present in two cellular locations: a major pool of the enzyme is found in the ER, where it is localized through determinants present in its transmembrane and cytoplasmic domains, and a minor pool is found at the plasma membrane. Whether cellular injuries that have been shown to induce *ECEL1* gene expression [10] influence the proportion of enzyme present in each pool remains to be determined.

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