Recombinant human endothelin-converting enzyme ECE-1b is located in an intracellular compartment when expressed in polarized Madin–Darby canine kidney cells

Arezou AZARANI, Guy BOILEAU and Philippe CRINE¹

Department of Biochemistry, Faculty of Medicine, University of Montréal, Montréal, Québec, H3C 3J7, Canada

Endothelin-converting enzyme (ECE) is a phosphoramidon-sensitive membrane-bound metalloprotease responsible for the conversion of big-endothelins into endothelins [Yanagisawa, Kurihara, Kimura, Tomobe, Kobayashi, Mitsui, Yazaki, Goto and Masaki (1988) *Nature* (London) **332**, 411–415]. Several distinct isoforms of ECE have been cloned and identified. ECE-1a, b and c have the same ectodomain and differ only by their cytosolic tails [Schweizer, Valdenaire, Nelböck, Deuschle, Edwards, Stumpf and Löffler (1997) *Biochem. J.* **328**, 871–877]. The ectodomain common to ECE-1 a, b and c shares extensive sequence similarities with neprilysin, a major kidney brush border metallopeptidase. To study the sorting of ECE in polarized cells, ECE-1b cDNA was expressed by transfection in polarized Madin–Darby canine kidney (MDCK) cells. Cell-surface biotinylation and immunofluorescence studies showed that ECE-1b is not expressed on the cell-surface but was rather located in intracellular compartments that could also be labelled with anti-Rab-5 and Rab-7 antibodies and was thus tentatively identified as early and late endosomes. Similar results were also obtained when ECE-1b was expressed in non-polarized Chinese hamster

ovary cells for comparison purposes. When MDCK or Chinese hamster ovary transfected cells were pre-treated with the ECE inhibitor phosphoramidon, a 3-fold increase in the level of ECE-1b was observed both by Western blotting and by enzymic activity. However, no change in the level of neprilysin or the β -chain of meprin, two apical membrane metallopeptidases, was observed in MDCK cells transfected under similar conditions. Northern blotting showed that the increase in the level of ECE-1b was not owing to changes in the ECE mRNA transcription rate or stability. Rather, pulse-chase experiments followed by immunoprecipitation showed a decrease in the rate of degradation of ECE-1b in phosphoramidon-treated cells. Half-lives were determined to be 2.8 and 7.5 h for non-treated and phosphoramidon-treated cells, respectively. Confocal microscopy showed accumulation of ECE-1b immunoreactive material in the lysosomes of phosphoramidon-treated cells. Taken together, these results suggest that ECE-1b turns over very rapidly between endosomal and lysosomal compartments and that lysosomal degradation of the enzyme is slowed down by phosphoramidon.

INTRODUCTION

Endothelins (ETs) are a family of peptides which play important roles in a variety of developmental processes [1–8], maintenance of vascular tone, cell proliferation and hormone production (for a review see [9]). A 21 amino acid peptide was originally isolated from cultured porcine aortic endothelial cells [10]. Three different ETs, called ET-1, ET-2 and ET-3, encoded by three distinct genes, have since been identified in human [11]. These ETs are produced as precursor forms and have to undergo a two-step processing to become fully active. The first step involves cleavage of the precursor at a dibasic amino acid sequence by furin [12,13], which results in the formation of an inactive intermediate form called pro-endothelin or big endothelin (big-ET). In the next step an enzyme called endothelin-converting enzyme (ECE) cleaves either a Trp-Val bond in big-ET-1 and big-ET-2 or a Trp-Ile bond in big-ET-3 to produce the mature active form of the peptide [10].

ECE is a 130 kDa type II integral membrane-bound protease with considerable sequence similarities to neprilysin (neutral endopeptidase-24.11, NEP) and Kell blood group proteins [14–16]. Similar to these enzymes, ECE consists of a short cytosolic N-terminal domain followed by a single hydrophobic transmembrane domain, and a large C-terminal extracellular domain (called the ectodomain) which contains the catalytic

Zn²⁺-binding motif (HEXXH). All three proteins also contain N-linked glycosylation sites as well as conserved cysteine residues. Several distinct isoforms of ECE have been cloned and identified, namely ECE-1 α , ECE-1 β and ECE-2. ECE-1 α was first cloned in rat and human by Shimada et al. [16,17], and in human by Schmidt et al. [18]. This isoform corresponds to human ECE-1b cloned by Valdenaire et al. [19] with the exception of a short N-terminal sequence missing from the ECE-1 α cytosolic domain. ECE-1 β was also cloned in rat and human by Shimada et al. [16,17,20] and corresponds to human ECE-1a cloned by Valdenaire et al. [19]. More recently, a third form of ECE-1, termed ECE-1c, has been cloned in humans [21]. The three isoforms of ECE-1 are derived from a single gene through the use of alternative promoters, whereas ECE-2 is encoded by a separate gene [14]. While ECE-1a, ECE-1b and ECE-1c differ only in their N-terminal cytosolic domains, ECE-1a and ECE-2 differ considerably over their entire sequence, including the catalytic extracellular domain, as they share only 59% amino acid identity [14]. ECE-1 isoforms and ECE-2 can also be distinguished on the basis of their cellular location, optimal pH, sensitivity to inhibitors such as phosphoramidon, and their preference for the processing of the three big-ETs [14,19,22–25].

The cell compartment where processing of the big-ETs by different ECEs occurs is still controversial, with evidence of both cell surface [20,24,26–28] and intracellular location [14,29,30].

Abbreviations used: ECE, endothelin-converting enzyme; MDCK, Madin–Darby canine kidney; CHO, Chinese hamster ovary; ET, endothelin; NEP, neprilysin, neutral endopeptidase-24.11; Endo H, endoglycosidase H; ER, endoplasmic reticulum.

¹ To whom correspondence should be addressed (e-mail crinep@BCH.UMontreal.CA).

Much effort has therefore been directed toward determining the specific location of different ECEs. While an intracellular location (possibly the *trans*-Golgi network) has been proposed for ECE-2 in transfected Chinese hamster ovary (CHO) cells [14], ECE-1 α was found to be expressed mostly on the cell surface of endothelial cells, expressing ECE endogenously, and in cells (i.e. COS and CHO) transfected with ECE-1 α cDNA [22,23,26,31].

Although ECE appears to be expressed on the luminal cell surface of polarized endothelial cells, very little is known about the mechanism of targeting ECE to its specific domain of the plasma membrane. NEP, which shares 37% amino acid identity and a similar membrane topology with ECE, is sorted to the apical membrane of kidney proximal tubule cells and transfected Madin–Darby canine kidney (MDCK) cells [32,33]. The aim of this study was to compare the sorting and turnover of ECE and NEP in polarized epithelial cells. To accomplish this we have expressed recombinant ECE-1b by transfection in MDCK cells. For comparative purposes, ECE-1b cDNA was also expressed in non-polarized CHO cells. Biotinylation and immunofluorescence studies showed that, contrary to what has been shown for NEP, ECE-1b is not expressed on the cell surface but is mostly located in intracellular compartments tentatively identified as early and late endosomes. Immunofluorescence studies performed on non-permeabilized cells on ice as well as determination of ECE-1b activity of non-permeabilized cells suggest that ECE-1b is not endocytosed from the cell surface but rather appears to be directly targeted from the Golgi to the endosomes. Unlike NEP, ECE was found to rapidly turn over in transfected MDCK cells (half-life of 2.8 h). However, when cells were treated with phosphoramidon, a potent metallopeptidase inhibitor, an increase in the level of ECE-1b protein and activity was observed. Combined data from Northern blotting, pulse-chase and immunofluorescence studies showed that this increase is not due to changes in mRNA levels but rather reflects a decrease in the rate of lysosomal degradation of ECE-1b protein.

MATERIALS AND METHODS

Materials

The human ECE-1b cDNA and the mouse antibody raised against the transmembrane domain of ECE-1 were provided by Dr. B. M. Löffler (F. Hoffmann–La Roche). The mouse antiserum against LAMP-2 (AC70) was kindly provided by Dr. R. Nabi (University of Montréal, Canada). The rabbit antisera raised against Rab 5 and Rab 7 were generous gifts from Dr. M. Desjardins (University of Montréal, Canada). The mouse monoclonal antibody (6H) against the α_1 subunit of Na/K-ATPase was kindly provided by Dr. M. J. Caplan (Yale University, U.S.A.). The rabbit polyclonal antibody against the β -subunit of meprin was a generous gift from Dr. A. J. Kenny (University of Leeds, U.K.). Detection of NEP was performed with a guinea-pig polyclonal anti-NEP antiserum for immunofluorescence and a mouse monoclonal antiserum (18B5) for Western blotting (both made in our laboratory). A rabbit polyclonal anti-ECE-1 (called #9) was raised against the ectodomain (Ser 188 to Ala 518) of ECE-1b in our laboratory. The antibody did not cross-react with NEP. Cross-reactivity with endogenous MDCK proteins was minimal as the antibody was found to bind weakly to lower relative molecular mass proteins in Western blots of non-transfected cell. When assayed in immunofluorescence confocal microscopy, the antibody did not stain any protein in non-transfected cells. The human actin cDNA was provided by Dr. L. DesGroseillers. Phosphoramidon was purchased from Sigma. Tissue-culture materials were obtained from Gibco/BRL.

Cell culture

MDCK cells were maintained in a 50:50 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium supplemented with 10% foetal bovine serum (FBS), 2 mM L-glutamine, 50 units/ml penicillin, 50 μ g/ml streptomycin, and 0.25 μ g/ml of fungizone. CHO cells were grown in Ham's F12 medium also supplemented with 10% FBS, 2 mM L-glutamine, 50 units/ml penicillin, 50 μ g/ml streptomycin, and 0.25 μ g/ml of fungizone. Stable transfected MDCK and CHO cells were maintained in their respective medium supplemented with 0.6 mg/ml of Geneticin. Cells were incubated in a humidified atmosphere of 95% air, 5% CO₂ at 37 °C.

Transfection and expression of human ECE-1b cDNA in MDCK and CHO cells

The full-length human ECE-1b cDNA was subcloned into the mammalian expression vector pRC/CMV (Invitrogen) that contains the aminoglycoside phosphotransferase 3' gene which confers resistance to the antibiotic Geneticin (G418 sulphate). The cDNA was transfected into MDCK and CHO cells using a calcium phosphate–DNA co-precipitation technique [34]. To select for stable expression of the ECE-1b protein, the cells were cultured in media containing G418 (0.6 mg/ml) starting 48 h after transfection and continued for a 1 to 2 week period. Surviving colonies were screened by Western blotting and immunofluorescence. The clone that expressed the highest level of ECE-1b was selected for further studies.

Cell surface biotinylation

Cells were grown on 24 mm diameter Transwell inserts (0.4 μ m pore, Costar) for 5 days and the medium was replaced with complete medium containing 10 mM sodium butyrate 24–48 h before the experiment. Steady-state biotinylation was carried out at pH 7.5 as described [35]. The biotinylated proteins were isolated with 100 μ l of packed immobilized streptavidin and analysed by SDS-PAGE and western blotting. The blot was incubated first with rabbit polyclonal antibody (#9) against ECE-1b (1:100 dilution) or with mouse monoclonal antibody (18B5) against NEP (1:200 dilution), then with a peroxidase-conjugated secondary antibody (Dako). The blots were then developed with Enhanced Chemiluminescence reagent (Dupont).

Immunofluorescence

Cells were grown on 24 mm diameter Transwell inserts or on 9 mm cell culture inserts (Gibco/BRL) and processed for indirect immunofluorescence [36]. Briefly, cells were rinsed with PBS (containing 1 mM MgCl₂ and 0.1 mM CaCl₂) (buffer A) and fixed in methanol for 10 min at room temperature. They were then washed in buffer A and permeabilized by incubation in a buffer A solution containing 0.3% Triton X-100 and 0.1% BSA (buffer B) for 15 min. After 30 min blocking incubation in buffer A solution containing 1% BSA and 0.3% Triton X-100 (buffer C), cells were incubated with primary antibodies diluted in the same blocking solution. The cells were then washed three times (5 min each) in buffer B and were incubated with fluorescein isothiocyanate-conjugated anti-rabbit IgG (Dako) (diluted 1:20) or the Texas-Red-conjugated anti-mouse IgG (diluted 1:1000) for 1 h. The cells were washed three times in buffer B for 5 min each and then once for 10 min in 5 mM NaP_i (pH 7.5) before mounting. Filters and coverslips were then mounted in a freshly prepared solution of 0.1% *p*-phenylenediamine (Sigma), 70% glycerol, 150 mM NaCl, 10 mM NaP_i (pH 7.4) and sealed with

rubber-cement. The cells were examined by a Zeiss Axiophot microscope and by a Bio-Rad laser scanning confocal microscope (MRC 600). Confocal images were generated using the following excitation/emission wavelengths: 488 nm/515–560 nm (for fluorescein-labelled antibodies), and 568 nm/590–640 nm (for Texas-Red-labelled antibodies). For single labelling of ECE-1b the rabbit polyclonal antibody #9 was used in a 1:50 dilution, for NEP the guinea-pig polyclonal antibody was used in a 1:100 dilution, and for Na/K-ATPase the mouse monoclonal antibody 6H was used in a 1:100 dilution. For double labelling of ECE-1b and Rab 5 and Rab 7, the mouse monoclonal antibody P4 was used in a 1:100 dilution for the detection of ECE-1b, and rabbit polyclonal antibodies Rab 5 and Rab 7 were used in a 1:100 dilution. For the double labelling of ECE-1b and LAMP-2 the rabbit polyclonal antibody #9 was used in a 1:50 dilution and the mouse monoclonal antibody, AC70, was used in a 1:200 dilution.

Pulse-chase study and immunoprecipitation

Cells were grown on 60 mm dishes and pulse-chase studies were performed as previously described [37]. In short, the cells were washed in DMEM lacking methionine and were 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 [³⁵S]methionine (200 µCi/ml, 1 ml/dish) (1328 Ci/mmol, ICN). After the pulse, the cells were rinsed twice in the same medium containing non-radioactive methionine in excess (0.15 mg/ml) (Sigma) and were chased for various periods of time in this new medium. The cells were then washed and scraped in PBS. The PBS was removed by centrifugation (10 min, 4000 r.p.m.). The cells were then dissolved in 200 µl of 1 % N-octylglucoside (Boehringer Mannheim). To this was added 200 µl of 2X IPP buffer (20 mM Tris, 2 % Triton X-100, 100 mM NaCl, 0.2 % SDS, 0.2 % BSA, 2 % sodium deoxycholate) and 10 µl of the rabbit polyclonal antibody #9. The samples were incubated overnight at 4 °C under constant agitation. The immune complexes were immunoprecipitated by adding protein A-Sepharose beads (Pharmacia). The recovered labelled molecules were analysed by SDS/PAGE and fluorography.

Northern blot analysis

Total RNA was extracted from cell cultures using Trisol (Gibco/BRL). The RNA preparation was denatured in 37 % formaldehyde and separated by electrophoresis in 1.5 % agarose gel, transferred onto nitrocellulose membrane filters and baked at 80 °C for 2 h. The RNA blots were then hybridized with human cDNAs encoding ECE-1b (nucleotides 1–2500) or actin (nucleotides 1–1199). Probes were labelled with [α -³²P]dCTP using an Amersham Multiprime DNA labelling kit. The filters were hybridized and washed as previously described [38]. The filters were then exposed to autoradiographic film with an intensifying screen for 2 days at –70 °C.

ECE enzymic activity

ECE-1b activity was measured by following the conversion of big-ET-1 into ET-1 with a specific radioimmunoassay (RIA) for ET-1 [39]. In brief, dissolved cells were incubated with big-ET-1 in a buffer containing 50 mM Tris/HCl, 0.1 % BSA, 0.1 % Na₂S₂O₈, pH 7.0 for a period of 30 min at 37 °C. The reaction was stopped by the addition of EDTA (150 mM). The tracer, ¹²⁵I-ET-1 (NEN, 2200 Ci/mmol), was then added to the samples (25000 cpm/reaction) as well as 50 µl of rabbit polyclonal antibody against ET (#1009), (1:1000) (provided by Dr. B.-M. Löffler,

Hoffmann-La Roche). The samples were then incubated overnight at 4 °C and the immune complexes were immunoprecipitated with Amerlex-M beads (Amersham). The radioactivity in supernatants was analysed by a γ -counter and transformed into pg of endothelin-1 by using a standard curve established with purified synthetic endothelin-1 (Alexis corporation). ECE-1b activity was measured by the difference between the amount of endothelin-1 immunoreactive material obtained in the presence and absence of 160 µM phosphoramidon (a concentration necessary for complete inhibition of ECE activity). The activity of this enzyme is reported in terms of production of ET-1 in pg·min⁻¹·µg⁻¹ of total protein. ECE-1b activity of non-permeabilized (whole) cells were also measured using the above procedure with the exception that cells were not solubilized with 1 % N-octylglucoside.

RESULTS

Expression of ECE-1b in CHO and MDCK cells

Human ECE-1b cDNA was expressed by stable transfection in polarized epithelial MDCK cells. These cells were then solubilized and analysed under reducing conditions by Western blotting

Figure 1 Expression of ECE-1b in MDCK cells

Wild-type MDCK cells were stably transfected with full-length human ECE-1b cDNA and analysed by SDS/PAGE and Western blotting. The blots were incubated first with rabbit polyclonal antibody (#9) raised against the ectodomain of ECE-1 (1:100) and then with a rabbit peroxidase-conjugated secondary antibody and developed with Enhanced Chemiluminescence reagent. In **A**, a diffused 130 kDa band corresponding to ECE-1b was observed under reducing conditions in transfected MDCK cells. No band was detected in non-transfected wild-type (W.T.) MDCK cells. In **B**, ECE-1b protein was digested by peptide-N-glycosidase F and Endo H. A sharp band of about 95 kDa was observed after Endo F digestion only.

Figure 2 Intracellular localization of ECE-1b in transfected MDCK cells

Cells were grown on Transwell inserts or on 24 mm cell culture inserts and processed for indirect immunofluorescence according to the procedure in the Materials and methods section. MDCK cells expressing NEP apically and Na/K-ATPase basolaterally were used as controls. *En face* (A, B and C) and *xz* section (D, E and F) confocal images show that NEP (A and D) and Na/K-ATPase (B and E) are expressed on the apical and basolateral surfaces respectively. In contrast ECE-1b (C and F) is expressed intracellularly. (Scale bars = 20 μ m).

using a rabbit polyclonal antibody raised against the ectodomain of ECE-1. As shown in Figure 1 (panel A), a double band of approximately 130 kDa was observed in transfected MDCK cells but not in non-transfected wild-type cells. When MDCK cell extracts were digested with peptide N-glycosidase F, this double band disappeared and was replaced by a sharp band of about 95 kDa (panel B). Endoglycosidase H (Endo H) failed to digest the 130 kDa band, suggesting that the ECE-immunoreactive protein carries complex oligosaccharides normally acquired after passage through the Golgi apparatus. Similar results were obtained in transfected CHO cells (not shown). The ECE enzymic activity found in cell extracts was estimated by measuring the conversion of big ET-1 into ET-1 using an RIA technique. Transfected MDCK and CHO cells produced 0.70 ± 0.09 pg and 0.57 ± 0.02 pg of ET-1 per min per μ g of total protein, respectively. This activity was fully abolished by 160 μ M phosphoramidon. No activity was found in non-permeabilized transfected MDCK or CHO cells or in wild-type cells.

Intracellular location of ECE-1b

The results of confocal immunofluorescence studies are shown in Figure 2. ECE-1b was conspicuously absent from both the apical

and basolateral surfaces of MDCK cells but was seen mostly as a punctuate signal in intracellular compartments (Figure 2C, *en face*, and 2F, *xz* section, confocal images). A similar intracellular ECE-1b localization was also observed in CHO cells (not shown). For comparison purposes, the localization of bona fide apical and basolateral proteins was also studied. The same MDCK cells were shown to express endogenous Na/K-ATPase on the basolateral surface (Figure 2B, *en face*, and 2E, *xz* section) whereas another NEP-transfected MDCK cell line exhibited a strong immunofluorescence on its apical membrane when stained with a NEP specific antibody (Figure 2A, *en face*, and 2D, *xz* section). Double immunofluorescence studies of transfected MDCK cells were then performed to better characterize the subcellular compartment where ECE accumulates. In Figure 3(A) (left hand side) cells were double stained to detect both Rab 5, a small relative molecular mass GTPase used as an early endosome marker [40], and ECE-1b (Figure 3A right hand side). The same experiment was repeated with Rab 7 (panel C), a late endosomal marker [41]. Superimposition of the signals for ECE and Rab proteins is shown in Figure 3 (panels B and D). These results clearly indicate a co-localization of ECE with Rab 5 and Rab 7 (yellow spots). When the transfected MDCK cells were double stained with antibodies for both ECE and LAMP-2, a lysosomal

Figure 3 Co-localization of ECE-1b with Rab 5 and Rab 7

In panels **A** and **C** cells were double stained with antibodies for Rab 5 (panel **A**, left hand side) and Rab 7 (panel **C**, left hand side) and visualized with a fluorescein-green-conjugated secondary antiserum. Simultaneously a mouse monoclonal antibody against ECE-1b was also used and visualized with Texas-Red secondary antibody. Co-localization of ECE-1b with either of these proteins is shown in **B** and **D**. These results show that ECE-1b is co-localized (evident from the presence of yellow spots) with Rab 5 and Rab 7. (**E**) (Left hand side) cells were double stained with a rabbit polyclonal antibody (#9) against ECE-1b and visualized with fluorescein-conjugated secondary antibody as well as with a Texas-Red conjugated antibody to detect LAMP-2 protein–antibody complexes (right hand side). Very little or no co-localization, however, was observed between ECE-1b and LAMP-2 (**F**). (**G**) Double immunofluorescence studies were performed on cells double stained with a fluorescein-conjugated antibody to detect ECE-1b protein–antibody complexes (left hand side) and a mouse monoclonal antibody against LAMP-2 visualized with Texas-Red secondary antibody (right hand side). Co-localization of ECE-1b and LAMP-2 after treatment of transfected MDCK cells with 100 μ M phosphoramidon for 48 h is shown in **H**. (Scale bars = 20 μ m).

Figure 4 Cell-surface biotinylation of ECE-1b and NEP in transfected MDCK cells

Transfected MDCK cells were grown on Transwell inserts and analysed by steady-state biotinylation according to the procedures in the Materials and methods section. In panel **A**, biotinylation was performed either on the apical (**A**) or basolateral (**B**) membrane on MDCK cells transfected (+) or non-transfected (–) with the ECE-1b vector. The presence of ECE in cell extracts was verified by performing the biotinylation on solubilized cells. In Panel **B**, the same experiment was performed on MDCK cells transfected with NEP cDNA.

protein [41] (panel E left and right hand sides, respectively), very little co-localization was observed (Figure 3F). Similarly ECE-1b was not found in the same compartment as AMF-R, a marker for microtubule-associated tubular organelles [41], or β -COP, a specific marker for the Golgi apparatus (results not shown).

Cell-surface biotinylation studies also failed to detect ECE-1b at the cell surface (Figure 4). The same results were obtained after incubation of non-permeabilized cells with the ECE-specific antibody on ice to prevent endocytosis. These results together with the fact that no ECE activity was detected on non-permeabilized transfected MDCK and CHO cells suggest that ECE-1b is transported directly from Golgi to the early and late endosomes and that virtually no enzyme reaches the cell surface.

The effect of phosphoramidon on the ECE-1b expression

Metallopeptidase inhibitors phosphoramidon and thiorphan were recently shown to cause a dose-dependent increase in the level of expression of ECE-1 α in endothelial cells [23]. Both agents were also found to cause the redistribution of ECE-1 α from the cell surface to an intracellular compartment. To find out whether these inhibitors had the same effects on MDCK cells transfected with ECE-1b, cells were treated with different concentrations (0.01 to 100 μ M) of these inhibitors for 48 h (Figure 5, panel A). A time course experiment was also performed to study the effect of the same inhibitors between 2 and 48 h (Figure 5, panel B). Phosphoramidon was found to cause a dose- and time-dependent increase (of up to 3 times) in the level of ECE activity present in the cells. A similar effect of phosphoramidon was observed in transfected CHO cells where phosphoramidon produced a 4-fold increase in the level of ECE activity (results not shown). The increase in the level of ECE-1b activity was observed as soon as two hours after the beginning of the pre-

Figure 5 Effect of phosphoramidon on ECE-1b protein and activity in MDCK cells

The activity of ECE-1b was measured by RIA techniques in terms of production of ET-1. Phosphoramidon sensitive ECE-1b activity was defined as the difference between its activity in the presence and absence of 160 μ M phosphoramidon (concentration necessary for complete inhibition of ECE activity). The activity of this enzyme is reported in terms of production of ET-1 in pg per min per μ g of total protein. (**A**) Different concentrations of phosphoramidon (0.01 to 100 μ M for 48 h) caused a significant increase ($P < 0.03$ as determined by a two-tailed Student's *t* test) in the level of the activity of ECE-1b in the transfected MDCK cells with the highest level obtained with a 0.1 μ M phosphoramidon concentration (causing an almost 3-fold increase). (**B**) 0.1 μ M concentration of phosphoramidon caused a time-dependent increase in the level of activity of ECE-1b in the MDCK cells ($P < 0.05$). No ECE-1b activity was observed in wild-type MDCK cells. (**C**) Level of ECE-1b protein was estimated from the results of western blotting experiments using a densitometer. The level of ECE-1b protein (measured from the intensity of each band and expressed in arbitrary units) is plotted against log [phosphoramidon]. A 3-fold increase was also observed in the level of ECE-1b protein. The results are expressed as the mean $\pm$ S.E. of up to four determinations. Values statistically different from control are indicated with asterisks.

treatment period. A 3-fold increase in the level of ECE-1b was also observed by Western blotting. These results are shown in Figure 5 (panel C) in which the level of ECE-1b protein estimated by a densitometer is plotted against the concentration of phosphoramidon. Parallel experiments showed that phosphoramidon treatment of cell cultures expressing either recombinant NEP or the β subunit of meprin in the same conditions failed to increase the level of these heterologous proteins. Contrary to previously published results [23], thiorphan had no effect on the level of ECE-1b expression in MDCK cells (results not shown). Taken together, these results suggest that phosphoramidon induces an increase in the intracellular level of ECE-1b, that this increase is specific to phosphoramidon as it is not observed with

Figure 6 Effect of phosphoramidon on ECE-1b protein expression observed by immunofluorescence

The increase at the level of the expression of ECE-1b protein upon its pre-treatment with 100 μ M phosphoramidon for a period of 48 h was observed by immunofluorescence. ECE-1b was labelled with the rabbit polyclonal antibody #9 in a 1:50 dilution. This protein was then visualized by fluorescein isothiocyanate-conjugated anti-rabbit IgG in a 1:20 dilution. Transfected MDCK cells were examined by a Bio-Rad laser scanning confocal microscope. In **A**, the transfected MDCK cells are untreated while in **B**, the cells are treated with phosphoramidon. (Scale bars = 20 μ m).

other metallopeptidase inhibitors and that phosphoramidon appears to affect ECE-1b and not other related metallopeptidases.

The increase in ECE-1b activity found in phosphoramidon-treated cells was also observed by confocal immunofluorescence studies. As shown in Figure 6, the intensity of the intracellular signal was increased after 100 μ M phosphoramidon treatment for 48 h. Moreover a substantial percentage of the ECE-1b immunoreactive material was still localized with Rab5 and Rab7 (not shown). However, some of the ECE-1b was now found in a location that also contained the lysosomal protein LAMP-2 (Figures 3G and 3H), whereas in the absence of phosphoramidon there was virtually no co-localization of LAMP-2 and ECE (Figures 3E and 3F). When transfected MDCK cells were treated with leupeptin (10 μ g/ml for 60 min) or ammonium chloride (15 mM for 90 min) (two inhibitors of lysosomal degradation) a pattern of co-localization between ECE-1b and LMAP-2 similar to the one seen when cells were treated with phosphoramidon was observed. ECE lysosomal localization, however, was not observed when these cells were treated with thiorphan (results not shown).

The effect of phosphoramidon on ECE-1b expression in transfected cells could either be due to an upregulation of the mRNA levels or to a decrease in the rate of degradation of the enzyme. To distinguish between these possibilities, both Northern blots and pulse-chase experiments were performed next. Northern blotting experiments showed that phosphoramidon does not change the mRNA levels for ECE-1b (Figure 7A). To find out whether phosphoramidon was affecting the rate of degradation of ECE-1b, cells were first pulsed for 15 min in the presence or absence of 100 μ M phosphoramidon. Cells were then washed and chase-incubated (in the presence or absence of phosphoramidon) for various times in a medium that contained excess methionine. The radioactive material immunoprecipitated throughout the pulse-chase experiment was analysed by SDS/PAGE and fluorography. The amount of ECE recovered in each sample was quantitated by microdensitometry and the results are plotted in Figure 7B. The results clearly indicate that the amount of ECE was greater in phosphoramidon-treated cells than in non-treated cells. The half-lives for ECE-1b in non-treated and phosphoramidon-treated cells were determined from Figure 7(B) and found to be 2.8 and 7.5 h respectively.

DISCUSSION

There appear to be several isoforms of human ECE-1. Shimada et al. [16,17,20] cloned two isoforms which they named ECE-1 α and ECE-1 β . Valdenaire et al. [19] also cloned initially two isoforms and named them ECE-1a and ECE-1b. The ECE-1b cloned by this group (and used by us in this paper) corresponds to ECE-1 α cloned by the two former groups with the exception of a small additional N-terminal sequence in the N-terminal cytosolic domain of ECE-1b. ECE-1 α cDNA thus appears to be missing an upstream in-frame start codon. More recently, a third isoform of ECE-1, termed ECE-1c has been cloned and shown to also arise through the use of an alternative promoter in the same gene [21].

ECE belongs to the same metallopeptidase family as NEP and shares extensive sequence similarities with this enzyme. NEP is expressed apically in epithelial cells. Angiotensin-converting enzyme, another related metallopeptidase has been shown to be concentrated along the luminal surface of the vascular endothelium where it metabolizes circulating vasoactive peptides, including angiotensin I and bradykinin [42,43]. Very little, however, is known about the targeting of ECE in polarized cells with distinct plasma membrane domains.

MDCK cells are one of the best models for studying protein sorting in polarized cells (for a review see [44]). In this study, we have transfected ECE-1b cDNA in MDCK cells. Parallel studies were also performed on non-polarized CHO cells for comparative purposes. ECE-1b was detected in Western blots with a rabbit polyclonal antibody. The enzyme was shown to be resistant to Endo H digestion. In contrast, endoglycosidase F digestion reduced its molecular mass from 130 to 95 kDa. Taken together, these results suggest that recombinant ECE has acquired terminal sugars after its passage in the Golgi apparatus. Folding of the protein into its native conformation was also thought to occur normally both in MDCK and CHO cells as the recombinant enzyme was found to be active and to catalyse the processing of big-ET into ET.

Biotinylation and immunofluorescence studies indicated that ECE-1b is expressed in intracellular compartments rather than at the cell surface. Double immunofluorescence studies suggest a predominant localization in the early and late endosomes in both

Figure 7 Northern blotting and pulse-chase experiments in phosphoramidon-treated cells

Panel **A**: MDCK cells were pretreated with different concentrations of phosphoramidon (0.01 to 100 μ M) for various periods of time. Total RNA was then extracted from cells and similar amounts of RNA were then analysed by Northern blotting as explained in the Materials and methods section. Human actin cDNA probe was used to show that the same amount of total RNA was loaded in each case, except for wild-type MDCK cells, where the RNA load was doubled. Panel **B**: cells were first pulsed for 15 min with [³⁵S]methionine and then chased for different periods of time in the presence or absence of 100 μ M phosphoramidon and the amount of the ECE-1b protein was estimated by measuring the intensity of each band with a densitometer and expressed in arbitrary units. These values were plotted against the time of chase. Results are expressed as the mean $\pm$ S.E. of up to three determinations from three separate experiments.

MDCK and CHO cells. Careful analysis of confocal immunofluorescence pictures gave no evidence for cell surface expression of ECE. The absence of ECE from the cell surface was also confirmed by the fact that no endothelin-converting activity was detected by the very sensitive RIA assay when the substrate was incubated with non-permeabilized transfected MDCK or CHO cells. There could be two explanations to account for the intracellular localization of the ECE-1b isoform: (i) ECE-1b is first targeted to the cell surface but rapidly endocytosed into endosomes where it resides; (ii) ECE-1b is directly targeted to and retained into its final endosomal compartment. Immuno-

fluorescence studies performed by incubation of non-permeabilized cells with the ECE-specific antibody on ice to prevent endocytosis failed to detect ECE-1b at the cell surface of transfected cells. Taken together, our results therefore suggest that ECE-1b is most probably not an endocytosed protein but rather a protein that is directly transported from Golgi to the early and late endosomes.

Recent studies by Barnes et al. [22,23] and Takahashi et al. [26] showed that 90 % of ECE-1 α is expressed on the cell surface of endothelial cells endogenously expressing this protein. The same observation was also made in COS and CHO cells transfected with expression vectors encoding this isoform [17,26]. In these studies, only about 10 % of this protein was reported to be present in an intracellular compartment identified as the Golgi apparatus. In a more recent study, ECE-1a and ECE-1c were found to be expressed at the cell surface of transfected CHO cells whereas ECE-1b was found to be intracellular and showed significant co-localization with a marker protein for the *trans*-Golgi network [21]. In the ECV304 endothelial cell line used by the same authors, the pattern of immunofluorescence labelling also suggests an intracellular localization for ECE-1b, thereby confirming in an endogenous system the distribution of ECE-1b found in transfected cells.

The difference in localization between ECE-1 α as observed by others, and ECE-1b in our study as well as in the study of Schweizer et al. [21] is puzzling. We cannot exclude the possibility that the targeting mechanism of ECE varies in some cell types. Another possible explanation might be the presence of an additional 17 amino acid N-terminal sequence in the cytoplasmic domain in ECE-1b compared with recombinant ECE-1 α . The integrity of the N-terminal cytosolic domain of ECE-1b might be crucial to determine the proper targeting of the protein. This hypothesis would have to be confirmed by introducing mutations and deletions in the cytosolic domains of the different ECE-1 isoforms to determine the specific amino acid(s) involved in the sorting of this type of protein.

ET, the most potent vasoconstrictor peptide identified to date, plays a crucial role in many normal developmental processes. Overproduction of this hormone, however, is believed to be responsible for numerous pathological conditions, such as cardiovascular (hypertension, stroke, and ischaemic heart diseases), renal and respiratory disorders. Its mitogenic role in promoting tumour growth has also been reported [15]. Since ECE plays a key role in the conversion of inactive big-ET into active ET, inhibition of ECE might be of some therapeutic value. Phosphoramidon, a potent NEP inhibitor, has been reported to inhibit the activity of ECE-1 with an EC₅₀ of 1 μ M [45]. However, when transfected MDCK and CHO cells were treated with concentrations of phosphoramidon in the μ M range, a 3–4-fold increase in the levels of ECE-1b protein and activity was observed. These results are in agreement with those obtained by Barnes et al. [23] who showed that pretreatment of cells expressing ECE-1 α with phosphoramidon also produced a dose-dependent increase in the level of ECE-1 α protein. Similarly, Matsumura et al. [46] showed that phosphoramidon also increases the release of big-ET-1 and ET-1 in human aortic endothelial cells. These authors proposed that while the later effect might be owing to the phosphoramidon-induced inhibition of ET-1 degradation by NEP, the former could be due partly to a transient stimulation of the expression of prepro ET-1 mRNA. Corder et al. [47] observed the same effect of phosphoramidon on ET-1 production in human endothelial cell line EA.hy 926, but not in cultured bovine aortic endothelial cells. They proposed that this cell-specific effect of phosphoramidon on the ET-1 production may be owing to the presence of distinct ECEs in these two cell types.

There are three possible explanations for the upregulation of ECE-1b by phosphoramidon. One possibility is that phosphoramidon can upregulate ECE mRNA transcription rate and stability. A second possibility is that phosphoramidon affects the structural stability of ECE-1b so that it is retained longer in the early and late endosomes before its transport and degradation in the lysosomes. The last possibility is that phosphoramidon inhibits lysosomal degradation of the ECE-1b. While Northern blotting experiments ruled out the first possibility, pulse-chase and immunofluorescence studies confirmed that phosphoramidon decreases the rate of degradation of ECE-1b. Confocal immunofluorescence studies showed that in phosphoramidon-treated cells there was a larger accumulation of ECE-1b protein in the lysosomes as evident by co-localization of ECE-1b and LAMP-2 (Figure 3G and 3H). Similar results were obtained when transfected MDCK cells were treated with leupeptin or ammonium chloride, which are inhibitors of lysosomal degradation (results not shown). Taken together, these results suggest that phosphoramidon promotes the intracellular accumulation of ECE-1b by interfering with lysosomal degradation of the enzyme. More studies, however, are needed to understand the specific effect of phosphoramidon on lysosomal functions. The effect of phosphoramidon on other ECE-1 isoforms and ECE-2 has to be determined as well.

Our results show that, in transfected cells, phosphoramidon caused a dose-dependent increase in the activity of ECE-1b to reach a maximum of a 3-fold increase at a concentration of 0.1 μ M. At higher concentrations, the effect started to level-off and even slightly decreased compared with the maximal effect observed at a concentration of 0.1 μ M. These results are somewhat different from those obtained when protein levels, rather than enzyme activities were measured. The level of ECE-1b protein increases 3-fold when the concentration of phosphoramidon increases from 0 to 100 μ M with no evidence of a decrease at higher concentrations. These differences could be explained by the fact that, at higher concentrations, an inhibitory effect of phosphoramidon on ECE-1b activity could partially counterbalance the increase in protein levels.

Barnes et al. [23], claimed that both thiorphan, a specific inhibitor of NEP, and phosphoramidon increased the levels of ECE-1 α . These authors proposed that both agents induce the internalization of ECE-1 α from the cell surface into an intracellular compartment tentatively identified as the Golgi apparatus. In our study, however, thiorphan, failed to alter the levels of ECE-1b and NEP in transfected MDCK cells. Moreover, we were unable to observe the internalization of ECE-1b from the cell surface to the Golgi compartment upon treatment of ECE-1b transfected MDCK or CHO cells with either phosphoramidon or thiorphan. Whether these differences are owing to variations in the cytosolic domains of these two isoforms of ECE, as discussed above, has to be studied further. All these results also clearly stress the need for further investigating the effect of various synthetic inhibitors of ECE that are presently produced in many laboratories.

This work was supported by a grant from the Medical Research of Canada to P.C.; A.A. is the recipient of a postdoctoral fellowship award from the MRC. We thank Dr. B.-M. Loeffler for providing antibodies, the material for enzymic assays and the cDNA for ECE-1b. Special thanks go to Drs. I. R. Nabi and J. Ahn for their advice. We are indebted to Drs. I. R. Nabi, M. J. Caplan, Desjardins, and A. J. Kenny for providing various antibodies and to Dr. L. DesGroseillers for providing the human actin cDNA. We thank L. Lépérance and C. Vezina for excellent technical assistance. Confocal microscopy was performed in the laboratory of Dr. M. Bendayan (University of Montréal) with the technical assistance of D. Gingras.

REFERENCES

- Chen, Y.-G., Danoff, A. and Shields, D. (1995) *J. Biol. Chem.* **270**, 18598–18605
- Kurihara, Y., Kurihara, H., Suzuki, H., Kodama, T., Maemura, K., Nagai, R., Oda, H., Kuwaki, T., Cao, W. H. and Kamada, N. (1994) *Nature (London)* **368**, 703–710
- Puffenberger, E. G., Hosoda, K., Washington, S. S., Nakao, K., DeWit, D., Yanagisawa, M. and Chakravart, A. (1994) *Cell* **79**, 1257–1266
- Baynash, A. G., Hosoda, K., Giald, A., Richardson, J. A., Emoto, N., Hammer, R. E. and Yanagisawa, M. (1994) *Cell* **79**, 1277–1285
- Hosoda, K., Hammer, R. E., Richardson, J. A., Baynash, A. G., Cheung, J. C., Giald, A. and Yanagisawa, M. (1994) *Cell* **79**, 1267–1276
- Kennedy, R. L., Haynes, W. G. and Webb, D. J. (1993) *Clin. Endocrinol.* **39**, 259–265
- Masaki, T. and Yanagisawa, M. (1992) *Essays Biochem.* **27**, 79–89
- Goto, K. and Warner, T. D. (1995) *Nature (London)* **375**, 539–540
- Rubanyi, G. M. and Polokoff, M. A. (1994) *Pharmacol. Rev.* **46**, 325–415
- Yanagisawa, M., Kurihara, H., Kimura, S., Tomobe, Y., Kobayashi, M., Mitsui, Y., Yazaki, Y., Goto, K. and Masaki, T. (1988) *Nature (London)* **332**, 411–415
- Inoue, A., Yanagisawa, M., Kimura, S., Kasuya, Y., Miyauchi, T., Goto, K. and Masaki, T. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 2863–2867
- Denault, J.-B., Claign, A., D'Orléans-Juste, P., Sawamura, T., Kido, T., Masaki, T. and Leduc, R. (1995) *FEBS Lett.* **362**, 276–280
- Laporte, S., Denault, J.-B., D'Orléans-Juste, P. and Leduc, R. (1993) *J. Cardiovasc. Pharmacol.* **22** (Suppl. 8), S7–S10
- Emoto, N. and Yanagisawa, M. (1995) *J. Biol. Chem.* **270**, 15262–15268
- Rawlings, N. D. and Barrett, A. J. (1993) *Biochem. J.* **290**, 205–218
- Shimada, K., Takahashi, M. and Tanzawa, K. (1994) *J. Biol. Chem.* **269**, 18275–18278
- Shimada, K., Takahashi, M., Ikeda, M. and Tanzawa, K. (1995) *FEBS Lett.* **371**, 140–144
- Schmidt, M., Kroger, B., Jacob, E., Seiberger, H., Subkowski, T., Otter, R., Meyer, T., Schmalzing, G. and Hillen, H. (1994) *FEBS Lett.* **356**, 238–243
- Valdenaire, O., Rohrbacher, E. and Mattei, M. G. (1995) *J. Biol. Chem.* **270**, 29794–29798
- Shimada, K., Matsushita, Y., Wakabayashi, K., Takahashi, M., Matsubara, A., Iijima, Y. and Tanzawa, K. (1995) *Biochem. Biophys. Res. Commun.* **207**, 807–812
- Schweizer, A., Valdenaire, O., Nelböck, P., Deuschle, U., Edwards, J. B., Stumpf, J. G. and Löffler, B. M. (1997) *Biochem. J.* **328**, 871–877
- Barnes, K., Murphy, L. J., Takahashi, M., Tanzawa, K. and Turner, A. J. (1995) *J. Cardiovasc. Pharmacol.* **26**, S37–S39
- Barnes, K., Shimada, K., Takahashi, M., Tanzawa, K. and Turner, A. J. (1996) *J. Cell Sci.* **109**, 919–928
- Corder, R., Khan, N. and Harrison, V. J. (1995) *Biochem. Biophys. Res. Commun.* **207**, 355–362
- Battistini, B. and Botting, R. (1995) *Drug News Perspect.* **8**, 365–391
- Takahashi, M., Fukuda, K., Shimada, K., Barnes, K., Turner, A. J., Ikeda, M., Koike, H., Yamamoto, Y. and Tanzawa, K. (1995) *Biochem. J.* **311**, 657–665
- Waxman, L., Doshi, K. P., Gaul, S. L., Wang, S., Bednar, R. A. and Stern, A. M. (1994) *Arch. Biochem. Biophys.* **308**, 240–253
- Matsumura, Y., Hisaki, K., Takaoka, M. and Morimoto, S. (1990) *Eur. J. Pharmacol.* **185**, 103–106
- Xu, D., Emoto, N., Giald, A., Slaughter, C., Kaw, S., DeWit, D. and Yanagisawa, M. (1994) *Cell* **78**, 473–485
- Gui, G., Xu, D., Emoto, N. and Yanagisawa, M. (1993) *J. Cardiovasc. Pharmacol.* **22** (Suppl. 8), 553–556
- Ingbar, D. H., Hepler, K., Dowin, R., Jacobsen, E., Dunitz, J. M., Nici, L. and Jamieson, J. D. (1995) *Am. J. Physiol. Lung Cell. Mol. Physiol.* **269**, L261–L271
- Jalal, F., Lemay, G., Zollinger, M., Berteloot, A., Boileau, G. and Crine, P. (1991) *J. Biol. Chem.* **266**, 19826–19832
- Jalal, F., Dehbi, M., Berteloot, A. and Crine, P. (1994) *Biochem. J.* **302**, 669–674
- Chen, C. and Okayama, H. (1987) *Mol. Cell. Biol.* **7**, 2745–2752
- Gottardi, C. J., Dunbar, L. A. and Caplan, M. J. (1995) *Am. J. Physiol.* **268**, F285–F295
- Gottardi, C. J. and Caplan, M. J. (1993) *J. Cell Biol.* **121**, 283–293
- Corbeil, D., Boileau, G., Lemay, G. and Crine, P. (1992) *J. Biol. Chem.* **267**, 2798–2801
- Azarani, A., Orlowski, J. and Goltzman, D. (1995) *J. Biol. Chem.* **270**, 23166–23172
- Löffler, B. M. and Maire, J. P. (1994) *Endothelium* **1**, 273–286
- De Hoop, M. J., Huber, L. A., Stenmark, H., Williamson, E., Zerial, M., Parton, R. G. and Dotti, C. G. (1994) *Neuron* **13**, 11–22
- Benlimame, N., Simard, D. and Nabi, I. R. (1995) *J. Cell Biol.* **129**, 459–471
- Takada, Y., Hiwada, K. and Kokubu, T. (1981) *J. Biochem. (Tokyo)* **90**, 1309–1319
- Caldwell, P. R., Seegal, B. C., Hsu, K. C., Das, M. and Soffer, R. L. (1976) *Science* **191**, 1050–1051

- 44 Mostov, K., Apodaca, G., Areoti, B. and Okamoto, C. (1992) *J. Cell Biol.* **116**, 577–583
- 45 Romero, G., Luttrell, L., Rogol, A., Zeller, K., Hewlett, E. and Lerner, J. (1988) *Science* **240**, 509–511
- 46 Matsumura, Y., Tsukahara, Y., Kojima, T., Murata, S., Murakami, A., Takada, K., Takaoka, M. and Morimoto, S. (1995) *Biol. Pharm. Bull.* **18**, 401–406
- 47 Corder, R., Harrison, V. J., Khan, N., Anggard, E. E. and Vane, J. R. (1993) *J. Cardiovasc. Pharmacol.* **22**, S73–S76

Received 8 December 1997/23 March 1998; accepted 16 April 1998