The N-terminal segment of endothelin-converting enzyme (ECE)-1b contains a di-leucine motif that can redirect neprilysin to an intracellular compartment in Madin–Darby canine kidney (MDCK) cells

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Endothelin-converting enzyme (ECE)-1 is a membrane-bound metallopeptidase of the neprilysin (NEP) family. ECE-1 is responsible for the conversion of inactive big-endothelins into active endothelins. Three different isoforms of human ECE-1 (ECE-1a, ECE-1b and ECE-1c) have been identified. They differ in their N-terminal cytosolic regions, have distinct tissue distribution and intracellular localization. ECE-1a and ECE-1c are both located at the cell surface whereas ECE-1b is targeted to an intracellular compartment. To better understand the nature of the signal responsible for the targeting of ECE-1b to the intracellular compartment, we have constructed several ECE/

NEP chimaeric proteins and expressed them by transfection into Madin–Darby canine kidney (MDCK) cells. This allowed us to identify a nine amino acid segment in the cytosolic tail of ECE-1b that is sufficient to relocate NEP from the cell surface to an intracellular compartment. Site-directed mutagenesis on these chimaeras led to the identification of two leucine residues as part of the intracellular retention signal.

Key words: Di-leucine, endothelin-converting enzyme, intracellular retention signal.

INTRODUCTION

Endothelin-1 was first described by Yanagisawa and co-workers in 1988 [1] as an endothelium-derived 21-residue vasoconstrictor peptide. Later studies demonstrated the existence of two other endothelins [2]: endothelin-2 and endothelin-3. These three peptides, encoded by three different genes, have been implicated in the maintenance of basal vascular tone and plasma volume, in the regulation of water balance, in hormone production (for a review see [3]), as well as in several developmental processes [4]. They are also involved in the pathophysiological mechanism of a number of vascular conditions [5].

Endothelins are processed from big endothelins by cleavage of the Trp²¹–Val/Ile²² bond. A putative endopeptidase activity responsible for this cleavage was described as endothelin-converting enzyme (ECE) by Yanagisawa and co-workers [1]. Complementary DNA encoding two ECE forms, termed ECE-1 and ECE-2 have been isolated [6–10]. Recently, a novel ECE specific for big endothelin-3 was purified from bovine iris microsome by Hasegawa and coworkers [11].

ECE-1 and ECE-2 are type II integral membrane-bound proteases with sequence similarities to neprilysin (neutral endopeptidase-24.11, NEP) and Kell blood group protein [6]. They consist of a short N-terminal cytosolic domain, a single transmembrane segment, and a large C-terminal ectodomain, which contains the catalytic Zn²⁺-binding motif (HEXXH). Three isoforms of human ECE-1 have been described to date: ECE-1a and ECE-1b [6], also termed ECE-1 β and ECE-1 α respectively [7], and more recently ECE-1c [8]. These three isoforms are encoded by the same gene with three distinct promoters [7,9]. ECE-2, however, is encoded by a distinct gene [10]. The three isoforms of human ECE-1 differ in their N-terminal cytosolic

sequence only. Whereas ECE-1a and ECE-1b have completely different N-terminal amino acid sequences, ECE-1c corresponds to a truncated form of ECE-1b, lacking its first 17 N-terminal amino acids (Figure 1) [6,8].

Besides having different tissue distributions ECE-1a, 1b and 1c also have major differences in their intracellular localization both in transfected Chinese hamster ovary (CHO) cells and in the ECV304 endothelial cell line [8]. Whereas ECE-1a and ECE-1c are expressed on the cell surface of these cells, ECE-1b was found predominantly intracellular. Similarly, human ECE-1b was also found in an intracellular compartment tentatively identified as early or late endosomes when transfected in Madin–Darby canine kidney (MDCK) cells [12].

Retention of a transmembrane protein along the secretory pathway as well as internalization of a protein from the cell surface requires some form of targeting or sorting information on the protein itself. Usually this information resides in sorting signals encoded in the cytoplasmic domain. These signals consist of sequences or structural motifs, many of which have either a critical tyrosine residue or a pair of leucine or bulky hydrophobic residues (reviewed in [13–15]). Tyrosine-based motifs have been identified in a variety of endocytosed molecules including the transferrin receptor [16]. It was proposed that one of the principal features of this motif is to induce the formation of a tight turn conformation. In 1992, Letourneur and Klausner first identified a di-leucine motif that could also mediate lysosomal targeting and endocytosis of CD3 chains [17]. Since then several such signals have been identified on the GLUT4 glucose transporter [18] and on the insulin receptor, for example [14,19].

The aim of this study was to identify a targeting signal in the N-terminal amino acid sequence of ECE-1b. The only difference between ECE-1b and ECE-1c being the 17 amino acids N-

Abbreviations: ECE, endothelin-converting enzyme; MDCK, Madin–Darby canine kidney; NEP, neprilysin (neutral endopeptidase 24.11).

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Figure 1 Schematic representation of the different ECE/NEP chimaeras

(A) CY corresponds to the cytosolic segment of the protein, and TM represents the transmembrane domain. Shaded boxes correspond to the ECE portion of the proteins, clear boxes represent the NEP portion of the proteins. The sequence of part of the cytosolic domain of ECE-1b is indicated, with Leu¹² and Leu¹³ in bold. (B) Sequences of the cytosolic segments of the chimaeras. Shaded and clear areas represent ECE and NEP residues respectively. Leu¹², Leu¹³ and Tyr²¹ and/or the corresponding alanine in the mutants are in bold.

terminal to ECE-1b, it was most likely that the putative intracellular retention signal would be located within these 17 amino acids. The identification of such a signal nevertheless required direct confirmation using two complementary strategies. First, the isolated internalization/intracellular retention signal must have been able to induce the intracellular targeting of an other protein normally expressed at the cell surface. Secondly, point mutations in the putative signal should have at least partially inhibited the internalization process.

To identify the ECE-1b internalization signal(s) we thus decided to study the fate of several chimaeric molecules containing the ectodomain of NEP fused to the cytoplasmic and transmembrane domains of ECE-1b. The choice of NEP as a reporter protein was dictated by three different considerations. First, as members of the same family of endopeptidases, ECE-1 and NEP have amino acid sequences with more than 50% identity [20]. Thus, fusion of the ectodomain of NEP with the cytoplasmic and transmembrane domains of ECE-1b was expected to result in a chimaeric protein with topological characteristics similar to those of their native natural parents. Secondly, previous studies on NEP expression in polarized epithelial cells clearly established the main features of the intracellular targeting of this protein [21–23]. Thirdly, specific high-affinity antibodies against the ectodomain of NEP are available, allowing immunohistochemical studies and immunoprecipitation experiments on NEP/ECE chimaeras.

Here, we have identified a nine amino acid segment present in the N-terminal portion of the ECE-1b cytosolic tail that appears to constitute a dominant signal for the intracellular retention of NEP/ECE-1b chimaeras. Site-directed mutagenesis then allowed us to identify two leucine residues as part of this signal.

MATERIALS AND METHODS

DNA constructions

Vector pCDNA3/RSV was a generous gift of Dr M. Bouvier (Department of Biochemistry, University of Montreal, Montreal, Quebec, Canada). The cytosolic and transmembrane domains of ECE-1b were then fused to the NEP ectodomain by PCR-based techniques, resulting in pCDNA3/RSV-ECE/NEP, coding for ECE/NEP. We then generated pCDNA3/RSV-Δ(1–8)-ECE/NEP from this template, by a two-step PCR strategy, using oligonucleotides carrying the appropriate deletion, thus obtaining Δ(1–8)-ECE/NEP.

Two complementary oligonucleotides corresponding to the coding sequence for the 17 amino acid N-terminal sequence of ECE-1b were then synthesized. After annealing of these oligonucleotides, the resulting fragment was ligated 5' from the complete coding sequence of NEP and the whole resulting DNA fragment was cloned into pCDNA3/RSV, thus giving pCDNA3/RSV-ECE(1–17)/NEP. The same strategy was used for the introduction of mutations into this chimaera, resulting in pCDNA3/RSV-Ala¹²-ECE(1–17)/NEP, pCDNA3/RSV-Ala¹³-ECE(1–17)/NEP, and pCDNA3/RSV-Ala^{12/13}-ECE(1–17)/NEP. These four vectors would produce ECE(1–17)/NEP, Ala¹²-ECE(1–17)/NEP, Ala¹³-ECE(1–17)/NEP and Ala^{12/13}-ECE(1–17)/NEP respectively.

All PCR products were confirmed by DNA sequencing after cloning in pCDNA3/RSV. Each of these expression vectors was then transfected into MDCK cells using the calcium phosphate precipitation method [24]. The neomycin analogue, geneticin, was used to select pools of resistant MDCK cells. Construction of the stable cell line expressing wild-type NEP is described in [25]. A schematic representation of all the constructs is shown in Figure 1.

Culture of MDCK cells

MDCK cells were a generous gift from Dr I. R. Nabi (Department of Pathology and Cellular Biology, University of Montreal, Montreal, Quebec, Canada). They were cultured in a medium consisting of 50% (v/v) Ham's F-12 medium and 50% (v/v) Dulbecco's modified Eagle's medium with 10% (v/v) fetal-bovine serum (Gibco), penicillin (100 units/ml), streptomycin (100 µg/ml), Fungizone (25 µg/ml), 15 mM Hepes, pH 7.4 and 5 mM selenium. Transfected cells were routinely cultured in atmosphere of 5% CO₂ in air at 37 °C in medium containing geneticin. Cells were plated at a density of approximately 10⁶ cells/Petri dish and the incubation medium was changed every second day. All the experiments on transfected cells were performed within the subsequent 50 passages, on confluent cells. Before each experiment, cells were incubated overnight in medium containing 10 mM sodium butyrate to enhance the production of the protein of interest.

Western blot analysis

MDCK cells expressing one of the chimaeras were cultured as described earlier. Cells were then lysed in PBS containing 1% (w/v) 1-*O*-*N*-octyl β-D-glucopyranoside, and the nuclei were removed by centrifugation at 1400 g for 20 min. Aliquots con-

taining 40 μ g of total proteins were then either incubated for 1 h at 37 °C with the endo- β -*N*-acetylglucosaminidase H (endo H) or with endo-peptide *N*-glycosidase F (endo F; New England Biolabs) as described by the manufacturer. Control experiments where the glycosidase was omitted were run in parallel. Protein samples were then analysed by SDS/PAGE [7.5% (w/v) acrylamide gel] before transfer on to a nitrocellulose membrane. Antibody 18B5 was then used (1:500 dilution) for the detection of ECE/NEP chimaeras using the Vectastain kit (Vector Laboratories, Burlingame, CA, U.S.A.).

Immunofluorescence

The guinea pig polyclonal antibody raised against rabbit NEP was described in [26]. The antibody recognizes all the chimaeras used in this study. Rhodamine-, FITC- or Texas Red-conjugated anti-guinea pig antibody was purchased from Jackson Laboratories (Bar Harbor, ME, U.S.A.). Confluent MDCK cells expressing the protein of interest were grown to confluence on Labtek chamber glass slides (Gibco). Cells were rinsed three times for 10 min in PBS containing 1 mM MgCl₂ and 0.1 mM CaCl₂ (Buffer A) and fixed for 20 min at room temperature in Buffer A containing 4% (v/v) paraformaldehyde. Cells were then washed in Buffer A and permeabilized by incubation in Buffer A containing 0.075% saponin and 1% (w/v) BSA (Buffer B) for 30 min. Cells were then incubated for 10 min at room temperature in Buffer C (Buffer B plus 50 mM NH₄Cl). After being washed in Buffer B, cells were then incubated for 30–60 min at room temperature with anti-NEP antibody diluted 1:200 in Buffer B. Cells were again washed three times, 10 min each, in Buffer B and incubated with either Texas-Red- or Rhodamine-conjugated anti-guinea pig secondary antibody (diluted 1:100) for 1 h in the dark. Finally, cells were washed three times, 10 min each in Buffer B, and one time in Buffer A before being mounted for immunofluorescence in Immuno Fluore Mounting Medium (ICN). Cells were then visualized using a dual channel laser scanning confocal microscope (Biorad MRC 600 Ar-Kr laser). For cell surface labelling, the same protocol was followed, except that saponin was omitted in all the solutions prior to incubation with the primary antibody.

Cell surface immunoprecipitation

MDCK cells expressing various chimaeras were cultured in 60 mm Petri dishes, and were metabolically labelled with 100 μ Ci Trans [³⁵S] label (ICN Biomedicals), before being submitted to various chase times. The cells were then washed twice with ice-cold PBS containing BSA (0.01 mg/ml) and incubated for 3 h at 4 °C in the same buffer with a 1:200 dilution of the NEP polyclonal antibody. This allowed the selective immunoprecipitation of the newly synthesized chimaeras that had reached the plasma membrane. After the incubation period, monolayers were rinsed three times with cold PBS and 40 μ l of cold lysate was added to remove excess antibody. Cells were then scraped from the Petri dishes and lysed in 500 μ l of PBS buffer containing 1% 1-*O*-*N*-octyl β -D-glucopyranoside, and the nuclei were removed by centrifugation at 1400 g for 20 min. The post-nuclear supernatants were diluted in 500 μ l of 2 $\times$ immunoprecipitation buffer [20 mM Tris/HCl, pH 7.4, 100 mM NaCl, 2% (w/v) sodium deoxycholate, 2% (v/v) Triton X-100, 0.2% SDS, 0.2% BSA] and transferred to a tube containing Protein A-Sepharose beads. This allowed the precipitation of radiolabelled ECE/NEP chimaeras present at the cell surface. The supernatant resulting from this first immunoprecipitation contained radioactive mol-

ecules present in intracellular compartments at the end of the chase incubation. These molecules were recovered by submitting this supernatant to an identical immunoprecipitation procedure. After subsequent rinsing in immunoprecipitation buffer and in PBS buffer, immunoprecipitates were boiled in electrophoresis sample buffer and analysed by SDS/PAGE (7.5% gels). The radiolabelled proteins on the gels were finally revealed by fluorography.

RESULTS

Expression of the ECE/NEP chimaeras in MDCK cells

All the vectors described in the Materials and methods section were transfected into MDCK cells and stable transfectants were isolated based on their resistance to the neomycin analogue, geneticin. Cells were then solubilized in PBS containing 1% 1-*O*-*N*-octyl β -D-glucopyranoside and analysed under reducing conditions by Western blot analysis using the 18B5 monoclonal antibody raised against the ectodomain of wild-type NEP [27]. In all cases a double band of approximately 95 kDa was observed in transfected MDCK cells. As seen in Figure 2, the chimaeras are expressed at comparable levels. When cellular extracts were digested with endo-peptide *N*-glycosidase F, the double band disappeared and was replaced in all cases by a sharp band of approx. 85 kDa. Treatment of cell extracts with endo- β -*N*-acetylglucosaminidase H (endo H) only digested a very small fraction of the total proteins, indicating that the majority of the produced chimaeras had acquired complex oligosaccharides after passing through the Golgi apparatus.

ECE-1b cytosolic tail is sufficient to retain NEP intracellularly

Previous studies showed that ECE-1b remains in an intracellular compartment when expressed in MDCK cells [12]. To examine the subcellular distribution of the different ECE/NEP chimaeras, indirect immunofluorescence microscopy was performed. Stable cell lines expressing either the chimaeras or wild-type NEP were fixed with paraformaldehyde and permeabilized with saponin to stain both intracellular and cell surface proteins. In some experiments, cells were not permeabilized in order to detect cell surface labelling only. A polyclonal antibody against wild-type NEP, that recognises an epitope in the NEP ectodomain, present

Figure 2 Expression of the different chimaeras in MDCK cells

Stably transfected MDCK cells expressing either wild-type NEP or one of the chimaeras were solubilized in PBS containing 1-*O*-*N*-octyl β -D-glucopyranoside. Total proteins (40 μ g) were incubated for 1 h at 37 °C (—), or treated for 1 h with endo- β -*N*-acetylglucosaminidase H (+H) or with endo-peptide *N*-glycosidase F (+F) before Western blot analysis with antibody 18B5, as described in the Materials and methods section. The level of expression is similar for every protein. The glycosylated proteins have a molecular mass of approx. 95 kDa and are resistant to endo- β -*N*-acetylglucosaminidase H digestion.

Figure 3 Intracellular localization of ECE/NEP and ECE(1-17)/NEP

Stably transfected MDCK cells expressing ECE/NEP or ECE(1-17)/NEP, as indicated on the Figure, were grown to confluency on Labtek chamber glass slides, fixed with paraformaldehyde, and either permeabilized (**A** and **B**, saponin) or not permeabilized (**C**, no saponin). They were then stained for the different chimaeric proteins, using a polyclonal antibody that recognizes an epitope in the ectodomain of NEP, and observed from the cell surface. ECE/NEP is located exclusively in intracellular vesicular structures, whereas a small proportion of ECE(1-17)/NEP is also visible on the cell surface, when no permeabilization is performed. (**B**) A horizontal section through the nucleus shows the perinuclear localization of the chimaeras. Scale bar: 20 μ m.

in all the different chimaeras, was used as a primary antibody. Non-transfected MDCK cells showed no staining with this anti-NEP antibody (results not shown).

As we showed previously [21], NEP in its native state is expressed at the surface of MDCK cells. By contrast, the ECE/NEP chimaera resulting from the fusion of entire cytoplasmic and transmembrane domains of ECE-1b to the NEP ectodomain failed to exhibit an immunofluorescence signal in non-permeabilized MDCK cells (Figure 3C). Immunofluorescence was observed only in cells permeabilized with saponin (Figures 3A and 3B) and was found associated with cytoplasmic vesicular structures, sometimes in a perinuclear location (Figure 3B). This observation suggests the presence of an intracellular retention signal in either the cytosolic or the transmembrane domain of ECE-1b, this signal being sufficient to mediate the intracellular retention of the NEP ectodomain.

Since ECE-1b differs from ECE-1c only by the presence of an additional sequence of 17 amino acid residues at its N-terminus, it is likely that this sequence is responsible for the intracellular retention of ECE-1b. We thus decided to fuse the 17 amino acid N-terminal segment of ECE-1b to the N-terminus of NEP and to look for the effect of this fusion on the intracellular transport of the protein. As shown on Figure 3, ECE(1-17)/NEP was mainly expressed inside the cell where it was found in vesicular structures similar to the ones identified for ECE/NEP. However, in this case, a faint staining was visible on the cell surface of non-

Figure 4 Cell surface immunoprecipitation of ECE/NEP and ECE(1-17)/NEP

Confluent MDCK cells expressing ECE/NEP or ECE(1-17)/NEP were labelled with 100 μ Ci [35 S]methionine before being submitted to various chase times. Cell surface immunoprecipitation of the chimaeras was then performed as described in the Materials and methods section. Proteins were resolved by SDS/PAGE and visualized by fluorography. Autoradiograms were scanned using HP Deskscan software. ECE/NEP is not visible in the cell surface fractions whereas some of the ECE(1-17)/NEP chimaeras appear at the cell surface between 20 and 40 min after the beginning of the chase, which corresponds to the kinetics of transport observed for NEP.

permeabilized cells. These observations indicate the presence of a dominant intracellular retention signal within the 17 amino acid sequence characteristic of ECE-1b.

In order to confirm the cellular localization of the chimaeric proteins and to determine the kinetics of their intracellular transport, MDCK cells expressing the various chimaeric proteins

Figure 5 Intracellular localization of the ECE(1–17)/NEP mutants

Stably transfected MDCK cells expressing Ala¹²-ECE(1–17)/NEP, Ala¹³-ECE(1–17)/NEP, or Ala¹²¹³-ECE(1–17)/NEP, as indicated on the Figure, were grown to confluence on Labtek chamber glass slides, fixed with paraformaldehyde, and either permeabilized (saponin) or not permeabilized (no saponin). They were then stained for the different chimaeric proteins, using a polyclonal antibody that recognizes an epitope in the ectodomain of NEP, and observed from the cell surface. The three chimaeras are present in intracellular vesicular structures, and are also found on the cell surface of MDCK cells when no permeabilization was performed prior to the incubation with primary antibody. Scale bar: 20 µm.

were pulsed for 15 min in medium containing [³⁵S]methionine and chased as indicated in Figure 4 before being submitted to cell surface immunoprecipitation (see the Materials and methods section). For ECE/NEP, no trace of the protein was detectable at the surface of the cells, even after a 180 min chase. However, in the case of ECE(1–17)/NEP, a small proportion of the chimaeric protein appeared at the cell surface between 20 and 40 min of chase. The kinetics of transport was identical with that previously observed for wild-type NEP in MDCK cells [21]. The fact that only a very small proportion of the total labelled ECE(1–17)/NEP proteins was found at the cell surface was consistent with the faint immunofluorescence labelling of non-

permeabilized cells, and confirmed that the majority of the chimaeric enzyme molecules were retained inside the cells.

Leu¹² and Leu¹³ are part of the intracellular retention signal of ECE-1b

From the results presented above, it appears that the stretch of 17 N-terminal amino acids that is specific to ECE-1b constitutes a dominant signal capable of mediating the intracellular retention of wild-type NEP, which would otherwise be targeted to the apical plasma membrane [21]. To determine more precisely which amino acids within this sequence were part of the

Figure 6 Cell surface immunoprecipitation of Ala^{12/13}-ECE(1-17)/NEP

Confluent MDCK cells expressing Ala^{12/13}-ECE(1-17)/NEP were labelled with 100 μ Ci [³⁵S]methionine before being submitted to various chase times, as indicated. Cell surface immunoprecipitation of the chimaera was then performed as described in the Materials and methods section. Proteins were resolved by SDS/PAGE and visualized by fluorography. Autoradiograms were scanned using HP Deskscan software.

internalization signal we first designed Δ (1-8)-ECE/NEP, a chimaeric construct identical with ECE/NEP except for the lack of the first 8 amino acid residues of the N-terminal cytosolic tail of ECE. This chimaera is also retained inside transfected cells (results not shown) suggesting that the signal is not located within the first 8 amino acid residues of the N-terminal segment. We thus turned our attention to other features, which might be located between residues 9 and 17.

Di-leucine sequences have previously been described as part of internalization signals in a number of proteins [17,22]. We thus decided to assess the role of Leu¹² and Leu¹³ in ECE-1b by mutating either one or both of these leucine residues in the ECE(1-17)/NEP chimaera. As shown in Figure 5, these mutations had a dramatic effect on the localization of the chimaera. Even though a large proportion of the protein was still present in the same cytosolic vesicular structures as those seen in Figure 3, a more intense immunofluorescence signal was observed on cells stained without prior permeabilization (no saponin). These results suggested that mutations in the di-leucine sequence considerably reduced the efficiency of the intracellular retention mechanism. It is also interesting to note that the cell surface fluorescent signal observed with these di-leucine mutants exhibited a punctate pattern similar to the one we previously observed for wild-type NEP [21]. MDCK cells join to form a continuous epithelial sheet on the dish surface when grown to confluence, as was always the case in our experiments. In these experimental conditions, we have shown previously that the antibody can neither reach the inside of the cells nor pass the tight junctions between two adjacent cells [25]. Thus in the conditions used, the cell surface proteins that can be reached by the antibody are only apical. The punctate staining that can be observed without saponin on Figure 5 thus concerns proteins that are present at the apical cell surface. Moreover, as was the case for Figure 3, the optical sections shown for the non permeabilized cells were taken at the cell surface, and an optical section through the centre of the cell showed no staining at all (results not shown).

To confirm the immunohistochemistry results we decided to monitor the kinetics of the apparition of Ala^{12/13}-ECE(1-17)/NEP at the cell surface during a pulse-chase experiment followed by cell surface immunoprecipitation. As shown in Figure 6, Ala^{12/13}-ECE(1-17)/NEP appeared at the cell surface between 20 and 40 min after the beginning of the chase, as previously observed for ECE(1-17)/NEP (Figure 4) and for wild-type NEP [21]. Thus, it appears that when the intracellular retention machinery fails, the endogenous apical targeting signal presumably present in the NEP ectodomain [23] still appears to be

Figure 7 Ala²¹-ECE/NEP localization

(A) Stably transfected MDCK cells expressing Ala²¹-ECE/NEP were grown to confluence on Labtek chamber glass slides. Cells were then fixed with paraformaldehyde before being permeabilized with saponin and subsequently stained immunocytochemically. The majority of the mutated chimaeric protein appears to be localized in the same cytosolic vesicular structure as ECE/NEP. (B) Stably transfected MDCK cells expressing Ala²¹-ECE/NEP were grown to confluence and labelled with 100 μ Ci [³⁵S]methionine before being submitted to various chase times. Cell surface immunoprecipitation of the chimaeras was then performed before the proteins were resolved by SDS/PAGE and visualized by fluorography. A very small proportion of the mutated chimaera appears at the cell surface between 20 and 40 min of chase.

functional in the chimaeric proteins. Taken together these results suggest that Leu¹² and Leu¹³ are part of a signal responsible for either the intracellular retention or the internalization of ECE-1b.

Tyr²¹ is part of the targeting signal for the intracellular localization of ECE-1b

Tyrosine residues have been previously reported to be part of several internalization signals [17,28-30]. In the endoprotease furin for example, which is involved in the first step of endothelin maturation, a tyrosine residue was shown to be responsible for the retrieval of the protein from the cell surface [31]. The amino acid sequence of the cytoplasmic tail of ECE-1b contains several tyrosine residues. One of those, Tyr²¹, is located only five residues from a stretch of acidic amino acids, and in the vicinity of several serine residues. This environment is very similar to the one of furin, where it was shown to be important for the proper targeting of the protein.

In order to study an eventual role of the Tyr²¹ residue in the targeting of ECE-1b, we mutated it into an alanine residue in the ECE/NEP chimaera, thus obtaining Ala²¹-ECE/NEP (see Figure 1). Immunohistochemistry experiments (Figure 7) showed that the mutated protein was mainly expressed intracellularly in

MDCK cells, apparently in the same cytosolic vesicles as ECE/NEP and ECE(1–17)/NEP. However, the immunoprecipitation experiments shown in Figure 7 indicated that some of the ECE/NEP chimaera was found at the cell surface after the mutation of Tyr²¹ to Ala. The quantity of protein mistargeted to the cell surface by this point mutation appears to be much less important than in the case of the Leu¹² and Leu¹³ mutations.

DISCUSSION

Several isoforms of ECE-1 have been cloned that differ exclusively in their cytosolic tail [6]. Considering the fact that the intracellular ECE-1b isoform is identical with the cell surface ECE-1c, except for an additional N-terminal 17 amino acid sequence, it is likely that the intracellular retention signal of ECE-1b is contained within this N-terminal extension.

When the cytosolic and transmembrane domains of ECE-1b were fused to the ectodomain of NEP there was a complete intracellular retention of this typical cell surface protein [27]. The nature of the intracellular compartment where the chimaeric protein was retained was not thoroughly studied in this paper. However the staining pattern, as observed in confocal microscopy, was identical with the one for ECE-1b, using the same experimental conditions, reported previously by Azarani et al. [12]. Thus it appears most likely that the chimaeric NEP/ECE-1b proteins studied in this paper localize to the MDCK endosomal compartment as indicated previously.

The fact that the first 17 amino acid residues of ECE-1b were able to prevent most of the ECE(1–17)/NEP chimaeras from reaching the cell surface indicates that a major component of the intracellular retention signal resides in this segment that is unique to ECE-1b. Since the $\Delta(1-8)$ -ECE/NEP chimaera remained intracellular, it appears unlikely that amino acids 1 to 8 are part of the targeting signal. Finally, mutations of Leu¹² and/or Leu¹³ to Ala, which restored partial cell surface expression of the chimaera, allowed the identification of these two residues as crucial components of the intracellular retention signal of ECE-1b.

A recent study by Marks et al. [32] showed that targeting through di-leucine- and tyrosine-based motifs used distinct saturable components. Rapoport and co-workers described the interaction of a di-leucine motif with the $\beta 1$ subunit of adaptor protein (AP)-1 [33]. These results, combined with previous observations [34], suggest that AP-1 and AP-2 act like differential filters for proteins with one or more sorting signals in their cytoplasmic tail. Transmembrane proteins with an AP-1 di-leucine or tyrosine signal would be captured by AP-1 and sorted to an endosomal/lysosomal compartment before continuing towards their final destination. A few transmembrane proteins would, however, bypass this filter and reach the plasma membrane. Those with AP-2 directed tyrosine signals would then be re-internalized.

The presence of a small amount of ECE(1–17)/NEP at the surface of MDCK cells, together with the internalization of small amounts of ECE-1c, as reported by Schweizer et al. [8], suggest the presence of another signal in the cytosolic or transmembrane domain of the chimaera that is common to ECE-1b and ECE-1c. Preliminary results shown in Figure 7 seem to indicate a role for a tyrosine residue in this phenomenon. This tyrosine could interact with AP-2 to redirect a certain amount of proteins to intracellular compartments. However, as the main role of this internalization machinery would be to redirect intracellularly the small amount of ECE-1b molecules that bypassed the Golgi/AP-1 retention system, it is most likely to be of low capacity. Thus

most of the ECE-1c proteins would escape it and remain at the cell surface.

The question remains about the putative role of an intracellular form of ECE. Endothelin-1 is first synthesized as an inactive precursor. It is then cleaved by the prohormone convertase furin in the trans-Golgi network [35], before the final maturation step by ECE-1. The cytoplasmic domain of furin was shown to mediate its localization to the trans-Golgi network where it fulfills its proteolytic processing functions [36]. It also recycles back and forth between the Golgi apparatus and the cell surface. Takahashi and co-workers [31] showed that the cytoplasmic domain of furin contains a unique tyrosine residue within a possible trans-Golgi network localization motif. When this tyrosine was mutated to alanine, retention of the mutated protein in the trans-Golgi network was not impaired, but retrieval of furin from the cell surface was significantly retarded. The same machinery could be involved in the retrieval of both furin and ECE-1b from the cell surface.

Although the experiments presented here do not allow precise identification of the intracellular vesicular compartment in which the different chimaeric proteins are located, recent observations propose that ECE-1b is located in endosomes when transfected into MDCK cells [12]. The presence of both furin and ECE-1b in two consecutive compartments of the secretory pathway could provide a very efficient way for rapidly processing pre-proendothelin into mature endothelin-1. Indeed, cell surface conversion of exogenous big endothelin-1 has been reported to be less efficient than intracellular conversion of endogenous big endothelin-1 [20]. Moreover, Russel et al. recently published observations concerning the presence of both endothelin and ECE in the cytoplasmic matrix, as well as in Weibel-Palade bodies and secretory vesicles of endothelial cells in the human coronary artery [37]. They propose that the intracellular maturation of the endothelin precursor and the subsequent release of endothelin-1, via the regulated secretory pathway, would induce a rapid constriction in response to compromised endothelial-cell integrity. Gui et al. also presented evidence for the intracellular localization of membrane-bound ECE from rat lung [38] and suggested that big endothelin-1 may be converted to endothelin-1 during its transit through the secretory pathway. We therefore suggest that ECE-1b could serve for the intracellular maturation of big-endothelin to endothelin in response to a yet unknown signal. ECE-1a and/or ECE-1c would themselves be implicated in the maturation of big endothelin-1 at the cell surface.

The use of specific antibodies to differentiate the three ECE-1 isoforms would allow the further testing of this hypothesis in endothelial cells. A more precise characterization of the molecular machinery involved in the intracellular retention process would also provide a better understanding of the intracellular trafficking of the various isoforms of ECE.

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