Alternative Splicing Regulates the Endoplasmic Reticulum Localization or Secretion of Soluble Secreted Endopeptidase*

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Sunu Budhi Raharjo‡, Noriaki Emoto§§, Koji Ikeda‡‡, Ryuichiro Sato, Mitsuhiro Yokoyama§§, and Masafumi Matsuo‡‡

From the ²Division of Molecular Medicine, International Center for Medical Research and the ³Division of Cardiovascular and Respiratory Medicine, Department of Internal Medicine, Kobe University Graduate School of Medicine, 7-5-1 Kusunoki, Chuo, Kobe 6500017, Japan, and the ⁴Department of Applied Biological Chemistry, Graduate School of Agriculture and Life Sciences, The University of Tokyo, Bunkyo, Tokyo 113-8657, Japan

A subfamily of zinc metalloproteases, represented by Neutral endopeptidase (EC 3.4.24.11) and endothelin-converting enzyme, is involved in the metabolism of a variety of biologically active peptides. Recently, we cloned and characterized a novel member of this metalloprotease family termed soluble secreted endopeptidase (SEP), which hydrolyzes many vasoactive peptides. Here we report that alternative splicing of the mouse SEP gene generates two polypeptides, SEPβ and SEPγ. After synthesis, both isoforms are inserted into the endoplasmic reticulum (ER) as type II membrane proteins. SEPβ then becomes an ER resident, whereas SEPγ, which differs by only the presence of 23 residues at the beginning of its luminal domain, is proteolytically cleaved by membrane secretase(s) in the ER and transported into the extracellular compartment. An analysis of the chimeric proteins between SEPβ and bovine endothelin-converting enzyme-1b (bECE-1b) demonstrated that the retention of SEPβ in the ER is mediated by the luminal domain. In addition, the dissection of the chimeric bECE-1b/SEP insertion showed that its insertion domain is obviously responsible for its secretion. A series of mutagenesis in this region revealed that the minimal requirement for cleavage was found to be a WDERTVV motif. Our results suggest that the unique subcellular localization and secretion of SEP proteins provide a novel model of protein trafficking within the secretory pathway.

Mammalian zinc metalloproteases have been implicated in a diversity of disease states because of their roles in the activation or inactivation of a variety of biologically active peptides. Therefore, they provide important therapeutic targets for certain diseases. Within this large group, nephrilysin (M13) constitutes a subfamily in which seven members have been identified to date, such as Neutral endopeptidase (EC 3.4.24.11) (NEP),1 Kell blood group protein, two different endothelin-converting enzymes (ECE-1 and ECE-2), PEX, which has been associated with X-linked hypophosphatemic rickets, endothelin-converting enzyme-like-1, and the recently identified soluble secreted endopeptidase (SEP). All these members are type II membrane glycoproteins, which display a single transmembrane stretch separating a short N-terminal cytoplasmic tail from a C-terminal extracellular/luminal domain. This luminal domain bears the enzyme active site, which includes HEXXXH, a highly conserved pentameric consensus sequence of a zinc binding motif. NEP is a metalloprotease with wide tissue distribution and is especially abundant in the brain and kidney. This endopeptidase has been shown to hydrolyze a wide range of small peptide mediators, such as enkephalins, substance P, atrial natriuretic peptide, neurotensin, bradykinin, angiotensin I and II, and endothelins (1). ECE-1 is primarily involved in the production of the vasoconstrictive peptide ET-1 by the cleavage of an inactive precursor, big ET-1. Two subisoforms of bovine ECE-1 termed ECE-1a and ECE-1b that differ from each other only in the N-terminal tip of their cytoplasmic tail showed distinct subcellular localization (2). ECE-2, which also produces ET-1, has an acidic pH optimum and may function intracellularly (3). Therefore, clarifying the precise subcellular localization of the protein would be favorable to characterize its physiological roles.

SEP, the most recently identified member of this family, shares higher structural and functional similarities with NEP than with other members of this metalloprotease family. Structurally, the sequence identity of SEP with respect to NEP is higher than those of the other members. Two arginine residues known to constitute the substrate binding sites in NEP (Arg102 and Arg104 in human NEP) are conserved in SEP (Arg121 and Arg122 in mouse SEP). Functionally, both SEP and NEP are promiscuous enzymes that hydrolyze a variety of physiologically active peptides. SEP has been implicated in the hydrolysis of angiotensin I, atrial natriuretic peptide, bradykinin, substance P, leucine-enkephalin, big ET-1, and ET-1. The activity of SEP is efficiently inhibited by the specific NEP inhibitor thiorphan but is not completely inhibited by the specific ECE inhibitor FR901533 (4).

Although SEP shares several important properties with other members of this metalloprotease family, it exhibits features unique to itself. First, two isoforms of SEP named SEP

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† To whom correspondence should be addressed: Division of Cardiovascular and Respiratory Medicine, Dept. of Internal Medicine, Kobe University Graduate School of Medicine, 7-5-1 Kusunoki, Chuo, Kobe 6500017, Japan. Tel.: 81-78-382-5846; Fax: 81-78-382-5859; E-mail: emoto@med.kobe-u.ac.jp.

‡ The abbreviations used are: NEP, Neutral endopeptidase; ECE, endothelin-converting enzyme; bECE-1b, bovine endothelin-converting enzyme-1b; SEP, soluble secreted endopeptidase; ET, endothelin; BAC, bacterial artificial chromosome; PCR, polymerase chain reaction; CHO, Chinese hamster ovary; MTP, microsomal triglyceride transfer protein; PAGE, polyacrylamide gel electrophoresis; Endo H, endo-β-N-acetylgalactosamidase H; PNGaseF, peptide-N-glycosidase F; Endo D, endo-β-N-acetylglucosaminidase D; TLCK, N,N′-tosyl-L-lysine chloromethyl ketone; ER, endoplasmic reticulum.
and SEP\(^9\) differ from each other in the presence of a 23-amino acid insertion region flanking the transmembrane domain of SEP. This feature was not found in other members of this metalloprotease family. Second, the membrane-bound SEP seems to localize in the early secretory pathway, which is unusual for these metalloprotease family members. Third, although all the other members discovered so far are membrane-associated proteins, SEP exists not only as a membrane-bound form but also as a circulating soluble form. This observation suggests that a proteolytic cleavage event occurred during the intracellular transport of SEP. These features make SEP unique among the members of this neprilysin family. Therefore, we designed the current study to investigate these special characteristics of SEP. In this report, we present evidence that two isoforms of SEP are generated via an alternative splicing mechanism. The membrane-bound SEP localized in the endoplasmic reticulum, and this ER localization is not attributed to misfolding. In addition, by making chimeric proteins between SEP and bovine endothelin-converting enzyme-1b (bECE-1b), another member of this metalloprotease family that is normally localized in the cell surface, the luminal domain of the SEP protein was identified as being important for retention. Furthermore, we also identified a specific motif in the SEP insertion region that is necessary for the cleavage process of this protein in the ER. This unique mechanism of localization and processing of SEP defines an interesting model for protein trafficking within the secretory pathway.

**EXPERIMENTAL PROCEDURES**

**Materials**—Enzymes used in molecular cloning were obtained from Roche Molecular Biochemicals or from New England Biolabs (Beverly, MA). Endo-\(\beta\)-N-acetylglucosaminidase H (Endo H) and peptide-N-glycosidase F (PNGase F) were from Roche Molecular Biochemicals. Endo D was from Seikagaku Co. Ltd. (Tokyo), \(\alpha\)-Protein A-Sepharose Fast Flow beads were from Amersham Pharmacia Biotech AB (Uppsala, Sweden).

**BAC Library Screening**—The mouse SEP gene locus was cloned by screening a BAC (bacterial artificial chromosome) library (Genome Systems, Inc., St. Louis, MO). A polymerase chain reaction (PCR)-generated radioactive probe, containing sequences within the mouse cDNA was used to probe a mouse genomic BAC library. The probe was generated from the following oligonucleotides, 5'-TATTTTGGGGACGGGATTTCCTC-3' and 5'-CATTATCATACAAAGCGTGT-3', which were chosen based on their likelihood to span a region within an SEP exon according to the genomic structure of SEP (6) and ECE-1 (7). One positive clone, pBAC-SEP, containing 150 kilobase pairs of genomic DNA was used for sequence analysis. This clone was purified and digested with several restriction enzymes and was run using a 0.7% agarose gel. The gel was Southern blotted and probed with \(^32\)P-labeled oligonucleotides, which were contained within the putative insertion exon and its immediate upstream and downstream exons.

**Long and Accurate PCR**—Long and accurate PCR was performed on pBAC-SEP DNA to determine the size and location of the insertions using Takara long and accurate PCR kit as described by the manufacturer. Oligonucleotides were derived from the mouse SEP cDNA sequence and the generation of the pME-S/S/E chimera, the cytoplasmic domain (amino acids 1–17) and the transmembrane helix (residues 18–40) of mouse SEP\(^9\) were fused in frame to the entire extracellular domain of bECE-1b (residues 78–758). Initially, site-directed mutagenesis was performed to introduce Eco47III and SalI sites at the beginning of the extracellular domain of mouse SEP\(^9\) and bECE-1b, respectively. The resulting pME-SEP\(^9\) plasmid was digested with Eco47III and NotI to remove its luminal domain, whereas the mutant pME-bECE-1b plasmid was digested with SalI and NotI to give a fragment containing its luminal domain. These two fragments were then ligated in the correct reading frame.

**PME-E/E/S** was constructed by fusing the cytoplasmic domain (amino acids 41–63) of mouse SEP to the transmembrane domain (residues 53–73) of bECE-1b to the whole extracellular domain of mouse SEP\(^9\) (residues 40–742) in two steps. The beginning of the extracellular domain of pME-bECE-1b and pME-SEP\(^9\) was first mutagenized to contain EcoRV sites, respectively. The mutant pME-bECE-1b plasmid was digested with EcoRV and XhoI to remove its luminal domain, whereas the mutant pME-SEP\(^9\) plasmid was digested with Eco47III and XhoI to isolate its luminal domain. These two fragments were then ligated to yield pME-E/E/S.

The plasmid expressing the E/E/oE chimeric protein was prepared by locating the insertion region (amino acids 41–63) of mouse SEP within the transmembrane region of bECE-1b as follows. First, pME-bECE-1b was mutagenized to create SalI site at the beginning of its extracellular domain. Second, PCR amplification of the insertion region of SEP was performed using a sense primer containing a SalI site (underlined), 5'-GTCGACAGGGAGGACGTGCG-3', and an antisense primer, 5'-GTGCAGCTTTTAAACGC-3', including a SalI site in the 5' end. The PCR product was digested with SalI and inserted into the plasmid pME-bECE-1b digested with the same enzyme.

All mutants were verified by sequencing at the level of the final plasmid.

**Mutagenesis of Mouse SEP**—Deletion constructs and amino acid-substituted constructs were made by site-directed mutagenesis (2) using the Muta-Gen Phagemid in vitro mutagenesis version 2 kit (Bio-Rad) as described by the manufacturer. All mutants were verified by DNA sequencing using primers both upstream and downstream of the insertion region.

**Cell Culture**—CHO-K1 cells were cultured as described previously (4). The coding region of mouse SEP or SEP\(^9\) was subcloned into the pME18Sf(+) expression vector under the control of the SR α promoter (4). Stable transfection of CHO cells and isolation of the transfectant clones (CHO/SEP and CHO/SEP\(^9\)) were performed as described previously (2). Transient transfections of SEP, SEP\(^9\), and mutant cDNA were carried out using LipofectAMINE Plus (Life Technologies, Inc.) as described by the manufacturer. The cells were cultured for 30 h after transfecting the plasmid into the cells.

**Immunoblotting**—Conditioned medium and postnuclear lysates were subjected to SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The membranes were probed with an antibody that recognized the C terminus of mouse SEP or mouse SEP\(^9\) as described previously (2). The membranes were incubated with 1:50 dilution of body against the C terminus of mouse SEP (4), bovine ECE-1 (2), or human microsomal triglyceride transfer protein (MTP) (8) and developed using the ECL Kit (Amersham Pharmacia Biotech) as recommended by the manufacturer.

**Fluorescent Immunocytochemistry**—Cells were seeded onto coverslips and cultured for 2 days. Fluorescent immunocytochemistry was performed as described previously (2, 4). Intracellular staining was performed as follows, cells were fixed and permeabilized in methanol for 5 min at −20 °C. After washing and blocking, the cells were probed with a polyclonal antibody directed against the mouse SEP C-terminal peptide (1:100), the bovine ECE-1 C-terminal peptide (1:200), or the human MTP (1:50). The cells were again washed before incubation with normal goat serum/phosphate-buffered saline containing 7.5 μg/ml fluorescein isothiocyanate-goat-anti-rabbit IgG (Zymed Laboratories, Inc.). The coverslips were mounted on microscope slides with 90% (v/v) glycerol, 50 mM Tris-HCl, pH 9.0, and 2.5% (w/v) 1,4-diazacyclo-(2.2.2)octane.

**Metabolic Labeling and Immunoprecipitation**—Metabolic labeling and immunoprecipitation were performed as described previously (2). However, the soluble proteins in the supernatant were precipitated by the method of Wessel and Flugge (9). The precipitates were then dissolved in 0.5% (v/v) of phosphoric acid and subjected to immunoprecipitation using SEP polyclonal antibody. Immunoprecipitates were analyzed on 7% SDS-PAGE and developed using the BAS2000 system.

**Endoglycosidase Digestion**—Conditioned medium and membrane fractions were used for digestion. Endo H and PNGaseF digestions were performed as described previously (4), and endoglycosidase D was done at 37 °C for 16 h in 0.2 mM phosphate buffer, pH 6.5, containing 0.1% Nonidet P-40. The samples were then subjected to immunoblotting.
FIG. 1. Organization of the region around the insertion exon of the mouse SEP gene. A, the partial nucleotide sequences and intron-exon junctions of the 5′ region of the mouse SEP gene. The insertion exon (dotted box), its immediate 5′ exon (closed box), and its next 3′ exon (open box) are shown. Intron-exon boundaries were determined by sequencing the SEP-BAC clone oligonucleotides as shown under “Experimental Procedures.” Upper and lowercase letters represent exon and intron sequences, respectively. Consensus splice donor and acceptor sites are underlined. The intron immediately located 5′ to the encoding insertion exon is ~9.5 kilobase pairs, and the length of its immediate 3′ intron is 555 base pairs. B, generation of SEP and SEP^D isoform mRNAs.

RESULTS

SEP and SEP^D Are Derived from a Single Gene by Alternative Splicing—Previously, we have isolated and characterized two isoforms of mouse SEP (4). SEP (765 residues) and SEP^D (742 residues) differ only in the absence of a 23-amino acid insertion immediately following the transmembrane domain in SEP^D, but both share the same N termini transmembrane domain and C-terminal residues.

To check whether these two isoforms were produced by the same gene via an alternative splicing mechanism, the mouse SEP gene was cloned from mouse BAC genomic library using a PCR-generated ^32P-labeled probe spanning a putative exon area of SEP that we predicted based on the structure of both the NEP (6) and ECE-1 gene (7). One positive clone, pBAC-SEP containing ~150 kilobase pairs of genomic DNA was obtained and subjected to sequence analysis. Exon-specific primers were designed based on the predicted intron-exon boundaries and used to directly sequence the BAC clone. All deduced intron-exon boundaries indicate the canonical consensus splice donor and acceptor sequences in accordance with the GT/AG rule (10).

The sequence analysis clearly showed that the exon encoding the 23-amino acid insertion of SEP is an independent exon separate from the immediate 5′ exon by a ~9.5-kilobase pairs intron and from the next 3′ exon by a 555-base pair intron (Fig. 1A). This transcript resulted in a 765-amino acid product of SEP. However, when this insertion exon skipped making its immediate 5′ exon by a ~9.5-kilobase pairs intron and from the next 3′ exon by a 555-base pair intron (Fig. 1A). This transcript resulted in a 765-amino acid product of SEP. However, when this insertion exon skipped making its immediate 5′ exon join directly to the next 3′ exon, the 742 amino acids of SEP^D were produced (Fig. 1B). It then became apparent that this insertion exon coincided with an intron-exon boundary.

These results suggest that these two isoforms of SEP mRNA originate from a single gene of the mouse genome by an alternative splicing mechanism.

SEP and SEP^D Expression Patterns Show ER Localization—To characterize the features of both SEP and SEP^D, we generated transfectant cells CHO/SEP and CHO/SEP^D by transfecting expression constructs driven by the SR α viral promoter. Immunoblot analysis with an anti-SEP C-terminal peptide antiserum showed that only CHO/SEP and not CHO/SEP^D cells release its soluble form with an apparent molecular mass of ~126 kDa into the culture medium, whereas both membrane-bound forms are expressed as an approximate 110-kDa protein in the membrane preparation of these cells (4).

To further analyze these proteins, we then examined their sensitivity to Endo H and PNGaseF. Endo H digests N-glycans of a high mannose type. Resistance to Endo H digestion indicates that a glycoprotein moved from the ER to the Golgi compartment in which further modification to complex oligosaccharides occurs, whereas PNGaseF completely removes all N-linked oligosaccharides. Treatment of solubilized membranes from both CHO/SEP and CHO/SEP^D cells with either Endo H or PNGaseF reduces the apparent molecular mass from 110- to 89-kDa, which corresponds to the calculated molecular mass of SEP, whereas N-linked glycosylation of the SEP soluble form was removed by PNGaseF and found to be resistant to Endo H (Fig. 2A). These observations confirm that the 110-kDa species observed in the membrane fraction of the cells is the partially glycosylated protein present in the early secretory pathway.

To precisely investigate the subcellular localization of the membrane-bound protein, we immunostained both CHO/SEP and CHO/SEP^D cells with antibodies that recognize the common C-terminal ectodomain of SEP. After permeabilization, both cells showed strong intracellular staining. This intracellular staining pattern is indistinguishable from the results observed when we stained the microsomal triglyceride transfer protein, an ER resident protein, using a polyclonal antibody against human MTP (8). These intracellular staining patterns were typical ER patterns (Fig. 2B). Taken together, these data demonstrated that the membrane-bound SEP and SEP^D are localized in the endoplasmic reticulum.

To determine whether the protein localized in the ER by a retention or a retrieval mechanism, both SEP and SEP^D proteins were subjected to endo-β-N-acetylglucosaminidase D digestion. Retention refers to the protein never being exported out of the ER. In the retrieval mechanism, however, proteins escape from the ER to the cis-Golgi where the oligosaccharide is converted from Man₉GlcNAc₂ to Man₉GlcNac by mannosidase-I and then retrieved to the ER (11). Although Endo H digests both ER and cis-Golgi types of glycosylation, Endo D uniquely hydrolyzes only N-linked oligosaccharides of the Man₉GlcNac₂ (12, 13). As shown in Fig. 2C, both SEP and SEP^D were resistant to Endo D. In addition, it has been accepted that the incubation of cells with the microtubular inhibitor nocodazole alters the distribution of ER proteins by recycling from the intermediate compartment (14). We observed that the distribution of both SEP and SEP^D were not altered by this treatment (data not shown). Therefore, these results suggest that these proteins localized in the ER by a retention mechanism.

The Luminal Domain of SEP/SEP^D Determines Its ER Localization—To analyze the features of the membrane-bound SEP/SEP^D responsible for ER retention, we substituted the cytoplasmic transmembrane and luminal domains of this protein with the corresponding domains of bECE-1b (Fig. 3A). The bECE-1b is another member of this metalloprotease family, and this protein is normally transported to the cell surface (2).
Because no differences were observed in the localization and/or retention of the membrane-bound SEP/SEP\(^\Delta\), only the findings with SEP\(^\Delta\) are presented.

Several reports have shown that the cytoplasmic domain of type II membrane proteins determine ER localization (15–17). Thus, we first analyzed a chimeric protein S/S/E (SEP\(^\Delta\) cytoplasmic, SEP\(^\Delta\) transmembrane, and bECE-1b luminal, Fig. 3A) in which the cytoplasmic tail and transmembrane domain of SEP\(^\Delta\) were fused to the luminal domain of bECE-1b. When the subcellular localization of the chimeric protein was analyzed in transfected CHO cells unexpectedly, the S/S/E chimera was expressed on the cell surface as detected by immunofluorescence in permeabilized cells (Fig. 3B). This surface labeling in permeabilized cells was also observed in the wild-type bECE-1b, suggesting that like bECE-1b this chimera exits from the ER very efficiently, which results in high concentrations of the chimera at the cell surface. Nontransfected control cells did not react with the anti-ECE-1 polyclonal antibody (data not shown), consistent with the evidence that CHO cells have little or no endogenous activity of ECE-1 (2). Therefore, it appears that in the absence of the SEP\(^\Delta\) luminal domain, the cytoplasmic SEP\(^\Delta\) tail in combination with its transmembrane domain is not sufficient to achieve retention.

We next asked whether only the luminal domain of SEP\(^\Delta\) is sufficient for the correct targeting of the protein. A chimeric construct in which both the cytoplasmic tail and transmembrane domain of SEP\(^\Delta\) were replaced by those of bECE-1b (E/E/S, bECE-1b cytoplasmic, ECE-1b transmembrane, and SEP\(^\Delta\) luminal, Fig. 3A) was thus created. Immunofluorescence analysis of transient transfectants expressing E/E/S chimera showed the same distribution as the wild-type SEP\(^\Delta\). This chimeric protein behaved similarly to wild-type SEP\(^\Delta\) as indicated by its typical internal staining pattern and its lack of cell surface staining (Fig. 3B). These data indicate that the SEP\(^\Delta\) luminal domain, independent of the transmembrane domain, determines the localization of SEP\(^\Delta\) protein in the ER.

We then confirmed these findings by endoglycosidase digestion. CHO-K1 cells were transfected with cDNAs encoding the wild-type SEP, bECE-1b, the chimera S/S/E, and E/E/S. After 30 h, the proteins were harvested and treated with Endo H and PNGaseF. SEP and E/E/S gave a single band of 110-kDa on SDS-PAGE, were sensitive to both Endo H and PNGaseF, and were converted to a band approximately 20 kDa smaller (Fig. 3C). On the other hand, bECE-1b and S/S/E gave a higher molecular mass band (126-kDa) aside from the 110-kDa band. The higher molecular mass bands were sensitive to PNGaseF but resistant to Endo H, whereas the smaller band was sensitive to both endoglycosidases as in the case of SEP, E/E/S, and E/E/S, indicating that proteins with higher molecular mass contain complex-type oligosaccharides (Fig. 3C). Together, these results clearly demonstrate that the luminal domain of SEP determines the localization of the SEP protein in the ER.

The ER Localization of Membrane-bound SEP Is Not Attributed to Misfolding—The abnormally assembled proteins (misfolded proteins) in the ER are rapidly destroyed with the half-life of less than 1 h (18). Using CHO/SEP\(^\Delta\) stable transfectant cells, we provide a biochemical analysis of SEP\(^\Delta\) trafficking and turnover by pulse-chase experiments. The CHO/SEP\(^\Delta\) cells were pulse-labeled with \(^{35}\)S-amino acids for 30 min and followed by chase periods at designated times. At specific time intervals, cell extracts were prepared and immunoprecipitated with an anti-SEP antibody. Before analysis by SDS-PAGE, half of the samples were treated with Endo H. During all the chase periods, the proteins remained fully sensitive to Endo H, suggesting that it never acquired any Golgi enzyme modifications (Fig. 4A). In addition, as shown in Fig. 4B, the membrane-bound SEP\(^\Delta\) was retained in the ER with a half-life of more than 2 h. Therefore, it appears that the retention of membrane-bound SEP in the ER is not because of gross misfolding. Our previous findings also support this conclusion. We have reported that both the membrane-bound and the soluble forms of SEP have an activity to hydrolyze big ET-1 (4).

The Minimal Determinant of SEP Cleavage Is Defined within the Insertion Domain—Because the SEP and SEP\(^\Delta\) polypeptides predicted from the cDNA are identical except for the 23 amino acids unique to SEP, we hypothesized that the structural determinants, which cause the release of the soluble form of SEP, must be embedded within these 23-amino acid insertion. To test this hypothesis, we made a construct (E/E/a/E) in which these 23 amino acids (residues 41–63 of SEP) were inserted in frame between the transmembrane and extracellular domains of bECE-1b. When cells expressing this construct were analyzed by immunoblotting, the chimeric protein was...
secreted efficiently as in the case of wild-type SEP (Fig. 5A), thus demonstrating that the insertion domain of SEP is necessary and sufficient for the cleavage process.

To determine the sequence in the 23-amino acid insertion of SEP responsible for its secretion, we initially speculated that the biologically active soluble form of SEP is proteolytically released from the membrane-bound SEP by a dibasic process ing endoprotease(s) (4). To test this point, we first deleted these dibasic amino acids (Lys62-Arg63) (D62–63) or mutated to Ser62-Ser63 (KR-SS) or Asn62-Gly63 (KR-NG), but all these mutant SEP forms were cleaved as efficiently as wild-type SEP in CHO cells (Fig. 5B).

Because we failed to prove that the dibasic residues are the cleavage site of SEP, we then constructed a series of deletion mutations spanning the insertion region and expressed them in CHO cells. Of this deletion mutation series, the deletion of six C-terminal amino acids (D58–63) completely prevented SEP cleavage as did the deletion of the C-terminal 7, 8, 9, 17, 19, and 21 amino acids (Fig. 5B). In contrast, the deletion of 14 amino acids at the N-terminal of the SEP insertion (D41–54) did not have any effect, and only after we deleted 17 amino acids (D41–57) was SEP secretion abolished. Therefore, the C-terminal of this insertion seems dispensable for the secretion of this protein. To further define the important amino acids in the C-terminal of the SEP insertion, we kept Arg58 in the C-terminal deletion mutant (D59–63) and observed that this mutant was 50% cleaved compared with the wild-type SEP in CHO cells (Fig. 5B).

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SEP Cleavage Occurs in the ER—Because we did not observe any Golgi modification of these glycoproteins during all the chase periods of membrane-bound SEP/SEP (Fig. 4A), we hypothesized that soluble SEP is cleaved in the ER and then transported to the downstream secretory pathway until it is secreted. For this purpose, we used a fusion protein secECE-1 in which the signal peptide of human placental alkaline phosphatase (PLAP) is joined in frame to the luminal domain of bECE-1b as a tool to compare the intracellular trafficking and turnover of the protein with SEP (19). The signal peptide of placental alkaline phosphatase has been shown to be cleaved by a signal peptidase in the ER (20).

We established stable transfectants of CHO/secECE-1, CHO/SEP, and CHO/bECE-1b. These cells were labeled with 35S-amino acids for 30 min, chased for 0, 1, 2, or 4 h, rinsed, and then cultured in medium without tracers. Both the cell extracts and medium were collected and immunoprecipitated using an anti-ECE-1 or anti-SEP antibody. Half of each sample was...
digested with Endo H and analyzed by SDS-PAGE. In the cells transfected with secECE-1, we could not observe any intracellular protein that received Golgi enzyme modification since at 4 times of chase periods the protein remained fully sensitive to Endo H. However, within 1 h, we already detected a significant amount of secreted protein that is resistant to Endo H in the conditioned medium, and this increased in a time-dependent manner (Fig. 6A). This means that before reaching the Golgi compartment, this protein was already cleaved so that it is easily secreted into the medium. Similar findings were observed when SEP was transfected into CHO cells. In the cell lysates, the proteins were sensitive to Endo H, whereas the observed proteins in the medium were resistant to Endo H during all the chase periods (Fig. 6B).

This finding is in agreement with the pulse-chase result of the intracellular SEP\(^*\) which lacks the insertion domain. This protein never received Golgi enzyme modifications (Fig. 4A). A strikingly different result was observed for the membrane-bound form of wild-type bECE-1b. It has been shown that the bECE-1b protein was transported to the down secretory pathway after synthesis and finally resided in the plasma membrane (2). At 0 h of chase (30 min of pulse), we already observed the Endo H resistant band in the membrane-bound bECE-1b, and after 1 h, a significant amount of protein was exposed to the Golgi or plasma membrane as observed by its resistance to Endo H. Meanwhile, the protein retained in the ER (Endo H sensitive, lower band) was gradually exported to the plasma membrane and disappeared from the ER within 4 h (Fig. 6C). These findings show that the trafficking of membrane-bound SEP is distinct from that of membrane-bound bECE-1b and behaved similarly to membrane-bound secECE-1. Furthermore, when we immunostained the E/E/E chimera (Fig. 5A) in CHO cells, we observed a typical ER-staining pattern (Fig. 6D). This suggests that to be secreted, this chimera protein is initially targeted to the ER from which proteolytic cleavage occurs and then transported to the down secretory pathway. Taken together, these data strongly indicate that SEP is cleaved in the ER and subsequently is secreted rapidly into the medium.

**DISCUSSION**

This study has established that mRNAs encoding the two isoforms of mouse SEP, the membrane-bound and the soluble forms, are generated by alternative splicing of a single copy gene. After synthesis, the newly synthesized SEP proteins are targeted to the ER as type II integral membrane proteins. The membrane-bound isoform retained in this compartment is mediated by the luminal domain. In contrast, the soluble isoform is cleaved and released into the extracellular compartment. We have identified and characterized the structural determinant required for the retention of the membrane-bound form and the release of the SEP luminal domain and also demonstrated that...
Subcellular Localization and Secretion of SEP

the proteolytic cleavage system operates in the ER. Therefore, the characterization of the membrane-bound and the soluble forms of SEP reveals a unique mechanism of subcellular localization and protein trafficking in the secretory pathway.

The finding that SEP can exist in both soluble and membrane-bound forms led us to ask about how these two forms are produced and how their production is regulated. There are several membrane-bound proteins that have soluble counterparts, one example is fibronectin (21). Fibronectin exists in both insoluble and soluble forms and is present in the extracellular matrix and plasma, respectively. This diversity is generated via alternative splicing of cassette exons, alternative donors and acceptors, and retained introns (22). The incorporation of a cassette exon in fibroblasts produces cellular fibronectin, whereas its exclusion in the liver generates plasma fibronectin (22). We hypothesized that SEP is probably generated by a similar mechanism as is this protein. The inclusion of a cassette exon immediately following the exon encoding the putative transmembrane domain of SEP protein introduces an in frame cleavable exon that allows the intracellular secretion machinery to cleave and secrete the encoded protein to the plasma while its exclusion gives rise to membrane-bound SEP. These findings indicate that the sites where SEP functions as a metalloprotease are determined, at least in part, by an alternative splicing mechanism.

The subcellular localization of nephrilysin family members is quite diverse. NEP (1), Kell (23), bECE-1b (2), and PEX (24) are expressed on the cell surface where they act as ectoenzymes. Another isoform of ECE-1, bECE-1a, resides in an intracellular compartment that largely overlaps with the Golgi apparatus (2), whereas ECE-2 seems to function in the trans-Golgi network based on its acidic activity (3). Valdenaire and Schweizer (25) recently showed that ECE-like-1 was localized in the ER when transfected into CHO cells. In the current study, we present the evidence to conclude that SEP is another member of this family showing ER localization when transfected in two distinct cell types, CHO cells and COS-7 cells (data not shown). This conclusion is based on two lines of evidence. First, indirect immunofluorescence experiments showed that the SEP-staining pattern was similar to that of MTP, a marker of the ER. Second, the intracellular form of SEP contains a high mannosetype oligosaccharide as defined by Endo H sensitivity, whereas the secreted SEP contained an Endo H resistant oligosaccharide. Nonetheless, the data presented in this study cannot explain whether the behavior of overexpressed SEP reflects that of endogenous SEP in mammalian cells. Considering the fact that the precise in vivo functions of SEP are unknown so far, the characterization of subcellular localization provides a clue for the identification of the physiological substrate.

In this report, we also demonstrated that ER localization of SEP appears to be mediated by the luminal domain, independent from its cytoplasmic domain and transmembrane domain. Thus, the luminal domain functions as an intracellular sorting signal. An important criterion to define an intracellular sorting signal is the ability of that signal to function in a heterologous system, i.e., whether it is transferable. Indeed, we showed that the luminal domain of SEP protein is obviously sufficient for inducing ER localization of the cell surface protein, bECE-1b. To our knowledge, the only type II membrane protein with an ER localization signal in the luminal domain is the one bearing the His-Asp-Glu-Leu (Lys-Asp-Glu-Leu in eukaryotes) motif. This motif is recognized by a receptor in the Golgi apparatus, which results in the recycling of KDEL proteins back to the ER (26). Thus, it acts as a retrieval signal. Because the luminal domain of SEP lacks a KDEL motif, it must contain a new ER localization determinant and/or motif. Therefore, the detailed characterization of this ER localization signal is a challenging work for further experiments aimed at understanding the molecular basis by which ER localization is achieved.

Both the retention and retrieval mechanisms are thought to be involved in keeping ER resident proteins from escaping down the secretory pathway. In the current paper, we suggest that the mechanism for ER localization of the SEP protein is

FIG. 6. Posttranslational modification of secECE-1, SEP, and bECE-1b and ER localization of membrane-bound E/E/a/E. Equivalent numbers of CHO cells stably expressing secECE-1 (A), SEP (B), or bECE-1a (C) were pulse-labeled with [35S]methionine for 30 min and then chased for a designated time in complete medium. SecECE-1 and SEP proteins were immunoprecipitated both from the supernatants (S) and cell lysates (C), whereas bECE-1b protein was immunoprecipitated only from cell lysates. Before analysis on SDS-PAGE, half of the samples were treated with Endo H. An autoradiograph from a representative experiment is shown. D, immunofluorescence microscopy of transiently transfected CHO cells expressing E/E/a/E chimera. Fixed and permeabilized cells were stained with a bovine ECE-1 antibody.
retention rather than retrieval. Several arguments support this idea. Although we used a strong SR \( \alpha \) viral promoter in our expression construct, we did not observe any leakage of the SEP protein in the downstream secretory pathway. This implies that this localization mechanism could not be saturated by overexpression, suggesting the retention mechanism (27). Moreover, our experiments with Endo D (specific for Man\(_{3}\)GlcNAc\(_{2}\) trimming) and nocodazole (data not shown) also provide further evidence. Nevertheless, the idea that SEP proteins localize in the ER by a retention mechanism remains a working hypothesis because the available results could not exclude a rapid recycling from the intermediate compartments.

There are two kinds of proteins retained in the ER, the misfolded and/or incompletely assembled proteins and the ER resident proteins. The misfolded proteins associate with ER chaperones and folding enzymes, such as calnexin or BiP, and are rapidly destroyed with a half-life of less than 1 h (11, 18). We feel that this is not the case for SEP since the membrane-bound SEP was fairly stable for at least 4 h with a half-life of more than 2 h (Fig. 4, A and B). A more attractive explanation for this localization is that the membrane-bound SEP is the ER resident protein, and the luminal domain is directly responsible for ER residency. A strong argument in favor of this view is our previous observation that not only the soluble form but also the membrane-bound form of SEP show enzymatic activity to hydrolyze big ET-1 (4). Thus, the maintenance of the enzymatic activity of the ER proteins strongly indicates that membrane-bound SEP is a properly folded protein (18, 28, 29).

The present results show that the insertional domain of SEP is obviously responsible for secretion, and the WDERTVV region (residues 55–61) is the critical part of the cleavage determinant. This finding is in conflict with the data published by Ghadhar and co-workers (5) who postulated that SEP was converted into a soluble form by the action of pro-hormone convertases at the dibasic residues (KR). There are at least two possible explanations for this discrepancy, the difference of the cell types and/or overexpression. We believe that the former is not the case for SEP because the AKR mutant protein was completely secreted when we transfected an expression construct of this mutant into CHO cells (Fig. 5B) as well as human embryonic kidney 293 cells (data not shown). Although we cannot exclude the possibility that the presence of SEP proteins in the supernatant is because of overexpression, it is worth noting that the expression level of SEP/AKR was not different as compared with the wild-type SEP. In addition, if overexpression is the reason, we should observe leakage from the ER in other mutants. In fact, we failed to detect any leakage of the other mutants. In fact, we failed to detect SEP secretion in the supernatant is because of overexpression, it is worth noting that the expression level of SEP/AKR was not different as compared with the wild-type SEP. In addition, if overexpression is the reason, we should observe leakage from the ER in other mutants. In fact, we failed to detect any leakage of the other mutants. However, we used a strong SR \( \alpha \) viral promoter in our expression construct, we did not observe any leakage of the SEP protein in the downstream secretory pathway. This implies that this localization mechanism could not be saturated by overexpression, suggesting the retention mechanism (27). Moreover, our experiments with Endo D (specific for Man\(_{3}\)GlcNAc\(_{2}\) trimming) and nocodazole (data not shown) also provide further evidence. Nevertheless, the idea that SEP proteins localize in the ER by a retention mechanism remains a working hypothesis because the available results could not exclude a rapid recycling from the intermediate compartments.

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