Conserved Cysteine and Tryptophan Residues of the Endothelin-converting Enzyme-1 CXAW Motif Are Critical for Protein Maturation and Enzyme Activity*

Kathryn J. MacLeod‡, Robert S. Fuller§, Jeffrey D. Scholten‡, and Kyyunghye Ahn‡

The neprilysin (NEP)/endothelin-converting enzyme (ECE) family of metalloproteases contains a highly conserved carboxyl-terminal tetrapeptide sequence, CXAW, where “C” is cysteine, “X” is a polar amino acid, “A” is an aliphatic residue, and “W” is tryptophan. Although this sequence strongly resembles a prenylation motif, human ECE-1 did not appear to be prenylated when labeled in vivo using various isoprenoid precursors in cell lines expressing ECE-1. We used site-directed mutagenesis to investigate the role of the CXAW motif and determined that the conserved cysteine residue of the CXAW motif in ECE-1, Cys755, is critical for proper folding of the enzyme, its export from the endoplasmic reticulum, and its maturation in the secretory pathway. In addition, site-directed mutagenesis revealed that the conserved tryptophan residue of the sequence CEVW appears to be important for endoplasmic reticulum export and is essential for enzyme activity. Deletion of Trp758 or substitution with alanine greatly slowed maturation of the enzyme, and resulted in more than a 90% loss of enzyme activity relative to the wild type. Conservative substitution of the tryptophan with phenylalanine did not reduce activity, whereas replacement with tyrosine, methionine, or leucine reduced enzyme activity by 50%, 75%, and 85%, respectively. Together, these data indicate that the conserved CEVW sequence does not serve as a prenylation signal and that both the conserved cysteine and tryptophan residues are necessary for proper folding and maturation of the enzyme. Furthermore, the conserved tryptophan appears to be critical for enzyme activity.

Neutral endopeptidase (neprilysin (NEP))1; EC 3.4.24.11) and ECEs are zinc-binding metalloproteases that belong to the M13 subfamily of neutral endopeptidases. The mammalian subfamily consists of seven members, including NEP (1), ECE-1 (2–5), ECE-2 (6), the erythrocyte cell-surface antigen Kell (KELL) (7), a phoshate regulating neutral endopeptidase (PEX) (8), and recently three members with yet undefined functions: ECE-like 1 endopeptidase (ECEL1; XCE) (9), soluble secreted endopeptidase (SEP) (10), and damage-induced neuronal endopeptidase (DINE) (11). The overall amino acid sequence identity of ECE-1 to ECE-2, NEP, KELL, and PEX is 59, 39, 31, and 37%, respectively, and increases to 74, 54, 36, and 49% when comparing only the last 250 carboxyl-terminal residues. The greatest amount of identity lies in the residues involved in zinc binding and catalysis. These proteins also contain 10 conserved cysteine residues in their carboxyl-terminal extracellular domain. The extent of amino acid similarity indicates a common structure and catalytic mechanism for these proteins.

The carboxyl terminus of each of these metalloproteases terminates in a highly conserved tetrapeptide sequence CXAW. Most frequently a charged (E/R) or uncharged polar residue (Q/S) follows the cysteine, and is in turn followed by a hydrophobic residue (V/I/L) preceding the Trp. The CXAW sequence resembles a possible CAAX prenylation motif where “C” is cysteine, “A” is an aliphatic residue, and “X” is any amino acid. A GenBank/E7/EMBL Data bank search for proteins terminating in CAAX, and containing the conserved zinc-binding motif of metalloproteases, HEXXH, revealed only members of the NEP/ECE family. The conservation of this sequence suggests that it might play an important role in these proteins.

We wanted to investigate the function of the CXAW motif for this family of proteases using ECE-1 as an example. ECE-1 is responsible for the final proteolytic processing step in the biosynthesis of endothelins (ETs) (5) and may be involved in the synthesis and/or degradation of other peptide hormones as well (12, 13). ECE-1 cleaves the biologically inactive big ET between Trp21 and Val22/Ile24 to generate the 21-amino acid mature peptide ET, a potent vasoconstrictor (4, 5). The enzyme therefore plays a key role in maintaining vascular tone by catalyzing the production of ET, making it an important target for the treatment of pathological conditions such as cardiovascular and renal diseases.

One potential role for the CXAW sequence is that it serves as a novel prenylation site, possibly involving a unique prenyltransferase. Prenylation, a post-translational lipid modification necessary for the association of proteins with membranes as well as for specific protein-protein interactions, involves the covalent attachment of a 15-carbon farnesyl or a 20-carbon geranylgeranyl isoprenoid moiety through a thioether linkage to the CAAX motif cysteine (for review, see Refs. 14 and 15). The nature of the isoprenyl group is primarily dependent upon the amino acid in the X position. Well characterized CAAX
motifs include the sequences CAAM, CAAS, or CAAA, which confer the addition of a farnesyl moiety to proteins, and CAAL and CAWL, specific for geranylgeranyl addition (reviewed in Refs. 14 and 15). The topology of the NEP/ECE family proteins suggests that the CXXW sequence would be an unusual location for prenylation to occur since they are type II transmembrane proteins with a luminal carboxyl terminus. The question of prenylation was particularly interesting because there are no known examples of prenylation of a luminal CAAX motif. In addition, we wanted to investigate the importance of the conserved cysteine residue in this motif. The sequence CAAX has not been identified as a common prenyltransferase substrate, yet the cysteine in this tetrapeptide is completely conserved throughout the NEP/ECE family of proteases.

In the present study, we wished to analyze the tetrapeptide CXXW sequence of ECE-1, CEVW, and determine a possible role for the motif. In order to investigate whether the sequence serves as a prenylation signal, in vitro and in vivo prenylation assays were developed to assess prenylation of ECE-1. Additionally, site-directed mutations of the CXXW motif of ECE-1 were constructed to characterize the role of the highly conserved cysteine and cysteine residues.

EXPERIMENTAL PROCEDURES

Materials—Phosphoramidon, Pefabloc, pepstatin, and leupeptin were purchased from Roche Molecular Biochemicals. [3H]Mevalonolactone (50–60 Ci/mmol), [3H]Farnesol (60 Ci/mmol), and [3H]Geranylgeraniol (60 Ci/mmol) were purchased from American Radiolabeled Chemicals (St. Louis, MO). Tran35S-label and [3H]mevalonolactone (20–40 Ci/mmol) were from PerkinElmer Life Sciences. [3H]Farnesyl pyrophosphate and [3H]Geranylgeranyl pyrophosphate were obtained from Amersham Pharmacia, Inc. Peptide N-glycosidase F (PNGase F) and endoglycosidase H (endo H) were purchased from New England Biolabs (Beverly, MA). Polyoxyethylene-10-lauryl ether (C12E10) was from Calbiochem (La Jolla, CA). Human big ET-1 (1–38) was purchased from Peptides International (Louisville, KY). Biotinylated hexapeptides were synthesized by American Peptide Co. (Sunnyvale, CA). Precast SDS-polyacrylamide gels were from Novex. All oligonucleotides were custom synthesized by Life Technologies, Inc.

Cell Culture—Chinese hamster ovary (CHO-K1) cells were cultured in DMEM (Life Technologies, Inc.) containing 10% fetal bovine serum (Life Technologies, Inc.). Cells were incubated in a humidified atmosphere at 37 °C with 5% CO2. ECE-1a was provided by M. Yanagisawa and transfected into CHO cells expressing ECE-1a, CHO/ECE-1a, was maintained in the same medium containing 10% fetal bovine serum, and labeled with Tran35S-label (100 Ci/ml) for 4 h. CHO cells, CHO/ECE-1a cells, or HUVECs were grown to 80% confluence and incubated with 50 µCi of [3H]farnesol (60 Ci/mmol) or 150 µCi of [3H]geranylgeraniol (60 Ci/mmol) in the presence of 50 µCi of Tran35S-label for 16 h. Plates were washed with cold PBS, and cells were lysed in radiouimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 10 mM Tris-HCl, pH 7.5, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 10 mM EDTA, 1 mM Pefabloc, 1 mM pepstatin A, and 50 µg/ml leupeptin). After preclearing samples with PBS, immunoprecipitation, and analysis of ECE and Ha-Ras was carried out as described above.

In Vivo Prenylation and Immunoprecipitation of ECE-1—CHO cells were transfected with the wild type and mutant pZeosSV/flag-ECE-1a constructs using LipofectAMINE 2000 following the manufacturer’s protocol (Life Technologies, Inc.). The post-nuclear supernatant was precleared by incubating with PBS beads for 1 h at 4 °C. Flag-ECE-1a was immunoprecipitated with an anti-flag M2-agarose affinity gel (Sigma) overnight at 4 °C. The beads were pelleted, and the pre-cleared lysate was incubated with the monoclonal anti-ECE-1 antibody (Upstate Biotechnology) overnight at 4 °C. Antibody conjugates were immunoprecipitated with PAS beads and incubated for 1 h at 4 °C. Beads were then washed three times in 10 mM NaH2PO4, pH 8.0, 1% Triton X-100 buffer, one time with 100 mM NaH2PO4, pH 8.0, and one time with 10 mM NaH2PO4 without detergents. Beads were resuspended in Laemmli sample buffer, and samples were subjected to 4–20% or 8% SDS-PAGE (Novex). Gels were fixed in 30% methanol, 7.5% acetic acid, soaked in Enlightening (PerkinElmer Life Sciences), dried, and exposed to BioMax MR film (Eastman Kodak Co.).

CHO cells, CHO/ECE-1a cells, or HUVECs were grown to 80% confluence and incubated with 50 µCi of Tran35S-label for 1 h. Cells were then incubated with 150 µCi of [3H]farnesol (60 Ci/mmol) or 150 µCi of [3H]geranylgeraniol (60 Ci/mmol) in the presence of 50 µCi of Tran35S-label for 16 h. Plates were washed with cold PBS, and cells were lysed in radiouimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 10 mM Tris-HCl, pH 7.5, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 10 mM EDTA, 1 mM Pefabloc, 1 mM pepstatin A, and 50 µg/ml leupeptin). After pre-clearing samples with PBS, immunoprecipitation, and analysis of ECE and Ha-Ras was carried out as described above.

In Vivo Prenylation and Immunoprecipitation of ECE-1—CHO cells were transfected with the wild type and mutant pZeosSV/flag-ECE-1a constructs using LipofectAMINE 2000 following the manufacturer’s protocol (Life Technologies, Inc.). Two days after transfection, cells were incubated for 1 h in methionine-free DMEM (Life Technologies, Inc.) containing 10% dialyzed fetal bovine serum, and then labeled with Tran35S-label methionine (50 µCi/ml) for 5 h. Plates were washed with cold PBS, and cells were lysed in RIPA buffer. Lysates were vortexed, and a post-nuclear supernatant was prepared by centrifugation at 13,000 × g. The post-nuclear supernatant was precleared by incubating with PBS beads for 1 h at 4 °C. Flag-ECE-1a was immunoprecipitated with an anti-flag M2-agarose affinity gel (Sigma) overnight at 4 °C. Beads were then washed three times in RIPA buffer and resuspended in Laemmli sample buffer. Samples were subjected to 8% SDS-PAGE and analyzed by autoradiography as described above. To monitor expression levels of flag-ECE-1a proteins, 10-µl aliquots of the supernatant were transferred to nitrocellulose filter (Novex), immunoblotted with a polyclonal anti-ECE antibody, and detected by ECL following the manufacturer’s protocol (Amersham Pharmacia Biotech).

Pulse-Chase Labeling—CHO cells were transfected with the WT and mutant pZeosSV/flag-ECE-1a constructs as described above. Two days following transfection, cells were incubated for 1 h in methionine-free DMEM (Life Technologies, Inc.) containing 10% dialyzed fetal bovine serum, and labeled with Tran35S-label (100 µCi/ml) for 20 min at 37 °C. Plates were washed twice with PBS and chased in DMEM/F-12 with 10% fetal bovine serum containing excess cold methionine and cysteine at 37 °C each. At the appropriate time points, plates were washed with PBS and cells were lysed with RIPA buffer. A control sample for each protein incubation was continuously labeled with 50 µCi/ml Tran35S-label for 4 h. Cell lysates were prepared with an anti-flag M2-agarose affinity gel, subjected to 8% SDS-PAGE, and analyzed by autoradiography as described above.

PNGase F and Endo H Treatment—Wild type flag-ECE-1a and the ECE-1a mutant, SEVW, were immunoprecipitated from transfected CHO cells 48 h after transfection using an anti-flag M2-agarose affinity gel. Proteins were then described above, and incubated in denaturing buffer (0.5% SDS, 1% β-mercaptoethanol) at 100 °C for 10 min. Samples were then incubated with and without 3,000 units of endo H or 3,000 units of PNGase F overnight at 37 °C following the manufacturer’s protocol (New England Biolabs). The reaction was stopped by the addition of sample buffer, and samples were run on 8% Novex SDS-PAGE, transferred to nitrocellulose, immunoblotted with a poly-
TABLE I  
Sequence homology of conserved carboxyl-terminal CXAW Motifs in ECE/NEP metalloproteases

| Metalloprotease   | CXAW sequence |
|------------------|---------------|
| ECE-1 (all isoforms) | CEVW          |
| ECE-2            | CEVW          |
| Neprilysin       | CVRW          |
| Keli Blood Group | CQLW          |
| PEX gene product | CRLW          |
| ECEL1 (XCE)      | CSNVW         |
| DINE             | CSNVW         |
| SEP              | CRIV          |
| C. elegans metalloprotease | CQVW |

clonal anti-ECE antibody, and detected by ECL following the manufacturer's protocol (Amersham Pharmacia Biotech).

**ECE-1 Membrane Preparation**—CHO cells were transfected with the wild type and mutant pZeoSV/flag-ECE-1a constructs as described above. Three days after transfection, cells were washed and scraped into PBS, collected by centrifugation at 2,000 \( \times g \), and then resuspended in homogenization buffer (50 mM Tris-HCl, pH 7.4, 0.1 M NaCl, 0.25% C12E10, 1 \( \mu \)M Pefabloc, and 50 \( \mu \)g/ml leupeptin). Dounce homogenized, and centrifuged at 100,000 \( \times g \) for 1 h. The pellet was resuspended in homogenization buffer and again centrifuged at 100,000 \( \times g \) for 1 h. The pellet was solubilized in solubilization buffer (50 mM Tris-HCl, pH 7.4, 0.1 M NaCl, 0.25% C12E10, 1 \( \mu \)M Pefabloc, 1 \( \mu \)g/ml pepstatin A, and 50 \( \mu \)g/ml leupeptin) at 4 °C overnight. Unsolubilized membranes were centrifuged at 100,000 \( \times g \) for 1 h. Aliquots were subsequently used for activity assays.

**ECE-1 Activity Assays**—Solubilized membrane fractions were diluted with 20 mM Tris-HCl, pH 7.4, 0.1% C12E10, and incubated with 0.1 \( \mu \)g big ET-1(1–38) in reaction buffer (100 mM MES-ROH, pH 6.8, 0.1% C12E10, 1 \( \mu \)g/ml Pefabloc, 50 \( \mu \)g/ml leupeptin, and 1 \( \mu \)g/ml pepstatin A). Reactions were carried out at 37 °C for 1 h and stopped by adding EDTA to give a final concentration of 5 mM. The final mixture was then analyzed for the amount of ET-1 by enzyme-linked immunosorbent assay following the manufacturer's protocol (Amersham Pharmacia Biotech).

**RESULTS**

**In Vitro Prenylation of the Hexapeptide HKCEVW**—The NEP/ECF subfamily of metalloproteases has considerable amino acid sequence homology, and all the members terminate in a unique tetrapeptide CXXA, which has 32% amino acid sequence identity to Caenorhabditis elegans \(^2\), which has 32% amino acid sequence identity to ECE-1. The conservation of this sequence indicated that it might play an important role in these proteins, possibly as a novel prenylation site. To test this, we performed in vitro prenylation assays using as substrate a biotinylated hexapeptide corresponding to the native sequence, HKCEVW. Briefly, biotinylated hexapeptides were incubated with CHO cell lysate, as a source of prenyltransferase enzymes, and either \(^{3}H\)farnesyl pyrophosphate or \(^{3}H\)geranylgeranyl pyrophosphate. Hexapeptides containing known prenylation sequences were included as positive controls (TKCVIM and TKCVLS for farnesyl transferase, and TKCVIL for geranylgeranyl transferase). A small percentage of the HKCEVW peptide (less than 5% relative to the control hexapeptide) was radio-labelled when incubated with \(^{3}H\)geranylgeranyl pyrophosphate (data not shown). The weak prenylation suggested that the sequence can serve as a prenyltransferase substrate. We therefore assessed whether ECE-1 could be prenylated in vivo.

**In Vivo Labeling of ECE-1a with Isoprenyl Precursors**—CHO cells stably expressing the ECE-1 isoform, ECE-1a (CHO/ECE-1a), were metabolically labeled with the isoprenoid precursor \(^{3}H\)mevalonolactone. To facilitate the cellular uptake of the mevalonolactone, a vector encoding the mevalonate

---

\(^2\) GenBank™/EMBL Data Bank accession no. AAC46806.1.

**Fig. 1. Effect of in vivo isoprenylation labeling of ECE-1.** CHO cells were transiently transfected with the mevalonate transporter, pMev, alone (lane 1) or pMev and Ha-Ras as a positive control (lane 2), and a stable cell line of CHO cells expressing ECE-1a was transfected with pMev (lane 3). Two days following transfection, cells were metabolically labeled with 150 \( \mu \)Ci of \(^{3}H\)mevalonolactone in the presence of lovastatin and cells were processed as described under “Experimental Procedures.” Ha-Ras and ECE-1a were immunoprecipitated from lysates and subjected to 4–20 SDS-PAGE and exposed to Biomax MR film (A). To examine the immunoprecipitation of ECE-1a, aliquots from samples were subjected to 8% SDS-PAGE, followed by Western blot analysis using a polyclonal anti-ECE-1 antibody (B). HUVECs were incubated with 150 \( \mu \)Ci of \(^{3}H\)farnesol in the presence of lovastatin, and immunoprecipitation and analysis of Ha-Ras (lane 2) and ECE-1 (lane 3) was carried out as described under “Experimental Procedures” (C). The control sample (lane 1) is without antibody.
served Trp758, the CEV mutant was constructed in which the nerylgeranylation (CEVL mutant) or methionine for farnesyl was replaced by a preferred residue, either leucine for geranylgeranylation (CEVW mutant). This is because the tryptophan residue has been replaced by a preferred residue, either leucine for geranylgeranylation (CEVL mutant) or methionine for farnesyl (CEVW mutant). To further evaluate the role of the conserved Trp758, the CEV mutant was constructed in which the tryptophan residue was deleted. Conservative substitutions of Trp758 were also produced with phenylalanine (CEVF) and tyrosine (CEVY). Finally, the aromatic residue was removed by replacing Trp758 with alanine (CEVA).

A construct of ECE-1a containing a flag epitope tag at the amino terminus was used for the mutagenesis. Constructs were subcloned into the mammalian expression vector pZeoSV and transfected into CHO cells. Two days following transfection, ECE-1a expression was determined by Western blot analysis of cell lysates using a polyclonal antibody to ECE-1 (data not shown), and by metabolic labeling of the cells with [35S]methionine and immunoprecipitation of ECE-1a (Fig. 2). Cells were transiently transfected with the WT and mutant flag-ECE-1a constructs, labeled with [35S]methionine for 5 h, cell lysates prepared, and flag-ECE-1a immunoprecipitated using an anti-flag M2-agarose affinity gel. The immunoprecipitates were then analyzed by SDS-PAGE and autoradiography.

With expression of WT, two 35S-labeled bands at 130 and 110 kDa were immunoprecipitated (Fig. 2A, lane 1). The identity of the two bands as ECE-1a was confirmed by Western blot analysis (data not shown). The ECE-1a mutants CEVL, CEVM, CEVF, and CEVY (Fig. 2A, lanes 3, 4, 7, and 8) all showed near WT expression levels of both bands, but the CEV and CEVA mutants (Fig. 2A, lanes 5 and 9) showed decreased levels of the upper 130-kDa band. In contrast, the two mutants in which the cysteine was replaced, SEVW and ΔCEVW, showed only the lower molecular weight form of the enzyme (Fig. 2A, lanes 2 and 6).

We wanted to determine whether the presence of the 130-kDa band was due to post-translational modification. In addition, we wanted to determine whether the 110-kDa band represented an immature form of the protein rather than a degradation product. The recent crystal structure of human NEP at 2.1 Å resolution shows that the cysteine of the CAXW motif in NEP, Cys746, is involved in an intramolecular disulfide bond with Cys705 (29). This result suggests that the homologous cysteine of the CAXW motif of ECE-1a, Cys755, may also be involved in an intramolecular disulfide bond important for proper folding of the enzyme. To test whether the 110- and 130-kDa forms of ECE-1a represent differences in protein folding or post-translational modification, two conserved cysteine residues were labeled in vivo by a mevalonolactone derivative.

Four isoforms of human ECE-1 (1a, 1b, 1c, and 1d) have been cloned. All of these are encoded by one gene and share a common carboxyl-terminal portion, but each is expressed from one of four distinct promoters, which regulate expression of the four unique amino termini (18–21). Although the carboxyl terminus of the ECE-1a CXAW sequence is identical in each of the isoforms, the amino-terminal sequences may be responsible for differences in tissue specificity of expression as well as differences in subcellular localization. The different isoforms are localized either to the cell surface or an intracellular compartment, possibly the Golgi (5, 19, 21–26).

In order to eliminate the possibility that prenylation of ECE-1 is isomeric-specific, HUVECs, which are known to express messages encoding each ECE-1 isoform (19, 21), were used for labeling. Cells were metabolically labeled with [3H]farnesol or [3H]geranylgeraniol as precursors for farnesyl phosphate and geranylgeranyl pyrophosphate, respectively (27, 28). Unlike mevalonolactone, the alcohol derivatives are readily taken up by the cells, providing a method to metabolically label the primary HUVECs without the need to transfect the pMev transporter. Cells were labeled overnight with [3H]farnesol or [3H]geranylgeraniol in the presence of lovastatin, lysed, ECE-1-immunoprecipitated, and analyzed by SDS-PAGE and autoradiography as described under “Experimental Procedures.” Immunoprecipitation of endogenous Ha-Ras was used as a positive control for labeling and prenylation from HUVECs labeled with [3H]farnesol. As shown in Fig. 1C, only Ha-Ras appears to be prenylated migrating as a single band of ~23 kDa (Fig. 1C, lane 2). ECE-1 did not appear to be labeled by either [3H]farnesol (Fig. 1C, lane 3) or [3H]geranylgeraniol (data not shown), indicating that it may not be modified by prenylation.

Effect of CAXW Motif Mutations on ECE-1a Protein Expression—Site-directed mutagenesis was used to assess the role of the CAXW motif and its effect on protein modification and enzyme activity. To simplify discussion of the mutants, the nomenclature used refers to the amino acid substitution in the CAXW sequence as summarized in Table II. The ECE-1a mutant, SEVW, refers to the replacement of Cys755 of the CAXW motif with serine. ΔCEVW refers to deletion of the CXAW sequence by introducing a stop codon at Cys755. The mutants CEVL, CEVM, and CEVF refer to mutations constructed to create possible prenylation sites where the tryptophan residue has been replaced by a preferred residue, either leucine for geranylgeranylation (CEVL mutant) or methionine for farnesylation (CEVM mutant). To further evaluate the role of the conserved Trp758, the CEV mutant was constructed in which the tryptophan residue was deleted. Conservative substitutions of Trp758 were also produced with phenylalanine (CEVF) and tyrosine (CEVY). Finally, the aromatic residue was removed by replacing Trp758 with alanine (CEVA).

| ECE-1a mutation | CXAW sequence |
|-----------------|---------------|
| Wild type       | CEVW          |
| C755S           | SEVW          |
| ΔC755-W758      | ΔCEVW         |
| W758L           | CEVL          |
| W758M           | CEVM          |
| ΔC23kDa         | C23kDa        |
| W758F           | CEVF          |
| W758Y           | CEVY          |
| W758A           | CEVA          |
residues other than Cys\(^{755}\) of ECE-1a, Cys\(^{632}\) and Cys\(^{743}\), were changed to serine creating two mutants (C632S and C743S) in addition to the SEVW (C755S) mutant. By sequence alignment with NEP, Cys\(^{632}\) in ECE-1a corresponds to the cysteine paired with Cys\(^{755}\), Cys\(^{743}\) is a conserved cysteine upstream of Cys\(^{755}\) and is believed to be disulfide-bonded with Cys\(^{110}\). Metabolic labeling and immunoprecipitation of ECE-1a from these mutants also resulted in a single band of \(\sim 110\) kDa (Fig. 3B, lanes 2 and 3). These results suggest strongly that the conserved cysteine residues in ECE-1a are necessary for protein folding or processing.

**Pulse-Chase Labeling of ECE-1a**—To further determine whether the conserved cysteine and tryptophan residues of the CEVW motif in ECE-1a are important for proper processing of the enzyme in the secretory pathway, we performed pulse-chase labeling of the WT enzyme and the ECE-1a mutants SEVW and CEV. Fig. 3 shows the results of the pulse-chase labeling in CHO cells transfected with WT, SEVW, or CEV. Briefly, cells were pulsed with \(^{35}\)S-methionine for 20 min and then chased for 0, 15, 30, 45, 60, 120, and 240 min in the presence of excess amounts of unlabeled methionine and cysteine as described under “Experimental Procedures.” A control sample for each was continuously labeled with \(250 \mu Ci\) of \(^{35}\)S-methionine for 4 h. Cells were lysed at the indicated time points and immunoprecipitated with an anti-flag M2-agarose affinity gel. After the pulse, a single band of 110 kDa was observed in each cell line (Fig. 3, A–C, lane 1). During the chase the WT enzyme undergoes post-translational modification, resulting in a mature form of the enzyme of 130 kDa by 30 min (Fig. 3A, lane 3). However, labeling of the SEVW mutant results in only the 110-kDa form of the enzyme even after 4 h of chase or in the control sample with continuous labeling (Fig. 3B). This result indicates that Cys\(^{755}\) of the CXAW motif is critical for proper processing and maturation of ECE. Additionally, post-translational processing of the ECE-1a mutant without the conserved Trp\(^{758}\), CEV, appears to be slower than for the WT, with the mature form of the enzyme not appearing until 120 min (Fig. 3C).

**Endoglycosidase Treatment of ECE-1a**—Treatment of WT and the SEVW mutant with glycosidases confirmed that the 110-kDa band is an immature form of the enzyme (Fig. 4). Treatment of glycoproteins with endo H removes immature but not medial Golgi-processed N-linked oligosaccharide side chains (30). Immunoprecipitated WT was treated overnight with endo H, and mobility of ECE-1a was analyzed by SDS-PAGE and immunoblot using a polyclonal anti-ECE-1 antibody. The lower 110-kDa band was endo H-sensitive and shifted to \(\sim 80\) kDa, consistent with the predicted molecular mass of ECE-1a (Fig. 4, lanes 1 and 2). The upper 130-kDa band was endo H-resistant, indicating that the two bands represent an immature endoplasmic reticulum-associated form and a mature form of the enzyme. Treatment of the mutant protein SEVW with endo H also resulted in a molecular mass shift from 110 to 80 kDa (Fig. 4, lanes 3 and 4). PNGase F, an enzyme that removes all N-linked oligosaccharide side chains, was used as a positive control for deglycosylation of ECE-1a. Treatment of both the WT and the SEVW mutant with PNGase F overnight completely deglycosylated both the mature and immature forms of ECE-1a and yielded one band of \(\sim 80\) kDa (Fig. 4, lanes 5–8). These results further demonstrate that Cys\(^{755}\) is critical for proper processing of the enzyme through the secretory pathway.

**ECE-1a Activities on Purified Membranes from ECE-1a CXAW Motif Mutants**—Enzyme activity assays were performed on the flag-ECE-1a mutants to determine what effect mutations of the CEVW sequence have on enzyme activity. ECE-1a activities were measured on purified membranes from CHO cells expressing WT and mutant flag-ECE-1a proteins using big ET-1 as substrate as described under “Experimental Procedures.” The amount of ECE-1a was normalized for the 130-kDa mature form of the protein by Western blot. Fig. 5 shows the relative ECE-1a activities of the WT and the CXAW mutants. Activities are expressed relative to the WT enzyme, which had a specific activity of \(1.9 \times 10^{-4} \ \mu mol/min/mg\) of the total membrane fraction defined as 100%. The CEVF mutant yielded a fully active enzyme, and CEVH had \(\sim 50\%\) activity of the WT enzyme. Surprisingly, deletion of the tryptophan residue, mutant CEV, or replacement with alanine, CEVA, resulted in more than a 95% loss in activity. The mutants CEVM...
TABLE III

| ECE-1a mutant | Phosphoramidon | PD 069185 |
|---------------|---------------|-----------|
| WT            | 2.2 ± 0.2     | 2.3 ± 0.2 |
| CEVF          | 7.3 ± 0.5     | 5.4 ± 0.3 |
| CEVY          | 0.57 ± 0.1    | 2.2 ± 0.5 |
| CEVL          | 5.6 ± 0.7     | 0.69 ± 0.3|
| CEVM          | 3.1 ± 1.1     | 1.8 ± 0.4 |

Kinetic analysis of ECE-1a and the ECE-1a mutants

ECE-1a activities were measured on membranes purified from CHO cells expressing the flag epitope-tagged constructs for WT and ECE-1a mutants as described under “Experimental Procedures.” ECE-1a activities were measured at 0.1 μM big ET-1 in the presence of the ECE-1 inhibitors phosphoramidon and PD 069185 over a range of concentrations from 0.1 to 100 μM. Kᵢ values were obtained using an equation, Kᵢ = IC₅₀/(1 + [S]/Kₘ), for competitive inhibitors phosphoramidon and PD 069185 under the assay conditions where the substrate concentration is well below the Kᵢ value of 2.7 μM for WT ECE-1a (37). These data represent the average of two to three separate experiments.

and CEVL had ~25% and 15% activity of WT levels, respectively. These two mutant proteins express both the 110- and 130-kDa bands at comparable levels to those of WT. These results suggest that an aromatic residue at the terminal position of the CXXAW motif is essential for activity, and that a hydrophobic residue only partially restores activity.

To determine whether these mutations affected binding to the active site, Kᵢ determinations for two competitive ECE-1a inhibitors, phosphoramidon and PD 069185 (31), were performed. As shown in Table III, the Kᵢ values for the mutants were not significantly different from WT for either compound. Only one mutant, CEVY, demonstrated a lower Kᵢ than the other mutants for phosphoramidon, and the Kᵢ for PD 069185 was reduced ~3-fold for mutant CEVL, suggesting a small increase in affinity for these compounds. Further kinetic work using purified enzyme will be needed to better characterize the function of the tryptophan residue on enzyme activity.

**DISCUSSION**

Currently there is no information concerning the role of the conserved terminal tetrapeptide CXXAW sequence of the NEP/ECE family of metalloproteases. The highly conserved sequence of cysteine-charged (E/R) or uncharged polar residue (Q/S)-hydrophobic residue (V/I/L)-tryptophan in this family of metalloproteases led us to investigate the function of this sequence. The sequence resembles a CAAX prenylation motif, making it a possible candidate for a novel prenylation signal. An aromatic residue in the carboxyl-terminal position of the CAAX motif is not a common feature, although the sequence CCIF, which is found at the carboxyl terminus of bovine brain Ras-related small G protein, G25K, is modified by all-trans-geranylgeranyl-Cys methyl ester (32). In addition, substrate specificity studies of farnesyltransferase modification of the precursor of the yeast a-mating factor demonstrated that the sequence CVIW can be farnesylated, although to a much lesser degree than the wild type sequence CVIA (33). We wanted to determine whether one function of the CXXAW motif is to serve as a prenylation signal for the NEP/ECE family that might involve a novel prenyltransferase.

The wild type sequence HKCEWV is weakly geranylgeranylated in vitro using the biotinylated hexapeptide as substrate for prenyltransferases. However, ECE-1 was not labeled in vivo by isoprenoid precursors in either of two cell lines tested: CHO cells stably expressing ECE-1a or HUVECs expressing endogenous isoforms of ECE-1. ECE-1 isoforms 1a, 1b, and 1c were shown to be palmitoylated (34), demonstrating that the enzyme undergoes post-translational modifications other than glycosylation. Nonetheless from the data presented in this study, it appears that ECE-1 is not prenylated.

In addition to investigating prenylation of ECE-1, we wished to further characterize the role of the CAAX motif through mutagenic analysis. Site-directed mutagenesis of the CEVW sequence demonstrated that the conserved Cys⁷⁵⁵ is critical for proper folding and maturation of the enzyme, possibly through intramolecular disulfide bond formation. Substitution of Cys⁷⁵⁵ with serine (mutant SEVW) or removal of the tetrapeptide by introduction of a stop codon at Cys⁷⁵⁵ (mutant ΔCEVW) resulted in a misfolded protein that possibly remained in an early secretory compartment, presumably the endoplasmic reticulum, as demonstrated by both pulse-chase labeling and endo H glycosidase treatment. The recent x-ray crystal structure of human NEP complexed with the inhibitor phosphoramidon shows that NEP contains 12 cysteine residues, all involved in disulfide bridges (29). Based on the NEP crystal structure and sequence alignment between NEP and ECE-1, Cys⁷⁵⁵ of ECE-1a is predicted to be disulfide-bonded with Cys⁶³². Site-directed mutagenesis of Cys⁶³² as well as Cys⁷⁴₃, the cysteine immediately upstream of Cys⁷⁵⁵, resulted in an immature form of the enzyme only. These results indicate that the conserved cysteines in ECE-1a, including Cys⁷⁵⁵ of the CEVW motif, are important for proper folding of the protein possibly through disulfide bonds making Cys⁷⁵⁵ unavailable for prenylation or modification.

Mutations of the conserved Trp of the CEVW sequence, Trp⁷⁵⁸, suggest that an aromatic or hydrophobic residue as the terminal amino acid of the enzyme is important for protein processing and for enzyme activity. The conservative mutation CEVY had little effect on enzyme activity, whereas the CEVY mutation reduced activity by 50%. A hydrophobic residue only partially substituted for Trp with the CEVL and CEVM mutants, resulting in 15% and 25% enzyme activity compared with wild type, respectively. These effects on activity do not appear to be due to changes in substrate affinity as Kᵢ determinations for two competitive inhibitors for ECE-1, phosphoramidon and PD 069185, did not show significant differences. Removal of
Trp758 in the CEV mutant or replacement with alanine, mutant CEVA, resulted in a completely inactive enzyme but appeared to affect protein processing and stability. These data suggest that an aromatic residue as the terminal amino acid of the protein is crucial for enzyme activity.

The recently released coordinates of human NEP reveal that the corresponding tryptophan, Trp749, resides in a hydrophobic pocket, which may be necessary to anchor the carboxyl terminus. Proper orientation of the carboxyl terminus would be required to affect protein processing and stability. These data suggest that considerable structural changes or the residue may be interacting with the conserved Cys746 and Trp758 residues are both critical for enzyme activity, as well as those of the aromatic or hydrophobic residue. However, alterations in substrate binding or catalysis (36). These data suggest that there may be structural differences between the two enzymes. In substrate binding or catalysis (36). These data suggest that there may be structural differences between the two enzymes.

It is likely that Trp758 of ECE-1a plays a similar structural role for the enzyme and that the effects observed on enzyme maturation and activity were consistent with the mutagenesis data, where the largest effects in substrate binding or catalysis (36). These data suggest that there may be structural differences between the two enzymes.

We have demonstrated that the conserved CXAW sequence of ECE-1a does not appear to be a prenylation signal, but that the conserved Cys746 and Trp758 residues are both critical for proper folding and maturation of the enzyme through the secretory pathway. We have also provided evidence for the importance of the terminal amino acid of ECE-1a, Trp758, in enzyme activity. It is clear from this study that the conserved CXAW motif plays an important role in enzyme function. This is the first work that identifies residues outside of the zinc-binding consensus sequence and catalytic site that influence activity, possibly giving new evidence for residues involved in formation or stability of the active site. Further work will be required to define more completely the function of these residues in the control of enzyme activity.

**Acknowledgments**—We thank Dr. Bernd-M Gollfier (F. Hoffman-La Roche, Basel, Switzerland), for the kind gift of the ECE-1 monoclonal antibody, ECE-6. We thank Dr. Gary V. Johnson (RED Systems, Inc., Minnetonka, MN) for the construction of the pZeroSV/Flag–ECE1a plasmid. We thank Dr. David Moreland for analysis of the neprilysin crystal structure. We also thank Douglass Fahnoe for many helpful discussions.

**REFERENCES**

1. Malfroy, B., Kuang, W. J., Seeburg, P. H., Mason, A. J., and Schofield, P. R. (1988) FEBS Lett. 229, 206–210
2. Ohnaka, K., Takayanagi, R., Nishikawa, M., Haji, M., and Nawata, H. (1993) J. Biol. Chem. 268, 26759–26766
3. Takahashi, M., Matsushita, Y., Iijima, Y., and Tanazawa, K. (1993) J. Biol. Chem. 268, 21394–21398
4. Shimada, K., Takahashi, M., and Tanazawa, K. (1994) J. Biol. Chem. 269, 18275–18278
5. Xu, D., Emoto, N., Giaid, A., Slaughter, C., Kaw, S., deWit, D., and Yanagisawa, M. (1994) Cell 78, 473–485
6. Emoto, N., and Yanagisawa, M. (1995) J. Biol. Chem. 270, 15262–15268
7. Lee, S., Zambas, E. D., Marsh, W. L., and Redman, C. M. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 6353–6357
8. Consortium, T. H. (1995) Nature 376, 130–136
9. Valdenaire, O., Richards, J. G., Fauli, R. L. M., and Schweizer, A. (1999) Mol. Brain Res. 64, 211–221
10. Ikeda, K., Emoto, N., Raharjo, S., Nurbantori, Y., Suika, K., Yokoyama, M., and Matsuo, M. (1999) J. Biol. Chem. 274, 32469–32477
11. Kiryu-Seo, S., Sasaki, M., Yokohama, H., Nakagomi, S., Hiyayama, T., Aoki, S., Wada, K., and Kiyama, H. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 4545–4550
12. Huang, M. V., and Turner, A. J. (1997) Biochem. J. 327, 23–26
13. Johnson, G. D., Stevenson, T., and Ahn, K. (1999) J. Biol. Chem. 274, 4053–4058
14. Zhang, F. L., and Casey, P. J. (1996) Annu. Rev. Biochem. 65, 241–269
15. Clarke, S. (1996) Annu. Rev. Biochem. 65, 159–196
16. Kim, C. M., Goldstein, J. L., and Brown, M. S. (1992) J. Biol. Chem. 267, 23115–23121
17. Hayes, J. S., Lawler, O. A., Walsh, M.-T., and Kinsella, B. T. (1999) J. Biol. Chem. 274, 23707–23718
18. Valdenaire, O., Rohrbacher, E., and Mattei, M. G. (1995) J. Biol. Chem. 270, 29794–29798
19. Schweizer, A., Valdenaire, O., Nelhock, P., Deuchelle, U., Dumas, J.-B., Stumpf, J. G., and Loffler, B.-M. (1997) Biochem. J. 328, 871–877
20. Orzechowski, H. D., Richter, C. M., Funke-Kaiser, H., Kroger, B., Schmidt, M., Menzel, S., Bohnemeier, H., and Paul, M. (1997) J. Mol. Med. 75, 512–523
21. Valdenaire, O., Lepailleur-Enouf, D., Egidy, G., Thouard, A., Barret, A., Vranckx, R., Tougard, C., and Michel, J.-B. (1999) Eur. J. Biochem. 264, 341–349
22. Barnes, K., Shimada, K., Takahashi, M., Tanazawa, K., and Turner, A. J. (1996) J. Cell Biol. 130, 919–928
23. Barnes, K., Eirolf, C. D., Lin, B., and Waechter, C. A. (1999) Methods Mol. Biol. 116, 107–123
24. Corsini, A., Farnsworth, O. C., McGeady, P., Gell, M. H., and Glomset, J. A. (1999) Methods Mol. Biol. 116, 124–144
25. Eofer, C., D’Arcy, A., Hennig, M., Winkler, F. K., and Dale, G. E. (2000) J. Biol. Chem. 275, 341–349
26. Oefner, C., and Kornfeld, S. (1980) J. Biol. Chem. 255, 18047–18057
27. Ahn, K., Sisneros, A. M., Herman, S. B., Pan, S. M., Hupe, D., Lee, C., Nikam, S., Cheng, X.-M., Doherty, A. M., Schroeder, R. L., Haleen, S. J., Kaw, S., Emoto, N., and Yanagisawa, M. (1998) Biochem. Biophys. Res. Commun. 243, 184–190
28. Yamane, H. K., Farnsworth, C. C., Xie, H., Evans, T., Howald, W. N., Gelb, M. H., Glomset, J. A., Clarke, S., and Fung, B. K. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 286–290
29. Trueblood, C. E., Boyartchuk, V. L., and Rine, J. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 10774–10779
30. Schweizer, A., Loffler, B.-M., and Eirolf, C. D. (1999) Biochem. J. 340, 649–656
31. Beaumont, A., Lemoual, H., Gioleau, G., Crine, P., and Roques, B. P. (1991) J. Biol. Chem. 266, 214–220
32. Shimada, K., Takahashi, M. T., and Tanazawa, K. (1996) Biochim. Biophys. Acta 1315, 863–867
33. Aitken, K., Herbiz, S. B., and Fahnoe, D. C. (1998) Arch. Biochem. Biophys. 358, 258–268

3 NEP coordinates are available from the Protein Data Bank (code 1DMT).
Conserved Cysteine and Tryptophan Residues of the Endothelin-converting Enzyme-1 C XAW Motif Are Critical for Protein Maturation and Enzyme Activity
Kathryn J. MacLeod, Robert S. Fuller, Jeffrey D. Scholten and Kyunghye Ahn

J. Biol. Chem. 2001, 276:30608-30614.
doi: 10.1074/jbc.M103928200 originally published online June 7, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M103928200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 37 references, 23 of which can be accessed free at
http://www.jbc.org/content/276/33/30608.full.html#ref-list-1