Characterization of Endothelin-converting Enzyme-2

IMPLICATION FOR A ROLE IN THE NONCLASSICAL PROCESSING OF REGULATORY PEPTIDES*

Received for publication, November 4, 2002, and in revised form, January 27, 2003
Published, JBC Papers in Press, January 30, 2003, DOI 10.1074/jbc.M211242200

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Most neuroendocrine peptides are generated by proteolysis of the precursors at basic residue cleavage sites. Prohormone convertases belonging to the subtilisin family of serine proteases are primarily responsible for processing at these “classical sites.” In addition to the classical cleavages, a subset of bioactive peptides is generated by processing at “nonclassical” sites. The proteases responsible for these cleavages have not been well explored. Members of several metalloprotease families have been proposed to be involved in nonclassical processing. Among them, endothelin-converting enzyme-2 (ECE-2) is a good candidate because it exhibits a neuroendocrine distribution and an acidic pH optimum. To examine the involvement of this protease in neuropeptide processing, we purified the recombinant enzyme and characterized its catalytic activity. Purified ECE-2 efficiently processes big endothelin-1 to endothelin-1 by cleavage between Trp72 and Val73 at acidic pH. To characterize the substrate specificity of ECE-2, we used mass spectrometry with a panel of 42 peptides as substrates to identify the products. Only 10 of these 42 peptides were processed by ECE-2. A comparison of residues around the cleavage site revealed that ECE-2 exhibits a unique cleavage site selectivity that is related to but distinct from that of ECE-1. ECE-2 tolerates a wide range of amino acids in the P1-position and prefers aliphatic/aromatic residues in the P1′-position. However, only a small fraction of the aliphatic/aromatic amino acid-containing sites were cleaved, indicating that there are additional constraints beyond the P1- and P1′-positions. The enzyme is able to generate a number of biologically active peptides from peptide intermediates, suggesting an important role for this enzyme in the biosynthesis of regulatory peptides. Also, ECE-2 processes proenkephalin-derived bovine adrenal medulla peptides, and this processing leads to peptide products known to have differential receptor selectivity. Finally, ECE-2 processes PEN-LEN, an endogenous inhibitor of prohormone convertase 1, into products that do not inhibit the enzyme. Taken together, these results are consistent with an important role for ECE-2 in the processing of regulatory peptides at nonclassical sites.

A wide variety of proteins in neuronal, endocrine, and immune tissues undergo proteolytic processing. Many of these proteins and peptides are intercellular messengers. Most neuroendocrine peptides are synthesized from precursor proteins. Post-translational processing of these precursors is a key step in the production of biologically active peptides. In the majority of cases, this occurs by proteolysis of the precursors at classical cleavage sites. These sites are usually multiple basic amino acids (1). Several neuropeptide-processing enzymes have been identified in mammalian cells (2–5). Initially the precursor is cleaved by endopeptidases of the subtilisin family of serine proteases, such as prohormone convertases (PCs)1 (for review, see Refs. 2 and 4). Following endopeptidase activity, carboxypeptidases such as carboxypeptidase E (CPE, also called CPH) remove the basic amino acids from the C termini of peptides (6). The peptides with C-terminal extended Gly residues are processed into C-terminal amidated peptides by peptidylglycine α-amidating monooxygenase (7). All of these enzymes involved in the generation of regulatory peptides exhibit a restricted neuroendocrine distribution as well as subcellular localization to peptides containing secretory vesicles; furthermore, they are optimally active at pH 5–6 (2–8). These properties are consistent with the involvement of these enzymes in the generation of a number of peptide hormones, neuropeptides, and other peptide neurotransmitters (8, 9).

A subset of bioactive peptides is generated by processing at nonclassical sites. These have been identified primarily by bulk purification from neuroendocrine tissues (10–12). Additionally, an examination of cleavage sites within the precursors for endogenous peptides showed that nonclassical processing is required to release the peptide from its precursor (13–15). Finally, mass spectrometric techniques to identify neuropeptides in the brains of mice lacking specific processing enzymes such as CPE have led to the identification of products of nonclassical cleavages (16).

Members of the metalloprotease family have been largely implicated in the processing of bioactive peptides at nonclassical sites (17). The majority of these proteases exhibit near neutral pH optima and a cellular and subcellular localization that is not consistent with a role for these enzymes in neuroendocrine peptide processing within the intracellular milieu (18). Among them ECE-1 exhibits a neutral pH optimum, a broad tissue distribution, and predominant cell surface expression (19, 20). ECE-1 converts big endothelin (ET) to endothelin through cleavage at a Trp-Val (endothelin-1 and -2) or a Trp-Ile (endothelin-3) site (19). ECE-2 was discovered in 1995 (21) as a

* This work was supported by National Institutes of Health Grants NS 26880 and DA00458 (to L. A. D.) and DA04494 (to L. D. F.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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‡ The abbreviations used are: PC, prohormone convertase; CPE, carboxypeptidase E; ECE, endothelin-converting enzyme; ET, endothelin; Dyn, dynorphin; BAM, bovine adrenal medulla; ACTH, adrenocorticotropic hormone; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight.
novel member of the ECE-1 gene family; the two gene products share 59% amino acid identity (18). In contrast to ECE-1, ECE-2 is optimally active at pH 5.5 and localized to an intracellular compartment (21–24). A recent study examining the isoforms of ECE-2 has found that the ECE-2b isoform is highly expressed in neuroendocrine tissues (brain, pituitary, and adrenal medulla) and poorly expressed in other tissues (25). These properties make ECE-2b an ideal candidate for intracellular processing of neuroendocrine precursors. Despite this, relatively little has been reported about the enzymatic properties of ECE-2b.

Here we describe studies characterizing the biochemical and kinetic properties of ECE-2. Because this enzyme is a transmembrane protein, purification of this protein in sufficient quantities to allow comprehensive studies could be labor intensive and time consuming. This problem has been overcome in the case of many Zn^{2+} metalloproteases, including ECE-1, through the expression of soluble secreted enzymes (26, 27). This has enabled abundant expression and rapid purification of these enzymes (26, 27). The resulting enzyme has been found to exhibit virtually identical biochemical properties as that of the endogenous enzyme (26, 27). We used a similar strategy to produce and purify the soluble catalytic portion of ECE-2 that is common to all isoforms (ECE-2a1, -2a2, -2b1, and -2b2). In this study, we show that purified ECE-2 exhibits an acidic pH optimum and a unique cleavage site selectivity. Furthermore, we show that ECE-2 is able to generate biologically active peptides from known precursors. These results support a role for ECE-2 in the nonclassical processing of regulatory peptides.

EXPERIMENTAL PROCEDURES

MATERIALS—Big endothelin-1 (human, 1–38), substance P, neuropeptide Y, [Arg^8]vasopressin, joining peptide (bovine), Zn^{2+}-endoproteinase (rat), dynorphin B (Dyn B), Dyn A, Dyn A-8, bovine adrenal medulla (BAM) peptide E, BAM 22, and luteinizing hormone-releasing hormone were purchased from Peninsula Laboratories, Inc. (San Carlos, CA). Adrenocorticotropic hormone (ACTH, rat), bradykinin, and angiotensin I, II, and III were acquired from Sigma. Little PEN-LEN was synthesized by Invitrogen, internally quenched fluorescent substrate McaBk2 (7-methoxycoumarin-4-yl)acetly-Arg-Pro-Pro-Gly-Phe-Ser-Ala-Phe-Lys-(4,2-dinitrophenyl)) was custom synthesized by Sigma-Genosys, and all other peptides were synthesized at the peptide synthesis facility at Albert Einstein College of Medicine. DEAE-Sepharose fast flow anion exchange column in 20 mM Tris-Cl, pH 7.1 (Buffer A). An ion exchange column was washed with 10 column volumes of Buffer A and eluted with a linear gradient of 0–0.5 M NaCl in Buffer A. The fractions were assayed for ECE-2 activity as described below and for protein levels using BCA reagent (Pierce). The peak of ECE-2 activity eluted around 0.25 M NaCl. Fractions containing the highest activity were pooled and loaded onto a TalonTM-Sepharose Co2+ affinity resin column equilibrated with Tris-Cl buffer, pH 7.1, containing 300 mM NaCl. Previously, we have found the Nia+ affinity resin to be unsuitable for purification of ECE-2. The enzyme bound to Co2+ resin was washed with the sodium acetate buffer, pH 6.0, containing 300 mM NaCl and was eluted with the same buffer adjusted to pH 5.0. Fractions containing ECE-2 activity were subjected to SDS-PAGE and visualized by silver staining.

**RESULTS**

**Eukaryotic Expression and Purification of ECE-2**—To facilitate the biochemical characterization, we expressed ECE-2 as a soluble secreted protein in a eukaryotic expression system. For this, the transmembrane domain (including the N-terminal region) was removed from ECE-2b secreted from baculovirus cells (Fig. 1B) as well as the 120-kDa form of endogenous ECE-2 (data not shown). The signal was blocked completely by the corresponding epitope peptide, suggesting that the antigen is specific to ECE-2.

**Identification of Peptide Hydrolysis Products by Mass Spectrometry**—Approximately 1 nmol of each peptide listed in Table II was incubated with purified ECE-2 in 0.2 mM sodium acetate, pH 5.5, at 37 °C. The reaction was carried out for various time periods and terminated by quenching. Peptides from samples were isolated using Zip-Tip hydrophobic chromatography columns and analyzed by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry on a Perceptive Biosystems Voyager-DE STR mass spectrometer as described (16, 30). Approximately 100 laser shots were summed per spectrum. n-Cyano-4-hydroxycinnamic acid saturated in 30% acetonitrile and 0.1% trifluoroacetic acid in water was used as a matrix. External calibration was performed with [des-Arg^1]bradykinin (Μ + H^+) = 904.4681) and neurotensin (Μ + H^+) = 1672.9170).

**Determination of Kinetic Constant for ECE-2 Hydrolysis of Different Peptides**—Approximately 0.2–100 μM peptides were incubated with 0.5–3 mM purified ECE-2 in 0.2 mM sodium acetate buffer, pH 5.5, containing 0.1% C_{18}F_{37}C_{8}H_{17} (v/v) at 37 °C. Reactions were carried out for various times and quenched by the addition of trifluoroacetic acid of 0.1%. Prior to injection, the trifluoroacetic acid and acetonitrile concentrations of the sample were adjusted to 0.05 and 15%, respectively. The samples were loaded onto a C_{18} column (0.46 × 15 cm) and eluted using a linear gradient of 15–65% acetonitrile in 0.05% trifluoroacetic acid. The peptides were detected by measuring absorbance at 215 nm. The initial rate of substrate hydrolysis (V_0) was determined by measuring the appearance of product under initial rate conditions (less than 10% substrate hydrolysis). V_0 values were plotted as a function of substrate concentration (S) and fit to the Michaelis-Menten equation using the PRISM package (version 2.0) software. V_0 values were determined using the equation k_{cat} = V_0[E] with a subunit molar mass of 100 kDa. Identity of peptide peaks was confirmed by MALDI-TOF mass spectrometry as described (16, 30).

**RESULTS**

**Eukaryotic Expression and Purification of ECE-2**—To facilitate the biochemical characterization, we expressed ECE-2 as a soluble secreted protein in a eukaryotic expression system. For this, the transmembrane domain (including the N-terminal region) was removed from ECE-2b to enable expression as a soluble enzyme and replaced with the signal sequence and prorogation of a secretory vesicle protein, CPE, to enable targeting of the recombinant enzyme to the secretory pathway. Thus the cDNA fragment encoding the entire luminal domain (that contains the catalytic region) of ECE-2b was fused in frame...
Substrate Specificity of ECE-2

Table I

<table>
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<tr>
<th>Purification steps</th>
<th>Total protein</th>
<th>Total activity</th>
<th>Yield</th>
<th>Specific activity</th>
<th>Purification</th>
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<td>0.28</td>
<td>13</td>
<td>0.22</td>
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</tr>
<tr>
<td>Talon affinity resin</td>
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<td>0.24</td>
<td>11</td>
<td>4.79</td>
<td>95.9</td>
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</tbody>
</table>

Fig. 1. Purification of ECE-2 activity from the media of Sf9 cells overexpressing ECE-2. A, fractions containing ECE-2 activity were subjected to purification by ion exchange and metal ion chromatography as described under “Experimental Procedures.” The resulting fractions were subjected to electrophoresis under denaturing conditions on an 8% polyacrylamide gel. B, Western blot analysis of ECE-2 using a polyclonal antiserum directed against the C-terminal region of ECE-2. The positions of protein standards (in kDa) are indicated.

with the cDNA encoding the signal sequence and proregion of CPE; the resulting sequence consisted of residues 1–42 of CPE, six His residues, and residues 81–766 of ECE-2b. The secreted medium from cells infected with recombinant virus was subjected to purification using anion exchange and metal ion affinity chromatography. For the determination of ECE-2 activity, the intramolecularly quenched fluorescent peptide substrate McaBk2 was used (31). This reagent originally was developed as a substrate for ECE-1 and has 7-methoxycoumarin-4-yl and dinitrophenyl as the donor and acceptor at the N and C termini, respectively. The substrate is readily hydrolyzable by ECE-2; this simple and fast assay proved to be ideal for the rapid purification and characterization of ECE-2.

ECE-2 was purified −100-fold from the secreted medium (Table I). The level of purification was judged by SDS-PAGE and visualized by silver staining (Fig. 1A). Western blotting analysis with a polyclonal antiserum directed against the C-terminal region of ECE-2 (Fig. 1B). The purified ECE-2 exhibits an apparent K_m of about 9 μM for the McaBk2 substrate.

Characterization of ECE-2 Activity: pH Optimum and Divalent Cation Sensitivity—We characterized the purified ECE-2 activity by examining its pH optimum and sensitivity to divalent cations. Previous studies with crude ECE-2 (membranes from cells expressing recombinant ECE-2) showed that the enzyme was optimally active at pH 5.5 (21). Our results with purified ECE-2 agree with this earlier observation in that the purified enzyme exhibits a sharp pH dependence (Fig. 2A). The pH optimum is 5.0–5.5, and the enzyme has <20% of the maximal activity at pH 4.5 or 6.0 and is inactive at pH 6.5 (Fig. 2A). This acidic pH optimum of ECE-2 contrasts with the near neutral pH optimum of ECE-1 (19, 20, 31). These results are consistent with a role in peptide processing for ECE-1 in the extracellular milieu and for ECE-2 in the intracellular milieu.

The sensitivity of ECE-2 to divalent ions was tested using ZnCl_2, CoCl_2, NiSO_4, CuSO_4, MnCl_2, MgCl_2, and CaCl_2. ECE-2 is activated to a small but significant extent by Co^{2+} ions at all concentrations examined (Fig. 2B). In response to Zn^{2+} and Cu^{2+}, the enzyme exhibits a biphasic response in that low concentrations (up to 10 μM) activate and higher concentrations inhibit ECE-2 activity. Ni^{2+} ions inhibit the activity at all concentrations tested. Mn^{2+}, Mg^{2+}, or Ca^{2+} ions had no significant effect on ECE-2 activity. Taken together, these results support the involvement of metal ions at the active site.

ECE-2 Hydrolyzes a Variety of Bioactive Peptides—Next we examined 30 distinct peptides and a pool of 12 peptides that differ only in the penultimate position for their ability to serve as substrates of ECE-2 (Table II), and we identified the products by MALDI-TOF mass spectrometry (Fig. 3). Among the biologically active peptides that were previously found to be cleaved by other related metalloproteases, big ET-1 and bradykinin are cleaved, respectively, at sites Trp^{21}-Val^{22} and Pro^{2}-Phe^{8}, which also are used by ECE-1 (31, 32). Interestingly,
[des-Arg^1]bradykinin is not cleaved even though the Pro^7-Phe^8 site is present in this peptide. This suggests that there is a minimum length requirement for cleavage by ECE-2 (discussed further below). Neurotensin, angiotensin I, and substance P are cleaved at sites (Pro^10-Tyr^11, Pro^7-Phe^8, and Gly^9-Leu^10, respectively) that also are cleaved by neprilysin. ECE-2 does not cleave luteinizing hormone-releasing hormone, which has been shown to be cleaved by neprilysin and ECE-1, albeit less efficiently (32). Furthermore, a number of peptides, such as angiotensin II and III, [Arg]vasopressin, ACTH, and /Hâ9252-neoen-dorphin, that are hydrolyzed by other metalloproteases are not cleaved by ECE-2 (Table II), suggesting that this enzyme exhibits unique substrate specificity.

Among the peptides derived from opioid peptide precursors, proopiomelanocortin-derived peptides (β-endorphin, ACTH, and J-peptide) are not cleaved by ECE-2 (Table II). In contrast, proenkephalin-derived peptides, namely peptide E, BAM 22, and BAM 18, were found to be good substrates for ECE-2 (Fig. 3 and Table II). The primary site of cleavage of peptide E is between Gly^23 and Phe^24 leading to the generation of BAM 23 (Fig. 3). Peptide E also is cleaved between Asp^16 and Tyr^17, albeit less efficiently, to generate BAM 16. Another proenkephalin A-derived peptide, BAM 22, also is cleaved by ECE-2 at a single site leading to the generation of BAM 12 (Table II). Finally, BAM 18 is cleaved by ECE-2 at Asp^16-Tyr^17 and at Glu^12-Trp^13 generating BAM 16 and BAM 12, respectively. Although the three substrates share substantial homology in their N-terminal region, the sites of processing of these peptides appear to have only a partial overlap. Furthermore, the fact that peptide E and BAM 22, which differ by only three amino acids at their C termini, are processed at entirely different sites suggests a role for peptide length and/or secondary structure in the recognition of the substrate by ECE-2.

Among the peptides derived from prodynorphin, Dyn B is cleaved by ECE-2, whereas Dyn A or Dyn A-8 is not (Table II). Among the pro-SAAS-derived peptides, only PEN-LEN is processed by ECE-2 (Fig. 3). The major cleavage is at Arg^21-Val^22 leading to the generation of PEN-21, and minor cleavages are at Ala^18-Leu^19 and Leu^20-Arg^21 (Fig. 3 and Table II). To address the amino acid requirement at and around the cleavage site, a number of synthetic peptides were tested as substrates. These 9–14-residue peptides represent various portions of carboxypeptidases A-5, D, and E. One set of peptides was a mixture containing 12 amino acid substitutions at the position...
Substrate Specificity of ECE-2

Big Endothelin-1  
Endothelin-1 1-21  
Big Endothelin-1 22-38  

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Theoretical mass</th>
<th>Mass (m/z)</th>
<th>% Intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>No enzyme</td>
<td></td>
<td>2142.45</td>
<td>100</td>
</tr>
<tr>
<td>ECE-2</td>
<td></td>
<td>1809.94</td>
<td>100</td>
</tr>
</tbody>
</table>

**Fig. 3. Cleavage site determination using MALDI-TOF mass spectrometry.** One nanomole of peptide was incubated in the presence or absence of purified ECE-2 (5 ng) in 0.2 M sodium acetate buffer, pH 5.5, for 100 min at 37°C. The reaction was terminated by quick freezing, and the samples were subjected to MALDI-TOF mass spectrometry as described (16, 28).
penultimate to the C terminus; the individual peptides present in the mixture were detectable by MALDI-TOF mass spectrometry. None of these peptides were cleaved by ECE-2.

From the analysis of sites within the 10 peptides that were cleaved (as well as those that were not), it appears that ECE-2 prefers cleaving at sites containing an aromatic residue (Trp, Tyr, or Phe) or an aliphatic residue with a large branched side chain (Ile, Val, or Leu) at the P1′ site. A wide range of amino acids is tolerated in the P1-position, although a Pro, Gly, or charged residue (Glu, Asp, Lys, or Arg) is often present at the P1 site of the most efficiently cleaved peptides. However, a notable exception is big ET-1, where the cleavage site presumably is exposed because of the cysteine bridge. Because ECE-2 does not cleave most of the potential sites that contain a P1′ aromatic or aliphatic residue, there must be additional constraints that limit the activity of this enzyme. Inspection of the hundreds of potential sites in the 42 peptides tested revealed several additional features that distinguish between cleaved and uncleaved sequences: cysteines are not present surrounding the cleavage site (from P3 to P3′), acidic residues are not present in the P3 to P3′ sites except at the P1-position, and there are no prolines in the P1′-to-P3′-positions. In addition, the cleavage sites are located between 7 and 23 residues from the N terminus and between 2 and 17 residues from the C terminus. Finally, if two sites are present that both fit these preferences, the enzyme appears to prefer the site closer to the C terminus. The peptidyldepeptidase-like activity seen with ECE-2 also has been reported for other metalloendoproteases, including neprilysin and ECE-1 (31, 32).

**Kinetic Analyses of ECE-2 Hydrolysis of Bioactive Peptides**

Initial velocity dependence on substrate concentration was determined for five representative peptides that serve as substrates for ECE-2 (Fig. 4 and Table III). These results show that the processing of substrates by ECE-2 follows typical Michaelis-Menten kinetics; this is shown for peptide E and bradykinin in Fig. 4. Comparisons of the kinetic parameters show that McaBk2 is the best substrate for ECE-1 and ECE-2. Although big ET-1 serves as a substrate for both ECE-1 and ECE-2, there is a substantial difference in catalytic rates between these two enzymes. Among the peptides processed by ECE-2, Dyn B exhibits the highest catalytic rate and lowest affinity, whereas big ET-1 exhibits the lowest catalytic rate and highest affinity. Furthermore, the fact that various peptides are processed at differing efficiencies suggests a role for ECE-2 in modulating the levels of biologically active peptides.

**DISCUSSION**

In this study, we have characterized the enzymatic properties of ECE-2. Using recombinant purified ECE-2, we show that the enzyme has an acidic pH optimum and is completely inactive at pH 7; neither the McaBk2 peptide nor larger peptide substrates including big ET-1 are cleaved by ECE-2 at neutral pH. This is in contrast to ECE-1, which exhibits maximal activity at pH 7.0 for the cleavage of larger peptides, including big ET-1, and a pH optimum of ∼6.0 for shorter peptide substrates (20, 33). Taken together, these results are consistent with the presence of at least two distinct substrates for ECE-2.

**Fig. 4. Kinetic analysis of ECE-2 hydrolysis of two representative bioactive peptides.** Purified ECE-2 (5 ng) was assayed using the indicated concentrations of the various peptides. The reaction mixtures were subjected to high pressure liquid chromatography analysis, and peptides were detected by absorbance at 215 nm. The initial rate of substrate hydrolysis was determined for five representative peptides that serve as substrates for ECE-2 (Fig. 4 and Table III). These results show that the processing of substrates by ECE-2 follows typical Michaelis-Menten kinetics; this is shown for peptide E and bradykinin in Fig. 4. Comparisons of the kinetic parameters show that McaBk2 is the best substrate for ECE-1 and ECE-2. Although big ET-1 serves as a substrate for both ECE-1 and ECE-2, there is a substantial difference in catalytic rates between these two enzymes. Among the peptides processed by ECE-2, Dyn B exhibits the highest catalytic rate and lowest affinity, whereas big ET-1 exhibits the lowest catalytic rate and highest affinity. Furthermore, the fact that various peptides are processed at differing efficiencies suggests a role for ECE-2 in modulating the levels of biologically active peptides.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_{m}$ ECE-2</th>
<th>$K_{m}$ ECE-1</th>
<th>$k_{cat}/K_{m}$ ECE-2</th>
<th>$k_{cat}/K_{m}$ ECE-1</th>
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</thead>
<tbody>
<tr>
<td>Big ET-1</td>
<td>0.4 ± 0.03</td>
<td>2.0 ± 0.3</td>
<td>0.0022 ± 0.000002</td>
<td>0.052 ± 0.0002</td>
</tr>
<tr>
<td>Peptide E</td>
<td>1.4 ± 0.17</td>
<td>NA</td>
<td>0.004 ± 0.0002</td>
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<tr>
<td>McaBk2</td>
<td>8.7 ± 0.7</td>
<td>6.0 ± 0.2</td>
<td>6.9 ± 0.4</td>
<td>110 ± 10</td>
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<tr>
<td>Bradykinin</td>
<td>27.4 ± 3.0</td>
<td>460.0 ± 10</td>
<td>5.8 ± 0.3</td>
<td>48 ± 2</td>
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<tr>
<td>Dynorphin B</td>
<td>48.4 ± 4.1</td>
<td>NA</td>
<td>8.8 ± 0.5</td>
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</table>

$^a$ Data from Johnson and Ahn (31).
$^b$ NA, not available.
with the processing of peptides at the cell surface by ECE-1 and in an intracellular compartment by ECE-2.

ECE-2b exhibits a neuroendocrine distribution (20, 21, 34, 35). A study examining the distribution of ECE-2b by in situ hybridization analysis has found that the localization is restricted to neurons and to areas of the central nervous system enriched in neuropeptides (34). Another study examining the distribution of ECE-2b within the endothelial cells by immunocytochemistry localized the enzyme to an intracellular compartment (23, 24). Consistent with this, the processing compartment of ECE-2b activity was found to be intracellular because co-expression of the enzyme with endothelin precursor was required for the generation of ET-1, whereas co-incubation of cells individually expressing the two was not sufficient to generate ET-1 (21). Taken together, the neuroendocrine distribution, acidic pH optimum, and subcellular localization to a peptide-containing compartment make ECE-2b an ideal candidate for an intracellular processing enzyme of neuroendocrine precursors.

Analysis of processing of biologically active peptides revealed that ECE-2 cleaves some but not all peptides that are processed by other Zn$^{2+}$ metalloendopeptidases (such as ECE-1 and nephrilysin). For example, some regulatory peptides (neurotensin, Bradykinin, angiotensin I, and substance P) that are cleaved by ECE-2 are also hydrolyzed by ECE-1 and nephrilysin. In contrast, a number of peptides that are cleaved by nephrilysin are not processed by either ECE-1 (32) or ECE-2 (Table II). Thus it appears that like ECE-1, ECE-2 is involved in the selective processing of specific peptides as opposed to the nonselective degradation of peptides. In the case of ECE-1, studies using big ET-1 and peptides derived from endothelins have suggested that hydrolysis of substrates by ECE-1 is highly dependent on substrate conformation (35, 36). In support of this, it was found that linear big ET-1, in which the formation of disulfide bonds was prevented by alkylation of the four cysteines, was cleaved at multiple sites; this is in contrast to the native big ET-1 that is cleaved at a single site by this enzyme (26). It is likely that the recognition of substrates by ECE-2 is also dependent on the substrate length and conformation because we find that not all structurally related peptides are recognized by ECE-2. Furthermore, the analysis of cleavage site selectivity of ECE-2 suggests that the peptide length as well as residues surrounding the cleavage site that would confer secondary structure play an important role in the recognition of the substrate by ECE-2.

A notable feature of ECE-2 is that the enzyme is able to process endoproteolytically peptide intermediates to generate biologically active peptides. Big ET-1 is processed at the Trp-Val site to generate ET-1; this site of cleavage is identical to the site used by ECE-1. However, the fact that ECE-2 processes big ET-1 at acidic pH (as described above) suggests that this enzyme is able to generate ET-1 intracellularly. Another potential biologically active peptide generated by ECE-2 is BAM 12 from the biologically active processing of BAM 22 (Table II). Both BAM 22 and BAM 12 were originally isolated as enkephalin-containing opioid peptides from bovine adrenal medulla (37) and later reported to be distributed in the substantia nigra and pallidum of rat and human brains (38, 39). BAM 12 exhibits κ opioid receptor selectivity that contrasts with the μ opioid receptor selectivity of BAM 22 (40, 41). These results imply that differential processing of these peptides would modulate selective activation of opioid receptor types. In addition, differential processing could also generate peptides that activate other G-protein-coupled receptors. A recent study has shown BAM 22 to be the most potent ligand for sensory neuron-specific G-protein-coupled receptors (SNSR-3 and SNSR-4) in the dorsal root ganglion; these receptors are thought to be involved in pain transmission (42). We find that ECE-2 efficiently processes peptide E to BAM 23, which contains a C-terminal Gly. Because peptides with C-terminal Gly are substrates for pep tidglycine α-amidating monoxygenase, BAM 23 would be converted rapidly into BAM 22 amide by this enzyme. Thus it appears that ECE-2 is capable of processing a number of peptide intermediates leading to the generation of a variety of endogenous ligands.

One of the peptide intermediates processed by ECE-2 is a pro-SAAS-derived peptide, PEN-LEN. We and others have shown previously that PEN-LEN peptides with intact C termini are inhibitors of the classical processing enzyme, pro hormone convertase 1 (29, 43). In this study, we find that ECE-2 cleaves PEN-LEN leading to the generation of shorter PEN peptides such as PEN-21. It should be pointed out that within the milieu of secretory vesicles, PEN-21 would be converted rapidly to PEN-20 by CPE. PEN-20 is an endogenous peptide found in both rat brain as well as pituitary (44). We also have shown that these shorter PEN peptides do not inhibit PC1 activity (29, 43). The endoproteolytic processing of PEN-LEN has functional implications because processing at internal sites, as seen with ECE-2, that results in the generation of shorter peptides (such as PEN-19 and PEN-20) would lead to a loss of PC1 inhibition (29, 43). Because PC1 is involved in the generation of a large number of neuroendocrine peptides, modulation of PC1 activity would have a significant impact on the levels of neuroendocrine peptides. Thus, the nonclassical processing of PEN-LEN by ECE-2 would affect the level of PC1 inhibitory peptides and is likely to play an important role in the regulation of neuroendocrine peptide levels in vivo.

Acknowledgments—We thank Dr. Kay Ahn for the gift of McaBk2 substrate and Chris Cardozo for help with high pressure liquid chromatography analysis of products.

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Substrate Specificity of ECE-2


Characterization of Endothelin-converting Enzyme-2: IMPLICATION FOR A ROLE IN THE NONCLASSICAL PROCESSING OF REGULATORY PEPTIDES

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doi: 10.1074/jbc.M211242200 originally published online January 30, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M211242200

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