Deposition of β-amyloid (Aβ) peptides in the brain is an early and invariant feature of all forms of Alzheimer’s disease. As with any secreted protein, the extracellular concentration of Aβ is determined not only by its production but also by its catabolism. A major focus of Alzheimer’s research has been the elucidation of the mechanisms responsible for the generation of Aβ. Much less, however, is known about the mechanisms responsible for Aβ removal. In this report, we describe the identification of endothelin-converting enzyme-1 (ECE-1) as a novel Aβ-degrading enzyme. We show that treatment of endogenous ECE-expressing cell lines with the metalloprotease inhibitor phosphoramidon causes a 2–3-fold elevation in extracellular Aβ concentration that appears to be due to inhibition of intracellular Aβ degradation. Furthermore, we show that overexpression of ECE-1 in Chinese hamster ovary cells, which lack endogenous ECE activity, reduces extracellular Aβ concentration by up to 90% and that this effect is completely reversed by treatment of the cells with phosphoramidon. Finally, we show that recombinant soluble ECE-1 is capable of hydrolyzing synthetic Aβ40 and Aβ42 in vitro at multiple sites.

Alzheimer’s disease (AD) is the most common cause of dementia in the elderly and is characterized pathologically by the accumulation of β-amyloid peptides (Aβ) in the brain in the form of senile plaques. Aβ is normally produced from the β-amyloid precursor protein (βAPP) through the combined proteolytic actions of β- and γ-secretase and is then secreted into the extracellular milieu (1, 2). The degree of Aβ accumulation is dependent not only on its production but also on the mechanisms responsible for its removal. While considerable effort has been directed at elucidating the enzymes and pathways contributing to the production of Aβ, much less is known regarding Aβ catabolism.

Aβ catabolism is likely to involve proteases at multiple sites, both intracellular and extracellular. Proteases acting at the site of Aβ generation and/or within the secretory pathway may degrade the peptide intracellularly, thus limiting the amount of the peptide available for secretion. The concentration of secreted Aβ may be further regulated by direct degradation by extracellular proteases and by receptor-mediated endocytosis or phagocytosis followed by lysosomal degradation. Catabolism of Aβ peptides at each of these steps would limit the accumulation of extracellular Aβ, and disruption of this catabolism may be a risk factor for AD. Additionally, the identification of enzymes that degrade Aβ intracellularly and extracellularly may lead to development of novel therapeutics aimed at reducing Aβ concentration by enhancing its removal.

Recent reports suggest a role for both insulin-degrading enzyme and nephrilysin (NEP) in the degradation of extracellular Aβ (3–10). Matrix metalloproteinase-9, EC 3.4.24.15, and α2-macroglobulin complexes have also been reported to play a role in Aβ degradation (11–13). In this report, we describe the identification of endothelin-converting enzyme-1 (ECE-1) as a novel Aβ-degrading enzyme. The endothelin converting enzymes are a class of type II integral membrane zinc metalloproteases (active site luminal) named for their ability to hydrolyze a family of biologically inactive intermediates, big endothelins (big ETs), exclusively at a Trp21–Val/Ile22 bond to form the potent vasoconstrictors endothelins (14). In addition to this specific cleavage event, ECE-1 has been reported to hydrolyze several biologically active peptides in vitro, including bradykinin, neurotensin, substance P, and oxidized insulin B chain by cleaving on the amino side of hydrophobic residues (15, 16).

Two different endothelin-converting enzymes have been cloned. The first identified, ECE-1, is abundantly expressed in the vascular endothelial cells of all organs and is also widely expressed in nonvascular cells of tissues including lung, pancreas, testis, ovary, and adrenal gland (17–19). A comprehensive analysis examining both ECE activity and expression in human brain has not been reported. Studies have, however, detected human ECE-1 immunoreactivity in fibers within the glial limitans and neuronal processes and cell bodies of the cerebral cortex (18). In rats, ECE-1 immunoreactivity has been detected in pyramidal cells of the hippocampus and in cultured primary astrocytes (20).

Four isoforms of human ECE-1 differing only in the cytoplasmic tail are produced by a single gene located on chromosome 1 (1p36) through the use of alternate promoters (17, 21–25). The four isoforms cleave big ETs with equal efficiency but differ primarily in their subcellular localization and tissue distribution (24, 25). Human ECE-1α is localized predominantly to the plasma membrane (24, 25). Human ECE-1c and ECE-1d have...
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also been reported to be localized predominantly to the plasma membrane with additional intracellular expression detected (24, 25). In contrast, human ECE-1b appears to be localized exclusively intracellularly. Co-immunolocalization studies performed by Schweizer et al. (24) on human ECE-1b-transfected CHO cells indicate the presence of this isoform in the trans-Golgi network (TGN). Azarani et al. similarly demonstrated that human ECE-1b was located in an intracellular compartment when expressed in Madin-Darby canine kidney cells (26), and Cailler et al. (27) demonstrated that a dileucine motif in the cytosolic tail of ECE-1b was probably responsible for its intracellular localization. In an endogenous ECE-1b- and ECE-1c-expressing cell line, ECV304, ECE-1 immunoreactivity was detected in intracellular Golgi-like structures as well as at the cell surface (24).

Bovine ECE-1b, which corresponds to human ECE-1c, is also localized predominantly on the plasma membrane (28). However, in contrast to human ECE-1a, bovine ECE-1a has convincingly been shown to be constitutively targeted to the lysosome (28). This difference between the localization of human and bovine ECE-1a may be due to the fact that the isoform-specific N-terminal region of ECE-1a is poorly conserved between the species. In fact, Emoto et al. (28) identified lysosome-targeting signals in the N-terminal tail of bovine ECE-1a that are not conserved in human ECE-1a.

ECE-2 is a homologous enzyme with catalytic activity similar to that of ECE-1. Bovine ECE-2 has been cloned and is encoded by a separate gene from ECE-1 (29). The sequence and chromosomal location of the human ECE-2 gene have not been reported. However, Nagase et al. (30) recently reported the cloning of an unidentified human brain cDNA, KIAA0604, that shares 93% identity with the bovine ECE-2 gene and is located on human chromosome 3. Given the similarity to bovine ECE-2, this cDNA probably represents human ECE-2.

ECE-2 is localized intracellularly and has an acidic pH optimum (29). Immunochemical analysis of endogenous ECE-2 in HUVeCs revealed a punctate pattern of staining with expression of ECE-2 in acidic intracellular vesicles of the constitutive secretory pathway (31). Northern blot analysis of bovine tissues revealed that ECE-2 is most abundantly expressed in neural tissues including cerebral cortex, cerebellum, and adrenal medulla, with low level expression detected in many other tissues (29). In mouse brain, ECE-2 is expressed in heterogeneous populations of neurons in the thalamus, hypothalamus, amygdala, dentate gyrus, and CA3 (32). Like ECE-1, ECE-2 cleaves big ET-1 most efficiently among the three big ETs (24, 29). Another member of the ECE family, ECE-3, has recently been purified from bovine iris microsomes and is highly specific for the conversion of big ET-3 (33). The enzyme responsible for this activity has not yet been cloned.

The role that ECE may play in Alzheimer's disease has not been previously explored. Here we present both pharmacological and biochemical evidence that ECE-1 can hydrolyze Aβ both in vitro and in vivo. These data indicate a potential role for this enzyme family in Aβ catabolism.

**EXPERIMENTAL PROCEDURES**

**Analysis of Aβ Concentration by Sandwich ELISA**—Human Aβ was measured by sandwich ELISA as previously described (34), using the BAN50/BA27 and BAN50/BC05 antibody systems (Takeda) to detect Aβ40 and Aβ42, respectively. Hamster Aβ derived from CHO cells was measured using the BNT77/BA27 and BNT77/BC05 antibody systems. The BNT77 antibody (Takeda) was raised against amino acids 11–28 and thus may recognize amino-terminally modified or truncated peptides (35). Aβ concentration was determined by comparing values obtained for samples with those obtained for synthetic Aβ40 and Aβ42 standards (Bachem).

**Cloning of Human ECE-1a and ECE-1b—**ECE-1a and ECE-1b were cloned by reverse transcription-polymerase chain reaction in two fragments (joined by a unique PeuII site) from human umbilical vein endothelial cell (HVEC, ATCC) RNA using the following primers (restriction sites underlined): 1) ECE-1a forward, 5'-CAGGAAATTCTGCACTGGCTGGTCGCACTTGTGAGGCGGG-3'; 2) ECE-1b forward, 5'-CAGGAAATTCTGCACTGGCTGGTCGCACTTGTGAGGCGGG-3'. ECE-1 PeuII reverse, 5'-AGAGCGCTCTCCAGGGCTGCTG-3'; 4) ECE-1 PeuII forward, 5'-CCACAGCTGGAGAGCTGCTG-3'; and 5) ECE-1 PeuII reverse, 5'-CTCTTACATTACAGCAGCTGGAGAGCTGCTG-3'. RNA was prepared from HUVeCs cells using the Qiagen RNeasy miniprep kit and was reverse transcribed using Superscrip II reverse transcriptase and an oligo(dT) primer (Roche Molecular Biochemicals). Amplification of ECE-1a-1c fragment was amplified using primers 1 and 5. The 5' fragment of ECE-1b was reverse amplified using primers 2 and 3. The 3' fragment of ECE-1, which is common to both isoforms, was amplified using primers 4 and 5. Pfu polymerase (Stratagene) was used for all amplifications. The 5' and 3' fragments were ligated together at the PeuII site and subcloned into pcDNAs (Invitrogen) using primer-encoded EcoRI and XbaI sites. The sequences of the constructs were confirmed by dideoxy sequencing by the Mayo Molecular Biology Core Facility.

**Cell Culture and Transfections—**Unless otherwise noted, all cell culture reagents were purchased from Life Technologies, Inc., and cell lines were purchased from ATCC. HUVeCs were cultured in Känigh's F12K medium (ATCC) supplemented with 10% fetal bovine serum, 0.1 mg/ml heparin, 100 mg/ml endotoxin-free penicillin, 100 μg/ml streptomycin, and 250 μM sucrose. CHO cells were cultured in Ham's F-12 medium (BioWhittaker) supplemented with 10% newborn calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. H4 cells (human neuroglioma origin) were cultured in Opti-MEM supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. For passaging of cells prior to experiments in which ECE activity was to be measured, a highly purified trypsin (Sigma T-7418) solution was used (17). CHO cells were transfected with FuGENE 6 (Roche Molecular Biochemicals) according to the directions of the manufacturer. Stable lines were generated by selecting pcDNAs-transfected cells (ECE-1a and ECE-1b) with 1 mg/ml Geneticin and pSecTag-transfected cells (solECE-1) with 0.8 mg/ml Zeocin (Invitrogen).

**Measurement of ECE Activity in Cell Membrane Fractions—**Cell membrane fractions were prepared as described by Xu et al. (17). Protein concentration was determined by BCA assay (Pierce) in membranes resuspended in buffer B (20 mM Tris-HCl, pH 7.4, containing 250 mM sucrose) or in membranes solubilized in buffer B containing 2.5% C8E6 (polyoxylene-ethylene-10-lauryl ether, Sigma). For measurement of ECE activities, cell membrane fractions (10–50 μg of protein) were incubated for 30 min at 37 °C with 0.1 μM big ET-1 (1–38) (American Peptide Co.) in 0.1 mM sodium phosphate buffer (pH 6.8) containing 0.5 mM NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF), 100 μM leupeptin, and 20 μM pepstatin. Duplicate reactions were carried out in the presence of phosphoramidon (100 μM). Reactions were stopped by the addition of EDTA (5 mM), and mature ET-1 (1–21) peptide was quantitated by sandwich ELISA (Amersham Pharmacia Biotech).

**Treatment of Cells with Metallopeptase Inhibitors—**Cells were passaged into six-well plates 1 day prior to treatment and were utilized at confluence. Triplicate wells were washed twice with Hank's balanced salt solution and then incubated for 17–24 h with 1 ml of growth medium containing phosphoramidon (Roche Molecular Biochemicals), thiorphan (Sigma), or captopril (Sigma) at the indicated concentrations. Control cells were incubated in growth medium containing an equal concentration of vehicle (phosphate-buffered saline). After treatment, the culture medium was harvested and spun at 14,000 × g, and the supernatant was analyzed for Aβ40 and Aβ42 by sandwich ELISA as described and for secreted APP by Western blot. To assess cellular toxicity of the compounds, MTS assays (CellTiter 96®, Promega), which measure the conversion of MTS to formazan by metabolically active cells, were performed on the cells after the indicated time points.

Culture medium was subjected to electrophoresis on 10–20% Tricine gels (Novex) and was subsequently transferred to Immobilon P (Millipore Corp.). Western blots on CHO cells were performed using 22C11 antibody (Roche Molecular Biochemicals) to detect secreted APP. Bound antibody was detected by incubation with the appropriate horseradish peroxidase-linked secondary antibody (Amersham Pharmacia Biotech) followed by ECL Western blotting reagents (Amersham Pharmacia Biotech) and exposure to x-ray film.

**Expression and Purification of Soluble ECE-1—**A construct encoding a soluble form of ECE-1 similar to that described by Ahn et al. (36) and Korth et al. (37) was generated by amplifying the extracellular domain of ECE-1a using the following primers: SolECE-1 forward, 5'-

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GAGAAGATCTCGAGTACGAGAAAGATTCC-3′; SolECE-1 reverse, 5′-CGTTCCTGTGCTCAGGGCGCCCGACGTGCTGTTGAGG-3′.

The solECE-1 construct was subcloned using primer-encoded EcoRI and NotI sites into pSecTag2B (Invitrogen), which incorporates a leader sequence onto the N terminus of the protein for secretion by mammalian vaccinia and c-Myc and HA epitopes at the C terminus and detects full-length peptides ending at position 40 (BA27) via binding of antibody BAN50 to the N terminus and tagging with synthetic Aβ assay.

The active enzyme concentration, precluding a determination of $K_m$ and $V_{max}$.

We next determined the second order rate constant, $k_{cat}/K_m$, for Aβ hydrolysis relative to that for big ET-1 hydrolysis by solECE-1. The second order rate for hydrolysis of each substrate was determined at substrate concentrations well below $K_m$. Specifically, Aβ40 (2.5 μM) and big ET-1 (0.1 μM) were incubated in triplicate alone or with solECE-1 (0.3–8.3 mM) at 37°C at either pH 6.5 or pH 5.6 in 50 mM MES buffer containing 100 mM NaCl, 0.05% bovine serum albumin, 1 mM PMSF, 100 μM leupeptin, and 20 μM pepstatin. The reactions were stopped by the addition of EDTA (5 mM), and mature ET-1 peptide was measured by sandwich ELISA (Amer sham Pharmacia Biotech). The second rate constant was determined as the apparent rate constant from Equation 1, $y = 1 - e^{-kt}$, where $y$ is the fraction of substrate hydrolyzed and $t$ is the time. Since $k = k_{cat}/K_m \times [E]$, the calculated $k$ was then divided by the reported $k_{cat}/K_m$ for big ET-1 hydrolysis by solECE-1 (2.52 × 10^4 M⁻¹ s⁻¹ at pH 6.5) (16) to determine the active enzyme concentration. The active enzyme concentration was estimated by this method throughout this report and was ~30-fold lower than that originally estimated from the protein concentration and Coomassie-stained gel, suggesting that our estimation of purity by Coomassie staining was incorrect or that a majority of the solECE-1 was in an inactive form. To control for the presence of co-purifying native CHO proteins, conditioned serum-free medium from nontransfected CHO cells was purified as above, and the eluted proteins were used in control experiments.

RESULTS

Phosphoramidon, but Not Thiorphan or Captopril, Increases Aβ Accumulation by H4 Neuroglioma Cells—Our group and others have previously shown that treatment with metalloprotease inhibitors, in particular phosphoramidon, results in a rapid 2–3-fold increase in the concentration of Aβ40 and Aβ42 in the conditioned medium of neuronal cell lines without affecting the concentration of secreted APP (sAPP) (39, 40). Importantly, this increase in Aβ concentration is as large or larger than that seen in cells expressing most AD-causing mutations (2). The enzyme(s) responsible for the phosphoramidon-induced elevations in Aβ has not previously been reported. Phosphoramidon is known to inhibit several metalloproteases including NEP (IC₅₀ = 0.034 μM), angiotensin-converting enzyme (ACE; IC₅₀ = 78 μM), ECE-1 (IC₅₀ = 1–3.5 μM), and ECE-2 (IC₅₀ = 0.004 μM) (29, 41) but does not inhibit insulin-degrading enzyme (42). A role for NEP in extracellular Aβ catabolism has been highlighted in a recent report from Iwata and colleagues (9). Infusion of the metalloprotease inhibitor thiorphan into the hippocampus of rats resulted in a significant increase in the amount of Aβ and in the deposition of the longer more amyloidogenic form, Aβ42, reportedly through the inhibition of Aβ degradation by NEP. NEP and ACE have been reported to reside predominantly on the cell surface, although a soluble form of NEP is also present in serum and cerebral spinal fluid (43–46). A recently identified thiorphan-sensitive NEP homo-
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Fig. 1. Effect of metalloprotease inhibitors on Aβ concentration in the culture medium of H4 cells. Confluent wells of H4 cells were treated with phosphoramidon, captopril (selective inhibitor of ACE), or thiorphan (selective inhibitor of NEP) for 24 h at the indicated concentrations. Aβ40 concentration in the conditioned medium was determined using the BAN50/BA27 sandwich ELISA. Data are plotted as mean ± S.E. of triplicate wells. MTS cell proliferation assays on the treated cells did not reveal cellular toxicity at any of the doses.

logue SEP/NL1/NEPII is expressed both as a membrane-bound and secreted protease (47–49). We evaluated a role for NEP and ACE in H4 neuroglioma cells using more selective inhibitors of these enzymes, thiorphan and captopril, respectively. We have not yet been able to similarly analyze ECE, since a more selective inhibitor of ECE is not commercially available.

Treatment of H4 cells with phosphoramidon (34 µM) resulted in a greater than 2-fold elevation in Aβ40 accumulation (Fig. 1), with a half-maximal effect occurring at a dose of ~7.5 µM (data not shown). However, treatment with thiorphan or captopril at concentrations greater than 1000 times the reported IC50 for the target enzymes in in vitro studies (50, 51), but less than that required to inhibit ECE, failed to result in increases in extracellular Aβ (Fig. 1), indicating that the phosphoramidon-induced effect in H4 cells is not likely to be due to inhibition of NEP or ACE. Similar results were obtained for Aβ42 (data not shown). Since NEP and ACE are localized mainly on the cell surface, the membrane permeability of these compounds is not relevant to the inhibition of the known protease, although we cannot rule out the possible presence of an intracellular form of NEP or ACE based on these results. Similarly, we cannot rule out the possibility that the phosphoramidon-induced effect in these cells is due to an as yet unidentified enzyme that is insensitive to treatment with thiorphan and captopril. We did, however, find endogenous ECE activity in solubilized membranes of H4 cells using a big ET conversion assay (17, 36) (data not shown). Collectively, these data led us to investigate ECE more closely.

Overexpression of Endothelin-converting Enzyme-1 Results in a Significant Decrease in Extracellular Aβ Concentration That Is Completely Reversed by Treatment with Phosphoramidon—Evidence implicating a potential role for ECE in modulating Aβ concentration came further from the casual observation that CHO cells, which have no endogenous ECE activity (17), produce very high levels of Aβ when compared with most other cell types and fail to respond to phosphoramidon (see Fig. 2). Conversely, HUVECs, which have high levels of endogenous ECE (52), accumulate very little Aβ unless treated with high concentrations of phosphoramidon (data not shown). To further investigate the role of ECE in Aβ accumulation, we cloned and stably transfected CHO cells with human ECE-1b and ECE-1a. ECE activity, determined using a big ET-1 conversion assay (17, 36), was confirmed to be present in solubilized membranes from the ECE-1-transfected cells and absent in vector-transfected cells (data not shown). The amount of

Fig. 2. Overexpression of ECE-1a or ECE-1b in CHO cells reduces extracellular Aβ concentration without affecting secretion of sAPP. Aβ40 (A) and Aβ42 (B) concentration in the conditioned media of stable ECE-1a- and ECE-1b-transfected CHO cell lines was determined by sandwich ELISA (BNT77/BA27 and BNT77/BC05, respectively) following a 24-h incubation with or without 100 µM phosphoramidon. Data are plotted as mean ± S.E. of triplicate wells. Western blot analysis (C) was also performed on the conditioned media of cells incubated with or without phosphoramidon (phos), using 22C11 antibody to detect sAPP.

ECE-1 activity in each of our stable ECE-1 lines was similar. Overexpression of either ECE-1a or ECE-1b in CHO cells, which lack endogenous ECE activity, resulted in a striking 75–90% reduction in Aβ40 and a 45–60% reduction in Aβ42 (Fig. 2). No significant changes were observed in the amount of sAPP accumulation in ECE-1-transfected cells compared with the vector controls, indicating that the cells were similarly viable and that general secretion is not affected by ECE-1 overexpression. The reduction in Aβ concentration in ECE-1a- and ECE-1b-transfected cells was completely reversed by treatment with phosphoramidon, indicating that the observed phenotype was probably due to the enzymatic activity of the overexpressed ECE-1.

Increased Removal of Exogenous Aβ Is Apparent Only in ECE-1a-transfected Cells—While the exact mechanism of the phosphoramidon-induced increase in Aβ concentration in neuronal cells was unknown, it has been suggested that it may be the result of inhibition of intracellular degradation of the peptide (40). Consistent with this hypothesis, treatment with phosphoramidon is reported to result in a 2-fold increase in extracellular Aβ concentration and an increase in cell-associated Aβ without affecting sAPP levels in SY5Y cells treated with the
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**Fig. 3. Effect of phosphoramidon on removal of exogenous Aβ by H4 cells.** Synthetic Aβ42 (150 pM) was added to confluent H4 cells in the presence (closed symbols) or absence (open symbols) of 34 μM phosphoramidon and incubated for 6 and 24 h. A second set of control H4 cells was incubated with or without phosphoramidon for the same time period. Aβ42 was measured in both sets at the indicated time points using the BANS/BA27 sandwich ELISA, and remaining synthetic Aβ concentration was determined by subtracting values obtained from control (endogenous) cells from those obtained from the cells incubated with spiked-in Aβ. The inset graph shows the accumulation of endogenous Aβ42 in the conditioned medium of H4 cells after 24 h of treatment with phosphoramidon. Data are plotted as mean ± S.E. of triplicate wells.

To determine whether extracellular Aβ removal could account, at least in part, for the dramatic decrease in extracellular Aβ concentration in the ECE-1-transfected cell lines, we next spiked synthetic Aβ40 into the culture medium in the presence or absence of phosphoramidon and determined the percentage of removal by sandwich ELISA at 6 and 24 h. After a 6-h incubation, removal of Aβ was similar in the culture medium of vector- and ECE-1-transfected CHO cells (Fig. 4A) and was not affected by phosphoramidon treatment (data not shown), although endogenous Aβ accumulation by phosphoramidon-treated ECE-1-transfected cells was increased 1.5–2-fold during the same time period (Fig. 4B).

Following a 24-h incubation, we did observe a significant increase (p = 0.0485) in the removal of the spiked-in Aβ in the medium of ECE-1a-transfected cells compared with the vector controls (Fig. 4A). No significant change in exogenous Aβ removal was observed in cells expressing ECE-1b. The ECE-1a-induced increase in Aβ removal could be completely attenuated by phosphoramidon treatment, indicating that the effect was probably due to the enzymatic activity of ECE-1a (Fig. 4A, inset). In the same ECE-1a cells, phosphoramidon treatment resulted in an ~600% increase in endogenous Aβ accumulation at the 24-h time point (Fig. 4B).

**A. Degradation of exogenous Aβ**

**B. Accumulation of endogenous Aβ**

**Fig. 4. Removal of exogenous synthetic Aβ by ECE-overexpressing CHO cells.** A, synthetic human Aβ40 (150 pM) was added to confluent CHO cells stably transfected with ECE-1a, ECE-1b, or the control vector and incubated for 6 and 24 h. Human Aβ40 was measured at the indicated time points using the BANS/BA27 sandwich ELISA, which does not detect endogenous CHO Aβ. Data shown represent the mean ± S.E. of triplicate wells that were incubated with synthetic Aβ40. The concentration of Aβ remaining after 24 h is significantly lower in ECE-1a cells than in vector controls (p = 0.0495, Mann-Whitney). The inset graph shows the percentage of exogenous Aβ removed by ECE-1a and vector-transfected cells after 24 h in the presence of phosphoramidon (34 μM). B, a second set of cells was incubated with or without phosphoramidon (34 μM) for the same time period to determine the accumulation of endogenous Aβ. Endogenous Aβ40 was measured at the indicated time points using the BNT77/BA27 sandwich ELISA. Data are plotted as mean ± S.E. of triplicate wells. Given that BNT77 was raised against amino acids 11–28 of Aβ, this assay can also detect amino truncated peptides and may lead to an overestimation of full-length Aβ40.

Partially Purified solECE-1 Degrades Aβ In Vitro—Recombinant, soluble forms of ECE-1 (solECE-1) lacking the intracellular and transmembrane domains have been reported to hydrolyze big ET-1 with activity comparable with that of membrane-bound ECE-1a (16, 36, 37). The soluble ECE-1 preparation described by Korth et al. (37) was active in a broad pH range from 5 to 7, with an optimum of pH 6.6–6.8 for big ET-1. Under the conditions assayed, Ahn et al. (36, 53) found that their soluble ECE-1 preparation had a somewhat narrower pH...
Fig. 5. Degradation of synthetic Aβ40 and Aβ42 by soluble ECE-1. Soluble ECE-1 (~6 nM), partially purified from transfected CHO cell medium, was incubated for 24 h at 37 °C with 0.01 μM Aβ40 (A) or Aβ42 (B). As a control, the enzyme was preincubated for 15 min with the ECE inhibitor PD069185 (150 μM) or phosphoramidon (150 μM) prior to the addition of Aβ40 or Aβ42, respectively. Closed bars, ECE inhibitor; open bars, no inhibitor. Identical reactions were carried out with co-purifying proteins isolated from nontransfected CHO cells (Control). After the incubation, the remaining Aβ was detected using the BAN50/BA27 and BAN50/BC05 sandwich ELISA systems, which detect full-length Aβ40 or Aβ42 peptides, respectively. Data are plotted as mean ± S.E. of triplicate reactions.

optimum, with an optimal pH for big ET-1 of 6.5. To examine whether ECE-1 is capable of direct catabolism of Aβ, we generated a soluble ECE-1 similar to those previously described. Incubation of synthetic Aβ40 and Aβ42 with this enzyme resulted in a nearly complete loss of the full-length peptides as detected by sandwich ELISA (Fig. 5). This reduction was completely blocked by incubation with phosphoramidon and also with a more selective ECE-1 inhibitor, PD069185. (This inhibitor, while very useful for in vitro studies, is not informative in cell-based studies due to its toxicity (38)). To confirm that the loss of Aβ was indeed due to Aβ catabolism and not to Aβ binding or some other phenomenon, we analyzed the effect of solECE-1 on Aβ by HPLC with a radiolabeled Aβ reporter molecule. Incubation of 3H-labeled Aβ40 with solECE-1 resulted in loss of the full-length peptide and formation of at least three novel peaks detected by reversed-phase chromatography (Fig. 6). The formation of these peaks was completely blocked by treatment with PD069185.

Determination of Cleavage Sites of Aβ40 by solECE-1—Since ECE-1 has been shown to cleave a number of biologically active peptides on the amino side of hydrophobic residues (16), there are multiple potential ECE-1 cleavage sites within the Aβ peptide. To determine the sites of Aβ40 cleavage, synthetic unlabeled peptide was digested with solECE-1, and the cleavage products were separated by reversed-phase HPLC. Digestion of Aβ40 in this experiment resulted in the formation of four major product peaks, similar to that which was observed with the 3H-labeled peptide. The major peaks were further analyzed by mass spectrometry and NH2-terminal sequencing, leading to the identification of three NH2-terminal fragments: Aβ−(1–16), Aβ−(1–17), and Aβ−(1–19). Only one C-terminal fragment was observed, which corresponds to Aβ−(20–40) (Fig. 7 and Table I).

Kinetic Analysis of Aβ40 Cleavage by solECE-1—SolECE-1 has been reported to hydrolyze big ET-1 with a Km of ~2–4 μM (16, 36, 37) and bradykinin with a Km of 340 μM (16). Despite a much higher Km, the catalytic efficiency of bradykinin hydrolysis by solECE-1 actually exceeds that of big ET-1, with a second order rate constant of 6.6 × 104 M−1 s−1 for bradykinin compared with 2.5 × 104 M−1 s−1 for big ET-1 at pH 6.5 (16). To attempt to determine the Km for Aβ40 hydrolysis by solECE-1, we initially examined the rate of hydrolysis of Aβ at various substrate concentrations up to 20 μM at the reported pH opti-
mum for big ET-1 cleavage. The rate of Aβ hydrolysis was linear with respect to substrate concentration up to 20 μM (data not shown). We were concerned that the use of significantly higher concentrations of Aβ in these experiments would complicate the kinetic measurements, since Aβ40 peptide is prone to aggregate and precipitate in the high micromolar range. Thus, we were unable to determine the $K_m$ and $V_{max}$. We were able, however, to calculate the $k_{cat}/K_m$ by measuring the rate of Aβ hydrolysis by solECE under second-order conditions. Under these conditions, when the substrate concentration is well below $K_m$, the rate of substrate hydrolysis is equal to the $k_{cat}/K_m$ multiplied by the enzyme concentration (see “Experimental Procedures”). Using this method, the $k_{cat}/K_m$ for Aβ40 hydrolysis by solECE-1 was determined to be $(1.7 \pm 0.6) \times 10^3$ M$^{-1}$ s$^{-1}$ at pH 6.5. This value is 15-fold lower than that for big ET-1 hydrolysis under the same conditions.

Intrigued by a recent report indicating that solECE-1 cleaves bradykinin and substance P with an acidic pH optimum of ~5.6 compared with the optimum of pH 6.5 for big ET-1 (53), we next compared the efficiency of solECE-1 hydrolysis of Aβ40 and big ET-1 at pH 5.6. Similar to bradykinin and substance P, solECE-1 cleaves Aβ40 more efficiently at pH 5.6, with a $k_{cat}/K_m$ determined to be $(2.0 \pm 0.5) \times 10^4$ M$^{-1}$ s$^{-1}$, ~12 times greater than the value determined at pH 6.5. The $k_{cat}/K_m$ for big ET-1 hydrolysis by our preparation of solECE-1 at pH 5.6 under these conditions was determined to be $\sim 6.1 \times 10^4$ M$^{-1}$ s$^{-1}$ at pH 5.6, slightly greater than that determined at pH 6.5. This value of $k_{cat}/K_m$ for big ET-1 hydrolysis at pH 5.6 is only 3-fold greater than that for Aβ40 at the same pH. It is important to note that the $k_{cat}/K_m$ values reported here rely on a determination of enzyme concentration based on the reported $k_{cat}/K_m$ of big ET-1 hydrolysis by solECE-1 at pH 6.5 (see “Experimental Procedures”), and thus the absolute values may differ based on any differences in actual enzyme concentration from our estimates. However, the relative $k_{cat}/K_m$ for Aβ40 hydrolysis versus big ET-1 hydrolysis was determined in parallel experiments with the same enzyme and would not change even if the estimated concentration of enzyme were slightly different.

### DISCUSSION

Recently, there has been considerable debate over the enzyme or enzymes that contribute most to Aβ catalysis in the human brain. Both insulin-degrading enzyme and nephrin have been argued to be major proteases involved in the degradation of secreted Aβ (5, 9). However, with the data available at this time, it is impossible to determine which, if any, of the identified proteases contributes most to Aβ degradation in the intact brain. It is likely that multiple proteases, both intracellular and extracellular, may play a role in determining Aβ concentration. The relative contribution of Aβ-degrading enzymes and other mechanisms of Aβ removal may vary in different regions of the brain and may also differ for Aβ40 and Aβ42. A decrease in the activity of any of these mechanisms, whether they are major or minor, may potentially result in increased Aβ accumulation and the development of AD pathology. Conversely, an increase in the activity of any enzyme capable of degrading Aβ may result in decreased accumulation of the peptide, potentially reducing the risk for AD.

Taken together, the data presented in this report indicate that ECE-1 activity can dramatically affect Aβ concentration, probably by direct degradation of the peptide. Recombinant soluble ECE-1 has a substrate specificity in non-ET peptides similar to that of NEP, with preferential cleavage on the amino side of hydrophobic residues (16). In contrast to NEP, however, ECE-1 appears not to cleave peptides smaller than 6 amino acids in length. Given this specificity, there are ~13 potential ECE/NEP cleavage sites in the Aβ40 peptide. At least five of these sites are reported to be cleaved by recombinant NEP in vitro (8). Using HPLC, mass spectrometry, and NH$_2$-sequence analysis, we have determined that soluble ECE-1 cleaves synthetic Aβ40 at least three sites, resulting in the formation of Aβ fragments 1-16, 1-17, 1-19, and 20-40. Consistent with the known substrate specificity of ECE-1, each of these observed cleavages by solECE-1 occurred on the amino side of hydrophobic residues (Leu$^{17}$, Val$^{18}$, and Phe$^{20}$). Given that ECE-2 is highly homologous to ECE-1 and shares similar catalytic activity (29), we hypothesize that ECE-2 may also be capable of degrading Aβ. Since ECE-2 is abundantly expressed in the central nervous system, this activity may also potentially be relevant to the accumulation of Aβ in the brain.

ECE-1 activity is localized both intracellularly and on the cell surface in many cell types, with ECE-2 appearing to reside exclusively within the cell. In CHO cells overexpressing human ECE-1a, we observed increased phosphoramidon-sensitive removal of exogenous Aβ after 24 h of incubation, indicating that ECE-1a may also contribute slightly to the extracellular degradation of the peptide. In CHO cells overexpressing ECE-1b and in H4 neuroglioma cells expressing endogenous ECE, degradation of exogenous Aβ was not sensitive to phosphoramidon. These results raise the possibility that the dramatic increase in Aβ accumulation by these cells in the presence of phosphoramidon may be due to inhibition of intracellular degradation of the peptide. Even in ECE-1a-expressing CHO cells, the dramatic increase in Aβ concentration upon treatment with phosphoramidon does not appear to be accounted for by the modest increase in exogenous Aβ degradation. We cannot, however, rule out the possibility of a local event at the cell surface upon secretion of endogenous Aβ that might not be evident in our spike experiments, where the peptide is diluted directly into the culture medium. While ECE-1a has been reported to be localized predominantly to the cell surface, this isoform has been shown to process big ET-1 intracellularly in CHO cells, most likely in secretory vesicles, as well as at the cell surface (54). Therefore, ECE-1a may similarly degrade Aβ intracellularly in CHO cells as it is being trafficked to the cell surface.

While detailed co-localization studies have not been performed, separate studies indicate that ECE and Aβ are present in the same cellular compartments. Human ECE-1b has been reported to be present in the TGN, a proposed site of Aβ generation in neuronal cells (24, 55). Interestingly, we found that solECE-1 hydrolyzed Aβ40 more efficiently at pH 5.6 than at pH 6.5, with a $k_{cat}/K_m$ at pH 5.6 only 3-fold lower than that for a known physiological substrate, big ET-1. This result may be particularly relevant, since the TGN, where ECE-1b appears to be expressed, has an acidic pH. ECE-2 is also likely to be present in the TGN and vesicles of the constitutive secretory pathway (29, 31). Consistent with the hypothesis that phosphoramidon may inhibit intracellular degradation of Aβ, Fuller et al. (40) have reported that phosphoramidon treatment of

### Table I

| Peak | Observed mass | N-terminal sequence | Assignment | Predicted mass |
|------|---------------|---------------------|------------|---------------|
| 1    | 1955.09       | DA                  | Aβ 1–16    | 1956.04       |
| 2    | 2068.72       | DA                  | Aβ 1–17    | 2069.2        |
| 3    | 2314.76       | DA                  | Aβ 1–19    | 2315.51       |
| 4    | 2033.56       | DA                  | Aβ 20–40   | 2034.39       |
| 5    | 4330.9        | DA                  | Aβ 1–40    | 4330.88       |

Identification of cleavage sites of Aβ40 by solECE-1

Synthetic Aβ40 was digested with solECE-1, and the resulting peptides were separated by HPLC as shown in Fig. 7A. Peaks 1–5 were collected and further analyzed by mass spectrometry and NH$_2$-terminal sequencing.
ECE activity does augment endothelin levels, endothelin receptors may be a target for the treatment of AD. One obvious concern with this treatment method is that patients may become hypertensive. Up-regulation of ECE activity in the periphery of cells injected with a construct to increase ECE expression, however, does not appear to result in increased circulating endothelin levels, indicating that ECE is not likely to be rate-limiting in the conversion of big ET to ET (61). Further, even if increased ECE activity does augment endothelin levels, endothelin receptor antagonists could be given in parallel to reduce or block any effect of increased endothelin levels.

Third, mutations in ECE may be identified that are causative of Alzheimer's disease in certain individuals. In this regard, it is worthwhile to note that the sib-pair analyses of genetic factors contributing to late onset AD have not excluded the region on chromosome 1 where the ECE-1 gene is located (62). Equally important, however, is the possibility that there may be individuals with normally high levels of ECE activity who are at a reduced risk for the disease. A careful analysis of ECE activity in AD and control individuals is necessary to determine the extent of the involvement of this enzyme family in the development of AD.

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REFERENCES

1. Checler, F. (1995) J. Neurochem. 65, 1431–1444
2. Golde, T. E., Eckman, C. B., and Younkin, S. G. (2000) Biochem. Biophys. Acta 1502, 172–187
3. Kurochkin, I. V., and Goto, S. (1994) FEBS Lett. 345, 33–37
4. Qiu, W. Q., Walsh, D. M., Ye, Z., Vekrellis, K., Zhang, J., Podlinsky, M. B., Rosner, M. R., Safavi, A., Hersh, L. B., and Selkoe, D. J. (1998) J. Biol. Chem. 273, 32730–32738
5. Vekrellis, K., Ye, Z., Qiu, W. Q., Walsh, D., Hartley, D., Chesneau, V., Rosner, M. R., and Selkoe, D. J. (2000) J. Neurosci. 20, 1057–1065
6. Perez, A., Morelli, L., Creto, J. C., and Castano, E. M. (2000) Neuroreer. Res. 25, 247–255
7. Chesneau, V., Vekrellis, K., Rosner, M. R., and Selkoe, D. J. (2000) Biochemistry 39, 509–516
8. Howell, S., Nairnzi, J., and Crine, P. (1995) Peptides 16, 647–652
9. Iwata, N., Tsukibi, S., Takakii, Y., Watanabe, K., Sekiguchi, M., Hosoki, E., Kawashima-Morishima, M., Lee, H. J., Hama, E., Sekine-Aizawa, Y., and Saigo, T. C. (2000) Nat. Med. 6, 143–150
10. Takaki, Y., Iwata, N., Tsukibi, S., Taniguchi, S., Toyoshima, S., Lu, B., Gerard, N. P., Gerard, C., Lee, H. J., Shiratori, K., and Saigo, T. C. (2000) J. Biochem. (Tokyo) 128, 897–902
11. Backstrom, J. R., Lim, G. P., Cullen, M. J., and Tookes, Z. A. (1996) J. Neurosci. 16, 7910–7919
12. Yamin, R., Malgeri, E. G., Shanne, J. A., McGraw, W. T., and Abraham, C. R. (1999) J. Biol. Chem. 274, 18777–18784
13. Qiu, W. Q., Borth, W., Ye, Z., Haass, C., Teplow, D. B., and Selkoe, D. J. (1996) J. Biol. Chem. 271, 8443–8451
14. Turner, A. J., and Murphy, L. L. (1996) Biochemistry 35, 1391–1400
15. Yoshinaga, Y., Hama, E., Sekine, A., Kurosawa, M., Lee, H.-J., Hama, E., Sekine-Aizawa, Y., and Saigo, T. C. (2000) FEBS Lett. 487, 423–428
16. Johnson, G. D., Stevenson, T., and Agha, K. (1999) J. Biol. Chem. 274, 4553–4558
17. Xu, D., Emoto, N., Giad, R., Cuilhar, C., Kawa, S., deWit, D., and Yanagisawa, M. (1994) Cell 78, 473–485
18. Davenport, A. P., Kue, R. E., Plumpson, C., Mockridge, J. W., Barker, P. J., and Huskinson, N. S. (1995) Eur. J. Biochem. 237, 570–577
19. Kloth, B., Soli, R., Corvol, P., and Pintet, F. (1999) J. Histochem. Cytochem. 47, 447–462
20. Barnes, R., Walken, B. J., Wilkinson, T. C., and Turner, A. J. (1997) J. Neurochem. 68, 570–577
21. Valdenaire, O., Rohrbacker, E., and Mategi, M. G. (1995) J. Biol. Chem. 270, 29774–29789
22. Shimada, K., Takahashi, M., Ikeda, M., and Tannawa, K. (1995) FEBS Lett. 371, 140–144
23. Schmidt, M., Kroger, B., Jacob, E., Seubelberger, H., Subkow, T., Otter, R., Meyer, T., and Hillen, H. (1994) FEBS Lett. 336, 238–244
24. Schweizer, A., Valdenaire, O., Nelbock, P., Deuchel, U., Dumas Milne Edwards, J. B., Stumpf, J. G., and Loffler, B. M. (1997) Biochem. J. 328, 871–877
25. 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
26. Aranci, A., Beilue, G., and Crine, P. (1999) Biochem. J. 339, 439–448
27. Cailler, F., Zappulla, J. P., Beilue, G., and Crine, P. (1999) Biochem. J. 341, 119–126
28. Emoto, N., Nishihata, Y., AliManserddo, H., Xie, J., Yamada, T., Yanagisawa, M., and Matsu, K. (1999) J. Biol. Chem. 274, 1509–1518
29. Emoto, N., and Yanagisawa, M. (1999) J. Biol. Chem. 270, 15262–15268
30. Nagase, T., Ishikawa, K., Miyajima, N., Tanaka, A., Katai, H., Nomura, N., and Ohara, O. (1996) DNA Res. 5, 31–39
31. Russell, B. D., and Davenport, A. P. (1999) Circ. Res. 84, 891–896
32. Yanagisawa, H., Hama, R. E., Richardson, J., Emeto, N., Williams, S. C., Takeda, S., Cloutier, D. E., and Yanagisawa, M. (2000) J. Clin. Invest. 105,
Degradation of Aβ by Endothelin-converting Enzyme

1373–1382
33. Hasegawa, H., Hiki, K., Sawamura, T., Aoyama, T., Okamoto, Y., Miwa, S., Shimoahama, S., Kimura, J., and Masaki, T. (1998) FEBS Lett. 428, 304–308
34. Suzuki, N., Cheung, T. T., Cai, X. D., Odaka, A., Otvos, L., Jr., Eckman, C., Golde, T. E., and Younkin, S. G. (1994) Science 264, 1336–1340
35. Asami-Odaka, A., Ishibashi, Y., Kikuchi, T., Kitada, C., and Suzuki, N. (1995) Biochemistry 34, 10272–10278
36. Ahn, K., Herman, S. B., and Fahnoe, D. C. (1998) Arch. Biochem. Biophys. 359, 258–268
37. Korth, P., Egidy, G., Parnot, C., LeMoullec, J. M., Corvol, P., and Pinet, F. (1997) FEBS Lett. 417, 365–370
38. Ahn, K., Siaseros, A. M., Herman, S. B., Pan, S. M., Hupe, D., Lee, C., Nikam, S., Cheng, X. M., Doherty, A. M., Schroeder, R. L., Halseen, S. J., Kaw, S., Emoto, N., and Yanagisawa, M. (1998) Biochem. Biophys. Res. Commun. 243, 184–190
39. Eckman, C. B., Prada, C.-M., and Younkin, S. G. (1995) in Society for Neuroscience, San Diego, CA
40. Fuller, S. J., Storey, E., Li, Q. X., Smith, A. I., Beyreuther, K., and Masters, C. L. (1995) Biochemistry 34, 8091–8098
41. Kukkola, P. J., Savage, P., Sakane, Y., Berry, J. C., Bilci, N. A., Ghai, R. D., and Jeng, A. Y. (1995) J. Cardiovasc. Pharmacol. 26, S65–S68
42. Ansorge, S., Bohley, P., Kirschke, H., Langner, J., and Wiederanders, B. (1984) Biomed. Biochim. Acta 43, 39–46
43. Turner, A. J., and Tanzawa, K. (1997) FASEB J. 11, 355–364
44. Corvol, P., Williams, T. A., and Soubrier, F. (1995) Methods Enzymol. 248, 283–365
45. Spillantini, M. G., Sicuteri, F., Salmon, S., and Malfroy, B. (1990) Biochem. Pharmacol. 39, 1353–1356
46. Soleilhac, J. M., Lafuma, C., Porcher, J. M., Auburtin, G., and Roques, B. P. (1990) Eur. J. Clin. Invest. 20, 1011–1517
47. Ikeda, K., Emoto, N., Raharjo, S. B., Nurhantari, Y., Saiki, K., Yokoyama, M., and Mateu, M. (1999) J. Biol. Chem. 274, 32469–32477
48. Ghaddar, G., Ruchon, A. F., Carpentier, M., Marcinkiewicz, M., Seidah, N. G., Crine, P., Desgroseillers, L., and Buileau, G. (2000) Biochem. J. 347, 419–429
49. Tanjo, O., Pacchinetti, P., Rose, C., Bonhomme, M. C., Gros, C., and Schwartz, J. C. (2000) Biochem. Biophys. Res. Commun. 271, 565–570
50. Roques, B. P., and Beaumont, A. (1992) Trends Pharmacol. Sci. 14, 11–14
51. Gronhagen-Riska, C., and Fyhrquist, F. (1980) Scand. J. Clin. Lab. Invest. 40, 711–719
52. Ahn, K., Pan, S., Beningo, K., and Hupe, D. (1995) Life Sci. 56, 2331–2340
53. Fahnoe, D. C., Knapp, J., Johnson, G. D., and Ahn, K. (2000) J. Cardiovasc. Pharmacol. 36, 522–525
54. Parnot, C., Le Moullier, M. J., Cousin, M. A., Guedin, D., Corvol, P., and Pinet, F. (1997) Hypertension 30, 837–844
55. Hartmann, T., Bieger, S. C., Bruhl, B., Tienari, P. J., Ida, N., Allsop, D., Roberts, G. W., Masters, C. L., Dotti, C. G., Unsicker, K., and Beyreuther, K. (1997) Nat. Med. 3, 1016–1020
56. Umezawa, S., Tatsuta, K., Izawa, O., and Tsujiya, T. (1972) Tetrahedron Lett. 1, 97–100
57. Yanagisawa, H., Yanagisawa, M., Goto, K., Masaki, T., and Kimura, S. (1991) Biochem. Biophys. Res. Commun. 174, 779–784
58. Yoshizawa, T., Iwashita, S., Mizusawa, H., Suzuki, N., Matsumoto, H., and Kanazawa, I. (1992) Neurosci. 22, 85–88
59. Yanagisawa, H., Yanagisawa, M., Kapur, R. P., Richardson, J. A., Williams, S. C., Clouthier, D. E., de Wit, D., Emoto, N., and Hammer, R. K. (1992) Development 115, 825–836
60. Gray, G. A., and Webb, D. J. (1996) Pharmacol. Ther. 72, 109–148
61. Telenouque, S., Emoto, N., deWit, D., and Yanagisawa, M. (1998) J. Cardiovasc. Pharmacol. 31, S54–S550
62. Kehoe, P., VanVrent-Vrieze, F., Crook, R., Wu, W. S., Holmans, P., Fenton, I., Spurlock, G., Norton, N., Williams, H., Williams, N., Lovestone, S., Perez-Tur, J., Hutton, M., Chartier-Harlin, M. C., Shears, S., Reo, K., Booth, J., Van Voorst, W., Ramic, D., Williams, J., Goate, A., Hardy, J., and Owen, M. J. (1999) Hum. Mol. Genet. 8, 237–245