Neprilysin Degradates Both Amyloid β Peptides 1–40 and 1–42 Most Rapidly and Efficiently among Thiorphan- and Phosphoramidon-sensitive Endopeptidases*

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To identify the amyloid β peptide (Aβ) 1–42-degrading enzyme whose activity is inhibited by thiorphan and phosphoramidon in vivo, we searched for neprilysin (NEP) homologues and cloned neprilysin-like peptidase (NEPLP) α, NEPLP β, and NEPLP γ cDNAs. We expressed NEP, phosphate-regulating gene with homologies to endopeptidases on the X chromosome (PEX), NEPLPs, and damage-induced neuronal endopeptidase (DINE) in 293 cells as 95- to 125-kDa proteins and found that the enzymatic activities of PEX, NEPLP α, and NEPLP β, as well as those of NEP and DINE, were sensitive to thiorphan and phosphoramidon. Among the peptidases tested, NEP degraded both synthetic and cell-secreted Aβ1–40 and Aβ1–42 most rapidly and efficiently. PEX degraded cold Aβ1–40 and NEPLP α degraded both cold Aβ1–40 and Aβ1–42, although the rates and the extents of the digestion were slower and less efficient than those exhibited by NEP. These data suggest that, among the endopeptidases whose activities are sensitive to thiorphan and phosphoramidon, NEP is the most potent Aβ-degrading enzyme in vivo. Therefore, manipulating the activity of NEP would be a useful approach in regulating Aβ levels in the brain.

Alzheimer’s disease (AD) is characterized by the accumulation of amyloid β peptide (Aβ) in the brain. Aβ is composed of 39–43 amino acids and is constitutively produced by proteolysis of the β-amyloid precursor protein (APP). Alterations in either synthesis or clearance of Aβ may potentially contribute to increased levels of Aβ and amyloid deposits. Although much attention has been focused on the production of Aβ, little is known about how Aβ is degraded and cleared, especially in the brain. Identification of the peptidases involved in Aβ catabolism in vivo is important for the development of therapeutics designed to prevent or treat AD.

Proteases, including cathepsin D (1), serine protease-α-macroglobulin complex (2) and insulin-degrading enzyme (3), were purified and identified as Aβ-degrading enzymes in vitro. Several other recombinant or purified peptidases have also been shown to degrade Aβ in vitro (4–14). We focused on the in vivo catabolism of Aβ42, because this specific form, rather than Aβ40, is considered to be the primary pathogenic agent in AD. Recently, we demonstrated that an endopeptidase(s) similar or identical to neprilysin (NEP), whose activity is sensitive to thiorphan and phosphoramidon, is involved in the catabolism of Aβ1–42 in vivo (15, 16).

NEP is a type II membrane protein on the cell surface and is classified as a member of the M13 family. NEP hydrolyzes and inactivates several circulating peptides, such as enkephalin, atrial natriuretic peptide, endothelin, and substance P, and has wide tissue distribution and substrate specificity (17). The M13 family comprises six zinc-dependent metalloproteases, NEP, endothelin-converting enzyme (ECE-1) (18), ECE-2 (19), KELL antigen (20), phosphate regulating gene with homologies to endopeptidases on the X chromosome (PEX) (21), and the recently identified damage-induced neuronal endopeptidase (DINE)/X-converting enzyme (XCE) (22, 23). Among these, only NEP and DINE (22) have been shown to be sensitive to both thiorphan and phosphoramidon. Because ECE-1 and ECE-2 are not inhibited by thiorphan (18, 19), they can be excluded as candidates for Aβ1–42-degrading enzymes in vivo. KELL is only partially inhibited by phosphoramidon (24) and is mainly present in erythroid tissues. Although PEX degrades parathyroid hormone-derived peptides (25), its sensitivity to thiorphan or phosphoramidon is unknown. These facts indicate that NEP, DINE, and PEX are candidates for Aβ1–42-degrading enzymes in vivo. We also need to consider the possible presence of an unidentified protease(s), whose activity is sensitive to thiorphan and phosphoramidon and responsible for Aβ1–42 degradation.

In this study, considering the redundancy of peptidases, we investigated the presence of unidentified protease(s), which is
homologous to NEP, DINE, or PEX. We cloned novel cDNAs, which were termed nephrilysin-like peptides (NEPLPs) and examined their sensitivities to thiorphan and phosphoramidon. We compared the ability to proteolyse Aβ among NEP, PEX, DINE, and NEPLPs and found that NEP was the most potent Aβ-degrading enzyme.

**EXPERIMENTAL PROCEDURES**

cDNA Cloning—To obtain cDNAs homologous to those of NEP and PEX, two degenerate oligonucleotides corresponding to amino acid residues 646–654 (GENA DNGG(2) and 685–696 (QLELRSAF AQVWC) in the mature NEP were used. 5'-GGGAAA(A/T)TATTTGCTGATAATGG-(A/G)GG-3' and 5'-CACCAT(A/T)(C/G)/(G/C/T)GAGCAATTTG(C/G)(A/G)-3' were sequenced.

PCR was performed under the same conditions described above. Products were excised from the gel, cloned into a pCR-TOPO vector (Invitrogen, San Diego, CA), and sequenced. One cDNA was named NEPLP α: 5' and 3' regions of the NEPLP gene were amplified by nested PCR with an Advantage cDNA PCR kit (CLONTECH, Palo Alto, CA) using AP-1/AP-2 as sense primers and 5'-GGGAAA(A/T)TATTTGCTGATAATGG-(A/G)GG-3' and 5'-GGGAGAAAA(C/T)ATTGCTGATAATGG-(A/G)GG-3' as antisense primers and 5'-GGGTG-GCCAGAGCCTAAGG-3' and 5'-TACTAGGTGTCGGTCTGAC-3' as primer pairs. Full-length NEPLP cDNAs were amplified by using the primer pairs 5'-GGGTTTCAGGGGAAATCTGAGGCTAGG-3' and 5'-TTGCTTACAGATGGACATCGCTT-3' and subcloned into the Smal site of pBluescript II KS (+).

A similar procedure was performed to obtain cDNAs homologous to DINE and DINEXCE. Briefly, two degenerate oligonucleotides (5'-AAAC(A/C/G/T)TTGGCTACCAGATGCGACATCGCTT-3' and 5'-AAAC(A/C/G/T)TTGGCTACCAGATGCGACATCGCTT-3') were used as sense primers and 5'-GGGTGGGG-3' and 5'-GGTGGGGCC-3' as sense primers and AP-1/AP-2 as antisense primers, respectively. Both products of the 5' and 3' regions were cloned into a pCR-TOPO vector and sequenced. Full-length NEPLP cDNAs were amplified by using the primer pairs 5'-GGGCTTACAGGAATCTGAGGCTAGG-3' and 5'-TTGCTTACAGATGGACATCGCTT-3' and subcloned into the Smal site of pBluescript II KS (+). A novel cDNA was named NEPLP β, NEPLP γ, or DINE cDNAs using a calcium phosphate method. Stable mouse neuroblastoma N2a cells expressing both human APP695 with the Swedish mutation and presenilin 2 (PS2) with the N141I mutation were obtained by selection with 250 μg/ml leupeptin and 50 μg/ml G418, respectively.

Digestion Assay of Cold Aβ1–42—Aβ1–42 (4.6 μM) was incubated with 10 μg of membrane fractions in 100 μl of 50 mM HEPES (pH 7.2) containing 1 μg/ml leupeptin, 1 μg/ml pepstatin A, and 1× complete without EDTA (Roche Molecular Biochemicals) at 37 °C for 1 h. The reaction was stopped by adding 250 μl of a solution containing 0.05% triethylamine, 10 mM betaine, and 0.1 mM EDTA, followed by boiling for 5 min, and was analysed using HPLC as described (15). For the inhibition studies, membrane fractions were preincubated with 10 μg/ml thiorphan for 5 min before adding the H'4'C/14'C-radiolabeled Aβ1–42.

**RESULTS**

cDNA Cloning of NEPLPs—To clone peptidases homologous to NEP and PEX, we designed degenerate oligonucleotide primers. With mouse brain cDNA as a template, the PCR resulted in the amplification of DNA with the predicted size (150 bp).
subcloning the PCR products and sequencing individual clones, we found that 11 of the 42 clones had a novel identical sequence that was similar to but distinct from members of the M13 family. The other 16 clones were identical to ECE-1, 11 clones were NEP, 2 clones were ECE-2, and 2 clones were PEX. During sequencing the full-length of the novel gene, we noted the presence of three isoforms, which probably resulted from alternative splicing. We termed them NEPLP α, NEPLP β, and NEPLP γ, and their structural characteristics are outlined in Fig. 1. Of 42 isolated full-length clones, 21 were NEPLP α, 20 were NEPLP β, and 1 was NEPLP γ. Compared with NEPLP α, NEPLP β and NEPLP γ had an insertion of 23 amino acids immediately following the putative transmembrane region and 37 amino acids near the center of the protein, respectively. All the isoforms were predicted to be type II transmembrane proteins with a putative zinc-binding domain (HEXXH) in the extracellular portion. NEPLPs showed the highest homology (~54%) to NEP among the members of the M13 family. The degenerate primers, which were designed to amplify the peptidases homologous to NEP and DINE/XCE, did not amplify a novel gene. Recently, three research groups independently reported a novel member of the M13 family (28–30). NEPLP α and NEPLP β were identical to SEP/ splice 1 and SEP/NL1/NPII, respectively. NEPLP γ was a novel isoform.

Expression of NEPLPs, NEP, PEX, and DINE in the 293 Cell Line—We stably transfected NEPLP cDNAs in 293 cells and determined the expression using the anti-FLAG antibody. In the 293 cells transfected with pcDNA vector alone (referred to as pcDNA cells), no endogenous NEPLP protein was detected (Fig. 2A, lane 5). In membrane fractions of 293 cells stably transfected with NEPLP α (NEPLP α cells), 100- and 120-kDa broad bands were detected (Fig. 2A, lane 6). Because NEPLP α was expressed as a single 100-kDa band in COS cells (not shown) and Chinese hamster ovary cells (28), the 120-kDa band probably represented a highly glycosylated or modified protein specific for the 293 cells. NEPLP β and NEPLP γ proteins were expressed in membrane fractions as 110- and 115-kDa bands, respectively (Fig. 2A, lanes 7 and 8). This corresponded to the increasing sizes of amino acids, although proteins with higher molecular mass did not accumulate as seen in NEPLP α cells. The amounts of NEPLP β and NEPLP γ proteins were lower than that of NEPLP α. NEPLP α, NEPLP β, and NEPLP γ were not detected in the cytosolic fractions (not shown).

When we expressed other members of the M13 family (NEP, PEX, and DINE) in the 293 cells, these proteins were detected as 110-, 110-, and 95-kDa bands in membrane fractions (Fig. 2A, lanes 2, 4, and 10), whereas they were not detected in the pcDNA cells (Fig. 2A, lanes 1, 3, and 9). NEP, PEX, and DINE were not detected in cytosolic fractions or CM (not shown). To estimate the relative amounts of the peptidases, we expressed each cDNA to which FLAG sequence was added at its C-terminal end. Because nearly identical intensities of bands were detected with anti-FLAG antibody (Fig. 2D), equal amounts of peptidases with FLAG were loaded onto the gels. Fig. 2E shows the results of loading the same amounts of FLAG-tagged peptidases as those in Fig. 2D. The gel shown in Fig. 2E was also loaded with 0.5 µg of lysates from NEP, PEX, and NEPLP α cells and 1.5 µg from DINE cells and was then stained with...
the corresponding antibodies. Almost identical band intensities resulted regardless of whether or not the peptidases contained FLAG. We estimated from the results that the relative amounts of peptidases were 1:1:1:1/3 for NEP:PEX:NEPLPα:DINE. The addition of FLAG to PEX and NEPLPα reduced their molecular weight compared with their weights in the absence of FLAG (Fig. 2E), suggesting immature glycosylation or modification resulting from the FLAG sequence at the C terminus. We also observed reduced proteolytic activity of NEP-FLAG than that of NEP (not shown). Therefore, we used each peptidase without FLAG in the following proteolytic analyses.

Proteolytic Activity to Synthetic Peptide (ZAAL-pNA) — We investigated the peptidase activity of NEPLPs, and their sensitivities to thiorphan and phosphoramidon. Membrane fractions of NEPLPs and other peptidases expressed in the 293 cells were incubated with ZAAL-pNA at pH 6.5 (Fig. 3, A and B). Membrane fractions of pcDNA cells showed a very low level of thiorphan- and phosphoramidon-sensitive activity due to the presence of an endogenous enzyme. A high degree of proteolytic activity was detected in the membrane fractions of the NEP cells (Fig. 3, A and B). PEX showed a low but significant proteolytic activity compared with that of the control (pcDNA) (Fig. 3A). Both NEPLPα and NEPLPβ proteolyzed the peptide, and their activities were almost the same and one-third compared with that of NEP, respectively. In contrast, NEPLPγ had no proteolytic activity compared with the control, although the level of the NEPLPγ protein was similar to that of NEPLPβ (Fig. 2A). This suggested that NEPLPγ protein has different substrate specificity or that it is a zymogen that requires proteolytic activation. It is also possible that the 37-amino acid sequence inserted into NEPLPγ inhibits its activity by interfering with proper folding. DINE had no proteolytic activity in this system (Fig. 3B), although its endopeptidase activity and sensitivity to thiorphan and phosphoramidon were demonstrated previously in a baculovirus expression system (22). Proteolytic activities of NEP, PEX, NEPLPα, and NEPLPβ were inhibited by thiorphan and phosphoramidon (Fig. 3A), suggesting that they could all be candidates for Aβ-degrading enzymes in vivo. The secreted form of NEPLPβ also had the
HPLC for degrading activity to cold Aβ1–42 affected with the cDNAs indicated in each panel were analyzed using peptidases. Intact Aβ, transfected cells (panels 8 and 9), membrane fractions (0.5 µg) were analyzed for their ability to proteolyze Aβ. Membrane fractions of the pcDNA cells exhibited almost no proteolytic activity to the radiolabeled Aβ1–42 (peak at 42 min in Fig. 4A, closed triangle) in 1 h at 37 °C. NEP decreased the radioactive peak at 42 min and increased the peaks at 7 and 37 min, suggesting that NEP efficiently proteolyzed the Aβ1–42 into free amino acids/small peptides and a catabolic intermediate (Fig. 4B, open triangles). This degradative activity by NEP was very similar to the in vivo proteolysis as described previously (15). The degradative activity was completely inhibited by thiorphan, suggesting that NEP directly proteolyzed the peptide (Fig. 4H). In 4 h, NEP almost completely digested the Aβ1–42 (not shown). In contrast PEX, NEPLP α, NEPLP β, NEPLP γ, and DINE did not degrade the Aβ1–42, because their HPLC profiles were almost the same as that of the control (pcDNA) (Fig. 4, C–G). HPLC profiles in the 3H mode were essentially identical to the 14C mode (not shown), as reported previously (15), suggesting that NEP proteolyzed the Aβ1–42 from both the N and C termini.

Degradation of Cold Aβ1–40 and Aβ1–42—We examined the degradation of cold Aβ1–40 and Aβ1–42 using HPLC under different conditions from those used for analyzing the degradation of 3H/14C-radiolabeled Aβ1–42 (see “Experimental Procedures”). Membrane fractions of the pcDNA cells were used as a negative control (Fig. 5A, panel 1). NEP almost completely degraded the Aβ1–40 peptide corresponding to the peak at 35.7 min (Fig. 5A, panel 2) in 2 h at 37 °C. The degradation was accompanied by the appearance of multiple peptides between 26 and 29 min (Fig. 5A, panel 4). Thorphan (Fig. 5A, panels 8 and 9) and phosphoramidon (not shown) completely abolished the proteolytic activity of NEP and NEPLP α. PEX, NEPLP β, NEPLP γ, DINE (Fig. 5A, panels 3, 5–7) and secreted form of NEPLP β (not shown) showed no proteolytic activity to Aβ1–40. Although we assayed 3-fold amounts of membrane fractions of NEPLP β, NEPLP γ, and DINE cells to normalize the amounts of peptidase activity (not shown). We obtained identical results at pH 7.2, too.

Degradation of Radiolabeled Aβ1–42—We characterized the Aβ-degrading activity of the peptidases using 3H/14C-radiolabeled Aβ1–42, which we had synthesized previously (15). To exclude the effects of endogenous peptidases, equal amounts of membrane fractions (0.5 µg) were analyzed for their ability to proteolyze Aβ. Membrane fractions of the pcDNA cells exhibited almost no proteolytic activity to the radiolabeled Aβ1–42 (peak at 42 min in Fig. 4A, closed triangle) in 1 h at 37 °C. NEP decreased the radioactive peak at 42 min and increased the peaks at 7 and 37 min, suggesting that NEP efficiently proteolyzed the Aβ1–42 into free amino acids/small peptides and a catabolic intermediate (Fig. 4B, open triangles). This degradative activity by NEP was very similar to the in vivo proteolysis as described previously (15). The degradative activity was completely inhibited by thiorphan, suggesting that NEP directly proteolyzed the peptide (Fig. 4H). In 4 h, NEP almost completely digested the Aβ1–42 (not shown). In contrast PEX, NEPLP α, NEPLP β, NEPLP γ, and DINE did not degrade the Aβ1–42, because their HPLC profiles were almost the same as that of the control (pcDNA) (Fig. 4, C–G). HPLC profiles in the 3H mode were essentially identical to the 14C mode (not shown), as reported previously (15), suggesting that NEP proteolyzed the Aβ1–42 from both the N and C termini.

**Degradation of Cold Aβ1–40 and Aβ1–42**—We examined the degradation of cold Aβ1–40 and Aβ1–42 using HPLC under different conditions from those used for analyzing the degradation of 3H/14C-radiolabeled Aβ1–42 (see “Experimental Procedures”). Membrane fractions of the pcDNA cells were used as a negative control (Fig. 5A, panel 1). NEP almost completely degraded the Aβ1–40 peptide corresponding to the peak at 35.7 min (Fig. 5A, panel 2) in 2 h at 37 °C. The degradation was accompanied by the appearance of multiple peptides between 26 and 29 min (Fig. 5A, panel 4). Thorphan (Fig. 5A, panels 8 and 9) and phosphoramidon (not shown) completely abolished the proteolytic activity of NEP and NEPLP α. PEX, NEPLP β, NEPLP γ, DINE (Fig. 5A, panels 3, 5–7) and secreted form of NEPLP β (not shown) showed no proteolytic activity to Aβ1–40. Although we assayed 3-fold amounts of membrane fractions of NEPLP β, NEPLP γ, and DINE cells to normalize the amounts of peptidase activity (not shown). We obtained identical results at pH 7.2, too.

**Table I**

| Kinetic analysis of cold Aβ1–40 and Aβ1–42 proteolysed by NEP |
|-------------------|--------|----------|----------|----------|
|                  | S₀     | Sₚ      | Kₘ       | Vₘₐₓ     |
|                  | µM     | µM      | µM       | nmol/min |
| Aβ40             | 4.6    | 1.18     | 11.2      | 158      |
| Aβ42             | 3.3    | 1.94     | 6.95      | 21.1     |

Data are representative of at least two independent experiments. C and D, time course of degradation of cold Aβ1–40 (C) or Aβ1–42 (D) by membrane fractions (10 µg) of each peptidase. The Y axis represents the remaining peak of the Aβ peptides compared with pcDNA cells (% of control) after indicated incubation periods.
FIG. 6. Proteolysis of cell-secreted Aβ1–40 and Aβ1–42 by the peptidases. Membrane fractions of 293 cells stably (A) or transiently (B) transfected with the cDNAs indicated below each lane were analyzed for degrading activity to Aβ secreted from N2a cells stably transfected with both the APP and PS2 mutants. After incubation with the membrane fractions (4 μg) and Aβ, quantitative ELISA analysis was performed to determine the Aβ1–40 (white columns) and Aβ1–42 (black columns). Data (Exp. 1 and 2) are representatives of at least three independent experiments and are shown with mean ± S.D.

In the case of cold Aβ1–42 was used as a substrate, NEP degraded it in a reproducible manner as demonstrated by the decrease of the original peak at 36.4 min and the appearance of a new peak at 26.4 min (Fig. 5B, panel 2) compared with the control (Fig. 5B, panel 1) in 4 h at 37 °C. In contrast, PEX, NEPLP α, NEPLP β, NEPLP γ, and DINE showed no detectable proteolytic activity to Aβ1–40 in 6–12 h (Fig. 5C).

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We determined Km and Vmax values for the proteolysis of Aβ by NEP using varying amounts of cold Aβ peptides (Table I). The Km values were 11.2 and 6.95 μM and Vmax values were 158 and 21.1 nm/min for Aβ1–40 and Aβ1–42, respectively. It is difficult to determine the Km and Vmax values for the other peptidases, presumably because the Km values are too large and/or the Vmax values are too small to measure.

Degradation of Cell-secreted Aβ1–40 and Aβ1–42. We investigated the degradation of cell-secreted Aβ by the peptidases. We chose the N2a cells as Aβ-producing cells because they showed stable co-transfection with APP with the Swedish mutation and PS2 with the N141I mutation and secreted large amounts of both Aβ1–40 and Aβ1–42. We tried to examine the effect of NEP expression by co-culturing the NEP-expressing 293 cells with the N2a cells or by culturing the NEP-expressing 293 cells in CM derived from the N2a cells. However, the 293 cells used in the present study by themselves possessed a potent activity that completely removed the Aβ in CM, presumably through internalization and/or extracellular degradation employed by endogenous proteins. To avoid cell-mediated removal of Aβ, the membrane fractions of the 293 cells were incubated with CM from the N2a cells at 37 °C for 16 h. NEP, which was expressed either stably or transiently, reproducibly degraded both Aβ1–40 and Aβ1–42 at concentrations ranging from picomolar to micromolar in vitro. Because picomolar concentrations of Aβ peptides are present in human cerebrospinal fluids and plasmas, NEP is most likely to be an Aβ-degrading enzyme in vivo.

We previously demonstrated that enzymatic activities of PEX and NEPLP α/SEP7/splice 1 were sensitive to thiorphan and phosphoramidon. Using different methods (HPLC and ELISA) and different substrates (synthetic and cell-secreted Aβ), we showed that among the peptidases tested NEP most rapidly and efficiently degraded not only Aβ1–42 but also Aβ1–40 at concentrations ranging from picomolar to micromolar in vitro. Because picomolar concentrations of Aβ peptides are present in human cerebrospinal fluids and plasmas, NEP is most likely to be an Aβ-degrading enzyme in vivo.

We previously reported that an endopeptidase(s) similar or identical to NEP is the most probable candidate for an Aβ1–42-degrading enzyme in vivo (15). In the present study we cloned NEPLP cDNAs and compared Aβ-degrading activity among NEP, PEX, NEPLPs, and DINE whose activities are sensitive to thiorphan and phosphoramidon. Using different methods (HPLC and ELISA) and different substrates (synthetic and cell-secreted Aβ), we showed that among the peptidases tested NEP most rapidly and efficiently degraded not only Aβ1–42 but also Aβ1–40 at concentrations ranging from picomolar to micromolar in vitro. Because picomolar concentrations of Aβ peptides are present in human cerebrospinal fluids and plasmas, NEP is most likely to be an Aβ-degrading enzyme in vivo.

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Aβ1–40 (7). Alternatively, NEP may mediate the initial cleavage of Aβ and another endogenous peptidase may further degrade Aβ. Although it is possible that NEP is indirectly involved in Aβ degradation through, for instance, the proteolytic activation of another peptidase, this is unlikely because NEP is capable of proteolysing peptides smaller than 4–5 kDa (31). In any case, it is noted that NEP is required for Aβ degradation.

We found that NEP degraded cold Aβ1–40 more rapidly than cold Aβ1–42 in vitro (Fig. 5). This may be due to a higher Vmax for Aβ1–40 than for Aβ1–42, although NEP had a slightly higher affinity to Aβ1–42 than to Aβ1–40 (Table I). The rate of degradation by NEP was different among various Aβ1–42 peptides (Fig. 4–6). The half-lives of radiolabeled Aβ1–42, cold Aβ1–42, and cell-secreted Aβ1–42 were ~1, 8, and more than 16 h, respectively. Moreover, the degraded products of radiolabeled 1–42 and cold Aβ1–42 may be different as evaluated by their retention times in HPLC. These different degrees of degradation are probably due to different conformations and concentrations of the peptides and/or different concentrations of the peptidases under the optimum conditions for each assay.

NEP is an ectoenzyme with a large extracellular domain containing a catalytic site. This indicates the direct involvement of NEP in Aβ degradation, because NEP can interact with and degrade Aβ with the correct topology on the cell surface. The fact that a soluble form of NEP is found in human plasma may reduce the Aβ degradation through, for instance, the proteolytic activation of another peptidase, this is unlikely because NEP is an ectoenzyme with a large extracellular domain.

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