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Acyl peptide hydrolase, a serine proteinase isolated from conditioned medium of neuroblastoma cells, degrades the amyloid-β peptide

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Abstract

Considerable evidence indicates that the amyloid-β (Aβ) peptide, a proteolytic fragment of the amyloid precursor protein, is the pathogenic agent in Alzheimer's disease (AD). A number of proteases have been reported as capable of degrading Aβ, among them: neprilysin, insulin-degrading enzyme, endothelin-converting enzyme-1 and -2, angiotensin-converting enzyme and plasmin. These proteases, originating from a variety of cell types, degrade Aβ of various conformational states and in different cellular locations. We report here the isolation of a serine protease from serum-free conditioned medium of human neuroblastoma cells. Tandem mass spectrometry (MS/MS)-based sequencing of the isolated protein identified acyl peptide hydrolase (APH; EC3.4.19.1) as the active peptidase. APH is one of four members of the prolyl oligopeptidase family of serine proteases expressed in a variety of cells and tissues, including erythrocytes, liver and brain, but its precise biological activity is unknown. Here, we describe the identification of APH as an Aβ-degrading enzyme, and we show that the degradation of Aβ by APH isolated from transfected cells is inhibited by APH-specific inhibitors, as well as by synthetic Aβ peptide. In addition, we cloned APH from human brain and from neuroblastoma cells. Most importantly, our results indicate that APH expression in AD brain is lower than in age-matched controls.

Keywords: acyl peptide hydrolase, Alzheimer's disease, amyloid-β degradation.
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Alzheimer's disease (AD) histopathology is characterized by the presence of senile plaques and neurofibrillary tangles in the brain. These two hallmarks are accompanied by massive neuronal loss. The major component of the senile plaques is the amyloid-β (Aβ) peptide (Glenner and Wong 1984; Masters *et al*. 1985). A 39–43-amino acid long, the Aβ peptide is produced during normal metabolism of a larger transmembrane protein, the amyloid precursor protein. Exposure of neurons, cell lines, or endothelial smooth muscle cells to high concentrations of Aβ causes cell death, while, interestingly, low concentrations of Aβ are neurotrophic (Yankner *et al*. 1990; Blanc *et al*. 1997). The steady-state levels of Aβ peptides *in vivo* are a direct consequence of the balance between their production and breakdown. The most described Aβ-degrading enzymes today are: neprilysin (Iwata *et al*. 2000) and its family member endothelin-converting enzyme-1 (ECE-1; Eckman *et al*. 2001), insulin-degrading enzyme (IDE; Vekrellis *et al*. 2000), angiotensin-converting enzyme (ACE; Hu *et al*. 2001), and plasmin (Van Nostrand and Porter 1999; Tucker *et al*. 2000).

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Abbreviations used: AANA, *N*-acetyl-alanyl-p-nitroanilide; Aβ, amyloid-β peptide; ACE, angiotensin-converting enzyme; AD, Alzheimer's disease; AEBSF, 4-(2-aminoethyl) benzenesulfonyl fluoride; APH, acyl peptide hydrolase; CL, cell lysate; CM, conditioned medium; DEAE, diethylamino ethanol; DFP, di-isopropyl fluorophosphates; ECE, endothelin-converting enzyme; IDE, insulin-degrading enzyme; IP, immunoprecipitation; MS, mass spectometry; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; TCA, trichloro acetic acid.

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We previously demonstrated a strong Aβ-degrading activity in serum-containing conditioned medium (CM) of human neuroblastoma cells that was completely inhibited by serine protease inhibitors, indicating that the activity was as a result of a serine protease secreted from these cells (Yamin *et al*. 1999). We isolated the serine protease from serum-free CM of the same neuroblastoma cells and identified it as acyl peptide hydrolase (APH; EC3.4.19.1) by tandem mass spectrometry (MS/MS)-based sequencing.

Here, we describe the isolation of APH as an Aβ-degrading enzyme, and provide evidence obtained from real-time PCR, indicating that AD brains express lower levels of APH than brains of age-matched controls. Further characterization of the role of APH in Aβ degradation could lead to a new pharmaceutical target for the treatment of AD.

Experimental procedures

APH protein isolation

Human neuroblastoma cell line, SK-N-MC (ATCC, Manassas, VA, USA) stably over-expressing metalloendopeptidase E.C.3.4.24.15 (also known as THOP1) was grown in culture until 80% confluent. Plates were washed with serum-free medium and incubated with serum-free opti-MEM (Gibco, Rockville, MD, USA) for 3 days. On the third day, the serum-free CM was collected, centrifuged for 30 min at 16 000 *g* (Sorvall RC-5B, Dupont, NJ, USA), and loaded on a diethylamino ethanol (DEAE) column equilibrated with 20 mM Tris-HCl (pH 7.9). The column was then washed with 10 × column volume of 20 mM Tris buffer (pH 7.9) and fractions were eluted with (3 × column volume each) increasing concentrations of NaCl (between 50 mM to 1 M) in 20 mM Tris (pH 7.9). Each fraction was assayed for protein concentration and for activity in the Aβ-degradation assay. The active fractions were also tested for Aβ-degrading activity in the presence of different protease inhibitors in order to identify the fraction containing the serine protease. The high molecular weight proteins (> 30 kDa) in the active fractions were concentrated further by passing through a Centricon 30 filter (Millipore, Billerica, MA, USA). The concentrated active fractions were labeled with [^{3}H]di-isopropyl fluorophosphates (DFP) and electrophoresed next to the same unlabeled fractions on 4–12% Nu-polyacrylamide gel electrophoresis (PAGE) gels (Invitrogen, Carlsbad, CA, USA). The gel portion containing the radioactive lanes was cut, treated with gel drying solution (Novex/Invitrogen, Carlsbad, CA, USA) for 45 min, and with Enhance solution (DuPont–NEN, Boston, MA, USA) for 20 min. Treated gels were dried and exposed to film. The other half of the gel containing the cold samples was stained with colloidal blue (Novex/Invitrogen) according to the manufacturer's protocol. Bands on the autoradiograms were aligned with the colloidal blue stained bands and the band corresponding to the radiolabeled band was excised and sent for sequencing. The sample was first subjected to trypsin digestion and then to a microcapillary reverse-phase HPLC run, directly coupled to the nano-electrospray ionization source of an ion trap mass spectrometer. The analysis was performed at the Harvard Microchemistry facility (Cambridge, MA, USA).

Aβ activity assays

Aβ-activity assays were performed by incubating 20 μL of each fraction with 10 000 cpm (7 pmol/L) of [^{125}I]Aβ1-40 peptide (Amersham Pharmacia Biotech, Piscataway, NJ, USA) for the designated time (usually overnight) at 37°C. After incubation, Laemmli sample buffer was added and samples were boiled and separated on 4–12% NU-PAGE gels (Novex/Invitrogen). Gels were then dried and exposed to film (Kodak BioMax XAR film). Bands on the autoradiogram indicate undegraded Aβ peptide. In some cases, the degradation of [^{125}I]Aβ1-40 was monitored by trichloro acetic acid (TCA) precipitation. Briefly, incubation of samples with [^{125}I]Aβ1-40 was terminated at different time points ranging from 30 min to 4 h. Samples (20 μL) were incubated on ice with 400 μL of 10% TCA solution and 20 μL of 10 mg/mL bovine serum albumin as carrier protein for 1 h. Undegraded [^{125}I]Aβ1-40 was precipitated by a 15-min spin at 4°C. Pellets were washed once with 400 μL of 10% TCA and then the radioactivity in the pellet and the supernatant counted in a gamma counter. Percent activity was calculated as follows: cpm counts obtained from the supernatant of the Aβ-alone sample were used as background counts and were subtracted from each samples' supernatant counts. These were defined as adjusted sup cpm. For each sample: % degradation = adjusted sup cpm/cpm in the pellet + adjusted sup cpm.

Inhibition assays

Samples were pre-incubated with the indicated inhibitors for 10 min at 37°C, then [^{125}I]Aβ1-40 was added for additional incubation of 18 h. Sample buffer was added and the samples were boiled and separated by Nu-PAGE gels as described above. The following inhibitors were tested: 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF), a serine protease inhibitor, at 2 mM; thiorphan, a neprilysin inhibitor, at 100 μM; insulin, an IDE inhibitor, at 20 μM; aprotinin a papain, trypsin and plasmin inhibitor, at 15 μM; EDTA/EGTA metalloprotease inhibitors at 1 mM each. The battery of APH-specific inhibitors that were synthesized include the competitive inhibitors Ac-Ala-OH and Ac-Met-OH, the irreversible inhibitor Ac-Leu-CH_2-Cl, as well as an inhibitor that acts by an unknown mechanism, 1-butane boronic acid. These APH inhibitors were used as previously published (Scaloni *et al*. 1992a, 1994).

Gel zymography

Samples from column fractions nos 13–16 and active plasmin (Sigma, St Louis, MO, USA), as control, were diluted in gel loading buffer without mercaptoethanol. Samples were loaded on 10% acrylamide gels containing 1% sodium dodecyl sulfate (SDS) and 0.1% gelatin and separated at 4°C. Gels were then washed at 4°C twice for 30 min in 2.5% Triton X-100 followed by two washes of 15 min each in H_2O at 4°C. Gels were incubated on a shaking incubator at 37°C for 40 h in phosphate-buffered saline in the presence or absence of 2 mM EDTA to inhibit metalloprotease activity. At the end of the incubation time, gels were stained with 1% amido black in 50% methanol, 10% acetic acid for 10 min at 25°C and de-stained in 50% methanol, 10% acetic acid until areas of degraded gelatin were visible.

[^{3}H]DFP labeling

Fraction nos 13 and 15 (18 μL) and a sample of the serine protease, cathepsin G, as control, were incubated in 20 mM Tris buffer pH 7.4

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with 3 μL of [^{3}H]DFP (Amersham Pharmacia Biotech) for 1 h at 37°C. After incubation, gel-loading buffer was added and the samples were separated on a 4–12% Nu-PAGE gel (Invitrogen). Gels were then treated with gel dry solution (Invitrogen) for 1 h, followed by 20 min incubation in Enlighting solution (DuPont–NEN). Gels were dried and exposed to X-ray film for 1 week at −80°C with intensifying screens.

Cloning by PCR

mRNA was prepared from two human brain gray matter samples, as well as from the human neuroblastoma cells SK-N-MC. Using the SuperScript first-strand synthesis system for RT-PCR kit (Invitrogen) and oligo dT primers, first-strand cDNA was synthesized from these samples. For the cloning of APH into the TOPO-V5/His tag mammalian expression vector (Invitrogen), we used the following primers: 5′-AGCTCGGATCCAGTACCCATGGAACGTCAGGTGCTGCTG-3′ as the forward primer, and 5′-GTGCTGGATATCTGCGCTGCCCAAGGTGTGTGCGTAGCCA-3′ as reverse primer. The forward primer has a *Bam*HI site and the reverse primer has an *Eco*RV site for subcloning.

In addition, a mutated APH clone was engineered in which the serine residue in the active site was substituted with an alanine. The mutant S587A was created using the QuickChange II XL site directed mutagenesis kit (Stratagene, La Jolla, CA, USA). The primers that were used for S587A mutant were: 5′-CCTTATGGGTGGTGCCCATGGTGGCTTCA-3′ as the forward primer, and 5′-TGAAGCCACCATGGGCACCACCCATAAGG-3′ as the reverse primer.

All APH clone bacterial cultures were grown in a 25°C shaker for 36 h to overcome the enzyme's toxicity for the bacteria when grown at 37°C.

Real-time PCR

Real-time PCR was done using TaqMan Gene Expression assay kits specific for human APH, glyceraldehydephosphate dehydrogenase and β-actin (Applied Biosystems, Foster City, CA, USA). Samples of frontal cortex from six AD and six age-matched controls were received from Drs A. McKee and N. Kowall from the ADRC Brain Bank at the Edith Nurse Rogers Memorial Veterans Hospital, Bedford, MA. Standard TaqMan default cycling protocol was used for PCR cycling (i.e. 2 min at 50°C; 10 min at 95°C; followed by 40 cycles of 15 s at 95°C; 2 min at 60°C). Each reaction was performed in triplicate in 25 μL total volume containing: 10 μL of a 1 : 50 dilution of cDNA sample, 12.5 μL of 2 × master mix (Applied Biosystems), 1.25 μL of primers and 1.25 μL H_2O. Analysis was performed with the ABI SDS (sequence detection system) software.

APH expression, immunoprecipitation, and western blots

The wild-type and mutant APH constructs were transfected into mammalian cells (COS 7 or SK-N-MC) using the Lipofectamine Plus reagent (Invitrogen) according to the manufacturer's protocol. 36–48 h after transfection, CM and cell lysates were harvested. Protein (20 μg) of each sample was loaded on a Nu-PAGE gel and transferred onto an immobilon-P membrane. Blots were blocked with 5% milk in TBST [10 mM Tris-Hcl pH 8.0, 150 mM NaCl, and 0.05% (v/v) Tween-20] and probed with a mouse monoclonal anti-V5 antibody (Invitrogen) targeting the V5 tag of the expressed protein, followed by rabbit anti-mouse HRP-conjugated secondary antibody, and detected by enhanced chemiluminescence (Pierce, Rockford, IL, USA). The same V5 antibodies were also used for immunoprecipitation (IP) of the expressed APH from the transfected cells as per Invitrogen's IP protocol (King of Prussia, PA, USA).

APH activity assay

APH activity was measured using the chromogenic substrate *N*-acetyl-alanyl-p-nitroanilide (AANA; Bachem) as previously described (Richards *et al*. 2000). Specifically, 10 μL of sample containing APH from cell lysates (CL), CM or IP pellets was added in a microtiter plate to 200 μL of substrate solution containing 0.2 M Tris-HCl, 1 mM dithiothreitol, pH 7.4 and 4 mM AANA. The absorbance at 405 nm was read after 5–20-min incubation at 37°C.

Results

APH isolation

We isolated APH from serum-free CM of the human neuroblastoma cell line SK-N-MC that over-expresses the metalloendopeptidase MP3.4.24.15 (THOP1). We previously showed that these CM contained a proteolytic activity that degrades Aβ (Yamin *et al*. 1999). In order to eliminate the potential contamination by serum proteins in the preparation, we used 3 days serum-free CM for the isolation process. As can be seen in Fig. 1, the serum-free CM was inactive in the Aβ degradation assay before separation on the ion exchange column (Fig. 1, sample marked O for original). Following separation on the DEAE column, we detected high Aβ-degrading activity in the fractions eluted with 250 mM NaCl. The protein concentration in the active fraction was less than 100 μg/mL (about 0.315 mg protein in total), i.e. 500 times less protein than the amount loaded on the column indicating a large enrichment in specific activity. In addition to the activity observed in the CM of EC3.4.24.15 transfected cells, a similar activity was isolated on a small-scale column (5 mL of CM on a 1-mL column) from mock-transfected SK-N-MC (data not shown). This result indicated, and was further confirmed by PCR, that the serine protease activity was not related to the EC3.4.24.15 over-expression in these cells, and that the enzyme described here is different also in size from those described by Yamin *et al*. (1999).

In order to identify the most active fraction and quantify the proteolytic activity following the DEAE column, we used the more sensitive assay, TCA precipitation of iodinated Aβ incubated with two different dilutions of the active fractions. The dilution in which loss of activity occurs indicates the fraction that has the highest activity. As seen in Fig. 2, the Aβ-degradation activity in fraction no. 14 was lost after a 2 × dilution, while the activity of fraction no. 15 was lost after a 4 × dilution, indicating that fraction no. 15 contains

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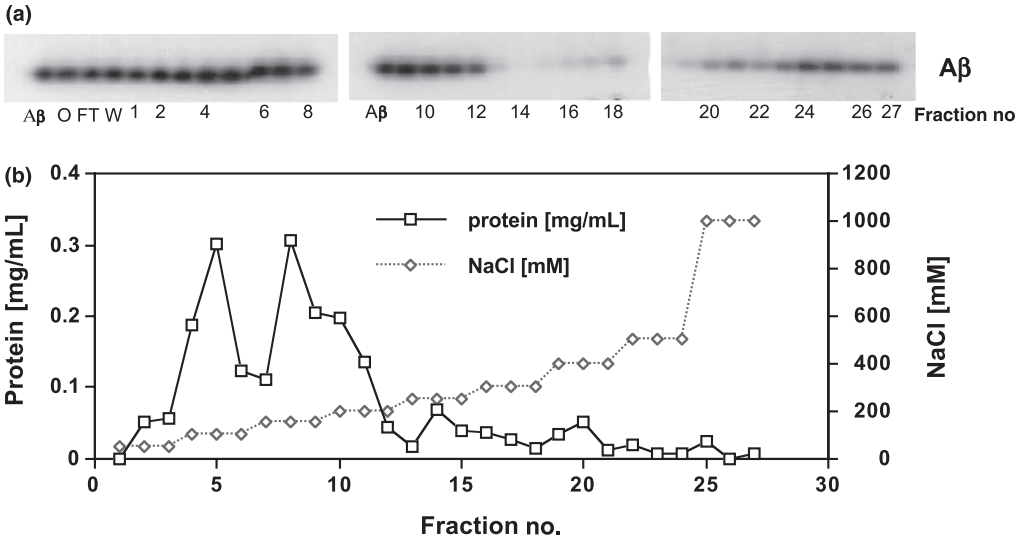

Fig. 1 [^{125}I]Aβ-degrading activity and protein concentration in the DEAE column fractions. Serum-free CM collected from SKNMC human neuroblastoma cells was loaded on a DEAE column. Fractions were collected with increasing salt concentrations and Aβ-degradation activity was assayed in each fraction. (a) Autoradiogram of undegraded [^{125}I]Aβ peptide remaining after incubation with the fractions as follows: Aβ, Aβ alone; O, original CM; FT, flow through; W, column wash; 1–3, 50 mM fractions; 4–6, 100 mM fractions; 7–9, 150 mM fractions; 10–12, 200 mM fractions; 13–15, 250 mM fractions; 16–18, 300 mM fractions; 19–21, 400 mM fractions; 22–24, 500 mM fractions; 25–27, 1-M fractions. (b) Protein elution profile.

the peak of activity. In addition, specific activity of fraction no. 15 is higher because the protein concentration in this fraction is lower than in the other fractions. Loss of activity after a higher dilution indicates enrichment in activity, but a more precise indication as to the level of enrichment is not possible because the original serum-free CM, pre-column, was inactive.

APH is a serine protease

To verify that the activity found is indeed that of a serine protease different from the activities of IDE, neprilysin and plasmin, or other metalloproteases such as ECE or ACE, we performed Aβ-degradation assays in the presence or absence of the following protease inhibitors: AEBSF and DFP, both serine protease inhibitors; aprotinin, a plasmin inhibitor; EDTA, a metalloprotease inhibitor; thiorphan, a neprilysin inhibitor, and insulin, an IDE inhibitor. As seen in Fig. 3, only AEBSF and DFP inhibited the activity of the fractions around the peak of activity (surrounding fraction no. 15). This inhibitory profile provides evidence that (i) the Aβ-degrading activity is that of a serine protease, and (ii) the activity in our CM is a result neither of the metalloproteases IDE, neprilysin, ECE and ACE, nor of the serine protease plasmin.

In addition to the inhibition assay we also performed SDS–PAGE zymography to compare the active fraction with purified activated plasmin. The plasmin band, indicating degraded gelatin, was observed at a lower molecular weight than the activity of the serine protease (data not shown). Furthermore, the activity of the active fraction was not inhibited when zymography gels were incubated in the presence of EDTA, indicating the activity was not the result of a metalloprotease action (data not shown).

MS/MS analysis

In order to visualize the active enzyme in the two most active fractions, we used a radiolabeled irreversible serine protease inhibitor, [^{3}H]DFP. As seen in Fig. 4(a), the two active fractions exhibited a distinguished pattern of labeling indicating that each contains different serine proteases. Fraction no. 14 (Fig. 4a, lane 3) displayed four radioactive bands, while fraction no. 15 (Fig. 4a, lane 4) demonstrated only one radioactive band (arrow in Fig. 4a). Of note, fraction no. 15 was inhibited only by the serine protease inhibitors AEBSF and DFP. Fraction no. 14 probably contained other proteases in addition to serine proteases because this fraction was inhibited by AEBSF, insulin and thiorphan. Therefore, a radioactively labeled and cold sample no. 15 that contained a serine protease activity and only one radiolabeled band with [^{3}H]DFP, were separated side by side on two lanes of a Nu-PAGE gel. The radioactive half of the gel was exposed to film while the cold half was stained with colloidal blue. The two halves of the gel were then aligned, and the colloidal blue stained band corresponding to the radioactive band was excised from the gel and submitted to mass spectrometry analysis.

APH (EC3.4.19.1) was identified as the only serine protease in the excised band. Figure 4(b) illustrates the

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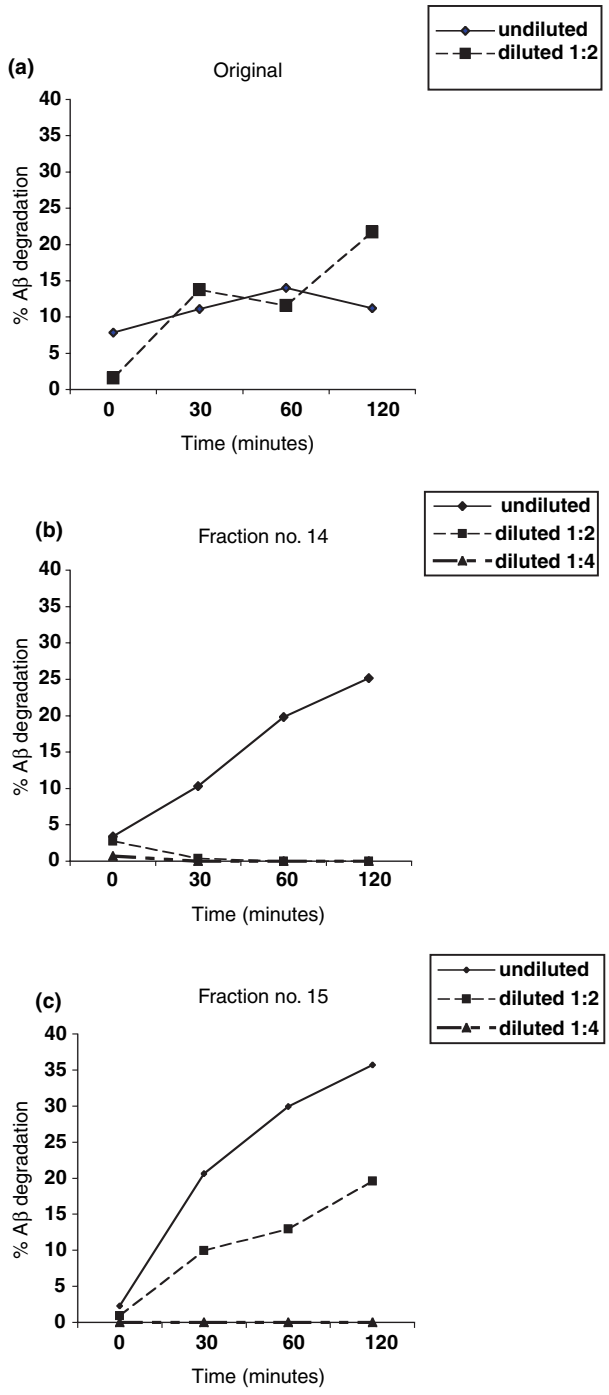

Fig. 2 [^{125}I]Aβ-degrading activity as measured by TCA precipitation. Samples of the original serum-free CM (a) and active fractions no. 14 (b) and no. 15 (c) were incubated with [^{125}I]Aβ (10 000 cpm). Each sample was assayed undiluted and at dilutions of 1 : 2 and 1 : 4. Incubation time was in minutes as indicated. Per cent Aβ-degradation activity was calculated as explained in Material and methods.

amino acid sequence of APH. The peptides sequenced by MS/MS are underlined and in bold, and the catalytic triad of the enzyme is in bold.

Cloning APH by PCR from cell lines and from AD and controls brain samples

We next cloned APH by PCR both from the SK-N-MC neuroblastoma cells and from human brain samples. The cloning was carried out using primers designed according to the Genebank sequence. In addition, real-time PCR was performed on mRNA samples from four cell lines and two samples of human frontal cortices. Compared with NIH H128 human small cell lung carcinoma cells that reportedly possess the lowest amount of APH expression among all cell lines tested (Scaloni *et al*. 1992b), APH relative expression levels in HEK293, SK-N-MC and SK-N-MC cells over-expressing the MP3.4.24.15 cell lines were similar to each other (data not shown). In contrast, there was an approximately five times lower relative expression of APH in six AD brain samples as compared with six samples of age matched controls brains as measured by real-time PCR. Figure 5 demonstrates the average for each group: AD, 0.5 ± 0.47 ($n = 6$), and control, 2.75 ± 2.5 ($n = 6$). The mean of individual ages of the AD and control samples was 81.3, and 77.5, respectively, indicating no significant difference in age between the two groups. The mean of post-mortem intervals was 9 h for the AD group, and 19.25 h for the control group, suggesting that the lower APH values in AD are not because of longer post-mortem intervals.

APH cDNA over-expression in cell lines

The 2.1-kb coding sequence of APH was cloned into the TOPO V5/His mammalian vector. The wild-type APH construct and the APH mutant, S587A, in which the serine in the active site has been substituted with alanine, were used in transfection experiments. Figure 6 shows the expression levels of the transfected APH constructs in SK-N-MC cell lysates and CM, and the activity of APH in the APH transfected cell lysate on the chromogenic substrate AANA in the presence or absence of APH inhibitors. The western blot (Fig. 6a) following immunoprecipitation of transfected APH with the anti-V5 antibodies provides evidence that a small amount of the enzyme is present in the CM. Activity was measured on the whole cell lysate without immunoprecipitation. In the CM, activity was much lower than in the cell extracts (data not shown), indicating that either only a small amount of the enzyme is secreted, or that the medium contains a potent inhibitor of APH. In order to distinguish the activity of the transfected APH from the endogenous enzyme, we performed APH activity assays on samples that were first immunoprecipitated with anti-V5 antibodies. Figure 6(b) demonstrates activity in cell lysates and CM after IP with the V5 antibodies.

Aβ inhibits recombinant APH activity on the specific substrate, AANA

The immunoprecipitated APH-containing pellet was re-suspended in 200 mM Bis-Tris buffer PH 7.4 and 10-μL

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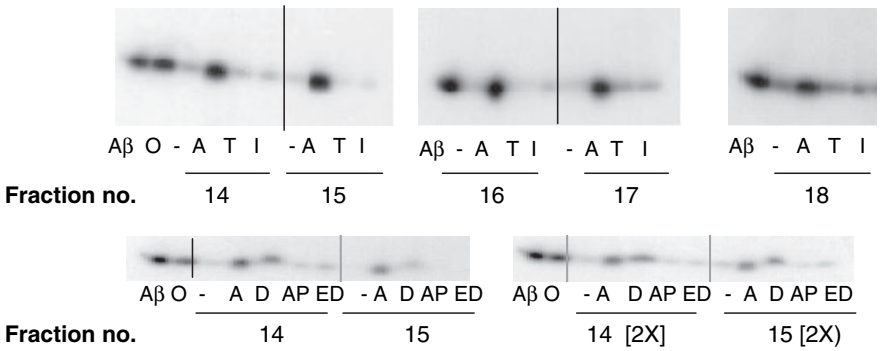

Inhibitor	Concentration	Class	Inhibition
AEBSF (A)	2 mM	serine	+
DFP (D)	6 μM	serine	+
Aprotinin (AP)	15 μM	serine (including plasmin)	-
Thiorphan (T)	100 μM	metalloprotease (including NEP)	-
Insulin (I)	26 μM	IDE	-
EDTA (ED)	1 mM	metalloprotease	-

Fig. 3 Inhibitory profile of the [^{125}I]Aβ-degrading activity. Active fractions from the ion exchange column were pre-incubated with the following inhibitors for 10 min before the addition of [^{125}I]Aβ peptide. Autoradiograms show undegraded [^{125}I]Aβ peptide. Lanes: Aβ, Aβ-alone; O, original serum-free CM; –, no inhibitor; A, + AEBSF; T, + thiorphan; I, + insulin; D, DFP; AP, aprotinin; ED, EDTA. Note that activity peaks at fraction no. 15 and is inhibited only by serine protease inhibitors.

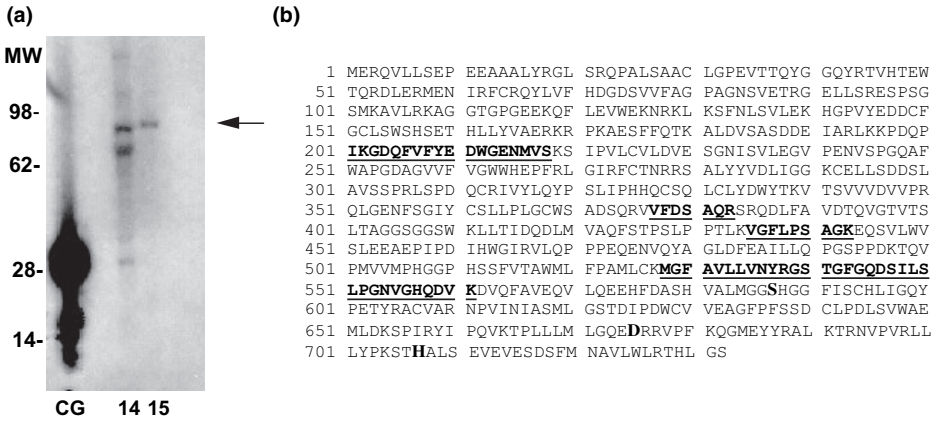

Fig. 4 [^{3}H]DFP labeling and the amino acid sequence of the tryptic peptides of APH identified by MS/MS. (a) Samples from two active fractions of the ion exchange column and cathepsin-G (a 28-kDa serine protease), as control, were incubated with the radiolabeled serine protease inhibitor [^{3}H]DFP for 15 min at 37°C. Samples were then run on a NU-PAGE gel, dried and exposed to film. Lanes are: CG, cathepsin-G; an empty lane; 14, fraction no. 14; 15, fraction no. 15. Note that the single band of fraction no. 15 was sent for mass spectrometry analysis. (b) Amino acid sequence of APH. The peptides sequenced by MS/MS are underlined and in bold. The active site catalytic triad Ser587, aspartic acid, D675, and His707, are in bold.

samples were added to the substrate solution in microtiter plates with or without inhibitors as described in Fig. 7. Figure 7(a) demonstrates activity in cell lysates after IP with the V5 antibodies in the absence or presence of several APH-specific inhibitors. In Fig. 7(b) we show that Aβ1-40 can inhibit APH activity from the cell lysates of APH transfected cells after IP at 10- and 1-μM concentration, while reversed Aβ peptide (Aβ40-1) at the same concentrations did not show any inhibitory effect. A similar inhibition pattern by Aβ and the APH inhibitor Ac-Leu-CK is also shown.

Aβ degradation by immunoprecipitated APH is specific and is inhibited by the APH-specific inhibitor Ac-Leu-CK

To confirm the direct activity of APH on Aβ, we isolated APH from COS7 cells using the ProBond His-tag purification system and incubated an estimated 1–1.5 ng of purified

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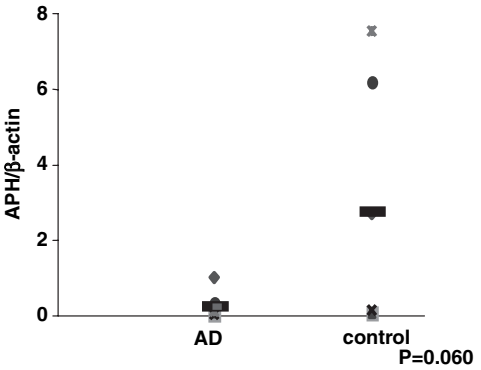

Fig. 5 Relative expression of APH in human brain samples. Relative expression of APH/β-actin as obtained by real-time PCR of mRNAs from six AD and six age-matched controls brain samples; *p*-value was calculated by Student's *t*-test.

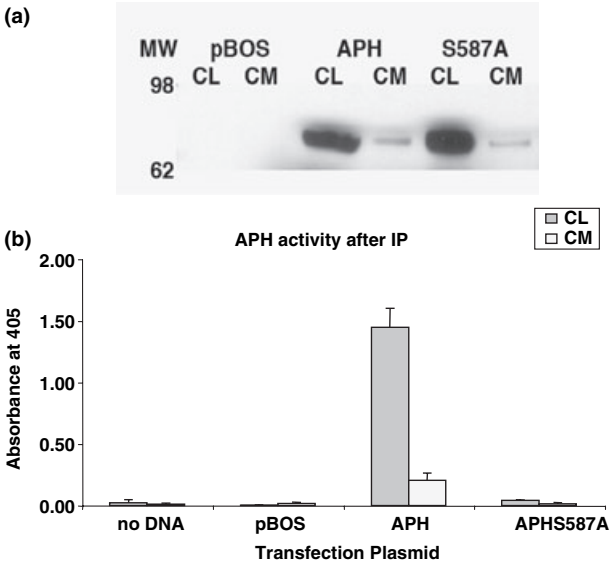

Fig. 6 APH expression and activity measured in transfected cells. Cells were transfected with pBos (the empty vector used for the cloning of APH), APH wild type or mutant APH S587A. Two days after transfection, cell lysate (CL) and conditioned medium (CM) were collected. (a) Western blot showing APH expression in CL and CM. (b) The IP pellets were re-suspended in 300 μL Bis-Tris buffer pH 7.4 and a 10-μL/well APH-containing sample was used in the APH activity assay. Assays were performed in triplicate on samples of CL or CM of the different transfected plasmids (bars represent readings of two experiments each carried out in triplicate; $n = 6$).

APH with iodinated Aβ peptide. We chose to use the COS7 cell line as these cells are more efficiently transfected and gave a better yield of isolated enzyme. The data are described in Fig. 8. Isolation of the enzyme by the His-tag from mammalian cells yielded a limited amount of pure protein as shown by the coomassie stained band in Fig. 8(a). The

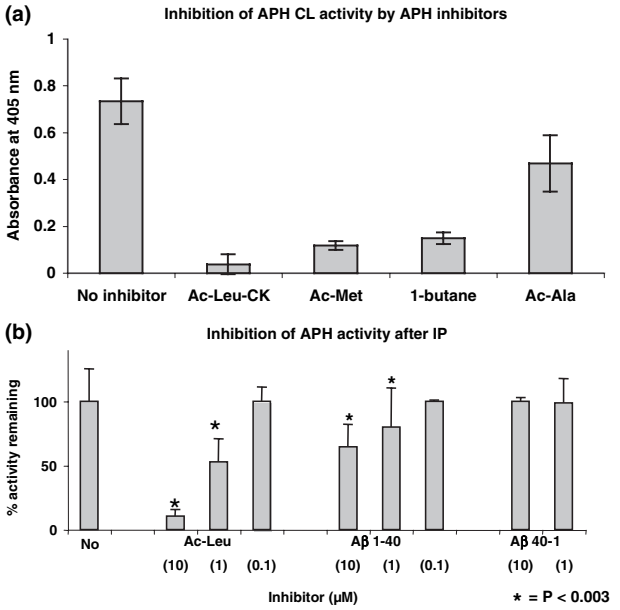

Fig. 7 Inhibition of APH activity by APH-specific inhibitors and by Aβ. APH was immunoprecipitated from COS7 transfected cells using the V5 antibody. The IP pellets were re-suspended in 300 μL Bis-Tris buffer pH 7.4 and a 10-μL/well APH-containing sample was used in the APH activity assay with or without inhibitors. Assays were carried out in triplicate. (a) Immunoprecipitated APH (10 μL) was incubated with 4 mM AANA with or without the following inhibitors: 5 μM Ac-Leu-CH_2Cl (Ac-Leu-CK); 5 μM Ac-Met-OH (Ac-Met); 5 μM 1-butane-boronic-acid (1-butane); 5 μM Ac-Ala-OH (Ac-Ala). Absorbance was read at 405 nm after 25-min incubation at 37°C. (b) Activity of immunoprecipitated APH from CL of the transfected cells. Absorbance was measured after 15-min incubation in the presence or absence of three different concentrations of the APH inhibitor Ac-Leu-CK, Aβ1-40 peptide or Aβ40-1.

isolated enzyme was incubated with [^{125}I]Aβ1-40 peptide and then separated on Nu-PAGE gels. Aβ degradation is demonstrated in Fig. 8(b, lane 2) and was inhibited by the APH-specific inhibitor Ac-Leu-CK. We are currently in the process of collecting larger amounts of purified protein for a more extended analysis of Aβ cleavage sites by APH.

Discussion

The levels of Aβ40 $_{\text{and}}$ Aβ42 in the AD brain are much higher than in the periphery (9.7 ng/mL in AD CSF vs. 0.14 ng/mL in plasma for Aβ40 and 0.61 ng/mL in AD CSF vs. 0.009 ng/mL in plasma for Aβ42; Mehta *et al*. 2001). This 70-fold difference reflects the balance between Aβ production and breakdown or clearance in the brain compared with the periphery. Accumulation of Aβ in the brain can be prevented by different mechanisms that include drainage from the extracellular space by receptor-mediated internalization via LRP, RAGE and other receptors (Weller

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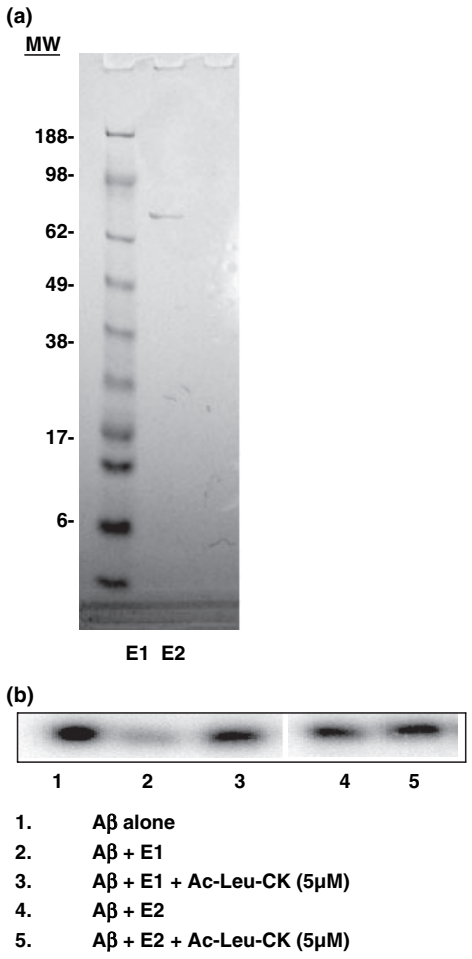

Fig. 8 Aβ degradation by purified APH. COS7 cells were transfected with wild-type APH cDNA. Two days after transfection, cells were collected and APH was purified on a nickel column using the ProBond purification system. Fractions eluted from the column were analyzed by SDS–PAGE for purity and then incubated with [^{125}I]Aβ1-40 peptide at 37°C. Mixtures were separated on Nu-PAGE gels and exposed to film. (a) Coomassie blue stained gel demonstrating the APH band eluted from the ProBond column. (b) Autoradiograph of Aβ degradation by fractions E1 and E2 in the presence or absence of 5 μM APH-specific inhibitor Ac-Leu-CK.

2002), and degradation by several peptidases reported to degrade Aβ *in vitro* and *in vivo* (Carson and Turner 2002).

Here, we report the isolation and identification of a serine protease that is different from the previously described Aβ-degrading enzymes. APH is a 75–80-kDa protease, one of four serine peptidases belonging to the prolyl oligopeptidase family. The family includes enzymes of different specificities: prolyl oligopeptidase, dipeptidyl peptidase IV, oligopeptidase B and acyl peptide hydrolase (Polgár 2002).

To date, human, porcine, and rat APH from various tissues has been characterized. All are 732 amino acids long, share 90% sequence homology, and are reported to form homotetramers (Tsunasawa *et al*. 1975; Raphel *et al*. 1999). The biological activity of APH has not been completely delineated. The enzyme is expressed in heart, liver, kidney, testis, brain, and also found in erythrocytes and plasma (Fujino *et al*. 2000a). Fujino and colleagues named it oxidized peptide hydrolase as it was found to degrade oxidized peptides (Fujino *et al*. 2000b). Shimizu *et al*. (2003, 2004) reported degradation of oxidized peptides *in vitro* by oxidized peptide hydrolase and demonstrated that APH activity was to protect cells under oxidative stress conditions by clearance of denatured proteins in coordination with the proteasome system. However, other studies, mostly carried out on APH isolated from the porcine gastrointestinal tract (Giardina *et al*. 1999), porcine liver, or human erythrocytes, describe APH as an enzyme that removes acetyl residues from *N*-acetylated peptides. APH was reported to be unique in the prolyl oligopeptidase family for its substrate preference. After cleavage by APH, the acetylated aminoterminal residue is removed, leaving the peptide shortened by a single amino acid (Rosenblum and Kozarich 2003).

As for substrate specificity, APH shows strong preference for acetylated N-termini of alanine, methionine, serine, and glycine. There is limited information available about the affinity for acetylated threonine, in that one peptide showed high activity and another no activity. Positively charged amino acids (lysine, histidine, arginine) have a negative effect on APH activity and, as the peptide length increases, the activity of APH decreases (Jones and Manning 1985; Scaloni *et al*. 1992a, 1994; Sokolik *et al*. 1994). The PH optimum was reported to depend on the substrate used. With acetyl-glutamate *p*-nitroanilide, the pH optimum is approximately pH 6, whereas for acetylalanine *p*-nitroanylide it is approximately pH 8.4 (Jones *et al*. 1986; Raphel *et al*. 1999). In contrast to prolyl oligopeptidase, which is a monomer, APH is composed of four identical subunits. Its structure shares the catalytic triad of other serine proteases composed of Ser-Asp-His, as well as the β-propeller domain found in prolyl oligopeptidase family structure for specific recognition of small peptides (Bartlam *et al*. 2004).

The catalytic triad of APH was determined to be near the C-terminus of the molecule and the enzyme was found to degrade peptides that are 20–50 residues long (Tsunasawa and Sakiyama 1984). APH is mapped to human chromosome 3p21 and it has been reported that six small-cell lung carcinoma cells lines undergo a deletion of the locus containing the APH gene (Jones *et al*. 1991; Scaloni *et al*. 1992b). In addition, APH was found to be a sensitive target for organophosphorus compounds and, as such, has been mentioned among the other proteases of the prolyl oligopeptidase family as a potential target for acetyl cholinesterase inhibitors treatment for cognitive enhancement in dementia patients (Richards *et al*. 2000).

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We isolated APH from serum-free CM of human neuroblastoma cells. Aβ-degrading activity was assayed following the fractionation of the medium on an ion exchange column, followed by concentration of the active fraction through a size filtration step. Finally, a colloidal blue-stained band from the purified fraction was aligned with a single [^{3}H]DFP-labeled band on the next lane in the gel, dissected and submitted for mass spectrometry. Several tryptic peptides corresponding to human APH were identified by MS/MS sequencing. As antibodies to APH are not commercially available, the presence of APH in SK-N-MC cells and in two samples from human frontal cortex was confirmed by cloning. Here, we describe, for the first time, the isolation of APH as an Aβ-degrading enzyme. Aβ is a 40–42-amino acid-long peptide and is oxidized on methionine 35 (Varadarajan *et al*. 2001; Schoneich 2005)**.** The Aβ peptide used in our assay is iodinated on tyrosine at position 10. Thus, if the N-terminus of Aβ is acylated, and if APH removes only the first N-terminal amino acid from the peptide, Aβ would still be radiolabeled and would be seen on our gels or by TCA precipitation. The fact that the Aβ band disappears from the autoradiogram after incubation with the active fraction indicates that at least the first 10 amino acids of the peptide are being cleaved. The finding that APH degrades oxidized peptides that are 20–50 residues long fits well with its potential role in Aβ degradation. The results presented here lead us into further studies on the mechanism of Aβ degradation by APH in the brain, such as cellular localization of the activity, sites of cleavage in Aβ, etc.

The real-time PCR data presented here indicate that APH expression in the AD brain is lower than in normal age-matched controls. APH expression was normalized to β-actin expression in each sample. The average ratio of APH/β-actin for controls is more than fivefold higher than the average APH/β-actin ratio for AD ($p = 0.0604$). The statistical significance of this difference needs to be determined on more samples. Such a determination will indicate a decrease in the expression level of APH in the AD brain and may serve as a new diagnostic tool for AD if it can be measured in plasma or CSF. In addition, we show here that the activity of APH on its specific chromogenic substrate, AANA, can be inhibited by specific synthetic APH inhibitors as well as by the Aβ peptide, likely acting as a competitive inhibitor. Interestingly, lower expression levels in AD samples were also seen with the two other Aβ-degrading enzymes, IDE and neprilysin (Yasojima *et al*. 2001; Cook *et al*. 2003), and work is now in progress to find ways to selectively activate IDE towards Aβ, relative to insulin and its other substrates (Song *et al*. 2003; Song and Hersh 2005).

To directly confirm the activity of APH on Aβ, recombinant APH was purified from transfected COS cells and incubated with iodinated Aβ. APH degraded Aβ and this degradation was inhibited by Ac-leucine-CK, a specific APH inhibitor as shown in Fig. 8. Work is now in progress to establish the APH-generated cleavage site(s) in Aβ by mass spectrometry as well as to determine the effect of APH overexpression on Aβ levels in tissue culture cells. Future experiments *in vivo* in transgenic mice will determine the effect of APH on Aβ levels and plaque load in brains of amyloid precursor protein × APH doubly transgenic mice.

In summary, we have identified a novel Aβ-degrading serine peptidase that is present in human brain but its expression level is lower in AD brains. More work is needed in order to determine whether Aβ is a more specific substrate of APH than of IDE or neprilysin. If this is the case, its activation may cause less side-effects than the activation of IDE or neprilysin, that are known to also degrade insulin and neuropeptides, respectively.

Finally, because APH is found both intracellularly and is secreted, its activation could be beneficial for reducing both the intracellular and extracellular Aβ, and for the prevention, delayed progression, and treatment of AD.

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