Biochemistry

 Volume 41, Number 4 January 29, 2002

Articles

Differential Effects of Proteases Involved in Intracellular Degradation of Amyloid β-Protein between Detergent-Soluble and -Insoluble Pools in CHO-695 Cells†

Shinji Sudoh,[‡,⊥] Matthew P. Frosch,[§,⊥] and Bryan A. Wolf*[,‡,#]

Department of Pathology and Laboratory Medicine, University of Pennsylvania, School of Medicine, Philadelphia, Pennsylvania 19104-6082, and Department of Pathology, Harvard Medical School and Brigham and Women's Hospital, Boston, Massachusetts 02115

Received June 8, 2001; Revised Manuscript Received November 1, 2001

ABSTRACT: The deposition of amyloid β-protein (Aβ or βA4) is a key feature of Alzheimer's disease. Most studies have focused on the generation of Aβ, but little is known about the degradation of Aβ. Recent reports suggest that insulin-degrading enzyme (IDE) and neutral endopeptidase (NEP) are involved in the extracellular degradation of Aβ. To date, however, far less is known about the degradation of intracellular Aβ. To elucidate the protease(s) responsible for the degradation of intracellular Aβ, we investigated the effect of various protease inhibitors on Aβ in two distinct intracellular pools (i.e., nonionic detergent-soluble and detergent-insoluble pools) in Chinese hamster ovary cells. Treatment with thiol and metal inhibitors resulted in the accumulation of intracellular Aβ and oligomers in detergent-soluble and -insoluble fractions. The overexpression of thiol-metalloprotease IDE resulted in a marked reduction in levels of detergent-soluble intracellular Aβ as well as extracellular Aβ40 and Aβ42. Moreover, intracellular Aβ in the detergent-insoluble fraction extracted with 70% formic acid or 6 M guanidine hydrochloride decreased markedly in the cells overexpressing IDE. In contrast, expression of NEP degraded the Aβ in the detergent-insoluble fraction markedly and partially degraded extracellular Aβ40 and Aβ42, but not intracellular soluble Aβ. Thiorphan, an inhibitor of NEP, accumulated, albeit to a lesser extent, in insoluble Aβ but not in soluble Aβ. Thus, IDE appears to degrade intracellular Aβ more effectively than does NEP in both the detergent-soluble and -insoluble fractions.

Alzheimer's disease (AD)[1] is a progressive neurodegenerative disorder characterized by senile plaques and neurofibrillary tangles. The major pathological component of senile plaque is amyloid β-protein (Aβ), which is usually a 40- or 42-amino acid peptide. Aβ is generated by proteolytic cleavage by two enzymes (β- and γ- secretase cleavage) from a larger type I integral transmembrane glycoprotein termed β-amyloid precursor protein (APP). The precise role of senile

† This work was supported by National Institutes of Health Grant AG09215 (B.A.W.).

* Address correspondence to Dr. Bryan A. Wolf, Department of Pathology and Laboratory Medicine, The Children's Hospital of Philadelphia, 5135 Main Building, 34th Street and Civic Center Blvd, Philadelphia, PA 19104, USA, Phone: 1-215-590-4446, Fax: 1-215-590-1021, E-mail: wolfb@mail.med.upenn.edu.

‡ University of Pennsylvania, School of Medicine.

⊥ Present Address: Department of Neurology, Center for Aging, Genetics and Neurodegeneration, Massachusetts General Hospital and Harvard Medical School, 114 Building, 16th Street, Charlestown, MA 02129, USA.

§ Harvard Medical School and Brigham and Women's Hospital.

Present Address: Department of Pathology and Lab Medicine, The Children's Hospital of Philadelphia, 5135 Main Building, 34th Street and Civic Center Blvd, Philadelphia, PA 19104, USA.

[1] Abbreviations: AD, Alzheimer's disease; Aβ, amyloid β-protein; IDE, insulin-degrading enzyme; APP, amyloid precursor protein; NEP, neutral endopeptidase; CHO, Chinese hamster ovary; NP-40, Nonidet P 40;

10.1021/bi011193l CCC: $22.00
Published on Web 01/03/2002

plaques in the pathogenesis of AD is unknown. Although it is widely accepted that Aβ plays a major role in the pathogenesis of AD, the relevance of Aβ to the degeneration of neurons in the brain remains unclear. Until recently, it was generally believed that extracellular accumulation of Aβ following secretion of soluble Aβ into the extracellular space was responsible for the AD pathology. However, recent reports identified intracellular Aβ-positive neurons in the brains of patients with sporadic AD (*1*) and more apoptotic neurons with intracellular Aβ deposits without extracellular amyloid plaque formation in mutant presenilin 1-transgenic mice (*2*). Thus, intracellular accumulation of Aβ may also be relevant to the pathogenesis of AD.

The exact mechanism by which Aβ is generated intracellularly before secretion has not been firmly established because of the difficulty involved in detecting very low levels of intracellular Aβ. Intracellular Aβ was recently shown to be located in two major pools defined by detergent solubility (*3*−*5*). One is a detergent-soluble pool in which Aβ is secreted from the cell into the extracellular space as a soluble Aβ during normal cellular metabolism. Several studies have reported that the Aβ in this pool is probably generated in the endocytotic pathway for Aβ40 (*6*), Golgi complex, or trans-Golgi network (TGN) for Aβ40 and Aβ42 (*4*, *7*). A second pool is a detergent-insoluble pool; the presence of such a pool is based on reports of considerable amounts of Aβ in a detergent-insoluble fraction extracted with formic acid in NT2N neuron-like cells or neuroblastoma cells (*3*−*5*). The intracellular accumulation of Aβ in the detergent-insoluble pool was found to increase in an age-dependent manner in NT2N cells (*5*), and Aβ in this pool is not secreted (*4*). In the brain, Aβ is present in the detergent-insoluble, glycolipid-enriched fraction (*8*). The increased accumulation of Aβ in the insoluble pool over time may implicate insoluble Aβ in the amyloidosis in AD, because the slow, progressive accumulation of Aβ over time in senile plaques is generally thought to be related to the age-dependency of AD.

Much attention has focused on the generation of Aβ; far less is known about its degradation. Several groups have addressed this topic. The thiol-metalloendopeptidase, insulin-degrading enzyme (IDE) (insulin protease, EC 3.4.22.11), purified from the cytosolic fraction of rat brain was capable of degrading synthetic Aβ (*9*), and synthetic Aβ peptide cross-linked to IDE in vitro (*10*). Extracellular secreted Aβ has also been shown to decrease in IDE-transfected cell lines (*11*). IDE is known to degrade several polypeptide hormones in vitro, including insulin, atrial natriuretic peptide (ANP), transforming growth factor α, and glucagon, suggesting a possible role of IDE in the regulation of small peptides (*12*). So far, IDE has been found primarily in the cytosol and in smaller amounts in plasma membrane and endosome (*13*). In contrast, Yamin et al. proposed that some serine proteases, but not IDE, are implicated in extracellular regulation of Aβ (*14*). Furthermore, Iwata et al. showed that infusion of rat brain with the selective inhibitors of neutral endopeptidase (EC 3.4.24.11) resulted in extracellular deposition of Aβ (*15*). In contrast to the reports regarding extracellular degradation of Aβ, there are few reports on the intracellular degradation of Aβ in living cells.

Identification of proteases responsible for the processing of peptides frequently has involved the use of exogenous synthetic peptides. However, the protease that cleaves synthetic peptide is sometimes not capable of cleaving natural peptides in normal cellular metabolism. This discrepancy is perhaps due to a lack of identity of conformation between the natural peptide in vivo and the synthetic peptide in vitro. Therefore, experiments assessing the degradation of endogenous peptides in living cells are necessary to confirm that the protease is actually involved in intracellular processing. To date, however, little is known about the degradation of naturally generated intracellular Aβ because of difficulty in its detection. In this study, we attempted to clarify the molecular mechanisms of intracellular Aβ degradation in normal cellular metabolism by investigating the effect of various inhibitors and overexpression of proteases on endogenous intracellular Aβ degradation in both detergent-soluble and detergent-insoluble fractions. For the detection of intracellular Aβ, we used our well-established highly sensitive immunoblotting technique (*7*, *16*) to distinguish very low level intracellular Aβ from other immuno-*cross*-reactive molecules such as abundant cell-associated APP, C99 (the β-secretase-cleaved C-terminal of APP), and other intermediate degradation products of APP that contain the common sequence of Aβ. Here, we report the results of inhibition and overexpression of IDE or NEP on intracellular Aβ in both detergent-soluble and -insoluble fractions.

EXPERIMENTAL PROCEDURES

Materials. Ham's F-12 medium and fetal bovine serum were obtained from Life Technologies (Grand Island, NY). Monoclonal antibody 9B12 (*17*) raised against human IDE was generously provided by Dr. Richard Roth (Stanford University). monoclonal antibody 56C6 raised against neprisylin (CD10) was obtained from Novocastra Laboratories (U.K.). The goat polyclonal antibody Karen raised against the ectodomain of APP (*18*) was generously provided by Dr. Virginia Lee (University of Pennsylvania). Monoclonal antibody 6E10 specific for human Aβ1−17 (*19*) was purchased from Senetek-PLC (Napa, CA). Anti-Aβ40 and Aβ42 antibodies were from Biosource International. All chemicals for the inhibitor experiments were purchased from Sigma Chemical (St Louis, MO).

Cell Culture. CHO cells stably transfected with human wild-type APP695 (CHO-695 cells) were kindly provided by Dr. Virginia Lee. CHO-695 cells were maintained in Ham's F-12 medium containing 10% fetal bovine serum, 1% L-glutamine, 250 μg/mL G418, 100 units/mL penicillin, and 100 mg/mL streptomycin.

Expression Vectors. The mammalian expression vectors encoding wild-type IDE pCMVhIDE or inactive-mutant IDE pH108L (*20*) were kindly provided by Dr. Wen-Liang Kuo (University of Chicago). The cDNA encoding rat neprilysin (*21*) was generated by reverse transcription polymerase chain reaction (RT-PCR) from rat brain total RNA (Clontech Laboratories, Palo Alto, CA) using SuperScript II (Life Technologies) and Pfx DNA polymerase (Life Technologies) with primers anchored with *Bam* HI (5′ end) and *Xba* I (3′ end) according to the manufacturer's protocol (forward primer: 5′-CGCGGATCCGCGGCTGAGCGGCTGAGGGAGGGATTT-3′; reverse primer: 5′-TGCTCTAGAGCATGTTTCTGTGGCTTTGGCGAGTCCT-3′). The amplified cDNA was purified and subcloned into pcDNA3.1 (Invitrogen, San Diego, CA) at *Bam* HI/*Xba* I sites. The resulting

plasmid was designated pcDNA3-NEP. The nucleic acid sequence of the construct was verified by DNA sequencing.

Pharmacological Treatment. Cells plated on 100-mm culture dishes were preincubated with the indicated concentration of protease inhibitor. After preincubation, the cells were washed twice with phosphate-buffered saline, and then further incubated under an atmosphere of 95% O_2/5% CO_2 at 37 °C for the appropriate time (up to 3 h) with the indicated protease inhibitor. Each chemical was used at the lowest concentration and the shortest treatment time needed to have an effect, as determined by the preliminary experiments, including the monitoring of cell viability with live/dead reduced biohazard viability/cytotoxity assay kit (Molecular Probes Inc.).

Transfection and Preparation of Samples. CHO-695 cells were grown in 100-mm culture dishes to 50% confluence, and then 4 μg of DNA of $pCMV_0$ or pcDNA3.1 (for control), pCMVhIDE (wild-type IDE), pH108L (mutant-type IDE), or pcDNA-NEP were transiently transfected with LipofectAMINE Plus reagent (Life Technologies) according to the manufacturer's instructions. At 24 h after transfection, the medium was changed to fresh medium, and the culture was incubated for an additional 24 h. At 48 h after transfection, the medium was collected, and the cells were washed twice with PBS and then harvested following the addition of 1 mL of STE buffer [150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 2 mM EDTA] and a protease inhibitor cocktail (Complete Mini, Roche Molecular Biochemicals, Indianapolis, IN). The medium was centrifuged at 10000*g* for 15 min at 4 °C to pellet the cell debris. The cells were then collected by centrifugation at 200*g* for 1 min and resuspended in five volumes of STE buffer containing a protease inhibitor cocktail and homogenized with a Dounce homogenizer. Proteins were solubilized by incubation with a nonionic detergent (1% Nonidet P-40 or Triton X-100) at 4 °C for 30 min. The resulting cell lysates were centrifuged at 105000*g* in a TLX ultracentrifuge (Beckman, Palo Alto, CA) for 60 min to obtain a clear supernatant. The remaining pellet was resuspended with 70% formic acid or 6 M guanidine hydrochloride and then sonicated to disperse the pellet. The formic acid-extracted samples were neutralized with Tris base. The homogenates were centrifuged at 105000*g* for 20 min to remove the pellet. For analysis of intracellular Aβ, the resultant supernatants were diluted 10-fold with RIPA buffer [150 mM NaCl, 10 mM Tris-HCl (pH 7.5), 1% Nonidet P-40, 0.1% SDS, and 0.2% sodium deoxycholate] and subjected to immunoprecipitation with 6E10 and a protein G Sepharose (Pharmacia Biotech) slurry.

Sandwich-ELISA. Sandwich-ELISA was performed as described previously (*22*) by using BAN50 specific for N-terminal Aβ as a capturing antibody and BA27 specific for Aβ40 or BC05 specific for Aβ42 as a reporter antibody.

Western Blot Analysis. For analysis of cell lysate-associated protein, the concentration of protein in cell lysates was determined with the bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL). Equal amounts of protein were subjected to 4−20% or 7.5% Tris-glycine gels and transferred to nitrocellulose membrane. The blot was incubated with primary antibody (9B12 against IDE, 56C6 against NEP, and Karen against APP) and secondary antibody, ^{125}I-conjugated protein A (NEN Life Science Products), was reacted for quantitative detection by PhosphorImager (Molecular Dynamics, Sunnyvale, CA) or visualized with an enhanced chemiluminescence method. For analysis of Aβ, immunoprecipitates were separated by 10−20% Tris/tricine gel and transferred to nitrocellulose membrane for the detection by highly sensitive immunoblotting as described previously (*7*, *16*). We designated the cell-associated Aβ obtained from this procedure as intracellular Aβ in our study, since trypsin treatment prior to lysis did not decrease the level of this cell-associated Aβ as previously reported (*16*). The intensity of bands corresponding to Aβ detected by immunoblotting was densitometrically qauntified. The statistical significance of the difference was evaluated by Student's two-tailed *t* test using StatView-J4.0 software.

RESULTS

Effects of Various Inhibitors on Intracellular Accumulation of Aβ. Since the protease responsible for intracellular degradation of Aβ has not yet been elucidated, we initially asked what type of protease(s) is primarily responsible for the degradation of intracellular Aβ in normal cellular metabolism. To explore this further, we first examined the effects of various protease inhibitors on levels of intracellular Aβ in CHO-695 cells. The cells were incubated for the appropriate time based on the preliminary experiments as described in Experimental Procedures. The treatment of the sulfhydryl agent *N*-ethylmaleimide (NEM), the metalloprotease 1,10-phenanthroline, and bacitracin each resulted in the accumulation of intracellular Aβ significantly ($p < 0.01$) in 1% NP-40-soluble fraction (Figure 1A). In contrast, the aspartyl protease inhibitor pepstatin A, the cysteine protease inhibitor E-64, the serine protease inhibitor—phenylmethylsulfonyl fluoride (PMSF) and leupeptin—and the metalloprotease inhibitors with a relatively narrow spectrum (potent inhibitors for NEP and NEP-like families)—thiorphan and phosphoramidon—had little or no significant effect on intracellular Aβ levels with various concentrations and various times examined. Accumulating evidence indicates that intracellular Aβ in intact cells can be divided into two different pools on the basis of solubility in the detergent (*3*−*5*). To elucidate the protease(s) responsible for the degradation of intracellular Aβ degradation in the detergent-insoluble pool, we further extracted the resultant pellet with 70% formic acid and assessed the Aβ levels in this pool, since a substantial amount of Aβ was reported to still exist in the detergent-insoluble pool following solubilization with nonionic detergent (*3*−*5*). As shown in Figure 1B, 1,10-phenanthroline and NEM resulted in accumulation of the Aβ significantly ($p < 0.01$) in the detergent-insoluble pool. These inhibitory profiles strongly suggest that thiol- and metal-dependent activities are implicated in the degradation of intracellular Aβ in both the detergent-soluble and -insoluble fractions. As for Aβ42, 1,10-phenanthroline and NEM, among the protease inhibitors examined, enhanced the levels of intracellular Aβ42, as assessed by the semiquantitative visual inspection of bands, due to the very low levels of intracellular Aβ42 (data not shown).

Effect of Overexpression of IDE on APP and Extracellular Aβ. The inhibitor profile of the accumulation of intracellular Aβ (Figure 1) was characteristic of the thiol-metalloprotease insulin-degrading enzyme (IDE) (*23*, *24*), raising the possibility that IDE is responsible for most intracellular Aβ degradation, although we cannot completely exclude the

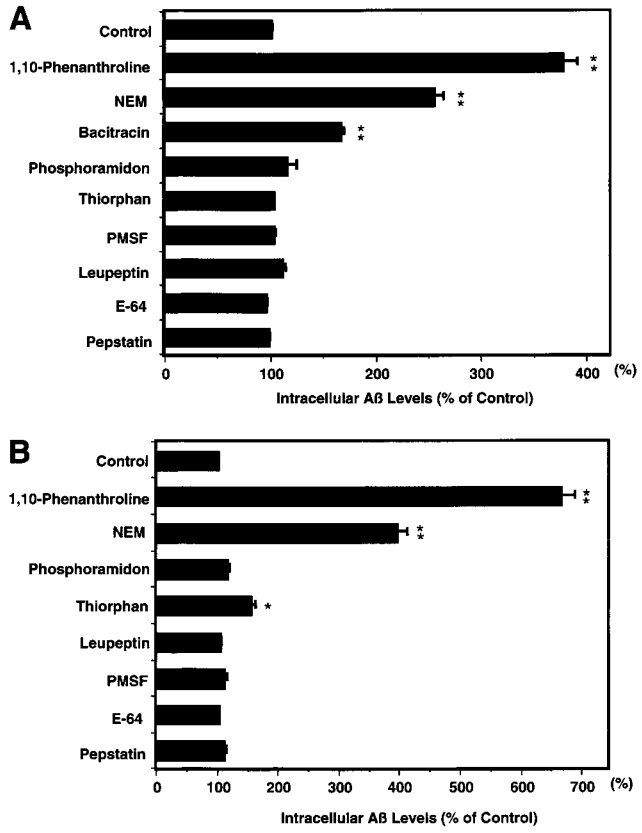

FIGURE 1: Effect of various inhibitors on intracellular Aβ levels in CHO-695 cells. CHO-695 cells plated on 100-mm culture dishes were incubated at 37 °C for up to 3 h with indicated reagents. (1) control (DMSO alone, final concentration was 0.5%), (2) 500 μM 1,10-phenanthroline, (3) 1 mM *N*-ethylmaleimide (NEM), (4) bacitracin 1 mg/mL, (5) 50 μM phosphoramidon, (6) 50 μM thiorphan, (7) 1 mM phenylmethylsulfonyl fluoride (PMSF), (8) 100 μM leupeptin, (9) 100 μM E-64, and (10) 50 μM pepstatin A. Note that final DMSO concentrations in medium were less than 0.5% in all experiments and only DMSO alone of these concentrations did not affect levels of intracellular Aβ. After incubation, the cells were washed twice, harvested with STE buffer containing protease inhibitor cocktail, and solubilized with 1% NP-40 for soluble fraction (A). Resultant pellets after ultracentrifugation were further extracted with 70% formic acid to obtain the insoluble fraction (B). Samples were immunoprecipitated with antibody 6E10. Immunoprecipitates were separated by 10−20% Tris/tricine gel and transferred to nitrocellulose membrane for detection by highly sensitive immunoblotting with antibody 6E10. The intensity of bands corresponding to intracellular Aβ was quantified densitometrically. The relative intensity values were calculated as a percentage of that from the control cells. The results are the mean (± SEM) of triplicate independent experiments. Asterisk marks indicates statistically significant difference from the control (* $P < 0.05$, ** $P < 0.01$).

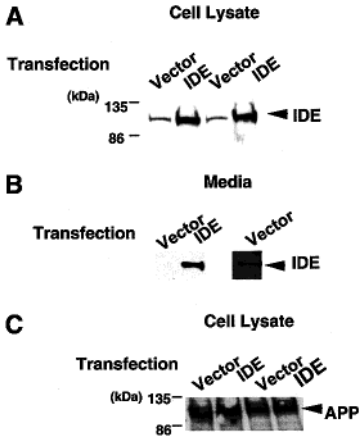

FIGURE 2: Expression of IDE and effect on APP in CHO-695 cells. CHO-695 cells were transfected with $pCMV_0$ (empty vector) or pCMVhIDE. Conditioned media or cell lysates were analyzed by 4−20% tris-glycine SDS−PAGE. IDE or APP was detected by immunoblotting with anti-IDE antibody 9B12 (panels A and B) or anti-APP antibody Karen (panel C). Resultant bands were quantified with PhosphorImager after reaction with ^{125}I-labeled secondary antibody. (A) Expression of IDE in cell lysates from $pCMV_0$ or pCMVhIDE-transfected CHO-695 cells. (B) Expression of IDE in conditioned media. Right column: long exposure of lane 1 of left column. (C) Effect of IDE overexpression on cell-associated APP.

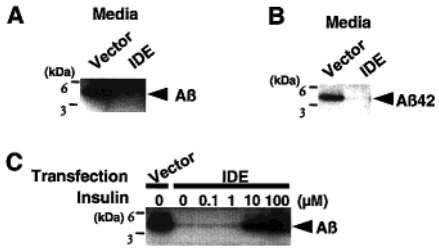

FIGURE 3: The effect of expression of IDE on levels of secreted Aβ. Cells were cultured for 24 h after transfection with $pCMV_0$ (for control) or pCMVhIDE and further incubated with fresh media for an additional 24 h and then collected. Conditioned media were collected and immunoprecipitated with 6E10 (A) or anti-Aβ42 specific antibody (B). Immunoprecipitates were separated by 10−20% Tris/tricine gel and transferred to nitrocellulose membrane for detection by immunoblotting with antibody 6E10. (C) The effect of insulin on IDE-mediated degradation. Various concentrations of exogenous insulin were added to culture media and incubated for 16 h. Conditioned media were collected and immunoprecipitated with antibody 6E10. Immunoprecipitates were separated by 10−20% Tris/tricine gel and transferred to nitrocellulose membrane for detection by immunoblotting with antibody 6E10. Data are representative of two independent experiments.

possibility that another known or unknown thiol-metalloprotease could perform this function. To obtain direct evidence that IDE is capable of degrading Aβ intracellularly, we investigated the effect of overexpression of IDE on Aβ levels. We first verified that the transfection of pCMVhIDE encoding wild-type human IDE increased the amount of 110-kDa IDE by approximately 4-fold, as compared with endogenous IDE in empty vector-transfected CHO-695 cells using the monoclonal antibody 9B12, which recognizes both the endogenous hamster IDE and the transfected human IDE (*25*) (Figure 2A). In contrast, the level of cell-associated APP was not significantly changed by overexpression of IDE (Figure 2C). We also ensured that C99 (the β-secretase-cleaved C-terminal of APP) was not affected by overexpression of IDE (data not shown).

The overexpression of IDE dramatically decreased levels of extracellular Aβ in conditioned media from CHO-695 cells (Figure 3A). Secreted Aβ42 also was degraded in IDE-transfected cells (Figure 3B). Similar results were obtained by sandwich-ELISA (Aβ40 and Aβ42 levels were decreased to 0.25 ± 0.15% and 0.14 ± 0.04% of control [$n = 2$], respectively). This reduction in secreted Aβ is in good agreement with the report by Vekrellis et al. (*11*), who used IDE-transfected CHO cells overexpressing V717F mutant APP751 (7PA2) cells. To see whether insulin competes with IDE in the reduction of extracellular Aβ, we next examined the dose response of the effect of insulin on IDE-mediated degradation of Aβ. Addition of 10 μM insulin almost completely reversed the IDE-mediated reduction of Aβ in

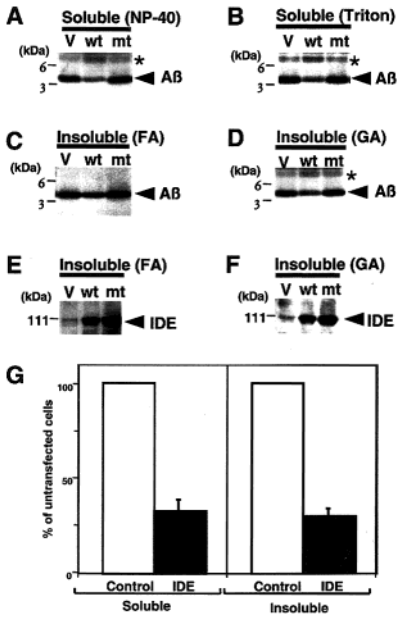

FIGURE 4: The effect of IDE expression on intracellular Aβ. CHO-695 cells were transfected with $pCMV_0$ (lane 1; control), pCMVhIDE (lane 2; wild-type human IDE), or pH108L (lane 3; an inactive human mutant IDE). Cells were solubilized with 1% NP-40 (A) or 1% Triton X-100 for soluble fraction (B). Resultant pellets after ultracentrifugation were extracted further with 70% formic acid (FA) (C) or with 6 M guanidine hydrochloride (GA) in Tris-HCl (D) and then immunoprecipitated with antibody 6E10. Immunoprecipitates were separated by 10−20% Tris/tricine gel and transferred to nitrocellulose membrane for detection by immunoblotting with antibody 6E10. (E, F) Cells were solubilized with 1% NP-40, and the resultant pellet was further extracted with 70% formic acid (E) or 6M guanidine hydrochloride (GA) (F). Equal amounts of protein were loaded onto 4−20% Tris/glycine gels and transferred to nitrocellulose membranes for detection by immunoblotting with anti-IDE antibody 9B20. The asterisk indicates Aβ dimer. Data are representative of three to eight independent experiments. (G) The intensity of bands corresponding to intracellular Aβ shown in panels A and C was quantified densitometrically. The relative intensity values were calculated as a percentage of Aβ relative to untransfected cells. Data are means ± SEM.

conditioned media from IDE-transfected CHO-695 cells (Figure 3C).

Effect of Overexpression of IDE on Intracellular Aβ in Detergent-Soluble and Detergent-Insoluble Fractions. Because the majority of insulin has been reported to be degraded by IDE in cytoplasm, we next investigated whether IDE is involved in the degradation of intracellular Aβ as well as secreted Aβ. Levels of intracellular Aβ in the NP40- or Triton X-100-soluble fraction were greatly reduced in wild-type IDE-transfected cells (Figure 4A,B). In contrast, there was no difference between levels of soluble intracellular Aβ in the empty vector-transfected cells and those in the cells transfected with the inactive form of mutant IDE (H108L), which eliminates Zn^{2+} binding (*20*). In contrast to the reduction of monomer Aβ, the ∼8 kDa band corresponding to Aβ dimers increased reproducibly (in eight independent experiments) in wild-type IDE-transfected cells as compared with levels in untransfected or mutant IDE-transfected cells.

While IDE has generally been reported to localize to the cytoplasm, our inhibitor study (Figure 1B) indicated that thiol and metal activity might be involved in the degradation in the detergent-insoluble fraction as well as in the detergent-soluble fraction, raising the possibility that IDE degrades Aβ in the detergent-insoluble fraction. To test this, we further examined the effect of IDE expression on Aβ in the detergent-insoluble pool using extraction with formic acid. A considerable amount of Aβ (∼40% of intracellular total Aβ) was recovered from the cell lysates extracted with 70% formic acid in CHO-695 cells in our assay system (Figure 4C, lane 1). Levels of intracellular Aβ in the detergent-insoluble pool that was extracted with formic acid were dramatically decreased in wild-type IDE-transfected cells compared with that from control and inactive mutated IDE (H108L)-transfected cells. For confirmation that IDE is capable of degrading detergent-insoluble Aβ, we attempted to extract Aβ with 6 M guanidine hydrochloride to achieve complete denaturation, a method that has also been used for extracting Aβ from brain (*26*) and neuroblastoma cells (*3*). We also demonstrated that the Aβ extracted with guanidine hydrochloride was significantly decreased in wild-type IDE-transfected cells (Figure 4D). The endogenous IDE in CHO-695 cells was observed in the nonionic detergent-insoluble fraction (Figure 4E, lane 1). The increase in IDE levels was similar (approximately 4-fold by densitometric analysis) to that in the detergent-soluble fraction after transfection with human IDE.

Effect of Zinc Inhibitor on Intracellular Aβ. IDE is a metalloendoprotease and requires Zn^{2+} to degrade insulin, and the Zn^{2+} chelator 1,10-phenanthroline inhibits the intracellular degradation of insulin in intact cells almost completely (*23*, *27*). To investigate whether IDE-mediated degradation of Aβ is reversed by the inhibition of IDE with 1,10-phenanthroline, we compared Aβ levels in IDE-transfected cells with or without 1,10-phenanthroline. Treatment with 1,10-phenanthroline completely reversed the IDE-induced degradation of intracellular Aβ in both the detergent-soluble and -insoluble fractions (Figure 5A) as well as in medium (Figure 5B). Yamin et al. (*14*) reported that some serine protease is involved in a major extracellular Aβ degradation pathway and that metalloproteases in the neuroblastoma cell line, including IDE, are less important contributors to the degradation of extracellular Aβ; they did not report on intracellular Aβ. In conditioned medium, treatment with 1,10-phenanthroline had only a slight low effect on levels of extracellular Aβ (Figure 5A, lane 2), a result consistent with those of Yamin et al. using synthetic Aβ in medium, although the 6-kDa band was increased in the cells treated with 1,10-phenanthroline. We found, however, that when the cells were incubated with 1,10-phenanthroline intracellular 4-kDa monomer Aβ, in contrast to the extracellular Aβ, gradually accumulated, starting as early as 15 min, in a time-dependent manner (Figure 5C). The time-dependent accumulation of intracellular Aβ also was observed in the NP-40-insoluble formic acid-extracted fraction. Densitometric quantitation of the band intensity of Aβ indicated that treatment with 1,10-phenanthroline increased intracellular the level of insoluble Aβ by ∼665%, intracellular soluble Aβ by ∼391%, and extracellular Aβ by ∼114%. In addition, an ∼6-kDa anomalous Aβ and an ∼8-kDa band corresponding to dimer Aβ markedly increased with time. The exact character of ∼6-kDa Aβ is unknown, although the major species has been found to be an Aβ peptide beginning at first Arg (*28*), and it is also recognized with antibody anti-Aβ40 specific for ending of Aβ40. Thus, it seems an anomalously comigrated Aβ1−40 in SDS−PAGE,

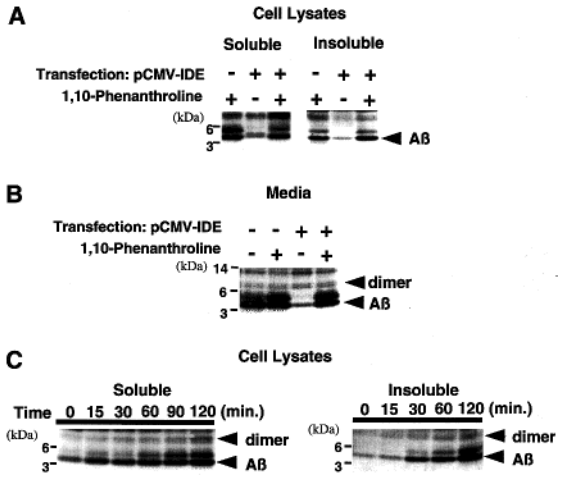

FIGURE 5: Effect of 1,10-phenanthroline on extracellular or intracellular Aβ degradation in IDE-transfected or untransfected cells. IDE- or empty vector-transfected cells were plated in 100-mm culture dishes and preincubated with or without 0.5 mM 1,-10-phenanthroline. After preincubation, the cells were washed twice and further incubated with fresh medium for up to 2 h. (A) For detection of intracellular Aβ, cells were solubilized with 1% NP-40 for the soluble fraction. The resultant pellet after ultracentrifugation was further extractedwith 70% formic acid for the insoluble fraction. (B) Aβ secreted during 2 h was collected. (C) Time-dependent accumulation of intracellular Aβ after treatment with 1,10-phenantholine. Each sample was immunoprecipitated with antibody 6E10, and then immunoprecipitates were separated by 10−20% Tris/tricine gel and transferred to nitrocellulose membrane for detection by immunoblotting with antibody. Data are representative of two or three independent experiments.

although we cannot rule out the possibility that it may undergo conformational transition or association with other molecules [Note that small, but significant, peaks were detected around molecular weight of 5.5 kDa (5590, 5842, 5943) in addition to the major peak, corresponding to Aβ1−40 in the combination of immunoprecipitation with antibody 6E10 and matrix-assisted laser desorption/ionization (MALDI) mass spectrometirc analysis (A. Khatri, S. Sudoh, and D. King, unpublished work)].

Effect of Neutral Endopeptidase on Extracellular and Intracellular Aβ. Recently, several groups reported that purified neutral endopeptidase (NEP, neprilysin) hydrolyzes synthetic Aβ1−40 on at least five cleaved sites in vitro (*29*) and that infusion of the inhibitor for NEP has been found to cause accumulation of Aβ42 in the extracellular space in rat brain homogenate (*15*). There is no direct evidence, however, that NEP actually acts on Aβ in normal cellular metabolism, and the location (i.e., extracellular or intracellular) of this degradation event has not been elucidated. This discrimination has not been possible because these brain homogenates contain whole components of both secreted Aβ and intracellular Aβ. Therefore, to address this issue, we transfected the expression vector encoding rat wild-type NEP cDNA sequence into CHO-695 cells. As expected, an ~97-kDa NEP protein was observed in the cell lysate from NEP-transfected cells probed with anti-NEP/CD10 antibody 56C6 (Figure 6A). The expression of NEP resulted in a partial decrease in extracellular Aβ40 (73 ± 4%, $n = 3$, by densitometiric analysis) and Aβ42 (62 ± 3%, $n = 3$) in conditioned medium (Figure 6B). As previously described (*29*), cell-associated APP and C99 was not affected in our experiments (data not shown). NEP had little effect on the

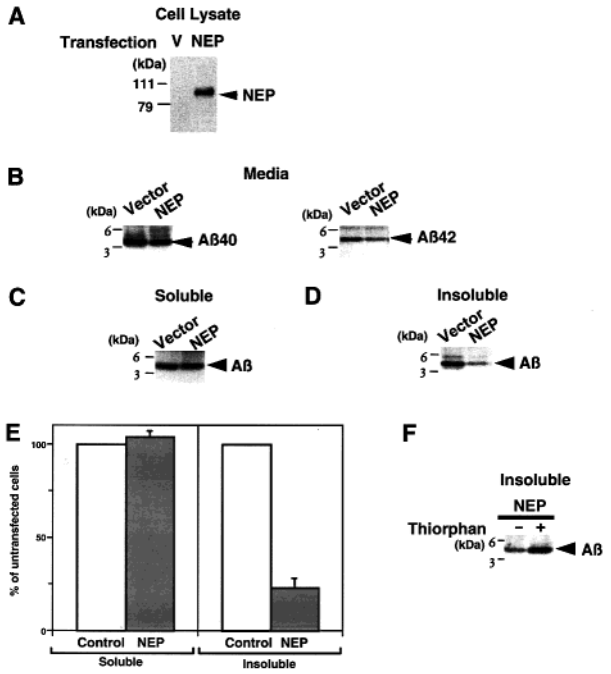

FIGURE 6: Effect of the expression of neutral endopeptidase (NEP) on extra- and intracellular Aβ. pcDNA3.1 encoding rat neprilysin (pcDNA3-NEP) or empty vector (pcDNA3.1) was transfected into CHO-695 cells. (A) Cell lysates were analyzed by 4−20% SDS−PAGE and probed with anti-CD10/NEP 56C6 antibody. (B) Conditioned media from CHO-695 cells overexpressing NEP were immunoprecipitated with antibody anti-Aβ40 or Aβ42 and then detected by highly sensitive immunobloting with antibody 6E10. (C) Cell lysates were extracted with 1% NP-40, immunoprecipitated with antibody 6E10, and detected with 6E10. (D) The resultant pellet after ultracentrifugation was extracted further with 70% formic acid for insoluble fraction. Samples were neutralized and subjected to immunoprecipitation with antibody 6E10 and detected by highly sensitive immunoblotting with antibody 6E10. (E) The intensity of bands corresponding to intracellular Aβ shown in panels C and D was quantified densitometrically. The relative intensity values were calculated as a percentage of Aβ relative to untransfected cells. Data are means ± SEM. (F) Effect of thiorphan on NEP-mediated decrease of Aβ. NEP-transfected cells were treated with or without 50 μM thiorphan for 3 h. The NP-40-insoluble fraction was immunoprecipitated with antibody 6E10 and detected by immunoblotting with 6E10. Data are representative of three independent experiments.

intracellular degradation of Aβ in the detergent-soluble fraction (Figure 6C, lane 2), while IDE clearly degraded Aβ in the same fraction (Figure 6C, lane 3). In contrast, NEP markedly degraded the Aβ in the detergent-insoluble fraction extracted with formic acid as well as the Aβ in IDE-transfected cells (Figure 6D). These differential effects of NEP and IDE on soluble and insoluble Aβ were clearly shown by densitometric quantitiation of the band of intracellular Aβ (Figure 4G and Figure 6E). Treatment with thiorphan, the inhibitor of NEP, reversed NEP-mediated reduction of intracellular Aβ in the detergent-insoluble fraction (Figure 6F), confirming that the decreased Aβ was modulated by NEP expression.

DISCUSSION

To date, there is no evidence of increased generation of Aβ in patients with sporadic AD (*30*), except in a small subset of patients with familial early-onset AD. Thus, attenuation of the function of the Aβ-degradation system

rather than upregulation of the Aβ-generation system could be related to the onset of sporadic cases of AD with aging. Several groups recently demonstrated the involvement of some peptidases in extracellular proteolysis of Aβ (*11*, *14*, *15*), but the mechanisms of intracellular degradation of Aβ remain to be elucidated. We do know, however, that some of the Aβ generated intracellularly remains in the intracellular pool and accumulates with time in the detergent-insoluble pool (*5*), suggesting the involvement of intracellular Aβ in the pathogenesis of AD. In this paper, we have focused on the intracellular degradation of endogenous Aβ by using CHO-695 cells.

Among the protease inhibitors examined, the sulfhydryl agent NEM, the metalloprotease inhibitor 1,10-phenanthroline, and bacitracin resulted in accumulation of intracellular Aβ in the detergent-soluble fraction. Other protein inhibitors (e.g., cysteine, serine, and aspartate proteases) had little or no effect on intracellular Aβ levels. We thus pursued the hypothesis that thiol-metalloprotease IDE is responsible for the degradation of intracellular Aβ, because these inhibitors have been shown to inhibit the acitivity of IDE (*23*, *24*, *27*, *31*). We clearly demonstrated that the expression of IDE markedly reduced the level of intracellular Aβ in the detergent-soluble fraction as well as the extracellular degradation of Aβ. This reduction of intracellular Aβ is not likely to be due to the altered processing of APP or degradation of APP modulated by IDE, since intracellular levels of full-length APP and C99 were not affected by overexpression of IDE. The finding of the preliminary experiment with trypsin treatment as described in Experimental Procedures indicated that this degradation of Aβ in this experiment reflects mainly the intracellular event by IDE. The complete reversal of the effect of IDE on intracellular Aβ with the IDE inhibitor 1,10-phenanthroline further confirmed these results. IDE is a unique thiol-metalloprotease, with no significant identity to any known mammalian protease (*24*). IDE is localized primarily in the soluble (100000*g* supernatant) cytosolic fraction (*13*). Indeed, once insulin is internalized and translocated to cytoplasm, the majority of insulin is degraded in the cytosol (*32*). Hence, IDE is probably capable of degrading Aβ as well as insulin in the nonionic detergent-soluble fraction.

Substantial amounts of intracellular Aβ also are present in the detergent-insoluble pool (*3*−*5*) both in the cells and in the brain (*8*). Some insoluble Aβ remained within the cells and accumulated with time (*5*). Therefore, to determine the mechanisms of Aβ accumulation related to AD pathogenesis, we investigated the mechanism of degradation of insoluble Aβ. The inhibitor profile, which indicated the involvement of a protease sensitive to both metal- and thiol-inhibitors in the degradation of insoluble Aβ, led us to hypothesize that the metallo-thiol protease IDE might be involved in the Aβ in this pool as well as that in the soluble fraction. We found that the expression of wild-type IDE remarkably reduced the insoluble Aβ levels extracted with different chemicals (e.g., either 70% formic acid or 6 M guanidine hydrochloride). Although degradation of insulin was found in cytosol, significant insulin-degrading activity was seen in other subcellular fractions including cell membranes (*33*). Endogenous IDE also was detected in the detergent-insoluble fraction after extraction with nonionic detergent. Thus, the IDE is likely to include the degradation of Aβ in the detergent-insoluble fraction. In this study, we cannot completely exclude the possibility of another known or unknown thiol-metalloprotease involved in the intracellular degradation. The creation of IDE-knock out mice would provide strong evidence that IDE is responsible for the degradation of Aβ.

Several reports have suggested that NEP is involved in the degradation of Aβ. Injection of thiorphan, a selective inhibitor for NEP (also NEP-like protease and to a lesser extent endothelin-converting enzyme) resulted in the accumulation of Aβ42 in the extracellular space of rat brain, suggesting that NEP is responsible for degradation of extracellular Aβ42 in rat brain in vivo (*15*). We present direct evidence that expression of NEP partially degraded secreted Aβ40 and Aβ42 and markedly degraded intracellular Aβ in the detergent-insoluble fraction, but not in the detergent-soluble fraction. NEP is a type II integral membrane glycoprotein consisting of a larger extracellular spanning domain and a shorter cytoplasmic domain; its active site is in the ectodomain (*34*). The cellular localization of NEP at the cell-surface plasma membrane suggests that it is involved regulation by inactivating small peptides on the cell-surface membrane (*35*, *36*). Interestingly, some membrane peptidases, including NEP, have been observed in the detergent-insoluble membrane fraction (*37*, *38*). In the brain, Aβ also is present in the detergent-insoluble glycolipid-enriched membrane fraction (*8*). From these results, taken together with ours, we could assume that NEP degrades primarily intracellular Aβ in the detergent-insoluble pool at the cell membrane.

The inhibitor experiments and overexpression experiments of IDE and NEP suggest that IDE is probably responsible for the degradation of intracellular Aβ in the detergent-soluble pool. In contrast, both IDE and NEP degraded Aβ in the detergent-insoluble fraction. We currently do not know the derivation of insoluble Aβ. Most APP and C99 are found in the detergent-soluble fraction, whereas minute amounts of APP and C99 were detected in the insoluble fraction, suggesting that most of Aβ is generated in the soluble fraction (*8*). Some of the Aβ generated in the soluble pool thus might be incorporated into the insoluble pool. If so, degradation of Aβ in the soluble pool at an early stage may be effective in preventing aggregation. In addition, thiorphan, the NEP inhibitor, had a small effect on accumulation of intracelluar Aβ (and Aβ42) in the insoluble fraction. These results, taken together, indicate that IDE plays a crucial role, as compared with NEP, in regulating intracellular degradation of Aβ.

It has not yet been resolved whether neuronal damage is induced by intracellular Aβ or by extracellular accumulation of Aβ after secretion. Studies with transgenic mice have shown little neuronal pathology as that seen in AD, even in the presence of accumulation of extracellular Aβ (*39*). In contrast, in older transgenic mice overexpressing presenilin 1 mutations, apoptotic neurons with intracellular Aβ deposits were observed without extracellular amyloid plaque formation, suggesting that intracellular Aβ accumulation correlates with neuronal death (*2*). One reason the importance of intracellular Aβ is controversial is that fibrillar amyloid has not yet been found intracellularly. However, a recent report suggested that the oligomer Aβ that may result in fibril formation arises first intracellularly, since incubation of extracellular Aβ did not lead to oligomer formation (*40*). In our current experiments using 1,10-phenanthroline, intrac-

ellular Aβ accumulated in a time-dependent manner in both soluble and insoluble fractions, and formation of oligomer Aβ was enhanced. Because of the small volume of cells, the concentration of intracellular Aβ would be much higher than that of extracellular Aβ (*41*). Since higher concentrations of Aβ enhance aggregation (*42*), subtle alterations in the intracellular degradation system might lead to the formation of aggregated Aβ. Although we cannot completely exclude the involvement of another, unknown thiol-metalloprotease in the accumulation of Aβ, our data suggest that attenuation of intracellular degradation might result in the intracellular aggregation of Aβ. The increase in extracellular Aβ by 1,-10-phenanthroline was low as compared with that of intracellular Aβ, suggesting that different protease(s) might be involved in the degradation activity of IDE in extracellular degradation of Aβ as discussed previously (*14*, *15*). Alternatively, another clearance mechanism, e.g., receptor-mediated internalization of Aβ (*43*), might mediate the extracellular level of Aβ. Levels of extracellular insulin of the brain using microdialysis were within nanomolar range (around 10 nM) (*44*) as well as that of cerebrospinal fluid (*45*). In our study, insulin at 10 μM was necessary to compete with an IDE-mediated decrease of extracellular Aβ. This concentration of insulin was somewhat higher than the concentration (1−10 μM) previously reported for results of an in vitro degradation-assay (*23*, *46*). This may due to our different assay system using intact cells, i.e., production (and secretion) of IDE or incorporation of insulin into the cells during the assay period might affect this discrepancy. The different approach may explain this discrepancy. In addition, previous in vivo study showed that injection of insulin (10 mg/mL, which corresponds to 20 μM in final concentration in vivo according to our calculation) into the brain did not inhibit Aβ catabolism (*19*). Thus, physiological insulin may be less relevant to IDE-mediated degradation of Aβ in extracellular space in vivo.

Several groups have reported a novel susceptibility locus for late-onset AD on chromosome 10q, where the IDE gene also is mapped (*47*−*49*). Although IDE has emerged as a potential candidate responsible for extracellular Aβ degradation (*11*), this remains controversial, since some investigators have shown that NEP (*15*) and some serine proteases (*14*) are responsible for extracellular Aβ degradation. However, our present study provides novel evidence of the probable involvement of IDE in the formation of intracellular soluble Aβ and in the degradation of insoluble Aβ. Accumulation of intracellular Aβ is considered harmful to cells. If the deposition of Aβ begins inside cells and if fibrillar Aβ accumulates in the detergent-insoluble fraction during aging, degradation of intracellular Aβ modulated by IDE could be a very crucial step in clearance of Aβ. One possible strategy for the treatment of AD would thus be promotion of the degradation of Aβ. We thus believe that our results showing the involvement of IDE and NEP in the degradation of intracellular Aβ provide new insights on the molecular mechanisms underlying amyloidogenesis.

ACKNOWLEDGMENT

We are grateful to Dr. Richard Roth (Stanford University) and Dr. Wen-Lingo Kuo (University of Chicago) for providing the antibody 9B12 and pCMV-hIDE, pH108L vector, respectively. We thank Dr. Virginia Lee (University of Pennsylvania) and Dr. Daniel Skovronsky (University of Pennsylvania) for constructive input and support on performing a sandwich-ELISA. We also thank Mr. Ashok Khatri (Massachusetts General Hospital) and Dr. David S. King (Howard Hughes Medical Institute, University of California, Berkeley) for performing a MALDI-TOF mass spectrometric analysis.

REFERENCES

1. Mochizuki, A., Tamaoka, A., Shimohata, A., Komatsuzaki, Y., and Shoji, S. (2000) *Lancet 355*, 42−43.
2. Chui, D.-H., Tanahashi, H., Ozawa, K., Ikeda, S., Checler, F., Ueda, O., Suzuki, H., Araki, W., Inoue, H., Shirotani, K., Takahashi, K., Gallyas, F., and Tabira, T. (1999*) Nat. Med. 5*, 560−564.
3. Morishima-Kawashima, M., and Ihara, Y. (1998) *Biochemistry 37*, 15247−15253.
4. Greenfield, J. P., Tsai, J., Gouras, G. K., Hai, B., Thinakaran, G., Checler, F., Sisodia, S. S., Greengard, P., and Xu, H. (1999) *Proc. Natl. Acad. Sci. U.S.A. 96*, 742−747.
5. Skovronsky, D. M., Doms, R. W., and Lee, V. M.-Y. (1998) *J. Cell Biol. 141*, 1031−1039.
6. Koo, E. H., and Squazzo, S. L. (1994) *J. Biol. Chem. 269*, 17386-17389.
7. Sudoh, S., Hua, G., Kawamura, Y., Maruyama, K., Komano, H., and Yanagisawa, K. (2000) *Eur. J. Biochem. 267*, 2036−2045.
8. Lee, S.-J., Liyanage, U., Bickel, P. E., Xia, W., Lansbury, P. T. Jr., and Kosik, K. S. (1998) *Nat. Med. 4*, 730−734.
9. McDermott, J. R., and Gibson, A. M. (1997) *Neurochem. Res. 22*, 49−56.
10. Kurochkin, I. V., and Goto, S. (1994) *FEBS Lett. 345*, 33−37.
11. Vekrellis, K., Ye, Z., Qiu, W. W., Walsch, D., Hartley, D., Chesneau, V., Rosner, M. R., and Selkoe, D. J. (2000) *J. Neurosci. 20*, 1657−1665.
12. Kurochkin, I. V. (1998) *FEBS Lett. 427*, 153−156.
13. Duckworth, W. C., Bennett, R. G., and Hamel, F. G. (1998) *Endocr. Rev. 19*, 608−624.
14. Yamin, R., Malgeri, E. G., Sloane, J. A., MaGraw, W. T., and Abraham, C. R. (1999) *J. Biol. Chem. 274*, 18777−18784.
15. Iwata, N., Tsubuki, S., Takaki, Y., Watanabe, K., Sekiguchi, M., Hosoki, E., Kawashima-Morishima, M., Lee, H.-J., Hama, E., Sekine-Aizawa, Y., and Saido, T. C. (2000) *Nat. Med. 6*, 143−150.
16. Sudoh, S., Kawamura, Y., Sato, S., Wang, R., Komano, H., and Yanagisawa, K. (1998) *J. Neurochem. 71*, 1535−1543.
17. Shii, K., and Roth, R. A. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 4147−4151.
18. Jolly-Tornetta, C., and Wolf, B. A. (2000) *Biochemistry 39*, 15282−15290.
19. Kim, K. S., Wen, G. Y., Banker, C., Chen, C. M. J., Sapienza, V. J., Hong, H., and Wisniewski, H. M. (1990) *Neurosci. Res. Commun. 7*, 113−122.
20. Gehm, B. D., Kuo, W.-L., Perlman, R. K., Rosner, M. R. (1993) *J. Biol. Chem. 268*, 7943−7948.
21. Malfroy, B., Schofield, P. R., Kuang, W.-J., Seeburg, P. H., Mason, A. J., and Henzel, W. (1987) *Biochem. Biophys. Res. Commun. 144*, 59−66.
22. Suzuki, N., Cheung, T. T., Cai, X. D., Odaka, A., Otvos, L. J., Eckman, C., Golde, T. E., and Younkin, S. G. (1994) *Science 264*, 1336−1640.
23. Harada, S., Smith, R. M., Smith, J. A., and Jarett, L. (1993) *Endocrinology 132*, 2293−2298.
24. Authier, F., Posner, B. I., and Bergeron, J. J. M. (1996) *Clin. Invest. Med. 19*, 149−160.
25. Seta, K. A., and Roth, R. A. (1997) *Biochem. Biophys. Res. Commun. 231*, 167−171.
26. Johnson-Wood, K., Lee, M., Motter, R., Hu, G., Gordon, R., Barbour, R., Khan, M., Gordon, H., Tan, D., Games, I., Lieberburg, D., Shenk, D., Seubert, P., and McConlogue, L. (1997) *Proc. Nalt. Acad. Sci. 94*, 1550−1555.

27. Kayalar, C., and Wong, W. T. (1989) *J. Biol. Chem. 264*, 8928−8934.
28. Podlisny, M. B., Ostaszewski, B. L., Squazzo, S. L., Koo, E. H., Rydell, R. E., Teplow, D. B., and Selkoe, D. J. (1995) *J. Biol. Chem. 270*, 9564−9570.
29. Howell, S., Nalbantoglu, J., and Crine, P. (1995) *Peptides 16*, 647−652.
30. Scheuner, D., Eckman, C., Jensen, M., Song, X., Citron, M., Suzuki, N., Bird, T. D., Hardy, J., Hutton, M., Kukull, W., Larson, E., Levy-Lahad, E., Viitanen, M., Peskind, E., Poorkaj, P., Schellenberg, G., Tanzi, R., Wasco, W., Lannfelt, L., Selkoe, D., and Younkin, S. (1996) *Nat. Med. 2*, 864−870.
31. Duckworth, W. C., Runyan, R. K., Write, T. K., Halban, P. A., and Solomon, S. S. (1981) *Endocrinology 108*, 1142−1147.
32. Harada, S., Smith, R. M., and Jarett, L. (1999) *Cell Biochem. Biophys. 31*, 307−319.
33. Duckworth, W. C. (1979) *Endocrinology 104*, 1758−1764.
34. Turner, A. J., and Tanzawa, K. (1997) *FASEB J. 11*, 355−364.
35. Lemay, G., Waksman, G., Roques, B. P., Crine, P., and Boileau, G. (1989) *J. Biol. Chem. 264*, 154620−15623.
36. Turner, A. J., Isaac, R. E., and Coates, D. (2001) *BioEssays 23*, 261−269.
37. Angelisova, P., Drbal, K., Horejsi, V., and Cerny, J. (1999) *Blood 93*, 1437−1439.
38. Riemann, D., Hansen, G. H., Niels-Christiansen, L.-L., Thorsen, E., Immerdal, L., Santos, A. N., Kehlen, A., Langner, J., and Danielsen, E. M. (2001) *Biochem. J. 354*, 47−55.
39. Guenette, S. Y., and Tanzi, R. E. (1999) *Neurobiol. Aging 20*, 201−211.
40. Walsh, D. M., Tseng, B. P., Rydel, R. E., Podlisny, M. B., and Selkoe, D. J. (2000) *Biochemistry 39*, 10831−10839.
41. Hartmann, T. (1999) *Eur. Arch. Psychiatry Clin. Neurosci. 249*, 291−298.
42. Jarrett, J. T., Berger, E. P., and Lansburry, P. T. Jr. (1993) *Biochemistry 32*, 4693−4697.
43. Kang, D. E., Pietrzik, C. U., Baum, L., Chevallier, N., Merriam, D. E., Kounnas, M. Z., Wagner, S. L., Troncoso, J. C., Kawas, C. H., Katzmann, R., and Koo, E. H. (2000*) J. Clin. Invest. 106*, 1159−1166.
44. Gerizussus, K., Rouch, C., Nicolaidis, S., and Orosco, M. (1998) *Physiol. Behav. 65*, 271−275.
45. Craft, S., Peskind, E., Schwartz, M. W., Schellenberg, G. D., Raskind, M., and Porte, D. Jr. (1998) *Neurology 50*, 164−168.
46. Qiu, W. Q., Walsh, D. M., Ye, Z., Vekrellis, K., Zhang, J., Podlisny, M. B., Rosner, M. R., Safavi, A., Hersh, L. B., and Selkoe, D. J. (1998) *J. Biol. Chem. 273*, 32730−32738.
47. Bertram, L., Blacker, D., Mullin, K., Keeney, D., Jones, J., Basu, S., Yhu, S., Mclnnis, M. C., Go, R. C. P., Vekrellis, K., Selkoe, D. J., Saunders, A. J., and Tanzi, T. E. (2000) *Science 290*, 2302−2303.
48. Ertekin-Taner, N., Graff-Radford, N., Younkin, L. H., Eckman, C., Baker, M., Adamson, J., Renald, J., Blangero Ertekin-Taner, N., Graff-Radford, N., Younkin, L. H., Eckman, C., Baker, M., Adamson, J., Ronald, J., Blangero, J., Hutton, M., and Younkin, S. G. (2000) *Science 290*, 2303−2304.
49. Myers, A., Holmans, P., Marshall, H., Kwon, J., Meyer, D., Ramic, D., Shears, S., Booth, J., DeVrieze, F. W., Crook, R., Hamshere, M., Abraham, R., Tunstall, N., Rice, F., Carty, S., Lillystone, S., Kehoe, P., Rudrasingham, V., Jones, L., Lovestone, S., Perez-Tur, J., Williams, J., Owen, M. J., Hardy, J., and Goate, A. M. (2000) *Science 290*, 2304−2305.

BI011193L