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Differential Degradation of Amyloid β Genetic Variants Associated with Hereditary Dementia or Stroke by Insulin-degrading Enzyme*

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Inherited amino acid substitutions at position 21, 22, or 23 of amyloid β (Aβ) lead to presenile dementia or stroke. Insulin-degrading enzyme (IDE) can hydrolyze Aβ wild type, yet whether IDE is capable of degrading Aβ bearing pathogenic substitutions is not known. We studied the degradation of all of the published Aβ genetic variants by recombinant rat IDE (rIDE). Monomeric Aβ wild type, Flemish (A21G), Italian (E22K), and Iowa (D23N) variants were readily degraded by rIDE with a similar efficiency. However, proteolysis of Aβ Dutch (E22Q) and Arctic (E22G) was significantly lower as compared with Aβ wild type and the rest of the mutant peptides. In the case of Aβ Dutch, inefficient proteolysis was related to a high content of β structure as assessed by circular dichroism. All of the Aβ variants were cleaved at Glu^3-Phe^4 and Phe^4-Arg^5 in addition to the previously described major sites within positions 13–15 and 18–21. SDS-stable Aβ dimers were highly resistant to proteolysis by rIDE regardless of the variant, suggesting that IDE recognizes a conformation that is available for interaction only in monomeric Aβ. These results raise the possibility that upregulation of IDE may promote the clearance of soluble Aβ in hereditary forms of Aβ diseases.

The accumulation of amyloid β peptide (Aβ)[1] in the brain is a central process in a number of human neurodegenerative disorders that may be grouped as "amyloid β diseases" (1). In Alzheimer's disease, Aβ is mainly found within senile plaques in the neurophil and vascular lesions, whereas in sporadic and hereditary amyloid angiopathies, Aβ deposits are mainly associated with cortical and leptomeningeal vessels leading to stroke or multi-infarct dementia. To a lesser extent, cerebral Aβ deposits are also present in normal aging (2). Autosomal dominant mutations in the amyloid β precursor protein (Aβ PP) gene result in amino acid substitutions at position 21, 22, or 23 of Aβ sequence. Although these Aβ variants present with a primarily vascular deposition, they translate into different clinical phenotypes. In this regard, Aβ Arctic (E22G) and Aβ Iowa (D23N) are characterized by presenile dementia and Aβ Flemish (A21G) is associated with early onset dementia and cerebral hemorrhage, whereas Aβ Dutch (E22Q) and Aβ Italian (E22K) variants have a predominant vascular phenotype characterized by massive strokes (3–7). The underlying mechanism of aggregation and deposition *in vivo* may be strongly influenced by the type of amino acid substitution as well as the location of mutations in the Aβ peptide. *In vitro* studies have shown that Aβ E22Q and Aβ D23N form typical amyloid fibrils at a higher rate than wild-type Aβ and that Aβ E22G assembles into unique protofibrils that may be toxic to neurons (3, 8–10). In the case of Aβ A21G, overproduction of the peptide may contribute as a pathogenic mechanism (11, 12). Regarding Aβ E22K, deposition seems not to be related with fibril formation rate, and yet this variant may be toxic to human cerebrovascular smooth muscle cells in culture (6, 13). In addition to the intrinsic aggregation properties of Aβ and its genetic variants or the rate of their production, recent studies (14, 15) in animal models have suggested that a defective clearance may influence the progressive accumulation of Aβ in the neurophil and cerebral vessels. Among the mechanisms that remove Aβ peptides from the brain, degradation by several proteases including neprilysin, endothelin-converting enzyme, and insulin degrading enzyme (IDE) is now being considered as an important component of the Aβ clearance process (16, 17, reviewed in Refs. 18 and 19). IDE is a highly conserved thiol metalloprotease with ubiquitous expression including the brain (20–22). Regarding its physiological role, IDE has been implicated in cellular growth and differentiation, modulation of proteasomal activity, and steroid signaling (23, reviewed in Ref. 24). In addition to insulin for which the protease has a K_m in the low nanomolar range, IDE is known to degrade several peptides capable of forming amyloid fibrils *in vitro* and *in vivo* including glucagon, amylin, atrial natriuretic peptide, calcitonin, and Aβ (25, 26, reviewed in Ref. 27). Studies using rat and human brain tissue homogenates, IDE-transfected cell lines, and primary neuronal cultures have supported a role of IDE in Aβ degradation *in vivo* (28–31). Moreover, IDE has been shown to

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[1] The abbreviations used are: Aβ, amyloid β; Aβ PP, amyloid β precursor protein; IDE, insulin-degrading enzyme; rIDE, recombinant insulin-degrading enzyme; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; WT, wild type; GST, glutathione *S*-transferase; CD, circular dichroism; MALDI-TOF MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; HCHWA-D, hereditary cerebral hemorrhage with amyloidosis, Dutch type.

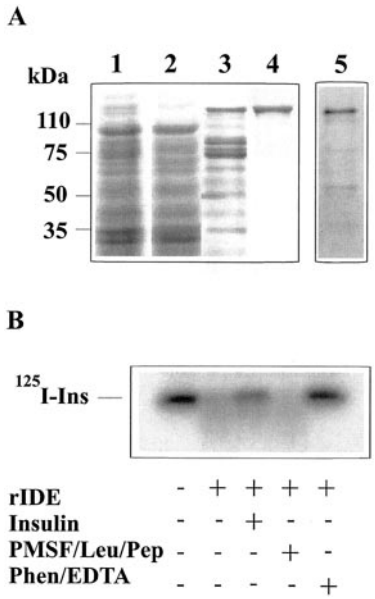

FIG. 1. **Characterization of recombinant rat IDE.** *Panel A*, SDS-PAGE and Coomassie Blue staining (*lanes 1–4*) of proteins obtained during the purification procedure of rIDE. *Lane 1*, total bacterial lysate; *lane 2*, proteins unbound to Ni column; *lane 3*, eluate with 100 mM imidazole; *lane 4*, eluate with 400 mM imidazole; *lane 5*, Western blot of purified rIDE using BC2 polyclonal antiserum. *Left*, molecular mass markers in kilodaltons. *Panel B*, SDS-PAGE and PhosphorImager analysis of [^{125}I]insulin degradation by rIDE in the presence or absence of different inhibitors as indicated. Unlabeled insulin was used at 1 μM. *Leu*, leupeptin; *Pep*, pepstatin; *Phen*, 1,10-phenantroline.

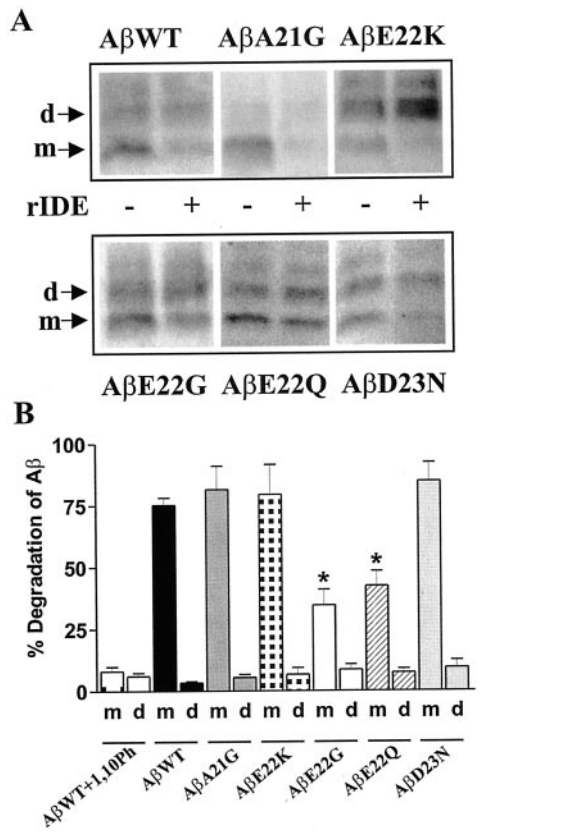

FIG. 2. **Degradation of Aβ variants by rIDE.** *Panel A*, Western blot analysis with monoclonal 6E10 of degradation of Aβ WT and Aβ genetic variants by rIDE. *m*, Aβ monomers; *d*, Aβ dimers. *Panel B,* densitometric quantitation of the immunoreactivity of Aβ as in *panel A*. *m,* monomers; *d*, dimers. *Bars* represent the mean ± S.E. of three independent experiments. *, $p < 0.01$ (Student's *t* test) as compared with Aβ WT.

protect cultured neurons from Aβ toxicity, indicating that the Aβ fragments generated by IDE are not themselves toxic to cells (32). Regarding the state of Aβ oligomerization and IDE specificity, it has been proposed that IDE is able to degrade efficiently monomeric Aβ as opposed to oligomeric Aβ species that are thought to be more toxic to neurons or vascular cells than typical amyloid fibrils (33). However, whether IDE is capable of degrading Aβ bearing pathogenic amino acid substitutions and how these changes may affect specificity is not known. The aim of our work was to study the degradation of Aβ genetic variants associated with human disease by recombinant IDE *in vitro* and to characterize the proteolytic products from each of these Aβ mutant peptides.

EXPERIMENTAL PROCEDURES

Expression and Purification of Recombinant Rat IDE—The plasmid pECE-IDE (kindly provided by Richard Roth, Stanford University), containing the coding region of rat IDE cDNA was used as a template for the PCR amplification of a truncated version of IDE using sense 5′-AGCACAGGATCCATGAATAATCCGGCCAT-3′ (nucleotides 139–155) and antisense 5′-TTCTCGAGGAGTTTTGCCGCCATGA-3′ (nucleotides 3072–3056) primers of the rat IDE cDNA sequence. Sites for the restriction enzymes *Bam*HI and *Xho*I, respectively, were introduced to facilitate cloning. The PCR DNA fragment was digested with *Bam*HI and *Xho*I and cloned into pET-30a(+) (Novagen) previously digested with the same restriction enzymes to generate the pET-IDE construct. Recombinant IDE 42-1019 (rIDE) was expressed in *Escherichia coli* BL21 and purified to homogeneity using a Hi Trap Ni-chelating column (Amersham Biosciences) following the method described by Chesneau and Rosner (34). Purity, as assessed by SDS-PAGE, was >95%. rIDE proteolytic activity was determined using [^{125}I]insulin as described below.

Production of Anti-IDE Polyclonal Antibodies—A region between amino acids 97 and 273 of the rat IDE sequence was amplified by PCR using as template pECE-IDE with the following primers: forward 5′-CTGAGCGGATCCCTGTCAGACCCTCCA-3′ and reverse 5′-CAATGTGAATTCCTTCACCACCAGATT-3′. After digestion with *Bam*HI and *Eco*RI, the 530-bp insert was subcloned into pGex2T vector (Amersham Biosciences). GST-IDE97–273 fusion protein was expressed and purified according to the manufacturer's suggestions. After immunization of New Zealand rabbits with GST-IDE97–273, the antiserum (BC2) was sequentially purified using a BL21/GST lysate coupled to CNBr-activated Sepharose (Amersham Biosciences) and a GST-agarose (Sigma) affinity columns, respectively. Specificity of anti-IDE antiserum BC2 was tested by Western blot against GST, GST-IDE97–273, purified rIDE (see above), and soluble fractions from human and rat brain and liver in which a single 115-kDa band was detected (data not shown).

Synthetic Peptides—Synthetic Aβ-(1–40)-peptides such as Aβ WT, Aβ A21G, Aβ E22Q, Aβ E22K, Aβ E22G, Aβ D23N, and Aβ-(1–40)/Aβ-(1–42) containing the rodent sequence (35) were synthesized by the W. M. Keck Foundation (Yale University, CT). All of the peptides were purified by reverse-phase high pressure liquid chromatography, and their purity was evaluated by amino acid sequence analysis and laser desorption mass spectrometry. Lyophilized aliquots of the peptides were dissolved at a concentration of 70 μM in distilled water as determined with a bicinchoninic acid assay (Pierce) after centrifugation at 10,000 rpm for 15 min to eliminate large aggregates. The supernatant was aliquoted and stored at −80 °C.

SDS-PAGE and Western Blot—Proteins obtained from *E. coli* BL21 lysates and after the different steps of purification were analyzed by 7.5% SDS-PAGE in Tris-Tricine gels. Aβ peptides were run on 12.5% Tris-Tricine SDS-PAGE. For Western blot analysis, proteins were transferred onto nitrocellulose membranes (Hybond ECL, Amersham Biosciences) and incubated with anti-Aβ monoclonal antibody 6E10 (Signet Laboratories) at 1:1000, anti-IDE monoclonal 9B12 (kindly provided by Richard Roth) at 1:1000, and polyclonal antibody BC2 at 1:1000. Immunoreactivity was detected with anti-mouse or anti-rabbit horseradish peroxidase-labeled IgG and ECL Plus (Amersham Biosciences). Immunoblots were scanned with STORM 840 and analyzed with ImageQuant 5.1 software (Amersham Biosciences).

In-gel Tryptic Digestion of rIDE and Amino Acid Sequencing—After SDS-PAGE and Coomassie Blue staining, the band of 125 kDa was cut and the gel slice was incubated in 100 mM ammonium bicarbonate, pH 8.3 containing 45 mM dithiothreitol for 30 min at 60 °C. The tube was cooled at room temperature, and 100 mM iodoacetamide was added

TABLE I

Mass spectrometry analysis of the cleavage products of Aβ variants by insulin-degrading enzyme

NF, not found; Obs., observed; Calc., calculated.

Aβ fragment	Molecular mass of Aβ variants													
	AβWT		AβA21G		AβE22K		AβE22Q		AβE22G		AβD23N		Aβrodent	
	Obs.	Calc.	Obs.	Calc.	Obs.	Calc.	Obs.	Calc.	Obs.	Calc.	Obs.	Calc.	Obs.	Calc.
	Da													
1–13	1561.8	1561.6	NF		1562.6	1561.6	1561.9	1561.3	1562.2	1561.6	1562.1	1561.6	1466.3	1465.5
1–14	1699	1698.3	1699.5	1693.7	1699.9	1698.7	1699.5	1698.7	1699.4	1698.7	1699.3	1698.7	1603.7	1602.6
1–18	2167.9	2167.3	NF		2165.6	2167.3	NF		2168.9	2167.3	2168.1	2167.3	2072.9	2071.2
1–19	2315.1	2314.5	2315.6	2314.5	NF		NF		2315.7	2314.5	2315.3	2314.5	2220.2	2218.4
1–20	2462.6	2461.7	NF		2462.9	2461.7	NF		NF		NF		NF	
1–26	NF		NF		NF		NF		NF		3019.2	3019.2	NF	
1–28	NF		NF		NF		NF		NF		3261.3	3261.5	3168.2	3166.5
4–13	1246.4	1246.3	1246.8	1246.3	1247	1246.3	1246.7	1246.3	1246.7	1246.3	1246.7	1246.3	1151.2	1150.2
4–14	1383.7	1383.5	1384	1383.5	1384.2	1385.5	1384	1383.4	1384	1383.5	1383.9	1385.5	NF	
4–19	2000	1999.2	NF		NF		NF		NF		NF		NF	
5–14	1236.3	1236.3	1236.8	1236.3	1237	1236.3	1236.6	1236.3	1236.6	1236.3	1236.6	1236.3	NF	
14–20	NF		NF		NF		918.9	918.1	NF		NF		NF	
14–28	1719.4	1718.9	1705.8	1704.9	NF		NF		1647.4	1646.9	NF		NF	
15–26	NF		NF		NF		NF		NF		1338.6	1338.5	NF	
15–28	1582	1581.8	1568.3	1567.8	NF		NF		1510.5	1509.7	NF		NF	
15–40	NF		NF		2650.5	2648.2	NF		2578.5	2579.1	NF		NF	
19–28	NF		1100.2	1099.2	NF		NF		NF		NF		NF	NF
19–40	2180.4	2180.6	NF		NF		NF		NF		NF		NF	
20–40	NF		2020.3	2019.4	2033.5	2032.4	2033.8	2032.4	1962.1	1961.3	2033.1	2032.3	2034.6	2033.5
21–40	NF		NF		1886.6	1885.3	1886.5	1885.2	1814.6	1814.1	1885.7	1885.2	1887.5	1886.2
27–40	NF		NF		NF		NF		NF		1327.7	1327.7	NF	
29–40	NF		1084.9	1085.4	NF		NF		1085.5	1086	NF		NF	

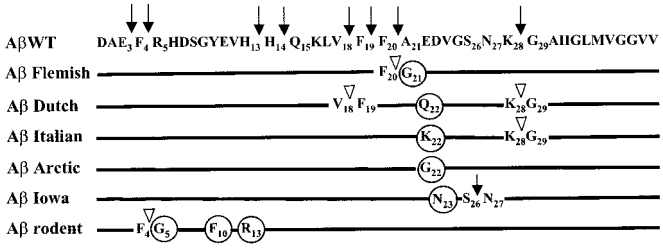

FIG. 3. **Schematic representation of cleavage sites within Aβ WT, Aβ rodent, and Aβ human genetic variants by rIDE.** *Arrows* show the peptide bonds hydrolyzed by rIDE. *Open arrowheads* (▽) indicate loss of cleavage under the experimental conditions tested. The *lines* represent identity of amino acid sequence and cleavage sites. Amino acid substitutions in each Aβ variant are shown in *circles*.

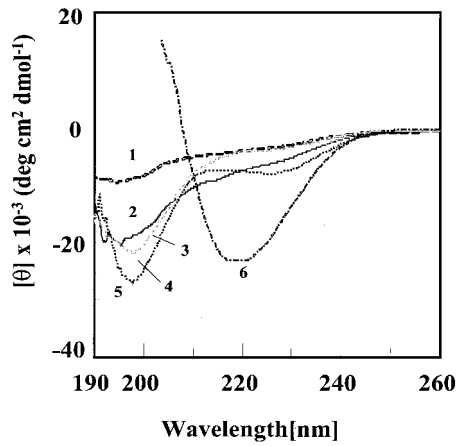

FIG. 4. **Circular dichroism spectra of Aβ variants.** Aliquots (400 μg) of each peptide were dissolved in 1.5 ml of 10 mM Tris-HCl buffer, pH 7.5. After centrifugation at 14,000 rpm for 5 min to remove aggregates, CD spectra were recorded in a JASCO spectropolarimeter J-720 in a 0.1-cm path length cell. Results are expressed as mean residue ellipticity in units of deg cm^2 $dmol^{-1}$ after substraction of buffer spectra and smoothing with algorithm provided by JASCO. Spectra: ***1***, Aβ E22G; ***2***, Aβ WT; ***3***, Aβ D23N; ***4***, Aβ A21G; ***5***, Aβ E22K and ***6***, Aβ E22Q.

followed by incubation for 30 min in the dark at room temperature. The gel was then washed in 50% acetonitrile, 100 mM ammonium bicarbonate with shaking for 1 h, cut in pieces, and transferred to a small tube. Acetonitrile was added to shrink the gel pieces, and the sample was dried in a rotatory evaporator. The gel pieces were re-swollen with 10 μl of 100 mM ammonium bicarbonate, pH 8.3, containing trypsin at a 10:1 ratio (w/w, substrate:enzyme). The sample was incubated overnight at 37 °C, and digestion products were extracted twice from the gel with 60% acetonitrile, 0.1% trifluoroacetic acid for 20 min. Combined extractions were loaded into a C_{18} high pressure liquid chromatography column (220 × 1 mm), and peptides eluted with a linear gradient from 0 to 100% acetonitrile, 0.1% trifluoroacetic acid. Selected peaks were applied to a 477A protein-peptide sequencer (Applied Biosystems) and subjected to Edman degradation sequence analysis at the Laboratorio Nacional de Investigacíon y Servicios en Péptidos y Proteinas facility (CONICET).

Degradation Assays—1 μg of each Aβ synthetic peptide was incubated alone or with 500 ng of purified rIDE in 10 μl of 100 mM sodium phosphate buffer, pH 7, in the presence or absence of 1 mM 1,10-phenantroline. After 30, 60, 90, and 120 min of incubation at 37 °C, samples were analyzed by SDS-PAGE and Western blot with 6E10 as described above. After each time point, degradation by rIDE was expressed as the percentage of remaining Aβ monomer or dimer as compared with Aβ incubated without the enzyme. [^{125}I]Insulin (specific activity 300 μCi/μg, kindly provided by Edgardo Poskus, University of Buenos Aires.) was incubated with rIDE alone or in the presence of the following sets of inhibitors: 1) 1 μM unlabeled insulin; 2) a mixture of 2 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml pepstatin A; 3) a mixture of 1 mM 1,10-phenantroline and 5 mM EDTA, respectively, in the same buffer as above. After incubation for various time points, the sample was run on SDS-PAGE, and the remaining intact [^{125} I]insulin was analyzed and quantitated with a STORM 840 PhophorImager (Amersham Biosciences).

Circular Dichroism (CD) Spectra—Aliquots of Aβ synthetic peptides freshly dissolved at 60 μM in 10 mM Tris-HCl, pH 7.5, were loaded into a 0.1-nm path length quartz cell, and the circular dichroism spectra were recorded in the far-ultraviolet light using a JASCO J-720 spectropolarimeter (JASCO Corporation). Forty scans for each experimental condition were obtained at 0.2-nm intervals over the wavelength range of 190–260 nm. Final spectra were obtained after the subtraction of background readings of blanks.

Mass Spectrometry Analysis—Molecular masses of intact Aβ peptides and the products of rIDE degradation were determined at the New York University Protein Analysis Facility. Aβ-containing samples (10 μl of final volume) were passed through reversed-phase ZipTip (Millipore) following manufacturer's instructions and analyzed on a Micromass TofSpec-2E (MALDI-TOF) mass spectrometer in linear mode using standard instrument settings. Internal and/or external calibration

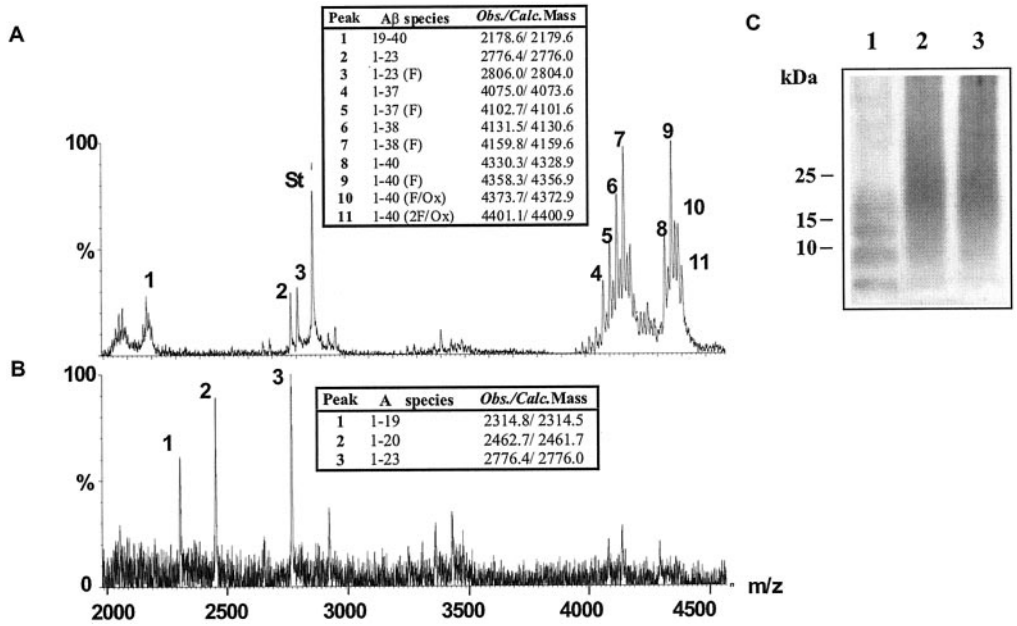

FIG. 5. **Degradation of Aβ E22Q from HCHWA-D leptomeningeal vessels by rIDE.** MALDI-TOF MS analysis of native Aβ E22Q extracted in formic acid after incubation for 1 h at 37 °C in the absence (*panel A*) or in the presence (*panel B*) of rIDE. *St*, standard (insulin mass/2H). *Insets*, calculated and observed masses of Aβ E22Q species and predicted peptides. The presence of one or two formic acid molecules is indicated by F or 2F, respectively. *Ox*, oxidized. *Panel C*, Western blot analysis with monoclonal 6E10 after incubation of Aβ E22Q from HCHWA-D leptomeningeal vessels with rIDE. Synthetic Aβ E22Q (*lane 1*) and HCHWA-D amyloid incubated for 1 h at 37 °C alone (*lane 2*) and with rIDE (*lane 3*) are shown. *Left*, molecular masses in kilodaltons.

was carried out using angiotensin I (average mass = 1296.5 Da) and insulin (average mass = 5733.5 Da).

Isolation of Aβ from Leptomeningeal Vessels—Leptomeningeal vascular amyloid from a patient with hereditary cerebral hemorrhage with amyloidosis Dutch type (HCHWA-D) was isolated as described previously (36). Leptomeninges were dissected from brain coronal sections, cut with scissors into 1–3-mm pieces, and placed in 0.1 M Tris-HCl, pH 8, on ice. Tissue was then washed by resuspension in 0.1 M Tris-HCl, pH 8, containing protease inhibitors (buffer A) and centrifuged at 800 × *g* for 5 min at 4 °C. The procedure was repeated 5 times. The material was collected by filtration through a 50-μm nylon mesh (Spectrum), washed with buffer A without inhibitors, and resuspended in 20 volumes of 2 mM $CaCl_2$ in 0.1 M Tris-HCl, pH 7.5, containing 0.3 mg/ml collagenase CLS-3 (Worthington) and 10 μg/ml DNase (Worthington). The mixture was incubated for 18 h at 37 °C. After digestion, the suspension was filtered through a 350-μm nylon mesh and the filtrate was centrifuged at 10,000 × *g* for 12 min. The pellet was resuspended in 2% SDS in 0.1 M Tris-HCl, pH 8, and incubated for 2 h at room temperature. The SDS-insoluble material was recovered by centrifugation as above and washed three times with distilled water. The pellet was dissolved in 10 volumes of 99% formic acid (Sigma) and incubated for 1 h at room temperature. After centrifugation at 10,000 × *g* for 10 min, the supernatant was then loaded on a Superose 12 column (10 × 300 mm) (Amersham Biosciences). Amyloid peptides were separated in 75% formic acid at 0.2 ml/min using a Bio-Cad Sprint chromatography system. Eluent was monitored at 280 nm, collected in 1-ml fractions, and analyzed as above.

RESULTS

Expression and Characterization of Recombinant Rat IDE—Our IDE bacterial expression vector pET-IDE encodes rat IDE (positions 42–1019) with a His_6 tag at its N terminus to facilitate the purification of the protease with a Ni-affinity columm. After elution with a step gradient of increasing imidazole concentrations, we obtained a highly purified recombinant protein with an apparent molecular mass of 125 kDa as assessed by SDS-PAGE. Western blot using our polyclonal antibody BC2 specifically recognized this 125-kDa fusion protein (Fig. 1*A*). Unexpectedly, the protein did not display immunoreactivity on Western blot with monoclonal 9B12, a well characterized anti-rat IDE antibody (data not shown) (37). To determine its identity, we performed "in-gel" digestion with trypsin followed by separation on microbore high pressure liquid chromatography and N-terminal amino acid sequence of a tryptic fragment. The sequence NVPLPEF matched positions 282–288 of rat IDE (20), confirming the identity of our recombinant protease and suggesting that 9B12 may indeed recognize a posttranslationally modified epitope in rat IDE that is not present in IDE expressed in bacteria. To characterize the activity of recombinant rIDE, we analyzed the degradation of [^{125}I]insulin. After 1 h of incubation, rIDE was able to digest [^{125}I]insulin and this activity was totally inhibited by EDTA-1,10-phenantroline and blocked to 80% in the presence of 1 μM unlabeled insulin (Fig. 1*B*). These results are fully consistent with the reported characterization of human recombinant IDE expressed in *E. coli* (34) and rat recombinant IDE produced in a baculovirus-insect cell system (32).

Degradation of Aβ WT and Aβ Genetic Variants by rIDE—We followed the degradation of Aβ WT and Aβ genetic variants by rIDE using SDS-PAGE and Western blot with monoclonal 6E10 that recognizes an epitope within positions 1–16 of Aβ (38) The relative amount of Aβ monomers and dimers that remained intact after incubation with rIDE was estimated by densitometry. This method allowed an accurate discrimination between monomeric and oligomeric Aβ as opposed to high pressure liquid chromatography or trichloroacetic acid precipitation (28, 29). The absence of Aβ degradation products on Western blots may reflect the loss of the 6E10 epitope attributed to rIDE activity. Alternatively, the Aβ N-terminal fragments generated by the protease may be too small to be detected on SDS-PAGE. Under the conditions of our assay, the quantitation of degradation showed linearity up to 90 min of incubation (data not shown); therefore, a 60-min incubation was the time selected for the comparative analysis. As indicated in Fig. 2, *A* and *B*, monomeric Aβ WT, Aβ A21G, Aβ E22K, and Aβ D23N were degraded at 75.2 ± 3, 81.5 ± 9.2, 79.5 ± 12, and 84.7 ± 7.6%, respectively. rIDE was substantially less efficient in degrading Aβ E22Q and Aβ E22G monomers (42.3 ± 6 and 34.6 ± 6%, respectively, $p < 0.01$ as compared with Aβ WT). In the presence of 1,10-phenantroline, degradation of Aβ WT was inhibited almost completely (Fig. 2*B*). In contrast to Aβ monomers, degradation of

dimers was consistently <10% for all of the variants studied (Fig. 2*B*), in agreement with the results reported recently on endogenous Aβ WT generated by Aβ PP-transfected cell lines (33).

Characterization of Aβ Proteolytic Products—Aβ WT proteolytic products generated by rIDE were consistent with previous reports with the major sites of cleavage at His^{13}-His^{14}, His^{14}-Gln^{15}, Val^{18}-Phe^{19}, Phe^{19}-Phe^{20}, Phe^{20}-Ala^{21}, and Lys^{28}-Gly^{29} (28, 32, 39). All of the Aβ fragments observed by MALDI-TOF MS are summarized in Table I, and Fig. 3 shows an schematic representation of the cleavage sites. Several fragments starting at Phe^4 and Arg^5 were found in Aβ WT and in all of the Aβ variants. These peptides were not the products of truncated synthesis as shown by MS analysis of undigested Aβ peptides and were not found when Aβs were incubated with rIDE in the presence of 1,10-phenantroline (data not shown). Moreover, Phe and Arg as P1′ residues are compatible with the known specificity of IDE (28, 41). Therefore, it seemed probable that rIDE was capable of hydrolyzing Aβ Glu^3-Phe^4 and Phe^4-Arg^5 peptide bonds. In Aβ A21G, there was a consistent absence of fragments ending at Phe^{20} or starting at Gly^{21}, suggesting the specific loss of a cleavage site. Notably, when we analyzed the digestion of rodent Aβ that present Gly instead of Arg at position 5, no fragments indicative of hydrolysis at the Phe^4-Gly^5 site were found, neither in rodent Aβ-(1–40) (Table I and Fig. 3) nor in rodent Aβ-(1–42) (data not shown), whereas all of the other cleavage sites present in human Aβ WT were conserved. The Dutch variant Aβ E22Q showed the apparent loss of sites at Val^{18}-Phe^{19} and Lys^{28}-Gly^{29}, whereas in the Italian variant Aβ E22K, the latter cleavage site was also absent. This peptide bond has been reported to be resistant to IDE in Aβ WT-(1–42) (32), and therefore, its hydrolysis by IDE may depend upon oligomerization rather than primary structure. Alternatively, the presence of Gly at P1′ may impose a subsite restriction as suggested by the loss of cleavage sites in the Aβ Flemish and rodent variants. The Iowa type Aβ D23N differed notably from all of the other Aβ peptides studied. Fragments consistent with positions 1–26, 15–26, and 27–40 were found, indicating the cleavage at the Ser^{26}-Asn^{27} bond (Table I and Fig. 3) and pointing to the possible importance of the Asp^{23} in the folding of the Aβ peptide.

Effect of Aβ Secondary Structure upon Degradation by rIDE—To study the possible influence of secondary structure of Aβ variants on the susceptibility to degradation by rIDE, we performed CD analysis of all of the peptides freshly dissolved in aqueous buffer at neutral pH without previous incubation and after the removal of large aggregates by centrifugation. As expected, Aβ E22Q displayed a negative peak at 220 nm and a positive peak at 195 nm consistent with a β structure in solution (6). In contrast, Aβ WT, Aβ A21G, Aβ E22G, Aβ E22K, and Aβ D23N showed a strong negative peak at 198 nm corresponding to unordered structures (Fig. 4).

Degradation of Aβ E22Q Purified from Leptomeningeal Vessels by riDE—To determine whether rIDE was capable of degrading the Dutch Aβ variant present in affected human tissue, we used purified Aβ from leptomeningeal vessels from a case of HCHWA-D that was extensively characterized previously (36). After the removal of formic acid by reverse-phase C_{18} Zip Tip and equilibration of the sample at neutral pH, Aβ Dutch was incubated with rIDE and degradation characterized as above. Western blot showed that Aβ Dutch was extensively aggregated into SDS-resistant oligomers of ~22–25 kDa that were unmodified in the presence of rIDE (Fig. 5*C*). However, MALDI-TOF MS analysis revealed the disappearance of monomeric Aβ Dutch and the generation of two fragments, positions 1–19 and 1–20 (Fig. 5, *A* and *B*), that were not seen in the presence of 1,10-phenantroline (data not shown).

DISCUSSION

Our results demonstrated that rIDE was capable of degrading all of the synthetic Aβ variants associated with human disease and Aβ Dutch purified from leptomeningeal vessels. However, the efficiency of degradation of synthetic Aβ monomeric species by rIDE was significantly lower for Aβ E22Q and Aβ E22G as compared with Aβ WT and the rest of the variant Aβ peptides studied. The concentrations of Aβ peptides used in our assays were above the critical concentration of Aβ-(1–40) for the formation of high molecular weight oligomers (41), and therefore, it is likely that aggregation of Aβ strongly influenced the rate of degradation by rIDE. In the case of the Aβ Dutch variant, a possible slower rate of degradation could be explained by the high content of β structure as shown by CD analysis. This result was fully consistent with several *in vitro* studies that have demonstrated the fast rate of assembly into stable fibrils of this Aβ mutant (8, 9, 36). The early deposition of Aβ E22Q and onset of symptoms in HCHWA-D patients strongly suggest that such mechanism may be relevant *in vivo*. Moreover, the amyloidogenic conformation of Aβ E22Q may be dominant because Aβ WT has been also found in the HCHWA-D vascular deposits (42). The Aβ Arctic variant peptide in turn has been shown to self-assemble rapidly into protofibrils that may be toxic to neural cells (3). Although the major quantitative differences in degradation were seen upon Aβ E22Q and Aβ E22G, it is unlikely that they reflect a protease-resistant conformation of these monomeric Aβ species. The models based on NMR have shown a similar coiled flexible structure in aqueous solution for Aβ E22Q and Aβ WT (43, 44). Moreover, we have previously demonstrated that at physiological concentrations in the low nanomolar range, Aβ WT and Aβ E22Q were degraded at a similar rate by endogenous brain IDE (29). Thus, it seems more likely that monomeric Aβ Dutch and Arctic variants were "hidden" from rIDE into higher oligomers or aggregates during incubation in physiological buffer that were then partially dissociated upon SDS treatment for Western blot analysis. It was notorious that all of the SDS-resistant Aβ dimers including those from Aβ WT were poorly degraded by rIDE, consistent with the resistance to proteolysis of native Aβ WT-soluble oligomers in cultured cells overexpressing Aβ PP (33). However, it cannot be concluded from these experiments whether Aβ dimers or higher oligomers are the resistant species to degradation by rIDE. The separation of stable Aβ oligomers by gel filtration under native conditions is needed to clarify this issue. In any case, our results and previous reports (28, 48) support that IDE recognizes a motif on monomeric Aβ within positions 18–22, a known hydrophobic stretch that is critical for amyloid formation (45–47). It may be possible that when these residues participate in peptide self-assembly, they are no longer accessible to IDE catalytic site. Our findings on Aβ D23N showed an apparent discrepancy with those presented recently by Van Nostrand *et al.* (10). In these reported experiments, Aβ Iowa and Aβ Dutch variants behaved similarly in terms of secondary structure and fibrillization rate after 48 h of incubation. Nevertheless, it is possible that at much shorter times of incubation, such as in our degradation and CD experiments, the rate of assembly into a β structure may be substantially faster for Aβ E22Q as compared with Aβ D23N, resulting in a higher resistance to proteolysis by rIDE. The Aβ Iowa peptide was different among all of the Aβ variants, because it presented a unique site of cleavage by rIDE at Ser^{26}-Asn^{27}. The possibility of multiple subsite interactions allowing different alignments of substrate relative to the site of catalysis in IDE has been recently demonstrated with the use of fluorogenic substrates (40). It remains to be addressed whether Asn instead of Asp at position 23 of Aβ, being at P_4

from Ser^{26}-Asn^{27}, may determine a new site of cleavage that seems atypical in light of the preference of IDE for hydrophobic and basic residues at P_1' (27, 40). In addition to overproduction and an enhanced aggregability imposed by amino acid substitutions, Aβ may accumulate in the brain due to defective clearance. In transgenic mice expressing human Aβ PP with pathogenic mutations that promote the activity of β-secretase such as the double K670N/M671L substitution, the time course of amyloid deposition shows an exponential increase after 9–10 months of age (15, 49). Yet, the rate of Aβ production and β-secretase activity seems to remain constant throughout the life span of these mice (14). A defect in reverse transport to the systemic circulation as shown for the Aβ Dutch variant (50, 51) together with inefficient local proteolysis may determine a rise in the levels of Aβ in the brain and therefore accelerate its rate of aggregation and deposition (16, 29, 52). In the case of human diseases associated with mutations clustered within the middle portion of Aβ, such accumulation shows a remarkable predisposition for vessels in which Aβ oligomers may be toxic to endothelial and smooth muscle cells. Our results *in vitro* raise the possibility that upregulation of IDE may promote the clearance of these Aβ genetic variants *in vivo*. In this regard, it is noteworthy that Aβ pathogenic mutants seem to be highly resistant to proteolysis by neprilysin *in vitro*, and therefore, increasing the activity of neprilysin may be inefficient to prevent Aβ deposition in certain hereditary forms of amyloid β diseases (53). Yet, the apparent resistance of Aβ oligomers to proteolysis by rIDE suggests that any strategy to lower Aβ based on the enhancement of this endogenous protease should aim at the degradation of Aβ at its monomeric state.

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