Purified recombinant insulin-degrading enzyme degrades amyloid β -protein but does not promote its oligomerization

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Amyloid β -protein ($A\beta$) has been implicated as an early and essential factor in the pathogenesis of Alzheimer's disease. Although its cellular production has been studied extensively, little is known about $A\beta$ clearance. Recently, insulin-degrading enzyme (IDE), a 110-kDa metalloendopeptidase, was found to degrade both endogenously secreted and synthetic $A\beta$ peptides. Surprisingly, IDE-mediated proteolysis of [¹²⁵I] $A\beta$ (1–40) in microglial cell-culture media was accompanied by the formation of ¹²⁵I-labelled peptides with higher apparent molecular masses, raising the possibility that the degradation products act as 'seeds' for $A\beta$ oligomerization. To directly address the role of IDE in $A\beta$ degradation and oligomerization, we investigated the action of purified recombinant wild-type and catalytically inactive IDEs. Our data demonstrate that (i) IDE alone is sufficient

to cleave purified $A\beta$ that is either unlabelled, iodinated or ³⁵S-labelled; (ii) the initial cleavage sites are His¹⁴–Gln¹⁵, Phe¹⁹–Phe²⁰ and Phe²⁰–Ala²¹; and (iii) incubation of IDE with [¹²⁵I] $A\beta$, but not with [³⁵S]- $A\beta$, leads to the formation of slower migrating species on gels. Since iodination labels N-terminal fragments of $A\beta$, and ³⁵S labels C-terminal products, we analysed unlabelled synthetic fragments of $A\beta$ and determined that only the N-terminal fragments migrate with anomalously high molecular mass. These results indicate that IDE alone is sufficient to degrade $A\beta$ at specific sites, and that its degradation products do not promote oligomerization of the intact $A\beta$ peptide.

Key words: Alzheimer's disease, $A\beta$ proteolysis, metalloendopeptidase, oligomer.

INTRODUCTION

Converging lines of evidence suggest that the amyloid β -proteins ($A\beta$ s) play a central role in Alzheimer's disease. The 40–42-amino acid peptide is the major component of the cerebral amyloid deposits characteristic of Alzheimer's disease pathology. Moreover, several genetic alterations associated with familial Alzheimer's disease have been shown to increase the production of $A\beta$ and/or its accumulation in amyloid plaques (for recent reviews see [1,2]). Decreasing the levels of $A\beta$ in the brain represents a potential therapeutic approach towards the disease. However, although the mechanism of $A\beta$ production and the pathways modulating the expression of the β -amyloid precursor protein have been investigated extensively, very little is known about the normal and pathological degradation processes of $A\beta$. Recently, studies conducted in cultured microglial cells, principal components of the cerebral immune system, have suggested that certain metalloproteases may be involved in the catabolism of $A\beta$ [3,4]. Subsequently, insulin-degrading enzyme (IDE, EC 3.4.22.11) has been shown to be present in the culture medium of microglial BV2 cells, where it participates in extracellular $A\beta$ degradation [5]. Moreover, soluble brain extracts containing IDE have been shown to exhibit $A\beta$ -degrading activity [6], and an *in vitro* study reported that IDE purified from rat liver could degrade $A\beta$ (1–28) and $A\beta$ (1–40) [7].

IDE is a 110-kDa neutral metalloendopeptidase with sensitivity towards thiol reagents (for a recent review see [8]). Human, rat and *Drosophila* IDE amino acid sequences all exhibit the HXXEH zinc-binding motif characteristic of the inverzincin family of

metallopeptidases, as well as a type-I peroxisomal targeting signal [9–14]. Consistent with this last feature, IDE has been shown to localize intracellularly in the cytosol and in peroxisomes in several cell types [15–17]. Numerous experiments suggest that IDE is the principal enzyme controlling insulin degradation in various cells. First, insulin fragments generated *in vitro* by the purified endopeptidase are identical to those isolated from intact liver [18] and hepatocytes [19]. Furthermore, inhibitors of IDE prevent insulin degradation in different cells [20,21], and the hormone can be cross-linked *in vivo* to the peptidase [22]. Moreover, monoclonal antibodies specific to IDE block insulin degradation in HepG2 cells [23]. Finally, overexpression of the endopeptidase in COS cells led to a several-fold increase in the intracellular rate of insulin degradation [24]. Although its affinity for insulin is especially high, IDE also degrades several other substrates *in vitro*, suggesting a potentially wider role for the protease in the clearance of hormones and bioactive peptides. These peptide substrates include glucagon, insulin-like growth factor I and II [25], atrial natriuretic factor [26,27], transforming growth factor- α [28] and, more recently, growth-hormone-releasing factor and β -endorphin [29].

Surprisingly, IDE-mediated proteolysis of [¹²⁵I] $A\beta$ (1–40) in the media of cultured cells was accompanied by the formation of higher-molecular-mass iodinated species reminiscent of $A\beta$ oligomers [5], raising the possibility that the degradation products act as 'seeds' for $A\beta$ oligomerization or oligomerize themselves. Several studies reported that IDE may interact intracellularly with specific proteins [8,16,30], and it is not clear yet whether the purified endopeptidase itself is able to reproduce the observations

Abbreviations used: $A\beta$, amyloid β -protein; DMEM, Dulbecco's modified Eagle's medium; IDE, insulin-degrading enzyme.

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in the cultures. To directly examine the role of IDE in A β degradation and oligomerization, we compared the action of a purified recombinant wild-type IDE with that of a catalytically inactive form of the enzyme. The high degree of purity of our two recombinant endopeptidases allowed us to establish that IDE, *per se*, is able to selectively degrade A β (1-40), whether it is unlabelled, iodinated or ³⁵S-labelled, mimicking the action of the A β -degrading IDE identified in cell-culture media. As IDE appears to rapidly cleave the peptide at multiple sites, the initial degradation fragments of A β were identified. Four of these degradation products were synthesized chemically and analysed further. The finding that two C-terminally truncated A β peptides exhibit abnormally slow electrophoretic migration in SDS/polyacrylamide gels is discussed with regard to the potential involvement of IDE in physiological clearance of the amyloid peptide.

EXPERIMENTAL PROCEDURES

Materials

Prestained standard SDS/PAGE molecular-mass markers were purchased from Gibco-BRL/Life Technologies (Grand Island, NY, U.S.A.). Iodinated A β (1-40) {[¹²⁵I]A β (1-40); $\approx$ 2000 Ci/mmol in 35 % (v/v) acetonitrile/0.1 % (v/v) trifluoroacetic acid} was generously provided by Dr John E. Maggio (University of Cincinnati College of Medicine, Cincinnati, OH, U.S.A.) or purchased from Amersham Life Science (Arlington Heights, IL, U.S.A.). [³⁵S]A β (1-40) [$\approx$ 8700 Ci/mol in 20 % (v/v) acetonitrile/5 mM sodium acetate, pH 8.0] was kindly given by A. Przybyla, University of Georgia (GA, U.S.A.). Initially polyhistidine-tagged, this peptide was produced and metabolically labelled in bacteria and subsequently purified by metal affinity chromatography. The histidine tag sequence was then proteolytically removed (A. Przybyla, personal communication). Unlabelled A β (1-40) and A β (1-20) were obtained from QCB (Quality Controlled Biochemicals, Hopkinton, MA, U.S.A.). The low-molecular-mass form of A β (1-40) was purified according to Walsh et al. [31]. A β (1-14), A β (15-40), A β (21-40) and non-radioactive iodinated A β (1-20) were synthesized by M. Condrón (Center for Neurologic Diseases, Brigham and Women's Hospital, Boston, MA, U.S.A.) on an Applied Biosystems 430A synthesizer using standard Fmoc (fluoren-9-ylmethoxycarbonyl) chemistry. Purity and sequence were confirmed by reversed-phase HPLC, MS and amino acid analysis. The purity of all peptides was found to be $\geq$ 85 %. Non-radioactive monoiodinated tyrosine was purchased from Advanced Chemtech (Louisville, KY, U.S.A.). Human insulin, BSA and trichloroacetic acid were from Sigma (St. Louis, MO, U.S.A.). Disuccinimidyl suberate was from Pierce (Rockford, IL, U.S.A.).

Expression and purification of the recombinant IDEs

The polyhistidine- and haemagglutinin-tagged wild-type and mutated IDEs were expressed in bacteria and purified by metal affinity chromatography, as described in Chesneau and Rosner [32]. IDE preparations were analysed by SDS/PAGE (10 % gels), and the proteins were visualized using GelCode Blue Stain reagent (Pierce).

Assaying the degradation of [¹²⁵I]A β (1-40)

[¹²⁵I]A β degradation was assayed by both trichloroacetic acid precipitation and gel fluorography [5]. Aliquots of the purified enzymes were incubated in 120 μ l of fresh Dulbecco's modified Eagle's medium (DMEM) containing 10 μ g/ml BSA, 65 nM unlabelled purified A β and 230 pM [¹²⁵I]A β at 37 °C for specific

times. Out of the 120- μ l reaction mixture, 100 μ l was then added to an equal volume of 15 % trichloroacetic acid, and incubated on ice for 20 min to precipitate undegraded A β (1-40). Full-length [¹²⁵I]A β and degradation products were counted separately as described previously [5]. The remaining 20 μ l of the incubation mixture was analysed by 10–20 % Tris/Tricine gel fluorography as described previously [5].

Assaying the degradation of [³⁵S]A β (1-40)

Aliquots of the purified IDE were incubated in 20 μ l of DMEM containing 10 μ g/ml BSA, 65 nM unlabelled purified A β and 1.9 μ M [³⁵S]A β for specific times at 37 °C. Reactions were terminated by the addition of SDS sample buffer, and analysed by 10–20 % Tris/Tricine gel fluorography as for [¹²⁵I]A β (above).

A β (1-40) affinity labelling

A β affinity labelling was performed by a modification of the method of Kurochkin and Goto [7]. Briefly, 60 ng of each purified IDE was incubated with 600 pM [¹²⁵I]A β in 50 mM Hepes, pH 7.5, 50 mM NaCl and 1 mg/ml BSA in a final volume of 30 μ l. Then 10 μ M unlabelled insulin or 1 mM (1,10)-phenanthroline were added to the reaction mixture as specified. After 10 min at room temperature, 1.5 μ l of a solution of disuccinimidyl suberate (3 mg/ml in DMSO) was added to each tube. After an additional 15 min at room temperature, reactions were terminated by the addition of SDS/PAGE sample buffer. Samples were heated, and electrophoresed on a 10–20 % Tris/Tricine denaturing polyacrylamide gel (Novex, San Diego, CA, U.S.A.). Cross-linked proteins were visualized by fluorography of the dried gel.

Identification of A β (1-40) early degradation products by IDE

Purified A β (1-40) (10 μ g) was incubated with 500 ng of wild-type or catalytically inactive mutant (E111Q) IDE in 100 μ l of 50 mM Tris/HCl, pH 7.5. After 30 min at 37 °C, the reaction mixtures were analysed by HPLC (219TP diphenyl reverse-phase column; 4.6 $\times$ 250 mm; Vydac, Hesperia, CA, U.S.A.) using a 20–45 % gradient of acetonitrile in 0.1 % trifluoroacetic acid over 50 min. Three peaks corresponding to early degradation products of A β (1-40) by IDE were collected, and their identities were determined by amino acid analysis and liquid chromatography MS. The A β products from a similar incubation were also analysed, without prior purification, by liquid chromatography MS with an LCQ ion trap spectrometer (Finnigan MAT, Manchester, U.K.).

SDS/PAGE and Western-blot analyses of A β peptides

A β peptide fragments were solubilized at a concentration of 50 μ M in 50 % DMSO. Equal amounts of each peptide, as well as a mixture, were then electrophoresed under denaturing conditions on a 10–20 % Tris/Tricine polyacrylamide gel (Novex). Peptides were either visualized using Novex Silver Express stain kit or GelCode Blue Stain reagent, or were transferred on to a 0.2- μ m nitrocellulose membrane (Schleicher & Schuell, Keene, NH, U.S.A.) for Western-blot analysis. The membrane was treated for 10 min with boiling PBS and immunoblotted with a mixture of A β monoclonal antibodies 2G3 [specific to A β (1-40)] and 3D6 (specific to the N-terminus; gifts of P. Seubert, Elan Pharmaceuticals, San Francisco, CA, U.S.A.).

Assaying the effect of $A\beta$ fragments on oligomerization of [125 I] $A\beta$ (1-40)

[125 I] $A\beta$ (1-40) (230 pM) was incubated for specific times at 37 °C in 20 μ l of DMEM containing 10 μ g/ml BSA, in the presence or absence of a cocktail of synthetic $A\beta$ fragments, each at 50 nM. Reactions were terminated by the addition of SDS sample buffer, and analysed by 10–20% Tris/Tricine SDS/PAGE and fluorography.

RESULTS

Purified wild-type IDE degrades [125 I] $A\beta$ (1-40)

To determine whether purified IDE alone was able to reproduce the observations about $A\beta$ degradation and oligomerization from our earlier cell-culture experiments [5], we used two highly purified recombinant endopeptidases. Wild-type human IDE and a catalytically inactive E111Q mutant (E111Q IDE) that were tagged at their N-termini with both polyhistidine and haemagglutinin were expressed in bacteria and purified by metal-affinity chromatography [32]. Under these conditions, other mammalian proteins that might associate with IDE *in vivo* are not present. An electrophoretogram of typical wild-type and E111Q IDE preparations is shown in Figure 1. The *in vitro* degradation of [125 I] $A\beta$ by the purified endopeptidases was assayed initially by trichloroacetic acid precipitation. To stay as close as possible to the assay conditions used in our culture studies [5], we performed all the incubations in DMEM. As shown in Figure 2(A), the isolated wild-type enzyme degrades the iodinated peptide in a time- and concentration-dependent manner. A high percentage of degradation is reached very rapidly, indicating that the velocity of the reaction is high. The levels of degradation obtained in the presence of an excess of unlabelled insulin, or with the inactive E111Q mutant, were similar to that of a control reaction with no enzyme, indicating that the reaction is specific for IDE.

Figure 1 SDS/PAGE analysis of the purified recombinant wild-type IDE

Purified recombinant wild-type (wt) or E111Q IDE (1 μ g) were electrophoresed on an SDS/10% polyacrylamide gel and visualized using the GelCode Blue Stain reagent.

Figure 2 *In vitro* degradation of [125 I] $A\beta$ (1-40) by IDE and affinity labelling of the purified recombinant IDEs

(A) *In vitro* degradation of [125 I] $A\beta$ was assayed by trichloroacetic acid precipitation as described in the Experimental procedures section. Incubations were performed using no enzyme (Δ), 25, 100 and 500 ng of wild-type IDE ($\circ$, $\blacklozenge$ and $\square$, respectively) and 500 ng of E111Q IDE ($\bullet$). Reactions were terminated after 0, 15, 30 and 60 min at 37 °C. (B) Each purified IDE (60 ng) was affinity-labelled with 600 pM [125 I] $A\beta$ (1-40) as described in the Experimental procedures section. Unlabelled ('cold') insulin (10 μ M) or 1 mM (1,10)-phenanthroline were included as indicated. Samples were electrophoresed on a 10–20% Tris/Tricine denaturing polyacrylamide gel, and the cross-linked products were visualized by fluorography of the dried gel.

To confirm that the inactivating E111Q mutation did not impair IDE protein structure, the ability of the mutant enzyme to bind the iodinated peptide was studied in cross-linking experiments. As seen in Figure 2(B), the E111Q IDE is capable of binding [125 I] $A\beta$ similarly to the wild-type enzyme (compare lanes 1 and 4, reading from the left). In both cases, the affinity labelling of the 110-kDa protein was displaced by the addition of an excess of unlabelled insulin (Figure 2B, lanes 2 and 5). Although (1,10)-phenanthroline is known to strongly inhibit IDE activity, it did not prevent the binding of $A\beta$ by either of the two enzymes (Figure 2B, lanes 3 and 6), as observed previously for insulin [33]. Taken together, the data presented in this section show that purified IDE is sufficient to degrade $A\beta$ in a concentration- and time-dependent manner.

The degradation of [125 I] $A\beta$ (1-40) by purified IDE is accompanied by the formation of higher-molecular-mass $A\beta$ -related species

In previous cell-culture experiments with [125 I] $A\beta$, we observed an IDE-mediated time-dependent appearance of $A\beta$ -related species with slower electrophoretic mobility that occurred concomitantly with the loss of the iodinated monomer [5]. Here, when we incubated purified IDE with synthetic [125 I] $A\beta$, we

Figure 3 Gel analysis of [^{125}I]A β (1-40) degradation by purified IDEs

In vitro degradation of [^{125}I]A β (1-40) was analysed by 10–20% Tris/Tricine SDS/PAGE and gel fluorography. Lanes 1–8 show the results of incubations performed with no enzyme (lane 1), 25, 100 and 500 ng of wild-type IDE (lanes 2–4, respectively), 500 ng of wild-type IDE in the presence of 10 μM insulin (lane 5), and 25, 100 and 500 ng of E111Q IDE (lanes 6–8, respectively). Incubations shown were terminated after 0, 30 and 90 min, as indicated.

Figure 4 *In vitro* degradation of [^{35}S]A β (1-40) by purified IDEs

The degradation of purified [^{35}S]A β (1-40) was assayed by 10–20% SDS/PAGE and fluorography. **(A)** Analysis of the reactions performed with no enzyme (lanes 1), 500 ng of wild-type IDE in the absence (lanes 2) or presence of 10 μM insulin (lanes 3), and 500 ng of E111Q IDE (lanes 4). Reactions were carried out for 90 min at 37 °C. **(B)** Time course of degradation of [^{35}S]A β (1-40) by 0, 25, 100 and 500 ng of wild-type IDE (lanes 1–4, respectively). Incubations were analysed after 0, 15, 45 and 180 min at 37 °C.

again observed the formation of iodinated species with apparent molecular masses of 6–19 kDa accompanying the disappearance of the 4-kDa full-length monomer (Figure 3). These species appeared in a time- and IDE-concentration-dependent manner. Incubations performed in the absence of the purified enzyme (Figure 3, lanes 1), in the presence of an excess of unlabelled insulin (Figure 3, lanes 5) or with increasing amounts of E111Q IDE (Figure 3, lanes 6–8) all yielded a single radiolabelled band

corresponding to the uncleaved [^{125}I]A β monomer. Although our aliquots at time point 0 were removed from the incubation mixture as soon as possible after the addition of enzyme, bands higher than the 4.3-kDa A β monomer were already detectable in the presence of wild-type, but not catalytically inactive E111Q, IDE (Figure 3, lanes 2–4, 0 min). This observation is consistent with the earlier evidence that IDE acts very rapidly on A β (Figure 2A). In reactions showing substantial peptidolysis (Figure

Table 1 Identification of the early degradation products of A β (1-40) by IDE

Purified A β (1-40) (10 μ g) was incubated with 500 ng of wild-type IDE for 30 min at 37 °C. The A β peptides in the crude reaction mixture were then identified by liquid chromatography MS. Also shown are the expected and observed molecular masses of the non-radioactive iodinated A β (1-20) control peptide.

A β fragment	Expected mass (kDa)	Observed mass (kDa)	Height (% of all products height)
Gln ¹⁵ -Val ⁴⁰	2649.1	2648.9	58%
Phe ²⁰ -Val ⁴⁰	2033.4	2032.5	8%
Ala ²¹ -Val ⁴⁰	1886.2	1885.3	14%
Asp ¹ -Val ⁴⁰	4329.9	4331.2	12%
Asp ¹ -Phe ²⁰	2461.7	2460	8%
Asp ¹ - ^[125I] Tyr ¹⁰ -Phe ²⁰	2587.7	2587	—

3, lanes 2–4, 90 min), radioactive fragments of molecular masses smaller than that of A β (1-40) were also detected. Although the addition of 65 nM unlabelled A β (1-40) to the reaction mixtures slowed the rate of degradation of [¹²⁵I]A β by IDE, similarly sized products were obtained in both assay conditions (results not shown). To prevent any artifactual oligomerization of the full-length [¹²⁵I]A β due to the addition of partially oligomerized unlabelled peptide, we first purified the low-molecular-mass form of unlabelled synthetic A β (1-40) as described previously [31]. Our results show that the purified recombinant wild-type IDE is able to generate labelled products of sizes similar to those of [¹²⁵I]A β generated by endogenous IDE released by microglial cells [5], and that the formation of the higher-molecular-mass A β species is directly dependent on IDE's proteolytic activity.

Purified wild-type IDE degrades [³⁵S]A β (1-40) without concomitant formation of higher-molecular-mass radiolabelled species

Since iodination occurs on Tyr¹⁰, it only allows the visualization of N-terminal fragments of A β generated by IDE-mediated

cleavage. In contrast, since [³⁵S]Met labels the sole methionine in A β (at residue 35), C-terminal fragments can be detected. We thus used gel fluorography to assay the action of purified IDE on [³⁵S]A β (1-40) that had been recombinantly expressed and metabolically labelled in bacteria and subsequently purified. As shown in Figure 4(A), wild-type IDE was able to degrade the radio-labelled peptide, generating only species smaller than [³⁵S]A β (1-40) (Figure 4A, 90 min, lane 2). Three radioactive bands (17–25 kDa) of constant intensity can be observed in all incubations, including the control reaction without enzyme. Whether they correspond to impurities obtained in the bacterially expressed [³⁵S]A β (1-40) or pre-existing A β oligomers is currently under investigation. Attempts to immunoprecipitate these proteins using antibodies specific to A β have been unsuccessful, suggesting that they are not related to the amyloid peptide (K. Vekrellis and D. Walsh, unpublished work). However, no new radioactive bands between 6 and 19 kDa (the size of the high-molecular-mass iodinated species) were generated by the action of IDE on [³⁵S]A β (1-40) (Figure 4A, 90 min, lane 2). As observed with the iodinated peptide, the degradation of the ³⁵S-labelled A β was inhibited in the presence of an excess of unlabelled insulin (Figure 4A, 90 min, lane 3). A time-course study showed that the disappearance of [³⁵S]A β (1-40) is accompanied by an increase in the intensity of lower-molecular-mass species in a time- and enzyme-concentration-dependent manner (Figure 4B). These observations show that the formation of higher-molecular-mass A β -derived species does not occur during the degradation of [³⁵S]A β (1-40) by IDE.

Identification of the initial degradation products of A β (1-40) generated by wild-type IDE

To identify the cleavage sites within synthetic A β (1-40) created by purified recombinant IDE on A β (1-40), unlabelled A β peptide was incubated with wild-type IDE or the inactive endopeptidase as a control for 30 min. Degradation products were separated by HPLC. N-terminal sequencing and MS analysis of the isolated proteolytic fragments identified His¹⁴-Gln¹⁵, Phe¹⁹-Phe²⁰ and

Figure 5 SDS/PAGE of synthetic A β fragments corresponding to those generated by IDE

A β (1-40), A β (1-14), A β (15-40), A β (1-20) and A β (21-40) (all 1 μ g) or a mixture of these four fragments of A β were electrophoresed on 10–20% Tris/Tricine SDS/polyacrylamide, and visualized by either silver staining (A) or Western blotting with the anti-A β monoclonal antibodies 2G3 and 3D6 (C). A β (1-40), A β (1-20) and unlabelled iodinated A β (1-20) (1 μ g each) were electrophoresed on a 10–20% Tris/Tricine SDS gel and visualized by GelCode Blue Stain reagent (B). Note that the iodinated A β fragment also migrates anomalously.

Table 2 Theoretical and experimental molecular masses of A β (1-40) and early degradation products

Experimental molecular masses of A β (1-40), A β (1-14), A β (15-40), A β (1-20), [Tyr-¹²⁵I]A β (1-20) and A β (21-40) were determined from their positions in 10–20% Tris/Tricine SDS gels, as they appear in Figure 5 compared with those of the molecular-mass markers.

	Molecular mass (kDa)		
	Expected	Observed	
		By silver staining	By Western blot
A β (1-40)	4.3	4.2	3.9
A β (1-14)	1.7	7.9	Not detected
A β (15-40)	2.65	3.5	3.7, 2.9
A β (1-20)	2.5	7.9, 4.6	6.9, 3.4
[Tyr- ¹²⁵ I]A β (1-20)	2.6	5.3	
A β (21-40)	1.9	3.6	3

Peptide²⁰–Ala²¹ as the early cleavage sites of A β (1-40) by IDE. The same degradation products were identified by MS analysis of the crude incubation mixture. The peptide identities and proportions are summarized in Table 1. This quantification indicates that the predominant site of cleavage by IDE is His¹⁴–Gln¹⁵. The low amount of A β (1-14) recovered from the reaction mixture suggests that this fragment is rapidly degraded further by the enzyme. As seen by HPLC analysis, longer incubations led to the complete disappearance of all original fragments, showing that A β can be cleaved at multiple sites by IDE (results not shown).

Two N-terminal degradation products of A β (1-40) migrate as higher-molecular-mass species in SDS/polyacrylamide gels

To further investigate the role of IDE in the formation of the higher-molecular-mass A β -related species detected in the incubations with [¹²⁵I]A β (1-40), we synthesized the major proteolytic fragments of the amyloid peptide generated by IDE: A β (1-14), A β (15-40), A β (1-20) and A β (21-40). In addition, to examine the effect that the iodine atom might have on the mobility of the N-terminal fragments, A β (1-20) with an iodinated Tyr (non-radioactive) instead of the normal Tyr at position 10 was also synthesized. Peptides were solubilized in 50% DMSO to prevent aggregation and analysed in parallel with A β (1-40) by 10–20% Tris/Tricine gel electrophoresis under denaturing conditions. Peptides were visualized by silver staining, with the GelCode Blue Stain reagent (Figures 5A and 5B), or by Western blotting using a mixture of the anti-A β monoclonal antibodies 2G3 [specific to A β (1-40)] and 3D6 (specific to the N-terminus; Figure 5C). Table 2 summarizes the expected and observed molecular masses of each peptide analysed. Although all samples contained similar amounts of material, some A β fragments were hardly detected by silver staining (Figure 5A). A β (1-14) appeared very weakly stained by silver, and was undetectable by Western blotting (Figures 5A and 5C), even when using various combinations of anti-A β antibodies (results not shown), suggesting that the epitopes in this fragment are not accessible to the antibodies. Two peptides also showed heterogeneity, migrating as two distinct species: A β (15-40) (Figure 5C, lane 3) and A β (1-20) (Figures 5A and 5C). Although not in complete agreement with their theoretical masses, both C-terminal fragments of A β [A β (15-40) and A β (21-40)] migrated with apparent molecular masses smaller than that of A β (1-40), as expected. In contrast, fragments A β (1-14) and A β (1-20) as well as the iodinated A β (1-20) control migrated predominantly as 5.3–7.9-kDa species,

Figure 6 Effect of IDE-mediated A β fragments on oligomerization of [¹²⁵I]A β (1-40)

[¹²⁵I]A β (1-40) was incubated for the indicated times in the absence (–) or presence (+) of a cocktail of unlabelled A β (1-14), A β (15-40), A β (1-20) and A β (21-40), each at 50 nM. Effects of the A β fragments on the oligomerization of [¹²⁵I]A β (1-40) were analysed by SDS/PAGE and fluorography.

which could reflect their altered mobility or their oligomerization (Figures 5A and 5B). No difference in the migration profiles was noted when all four truncated peptides were mixed together, suggesting that they do not oligomerize together (Figure 5).

Considering that iodination labels N-terminal fragments (at Tyr¹⁰) whereas ³⁵S labels C-terminal products (at Met³⁵), these observations suggest that the higher-molecular-mass species observed in the IDE incubations with [¹²⁵I]A β correspond to N-terminal products of A β , and not to oligomers of the full-length (1-40) peptide. To further test this hypothesis, a cocktail of the unlabelled A β peptide fragments was added to [¹²⁵I]A β (1-40) in solution in DMEM, and incubated at 37 °C for up to 90 min. Only one band corresponding to monomeric [¹²⁵I]A β (1-40) was detected by gel fluorography and this was not affected by the presence of the four A β fragments (Figure 6). In experiments where the non-radioactive iodinated A β (1-20) was also included in the peptide mix, it did not affect the migration of the [¹²⁵I]A β (1-40) (results not shown). Taken together, these results suggest that the degradation products generated by IDE do not act as nucleating seeds to promote oligomerization of monomeric A β (1-40), and that those lacking the C-terminus migrate anomalously high in SDS/PAGE.

DISCUSSION

Progressive accumulation of A β in senile plaques is regarded as a major precipitating factor in the onset of Alzheimer's disease. Consequently, reducing levels of cerebral A β represents a logical therapeutic approach towards the disease. Although the production of the peptide is actively studied, little is known about A β clearance and the endoprotease(s) implicated in the process. Two different approaches have recently identified IDE as a possible A β -degrading enzyme [5,6]. However, the formation of higher-molecular-mass A β species appeared to occur concomitantly with the degradation of [¹²⁵I]A β (1-40) by the IDE released by BV2 microglial cells, raising the possibility that the A β fragments generated act as 'seeds' for the oligomerization of the full-length peptide [5]. The latter effect of IDE would be potentially pathogenic if it occurred *in vivo*. The present studies using two highly purified recombinant human IDEs [32] allowed us to clarify the role of the metalloendopeptidase in A β degradation and oligomerization.

First, we demonstrate that the enzyme itself is able to degrade unlabelled, iodinated or ³⁵S-labelled A β (1-40), reproducing the action not only of the natural endopeptidase released by microglial cells [3,5] but also of that detected in crude brain extracts [6].

Our data strongly suggest that the iodinated species migrating at 6–19 kDa in denaturing polyacrylamide gels correspond to N-terminal degradation products of [125 I]A β . These species, which we first observed in conditioned media of BV2 cells containing [125 I]A β [5], are also generated by the action of the purified recombinant wild-type IDE (Figure 3). Moreover, A β (1–40) degradation products generated *in vitro* by soluble fractions from human and rat cerebral cortex that contain a 110-kDa protein immunologically related to IDE have been identified as the (1–14), (1–15), (1–18), (1–19), (1–20) and (22–31) peptides [6]. In our study, short incubations of the unlabelled peptide with the purified endopeptidase allowed the initial cleavage sites to be identified as His¹⁴–Gln¹⁵, Phe¹⁹–Phe²⁰ and Phe²⁰–Ala²¹, all in agreement with the results of the *in vitro* study performed on crude brain extracts [6].

In addition to its well-documented cytosolic and peroxisomal localizations, IDE has been detected in the culture medium of lymphocyte and microglial cells, as well as at the cell surface of several cell types [5,29,34,35]. However, the mechanism by which the protein reaches the outside of the cell is unclear. Indeed, although in both human and rat, IDE mRNA exhibits two in-frame potential initiation codons and thus possibly encodes two proteins differing by their N-terminus, none of the four predicted N-terminal amino acid sequences possess the characteristics of a potential signal peptide. The hypothesis that there is a second mRNA encoding a 'secreted' IDE is reasonable, given that mRNAs of different lengths have been identified in some tissues [12,36]. However, a fraction of the rat IDE translated from the second methionine and overexpressed in Chinese hamster ovary cells can be labelled by a membrane-impermeable biotinylation agent, suggesting that it reaches the cell surface [34]. The cDNAs encoding the recombinant human IDEs used in the present study also lack the first initiation codon [32]. The resulting wild-type endoprotease, indistinguishable in size (110 kDa) from the mammalian enzyme produced in COS cells as a mainly intracellular protein and in microglial cells as a secreted protein, is able to bind and degrade A β . Although a slightly longer isoform of IDE may exist [35], these observations suggest that the metalloendopeptidase known to degrade insulin intracellularly is also capable of cleaving A β outside the cell.

We also show that two C-terminally truncated A β peptides, chosen because they were generated early in the degradation of A β by IDE, can migrate anomalously as 6.9–7.9-kDa species in SDS/PAGE (Figure 5). Mixed in similar proportions, the four synthetic peptides analysed did not seem to affect each other's migration in polyacrylamide gels (Figure 5), nor did they seem to lead to oligomerization of the iodinated full-length A β (Figure 6). Modification of tyrosine at position 10 with unlabelled iodine also did not affect this anomalous migration. Consequently, the high-molecular-mass A β -related species detected after incubating [125 I]A β (1–40) with the conditioned BV2 media [5] or with the purified wild-type IDE (Figure 3) are likely to correspond to N-terminal fragments of A β , and not oligomers of the full-length peptide, as their behaviour in SDS/PAGE first suggested. Furthermore, the fact that no high-molecular-mass species are detected with the [35 S]A β (1–40) substrate, which is labelled at its C-terminus, suggests that only N-terminal peptides have this anomalous behaviour in SDS/PAGE under our assay conditions. However, whether these higher-molecular-mass species represent homo-oligomers of each fragment or partially folded monomers migrating aberrantly remains to be investigated. It has been shown that endogenous, secreted A β (1–40) itself can migrate as at least three distinct species in SDS/PAGE: the 4-kDa monomeric form, the 8-kDa dimer and a 6-kDa form thought to correspond to an anomalously migrating monomer [37] (R.

Wang, D. Walsh and D. J. Selkoe, unpublished work). If synthetic A β (1–14) and A β (1–20) do oligomerize under our assay conditions, the high-molecular-mass species detected for these two peptides would correspond to apparent tetramers or trimers, respectively (Table 2). Analysed by SDS/PAGE, newly dissolved A β (1–42) similarly migrates not only as a predominant monomeric band ($\approx$ 4.5 kDa), but also as an apparent tetrameric band (18 kDa) [38]. Although the hypothesis that the migration of these potential oligomers is not linearly related to their molecular mass cannot be excluded, this observation also raises the possibility that oligomerization occurs by passage through thermodynamically stable stages. Also, several structural and biochemical studies suggest that the region of A β containing residues 19–35 is essential for both neurotoxicity [39] and aggregation properties of the peptide [38,40]. The N-terminus of A β , far less hydrophobic than the central and C-terminal regions of the peptide, and thus less susceptible to aggregation, has received less attention. Tested for its potential neurotrophic and neurotoxic actions on rat hippocampal cells, A β (1–16) showed no activity at 20 μ M when the toxic response of A β (1–40) was first observed at 40 nM [39]. When analysed by light microscopy, sedimentation and SDS/PAGE, A β (1–15) incubated at 37 °C for 7 days neither showed aggregation nor had a toxic effect on hippocampal cultures [38]. However, it is intriguing that out of the four peptides studied in our experimental conditions, only the two N-terminal peptides have shown anomalous migration profiles in SDS/PAGE. It thus remains to be determined whether the fragments of A β released by IDE may themselves oligomerize and contribute to plaque formation.

We reported recently that primary neuronal cultures degrade monomeric A β via endogenous IDE and that IDE overexpression markedly reduces the endogenous levels of both extracellular and intracellular A β_{40} and A β_{42} [35]. Recently, Iwata et al. reported that endopeptidase 24.11 (nepyrlisin) is involved selectively in the catabolism of A β_{42} in rat brain parenchyma [41]. It is therefore possible that, in addition to IDE, other peptidases may contribute to the degradation of pathogenic A β species. Regardless, the data presented in this study support the hypothesis that IDE may play a role in A β clearance. Moreover, although the degradation of A β by certain other proteases does not seem to lead to the formation of higher-molecular-mass species [42], our data point out the possibility of confusing oligomers of A β (1–40) with IDE-mediated degradation products of A β . Particular caution should thus be taken in analysing results from experiments using the iodinated peptide in whole-cell cultures or their conditioned media when this endopeptidase is present.

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