Identification of a Mutant Amyloid Peptide That Predominantly Forms Neurotoxic Protofibrillar Aggregates

Received for publication, December 31, 2002, and in revised form, April 7, 2003
Published, JBC Papers in Press, April 8, 2003
DOI 10.1074/jbc.M213298200

Isam Qahwash‡, Katherine L. Weiland‡, Yifeng Lu‡, Ronald W. Sarver§, Rolf F. Kletzien‡, and Riqiang Yan‡†‡

From the Departments of ‡Cell & Molecular Biology and §Analytical Chemistry, Pharmacia Corporation, Kalamazoo, Michigan 49007

The amyloid peptide (Aβ), derived from the proteolytic cleavage of the amyloid precursor protein (APP) by β- and γ-secretases, undergoes multistage assemblies to fibrillar deposits in the Alzheimer’s brains. Aβ protofibrils were previously identified as an intermediate preceding insoluble fibrils. While characterizing a synthetic Aβ variant named EV40 that has mutations in the first two amino acids (D1E/A2V), we discerned unusual aggregation profiles of this variant. In comparison of the fibrillogenesis and cellular toxicity of EV40 to the wild-type Aβ peptide (Aβ40), we found that Aβ40 formed long fibrillar aggregates while EV40 formed only protofibrillar aggregates under the same in vitro incubation conditions. Cellular toxicity assays indicated that EV40 was slightly more toxic than Aβ40 to human neuroblastoma SHEP cells, rat primary cortical, and hippocampal neurons. Like Aβ40, the neurotoxicity of the protofibrillar EV40 could be partially attributed to apoptosis since multiple caspases such as caspase-9 were activated after SHEP cells were challenged with toxic concentrations of EV40. This suggested that apoptosis-induced neuronal loss might occur before extensive depositions of long amyloid fibrils in AD brains. This study has been the first to show that a mutated Aβ peptide formed only protofibrillar species and mutations of the amyloid peptide at the N-terminal side affect the dynamic amyloid fibrillogenesis. Thus, the identification of EV40 may lead to further understanding of the structural perturbation of Aβ to its fibrillation.

Alzheimer’s disease (AD) is the most common age-related neurodegenerative disorder. Extracellular amyloid plaques and intracellular neurofibrillary tangles are two typical pathological lesions of AD brains. Amyloid plaques, or neuritic plaques, which are more related to the AD pathogenesis, mainly consist of a cluster of heterogeneous amyloid peptides (Aβ) ranging from 39 to 43 amino acids (1–2). Among these, the Aβ with 40 amino acids (Aβ40) accounts for about 90% while the less soluble C-terminally extended Aβ42 is close to 10%. The hyperphosphorylation of tau, a microtubule binding protein, leads to the formation of neurofibrillary tangles (3–6). Studies with transgenic mice show that Aβ promotes the formation of paired helical tau filaments (7–8). Thus far, considerable experimental data favor the hypothesis that amyloid depositions in patient brains are one of the etiological factors causing AD dementia (9).

Aβ peptides are derived from consecutive processing of the amyloid precursor protein (APP) by two endopeptidases: β- and γ-secretases. A membrane-bound aspartyl protease, named BACE1, was simultaneously identified as the β-secretase (10–13). The molecular identity of γ-secretase has not been fully revealed yet. Nevertheless, the transmembrane protein presenilin 1 seems indisputably required for the release of the amyloid peptide from its precursor (see reviews in Ref. 14). Pathogenic studies have manifested that the majority of mutations in APP, presenilin 1, or presenilin 2, identified from the early-onset familial AD patients, increase either total production of Aβ or the proportion of Aβ42 (reviewed in Ref. 15).

Monomeric Aβ, when the critical concentration is reached, quickly folds into aggregated intermediate species such as oligomeric and protofibrillar forms, and finally, into insoluble fibrillar aggregates (16). Biophysical studies suggest that all these forms of Aβ are in equilibrium (17–18). Increased production of Aβ peptides, particularly Aβ42, promotes amyloid fibrillogenesis and deposition in the limbic system (19–20). In situ characterizations of brain tissues confirm the presence of aggregated Aβ fibrils (21) and SDS stable Aβ oligomers have been found in brains of Alzheimer patients (22). Earlier studies showed that insoluble Aβ fibrils are toxic to neurons in vitro and are associated with neuronal damage in vivo (23–26). Recently, studies using different approaches have demonstrated that both Aβ oligomers (27–28) and protofibrils (29–30) are neurotoxic as well.

During the optimization of substrates for measuring β-secretase activity in cells, we generated several APP mutants including APPΔ57 (K670N/M671I/D672E/A673V) and APPΔ85 (V699F/K670S/M671L/D672E/A673V). Expression of these two constructs in cells showed increased processing of mutant APP at the β-secretase site (31) and produced mutated (D1E/A2V) amyloid peptides that we named EV40 and EV42 to distinguish them from the wild-type Aβ40 and Aβ42. By examining the cell culture carefully, we found that stable cell lines expressing high levels of these two APP variants were less healthy than those expressing wild-type or Swedish APP. To understand whether the mutated residues in Aβ would change the properties of amyloid peptides and therefore cause the stress to the cell growth, we examined morphological structures and cellular toxicity of EV40 and EV42. We found that EV40 predominantly formed short, curvy, and sticky protofibrils that were similar to the intermediates of natural Aβ40 fibrils. Furthermore, this form of protofibrils was slightly more toxic to
cultured SHEP cells and neurons than Aβ40. The work reported here has been the first to show that the N-terminal sequence of Aβ affects the rate and state of amyloid fibrillogenesis. This may lead to further exploration of the kinetics of amyloid aggregation affected by cellular factors that may potentially interact with the N-terminal end of Aβ.

**MATERIALS AND METHODS**

**Peptide Synthesis—**Amyloid β-(1–40) (Aβ40) was either purchased from Polypeptide Laboratories (Torrance, CA), or together with its mutant, EV40 (DIE/A2V), were synthesized by solid-phase methods employing an Applied Biosystems Model 433A Peptide Synthesizer. Crude peptide was dissolved in 0.05% trifluoroacetic acid in water and loaded onto a preparative reverse phase HPLC column (Vydac C-18, 22 × 250 mm, 10 micron) with a flow rate of 4 mL/min and equilibrated with solvent A (0.1% trifluoroacetic acid in water). The column was developed with a linear gradient employing solvents A and B (0.07% trifluoroacetic acid in solvent A (0.1% trifluoroacetic acid in water). The column eluent was monitored by absorbance at 220 and 280 nm. Fractions were monitored on an analytical reverse phase system (Vydac C18, 4.6 × 250 mm, 5 micron); solvents and conditions were as above. A linear gradient from 0–70% B over 20 min at 1.0 mL/min was employed for this purpose. The chemical authenticity of each peptide was established by mass spectrometry employing Micromass Platform II mass spectrometer equipped with a Hewlett Packard Series 1050 HPLC system. The identity of the peptide was confirmed by injecting 5 μL of sample into the flow of 100 μL/min of 1:1 methanol/water. The mass spectrometer was operated in electrospary ionization mode with needle voltage 3 kV, temperature 120 °C, and cone voltage 30 V. The identity of the each peptide was confirmed by amino acid sequencing of the synthetic peptides.

**Turbidity Assays of Amyloid Aggregation—**Turbidity assays were carried out as previously described (32). Briefly, a 1 mM stock was made by dissolving the peptide in 0.22-μm filter-sterilized 0.1% acetic acid then diluted 1:20 in calcium and magnesium-free Dulbecco’s phosphate buffered saline to a final concentration of 50 μM. Aliquots of the peptide solutions (250 μL) were transferred to wells of Corning 96-well tissue culture plates, and the wells were tightly sealed with an adhesive sealer. The plates were constantly shaken at 800 oscillations/min on a Titer Plate Shaker (Lab-Line Instruments, Inc., Melrose Park, IL) to induce aggregation. Monitoring the aggregation process was accomplished by measuring the optical density of each well at 405 nm using a SpectraFluor Plus plate reader exciting at 485 nm and emitting at 535 nm. Alternatively, cellular toxicity was also determined by LDH assay (Promega, Madison, WI), measuring the release of the cytosolic lactate dehydrogenase at 492 nm. Western Blot Analysis of Caspase Activation—Following amyloid challenge, cells were harvested and lysed in MAFK lysis buffer on ice (20 mM HEPES (pH 7.3), 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM sodium orthovanadate, 0.1 mM molybdc acid, 10 mM MgCl2, 10 mM β-glycerophosphate, 5 mM p-nitrophenylphosphate, 1 mM phenylmethylsulfonfluoride, 0.5% Nonidet P-40, 1 mM Na3 VO4, 5 μg/mL leupeptin, 5 μg/mL pepstatin, 0.05% 2-mercaptoethanol). The lysed cells were centrifuged at 10,000 × g for 10 min to remove cellular debris, and nuclei and cell lysates were then quantified based on protein concentrations. Equivalent protein samples, representing ~7 × 104 cells, were electroblotted on 4–12% Bis-Tris NuPAGE gel (Invitrogen, Carlsbad, CA). Following electrophoresis, proteins were transferred to an Immobilon-P membrane for Western analysis (Millipore, Bedford, MA). Processing/activation of caspases was evaluated by incubating with anti-caspase antibody (0.5 μg/mL, Santa Cruz Biotechnology, Santa Cruz, CA) or anti-caspase-9 (1:1000 dilution, PharMingen, San Diego, CA). Following incubation, the appropriate horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology), immunoreactivity was detected by chemiluminescence using SuperSignal West PICO reagent (Pierce).

**RESULTS**

**EV40 Displayed Different Aggregative Profiles from Aβ40 Based on Turbidimetric Assays—**Aβ fibrillogenesis is a multi-step process involving nucleation, elongation, and aggregation. The kinetics of Aβ fibrillogenesis has been previously monitored by various approaches including turbidimetry (32). To gain a quick assessment of the EV40 aggregation, we compared kinetic aggregation profiles of EV40 to Aβ40 by turbidimetric assays. As shown in Fig. 1, after a short duration of lag phase (~40 min) under constantly controlled shaking, Aβ40 solutions quickly became turbid. The peak of the turbidity, which reflects larger aggregation of the testing peptide, was reached at about 180-min postinitiation of shaking. Nevertheless, turbidity in
the wells containing EV40 increased at a much slower rate (Fig. 1), suggesting that these two peptides aggregated with distinct kinetics of nucleation and oligomerization. At 360-min postinitiation of shaking, the maximum turbidity of the EV40-containing wells was significantly less than that of the Aβ40-containing wells. This observation implicated that both peptides had distinct aggregation rates, and, therefore, prompted us to examine the properties of EV40 more carefully.

**EV40 Predominantly Formed Protofibrils While Aβ40 Assembled to Long Fibrils**—The morphology of Aβ fibrils has been well documented by the approaches of electron microscopy, atomic-force microscopy, etc. Since EV40 had a much slower aggregation rate based on the turbidimetric assays, we suspected that the biophysical parameters of EV40 might be different from Aβ40. To address this, we compared the morphological structures of these two peptides in parallel by electron microscopy. In our initial experiment, we allowed the peptides to aggregate at 37 °C for 48 h followed by examining the fibrillar formation. Under these conditions, Aβ40 formed long, rigid, and extended fibrils ~5–10 nm in diameter that could be longer than 500 nm in length (Fig. 2A), consistent with the observations summarized previously (17). Under the same conditions, EV40 did not form long and rigid fibrils as seen in the Aβ40 preparations (comparing Fig. 2, A with B). Instead, the morphological structures found in the EV40 preparations were short and curvy with ~5 nm in diameter (Fig. 2B), and ring-like structures were evident. Frequently, these short protofibrils tended to associate with each other, but did not assemble into long fibrils. In general, the morphological structure of EV40 was similar to that of the Aβ protofibrils, identified as an intermediate that will progress to long and straight fibrils after longer incubation (17, 34). The EM morphology of EV40 also resembled the short protofibrils reported for a sample of Aβ40 in the presence of apomorphine (35). Thus, EV40 seemed to form an aggregated structure resembling the intermediate protofibrillar aggregates of natural Aβ40.

We then conducted more careful studies focusing on the dynamic protofibrillar progression of EV40 and Aβ40. Without incubation at 37 °C, no identifiable protofibrils or long fibrils were observed from freshly dissolved and negatively stained Aβ40 (Fig. 2C). Occasionally, we noticed a bead-like structure in freshly dissolved Aβ40 (similar to that seen in the background of Fig. 2A). It is unclear whether it is related to the low molecular weight (LMW) oligomers of Aβ. On the contrary, EV40 formed smaller protofibrils (<100 nm in length) even without intentional incubation at 37 °C (Fig. 2D). If incubated for 4 h at 37 °C, Aβ40 proceeded to form short and irregular protofibrils (Fig. 2E), consistent with prior observations (17, 30, 34). Incubation of the EV40 solution at 37 °C for 4 h would allow short protofibrillar EV40 to grow into longer protofibrils (Fig. 2F). Interestingly, even being incubated up to 8 days at 37 °C, EV40 still formed curvy protofibrillar aggregates and never formed long and straight fibrils (shown in Fig. 2G) while Aβ40 typically formed rigid fibrils after incubation for about 48 h (Fig. 2A). It appeared that EV40 had a very short duration of nucleation and oligomerization, but quickly locked into the protofibrillar state during prolonged incubation.

Since synthetic peptides have been shown to have slight biophysical variations in batch-to-batch preparations, we repeated electron microscopy experiments using three different batches of EV40 to verify the above results. Again, we observed similar protofibrillar patterns using various batches of EV40, suggesting that the protofibrillar form of EV40 was not due to an unusual synthesis of this peptide. Aβ40 always produced fibrillar aggregates no matter whether Aβ40 peptide was purchased from a commercial source or synthesized in house employing the same preparative procedures for producing EV40.

**The Aggregative Progression Conformation of EV40 and Aβ40 by Circular Dichroism Spectroscopy**—To determine the biophysical natures of Aβ40 and EV40 peptides, we employed circular dichroism to monitor the structural transition of these two peptides during aggregation. A solution of freshly prepared Aβ40-contained peptide in a mostly random coil and antiparallel β-sheet conformation as determined by principal component analysis of the CD spectrum, 47% random coil, 21% β-turns, 31% antiparallel β-sheet, <5% parallel β-sheet, and α-helical structure. Little change in the CD spectrum of the solution was detected for 2 h at room temperature but incubation at 37 °C for 24 h resulted in slightly greater negative ellipticity at 215–230 nm as shown in Fig. 3A. Incubation for another 24 h produced additional spectral alterations consistent with increased antiparallel β-sheet.

Unlike Aβ40, a freshly prepared solution of EV40 showed little conformational change for 72 h at 37 °C (Fig. 3B). Interestingly, the CD spectra of EV40 were also very similar to the spectrum of Aβ40 collected after 24 h at 37 °C. Similar conformation of EV40 with that of a protofibrillar aggregate of Aβ40 was consistent with the electron microscopy observations sug-
Mutant Amyloid Peptide Forms Toxic Protofibrils

23190

suggesting that EV$_{40}$ formed an aggregated structure similar to the intermediate protofibrillar aggregates of A$\beta_{40}$.

A comparison of CD spectra for A$\beta_{40}$ and EV$_{40}$ dissolved in water at 200 $\mu$m is shown in Fig. 3C. Initial spectra and spectra collected after 72 h incubation at 37 °C are shown. Using a shorter pathlength than used for the previous experiments, spectral data could be collected to lower wavelengths for these solutions Me$_2$SO that absorb at lower wavelengths. The same trend was evident as was detected at lower peptide concentrations. A large reduction in random coil with concomitant increase in $\beta$-sheet conformation was detected for A$\beta_{40}$ upon incubation and although there was less random coil conformation in EV$_{40}$ initially, there was less change in conformation after incubation. EV$_{40}$ contained more $\beta$-sheet structure than A$\beta_{40}$ initially as indicated by the ratio of ellipticities at 208 compared with 215 nm. Again, this is consistent with the other data that indicates EV$_{40}$ forms protofibrils soon after dissolution but then undergoes a delay in elongated fibril formation.

$EV_{40}$ Suppresses Fibrillation of A$\beta_{40}$—Growth of A$\beta_{40}$ fibrils can be affected by the presence of various reagents including short peptides (35–36) and modification of residues within the A$\beta$ region (37). To determine whether EV$_{40}$ would interfere with the fibrillar assemblies of A$\beta_{40}$, we mixed different ratios of A$\beta_{40}$ to EV$_{40}$ in the same tube and examined the morphological structures of the mixtures. When an equal amount of EV$_{40}$ and A$\beta_{40}$ powders were mixed in a tube and dissolved using the same procedure described for the above electron microscopy experiment, we found that the morphological structure of the mixture was more similar to EV$_{40}$ preparations (Fig. 4A). The purities of each individual peptide and the mixed peptides used for the above EM experiments were verified by MALDI-TOF mass spectra (Fig. 4, B–D). This suggested that EV$_{40}$ might have suppressed fibrillation of A$\beta_{40}$. However, this suppressive effect was not dominant since an increased proportion of A$\beta_{40}$ (e.g. 80% of A$\beta_{40}$) would increase the long fibrillar aggregates in the preparations (data not shown). Thus, EV$_{40}$ could interfere with the assembly of A$\beta_{40}$ into long fibrils.

$EV_{40}$ Is Slightly More Neurotoxic Than A$\beta_{40}$—Previous experiments demonstrate that both A$\beta$ protofibrils and fibrils induce acute electrophysiological changes and are toxic to cortical neurons (30). However, it was difficult to compare A$\beta$ protofibrils and fibrils side by side due to limitations in the preparations of these materials. Here, we could compare the cellular toxicity of EV$_{40}$ protofibrils with A$\beta_{40}$ fibrils at the same concentrations. Prior to the treatment, we first incubated solutions of EV$_{40}$ and A$\beta_{40}$ at 37 °C for 48 h to allow aggregation, and then added the aggregated peptides to cultured human neuroblastoma SHEP cells. After cells were treated for 2 days, the cell viability was examined by assays using Sytox green nucleic acid fluorescent stains (38–39). With the increase of either EV$_{40}$ or A$\beta_{40}$ concentrations, more cells showed toxicity (Fig. 5A, ** indicates $p < 0.0001$ and * indicates $p < 0.005$ relative to control). Cells treated with A$\beta_{40}$ (50 $\mu$m) had $\sim$78% cell death, whereas the cells treated with the same concentration of EV$_{40}$ showed $\sim$86% cell death ($p = 0.2$, A$\beta_{40}$ versus EV$_{40}$).

We also compared cellular toxicity in SHEP cells treated with either freshly dissolved (without incubation as indicated with preaggregated) or aggregated (preincubation at 37 °C for 48 h) peptide solutions. Cells treated with freshly dissolved EV$_{40}$ showed significantly higher cellular toxicity than A$\beta_{40}$ to

Fig. 2. Electron micrographs of A$\beta_{40}$ or EV$_{40}$ aggregates. Negatively stained samples were prepared for examination by electron microscopy after incubation of A$\beta_{40}$ for 48 h (A), 0 h (C), and 4 h (E) or EV$_{40}$ for 48 h (B), 0 h (D), 4 h (F), and 8 days (G). The scale bar represents 50 nm. The inserted image in panel F reflects another typical structure under this condition. A negative surface image was presented in panel H.
SHEP cells (p < 0.0001, Aβ40 versus EV40), determined both by LDH release into the medium (Fig. 5B) as well as Sytox green fluorescence (Fig. 5C). This difference was clearly consistent with the EM experiments that the non-incubated Aβ40 presumably consists of both monomers and oligomers while EV40 contains mainly short protofibrils (see Fig. 2, C and D).

Addition of aggregated Aβ40 into cultured SHEP cells showed a large increase in cellular toxicity compared with non-aggregated Aβ40, suggesting that SHEP cells were more vulnerable to aggregated Aβ40 than monomeric Aβ40. Further incubation of EV40 only slightly increased its cellular toxicity (Fig. 5B and C), indicating that short protofibrils of EV40 were sufficient to kill SHEP cells. Altogether, these assays suggested that protofibrillar EV40 exhibited modest but consistently higher toxicity than fibrillar Aβ40 to SHEP cells in multiple independent experiments.

To confirm that EV40 is slightly more toxic to cells than Aβ40, we repeated the study with cultured rat primary neurons prepared from the cortex or hippocampus. Similar to the observations in SHEP cells, we found that EV40 treatment caused an average of 85% death of rat primary neurons (p < 0.0001) while the same concentration of fibrillar Aβ40 resulted in 67% neuronal death on average (p < 0.0001) (Fig. 5D). Hence, EV40 did appear to be more toxic, although not substantially, to both human neuronal cells and rat primary neurons when compared with the Aβ40 treatment.

Activation of Caspases by EV40 Protofibrils—Neuronal toxicity induced by amyloid peptides has been partially attributed to programmed cell death (40–41). Activation of caspase-2, -3, -6, -8, -9, and -12 in different neuronal cells by amyloid peptides have been demonstrated (42–45). Although we observed cellular toxicity after SHEP cells were treated with either Aβ40 or EV40, it was unclear whether this toxicity could be ascribed to apoptotic death. To explore this, we treated SHEP cells with either Aβ40 or EV40 in parallel and examined the activation of multiple caspases. Under our treatment conditions, we found that dramatic reductions of procaspase-9 with concomitant increases in the smaller active form were induced by either fibrillar Aβ40 or protofibrillar EV40 (Fig. 6), suggesting that both protofibrillar and fibrillar forms of amyloid peptides similarly activated caspases in the apoptotic pathway. We also observed similar activations of caspase-2, -3, and -8 by both Aβ40 and EV40 (data not shown). Likely, activation of caspase-9 occurred first and the activated caspase-9 in turn triggered sequential activation of downstream caspase-3, -2, and -8 (46). Apoptosis induced by protofibrillar amyloid species might implicate that AD neurons could be vulnerable to insult from intermediate amyloid aggregates, probably before the formation of amyloid plaques.

**DISCUSSION**

Assembly of amyloid fibrils occurs in multiple stages: monomers, dimers, tetramers, oligomers, protofibrils, and fibrils. Turbidimetric assays that were previously used to monitor the progression of amyloid fibrillogenesis (32, 47) may not fully reflect the authentic kinetics of Aβ fibrillogenesis. It has been shown that partial monomeric Aβ quickly folded into dimers, trimers, and oligomers during the period of preparations for the
assays at either room temperature or 37 °C after the peptide was freshly dissolved. Assembly of EV₄₀ to protofibrils occurred almost immediately after the EV₄₀ peptide was dissolved in 0.1% acetic acid solution followed by dilution with either phosphate-buffered saline (Fig. 2) or serum-free conditioned medium (data not shown). However, there was no significant increase in turbidity of the EV₄₀ solution even 6-h post-constant shaking while turbidity of the Aβ₄₀ solution peaked after 3 h (Fig. 1). Likely, short protofibrils should be considered to be partially soluble. Our data implicated that the increased turbidity might only reflect the formation of large aggregated amyloid fibrils while the increased formation of oligomers

**Fig. 4.** EV₄₀ interferes with formation of long Aβ₄₀ fibrils. A, electron micrograph of Aβ₄₀ and EV₄₀ aggregates. Equal amounts of EV₄₀ and Aβ₄₀ powders were mixed, dissolved, and then incubated at 37 °C for 48 h. The sample was negatively stained for electron microscopy. The scale bar represents 50 nm. B–D, MALDI/MS analysis of Aβ₄₀, EV₄₀, and a 1:1 Aβ₄₀ to EV₄₀ mixture.
Mutant Amyloid Peptide Forms Toxic Protodfibrils

A human neuroblastoma SHEP cells were treated with three concentrations (50, 25, and 12.5 μM) of either EV40 or Aβ40 that was preincubated at 37°C for 48 h to allow aggregation. The viability of cells was determined using the Sytox Green fluorescence assay that is based on the permeability of fluorescent stain into dying cells. (**, p < 0.001; *, p < 0.005).

B and C, freshly dissolved peptide solutions (50 μM) either without incubation (0 h incubation) or preaggregated (incubation at 37°C for 48 h) were added to cultured human SHEP cells, and this treatment was allowed to proceed for 2 days. The neurotoxicity was measured by LDH assay that is based on the release of the enzyme into the cultured media or Sytox green fluorescence assay (**, p < 0.0001; *, p < 0.001).

D, rat primary cortical and hippocampal neurons were treated with preaggregated EV40 and Aβ40 (50 μM, incubation at 37°C for 48 h) for 2 days. The viability of the primary neurons was determined by Sytox Green fluorescence assay (**, p < 0.0001; *, p < 0.001).

Many factors including pH, temperature, salt, and changes of residues have been shown to affect the course of Aβ fibrillogenesis (18, 53). Earlier studies focusing on the C-terminus of Aβ show that residues 34–42 are critical for the seeding and subsequent evolution of amyloid aggregates (19, 32, 54–55). The central region of Aβ also plays a critical role in the progression to mature insoluble fibrils. Residues 16–20 were identified to serve as a binding motif during polymerization (36). Studies of peptides containing Flemish (A21G), Arctic (E22G), and Dutch (E22Q) mutations suggest that these residues greatly affect the rate or the state of fibrillar aggregations (28, 34, 45, 56–58). For example, Flemish Aβ40 (A21G) does not form protodfibrils (54) while Arctic Aβ forms short and straight protodfibrils (58). Previously, the N-terminus of Aβ has been considered less crucial in affecting the course of fibrillogenesis. For example, N-terminally truncated Aβ (starting at Glu3, Phe4, Arg5, Gly8, Glu11, Val12, or Leu17) does not suppress, but instead enhances in some cases, aggregation in vitro (59–60). In this study, we found that mutation of the first two amino acids of Aβ (D1E/A2V) had a profound effect on the course of amyloid fibrillogenesis as shown in Fig. 2 (Aβ40 versus EV40). Although peptides with the first few N-terminal residues deleted still form long amyloid fibrils as shown by Tekirian et al. (61), the presence of a bulky side chain at Aβ residue 2 could potentially alter the rate of protodfibrillar extension as discussed in this study.

Nichols et al. (62) have suggested that growth of amyloid protodfibrils can be achieved in two ways: elongation of protodfibrils by monomer deposition and lateral association of protodfibrils. An increase in NaCl concentration promotes protodfibril
of SHEP cells grown in the presence of 10% serum (apoptosis, one plate of SHEP cells was irradiated with UV light. A plate from the resin in two peptide synthesis facilities where A explain our earlier observations that cells expressing high lev-

aggregation in the peptide synthesis resin. This could also be detrimental to cell growth considering the likelihood of its properties of EV40. It is likely that EV 42 could be even more prone to die when they reached moderately high confluency.

A residue (Val) to this mutant peptide had a profound effect on the solubility or folding of this mutated peptide was at least altered. We found that both EV41 and EV42 could be synthesized by standard solid-phase methods, but could not be purified from the resin in two peptide synthesis facilities where 3 represents anti, and * represents an alternative splicing procaspase-9 variant in SHEP. Serum starvation could trigger weak activation of caspase as shown in Opti-MEM I control lane.

lar extension via lateral association of protofibrils while addition of monomeric Aβ favors elongation of protofibrils by monomer deposition. We found that EV40 forms smaller protofibrils almost immediately after the peptides were dissolved. It is likely that the concentration of monomeric EV40 became scarce shortly after the peptide was dissolved and this might suppress the elongation of EV40 protofibrils. Alternatively, the salt concentration was not optimal for promoting lateral asso-

association of EV40 protofibrils. Thus, more careful studies are necessary to gain insight into the mechanism of protofibrillar extension.

Although it still remains unclear how the bulkier Glu-Val side chains impact the structure of Aβ in this region, the solubility or folding of this mutated peptide was at least altered. We found that both EV41 and EV42 could be synthesized by standard solid-phase methods, but could not be purified from the resin in two peptide synthesis facili-

ties where 3 reports anti, and * represents an alternative splicing procaspase-9 variant in SHEP cells, and the same blot was subsequently stripped and re-probed with anti-actin. The levels of α-actin (43 kDa) verified equal loading of individual samples. Serum starvation could trigger weak activation of caspase as shown in Opti-MEM I control lane.

and to dissolve already formed fibrils (63).

Moreover, the fibrillogenesis of EV40 apparently differs from Arctic Aβ40. Arctic Aβ40 (E22Q) was reported to increase the rate and quantity of protofibrillar species compared with wild-type Aβ40 (58). Nonetheless, Arctic Aβ40 continues to form long fibrillar species during longer incubation while EV40 did not form long and straight fibrils during extended incubation for up to 8 days.

One of the key issues in validating the “amyloid hypothesis” is whether Aβ is detrimental to neurons. The proteinaceous components and compositions in the neuritic plaques are highly complicated. Technically, it is not possible yet to perform a neurotoxicity study by reconstituting in vivo situations using synthetic materials. Transgenic mice expressing only human APP variants fail to replicate AD pathological lesions (64). However, cellular toxicity studies using a single component (e.g. Aβ30 or Aβ40) have been extensively addressed to link Aβ fibrils to neuronal or synaptic loss seen in AD brains. Aβ in the range of 20–100 μM has been shown to be toxic to neurons (23–26), but no single mechanism can account for this induced neuronal death (66). In this study, we found that EV40 was moderately more toxic to both human neuroblastoma SHEP cells and rat primary neurons than wild type Aβ40. Hartley et al. (30) treated mixed brain cultures with protofibrillar Aβ40 that was prepared from size-exclusion chromatography and found that their protofibrillar Aβ40 was not as toxic as their long fibrillar Aβ40. This discrepancy could be related to the differences in initial doses of protofibrillar Aβ40 and fibrillar Aβ40 in their treatments. However, they demonstrate that protofibrillar Aβ40 causes increased frequency of action potentials and membrane depolarizations (30). Here we reported that multiple caspases in SHEP cells were activated by protofibrillar EV40. Since the protein levels of caspase-12 in SHEP cells are below our detection limit, we treated breast carcinoma MCF-7 cells with aggregated peptides and found that both protofibrillar EV40 and long fibrillar Aβ40 similarly activated caspase-12 (data not shown). Altogether, these results implicated that protofibrillar Aβ40 might play similar roles to fibrillar Aβ40 in affecting neuronal functions such as neurotransmitter release/uptake, long term potentiation, oxidative stress and neuronal survival. Consistent with this, Aβ oligomers have been shown to disrupt synaptic plasticity and cause neuronal toxicity (27–28). Thus, blocking initial nucleation of Aβ, rather than dissolution of amyloid fibrils or inhibiting later stages of fibrillogenesis, may be a more relevant approach for AD treatment.

Thus far, clinical APP mutations at the N-terminal end of Aβ region have not been identified yet. The data from this study suggested that a D1E/A2V mutation in the Aβ peptide could be severe. Mouse genetic studies with this mutation would provide valuable insight in this aspect as well.

**Acknowledgments**—We thank Dr. Dennis J. Selkoe (Brigham and Women’s Hospital) for helpful suggestions and Dr. Hilal A. Lashuel (Brigham and Women’s Hospital) for the critical reading of the manuscript; Nancy C. Stratman and Donald B. Carter (Pharmacia Corp.) for the help in turbidimetric assay; Carol A. Bannow and Clark W. Smith (Pharmacia Corp.) for the synthesis of amyloid peptides; Eric T. Lund (Pharmacia Corp.) for the assistance in MALDI-TOF mass spectra; John T. Stout and Robert R. Eversole (Western Michigan University) for the assistance in electron microscopy experiments.

**REFERENCES**

1. Glenner, G. G., and Wong, C. W. (1984) Biochem. Biophys. Res. Commun. 122, 1131–1135
2. Masters, C. L., Simms, G., Weinman, N. A., Multhaup, G., McDonald, B. L., and Beyreuther, K. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 4245–4249
3. Grundke-Iqbal, I., Iqbal, K., Quinlan, M., Tung, Y. C., Zaidi, M. S., and Wisniewski, H. M. (1986) J. Biol. Chem. 261, 6084–6089
4. Ihara, Y., Nukina, N., Miura, R., and Ogawara, M. (1986) J. Biochem. (Tokyo) 99, 1807–1810
Mutant Amyloid Peptide Forms Toxic Protofibrils

5. Kosik, K. S., Joachim, C. L., and Selkoe, D. J. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 4044–4048
6. Wood, JG., Mirra, S. S., Pollock, N. J., and Binder, L. I. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 4040–4043
7. Gotz, J., Chen, F., van Dorpe, J., and Nitsch, R. M. (2001) Science 293, 1491–1495
8. Lewis, J., Dickson, D. W., Lin, W. L., Chisholm, L., Corral, A., Jones, G., Yen, S. H., Sahara, N., Skipper, L., Yager, D., Eckman, C., Hardy, J., Hutton, M., and Mckewn, E. (2001) Science 293, 1487–1491
9. Hardy, J., and Selkoe, D. J. (2002) Science 297, 353–357
10. Vassar, R., Bennett, B. D., Babi-Khan, S., Kahn, S., Mendiaz, E. A., Denis, P., Teplow, D. B., Ross, S., Amarante, P., Leof, R., Luo, Y., Fischer, S., Fuller, J., Edendorf, S., Lile, J., Jaroniszki, M. A., Bierie, A. L., Curran, E., Burgess, T., Louis, J. C., Collins, P., Treanor, J., Rogers, G., and Citron, M. (1999) Science 286, 735–741
11. Yan, R., Bienkowski, M. J., Shuck, M. E., Xiao, H., Tomy, M. C., Paul, A. M., Braschler, J. R., Stratman, N. C., Mathews, W. B., Buhl, A. E., Carter, D. B., Tomasselli, A. G., Parodi, L. A., Heinrikson, R. L., and Gurney, M. E. (1999) Nature 402, 533–537
12. Hussain, I., Powell, D., Howlett, D. R., Tew, D. G., Meek, T. D., Chapman, C., Gloger, I. S., Murphy, K., Southan, C. D., Ryan, D. M., Smith, T. S., Simmons, D. L., Walsh, F. S., Dingwall, I. C., and Christie, J. (2001) Mol. Cell Neurosci. 14, 419–427
13. Sinha, S., Anderson, J. P., Barbour, R., Basi, G. S., Casaccella, R., Davis, D., Dean, M., Doeye, H. F., Frigon, N., Hong, J., Jacobson-Croak, K., Jewett, N., Keim, P., Knops, J., Lieberburg, I., Power, M., Tan, H., Tatsuno, G., Tung, J., Schenk, D., Seubert, P., Soumassa, S. M., Wang, S., Walker, D., and John, Y. (1999) Nature 402, 537–540
14. Sisodia, S. S., and St George-Hyslop, P. H. (2002) Nat. Rev. Neurosci. 3, 281–290
15. Haass, C. (1996) Curr. Opin. Neurol. 9, 254–259
16. Koo, E. H., Lansbury, P. T., Jr., and Kelly, J. W. (1999) Science 285, 1336–1340
17. Harper, J. D., Wng, S. S., Lieber, C. M., and Lansbury, P. T., Jr. (1997) Biochemistry 36, 8972–8980
18. Teplow, D. B. (1998) Amyloid 5, 121–142
19. Suzuki, N., Chong, T. T., Cai, X. D., Odaka, A., Orlos, L. J., Eckman, C., Golde, T. E., and Younkin, S. G. (1994) Science 264, 1336–1340
20. Tomasselli, A. G., Qahwash, I., Emmons, T. L., Lu, Y., Leune, J. W., Lull, J. M., Fok, K. F., Bannow, C. A., Smith, C. W., Bienkowski, M. J., Heinrikson, R. L., and Yan, R (2003) J. Neurochem. 84, 1006–1017
21. Jarrett, J. T., Berger, E. P., and Lansbury, P. T. Jr. (1993) Biochemistry 32, 4693–4697
22. Hennessey, J. P., Jr., and Johnson, W. C., Jr. (1991) Biochemistry 20, 1085–1094
23. Walsh, D. M., Lomakin, A., Benedek, G. B., Condron, M. M., Selkoe, D. J., and Teplow, D. B. (1997) J. Biol. Chem. 272, 22364–22372
24. Lashuel, H. A., Hartley, D. M., Balakhranoez, A., Aggarwal, A., Teiehbier, S., and Teplow, D. B. (2001) J. Biol. Chem. 276, 8545–8548
25. Tjernberg, L. O., Naslund, J., Lindquist, F., Johansson, K., Karlstrom, A. R., Thyberg, J., Tenerz, L., and Nordstedt, C. (1996) J. Biol. Chem. 271, 1710–1719
26. Hou, L., Kang, I., Marchant, B. E., and Zegerski, M. G. (2002) J. Biol. Chem. 277, 40173–40176
27. Cheung, N. S., Beart, P. M., Pascoc, C. J., John, C. A., and Bernard, O. (2000) Science 288, 1711–1714
28. Luo, J. L., Wallace, M. S., Hawver, D. B., Kusiak, J. W., and Wallace, W. C. (2001) J. Neurosci. Res. 63, 410–420
29. Anderson, A. J., Su, J. H., and Cotman, C. W. (1996) J. Neurosci. 16, 1710–1719
30. Estus, S., Tucker, H. M., van Rooyen, C., Wright, S., Brigham, E. F., Wogulis, M., and Rydel, R. E. (1997) J. Neurosci. 17, 7736–7745
31. Allen, J. W., Eldelah, B. A., Huang, X., Knochel, S. M., and Faden, A. I. (2001) J. Neurosci. Res. 65, 45–53
32. Marin, N., Romero, B., Bosch-Morell, P., lanzilla, M., Felipo, V., Roma, J., and Romero, F. J. (2000) Mech. Ageing Dev. 119, 63–67
33. Nakagawa, T., Zhu, H., Morishima, N., Li, E., Xu, Y., Yankner, B. A., and Yuan, J. (2000) Nature 403, 98–103
34. Tran, C. M., Rabacchi, S. A., Friedman, W. J., Frappier, T. F., Brown, K., and Shelanski, M. L. (2000) J. Neurosci. 20, 1386–1392
35. Soto, C., Kindy, M. S., Baumann, M., and Frangione, B. (1996) Biochem. Biophys. Res. Commun. 226, 1072–1080
36. Barlow, J., and Selkoe, D. J. (1993) Biochemistry 32, 1427–1434
37. Romaneu, M., and Labeur, C. (2001) J. Neurosci. Res. 71, 144–152
38. Price, D. L., and Sisodia, S. S. (1998) Annu. Rev. Neurosci. 21, 493–505
39. Yu, J., and Yankner, B. A. (2000) Nature 407, 802–809
Identification of a Mutant Amyloid Peptide That Predominantly Forms Neurotoxic Protofibrillar Aggregates
Isam Qahwash, Katherine L. Weiland, Yifeng Lu, Ronald W. Sarver, Rolf F. Kletzien and Riqiang Yan

J. Biol. Chem. 2003, 278:23187-23195.
doi: 10.1074/jbc.M213298200 originally published online April 8, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M213298200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 63 references, 28 of which can be accessed free at http://www.jbc.org/content/278/25/23187.full.html#ref-list-1