Neurodegeneration Induced by β-Amyloid Peptides in vitro: The Role of Peptide Assembly State

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The progressive neurodegeneration of Alzheimer's disease has been hypothesized to be mediated, at least in part, by β-amyloid protein. A relationship between the aggregation state of β-amyloid protein and its ability to promote degeneration in vitro has been previously suggested. To evaluate this hypothesis and to define a structure–activity relationship for β-amyloid, aggregation properties of an overlapping series of synthetic β-amyloid peptides (βAPs) were investigated and compared with βAP neurotoxic properties in vitro. Using light microscopy, electrophoresis, and ultracentrifugation assays, we found that few βAPs assembled into aggregates immediately after solubilization, but that over time peptides containing the highly hydrophobic β29–35 region formed stable aggregations. In short-term neuronal cultures, toxicity was associated specifically with those βAPs that also exhibited significant aggregation. Further, upon the partial reversal of β1–42 aggregation, a concomitant loss of toxicity was observed. A synthetic peptide derived from a different amyloidogenic protein, islet amyloid polypeptide, exhibited aggregation but not toxicity, suggesting that βAP-induced neurotoxicity in vitro is not a nonspecific reaction to aggregated protein. The correlation between βAP aggregation and neurotoxicity was also observed in long-term neuronal cultures but not in astrocyte cultures. These data are consistent with the hypothesis that β-amyloid protein contributes to neurodegeneration in Alzheimer's disease.

[Key words: Alzheimer's disease, β-amyloid, neurotoxicity, peptide assembly, aggregation, islet amyloid, hippocampal culture]

Alzheimer's disease (AD) is characterized neuropathologically by senile plaques, cerebrovascular amyloidosis, neurofibrillary tangles, and selective neuronal loss. Classic senile plaques, insoluble extracellular deposits primarily comprised of β-amyloid protein aggregates, are typically surrounded by dystrophic neurites and thus may facilitate neurodegeneration (Tanzi et al., 1989; Selkoe, 1991). In support of this position, recent evidence suggests that β-amyloid may contribute to the progressive neuronal loss of AD. Several reports indicate that the familial form of AD is associated with and presumably may be precipitated

by genetic mutations that yield amino acid substitutions adjacent to the β-amyloid region of the amyloid precursor protein (APP) (Chartier-Harlin et al., 1991; Goate et al., 1991; Murrell et al., 1991). These changes may facilitate the abnormal proteolysis of APP that is believed to result in β-amyloid deposition. In addition, β-amyloid protein has been shown to have neurotoxic properties both in vitro and in vivo.

In general, studies of β-amyloid's biological activities suggest that it may increase neuronal risk for degeneration and/or may be directly neurotoxic. Thus far, however, data are unavailable to define convincingly either the features of β-amyloid salient to its degenerative abilities or the mechanism(s) by which it acts. The β-amyloid protein consists of approximately 42 amino acid residues (Masters et al., 1985b; Kang et al., 1987) and is derived from both extracellular and transmembrane domains of APP, a normally occurring and widely distributed protein (Fig. 1). Soluble, synthetic β-amyloid peptides (βAPs) of varying lengths are reported to yield both trophic and toxic responses on CNS neurons in vitro. Hippocampal cultures treated with both truncated and full-length βAPs initially exhibit enhanced survival (Whitson et al., 1989, 1990; Yankner et al., 1990) and increased neurite outgrowth (Whitson et al., 1990; Pike et al., 1991b), but after several days in vitro can show greater neuronal degeneration than untreated controls (Yankner et al., 1990). In mature cortical cultures, soluble βAPs, although not directly toxic, do potentiate the toxicity caused by both exposure to glutamate (Koh et al., 1990; Mattson et al., 1992) and transient glucose deprivation (Copani et al., 1991). In vivo, β-amyloid is reported to be directly neurotoxic. Introduction of purified senile plaque cores into the rat hippocampus causes focal neuronal damage (Frautschy et al., 1991). Similarly, in vivo injection of β1–40 is reported to result in stable β-amyloid deposits accompanied by localized neurodegeneration (Kowall et al., 1991). This last finding is consistent with the observation that insoluble β-amyloid prevails within the AD brain (Wisniewski et al., 1989) and underscores the relevance of evaluating the activity of the insoluble form of β-amyloid.

Recently, several laboratories have reported that various synthetic βAPs form filamentous, β-sheet assemblies in vitro (Kirschner et al., 1987; Halverson et al., 1990; Barrow and Zagorski, 1991; Hilbich et al., 1991). The assembly phenomenon is reported to be dependent on the pH of βAP solutions (Barrow and Zagorski, 1991; Burdick et al., 1992), βAP concentration, and length of time in solution (Burdick et al., 1992). Although several β-amyloid fragments have been reported to form such assemblies, only βAPs that include a substantial portion of the transmembrane sequence assemble into aggregates that are stable at pH 7.4 and resistant to disruption by SDS (Burdick et
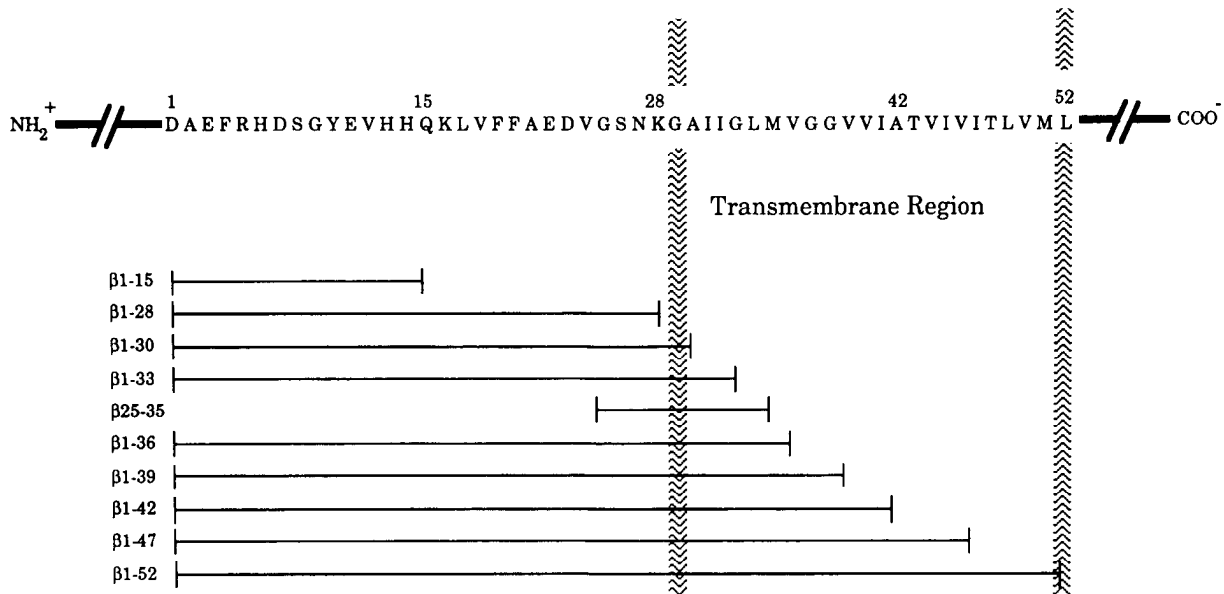
Figure 1. Utilized synthetic peptides correspond to portions of the β-amyloid protein. APP exists in several isoforms (695–751 amino acid residues), all of which contain the β-amyloid region that begins approximately 99 residues from the C-terminus (Kang et al., 1987). The 42 amino acid β-amyloid protein contains both hydrophilic (extracellular residues 1–28) and hydrophobic (transmembrane residues 29–42) domains. In this study, we employed an overlapping series of synthetic βAPs, ranging from a small 11 residue fragment (β25–35) to peptides extending beyond the full β-amyloid sequence (β1–47 and β1–52).

al., 1992). Thus, interactions between the hydrophobic transmembrane residues have been postulated to function in the stable formation of insoluble β-amyloid aggregates *in vitro* and may be related to *in vivo* amyloid deposition (Hilbich et al., 1991; Burdick et al., 1992).

Recently, we have also observed *in vitro* formation of insoluble β-amyloid assemblies, which resulted following incubation of β1–42 solutions (Pike et al., 1991b). Further, we reported a change in the biological activity of β1–42 solutions after this period of incubation. In agreement with a previous finding (Whitson et al., 1990), we found that initially soluble β1–42 enhanced neurite outgrowth in immature hippocampal cultures but was not toxic. However, β1–42 that had been preincubated not only exhibited considerable aggregation, but also caused a dose-dependent toxicity (Pike et al., 1991b). Similar incubation of β1–28 solutions resulted in neither stable aggregate formation nor toxicity, suggesting that both properties may be specific to hydrophobic βAPs (Pike et al., 1991a). In addition, we recently reported that the neurites of cultured neurons surround and infiltrate β1–42 aggregates and subsequently adopt dystrophic morphology similar to that observed in neurites associated with senile plaques in the AD brain (Pike et al., 1992). Although these findings suggest a potential link between βAP assembly and neurotoxicity, this relationship requires further investigation. In this study, we have examined the structure–function relationship between the aggregation and toxic properties of βAPs using a series of analogs. The presented data not only support the hypothesis that aggregated forms of βAPs contribute to neuronal degeneration, but also more clearly define the primary structures necessary for β-amyloid self-assembly.

Materials and Methods

Synthetic peptides. Peptides were synthesized by solid-phase F-moc amino acid substitution and purified by reverse-phase HPLC, as described elsewhere (Burdick et al., 1992). As previously discussed, high peptide

purity was attained by use of high coupling efficiencies during synthesis, and confirmed by peptide sequencing and electrospray mass spectrometry analyses following purification (Burdick et al., 1992). Peptides were lyophilized as HCl salts and stored in aliquots at −20°C until solubilization at a concentration of 250 μM in double-deionized water (ddH$_2$O). Selected samples of β25–35 were solubilized at 2.5 mM in 35% acetonitrile, 0.1% trifluoroacetic acid (TFA) and ddH$_2$O. Due to solubility constraints, the β1–47 and β1–52 peptides were initially solubilized at a concentration of 40 mg/ml in 1,1,1,3,3,3-hexafluoro-2-propanol (HFP; Sigma) and then diluted with ddH$_2$O into 250 μM stock solutions. The 20–29 fragment of islet amyloid protein (NH$_2$-SNNFGAILSS-COOH) was solubilized at 2.5 mM in ddH$_2$O. Peptide solutions were used immediately following solubilization (New condition) and or were put into capped vials and placed in a 37°C incubator for a 7 d period (Aged condition).

For the purpose of studying aggregation reversal, some New and Aged β1–42 stock solutions were frozen in liquid nitrogen and lyophilized. The dried peptides were resuspended at 250 μM in either ddH$_2$O or HFP for a 1 hr period, and then lyophilized a second time, and finally resuspended in ddH$_2$O at 250 μM before use.

Aggregation assessment. The aggregation state of both New and Aged βAPs was assessed in three ways. First, light microscopy was used to identify the presence of precipitated peptide both in stock solutions and after their addition to tissue culture wells; observations were confirmed by three observers. Second, migration patterns following sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) were examined using the gel and buffer systems described by Laemmli (1970). Samples of βAP stock solutions were added to reducing buffer, heated at 100°C for 3 min, and electrophoresed on SDS, 15% polyacrylamide gels at 70 V. Third, the sedimentable portion of βAP solutions was quantified using the centrifugation protocol outlined below.

βAPs were radioiodinated to a specific activity of ∼2.6 × 10⁶ cpm/μg, as previously described (Burdick et al., 1992). Solubility constraints required that β1–47 and β1–52 be radioiodinated in 20% dimethyl sulfoxide. Radiolabeled stock solutions (250 μM) were prepared by combining 70–90 nmol of radioiodinated peptide with 160–180 nmol of unlabeled peptide per 1 ml of ddH$_2$O. Both New and Aged radiolabeled stock solutions were diluted to 25 μM in Dulbecco's modified Eagle's medium (DMEM; pH 7.3), equilibrated at room temperature for 2 hr, and then loaded dropwise (50 μl/tube) into microfuge tubes above 250 μl 20% sucrose pads (pH 7.3). Due to an inability to sediment the β1–47 and β1–52 solutions through sucrose, 10 mM HEPES (pH 7.3) pads were used instead. Samples were centrifuged (Beckman TL-100 ultra-

centrifuge, TLA 100.1 rotor) at 100,000 × g for 1 hr. Pellet and supernatant fractions were separated and their radioactive counts quantified using a Beckman Gamma 5000 gamma counter. Thin-layer chromatography in a 100% ethanol solvent system was used to correct supernatant values for free iodine and iodination breakdown products. Percentage sedimentation was calculated as $[\Sigma(\gamma_{pellet}/\gamma_{corrected\ supernatant} + \gamma_{pellet})]/n \times 100$, where n represents number of observations. Due to an inability to iodinate the β25–35 and islet amyloid peptides using standard procedures, sedimentation of these peptides was quantified using a Lowry protein determination (Waterborg and Matthews, 1984). To accommodate limitations of this assay, stock solutions were diluted to 25 μM in 500 μM HEPES (pH 7.3) and centrifuged (250 μl/tube) through ddH$_2$O pads. In comparison tests using iodinated βAPs, this protein assay yielded results nearly identical to those obtained from gamma counting.

Tissue culture. Short-term neuronal cultures were prepared according to previously described methods with minor modifications (Brewer and Cotman, 1989). Hippocampal tissue from embryonic day 18 Sprague–Dawley rat pups was dissected and then rinsed in cold Ca^{2+}/Mg^{2+}-free Hanks' balanced salt solution supplemented with 20 mM HEPES, 4.2 mM sodium bicarbonate, 1 mM pyruvate, and 3 mg/ml bovine serum albumin (BSA). Following mechanical dissociation of the tissue with a constricted pipette in cold buffer, two volumes of 10% fetal bovine serum (FBS) in DMEM were added to the suspension. After the suspension settled for 2 min, the supernatant was collected and centrifuged for 2 min at 200 × g. The cell pellet was resuspended in serum-free DMEM (pH 7.3) supplemented with 2.4 mg/ml BSA and a modification of Brewer's B16 defined components (with 250 nM vitamin B12 and without catalase, glutathione, and superoxide dismutase) (Brewer and Cotman, 1989). Cells were plated at a density of 15,000 cells/cm^2 and grown in a humidified incubator at 37°C in 5% CO$_2$. Peptide solutions were diluted from stock samples into defined medium to yield a final concentration of 25 μM for βAPs and 25–250 μM for islet amyloid peptide. Peptides were added to cultures either at time of plating or 1 hr later. In control experiments addressing the possibility of substrate-adhesion interference, β25–35 and β1–42 were added to cultures 24–48 hr after cell plating. To ensure use of a highly neuronal culture system, some cultures were fixed with 2% glutaraldehyde and immunostained (Vector Elite ABC kit, Vector diaminobenzidine visualization kit) with antibodies against glial fibrillary acidic protein (1:2500; Dako) and neuron-specific neurofilaments (1:1000; SMI-311, Sternberger Monoclonals, Inc.).

Long-term neuronal cultures utilized hippocampal neurons that were prepared as described above and plated onto purified, secondary astrocyte cultures. Astrocyte cultures were prepared using a modification of the McCarthy and de Vellis (1980) method as previously described (Bridges et al., 1991). Briefly, dissected and triturated neocortical tissue from 2–4 d postnatal Sprague–Dawley rat pups was plated in 75 cm^2 flasks and maintained in 10% FBS supplemented DMEM with three medium changes per week. After 7–10 d, astrocytes were purified from cultures by overnight shaking (280 rpm) followed by trypsinization of remaining cells. Harvested astrocytes were plated (2.5 × 10^4 cells/cm^2) onto polylysine-coated 16 mm culture wells and grown to confluency (3–5 d). Neurons were plated (1 × 10^5 cells/cm^2) onto the astrocyte monolayer and maintained in DMEM supplemented with 10% FBS. Three days after plating neurons, cultures were given fresh medium and exposed to 10 μM cytosine arabinoside (Sigma) for 3 d to halt glial division. The cultures were then shifted into serum-free DMEM supplemented with N2 components (Bottenstein and Sato, 1979), and 40% of the medium was subsequently replaced twice per week. Twelve days after plating, neurons were treated with 25 μM β25–35 initially solubilized to 2.5 mM in either ddH$_2$O or 35% acetonitrile, 0.1% TFA. For some experiments, secondary astrocyte cultures were treated as described above except that they were not plated with neurons.

Viability assessment and statistics. Cell viability of short-term cultures was assessed according to morphological characteristics as previously described (Pike et al., 1991b). Cultures were photographed (3 fields/well in a constant pattern) under phase-contrast microscopy (37.5×; Olympus IMT-2 inverted microscope) 24 hr after plating. Projections of photo negatives onto a 29 × 31 cm screen were used to quantify cell survival on the basis of morphological criteria: smooth, round, and vacuole-free phase-bright cells were scored as alive. Control wells always had scored survival levels in excess of 150 cells/well. Each condition was represented in four wells per experiment and repeated at least three times in separate experiments using different peptide stock solutions. Since the

number of surviving untreated cells varies across preparations, statistical comparisons were made using raw data in a nested analysis of variance (ANOVA) design with preparations as the nested factor. Survival data is represented graphically as percentage of cell loss in each condition relative to untreated controls using the following formula: % cell loss = $-100[\Sigma(\text{condition survival/control survival})/n] + 100$, where n represents the number of observations.

Determination of cell viability in long-term neuronal cultures was based on the exclusion of trypan blue dye. Cultures were exposed to 0.2% trypan blue (GIBCO) for 15 min and then rinsed with maintenance medium. Stained cultures were photographed (50×) as described above. The number of both trypan-positive and trypan-negative cells was counted from projected negatives; cell counts always exceeded 150 cells/well. Conditions were represented in at least three wells per experiment and repeated in at least three separate experiments. In order to analyze the combined data from all experiments, raw data was converted to z-scores and analyzed by one-way ANOVA with Scheffé F test post hoc analysis. Cell count data is represented graphically as the percent of control values.

Cell viability of β25–35 treated secondary astrocyte cultures was determined by lactate dehydrogenase assay using the method described by Koh and Choi (1987).

Results

βAP aggregation

Based on our previous findings with the β1–28 and β1–42 peptides, we expanded our investigation of differential βAP assembly and its relationship with neurotoxicity using several synthetic βAPs (Fig. 1). The peptide β1–15 represents the fragment that may result from the predicted normal cleavage of APP near residue 16 (Esch et al., 1990). The truncated β25–35 fragment includes extracellular and transmembrane residues and has been reported to represent an active region of β-amyloid that shows some homology with tachykinin neuropeptides (Yankner et al., 1990). The remaining series of peptides beginning with β1–28 are progressively longer at their C-termini and thus include increasingly longer portions of the transmembrane domain: β1–30, β1–33, β1–36, β1–39, β1–42, β1–47, β1–52.

Upon light microscopic examination of freshly prepared (New) βAP stock solutions, most peptides appeared completely soluble, lacking any visible indication of precipitated aggregates (Table 1). In contrast, the New β25–35, β1–47, and β1–52 stock solutions exhibited both fine particulate and larger sheet-like precipitates consistent in appearance with our previous observations (Pike et al., 1991a,b). Dilution of βAPs into tissue culture medium creates conditions less suitable for aggregation, since the result is a 10-fold decrease in molar concentration and a pH increase from ~5 to 7.3 (Burdick et al., 1992). Despite these changes, microscopic examination of New βAPs added to culture wells mirrored observations of stock solutions, with aggregates still visible in the β25–35, β1–47, and β1–52 conditions (Table 1).

Following a 7 d incubation of the stock solutions at 37°C (Aged), aggregates were visible under the light microscope in all of the stock solutions with the exception of β1–15. Following dilution into DMEM, however, stable aggregate structures were visible only for β25–35, β1–36, β1–39, β1–42, β1–47, β1–52, and, to a lesser extent, β1–33 (Table 1).

The light microscopic observations agree well with SDS-PAGE analysis of the New βAPs (Fig. 2A). The β1–15, β1–28, β1–30, β1–33, β1–36, and β1–39 peptides show single, apparently monomeric bands. The broad and higher than expected molecular weights of some monomeric bands likely represents an artifact of reducing gels, as previously reported (Burdick et al., 1992).

Table 1. Visualization of precipitated βAP aggregates under light microscopy

βAP	New		Aged	
	Stock solution	DMEM	Stock solution	DMEM
β1–15	–	–	–	–
β1–28	–	–	+	–
β1–30	–	–	+	–
β1–33	–	–	+	+/–
β25–35	+	+	+	+
β1–36	–	–	+	+
β1–39	–	–	+	+
β1–42	–	–	+	+
β1–47	+	+	+	+
β1–52	+	+	+	+

βAP fragments were examined under light microscopy both in stock solutions (250 μM, pH ~5) and following addition to culture medium (25 μM, pH 7.3) for the presence of particulate precipitate and sheet-like structures characteristic of aggregation. Observations were made after both initial peptide solubilization (New) and 7-d incubation of stock solutions (Aged). –, no visible aggregates; +, visible aggregates; +/–, limited, inconsistent visualization of aggregates.

The β1–42 peptide exhibits not only a predominant monomeric band (~4.5 kDa) but also an apparent tetrameric band (~18 kDa) suggesting an early aggregated component. In contrast, β25–35 will not enter the gel and is recognized only as a strongly insoluble band at the top of the stacking gel. The β1–47 and β1–52 peptides also exhibit high-molecular-weight bands that are inconsistent with denatured monomers. In agreement with previous reports (Hilbich et al., 1991; Burdick et al., 1992), we interpret bands of much higher than expected molecular weight that are located at the stacking/separating gel interface and or at the top of the stacking gel as insoluble forms of aggregated β-amyloid. A similar electrophoretic pattern has also been described for insoluble β-amyloid purified from AD brain tissue (Masters et al., 1985a; Selkoe et al., 1986).

Electrophoretic patterns of Aged β1–15, β1–28, β1–30, β1–33, and β1–36 suggest monomeric peptides under reducing conditions (Fig. 2B). In three out of six experiments, Aged β1–39 exhibited a high-molecular-weight band characteristic of aggregated peptides. This variability may reflect a transition region in βAP after which the aggregated forms of full-length βAPs become the predominant species. Aged β1–42 consistently exhibits several bands, suggesting multiple stable aggregate intermediates. Following aging, the β1–47 and β1–52 peptides show increased aggregation evidenced by predominant stacking gel bands, whereas β25–35 remains a single, insoluble band.

Since neither microscopic examination nor SDS-PAGE yields quantitative aggregation data and because the peptide conditions in electrophoresis do not parallel those in tissue culture, we employed a centrifugation assay to assess βAP aggregation further under more suitable conditions. Radioiodinated βAP stock solutions were diluted to 25 μM, equilibrated to pH 7.3, and then centrifuged at 100,000 × g for 1 hr. Quantitation of peptide amounts in both the pellets and supernatants permitted calculation of the percentage of sedimentable material. As shown in Figure 3, β25–35, β1–47, and β1–52 are the only New βAPs that have a statistically significant level of sedimentation. Following a 7 d aging period, β1–36, β1–39, and β1–42 also demonstrate aggregation with significant levels of sedimentation.
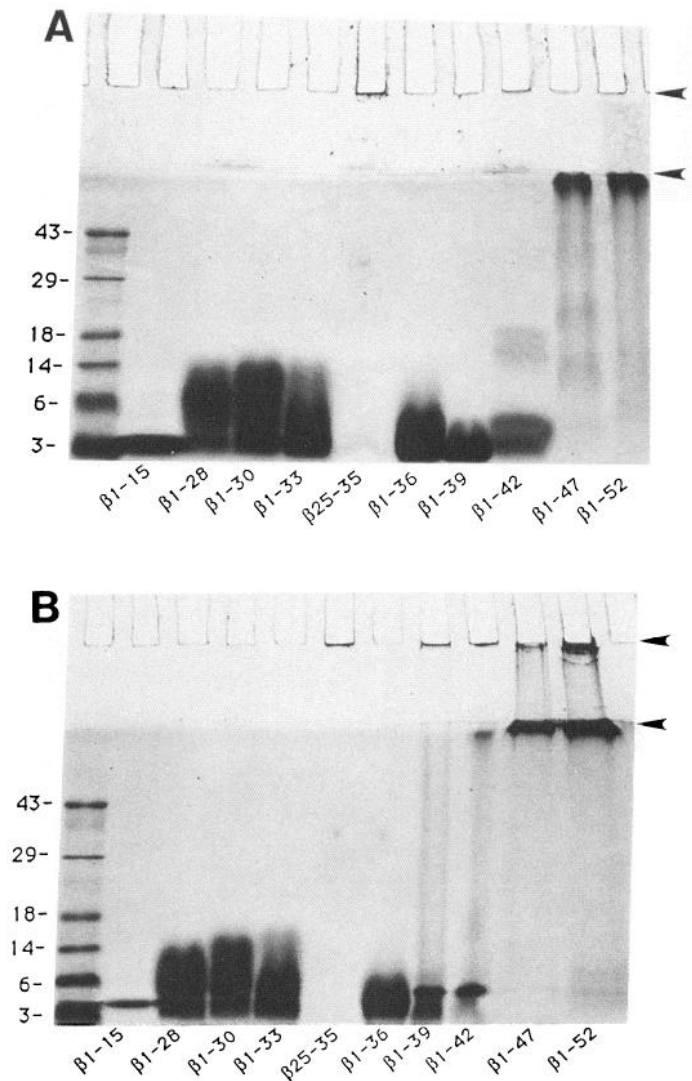

Figure 2. SDS-PAGE analyses indicate that several New and Aged βAPs form aggregates resistant to denaturation. *A,* Among the New βAPs, only β25–35, β1–47, and β1–52 exhibit high-molecular-weight bands (*arrowheads*) characteristic of aggregation. *B,* Aggregation bands are apparent in several βAPs following incubation of peptide solutions: Aged β25–35, β1–39, β–42, β1–47, and β1–52. Protein standards (GIBCO–Bethesda Research Laboratories, low range) with indicated molecular masses (in kilodaltons) were run in the *far left lane*. Twelve micrograms of βAP/lane were run in 15% polyacrylamide gels, followed by staining with Coomassie brilliant blue.

βAP neurotoxicity in short-term cultures

Since our previous studies had documented neuronal loss only in the presence of aggregated βAPs, we hypothesized that *in vitro* neurodegeneration should result specifically following treatment with any βAPs, New or Aged, that demonstrate significant levels of aggregation. In order to test this prediction, we treated short-term hippocampal cultures with both New and Aged βAPs at the previously established effective concentration of 25 μM (Pike et al., 1991b) and determined cell viability 24 hr later. Immunostaining of selected cultures confirmed that neurons comprised approximately 95% of the viable cells in untreated wells, a finding consistent with previous reports (Banker and Cowan, 1977; Barbin et al., 1984). Use of this highly neuronal, short-term culture system simplifies interpretation of toxicity data

Figure 3. Sedimentation assays measure βAP aggregation under culture-like conditions. Aggregation of New (*hatched bars*) and Aged (*solid bars*) βAPs was assessed by quantifying the percentage of peptide that sedimented following 1 hr of ultracentrifugation at 100,000 × g. Bars represent mean percentage sedimentation values (±SEM) of three or four observations. Statistical analyses were performed using one-way ANOVA [$F(19,53) = 60.39$, $p = 0.0001$] and the Scheffé F test for post hoc between-group comparisons. *, $p < 0.05$ relative to the nonaggregating peptide New β1–15.
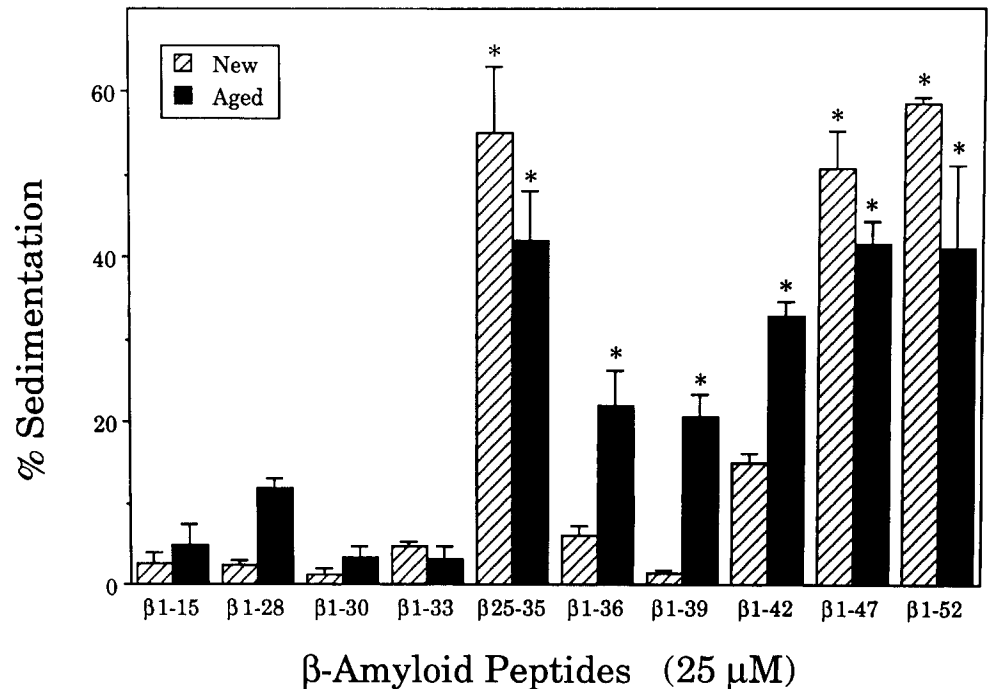
since the potential contributions of both glia–neuron interactions and glutamate receptor activation are minimized.

Although βAPs were not observed to interfere with cell–substratum adhesion, they were typically added to cultures 1 hr after plating to avoid this potential pitfall. Microscopic examination of βAP-treated culture wells revealed no floating cells or other visible indications of cell detachment. To address this issue further, selected aggregated βAPs were added to cultures 24 hr and 48 hr after plating. This delayed treatment did not affect the magnitude of βAP-induced toxicity (data not shown).

Hippocampal cultures responded to most New βAPs with

survival levels comparable to untreated controls (Fig. 4). However, cultures treated with three of the New βAPs demonstrated significant reductions in survival: β25-35 showed 64.2% ± 2.7% cell loss in comparison to controls; β1–47, 64.0% ± 4.7%; and β1–52, 71.8% ± 3.7%. Since β1–47 and β1–52 were initially solubilized in HFP, the appropriate HFP dilution (~1:400) was added directly to cultures but was not found to be toxic. Because some New βAPs displayed both significant aggregation and toxicity, their toxicity could not be definitively related to aggregation without demonstration that nonaggregated forms of the βAPs also lack toxicity. Thus, we solubilized separate β25-35

Figure 4. Significant cell loss is observed following treatment with aggregated βAPs. The effect of New (*hatched bars*) and Aged (*solid bars*) βAPs on cell survival was determined 24 hr after their addition to short-term neuronal cultures. Bars represent mean percentage cell loss (±SEM). Significant cell loss was observed for New β25-35, β1–47, ad β1–52 and Aged β25-35, β1–36, β1–39, β1–42, β1–47, and β1–52. Statistical analyses were conducted for each condition using raw data and nested ANOVAs. *, $p < 0.05$ relative to untreated control groups.
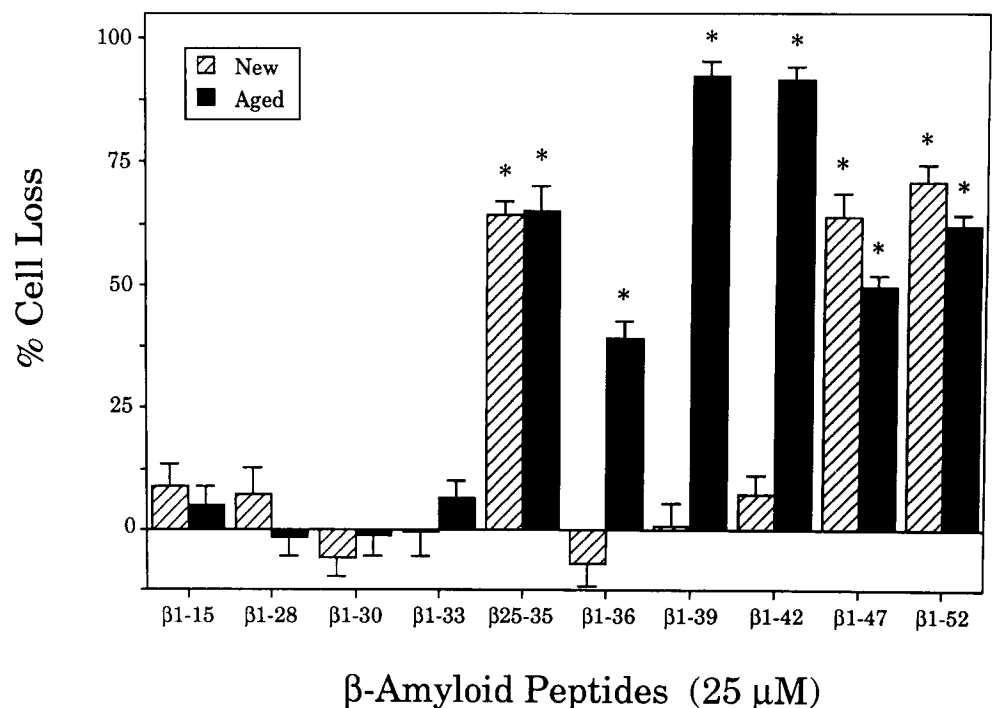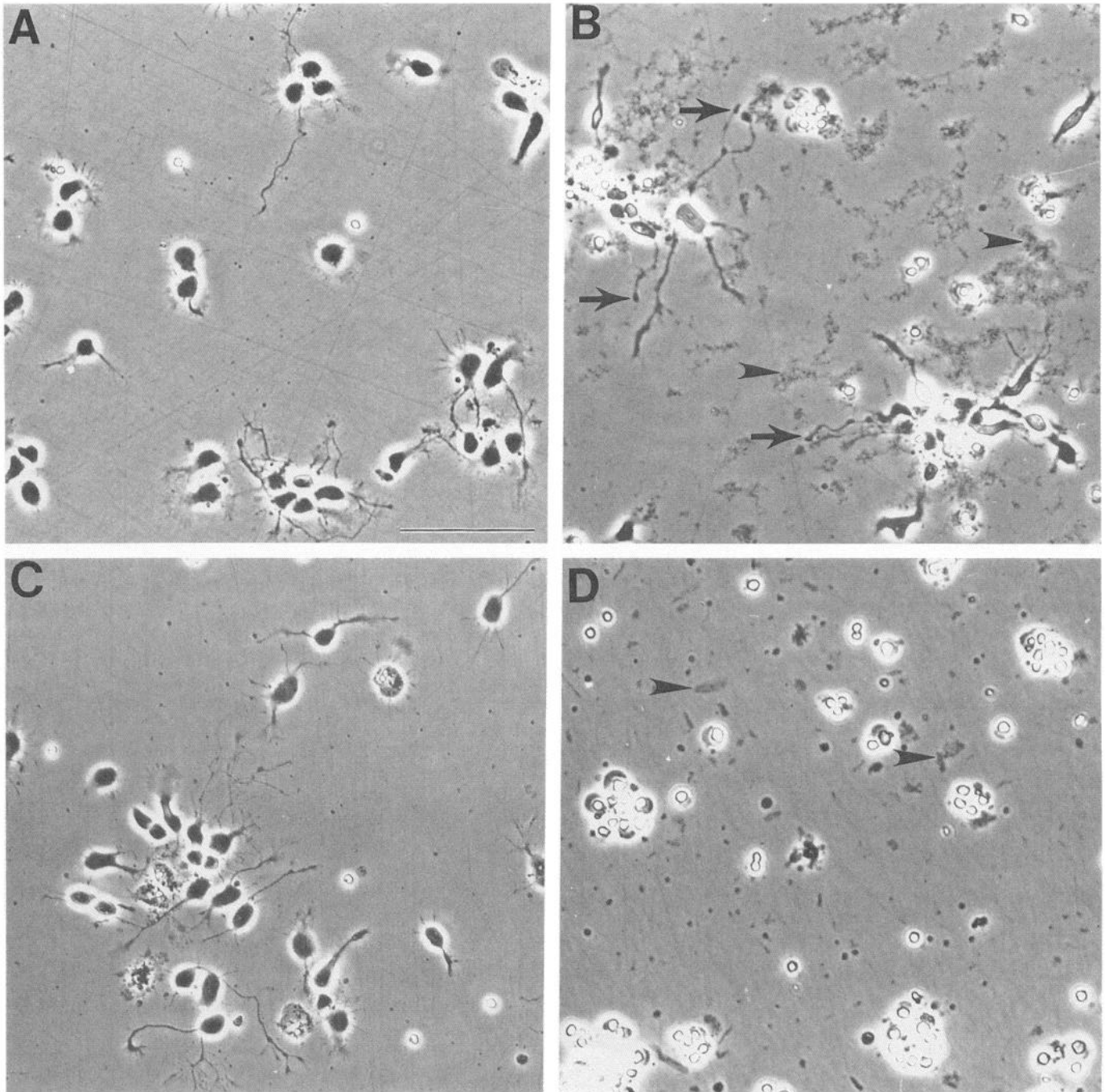
Figure 5. Aggregated βAPs cause degenerative changes in cultured neurons. Treatment of short-term hippocampal cultures with aggregated βAPs (25 μM) results in distinct morphological changes within 24 hr. *A*, Untreated control cells appear healthy and extend short processes. *B*, In the New β25–35 condition, most cells exhibit either swollen, dystrophic neurites (*arrows*) or complete degeneration (cellular collapse); many large aggregate precipitates are also visible (*arrowheads*). *C*, In wells with New β1–39, no aggregates are visible and, as in the control condition, cells show no apparent degeneration. *D*, However, in the Aged β1–39 condition both aggregates (*arrowheads*) and severe cell loss are observed. Scale bar, 100 μm.

samples in 35% acetonitrile, 0.1% TFA rather than in ddH$_2$O alone, a procedure previously demonstrated to yield soluble βAPs (Yankner et al., 1990). We found that New β25–35 (35% acetonitrile, 0.1% TFA) was absent of significant aggregation [7.8% ± 2.8% sedimentation; in comparison to New β1–15, $F(1,7) = 2.9$, $p = 0.14$] and did not induce toxicity [105.3% ± 5.0% cell survival in comparison to vehicle-treated controls; $F(1,20) = 0.71$, $p = 0.41$].

Hippocampal cultures were also treated with Aged βAPs. Following the peptide aging period, β1–15, β1–28, β1–30, and β1–33 continued to exert no significant effect on the survival of hippocampal neurons whereas β25–35, β1–47, and β1–52 still exhibited significant toxic effects (Fig. 4). Unlike their relatively benign influences in the newly solubilized condition, Aged β1–36, β1–39, and β1–42 caused widespread and, in some instances, nearly total neuronal degeneration.
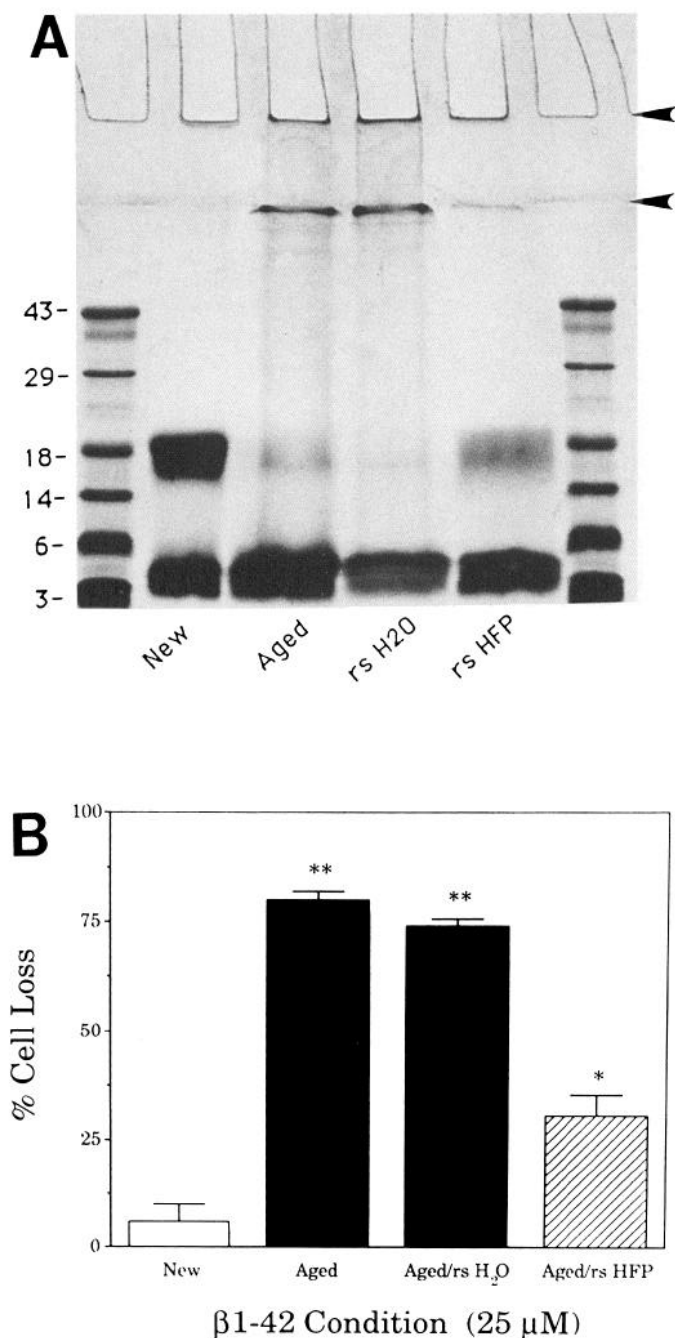
A

B

β1-42 Condition (25 μM)

Figure 6. Both the aggregation and neurotoxicity of Aged β1–42 are partially reversed by HFP-resolubilization treatment. *A*, Analysis by SDS-PAGE shows strong aggregation bands (*arrowheads*) in the lanes corresponding to Aged β1–42 (*Aged*) and Aged β1–42 resolubilized in ddH₂O (*rs H2O*). Aged β1–42 resolubilized in HFP (*rs HFP*) exhibits less intense aggregation bands and a stronger tetramer (~18 kDa) band, similar to the profile of newly solubilized β1–42 (*New*). Molecular masses (in kilodaltons) of protein standards are indicated. *B*, Both Aged β1–42 and Aged β1–42 resolubilized in ddH₂O cause severe cell loss in short-term neuronal cultures. Significantly less cell loss is caused by Aged β1–42 resolubilized in HFP. *, $p < 0.05$ relative to untreated controls; **, $p < 0.05$ relative to both untreated controls and Aged β1–42/rs HFP.

The βAP-induced degeneration resulted in obvious changes in neuronal morphology, including swollen and beaded neurites, vacuolar inclusions, and cellular collapse (Fig. 5). These degenerative events exhibited a fairly rapid onset, with observable morphological changes typically beginning within 4–8 hr of βAP treatment. We have recently reported that these morphological changes are an initial and definitive stage in the process of βAP-induced degeneration *in vitro* (Copani et al., 1992; A. Copani, D. T. Loo, C. J. Pike, A. J. Walencewicz, and C. W. Cotman, unpublished observations). The morphological changes are succeeded by a significant decrease in metabolic activity [as assayed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT)], condensation of nuclear chromatin, pronounced fragmentation of DNA, membrane blebbing, and eventually cell lysis (as determined by lactate dehydrogenase release and positive trypan blue staining).

Aggregation reversal

A corollary of our hypothesis of aggregation-related toxicity is that the reversal of peptide aggregation should be accompanied by a corresponding attenuation of toxicity. To investigate this possibility, we attempted to reverse the aggregation state of Aged β1–42 with the α-helix–promoting solvent HFP, since previous studies have demonstrated that βAPs insoluble in aqueous media show increased solubility in this solvent (Halverson et al., 1990; Hilbich et al., 1991; Burdick et al., 1992). Sister samples of Aged β1–42 were lyophilized and then temporarily resuspended at 250 μM: one in ddH₂O (β1–42/H₂O), the other in HFP (β1–42/HFP). The samples were lyophilized again and resuspended in ddH₂O at 250 μM. Aliquots of these two final β1–42 solutions were compared in SDS-PAGE with an aliquot of the original, untreated Aged β1–42 and a New β1–42 sample (Fig. 6A). Although the New β1–42 lane shows a strong ~18 kDa band in addition to its monomeric band, it does not exhibit the characteristic aggregation bands visible in the Aged β1–42 lane. Aggregation bands are also apparent for Aged β1–42/H₂O, but are substantially decreased in Aged β1–42/HFP, suggesting a partial reversal of aggregation as a result of HFP exposure.

Samples from these four β1–42 solutions were also assayed for toxicity using the short-term hippocampal culture paradigm. Consistent with our previous data, Aged β1–42 but not New β1–42 caused significant neuronal degeneration. Aged β1–42/H₂O (74.0% ± 1.7% cell loss) exacted levels of cell damage nearly identical to the untreated Aged β1–42 (80.1% ± 1.7% cell loss). Cultures exposed to the Aged β1–42/HFP (30.6% ± 4.7% cell loss) exhibited significantly less degeneration than both Aged β1–42/H₂O and untreated Aged β1–42, but still significantly more than New β1–42 and the untreated controls (Fig. 6B). Because the toxicity caused by Aged β1–42/HFP could have been due to incomplete aggregation reversal and/or an effect of HFP, we then treated cultures with New β1–42 samples that had been subjected to the HFP resolubilization procedure. New β1–42/HFP (30.1% ± 3.0% cell loss) caused a response commensurate with that of Aged β1–42/HFP. Since direct addition of HFP to culture medium (~1:400 final dilution) did not affect cell survival, these data suggest that the neurodegeneration observed in β1–42/HFP conditions may be related to an indirect effect of HFP (e.g., HFP-induced conformational changes).

Islet amyloid polypeptide

Islet amyloid polypeptide (IAP) is a 37 amino acid protein that forms amyloid deposits in the pancreases of type 2 diabetes patients (Cooper et al., 1987; Westermark et al., 1987). Previous work has demonstrated that an internal sequence of IAP (residues 20–29) is likely responsible for IAP amyloidogenicity since a synthetic peptide homologous to this region forms twisted

β-pleated sheet fibrils *in vitro* (Glenner et al., 1988) and exhibits positive Congo red staining (Glenner et al., 1988; Westermark et al., 1990). In order to address the possibility that the neurotoxicity associated with aggregated βAPs may result from a nonspecific neuronal interaction with aggregated, amyloidogenic proteins in general rather than a more specific response to aggregated βAP, we examined the aggregation and biological activities of the 20–29 region of IAP (IAP20–29).

Both New and Aged stock solutions of synthetic IAP20–29 exhibited particulate matter that was similar in appearance to aggregates of βAP. These IAP aggregates stained positively with thioflavin-S (data not shown) and remained stable following addition to culture medium. Upon ultracentrifugation, IAP20–29 showed sedimentation values (18.4% ± 3.2% for newly solubilized, 25 μM solution) similar to some aggregating βAPs. In contrast to aggregating βAPs, addition of 25 μM IAP20–29 to short-term hippocampal cultures did not induce degenerative morphological changes (Fig. 7A). In fact, IAP20–29 did not significantly affect neuronal survival at any of the tested concentrations, which ranged from 25 to 250 μM (Fig. 7B).

βAP neurotoxicity in long-term cultures

Although short-term cultures are an advantageous paradigm for this study, it is important to demonstrate that findings derived from them can be extrapolated to fully developed long-term cultures containing both neuronal and glial elements, a paradigm that more closely parallels *in vivo* conditions. Thus, we examined the effects of 25 μM β25–35, the proposed active fragment of β-amyloid, in both its soluble and aggregated forms on long-term cultures consisting of hippocampal neurons plated on top of a confluent astrocyte monolayer. As described above in short-term culture experiments, soluble β25-35 was attained by initial solubilization in 35% acetonitrile, 0.1% TFA whereas aggregated β25-35 resulted from solubilization in ddH₂O.

Cultures were treated with β25–35 after the neurons had been cultured for 12 d; untreated neurons were observed to retain viability for at least 21 d in this culture system. Similar to results with short-term cultures, neurons in long-term cultures treated with soluble β25–35 appeared morphologically normal after 24 hr whereas those exposed to aggregated β25–35 exhibited morphological indications of degeneration, including somal shrinkage and neuritic regression. Cell viability was assessed by trypan blue exclusion rather than morphological criteria due to the difficult nature of quantifying morphological degeneration by phase-contrast microscopy in high-density, mature cultures. Since cell lysis is a late event in βAP-induced degeneration (Copani et al., 1992; Copani, Loo, Pike, Walencewicz, and Cotman, unpublished observations), viability determinations were made 72 hr after β25–35 treatment. Similar to control wells, those treated with soluble β25–35 exhibited very few trypan-positive cells, indicating a general absence of cell lysis. In contrast, neurons exposed to aggregated β25–35 showed a significant level of degeneration with approximately 30% staining trypan positive (Fig. 8). Because the total number of cells (sum of trypan-positive and trypan-negative cells) did not significantly vary across conditions, cell detachment can be excluded as the primary contributor to the observed degeneration.

The observed degeneration was limited to neurons since sister astrocyte cultures containing no neurons did not show significant degeneration following 72 hr exposure to 25 μM concentrations of either soluble or aggregated β25–35. Although βAP treatment induced reactive morphology in astrocytes within 24 hr, at 72
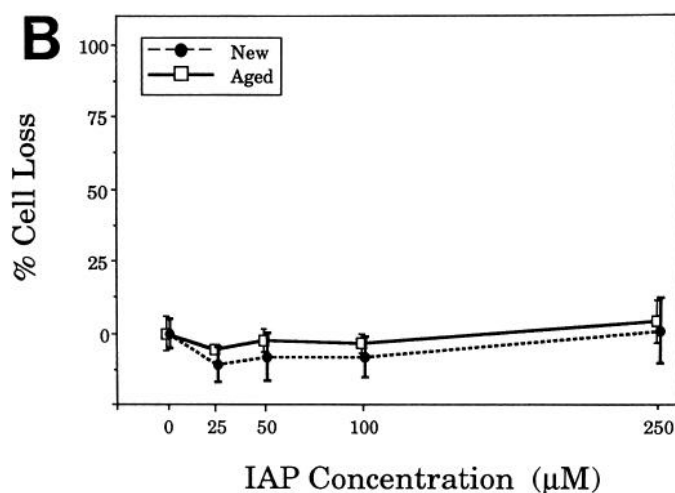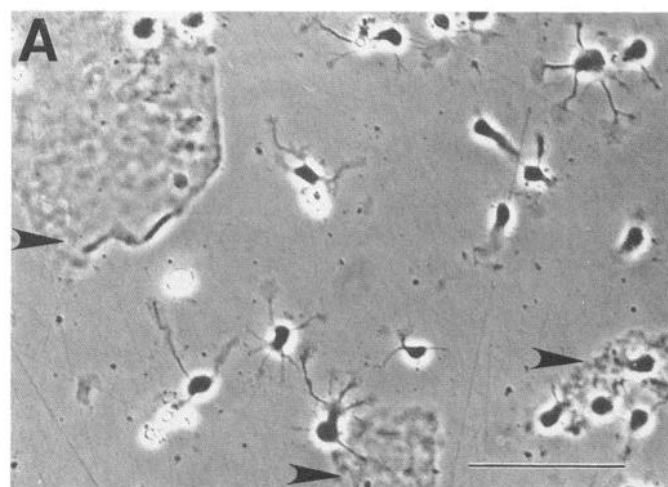

Figure 7. IAP20–29 exhibits aggregation but does not induce toxicity. *A*, After 24 hr *in vitro*, short-term cultured neurons exposed to 25 μM New IAP20–29 do not exhibit any morphological indications of degeneration despite the presence of stable peptide aggregates (*arrowheads*). *B*, In concentrations from 25–250 μM, neither New (*broken line*) nor Aged (*solid line*) IAP20–29 significantly affected cell survival. New conditions: $F(4,50) = 0.37$, $p = 0.83$; Aged conditions: $F(4,57) = 1.4$, $p = 0.25$. Scale bar (*A*), 100 μm.

hr β25–35 treated cultures did not exhibit significant cell lysis in comparison to control cultures, as determined by lactate dehydrogenase release (one-way ANOVA, $F(3,20) = 2.34$, $p > 0.10$).

A previous study reported that the βAP potentiation of an endogenous glutamate insult in long-term cortical cultures was markedly attenuated by addition of MK801, a selective, noncompetitive antagonist for the NMDA class of glutamate receptor (Copani et al., 1991). Other reports suggest that excitotoxic degeneration of long-term hippocampal cultures is effectively prevented by MK801 (Rondouin et al., 1988; Abele et al., 1990). Thus, we investigated the possibility that this glutamate antagonist would protect against βAP-induced degeneration in our long-term cultures. Although 3 μM MK801 reduced by nearly 50% the toxicity induced by 1 mM glutamate over 72 hr (Scheffé *F* value = 6.24, $p < 0.01$), it did not significantly affect the cell loss caused by exposure to 25 μM aggregated β25–35 (Scheffé *F* value = 0.35, $p > 0.1$).
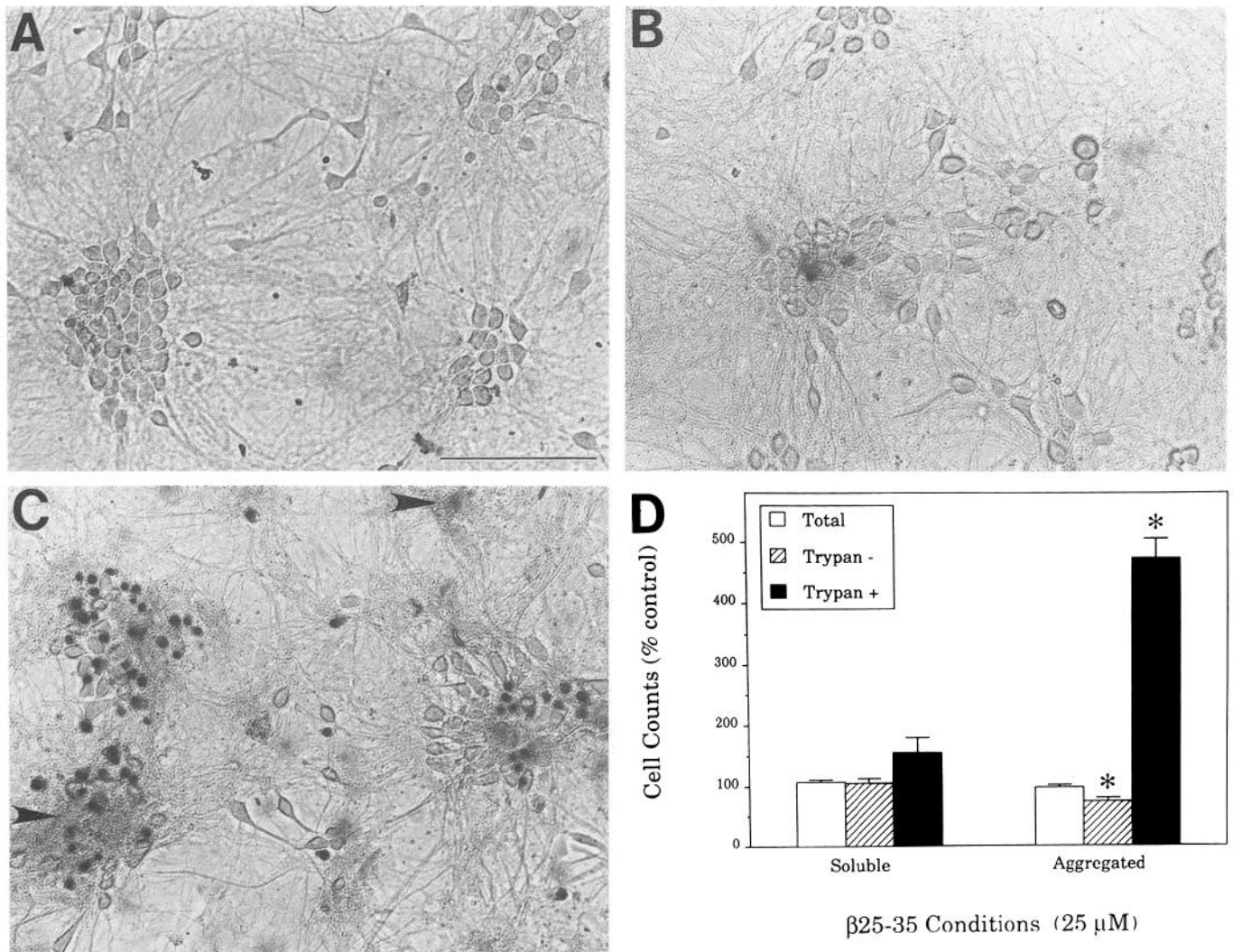
Figure 8. Aggregated βAP induces neurodegeneration in long-term cultures. *A,* Untreated long-term cultured neurons exhibit good viability as evidenced by intact neurites and exclusion of trypan blue dye. Cultures are viewed under bright-field microscopy. *B,* Similarly, neurons treated with soluble 25 μM β25–35 exclude trypan blue. *C,* Conversely, positive trypan staining of neurons treated with 25 μM aggregated β25–35 indicates extensive cell lysis. *Arrowheads* indicate β25–35 aggregates that show light, nonspecific trypan staining. *D* Cell counts of trypan-stained cultures demonstrate that all conditions have the same number of cells, but that the aggregated β25–35 condition has significantly fewer viable (trypan negative) and significantly more degenerated (trypan-positive) neurons. *, $p \leq 0.01$ in comparison to control condition. Scale bar (*A*), 100 μm.

Discussion

In this article, we have evaluated the hypothesis that the state of β-amyloid aggregation is related to its neurotoxic properties. The data support our prediction that those βAPs exhibiting stable aggregates are neurotoxic. The state of βAP aggregation, assayed by sedimentation studies to provide quantitative data under conditions parallel to those in culture, predicts the documented toxicity. Significant aggregation was found for New β25–35, β1–47, and β1–52 and Aged β25–35, β1–36, β1–39, β1–42, β1–47, and β1–52. Similarly, significant toxicity was limited to these aggregating peptides. Further, we demonstrate that the reduction of βAP aggregation by specific solvents is accompanied by corresponding reductions in toxicity.

βAP aggregation results from the tendency of β-amyloid to adopt solution conformations that promote its self-association into dimer, tetramer, and multimer peptide assemblies. These assemblies exhibit β-sheet conformation (Kirschner et al., 1987; Halverson et al., 1990; Barrow and Zagorski, 1991), high in-

solubility (Halverson et al., 1990; Hilbich et al., 1991; Burdick et al., 1992), and positive staining with Congo red (Hilbich et al., 1991; Burdick et al., 1992) and thioflavin (Burdick et al., 1992), similar to β-amyloid isolated from the senile plaques of AD brains. Deposition of β-amyloid in AD, and thus senile plaque formation, likely depends upon the self-assembly of β-amyloid protein, which presumably results from an abnormal proteolysis of APP. On the basis of our data, only βAPs containing the hydrophobic β29–35 sequence are predicted to form stable assemblies that are resistant to unfavorable conditions (e.g., low βAP concentration, solution pH > 7). In agreement with this prediction, aqueous solutions of β29–42 are reported to be entirely β-sheet in structure whereas β1–28 solutions are random coil (Barrow and Zagorski, 1991). These data suggest that a variety of hydrophobic βAPs could be deposited in the AD brain, a possibility consistent with reports of ragged N-termini in βAPs isolated from senile plaques (Masters et al., 1985a,b). Together, our aggregation data more clearly define the sequence requirements for stable βAP aggregates and serve as

a basis for predictions concerning assembly-related neuronal degeneration.

We show that following self-assembly into stable aggregates, βAPs induce neurodegeneration in both short-term and mature cultures. Thus, the state of βAP assembly appears to influence their biological activities. Accordingly, solvation conditions that alter βAP conformation and assembly may also affect βAP-induced degeneration. Consistent with this position, we observed that, in comparison to water-solubilized β25–35, solubilization of β25–35 in 35% acetonitrile, 0.1% TFA significantly reduced both its level of measurable aggregation and its neurotoxic impact. Similarly, resolubilization of aggregated β1–42 in HFP increased the peptide's solubility and lessened its degenerative effects. Interestingly, soluble β1–42 resolubilized in HFP induced toxicity equivalent with that caused by aggregated β1–42 resolubilized in HFP. Combined with our other observations, this finding suggests that βAPs in solution may reach a state of equilibrium along a continuum between solubility and aggregation. The equilibrium levels of unassembled monomers (possibly in different conformations) and assembled dimers and multimers may be determined in part by the solvent (e.g., water, HFP, or 35% acetonitrile, 0.1% TFA).

Since aggregated βAPs induce neurotoxicity, other stable protein aggregates exhibiting β-sheet structure may share this property. This possibility does not weaken the theory that βAP contributes to AD neurodegeneration, since it is βAP, not other proteins, that is deposited within the AD brain. Regardless of this argument, the notion of a generalized aggregation-related toxicity is not supported by our data: the aggregating peptide IAP20–29 does not affect neuronal survival in the defined paradigm. Although this observation does not rule out the potential for toxicity by other aggregated proteins, it does suggest that the mechanism of aggregated βAP-induced toxicity may be relatively specific.

The mechanism(s) responsible for the biological activities of βAPs has not been clearly defined. Yankner and colleagues hypothesized that βAP-induced effects may be mediated by a receptor pathway since equimolar addition of substance P and other tachykinins blocked both trophic and toxic responses to βAPs (Yankner et al., 1990). More recently, the serpin-enzyme complex receptor was reported to bind both βAPs and substance P and thus was suggested to represent the βAP site of action (Joslin et al., 1991). However, the toxicity reported here likely results from a mechanism unrelated to the one proposed by Yankner and colleagues, since we have not observed protection against the toxicity of New β25–35 by equimolar addition (25 μM) of substance P (p = 0.78 by one-way ANOVA, n = 3). Similarly, recent *in vivo* studies of βAP-induced toxicity have not demonstrated protection by substance P (Rush et al., 1992; Waite et al., 1992).

Toxicity mediated by excitatory amino acids (EAAs) is a well-documented mechanism of neurodegeneration (see Rothman and Olney, 1987; Choi, 1991) that has also been postulated to function in βAP-induced toxicity. For example, Koh et al. (1990) reported that soluble βAPs are not directly neurotoxic to mature cortical cultures, but do potentiate EAA-mediated toxicity. A study by Copani et al. (1991) substantiated the EAA/βAP link by demonstrating that full-length βAP exacerbates the neurodegeneration caused by transient glucose deprivation, an EAA-mediated neurotoxic event. The EAA/βAP toxicity relationship has been repeated and extended in a recent publication by Mattson et al. (1992). Mattson et al. also reported that βAPs increase

the resting intraneuronal concentration of calcium, suggesting that the mechanism of βAPs' effects includes destabilization of calcium levels. In the present study, a similar disruption of the tightly regulated intracellular calcium levels remains a potential mechanism in the toxicity of aggregated βAPs. However, the same EAA/βAP toxicity relationship may not apply to the reported toxicity for the following three reasons: (1) in our short-term culture studies toxicity is elicited during the first day *in vitro*, a period when hippocampal neurons are insensitive to EAA-mediated toxicity (Mattson et al., 1988); (2) EAA-mediated toxicity is accompanied by marked cellular swelling, whereas aggregation-related βAP toxicity is characterized morphologically by cellular collapse; (3) MK801, a potent inhibitor of glutamate-mediated toxicity *in vitro*, does not significantly attenuate β25–35 toxicity in our long-term cultures.

Since β-amyloid exists in an insoluble, aggregated state within senile plaques, determination of how cells interact with aggregated βAPs *in vitro* may be an important step in understanding how β-amyloid may contribute to neurodegeneration within the AD brain. Although mechanisms to account for the toxicity of soluble βAPs have been proposed, they do not appear to explain the neurodegeneration caused by aggregated βAPs. Recently, we and others have reported that the βAP-induced degeneration of both short-term (Copani et al., 1992; Copani, Loo, Pike, Whittemore, Walencewicz, and Cotman, unpublished observations) and long-term (Forloni et al., 1992) neuronal cultures appears to follow an apoptotic pathway of cell death. Apoptotic degeneration involves cellular processes believed to be distinct from those occurring during necrotic cell death, which can be induced by acute excitotoxic insults (Masters et al., 1989; Ignatowics et al., 1991). Although how βAPs may trigger apoptosis is not known, we hypothesize that a death program would be stimulated by compromised cellular functions that could result from intraneuronal accumulation of aggregated βAPs. Consistent with this possibility, a recent study by Glabe and colleagues found that cultured fibroblasts uptake and collect β1–42 within lysosomes, where it accumulates in aggregated forms but is not degraded; nonaggregating βAPs were rapidly degraded and did not accumulate intracellularly (Knauer et al., 1992).

In conclusion, we have demonstrated that hydrophobic sequences appear to be necessary for stable *in vitro* aggregation of βAPs, and that this aggregation, in turn, is associated with toxic properties of βAPs. The exact mechanism(s) responsible for the degenerative effects of aggregated βAPs remains undefined, but may ultimately involve an apoptotic pathway. We suggest that conformational changes in βAPs promote self-assembly into insoluble aggregates and may confer quantitative and/or qualitative changes in β-amyloid's biological activities. The result may be an enhancement in the degenerative effects of β-amyloid by aggregated forms: soluble βAPs appear to induce degeneration by exacerbating other forms of toxicity whereas aggregated βAPs can induce toxicity in the absence of additional insults. Thus, in the AD brain, neurons associated with β-amyloid deposits are predicted to be vulnerable to degeneration, a risk that may be reduced by measures that can lessen or prevent the protein's self-assembly.

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