0026-895X/03/6405-1160–1168$7.00
MOLECULAR PHARMACOLOGY
Vol. 64, No. 5
2702/1104026
*Mol Pharmacol* 64:1160–1168, 2003
*Printed in U.S.A.*


# The Peptide KLVFF-$K_6$ Promotes β-Amyloid(1–40) Protofibril Growth by Association but Does Not Alter Protofibril Effects on Cellular Reduction of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT)

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Received June 6, 2003; accepted August 6, 2003

This article is available online at http://molpharm.aspetjournals.org

## ABSTRACT

The peptide KLVFF-$K_6$ was observed by Lowe et al. (*Biochemistry* **40:**7882–7889, 2001) to simultaneously enhance amyloid β-protein (Aβ) fibrillogenesis and decrease cellular toxicity, as measured in a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay. It was postulated that accelerated Aβ aggregation and precipitation induced by KLVFF-$K_6$ may lead to an increase in less toxic insoluble fibrils at the expense of more toxic soluble protofibrils. In a previous study, we distinguished between two modes of protofibril growth: elongation by monomer deposition and direct protofibril-protofibril association. These growth mechanisms could be resolved by varying Aβ monomer and NaCl concentrations. Using assays designed to isolate these distinct modes of protofibril growth, we report here that larger Aβ aggregates formed in the presence of KLVFF-$K_6$ resulted from enhanced protofibril association. $^3$H-Radiomethylated KLVFF-$K_6$ bound to associated protofibrils with an apparent $K_d$ of 180 nM, and concentrations of free [$^3$H]KLVFF-$K_6$ in this range were sufficient to convert soluble protofibrils to sedimentable fibrils. However, promotion of Aβ protofibril association by KLVFF-$K_6$ had no effect on Aβ-induced decreases in cellular MTT reduction. Therefore, our data do not support the proposal that insoluble fibrils formed with KLVFF-$K_6$ are less toxic than soluble protofibrils. KLVFF-$K_6$ did not alter rates of protofibril elongation by monomer deposition. In contrast, when added to Aβ monomers isolated with the use of size-exclusion chromatography, KLVFF-$K_6$ inhibited fibrillogenesis, as measured by thioflavin T fluorescence, and this inhibition was paralleled by a failure to alter cellular MTT reduction.

Amyloid plaques in brain tissue are a hallmark of Alzheimer's disease (AD). Primary components of these plaques are 40- and 42-residue peptides, denoted Aβ(1–40) and Aβ(1–42), that are derived by proteolysis of cellular amyloid precursor protein (Miller et al., 1993; Yankner, 1996b). Monomeric amyloid β-protein (Aβ) self-associates to form fibrillar Aβ, which deposits to yield amyloid plaques. It has been proposed that the accumulation of fibrillar Aβ in the brain initiates a cascade of events that results in neuronal cell death and leads to cognitive decline (Yankner, 1996b). This hypothesis, known as the amyloid hypothesis, has gained support over the last decade. Multiple lines of evidence suggest a role for Aβ in disease progression. Several mutations in both the amyloid precursor protein and the presenilin genes (PS1 and PS2) have been linked to early-onset AD. In each case, these mutations lead to the elevated production of Aβ or an increase in the relative amount of the longer, more fibrillogenic form of Aβ, Aβ(1–42) (Duff et al., 1996). Overexpression of these mutant genes in transgenic mice results in an age-dependent development of Aβ fibril deposition (Hsiao et al., 1996; Kawarabayashi et al., 2001). Furthermore, whereas freshly dissolved, monomeric Aβ is inert, aged Aβ preparations, which have had the opportunity to aggregate into fibrillar from, evoke neurotoxicity in culture (Yankner, 1996a). Consequently, the development of compounds that interfere with the fibril formation process remains a viable option for drug development.

Point mutations within the hydrophobic core of Aβ have identified residues 16–20 (KLVFF) as essential for fibril formation (Hilbich et al., 1992; Esler et al., 1996). The KLVFF pentapeptide was found to be the minimum sequence required to bind Aβ(1–40) when a library of trimeric to decam-

This work was supported by an award from the American Heart Association, Florida/Puerto Rico Affiliate (to M.A.M.).

**ABBREVIATIONS:** AD, Alzheimer's disease; Aβ, amyloid β-protein; DLS, dynamic light scattering; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate-buffered saline; AFM, atomic force microscopy; TFA, trifluoroacetic acid; SEC, size-exclusion chromatography.

eric peptides spanning the entire Aβ(1–40) sequence was synthesized and screened for their ability to bind the full-length peptide, and alanine substitution subsequently showed that Lys16, Leu17, and Phe20 are critical for this interaction (Tjernberg et al., 1996). The stereospecific binding of KLVFF to the homologous sequence in Aβ was later confirmed and shown to result from specific hydrophobic and electrostatic interactions (Tjernberg et al., 1997). Murphy and colleagues (Ghanta et al., 1996; Pallitto et al., 1999; Lowe et al., 2001) developed the hybrid peptide KLVFFKKKKKK (KLVFF-$K_6$), consisting of KLVFF as a 'recognition element' coupled to a C-terminal hexalysine 'disrupting element' designed to interfere with Aβ self-assembly. However, using dynamic light scattering (DLS) to monitor aggregate size, these workers reported that the hybrid peptide increased the rate of Aβ fibril formation and led to an alteration in aggregate morphology toward a more branched structure. These changes correlated with a decrease in Aβ toxicity as inferred from an MTT reduction assay (Ghanta et al., 1996; Pallitto et al., 1999; Lowe et al., 2001). Hexalysine was not a unique 'disrupting element', because a negatively charged C-terminal tetraglutamate segment resulted in similar effects on Aβ fibril formation and MTT reduction (Lowe et al., 2001). These results seem paradoxical, because the amyloid hypothesis proposes that Aβ fibril formation leads to neuronal death. Furthermore, other variations on the KLVFF peptide inhibit Aβ fibrillogenesis. These include the C-terminal addition of short hydrophilic polymers as 'disrupting elements' (Watanabe et al., 2002), *N*-methylation of alternate amide linkages (Gordon et al., 2001), and replacement of alternate amide linkages with ester bonds (Gordon and Meredith, 2003). One explanation for the observations of Murphy and colleagues might be that the acceleration in Aβ aggregation rate leads to an increase in less toxic insoluble fibrils at the expense of toxic soluble protofibrils. Increasing evidence suggests that soluble protofibrils or oligomers are the primary toxic species and that the fibril itself may be protective (Kirkitadze et al., 2002). Aβ protofibrils and oligomers have been shown to induce neurotoxicity (Lambert et al., 1998; Hartley et al., 1999) and to inhibit hippocampal long-term potentiation (Walsh et al., 2002). One report found that Aβ preparations optimized for high oligomeric and protofibrillar content induced significantly greater effects in the MTT reduction assay than fibrillar preparations (Dahlgren et al., 2002), although another found no difference between Aβ protofibrils and fibrils in this assay (Walsh et al., 1999). The recently characterized Arctic mutation in APP, which leads to early-onset AD, resulted in increased production of Aβ protofibrils, whereas the overall rate of fibrillogenesis was unaltered (Nilsberth et al., 2001). Finally, although plaque density fails to predict disease progression, levels of soluble Aβ have been correlated with measures of disease severity (Lue et al., 1999; McLean et al., 1999).

In a previous study, we distinguished between two modes of protofibril growth: elongation by monomer deposition and direct protofibril-protofibril association. These growth mechanisms could be resolved by varying Aβ monomer and NaCl concentrations (Nichols et al., 2002). This study uses these distinct protofibril growth assays to investigate whether KLVFF-$K_6$ can differentially affect protofibril growth and to establish the stoichiometry for these interactions. Furthermore, this study investigates whether the effects of KLVFF-$K_6$ on protofibril growth are sufficient to alter the effects of Aβ protofibrils on cellular MTT reduction.

## Materials and Methods

**Materials.** Aβ(1–40) peptide was obtained from QCB (Hopkinton, MA). Peptides KLVFFKKKKKK (KLVFF-$K_6$) and KKKKKK ($K_6$) were synthesized by the protein and peptide core facility at Mayo Clinic (Rochester, MN) using standard 9-fluorenylmethoxycarbonyl techniques. [$^3$H]HCHO (10 Ci/mmol) was from American Radiolabeled Chemicals, Inc. (St. Louis, MO). [$^3$H]HCHO (85.5 mCi/mmol), [$^{14}$C]HCHO (56 mCi/mmol), and scintillation cocktail (Ultima Gold) were from PerkinElmer Life Sciences (Boston, MA). Bovine serum albumin, horse serum, and thioflavin T were from Sigma (St. Louis, MO). RPMI 1640 media, fetal bovine serum, penicillin/streptomycin/glutamine, and phosphate-buffered saline (PBS) were from Invitrogen (Carlsbad, CA). MTT was from Molecular Probes (Eugene, Oregon).

**Preparation of Aβ Peptides.** Aβ(1–40) peptide was obtained in lyophilized form and stored at −20°C desiccated until reconstitution in deionized water (Nichols et al., 2002). Before use in aggregation or elongation assays, stock monomer was purified by SEC on a 1 × 30-cm Superdex 75 HR 10/30 column (Amersham Biosciences, Piscataway, NJ), and concentrations of monomeric Aβ were determined with an extinction coefficient of 1450 $cm^{-1}$ $M^{-1}$ at 276 nm (Nichols et al., 2002). Aβ(1–40) and KLVFF-$K_6$ were radiolabeled by reductive methylation as described previously (Nichols et al., 2002). [$^3$H]HCHO or [$^{14}$C]HCHO was used directly or after dilution with unlabeled HCHO to lower specific activity. $^3$H- or $^{14}$C-labeled Aβ was separated from excess HCHO and side reaction products by SEC on Superdex 75, whereas $^3$H-labeled KLVFF-$K_6$ was separated by SEC on a 1 × 30-cm Superdex Peptide HR 10/30 column (Amersham Biosciences). The Aβ peptide was completely methylated (Nichols et al., 2002), with specific activities ranging from 300 to 1050 dpm/pmol for $^3$H and 18 dpm/pmol for $^{14}$C. Electrospray ionization-mass spectrometry with a Deca XP Plus quadrupole ion trap mass spectrometer (Thermo Finnigan, San Jose, CA) demonstrated that of the 16 available amine methylation sites in the KLVFF-$K_6$ peptide, an average of five or six were methylated. Further tandem mass spectrometry analysis indicated that the percentage methylation of the N-terminal α-amino group was about twice that of the ε-amino groups of the lysine residues. This result is consistent with previous observations that ε-amino groups do not reductively methylate as readily as α-amino groups (Sherman et al., 1983). Amino acid analysis established a specific activity of 190 dpm/pmol.

**Fluorescence Determinations of the Binding of Thioflavin T to Aβ Amyloid.** Thioflavin T fluorescence measurements were made as described previously (LeVine, 1993; Walsh et al., 1999). Fluorescence was monitored by diluting Aβ samples into comparable buffer containing 5 μM thioflavin T at 23°C on an LS 50B luminescence spectrometer (PerkinElmer Life Sciences) with excitation at 450 nm, emission from 470 to 500 nm, and slits of 10 nm (Nichols et al., 2002).

**Preparation of Aβ(1–40) Protofibrils and Fibrils.** Aβ(1–40) protofibrils and fibrils were prepared as described previously (Nichols et al., 2002). Briefly, unlabeled or radiomethylated Aβ(1–40) monomer (final concentration, 70–120 μM), freshly isolated by SEC on Superdex 75 in 0.5 to 1 ml of 50 mM Tris-HCl, 5 mM EDTA-NaOH, pH 8.0 (denoted 50 mM Tris-EDTA) with 0 to 150 mM NaCl at room temperature, was agitated vigorously by continued vortexing to promote aggregation. Aggregation was monitored by thioflavin T fluorescence until a fluorescence increase estimated to be 20 to 100% of the maximum final fluorescence was observed. The sample was spun for 10 min in a tabletop Microfuge (Beckman Coulter, Fullerton, CA) at 18,000*g*. The pellet obtained from this centrifugation was defined as the fibril fraction. The supernatant was chromatographed on Superdex 75, and Aβ eluting in the void volume was defined as the protofibril fraction. Unlabeled fibril and protofibril concentrations were estimated by thioflavin T fluorescence combined with UV ab-

sorbance at 280 nm corrected for light scattering as described previously (Nichols et al., 2002). Protofibril and fibril concentrations are expressed in monomer concentration units. Fibrils and protofibrils were used immediately or stored at 4°C for up to several days. This storage had no apparent effects on fibril or protofibril behavior in the assays discussed below.

**Protofibril Association Assay.** Protofibrils isolated on Superdex 75 were diluted to 0.1 to 2 $\mu$M in 50 mM Tris-EDTA with either no peptide (control), KLVFF-$K_6$ (0.1–20 $\mu$M), or $K_6$ (1–2 $\mu$M). In some studies, 150 mM NaCl was included in the reaction mixture. Association reactions were incubated without agitation at room temperature, and DLS intensity and aggregate size were measured as described previously (Nichols et al., 2002) with a DynaPro MSX instrument (Protein Solutions Inc., Piscataway, NJ). Reactions were also monitored for thioflavin T fluorescence by periodic dilution of aliquots into 5 $\mu$M thioflavin T.

**Protofibril Elongation Assay.** Isolated protofibrils and freshly isolated A$\beta$ monomer were diluted to final concentrations of 2 $\mu$M and 20 $\mu$M, respectively, in 50 mM Tris-EDTA with or without the KLVFF-$K_6$ or $K_6$ peptides. A$\beta$ protofibrils and monomer were either both unlabeled or both radiomethylated. Elongation reactions were incubated without agitation at room temperature, and thioflavin T fluorescence was continuously monitored in situ by inclusion of 5 $\mu$M thioflavin T in the reaction. Elongation rates were determined by linear regression of the initial increase in thioflavin T fluorescence.

**Monomer Aggregation Assay.** A$\beta$(1–40) monomer freshly isolated on Superdex 75 was diluted to 100 $\mu$M in 50 mM Tris-EDTA alone or with the KLVFF-$K_6$ or $K_6$ peptides. Aggregation reactions were incubated at room temperature either still or under continuous agitation imposed using a platform shaker (Lab-line Instruments, Melrose Park, IL) at approximately 1000 rpm, and thioflavin T fluorescence was monitored by periodic dilution of an aliquot into 5 $\mu$M thioflavin T.

**Characterization of the Binding of [$^3$H]KLVFF-$K_6$ to A$\beta$ Protofibrils.** Unlabeled A$\beta$(1–40) protofibrils isolated on Superdex 75 were diluted to 1 $\mu$M in 50 mM Tris-EDTA with [$^3$H]KLVFF-$K_6$ at final concentrations ranging from 0.06 to 4 $\mu$M. Reactions were incubated without agitation at room temperature for 1 h and microcentrifuged for 10 min at 18,000*g*. The concentrations of peptide and protofibril were measured by radioactivity and thioflavin T fluorescence, respectively, before centrifugation and in the supernatant. The concentrations of bound peptide ($[L]_{bound}$) and associated protofibril ($[PF]_{assoc}$, in monomer units) in the sedimented fraction were determined by difference. Parallel samples containing [$^3$H]KLVFF-$K_6$ alone were measured before and after incubation to determine peptide loss caused by adsorption, and the final quantity of sedimented peptide was corrected for this loss. Three separate experiments were performed, and the compiled data were fit to the single-site ligand binding model (Langmuir, 1918) in eq. 1 using a nonlinear least-squares regression analysis (SigmaPlot 2001).

$$\frac{[L]_{bound}}{[PF]_{assoc}} = \frac{r([L])}{n(K_d + [L])} \quad (1)$$

where [L] represents the concentration of the free ligand [$^3$H]KLVFF-$K_6$, $K_d$ is the intrinsic dissociation constant for peptide binding to a monomer unit within the protofibril, $n$ is the number of monomer units per protofibril, and $r/n$ is the fraction of monomer units bound to ligand at saturating ligand concentrations. The extent of ligand binding to soluble protofibrils in the supernatant fraction was assumed to be identical to that in the sedimented fractions, and values of [L] were calculated as the difference of the total ligand and the bound ligand in the supernatant fraction.

**Atomic Force Microscopy.** Samples were prepared for AFM on mica as described previously (Nichols et al., 2002). Images (10 × 10 $\mu$m, 512 × 512 pixels) were analyzed for particle height distributions using NanoScope III (Nichols et al., 2002). Heights were measured on a grid of regularly spaced horizontal line sections through the image.

**MTT Reduction Assay.** This assay was adapted from the procedure of Lowe et al. (2001). PC-12 cells were grown on 75-cm$^2$ polystyrene tissue culture flasks (Corning Glassworks, Corning, NY) in RPMI medium supplemented with 5% fetal bovine serum, 10% heat-inactivated horse serum, 100 U/ml penicillin, 100 $\mu$g/ml streptomycin, and 300 $\mu$g/ml glutamine. Cells were maintained in a humidified incubator that provided an atmosphere of 5% $CO_2$ and 95% air at a constant temperature of 37°C. Cells were harvested from 75-cm$^2$ flasks by washing with PBS followed by resuspension via agitation in fresh media. Cells were plated onto 96-well, flat-bottomed tissue culture treated plates (Costar, Cambridge, MA) at a density of approximately 6000 cells/well (100 $\mu$l/well). Plates were incubated at 37°C for 24 h to allow cells to attach. A$\beta$(1–40) preparations in 50 mM Tris-HCl, pH 8.0, or 50 mM Tris-EDTA were diluted to a final concentration of 0.01 nM to 2 $\mu$M in cell culture media without phenol red. This provided a 10-fold or larger dilution, which was sufficient to minimize buffer-induced changes in MTT reduction. Medium (80 $\mu$l) was removed from each well and replaced with 80 $\mu$l of medium containing A$\beta$ alone (positive control), medium containing A$\beta$ and peptide, or medium containing buffer (negative control). Each treatment was performed in six replications, and values are presented as the mean ± S.E. Plates were incubated at 37°C for 24 h. This treatment time was sufficient for control samples with A$\beta$ to induce optimal changes in MTT reduction in PC-12 cells. 10 $\mu$l of 5 mg/ml MTT (in PBS) was added to each well, and plates were incubated at 37°C for 4 h, an incubation time sufficient to induce an optimal signal for MTT reduction. One hundred microliters of 10% SDS in 0.01 M HCl was then added to each well, and plates were incubated overnight at 37°C. The absorbance of the formazan product of MTT reduction was measured at 570 nm using a microplate reader (Molecular Devices, Sunnyvale, CA) with background subtraction. Values were reported as percentage of negative control $[(100\%)(Abs_{sample} - Abs_{background})/(Abs_{negative\ control} - Abs_{background})]$.

## Results

We first examined KLVFF-$K_6$ in assays on A$\beta$ protofibril growth and monomer aggregation that we developed previously (Nichols et al., 2002). Unlabeled and radiomethylated A$\beta$(1–40) react similarly in these assays except that rates of aggregation and growth are slower with the radiolabeled A$\beta$. To avoid complications resulting from trace labeling, we employed either unlabeled or fully methylated A$\beta$. Furthermore, the effects of KLVFF-$K_6$ were the same with labeled and unlabeled A$\beta$; experiments with both preparations are illustrated in the following sections.

**KLVFF-$K_6$ Promoted A$\beta$ Protofibril Association.** Isolated A$\beta$(1–40) protofibrils grow by direct protofibril-protofibril association on addition of NaCl in the absence of monomer (Nichols et al., 2002). Association was monitored by DLS and corresponded to an increase in light scattering intensity and aggregate size at a constant A$\beta$ protofibril concentration. To determine whether KLVFF-$K_6$ influenced the association reaction, radiomethylated A$\beta$(1–40) protofibrils were isolated in 50 mM Tris-EDTA buffer and incubated in the presence and absence of KLVFF-$K_6$. As shown in Fig. 1, no increase in light scattering intensity was observed in the buffer alone, but a significant increase was apparent in the presence of KLVFF-$K_6$. Higher rates of association were observed with increasing equimolar concentrations of KLVFF-$K_6$ and A$\beta$ protofibrils and with higher ratios of peptide to A$\beta$ (data not shown), but association was detected at ratios of peptide to A$\beta$ as low as 1:10. Background rates of protofibril association

in 150 mM NaCl, were accelerated by equimolar KLVFF-$K_6$, but the increase was less pronounced than that observed in the absence of NaCl. To rule out any possibility that hexalysine polycations alone were responsible for the increase in protofibril association, Aβ protofibrils were incubated in the presence of the peptide $K_6$. This peptide failed to induce an increase in light scattering intensity (Fig. 1), indicating that interaction between the peptide KLVFF motif and Aβ is required for the promotion of protofibril association. However, KLVFF alone is not sufficient to affect Aβ assembly, because this pentapeptide failed to increase the growth rates or alter the morphology of Aβ aggregates (Pallitto et al., 1999).

Images of Aβ protofibrils associated for 1 h in the presence or absence of KLVFF-$K_6$ were obtained by AFM and confirmed the DLS observations. The presence of equimolar KLVFF-$K_6$ led to the recruitment of Aβ protofibrils into large aggregates, as evidenced by the simultaneous appearance of larger structures and disappearance of smaller structures (Fig. 2, A and B). The aggregates obtained with KLVFF-$K_6$ featured disordered clumps of protofibrils, a morphology reminiscent of that observed after NaCl-induced protofibril association, whereas the protofibrils incubated in the absence of peptide retained the discrete morphology of the initial protofibrils (Nichols et al., 2002). Quantitative analysis of the AFM images revealed a shift in the height distribution consistent with the increase in aggregate size. Aβ protofibrils incubated in buffer alone showed a range of protofibril heights with a peak around 3 nm (Fig. 2C). After association with equimolar KLVFF-$K_6$, a substantial loss in the 3-nm protofibril peak was observed, and this was accompanied by the appearance of large clusters with heights exceeding 9 nm (Fig. 2D).

To further confirm that the larger aggregates observed by DLS and AFM were a product of the coalescence of existing protofibrils, amyloid content was assessed with thioflavin T, a fluorophore that shows greatly enhanced fluorescence on binding to amyloid fibrils (LeVine, 1993). Aβ protofibrils incubated in the presence and absence of equimolar KLVFF-$K_6$ exhibited similar thioflavin T fluorescence, indicating that the two samples contained equivalent amounts of amyloid. However, the relative amount of soluble protofibril versus insoluble fibril was influenced by the presence of KLVFF-$K_6$. Overnight incubation of Aβ protofibrils with equimolar KLVFF-$K_6$ resulted in the sedimentation of >90% of material staining with thioflavin T, whereas protofibrils incubated in buffer alone remained 70 to 100% soluble. Thus, protofibril association induced by KLVFF-$K_6$ does not lead to de novo amyloid formation but instead results in the formation of sedimentable fibrils via conversion of soluble protofibrils. Protofibrils associated with NaCl exhibited a similar increase in sedimentable material (Nichols et al., 2002).

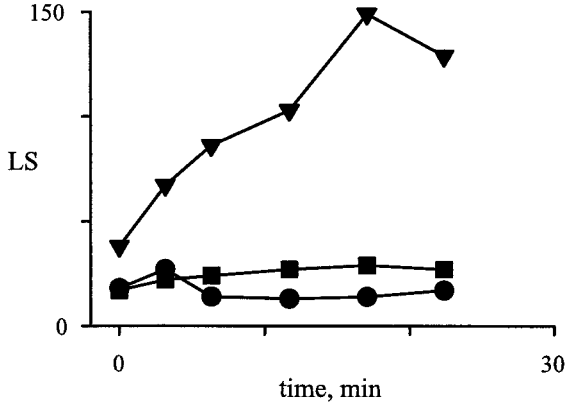


**Fig. 1.** The peptide KLVFF-$K_6$ promotes protofibril association. Freshly isolated $^{14}$C-Aβ(1–40) protofibrils diluted to 2 μM in 50 mM Tris-EDTA were incubated alone (●), with 2 μM KLVFF-$K_6$ (▼), or with 2 μM $K_6$ (■) at room temperature. Light scattering intensity (LS) was monitored by DLS. Results are representative of six experiments.

**KLVFF-$K_6$ Failed to Alter Rates of Aβ Protofibril Elongation by Monomer Deposition.** Aβ protofibril elongation by monomer deposition can be resolved by incubation of purified Aβ protofibrils in the presence of excess monomer and the absence of NaCl (Nichols et al., 2002). To ascertain the effect of KLVFF-$K_6$ on Aβ protofibril elongation, reactions were monitored for increases in thioflavin T fluorescence (F) (Fig. 3A). When Aβ protofibrils were incubated with an excess of Aβ monomer, elongation proceeded as observed previously (Nichols et al., 2002). The KLVFF-$K_6$ peptide did not alter the rate of elongation, even when the peptide was present in 10-fold molar excess of protofibril (Fig. 3A), and the $K_6$ peptide also was without effect (data not shown).

**Aβ Monomer Aggregation was Inhibited by KLVFF-$K_6$.** Aggregation of isolated Aβ monomer was induced by continuous agitation and monitored by thioflavin T fluorescence. The time course followed the pattern observed previously, with a lag time followed by a period of steady growth and then a plateau (Nichols et al., 2002). Addition of equimolar amounts of KLVFF-$K_6$ to the Aβ monomer aggregation

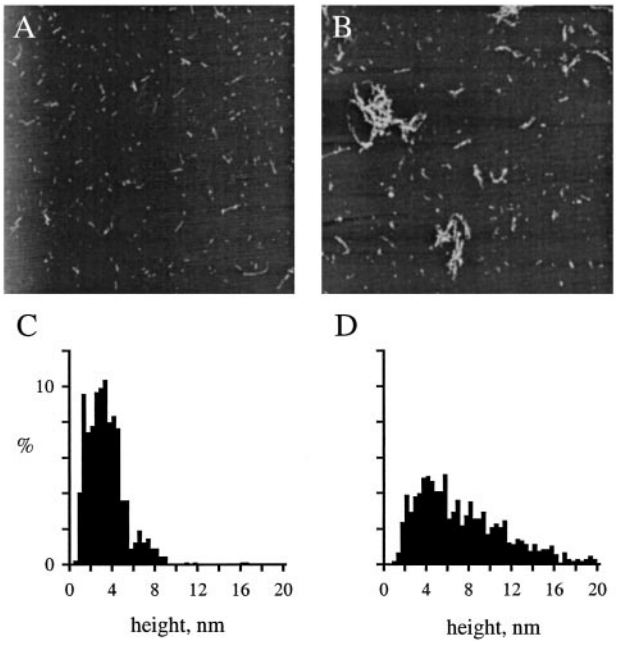


**Fig. 2.** Aβ(1–40) protofibril association induced by KLVFF-$K_6$ as monitored by AFM. Freshly isolated, unlabeled Aβ(1–40) protofibrils diluted to 2 μM in 50 mM Tris-HCl, pH 8.0, were incubated alone (A and C) or with 2 μM KLVFF-$K_6$ (B and D) for 1 h at room temperature without agitation. Samples were diluted 5-fold in 50 mM Tris-HCl, pH 8.0, applied to mica stubs, and analyzed by AFM as outlined under *Materials and Methods*. A and B, representative 10 × 10-μm images. C and D, height distributions of all particles observed on regularly spaced horizontal lines through the AFM images. These distributions tabulate the frequency of particle heights in 0.4-nm increments and represent 900 to 1100 measurements from four images.

led to a substantial increase in the lag time to fluorescence (Fig. 3B). Others have shown that the peptide KLVFF has no effect on aggregation of Aβ(1–40) monomer (Findeis et al., 1999). In contrast, addition of equimolar amounts of the $K_6$ peptide slightly promoted aggregation, as evidenced by a decrease in the lag time. A similar effect was observed when NaCl was added to the aggregation mixture (Nichols et al., 2002). When Aβ monomer aggregation was allowed to proceed in the absence of agitation, a similar inhibition by KLVFF-$K_6$ was observed, albeit over a longer time course (data not shown).

Despite the slower Aβ aggregation in the presence of KLVFF-$K_6$, aggregates that did form were rapidly converted to fibrils, with >90% of the thioflavin T-binding material sedimenting as quickly as it was formed. Therefore, no protofibril fraction could be generated when KLVFF-$K_6$ was present, as expected from the strong promotion of protofibril association by KLVFF-$K_6$ reported above.

**[$^3$H]KLVFF-$K_6$ Was Bound to Aβ Protofibrils.** To accelerate the association of Aβ protofibrils, KLVFF-$K_6$ must bind to sites on the protofibril. This binding was quantified after radiolabeling KLVFF-$K_6$ with a reductive radiomethylation procedure similar to that employed for Aβ monomer (Nichols et al., 2002). [$^3$H]KLVFF-$K_6$ promoted Aβ(1–40) protofibril association in the same concentration-dependent manner as the unlabeled peptide, and AFM images confirmed that Aβ protofibrils associated in the presence of [$^3$H]KLVFF-$K_6$ exhibited structures similar to protofibrils associated in the presence of the unlabeled peptide (data not shown). After 1 h of incubation, a free [$^3$H]KLVFF-$K_6$ concentration of about 100 nM was sufficient to convert 50% of the soluble protofibrils to sedimentable fibrils, and higher concentrations resulted in complete sedimentation. Binding was measured by exploiting this conversion. Mixtures of [$^3$H]KLVFF-$K_6$ and Aβ protofibrils were microcentrifuged, and sedimented peptide (measured by radioactivity) and sedimented Aβ (measured by thioflavin T fluorescence) were determined over a range of peptide concentrations. The results of three separate experiments are plotted in Fig. 4 as the ratio of sedimented [$^3$H]KLVFF-$K_6$ to sedimented protofibril (measured in monomer units) versus the free [$^3$H]KLVFF-$K_6$ concentration. Binding was assumed to involve a single class of sites of equal affinity on the protofibrils, and the data in Fig. 4 were fit to eq. 1 to yield a dissociation constant $K_d$ of 180 ± 90 nM. This value may be an overestimate because electrostatic interactions between bound [$^3$H]KLVFF-$K_6$ ligands may progressively decrease ligand affinities at higher levels of saturation. The fraction of monomer units bound to ligand at saturating concentrations of [$^3$H]KLVFF-$K_6$ ($r/n$) extrapolated to 0.4, revealing that less than half of the Aβ monomer units in protofibrils were available to bind peptide. This value suggests that peptide binding sites may become inaccessible either as Aβ monomer is incorporated into fibrillar β-sheets or as protofibrils associate laterally. Because our Aβ(1–40) protofibrils typically contain between 1000 and 3000 monomer units (Nichols et al., 2002),

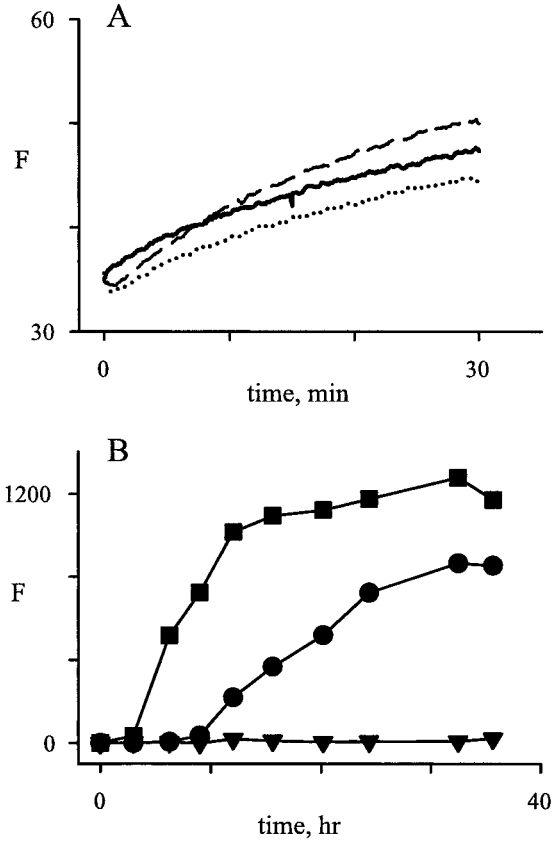


**Fig. 3.** The peptide KLVFF-$K_6$ inhibits Aβ monomer aggregation. A, elongation by monomer deposition. Freshly isolated $^{14}$C-Aβ(1–40) protofibrils diluted to 2 μM and freshly isolated $^{14}$C-Aβ(1–40) monomer diluted to 20 μM in 50 mM Tris-EDTA were incubated together (solid line), with 2 μM KLVFF-$K_6$ (dashed line), or with 20 μM KLVFF-$K_6$ (dotted line) at room temperature. Thioflavin T fluorescence (F) was monitored in situ by inclusion of 5 μM thioflavin T in the reaction mixture. Results are representative of four experiments. B, monomer aggregation. Freshly isolated $^3$H-Aβ(1–40) monomer diluted to 100 μM in 50 mM Tris-EDTA was incubated alone (●), with 100 μM KLVFF-$K_6$ (▼), or with 100 μM $K_6$ (■) at room temperature and under constant agitation. Thioflavin T fluorescence (F) was monitored by periodic dilution of a 10-μl aliquot into 5 μM thioflavin T (150 μl); fluorescence values were corrected to account for Aβ adsorption, which was measured by loss of radioactivity. Results are representative of three experiments.

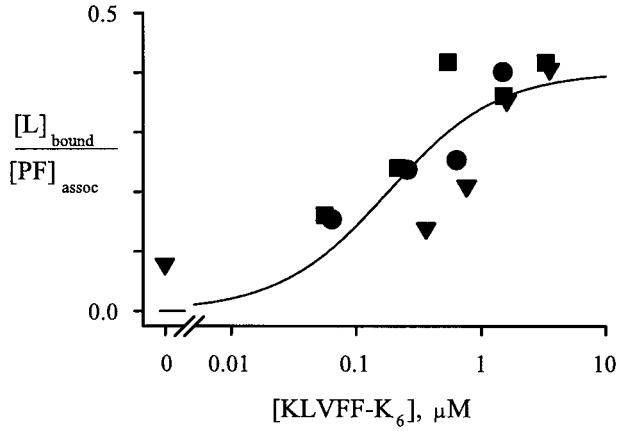


**Fig. 4.** Binding of [$^3$H]KLVFF-$K_6$ to Aβ protofibrils. Freshly isolated, unlabeled Aβ(1–40) protofibrils were diluted to 1 μM in 50 mM Tris-EDTA and [$^3$H]KLVFF-$K_6$ was added at concentrations ranging from 6.25 nM to 4 μM for 1 h at room temperature without agitation. Samples were microcentrifuged at 18,000*g* and supernatants were measured for peptide (by radioactivity) and Aβ protofibril (by dilution into 5 μM thioflavin T). The amounts of peptide and protofibril in the sedimented fraction were determined by difference, and the results were plotted as a ratio versus the free peptide concentration. Binding was analyzed with a model that incorporates multiple binding sites with equal affinity (eq. 1). Data from three separate experiments, indicated by the different symbol types, were fit to eq. 1 to describe binding ($K_d$ = 180 ± 90 nM).

binding at sites on a few hundred of these units resulted in the observed protofibril association.

KLVFF-$K_6$ could slow monomer aggregation by also binding to Aβ monomers and preventing their incorporation into higher order structures. No high-affinity binding was evident, however, when mixtures of [$^3$H]KLVFF-$K_6$ and unlabeled Aβ monomer were resolved by SEC on Superdex 75. Ratios of peptide to Aβ in the monomer fraction were less than 1:1000, indicating that virtually no binding survived SEC separation. Murphy and colleagues previously observed that Aβ aggregated in the presence or absence of hybrid peptide and resolved by SEC failed to show any shift in the monomer retention time or any differences in the distribution of Aβ monomer, dimer, and fibril (Lowe et al., 2001). Thus, it is not likely that KLVFF-$K_6$ inhibits Aβ monomer aggregation by trapping Aβ monomer.

**Promotion of Protofibril Association by KLVFF-$K_6$ Failed to Reverse Aβ Effects on Cellular MTT Reduction.** To explore whether the Aβ protofibril association induced by KLVFF-$K_6$ alters the activity of Aβ protofibrils on cultured cells, an MTT reduction assay was employed. This assay is not a direct measure of cell survival but instead identifies changes in cellular redox activity that may correlate with cell viability (Shearman et al., 1994). Because decreases in MTT reduction can be observed at Aβ concentrations below those that compromise cell survival (Shearman et al., 1994), this assay is more widely used than direct measures of cell death to investigate the neurotoxicity of Aβ fibrils, protofibrils, and oligomers (El-Agnaf et al., 2000; Ward et al., 2000). However, concerns about the correlation of MTT reduction with cell viability (see *Discussion*) should be considered when interpreting MTT results.

PC-12 cells exhibited a decrease in the reduction of MTT after treatment with Aβ protofibrils, and a similar decrease was observed when protofibrils were incubated overnight with KLVFF-$K_6$ (Fig. 5A). The incubation with KLVFF-$K_6$ converted all of the protofibrils to sedimenting fibrils (Fig. 5B). Furthermore, KLVFF-$K_6$ did not alter the effects of Aβ protofibrils on MTT reduction even in the presence of a 100-fold excess of peptide or when the incubation time was extended up to 15 days. Thus, promotion of Aβ protofibril association by KLVFF-$K_6$ was not sufficient to prevent or reverse the effect of Aβ protofibrils on this cellular redox activity. To further examine whether aggregate size affects this activity, the dependence of MTT reduction on the concentration of both Aβ fibrils and Aβ protofibrils was compared. Fibrils and protofibrils exhibited a similar dose-response curve for MTT reduction: both decreased MTT reduction at nanomolar levels (Fig. 6).

In contrast to KLVFF-enhanced protofibril association, the inhibition of Aβ monomer aggregation by KLVFF-$K_6$ (as in Fig. 3B) resulted in the absence of an Aβ effect on cellular MTT reduction. Whereas a control Aβ monomer aggregation mixture decreased MTT reduction to an extent similar to that of isolated Aβ protofibrils, Aβ monomer incubated in the presence of equimolar KLVFF-$K_6$ under constant agitation for 12 h failed to induce a decrease in MTT reduction (data not shown). However, agitation with KLVFF-$K_6$ for times longer than the lag time in Fig. 3B resulted in thioflavin T-binding aggregates that exhibited MTT reduction similar to control samples. Thus, complete inhibition of Aβ monomer aggregation, as measured by thioflavin T fluorescence, was necessary for KLVFF-$K_6$ to prevent Aβ effects on cellular activity.

Murphy and colleagues reported that Aβ(1–40) aggregates formed in the absence of KLVFF-$K_6$ decreased cellular MTT reduction, whereas those formed in the presence of this peptide did not (Lowe et al., 2001). In an effort to reconcile our contrasting observations, we examined their Aβ aggregation conditions. In their studies, Aβ was freshly dissolved in 0.1% TFA at 5 to 10 mg/ml before dilution into PBS. With our Aβ(1–40), DLS measurements of similar stock solutions in 0.1% TFA revealed a substantial number of aggregates with $R_H$ values in the range of 20 and 200 nm. Dilution into PBS resulted in an instantaneous increase in light scattering to intensities that were offscale, in both the presence and absence of KLVFF-$K_6$. Before their light scattering studies, the solutions of Aβ in PBS were filtered through 0.45-μm filters. Similar filtration of our solutions brought the DLS intensities back on scale and allowed us to confirm that KLVFF-$K_6$

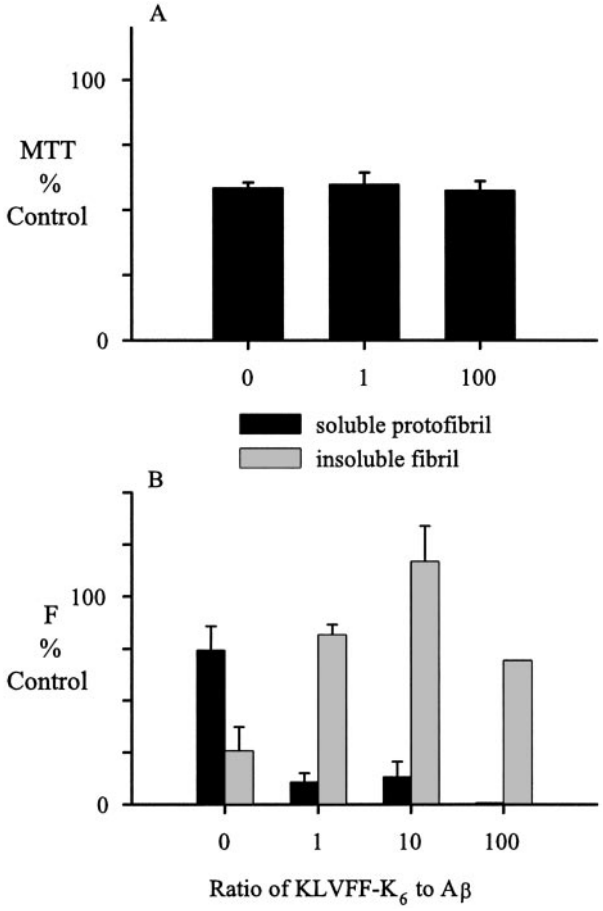


**Fig. 5.** Cellular MTT reduction after incubation of KLVFF-$K_6$ with Aβ protofibrils. A, MTT reduction assay. Freshly isolated, unlabeled Aβ(1–40) protofibrils (2 μM) were incubated as in Fig. 1 with varying molar ratios of KLVFF-$K_6$ to Aβ (0, 1, or 100) overnight. An aliquot of each reaction was diluted 20-fold into cell culture media and applied to PC-12 cells for an MTT reduction assay as outlined under *Materials and Methods*. MTT results are expressed as the percentage of MTT reduction compared with negative control wells containing an equivalent dilution of buffer into media. Error bars indicate standard error ($n = 6$). B, thioflavin T measurements. Fluorescence measurements with thioflavin T after the initial overnight incubation confirmed that protofibrils associated by KLVFF-$K_6$ were converted to sedimentable fibrils with little change in thioflavin T fluorescence. Error bars indicate standard error (ratio of KLVFF-$K_6$ to Aβ of 0 (Control) and 1, $n = 4$; 10, $n = 2$; 100, $n = 1$).

sharply accelerated subsequent increases in DLS intensities (Lowe et al., 2001). The presence of pre-existing aggregates in these Aβ stocks explains why no inhibition of Aβ aggregation by KLVFF-$K_6$ like that in Fig. 3B was observed. Instead, a KLVFF-$K_6$ promotion of interaction between Aβ aggregates like that in Fig. 1 was the predominant effect. The TFA-PBS aggregates employed in their MTT reduction assays were similar to those used in the DLS studies except that no filtration was performed before 48-h incubation without agitation at 37°C. In our incubations, the fluorescence was similar with and without KLVFF-$K_6$ and remained roughly constant throughout the 48 h. After incubation, the large size of the aggregates in both preparations was confirmed by sedimentation of >95% of the fluorescence. When observed by electron microscopy, these aggregates displayed a finer and more tangled network than fibrils aggregated under our standard conditions (Fig. 7). After dilution and 24-h incubation of these aggregates in cell culture media at 37°C, 50 to 60% of the thioflavin T fluorescence was lost, and a substantial amount of the remaining fluorescence was transferred from the sedimentable to the soluble fraction. In contrast, protofibrils aggregated under our standard conditions retained thioflavin T fluorescence, and >70% of this fluorescence became sedimentable. The combination of the large size, altered morphology, loss of aggregate mass, and change in aggregate composition appeared to render the TFA-PBS aggregates less active in the MTT assay. The dependence of MTT reduction on Aβ concentration was markedly shifted for these aggregates, with decreases in MTT reduction apparent only at micromolar levels (based on the original amounts of stock Aβ) (Fig. 6), about 2 to 3 orders of magnitude higher than for protofibrils and fibrils aggregated under our standard conditions.

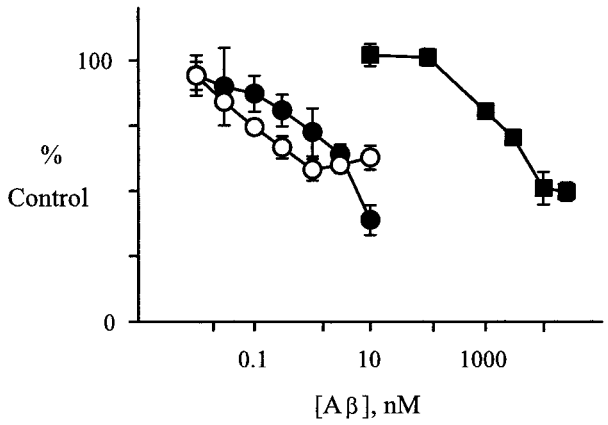


**Fig. 6.** Preparations of Aβ aggregates differ in their potencies in the MTT reduction assay. Unlabeled Aβ(1–40) fibrils (●) and protofibrils (○) were prepared and isolated as described under *Materials and Methods*. These preparations were diluted in 50 mM Tris-HCl, pH 8.0, and then further diluted 10-fold into cell culture media to give concentrations ranging from 0.01 to 10 nM. To prepare Aβ aggregates in PBS by the procedure of Murphy and colleagues (Lowe et al., 2001) (■), Aβ was freshly dissolved in 0.1% TFA for 1 h at room temperature, diluted into PBS, pH 7.4, at a concentration of 115 μM, and incubated without agitation for 48 h at 37°C. Aggregates were diluted approximately 5-fold into cell culture media to give concentrations ranging from 10 nM to 25 μM. Diluted solutions were applied to PC-12 cells for MTT assays as outlined under *Materials and Methods*. Results are expressed as percentage of MTT reduction compared with negative control wells containing an equivalent dilution of buffer into media. Error bars indicate standard error ($n$ = 6). Some error bars lie within symbols.

## Discussion

Murphy and colleagues previously reported that the hybrid peptide KLVFF-$K_6$ simultaneously increased the rate of Aβ fibril formation and decreased cell toxicity (Ghanta et al., 1996; Pallitto et al., 1999; Lowe et al., 2001). The amyloid hypothesis proposes that fibrillar forms of Aβ initiate a chain of events that culminate in neuronal death. In this context, the experimental observations of Murphy and colleagues seem paradoxical. However, increasing evidence suggests that soluble Aβ aggregates may be the more toxic Aβ species, whereas larger aggregates and plaques may, in fact, be protective (Kirkitadze et al., 2002). It was thus postulated that accelerated Aβ aggregation and precipitation induced by KLVFF-$K_6$ may lead to an increase in less toxic insoluble fibrils at the expense of more toxic soluble protofibrils (Lowe et al., 2001). In a previous study, we identified two distinct modes of protofibril growth: elongation by monomer deposition and direct protofibril-protofibril association. These growth mechanisms could be resolved by appropriately adjusting the Aβ monomer and NaCl concentrations (Nichols et al., 2002). This study used these assays to determine whether accelerated fibril formation induced by the hybrid peptide KLVFF-$K_6$ occurred specifically at the level of protofibril growth. We then further tested the hypothesis that decreased toxicity resulted from the KLVFF-$K_6$-promoted growth of soluble protofibrils into insoluble fibrils.

When direct protofibril-protofibril association was induced by incubating purified Aβ protofibrils in the absence of Aβ monomer, KLVFF-$K_6$ markedly increased the rate of Aβ protofibril association in a concentration-dependent manner (Fig. 1). AFM images confirmed the appearance of larger structures accompanied by a shift in the height distribution to particles with increased heights (Fig. 2). In contrast, protofibril elongation by monomer addition, initiated by addition of Aβ monomer to purified protofibrils, was unaltered by the presence of KLVFF-$K_6$ (Fig. 3A). Murphy and colleagues concluded from static light scattering measurements that Aβ aggregated in the presence of KLVFF-$K_6$ seemed to form more branched structures than did Aβ aggregated alone (Pallitto et al., 1999; Lowe et al., 2001). They proposed that this could be caused by either a disruption of linear elongation, via the creation of multiple loci for addition of monomer, or to an increase in aggregate association (Pallitto et al., 1999). If KLVFF-$K_6$ increased branching via the creation of additional

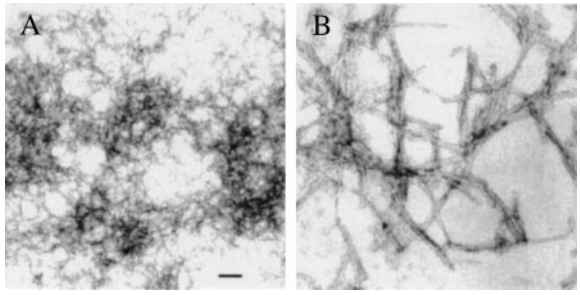


**Fig. 7.** Electron micrographs of Aβ(1–40) fibrils prepared using different solution conditions. A, Aβ(1–40) fibrils aggregated after the procedure of Murphy and colleagues (see legend to Fig. 6 and Lowe et al., 2001). B, Aβ(1–40) fibrils aggregated under our standard conditions with buffer containing 150 mM NaCl (see *Materials and Methods* and Nichols et al., 2002). At 144 h, fibrils were sedimented by centrifugation at 18,000*g*, the supernatant was removed, and the pellet was washed and gently suspended in water. Images are shown relative to a calibration bar of 0.1 μm.

elongation loci, an increase in the elongation rate would have been expected. Because we observed that protofibril elongation by monomer addition was unaltered in the presence of KLVFF-$K_6$, any branched morphology would seem to result from increased interactions between aggregates and not from creation of new loci for branched addition of monomer.

It is widely reported that agents blocking Aβ aggregation also prevent the cellular toxicity of Aβ (Soto et al., 1998; Hughes et al., 2000; Reixach et al., 2000). Several studies have shown that KLVFF derivatives inhibit the aggregation of Aβ as monitored by thioflavin T fluorescence (Gordon et al., 2001; Watanabe et al., 2002; Gordon and Meredith, 2003) and inhibit cellular MTT reduction (Watanabe et al., 2002). Our data showing that the addition of KLVFF-$K_6$ to purified Aβ monomer both inhibited aggregation and blocked a response in the MTT assay are consistent with these reports. The observations of Murphy and colleagues raised the further important possibility that larger Aβ aggregates formed in the presence of KLVFF-$K_6$ were less active in the MTT reduction assay than smaller aggregates formed in the absence of this peptide (Lowe et al., 2001). However, our data do not support this conclusion. Although our protofibril growth assays showed that KLVFF-$K_6$ induced an increase in aggregate size by promoting protofibril association, this increase in size did not alter protofibril effects on PC-12 cells (Fig. 5A). All Aβ aggregates were observed to decrease MTT reduction, regardless of their size. Moreover, the changes in MTT reduction showed a similar dependence on fibril and protofibril concentrations down to nanomolar levels (Fig. 6).

We investigated differences in Aβ aggregation procedures that could account for the discrepancy between our results and those of Murphy and colleagues. When we applied their procedure, aggregates present in the initial stocks of Aβ in 0.1% TFA seemed to act as seeds that induced virtually instantaneous aggregation on dilution into PBS, regardless of the presence or absence of KLVFF-$K_6$. The resulting aggregates were larger and less stable after dilution into media than protofibrils generated in our standard protocol. As an apparent consequence of these differences, these aggregates were less potent in the MTT assay than our protofibrils or fibrils (Fig. 6). When we added an equimolar amount of KLVFF-$K_6$ to incubations generating these aggregates and tested them at the highest Aβ concentration in Fig. 6, MTT reduction returned to the control levels observed in the absence of Aβ (data not shown). In contrast, our standard protofibrils exhibited no shift in the dose-response curve in the presence of KLVFF-$K_6$. It is noteworthy that the dose-response curves for aggregates prepared in TFA-PBS shifted with different Aβ preparations, to the extent that a concentration of 25 μM Aβ did not consistently give the decrease in MTT reduction shown in Fig. 6. This variability in response complicates the significance of the KLVFF-$K_6$ effect on these Aβ preparations.

Conclusions about the comparable neurotoxicity of protofibrils and fibrils in our studies must be tempered, however, because questions have been raised about the relevance of MTT reduction to cell viability (Patel et al., 1996; Abe and Saito, 1999). Several studies have found that cells treated with aggregated Aβ show a decrease in MTT reduction but fail to exhibit other indicators of toxicity, including the reduction of other metabolic dyes (Shearman et al., 1995; Hertel et al., 1996), lactate dehydrogenase release (Hertel et al., 1996; Patel et al., 1996), and trypan blue exclusion (Patel et al., 1996). MTT is taken up by cells through endocytosis, and the formazan product of MTT reduction is transported to the cell surface by exocytosis, where it appears as a crystalline product (Liu and Schubert, 1997). MTT reduction stops when the crystalline product appears (Hertel et al., 1996; Abe and Saito, 1999), either from blockage of MTT endocytosis (Liu and Schubert, 1997) or from cell death arising from membrane damage induced by the crystalline product (Hertel et al., 1996). Aggregated Aβ increases the rate of formazan exocytosis and deposition (Liu and Schubert, 1997) and halts MTT reduction at an earlier time when less formazan has been produced. Therefore, Aβ-induced changes in MTT reduction involve alterations in exocytosis. However, Aβ-induced decreases in MTT reduction also are inhibited by antioxidants (Munoz et al., 2002; Ito et al., 2003), suggesting that Aβ also may alter some cellular redox activity. At the concentrations of aggregated Aβ and treatment times employed here (Fig. 6), no loss of cellular redox activity was detected with assays using either 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide (XTT) (Roehm et al., 1991) or Alamar Blue (Nociari et al., 1998) (data not shown). Primary neuronal cultures exposed to higher concentrations of Aβ (>10 μM) and for longer periods of time (2–4 days) have been shown to exhibit a loss of cell viability (Abe and Saito, 1999; Hartley et al., 1999). It is unclear whether such losses involve Aβ-accelerated exocytosis or Aβ-induced redox changes as a precursor to toxicity. The determination of whether the enhancement of Aβ protofibril association by KLVFF-$K_6$ alters the cellular toxicity of Aβ aggregates will require more direct assays of cell viability.

Although the effects of KLVFF-$K_6$ on Aβ toxicity remain unresolved, the data presented here show that the peptide KLVFF-$K_6$ alters Aβ assembly in two ways: it selectively promotes Aβ protofibril association without altering Aβ protofibril elongation and it inhibits Aβ monomer aggregation. These observations suggest that ligand binding to multiple binding surfaces within the Aβ peptide and protofibril structure affect protofibril formation and growth in different ways. Future experiments will be needed to discern whether targeting potential drugs to one of these binding surfaces is more effective in preventing fibril formation or in providing a therapeutic benefit.

**Acknowledgments**

We are grateful to Kristy Greene (Mayo Clinic) for compilation of AFM measurements and to Stephanie Cratic-McDaniel (Johns Hopkins University School of Medicine) for assistance with AFM imaging. We also recognize Wen-Lang Lin (Mayo Clinic) for technical support with electron microscopy imaging and Bernadette Cusack (Mayo Clinic) for acquisition of mass spectrometry measurements.

**References**

Abe K and Saito H (1999) Both oxidative stress-dependent and independent effects of amyloid β protein are detected by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay. *Brain Res* **830:**146–154.

Dahlgren KN, Manelli AM, Stine WB Jr, Baker LK, Krafft GA, and LaDu MJ (2002) Oligomeric and fibrillar species of amyloid-β peptides differentially affect neuronal viability. *J Biol Chem* **277:**32046–32053.

Duff K, Eckman C, Zehr C, Yu X, Prada CM, Perez-tur J, Hutton M, Buee L, Harigaya Y, Yager D, et al. (1996) Increased amyloid-β42(43) in brains of mice expressing mutant presenilin 1. *Nature (Lond)* **383:**710–713.

El-Agnaf OM, Mahil DS, Patel BP, and Austen BM (2000) Oligomerization and toxicity of β-amyloid-42 implicated in Alzheimer's disease. *Biochem Biophys Res Commun* **273:**1003–1007.

Esler WP, Stimson ER, Ghilardi JR, Lu YA, Felix AM, Vinters HV, Mantyh PW, Lee

JP, and Maggio JE (1996) Point substitution in the central hydrophobic cluster of a human β-amyloid congener disrupts peptide folding and abolishes plaque competence. *Biochemistry* **35:**13914–13921.
Findeis MA, Musso GM, Arico-Muendel CC, Benjamin HW, Hundal AM, Lee JJ, Chin J, Kelley M, Wakefield J, Hayward NJ, and Molineaux SM (1999) Modified-peptide inhibitors of amyloid β-peptide polymerization. *Biochemistry* **38:**6791–6800.
Ghanta J, Shen CL, Kiessling LL, and Murphy RM (1996) A strategy for designing inhibitors of β-amyloid toxicity. *J Biol Chem* **271:**29525–29528.
Gordon DJ and Meredith SC (2003) Probing the role of backbone hydrogen bonding in β-amyloid fibrils with inhibitor peptides containing ester bonds at alternate positions. *Biochemistry* **42:**475–485.
Gordon DJ, Sciarretta KL, and Meredith SC (2001) Inhibition of β-amyloid(40) fibrillogenesis and disassembly of β-amyloid(40) fibrils by short β-amyloid congeners containing *N*-methyl amino acids at alternate residues. *Biochemistry* **40:**8237–8245.
Hartley DM, Walsh DM, Ye CP, Diehl T, Vasquez S, Vassilev PM, Teplow DB, and Selkoe DJ (1999) Protofibrillar intermediates of amyloid β-protein induce acute electrophysiological changes and progressive neurotoxicity in cortical neurons. *J Neurosci* **19:**8876–8884.
Hertel C, Hauser N, Schubenel R, Seilheimer B, and Kemp JA (1996) β-amyloid-induced cell toxicity: enhancement of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide-dependent cell death. *J Neurochem* **67:**272–276.
Hilbich C, Kisters-Woike B, Reed J, Masters CL, and Beyreuther K (1992) Substitutions of hydrophobic amino acids reduce the amyloidogenicity of Alzheimer's disease βA4 peptides. *J Mol Biol* **228:**460–473.
Hsiao K, Chapman P, Nilsen S, Eckman C, Harigaya Y, Younkin S, Yang F, and Cole G (1996) Correlative memory deficits, Aβ elevation and amyloid plaques in transgenic mice. *Science (Wash DC)* **274:**99–102.
Hughes E, Burke RM, and Doig AJ (2000) Inhibition of toxicity in the β-amyloid peptide fragment β-(25–35) using *N*-methylated derivatives: a general strategy to prevent amyloid formation. *J Biol Chem* **275:**25109–25115.
Ito Y, Kosuge Y, Sakikubo T, Horie K, Ishikawa N, Obokata N, Yokoyama E, Yamashina K, Yamamoto M, Saito H, et al. (2003) Protective effect of *S*-allyl-L-cysteine, a garlic compound, on amyloid β-protein-induced cell death in nerve growth factor-differentiated PC12 cells. *Neurosci Res* **46:**119–125.
Kawarabayashi T, Younkin LH, Saido TC, Shoji M, Ashe KH, and Younkin SG (2001) Age-dependent changes in brain, CSF and plasma amyloid β protein in the Tg2576 transgenic mouse model of Alzheimer's disease. *J Neurosci* **21:**372–381.
Kirkitadze MD, Bitan G, and Teplow DB (2002) Paradigm shifts in Alzheimer's disease and other neurodegenerative disorders: the emerging role of oligomeric assemblies. *J Neurosci Res* **69:**567–577.
Lambert MP, Barlow AK, Chromy BA, Edwards C, Freed R, Liosatos M, Morgan TE, Rozovsky I, Trommer B, Viola KL, et al. (1998) Diffusible, nonfibrillar ligands derived from $A\beta_{1-42}$ are potent central nervous system neurotoxins. *Proc Natl Acad Sci USA* **95:**6448–6453.
Langmuir I (1918) The adsorption of gases on plane surfaces of glass, mica and platinum. *J Am Chem Soc* **40:**1361–1403.
LeVine H (1993) Thioflavine T interaction with synthetic Alzheimer's disease β-amyloid peptides: detection of amyloid aggregation in solution. *Protein Sci* **2:**404–410.
Liu Y and Schubert D (1997) Cytotoxic amyloid peptides inhibit cellular 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction by enhancing MTT formazan exocytosis. *J Neurochem* **69:**2285–2293.
Lowe TL, Strzelec A, Kiessling LL, and Murphy RM (2001) Structure-function relationships for inhibitors of β-amyloid toxicity containing the recognition sequence KLVFF. *Biochemistry* **40:**7882–7889.
Lue LF, Kuo YM, Roher AE, Brachova L, Shen Y, Sue L, Beach T, Kurth JH, Rydel RE, and Rogers J (1999) Soluble amyloid β peptide concentration as a predictor of synaptic change in Alzheimer's disease. *Am J Pathol* **155:**853–862.
McLean CA, Cherny RA, Fraser FW, Fuller SJ, Smith MJ, Beyreuther K, Bush AI, and Masters CL (1999) Soluble pool of Aβ amyloid as a determinant of severity of neurodegeneration in Alzheimer's disease. *Ann Neurol* **46:**860–866.
Miller DL, Papayannopoulos IA, Styles J, Bobin SA, Lin YY, Biemann K, and Iqbal K (1993) Peptide compositions of the cerebrovascular and senile plaque core amyloid deposits of Alzheimer's disease. *Arch Biochem Biophys* **301:**41–52.
Munoz FJ, Opazo C, Gil-Gomez G, Tapia G, Fernandez V, Valverde MA, and Inestrosa NC (2002) Vitamin E but not 17β-estradiol protects against vascular toxicity induced by β-amyloid wild type and the Dutch amyloid variant. *J Neurosci* **22:**3081–3089.
Nichols MR, Moss MA, Reed DK, Lin W-L, Mukhopadhyay R, Hoh JH, and Rosenberry TL (2002) Growth of β-amyloid(1–40) protofibrils by monomer elongation and lateral association. Characterization of distinct products by light scattering and atomic force microscopy. *Biochemistry* **41:**6115–6127.
Nilsberth C, Westlind-Danielsson A, Eckman CB, Condron MM, Axelman K, Forsell C, Stenh C, Luthman J, Teplow DB, Younkin SG, et al. (2001) The 'Artic' APP mutation (E693G) causes Alzheimer's disease by enhanced Aβ protofibril formation. *Nat Neurosci* **4:**887–893.
Nociari MM, Shalev A, Benias P, and Russo C (1998) A novel one-step, highly sensitive fluorometric assay to evaluate cell-mediated cytotoxicity. *J Immunol Methods* **213:**157–167.
Pallitto MM, Ghanta J, Heinzelman P, Kiessling LL, and Murphy RM (1999) Recognition sequence design for peptidyl modulators of β-amyloid aggregation and toxicity. *Biochemisty* **38:**3570–3578.
Patel AJ, Gunasekera S, Jen A, and Rohan de Silva HA (1996) β-Amyloid-mediated inhibition of redox activity (MTT reduction) is not an indicator of astroglial degeneration. *Neuroreport* **7:**2026–2030.
Reixach N, Crooks E, Ostresh JM, Houghten RA, and Blondelle SE (2000) Inhibition of β-amyloid-induced neurotoxicity by imidazopyridoindoles derived from a synthetic combinatorial library. *J Struct Biol* **130:**247–258.
Roehm NW, Rodgers GH, Hatfield SM, and Glasebrook AL (1991) An improved colorimetric assay for cell proliferation and viability utilizing the tetrazolium salt XTT. *J Immunol Methods* **142:**257–265.
Shearman MS, Hawtin SR, and Tailor VJ (1995) The intracellular component of cellular 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction is specifically inhibited by β-amyloid peptides. *J Neurochem* **65:**218–227.
Shearman MS, Ragan CI, and Iversen LL (1994) Inhibition of PC12 cell redox activity is a specific, early indicator of the mechanism of β-amyloid-mediated cell death. *Proc Natl Acad Sci USA* **91:**1470–1474.
Sherman G, Rosenberry TL, and Sternlicht H (1983) Identification of lysine residues essential for microtuble assembly. Demonstration of enhanced reactivity during reductive methylation. *J Biol Chem* **258:**2148–2156.
Soto C, Sigurdsson EM, Morelli L, Kumar RA, Castano EM, and Frangione B (1998) β-sheet breaker peptides inhibit fibrillogenesis in a rat brain model of amyloidosis: implications for Alzheimer's therapy. *Nature (Lond) Med* **4:**822–826.
Tjernberg LO, Lilliehook C, Callaway DJE, Naslund J, Hahne S, Thyberg J, Terenius L, and Nordstedt C (1997) Controlling amyloid β-peptide fibril formation with protease-stable ligands. *J Biol Chem* **272:**12601–12605.
Tjernberg LO, Naslund J, Lindqvist F, Johansson J, Karlstrom AR, Thyberg J, Terenius L, and Nordstedt C (1996) Arrest of β-amyloid fibril formation by a pentapeptide ligand. *J Biol Chem* **271:**8545–8548.
Walsh DM, Hartley DM, Kusumoto Y, Fezoui Y, Condron MM, Lomakin A, Benedek GB, Selkoe DJ, and Teplow DB (1999) Amyloid β-protein fibrillogenesis: structure and biological activity of protofibrillar intermediates. *J Biol Chem* **274:**25945–25952.
Walsh DM, Klyubin I, Fadeeva JV, Cullen WK, Anwyl R, Wolfe MS, Rowan MJ, and Selkoe DJ (2002) Naturally secreted oligomers of amyloid β protein potently inhibit hippocampal long-term potentiation *in vivo. Nature (Lond)* **416:**535–539.
Ward RV, Jennings KH, Jepras R, Neville W, Owen DE, Hawkins J, Christie G, Davis JB, George A, Karran EH, et al. (2000) Fractionation and characterization of oligomeric, protofibrillar and fibrillar forms of β-amyloid peptide. *Biochem J* **348:**137–144.
Watanabe K, Nakamura K, Akikusa S, Okada T, Kodaka M, Konakahara T, and Okuno H (2002) Inhibitors of fibril formation and cytotoxicity of β-amyloid peptide composed of KLVFF recognition element and flexible hydrophilic disrupting element. *Biochem Biophys Res Commun* **290:**121–124.
Yankner BA (1996a) Mechanisms of neuronal degeneration in Alzheimer's disease. *Neuron* **16:**921–932.
Yankner BA (1996b) New clues to Alzheimer's disease: unraveling the roles of amyloid and tau. *Nat Med* **2:**850–852.

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