Acceleration of Amyloid Fibril Formation by Specific Binding of Aβ-(1–40) Peptide to Ganglioside-containing Membrane Vesicles*

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The interaction of Alzheimer’s Aβ peptide and its fluorescent analogue with membrane vesicles was studied by spectrofluorometry, Congo Red binding, and electron microscopy. The peptide binds selectively to the membranes containing gangliosides with a binding affinity ranging from 10⁻⁶ to 10⁻⁷ m depending on the type of ganglioside sugar moiety. This interaction appears to be ganglioside-specific as under our experimental conditions (neutral pH, physiologically relevant ionic strength), no Aβ binding was observed to ganglioside-free membranes containing zwitterionic or acidic phospholipids. Importantly, the addition of ganglioside-containing vesicles to the peptide solution dramatically accelerates the rate of fibril formation as compared with that of the peptide alone. The present results strongly suggest that the membrane-bound form of the peptide may act as a specific “template” (seed) that catalyzes the fibrillogenesis reaction in vitro.

One of the histopathological hallmarks of Alzheimer’s disease is the presence of insoluble amyloid deposits within the gray matter regions of the brain and the vascular walls of cerebral blood vessels (1). The principal component of these deposits is the ~4-kDa amyloid β peptide (Aβ), a product of proteolytic processing of a much larger amyloid precursor protein (2). While biological functions of Aβ are still poorly understood, rapidly accumulating evidence points to a causative (rather than merely consequential) role of the peptide in the pathogenesis of Alzheimer’s disease. Such a causative link between Aβ and Alzheimer’s disease is indicated by genetic studies which identified specific mutations in amyloid precursor protein (in close proximity to the amino or carboxyl terminus of Aβ or within the Aβ region) that are tightly linked to heritable forms of Alzheimer’s disease (3–5). Further support is derived from in vitro studies which show that synthetic Aβ peptide is toxic to neuronal cells in culture (6–9). However, despite recent important advances, the molecular mechanisms of Aβ-induced neuronal cell death remain largely unknown.

To understand the neurotoxic action of Aβ, it is essential to identify specific cellular components that interact with the peptide and mediate a biological response of the affected cells. A likely primary target of Aβ is the neuronal plasma membrane. Indeed, a rapidly growing number of observations indicate that the peptide may alter important physical and biological properties of the membrane (10–17). The mechanisms of Aβ-membrane interactions remain, however, elusive. Whereas some investigators have proposed the involvement of specific proteinaceous receptors (18, 19), other studies postulate models based on the interaction of Aβ with the lipid bilayer matrix of the plasma membrane (14, 15). Our present data show that Aβ binds with high affinity and selectivity to gangliosides. Furthermore, in the presence of ganglioside-containing membrane vesicles, there is a dramatic increase in the rate of fibril formation by the peptide. We postulate that the membrane-bound Aβ may act as a template that catalyzes the fibrillogenesis reaction in vivo.

EXPERIMENTAL PROCEDURES

Materials—Aβ-(1–40) was purchased from American Peptide Co. [Trp(7)Aβ-(1–40)] was prepared as described previously (20). Phospholipids were obtained from Avanti Polar Lipids, and gangliosides GMI, GMI, and GT1b were from Calbiochem; ganglioside GM1, asialoganglioside GM1, N-acetylneuramidic acid, and HFIP were from Sigma. The pentasaccharide II’SNeuAc-GgOse4 was obtained from BioCarb Chemicals. Prior to the experiments, the peptides were dissolved to 1 mg/ml in HFIP and stored at −20 °C (21).

Preparation of Membrane Vesicles—Small unilamellar phospholipid vesicles were prepared as described previously (22). Vesicles were kept at room temperature and used within 12 h after preparation. Ganglioside-containing vesicles were obtained by adding to sonicated POPC vesicles an appropriate amount of micellar ganglioside in buffer and incubating the mixture for several hours (23).

Peptide Binding Experiments—Peptide-membrane binding experiments were performed with [Trp(7)Aβ-(1–40)] by following changes in the fluorescence spectra of the sole tryptophan residue of the peptide upon its incubation with lipid vesicles. For this purpose, small aliquots of concentrated vesicle suspension were successively added to peptide solution in buffer (1.3 μM peptide in PBS if not stated otherwise). After each addition of lipid the solution was thoroughly mixed and left to equilibrate for 10 min at room temperature (such an incubation period was found to be sufficient to establish equilibrium). Fluorescence spectra were measured on an SLM 8100 spectrofluorometer using a 3-mm quartz cuvette and an excitation wavelength of 280 nm. Each spectrum was corrected for light scattering effects (by subtracting lipid blanks in the same buffer) and for wavelength-dependent efficiency of the detection system. Fluorescence titration curves were analyzed in terms of the peptide-ganglioside dissociation constant, Kd, defined as: Kd = [free peptide] [free ganglioside]/complex. This equation was transformed into the following form containing directly measurable quantities,

\[ y = \frac{n[K_x + p_x - (p_x^2)(X_{\text{max}})]}{X_{\text{max}} - x} \]  

(adapted from Ref. 24) and fitted to the experimental data with a nonlinear regression analysis. Parameter p in the above equation denotes total peptide concentration, y is the ganglioside concentration, and n is the stoichiometry of binding. The quantity x represents the change (either the wavelength shift in the fluorescence emission maximum or the change in fluorescence intensity) of the fluorescence spec-

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Ganglioside-containing Vesicles Promote Aβ Fibril Formation

**FIG. 1.** Titration curves of 1.3 μM [Trp₁₀]Aβ-(1–40) in PBS with POPC vesicles (●), POPS vesicles (X), POPC vesicles containing 3 mol % G₅₁, ganglioside (○), and POPC vesicles containing 3 mol % asialoganglioside (▲). The dotted line (- - -) indicates the curve fitted by nonlinear regression analysis. The upper ordinate axis refers to the phospholipid (POPC and POPS) concentration whereas the lower ordinate axis refers to the concentration of ganglioside G₅₁ and asialoganglioside G₅₁. Inset, fluorescence emission spectra of free [Trp₁₀]Aβ-(1–40) (——) and the peptide bound to ganglioside-containing POPC vesicles (----).

**RESULTS**

Membrane Binding—To study the interaction of Aβ-(1–40) with membrane vesicles of different lipid composition, we prepared a peptide analogue in which Tyr at position 10 was replaced by Trp. Tryptophan residue provides a convenient spectroscopic probe which allows the measurement of peptide-membrane binding by fluorescence spectroscopy. The properties of the fluorescent peptide were found to be essentially identical to those of the parent molecule (20).

The fluorescence emission spectrum of HFIP-disaggregated peptide in PBS has a maximum at 347 nm and is indicative of a polar environment of the Trp residue. Incubation of the peptide with membrane vesicles consisting solely of phosphatidylcholine did not result in any measurable spectral change, suggesting the lack of peptide interaction with these vesicles. Similarly, no alterations in peptide fluorescence (in PBS) were observed upon addition of vesicles prepared from phosphatidylserine or phosphatidylylycerol (Fig. 1).² However, the fluorescence spectra changed drastically when the vesicles were doped with gangliosides. As shown in the inset within Fig. 1, upon addition of POPC vesicles containing 3 mol % ganglioside G₅₁ to peptide solution in PBS, the emission maximum is shifted to a shorter wavelength, and there is an enhancement of the fluorescence intensity. The observed blue shift reflects the increase in hydrophobicity of the tryptophan microenvironment and is indicative of peptide binding to the membrane. The titration curve obtained by measuring changes in the wavelength of the fluorescence emission maximum at increasing concentrations of vesicles shows that peptide binding to ganglioside G₅₁ is saturable (Fig. 1) and can be characterized by 1:1 stoichiometry with a dissociation constant of 1.4 × 10⁻⁶ M. The binding affinity did not change appreciably when PBS was replaced with low ionic strength buffer (Table I). Effects qualitatively similar to those illustrated in Fig. 1 were also observed for other members of the ganglioside family, including gangliosides G₁₀, G₇, and G₃. However, the dissociation constants were found to differ significantly (in the range between 2 × 10⁻⁷ M to 5 × 10⁻⁸ M), indicating the following order of peptide affinity for different gangliosides: G₁₀ > G₇ > G₃ (Table I).

The results described above indicate that peptide binding to membrane vesicles is mediated through specific recognition by the gangliosides. Consistent with this, no peptide association with the membranes was detected when ganglioside G₅₁ was replaced by asialoganglioside G₅₁ (glycolipid lacking sialic acid portion of the head group) (Fig. 1). To further test the role of the sugar moiety in peptide binding to ganglioside-containing membranes, we titrated [Trp₁₀]Aβ-(1–40) with free G₅₁-pentasaccharide, as well as with sialic acid. While in these cases the position of the emission maximum remained unchanged, a concentration-dependent, saturable quenching of tryptophan fluorescence was observed clearly indicating Aβ peptide interaction with the free sugars (Fig. 2). (The different response of peptide fluorescence upon binding to free sugars and gangliosides is understandable since only the latter interaction leads to membrane-dependent increases in the hydrophobicity of the Trp microenvironment.) Analysis of the titration curve revealed that the affinity of the peptide for free G₅₁-pentasaccharide is only modestly (5 times) lower than that for membrane-associated G₅₁-ganglioside. Much weaker, although measurable, peptide binding was observed when sialic acid was titrated into the peptide solution (Fig. 2 and Table I).

**Effect of Membrane Binding on Aβ Fibrillation—**The Congo Red assay is based on the observation that the dye binds to amyloid fibrils, shifting toward higher wavelength the maximum of its absorption spectrum (21, 25). In this study, we have used the ratio of the absorbance at 540 and 480 nm as a measure of Aβ fibril formation. The ratio parameter increases linearly with the amount of fibrillar peptide and, in our experience, is more reproducible than the absorbance difference-based parameters used in other studies (21, 25).

In agreement with previous reports (21, 25), the kinetics of fibril formation by HFIP-disaggregated Aβ-(1–40) is very slow.

**Table I**

| Ganglioside or sugar moiety | Binding constant, Kᵢ (× 10⁻⁶ M) |
|----------------------------|----------------------------------|
| G₅₁                       | 1.4                             |
| G₃₁                       | 1.1                             |
| G₅₂                       | 3.7                             |
| G₁₀                       | 0.22                            |
| G₇                        | 0.26                            |
| Sialic acid               | 218                             |
| G₅₁-pentasaccharide       | 7                               |

² Consistent with previous data (22, 31), binding of Aβ-(1–40) to acidic phospholipids could be detected by fluorescence spectroscopy only under the conditions of very low ionic strength (10 mM phosphate buffer, no NaCl) or at acidic pH. However, this nonspecific, purely electrostatic interaction is beyond the scope of the present study.
Ganglioside-containing Vesicles Promote Aβ Fibril Formation

No fibrils were formed up to 5–6 days of peptide incubation in PBS (Fig. 3). However, the rate of fibrillogenesis was greatly increased in the presence of ganglioside GM1-containing membrane vesicles (Fig. 3). In the latter case, massive Congo Red binding (corresponding to approximately 56% of the maximum binding) was detected already after 1 day of incubation. Simultaneous experiments performed in the presence of ganglioside-free POPC vesicles did not indicate any increase in fibril formation (data not shown for brevity). In preliminary studies, we noted that ganglioside-containing POPC vesicles alone can bind a limited amount of the Congo Red dye. However, this binding is negligible at the ganglioside concentrations used in the studies presented herein.

Aβ-(1–40) fibril formation was also studied by transmission electron microscopy. Consistent with the Congo Red binding data, no fibrillar structures were detected following 1-day peptide incubation in PBS alone (Fig. 4A) or in the presence of ganglioside-free POPC vesicles (data not shown). However, following 1 day of incubation, mixtures in the presence of ganglioside GM1-containing membranes exhibited, in addition to the vesicles, numerous fibrillar structures. The fibrils varied in length and had an average diameter of approximately 9 nm. Notably, the fibrils were for the most part associated with the membrane vesicles, and many of them appeared to originate directly from the vesicular surface (Fig. 4B).

DISCUSSION

A growing number of observations indicates that the neurotoxic action of Aβ is mediated by peptide-induced perturbation of the functional and structural properties of neuronal plasma membranes. Some of the reported membrane effects of the peptide include changes in bulk membrane fluidity, perturbation of the interface between lipids and proteins, inactivation of membrane-bound enzymes, formation of new or modulation of pre-existing membrane channels, and activation of free radical-generating pathways (10–17, 26–30). However, the molecular mechanisms of Aβ-membrane interactions as well as the nature of acceptor molecules responsible for Aβ binding to the membrane surface remain largely unknown. The goal of this study was to characterize the interaction of Aβ with the lipid components of neuronal plasma membrane. To this end, we have used a fluorescent analogue of Aβ-(1–40) in which the sole Tyr residue was substituted with Trp. Given the similarity of the aromatic residues, such a substitution is usually considered to have minimum effect on the properties of proteins and peptides. Indeed, no differences were found in the biophysical properties of Aβ-(1–40) and [Trp10]Aβ-(1–40) (20). The advantage of using a Trp-labeled peptide is that its membrane binding can be assessed directly from changes in fluorescence spectra upon addition of membrane vesicles, with no need for physical separation of the free and bound species. Furthermore, from a structural point of view, Tyr → Trp substitution is less perturbing compared with other chemical modifications commonly used for Aβ labeling, including radioiodination and attachment of extrinsic fluorescent probes.

The key finding of the present study is that Aβ peptide interacts selectively with membrane gangliosides. This interaction is characterized by a relatively high affinity and a considerable degree of specificity with respect to the structure of the glycolipid oligosaccharide moiety. In addition to the sialic acid group, which is a prerequisite for the effective recognition of Aβ, other structural elements of the glycolipid appear to play a role in the peptide-ganglioside interaction. Thus, the observed 3-fold tighter binding of Aβ to GM1 as compared with GM2 points to a stabilizing role of the terminal galactose residue (which is absent in GM2). The interaction is further strengthened (by a factor of approximately 6) in the presence of a second sialic acid residue, as in GD1a. While further studies are needed to fully elucidate structural and mechanistic aspects of Aβ-ganglioside binding, it is notable that this binding shows very little sensitivity to ionic strength. This characteristic clearly differentiates Aβ interaction with gangliosides from that observed between the peptide and acidic phospholipids such as phosphatidylserine or phosphatidylglycerol. The latter interaction appears to be driven by nonspecific electrostatic effects; it is completely abolished in the presence of

![Fig. 2. Titration curves of [Trp10]Aβ-(1–40) in PBS with free sialic acid (●) and GM1-pentasaccharide (○). The dotted lines indicate the curves fitted by non-linear regression analysis.](image)

![Fig. 3. Congo Red binding of 58 μM Aβ-(1–40) in PBS alone (●) and in the presence of POPC vesicles containing 9 mol % ganglioside GM1 (○). The molar ratio of ganglioside GM1 to peptide was 1:2.](image)

![Fig. 4. Electron micrographs taken following 1 day of incubation in PBS at 37 °C of 58 μM Aβ-(1–40) alone (A) and in the presence of POPC vesicles containing 9 mol % GM1 (GM1) ganglioside (B). The molar ratio of ganglioside GM1 to peptide monomer was 1:1. Magnification × 116,000, scale bar = 60 nm.](image)
higher (150 mM) salt concentration (22, 31). The apparent lack of Aβ-(1–40) binding to phospholipids under physiologically relevant conditions is at odds with the recent hypothesis that Aβ peptide exerts its neurotoxic effect by a relatively nonspecific mechanism which involves direct interaction with the phospholipid bilayer to form Ca2+ channels (14, 15). However, the general “channel hypothesis” is not necessarily without merit. Experiments are currently under way to explore whether peptide incorporation into the membrane could be mediated by specific binding to gangliosides or other surface receptors.

While present only in relatively small quantities in most tissues, gangliosides are abundant components of neurons. They constitute about one-tenth of total neuronal membrane lipids (32, 33) and appear to be especially highly concentrated in pre- and postsynaptic membranes (34). Functionally, gangliosides have been implicated in a number of important neurobiological events such as neuritogenesis, synaptogenesis, synaptic transmission, and neuronal survival after injury. We postulate that oligosaccharide-specific interaction of Aβ with gangliosides may play a role in Aβ-induced neuronal degeneration. In particular, gangliosides are likely to function as high avidity “receptors” that capture the peptide and tether it to the cell surface. Once bound to the membrane surface, the peptide may engage in relatively non-specific interactions with other membrane components, initiating the cascade of events that lead to membrane pathology (35) and eventually, neuronal cell death. It should be noted that Aβ-ganglioside binding affinity is somewhat (4–5 times) lower than that reported for peptide binding to putative proteinaceous receptors such as the receptor for advanced glycation end products or serpin-enzyme complex receptor (18, 19). However, the modestly lower affinity could be easily compensated by a very high surface density of gangliosides. The proposed role of gangliosides as Aβ receptors is consistent with the finding that treatment with neuroaminidase greatly decreases binding of Aβ peptides to PC12 cells (36).

A striking consequence of ganglioside-mediated binding of Aβ to the membrane is the rapid acceleration of β-amyloid fibril formation. We suggest an important significance of this finding because a correlation appears to exist between biological effects of Aβ and its aggregation state (7–10). Furthermore, it is believed that the fibrillar peptide itself represents the neurotoxic species. The mechanism of ganglioside-mediated Aβ fibrilization likely involves an initial step in which the glycolipid-bound peptide self-associates on the membrane surface, undergoing a conformational transition to a β-sheet structure. Such a conformational transition has indeed been demonstrated in our recent circular dichroism study (23). Surface-associated (β-sheet-rich) peptide microaggregates could then act as specific template (“seeds” (37)) which recruit peptide molecules from solution and promote fibril formation by the β-sheet augmentation mechanism. The role of ganglioside-bound Aβ as a physiologic “seeding agent” is strongly supported by the recent observation that ganglioside GMI-bound peptide constitutes an integral component of diffuse plaques associated with early stages of Alzheimer’s disease (38). Furthermore, the proposed involvement of the membrane surface in Aβ fibrillogenesis is consistent with the in situ observation that Aβ is localized along neuronal plasma membranes (especially pre-synaptic regions) in early diffuse plaques (39).

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