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Published on Web 08/17/2006

Phage Display Affords Peptides that Modulate β-Amyloid Aggregation

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Abstract: As the population ages, the need to develop methods to understand and intercept the processes responsible for protein aggregation diseases is becoming more urgent. The aggregation of the protein β-amyloid (Aβ) has been implicated in Alzheimer's Disease (AD); however, whether the toxic species is a large, insoluble aggregate or some lower order form is not yet known. Agents that can modulate the aggregation state of Aβ could resolve this controversy by facilitating our understanding of the consequences of aggregation and its underlying mechanism. To date, however, ligands that bind to specific forms of Aβ have not been identified. To address this deficiency, we tested whether phage display could yield such ligands by screening libraries against Aβ in two different states: monomeric or highly aggregated. Intriguingly, the peptides selected had different effects on Aβ aggregation. Peptides selected for binding to monomeric Aβ did not perturb aggregation, but those selected using highly aggregated Aβ increase the rate of aggregation drastically. The latter also alter the morphology of the resulting aggregate. The ability of a peptide to promote aggregation correlated with its affinity for the N-terminal 10 residues of Aβ. This result indicates that the mechanism by which the peptides influence aggregation is related to their affinity for the Aβ N-terminus. Thus, the identification of compounds that bind to this Aβ section can afford agents that affect aggregation. Moreover, the data suggest that endogenous ligands that interact with the N-terminal region can influence the propensity of Aβ to form aggregates and the morphology of those that form. Our data highlight the utility of phage display for identifying ligands that bind to target proteins in different states, and they indicate that such agents can be used to perturb protein aggregation.

Introduction

In the United States, the neurodegenerative disorder Alzheimer's Disease (AD) currently afflicts as many as 6 million people; recent projections suggest that this number will soar by 2050 to 16 million.[1] The need to develop methods to understand and intercept the molecular interactions that precipitate this disease is becoming more urgent. The discovery and development of compounds that affect protein aggregation processes can facilitate this understanding and guide the design of therapeutic agents.

Different protein aggregates have been linked to the development of dementia in AD. Among these are neurofibrillary tangles, composed mainly of the actin-associated protein tau, and amyloid plaques, composed mainly of the amyloid beta (Aβ) protein. Aβ is a peptide composed of 40−43 amino acid acids (see Chart 1A for sequence**)** generated by proteolysis of the membrane-spanning protein amyloid precursor protein (APP). These proteolytic products can form toxic aggregates in vitro.[2]

The structure of Aβ in the aggregated state is that of a cross β-strand with a turn near residues 24−30.[3,4] It has been proposed that the central hydrophobic core, including Phe19 and Phe20, contributes to fibril formation through π-stacking interactions.[5] Additionally, some studies indicate that divalent metal cations, such as Zn^{2+} and Cu^{2}, can promote Aβ aggregation and its associated toxicity. Other studies indicate metal cations can disrupt aggregation.[6−8] Whatever the chemical basis for the stability of the aggregates, both large aggregates and smaller oligomeric intermediates have been investigated in an effort to ascertain their role in AD.

Aβ monomer rapidly self-assembles into a soluble oligomeric intermediate that eventually matures into an insoluble aggregate.[2,9] Evidence is emerging that the toxicity resulting from Aβ aggregation is not due to the insoluble fibrils, but rather from soluble, protofibrillar aggregates composed of a small number of monomers[10−13] (Figure 1). Indeed, plaque formation and higher-order aggregation may capture the lower order, toxic

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10.1021/ja0619861 CCC: $33.50

Chart 1. (A) Amino Acid Sequence of the β-Amyloid Peptide. (B) Amino Acid Sequence of Parent Peptide from Previous Studies.[24] (C) Sequence of Phage Display Libraries[a] and Theoretical and Actual Diversities of the Libraries. (D) Aβ Preparations Used in Affinity Screens of Phage Display Libraries[b]

A

Aβ 1-40

DAEFR HDSGY EVHHQ KLVFF AEDVG SNKGA IIGLM VGGVV

B

Parent molecule

KLVFFKKKKKK

C

Library	Sequence	Theoretical Diversity (unweighted)	Transformants
PoPo	PoPoPoKLVFFPoPoPoPo	2.8×10^5	3.6×10^8
LA	XXXKLpLpArArPoPoPoPo	1.3×10^{10}	1.3×10^5

D

Affinity Screens

Aggregated: Aβ aggregated 24 hr at 37 °C and adsorbed to polystyrene

Monomeric: Aβ immobilized in DMSO and washed with GnHCl

Zn^{2+}: Aβ aggregated in the presence of 50 mM $ZnCl_2$

EDTA: Aβ aggregated in the presence of 3 mM EDTA

[a] X=A(6.2%), C(3.1%), D(3.1%), E(3.1%), F(3.1%), G(6.2%), H(3.1%), I(3.1%), K(3.1%), L(9.4%), M(3.1%), N(3.1%), P(6.2%), Q(3.1%), R(9.4%), S(9.4%), T(6.2%), V(6.2%), W(3.1%), Y(3.1%), stop(3.1%); Po=polar: N(8.3%), S(8.3%), K(8.3%), R(25%), H(8.3%), Q(8.3%), D(8.3%), G(16%), E(8.3%); Lp=lipophilic: T(16%), M(8.3%), I(8.3%), P(16%), L(16%), A(16%), V(16%); Ar=favoring aromatic: C(13%), Y(13%), F(13%), S(25%), W(13%), L(13%), stop(13%). [b] See text for additional details.

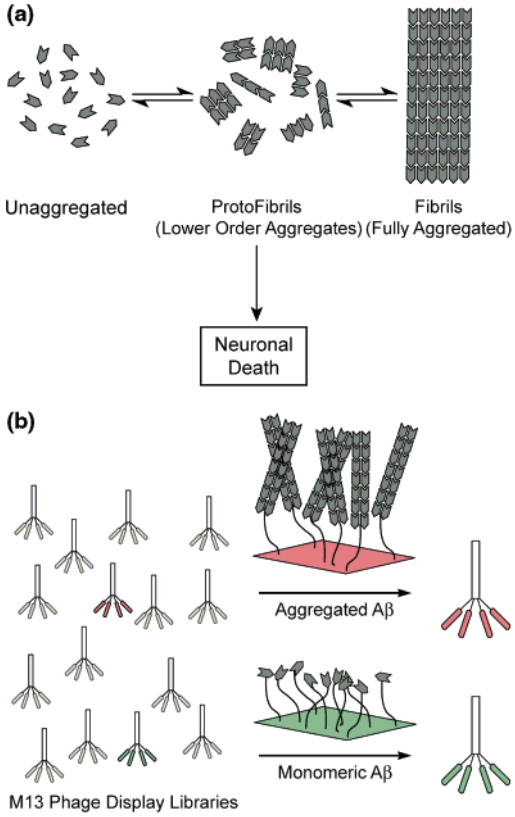

Figure 1. (a) The protofibrillar intermediate, not fibrils, is believed to be the toxic species. (b) General scheme for identifying phage that bind to different aggregation states of Aβ. Red phage represent those that bind aggregated Aβ; green phage represent those that bind monomeric Aβ.

species. By manipulating solvent conditions, it is possible to form amyloid aggregates from globular proteins that are not normally amyloidogenic, suggesting that the ability to form amyloid aggregates is a property shared by a majority of proteins.[14,15] Thus, in the event of protein misfolding, the rate of amyloid formation may be an important variable controlling toxicity: proteins that form persistent soluble aggregates should be more toxic than those that form fully aggregated amyloid.

Evidence has emerged that subdomains within Aβ influence its propensity to aggregate. The N-terminal domain of Aβ has been implicated in controlling interactions between fibers. Moreover, its inherent dynamics implicate it in the conformational switch between α-helix and β-sheet;[16] this region also seems to be important in the transition from soluble aggregates to insoluble plaques (vide infra).[17] Intriguingly, antibodies directed against N-terminal residues 3−6 (EFRH) were shown to reverse the aggregation of Aβ. Together, these results suggest a role for the N-terminus in amyloid formation.[18,19]

The central region of the Aβ peptide is also important in aggregation, and it has been a target for the development of Aβ aggregation effectors. Specifically, it has been demonstrated that the pentapeptide corresponding to residues 16−20 (KLVFF) can bind to Aβ.[20] Previously, we described a strategy to inhibit Aβ aggregation by linking this binding domain to a "solubilizing domain," composed of polar amino acids on the C-terminus. The resulting "composite peptides" decrease the cellular toxicity of Aβ; yet, surprisingly, they increase the rate of Aβ aggregation.[13,21,22] They also alter the morphology of the aggregates

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by promoting lateral growth.[23] Using a surface plasmon resonance-based (SPR) affinity assay, we found that the strongest Aβ binders (e.g., KLVFF$-K_6$) elicit the most pronounced increases in the rate of aggregation and possess the greatest ability to inhibit Aβ toxicity.[24] These results constitute another line of evidence that higher-order, insoluble aggregates are not the toxic species.

Having established a link between a compound's affinity for Aβ and its ability to alter aggregation, we sought to find molecules that alter the aggregation state of Aβ. Small molecules that stabilize the folded states of proteins have been shown to prevent aggregation;[25] however, Aβ is not known to have a monomeric folded state. This property might confound efforts to identify compounds that bind to different Aβ aggregation states. We reasoned, however, that such compounds might be found by exploring a large swath of sequence space. Phage display is a powerful method to identify, from large libraries, peptide sequences with desirable attributes.[26−28] We therefore tested whether it could be used to identify molecules that modulate Aβ aggregation (Figure 1).

Phage display has been used to find effectors and selective reporters for aggregation diseases. Solomon and co-workers[15] have used a random phage display library to determine the binding region of an *anti*-Aβ antibody. The resulting epitope (from the N-terminus of Aβ, vide supra) was displayed on phage and used for adjuvant-free immunization; this protocol resulted in a significant reduction in Aβ plaques in transgenic mice.[29] A random library of 20-mers has been screened against Aβ for the purpose of developing delivery agents and reagents for detection of Aβ aggregates.[30] A random 12-mer library also was screened against enantiomeric Aβ (composed of only D-amino acids) with the goal of identifying protease-resistant D-peptides that bind Aβ.[31] Additionally, Nagai et al.[32] have used X_5-fixed-X_5 phage-display libraries to screen against polyglutamine proteins. Like Aβ, these proteins aggregate and are implicated in neurodegenerative diseases. The resulting peptides reduce aggregation and co-localize with the aggregated protein when produced in cells. Because proteins that aggregate exist in at least two different states (monomeric and aggregated), ligands that bind these different states can serve as valuable probes. Although there are no reports of phage display being used to find such compounds, we reasoned that this method might yield Aβ ligands with different binding properties. Here, we report that screening libraries against different types of Aβ preparations can afford peptides with different propensities for altering Aβ aggregation.

Results and Discussion

Phage Display Library Design. The design of our libraries was guided by our previous studies using KLVFFK_6. Because this peptide has affinity for Aβ, we reasoned that variants of this sequence might bind the different Aβ forms (Chart 1). We designed two different libraries on this basis; both of these were generated such that the peptides were displayed as fusions to the minor coat protein pIII of the bacteriophage M13. The first, referred to as "PoPo", displays sequences of the form PoPoPoKLVFFPoPoPoPo, wherein Po indicates a residue with a polar side chain. We choose to focus on sequences bearing polar residues at these positions because the six C-terminal lysine residues of the parent KLVFFK_6 peptide contribute to its affinity for Aβ.[24] Accordingly, in the PoPo library, the KLVFF sequence is retained but appended C- and N-terminal sequences are diversified. The second library was designed to investigate whether changes in the core KLVFF sequence might lead to more potent ligands. To this end, we generated an "LA" library of the form XXXKLpLpArArPoPoPoPo, where X is any amino acid and Lp and Ar indicate a residue with a lipophilic or aromatic side chain. The two lipophilic residues were chosen to correspond to the Leu and Val residues in the KLVFF sequence and the two aromatic residues to the two Phe residues. For the less diverse PoPo library, complete sequence coverage (based on transformation efficiency) was attained, but this level of coverage was not achieved for the more diverse LA library (Chart 1). Although the LA library was less diverse than was theoretically possible, characterization of both phage libraries[33] indicates that they explore a distribution of the restricted sequence space; they possess only the slight biases typically seen.[34]

Phage Display Screens. To identify sequences that bind to Aβ in different states, each library was screened against monomeric or aggregated Aβ (Chart 1D). Although protofibrils may contribute to Aβ toxicity, these cannot readily be isolated; therefore, they were not targets of the screens. In contrast, monomeric Aβ and aggregated Aβ are stable. Moreover, we reasoned that compounds that bind to monomeric Aβ might prevent its conversion to protofibrils; alternatively, compounds that bind aggregated Aβ could drive the equilibrium from protofibrils toward the fully aggregated state. To test whether sequences with the desired attributes could be identified, we screened the libraries against both aggregated Aβ adsorbed to polystyrene (referred to as the "aggregate screen") or Aβ immobilized to favor the monomeric species (the "monomer screen").[24]

We conducted 4−6 rounds of screening and then determined the sequences of the selected clones. This selection procedure was developed to afford affinity-matured libraries that were partially convergent (see Table 1 and Figure S21, Supporting Information). These criteria resulted in a number of sequences whose relative populations in each of the screens could be used to derive a predicted selectivity for different aggregation states. From the LA library,[33] for example, the peptide sequences FYLKVPSLHHHH and NYSKMIFSHHHH were selected in both the aggregate and monomer screens. Their relative populations in the mature libraries, however, suggest that they exhibit

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Table 1. Encoded Peptide Sequences from Phage Clones Derived from Affinity Maturation

		selectivity[c]	
sequence[a]	library[b]	aggregate %	monomer %
FYLKVPSLHHHH[d]	LA	23	61
GRDKLVFFHHHH[d]	PoPo	0	18
NYSKMIFSHHHH[e]	LA	23	8
HNHKLVFFHHQH[e]	PoPo	27	0
		EDTA %	Zn^{2+} %
DFRKLLLSGQSQ[f]	LA	0	100
		aggregate %	monomer %
RHEKLVFFHHNH	PoPo	13	0
GDQKLVFFHHHH	PoPo	0	36
HHNKLVFFQDRH	PoPo	0	18
VSLKTLSLHHHH	LA	15	0
SSLKPPSLHHHH	LA	0	5
		EDTA %	Zn^{2+} %
ADYKAPSYNEGR	LA	71	0
SSDKTPYYKNEE	LA	29	0

[a] Top: Synthesized peptides. Bottom: Peptide sequences either found to be insoluble or not synthesized. [b] The sequence of each library is defined in Chart 1C. [c] Selectivity for sequences[d,e] is defined as a comparison between the percentages of the clones found the in the aggregated screen versus the percentages of those found in the monomeric screen, whereas selectivity for the sequence[f] is determined by the comparison between the percentage of the clone found in the EDTA screen versus the percentage found in the Zn^{2+} screen. (See Chart 1D for screening conditions.[33]) [d] Clones predicted to have selectivity for monomeric Aβ. [e] Clones predicted to have selectivity for aggregated Aβ. [f] Clone predicted to have selectivity for Aβ aggregated with Zn^{2+}.

preferences for different Aβ preparations. Specifically, the former sequence predominated in the monomer screen; the latter composed a greater proportion of the sequences found in the aggregate screen. These results suggest that each sequence binds preferentially to Aβ in a particular state (Table 1)[33] For example, of the 38 clones isolated from the monomer screen, 23 (i.e., 61%) had the sequence FYLKVPSLHHHH (Table 1). This sequence was also isolated in the aggregate screen; it was displayed by 23% of the total clones. These data suggest that the peptide displayed by this phage clone has selectivity for monomeric Aβ.

When the PoPo library was screened, the population of clones selected for monomeric versus aggregated Aβ was even more skewed (Table 1). Specifically, phage clones displaying the peptide sequence GRDKLVFFHHHH were detected only from the screen employing monomeric Aβ; HNHKLVFFHHQH was identified only from the screen using aggregated Aβ. These data suggest that peptide sequences with selectivity for monomeric versus aggregated Aβ can be identified.

It is intriguing that phage presenting peptides with sequences containing multiple histidine residues were obtained from the aforementioned screens. These findings led us to explore whether divalent metal cations influence aggregation. Oligo-His sequences are known to bind metal ions and the aggregation of Aβ can be influenced by divalent cations (vide supra). To test for a role of metal cations in Aβ binding, we conducted screens of the LA library using Aβ aggregated in the presence of Zn^{2+}, a cation that has been shown to effect the aggregation of Aβ.[6] Surprisingly, this screen did not result in phage clones displaying sequences encoding His residues. Indeed a clone displaying the sequence DFRKLLLSGQSQ was obtained. (Table 1) This sequence, which contains no His residues, was identified with extremely high convergence (Table 1). These data suggest that the His-rich sequences identified in the two previous screens are not interacting with Aβ via Zn^{2+}. Although these results do not preclude the possibility that the peptides identified in the first screens bind through other divalent cations, they indicate that such a binding mode is unlikely.

The Affinities of the Synthetic Peptides for Aβ. On the basis of their aqueous solubility (determined or predicted insolubility), we synthesized several peptides identified in our various screens for further evaluation (Table S21, Supporting Information). To assess the ability of these sequences to bind Aβ, we employed a surface plasmon resonance (SPR) assay[24] (Table 2). This assay involves monitoring the SPR response after immobilized Aβ (or Aβ fragment) is treated with peptide solutions at various concentrations and the mixture is allowed to equilibrate. With immobilized $A\beta_{10-35}$ as the target, most of the peptides, with the exception of Ac−HNHKLVFFHHQH−NH_2, bound with higher affinity than the parent peptide Ac−KLVFFKKKKK−OH. This result highlights the value of phage display to identify effective peptide ligands for Aβ. The peptides derived from screening the LA library bound the strongest, suggesting that more potent ligands can be found through exploring variations of the naturally occurring KLVFF sequence.

Thioflavin T Aggregation Assay. Given that the peptides identified from our screens bind Aβ, we tested their abilities to alter Aβ aggregation. The steady state level of amyloid aggregates was measured using the small molecule dye thioflavin T (ThT). ThT can bind amyloid aggregates; when it does, its fluorescence emission intensity greatly increases.[43] Aβ and the peptide were allowed to aggregate in the presence of ThT, and the ThT emission intensity of the sample was compared to that of ThT in buffer or peptide alone (Figure 2). For the samples containing only Aβ, we observed an increase in the ThT signal of approximately 20-fold. The peptide we had designed previously (KLVFFK_6) appeared to cause a modest decrease in ThT fluorescence emission. Adding the peptides identified in our screen against monomeric Aβ appeared to have little effect on Aβ aggregation. In contrast, when samples containing peptides identified from the screen using aggregated Aβ were tested, we detected an increase in amyloid formation over that due to Aβ alone. Strikingly, the peptide predicted to have the greatest selectivity for aggregated Aβ, Ac-HNHKLVFFHHQH-NH_2, caused an increase in ThT fluorescence over PBS of almost 60-fold. These data suggest that compounds that promote or stabilize the formation of large aggregates can be readily identified.

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Table 2. Dissociation Constants (K_d (μM))[a] for Phage Display-Derived Peptides Binding to Truncations of Aβ as Determined by SPR

Aβ truncations	Ac-FYLKVQS-LHHHH-NH_2[b]	Ac-GRDKLV-FFHHHH-NH_2[b]	Ac-NYSKMIF-SHHHH-NH_2[d]	Ac-HNHKLV-FFHHQH-NH_2[d]	Ac-DFRKLLL-SGQSQ-NH_2[e]	NH_2-KLVFFK-KKKKK-OH
Aβ_{1-10}	N. D.[c]	50 ± 10	47 ± 7	25 ± 4	N. D.	100 ± 12
Aβ_{11-20}	N. D.	N. D.	N. D.	N. D.	N. D.	N. D.
Aβ_{16-25}	N. D.	N. D.	N. D.	N. D.	N. D.	N. D.
Aβ_{21-30}	4 ± 1	28 ± 4	15 ± 6	2 ± 0.4	85 ± 2	N. D.
Aβ_{31-40}	N. D.	N. D.	N. D.	N. D.	30 ± 1	N. D.
Aβ_{10-35}	7 ± 2	15 ± 4	6 ± 3	46 ± 6	18 ± 5	30 ± 2

[a] Errors are ± standard error. [b] Peptide sequences derived from clones predicted to have selectivity for monomeric Aβ. [c] N. D.=no detectable saturation binding. Because the peptides are approximately the same molecular weight, the detection limit is determined by the shape of the saturation binding isotherm. [d] Peptide sequences derived from clones predicted to have selectivity for aggregated Aβ. [e] Peptide sequence derived from clone predicted to have selectivity for Aβ aggregated with Zn^{2+}.

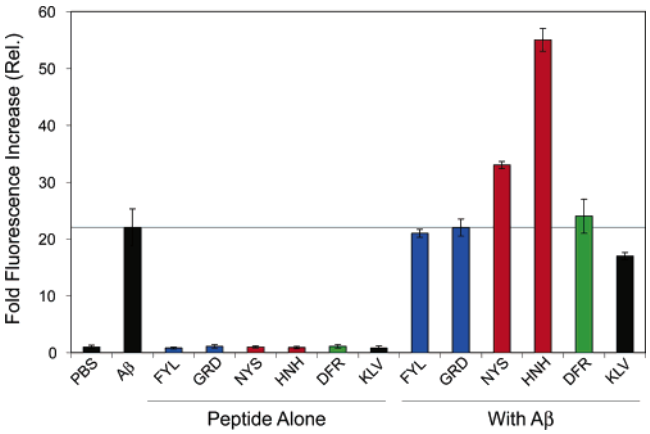

Figure 2. Thioflavin T (ThT) fluorescence emission of samples containing Aβ alone or Aβ and phage-display-derived peptides. For clarity, the peptide sequences are indicated by the first 3 amino acids (see Table 2 for full sequences). The samples were normalized to the emission intensity of ThT in PBS. (Blue) Peptides derived from clones identified in the screen against monomeric Aβ. (Red) Peptides identified from clones identified in the screen against aggregated Aβ. (Green) Peptide derived from clones identified in the screen against Aβ aggregated in the presence of Zn^{2+}. The horizontal line indicates the relative level of ThT fluorescence obtained for aggregated Aβ alone. Error bars represent ± the standard error.

Dynamic Light Scattering. The kinetics of the Aβ aggregation process can be followed by monitoring changes in light scattering.[35] An advantage of using light scattering is that it does not require a reporter dye or label to follow aggregation. Thus, dynamic light scattering experiments were performed using the peptides derived from phage display (Figure 3). The trends observed in the ThT equilibrium binding assay are manifested, but, in this kinetic assay, the differences between peptides are even more pronounced. Peptides derived from the screens for binding to Aβ in the presence of Zn^{2+} or monomeric Aβ caused a slight increase in the rate of aggregation over that of Aβ alone. In contrast, when Aβ was treated with a peptide derived from the screen using aggregated Aβ, the increase in the rate of aggregation was dramatic. This result was obtained regardless of the library from which the peptide was identified. The peptide predicted to have the highest selectivity for the aggregated state (Table 1), Ac-HNHKLVFFHHQH-NH_2, promoted rapid aggregation: After approximately 5 h, the sample contained macroscopic aggregates that began to precipitate. Thus, the compounds identified from the screen for ligands that bind monomeric Aβ have little effect on Aβ aggregation, but those derived from the screen with aggregated Aβ, promote Aβ aggregation. These results provide further evidence that different preparations of the target can yield selected peptides that have markedly different effects on aggregation.

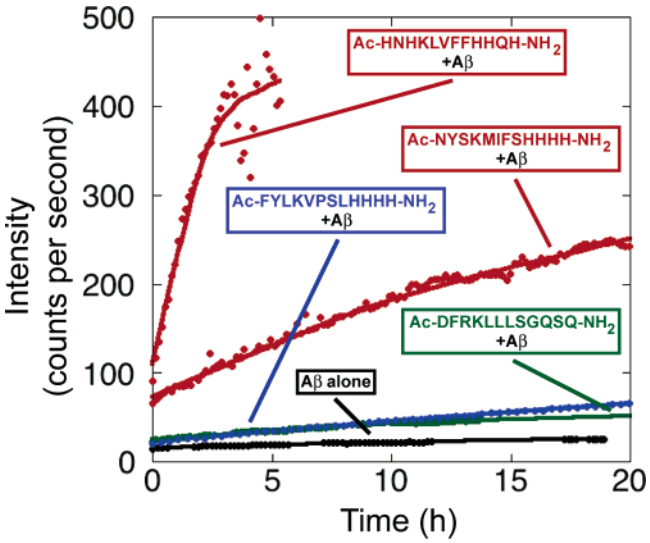

Figure 3. Results from dynamic light scattering analysis of aggregates formed in the presence and absence of phage display-derived peptides. (Blue) Data from sample containing peptide derived from clone predicted to have selectivity for monomeric Aβ. (Green) Data from a sample containing a peptide derived from a clone predicted to have selectivity for Aβ aggregated in the presence of Zn^{2+}. (Red) Data from samples containing peptides derived from clones predicted to have selectivity for aggregated Aβ. In the absence of Aβ, no aggregates were detected.

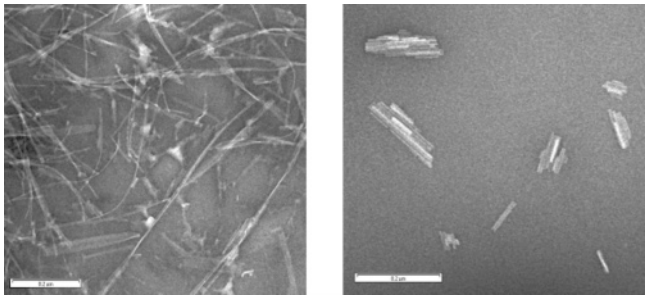

Figure 4. Representative[33] TEM micrographs of Aβ aggregated (left) alone and (right) in the presence of Ac−HNHKLVFFHHQH−NH_2. Scale bars = 0.2 μm.

Electron Microscopy. The ability of the peptides to affect the extent and rate of Aβ aggregation prompted us to examine whether the morphology of the resulting aggregates was perturbed. We used transmission electron microscopy to visualize the products resulting from the aggregation of Aβ alone or in the presence of the phage display-derived peptides. Each of the peptides caused morphological changes in the aggregate structure. The peptide predicted to have the greatest selectivity for aggregated Aβ, however, had the most pronounced effect. As in other assays, the addition of Ac−HNHKLVFFHHQH−

NH_2, elicited dramatic changes (Figure 4). The observed aggregates in the presence of peptide were much shorter and wider than those arising from Aβ alone. They exhibit a substructure that is similar in width to fibrils arising from Aβ aggregated alone, suggesting that they are bundles of short fibrils. We hypothesize that the peptides promote lateral aggregation at the expense of longitudinal extension. Interestingly, Kim and Murphy established a mathematical model for light scattering data that suggests the parent molecule, KLVFF−K_6, acts by lateral alignment of the Aβ aggregate.[23] Our results support this model; moreover, they suggest that HNHKLVFF-HHQH-NH_2 acts through a similar mode.

Affinity of selected peptides for different regions of Aβ. Through the use of our SPR-based affinity assay (vide supra) we sought to determine whether the selected peptides interact with specific regions of full length Aβ. If such "hot spots" could be identified, they could guide the identification of agents that modulate Aβ aggregation. Because Aβ can adopt an extended β-sheet conformation in the aggregate, it is likely that any identified peptide sequence would bind consecutive amino acids. To this end, we immobilized the peptides corresponding to 10-mer truncations of Aβ (1−10, 11−20, 21−30, and 31−40) and used SPR to monitor the ability of the selected peptides to bind these sequences (Table 2).

We first explored the binding of our peptides to the region of Aβ that contains the KLVFF (Aβ_{16-20}) sequence. It has been suggested that the KLVFF sequence on one copy of Aβ binds the same region on another copy.[36] Due to the presumed importance of this domain, Aβ_{16-25}, which places the key pentamer at the N-terminus, and Aβ_{11-20}, which has the pentamer at the C-terminus, were both immobilized. Interestingly, none of the phage derived-peptides or the rationally designed parent peptide show appreciable affinity for either Aβ_{11-20} or Aβ_{16-25}. Thus, the KLVFF sequence within Aβ is not what is recognized. This result is not obvious, as the aromatic side chains of KLVFF might be expected to engage in $\pi-\pi$ interactions within the aggregate. Indeed, π-stacking between monomers has been presumed to stabilize the Aβ aggregate, and stacking is a major component of many models of Aβ aggregates.[36−38] A recent report, however, suggests that such π-stacking interactions are not energetically significant across β-strands.[39] Although the parent peptide does not bind the Aβ_{21-30} sequence, all phage-derived peptides do, suggesting this region can engage in binding interactions. The phage display method therefore can be used to identify favorable regions on Aβ for interaction.

Other sites within Aβ were also found to engage in binding interactions with the selected peptides. For example, only the peptide derived from the screen containing Zn^{2+} demonstrated measurable binding to the C-terminal peptide corresponding to Aβ_{31-40}. A more favorable region for binding is the N-terminus (Aβ_{1-10}). This region serves as a binding site for the parent peptide, one peptide derived from the monomer screen, and all of the phage display-derived peptides obtained from screening over aggregated Aβ.

Extent of Amyloid Content and Degree of Aggregation Correlate with Peptide Affinity for the N-Terminus of Aβ. We sought to determine whether we could identify a relationship between the ability of a peptide to bind a specific site on Aβ and its ability to influence aggregation. If ligand binding to a specific region can be correlated with its effects on aggregation, the identified region could serve as control point for aggregation modulators. To this end, we compared a peptide's effect on Aβ aggregation (as determined by ThT fluorescence emission, Figure 2) to its affinity for different sites on Aβ. Although several of the peptides bound to Aβ_{21-30}, there was no relationship between a peptide's affinity for this region and its ability to influence aggregation. Indeed, correlation to all the sites (including Aβ_{10-35}) was poor with one exception: Activity could be correlated with affinity for the Aβ_{1-10} sequence (R = 0.82) (Figure 5). This result suggests that a ligand's affinity for Aβ_{1-10} is a better indicator of the degree to which it can affect aggregation than its ability to interact with other regions of Aβ. Accordingly, we anticipate that the Aβ_{1-10} sequence serves as an excellent target for compounds that alter Aβ aggregation. Our results also indicate that naturally occurring protein or peptide sequences that interact with the Aβ_{1-10} can alter its in vivo aggregation properties. These results are consistent with the report that antibodies directed against the N-terminus can affect Aβ aggregation (vide supra). The difference in the effects of this antibody and our peptides on Aβ aggregation could arise from the relative sizes of the different molecules[40] or a more complex mechanism of action.

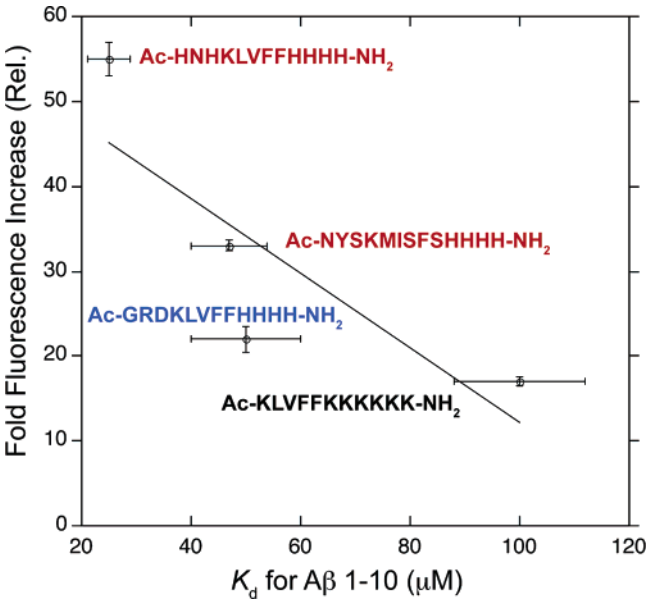

Figure 5. Correlation of the affinity of phage display-derived peptides for Aβ_{1-10} with ThT fluorescence emission intensity (Figure 2). R = 0.82.[33] (Black) Lead peptide sequence identified as a ligand for Aβ in previous studies. (Blue) Peptides derived from clone predicted to have selectivity for monomeric Aβ; (Red) Peptide sequences derived from clones predicted to have selectivity for aggregated Aβ. Error bars represent ± S. E.

Conclusions

Compounds that affect amyloid formation can serve as valuable probes of the aggregation process. Given the evidence implicating Aβ aggregation in Alzheimer's Disease, we sought to identify compounds that perturb Aβ aggregation. We postulated that phage display could afford such compounds. To test this hypothesis, we designed and screened libraries against Aβ in different states (monomeric, aggregated, and aggregated in the presence of either Zn^{2+} or ethylenediaaminetetraacetic acid (EDTA). These different screens afforded different populations of hit sequences. To characterize whether the predominant

sequences obtained from the different screens have different effects on Aβ aggregation, we tested them in a battery of assays.

From analyzing the properties of the identified peptides, insights into altering Aβ aggregation emerged. First, we found that screening against amyloid in an aggregated state (as compared to screening over monomeric Aβ or Aβ aggregated in the presence of Zn^{2+}) results in molecules that dramatically increase the rate of Aβ aggregation. Thus, phage display can be used to identify ligands that bind to different aggregation states and influence aggregation. Second, our data indicate that the higher the affinity of a compound for the N-terminus of Aβ, the greater its ability to affect Aβ aggregation. This correlation suggests a common mechanism underlying the effect of the peptides on Aβ aggregation. There is mounting evidence that the toxic species in Alzheimer's Disease are the soluble, not insoluble, Aβ aggregates. Thus, it is possible that compounds that *promote* the formation of insoluble Aβ aggregates may alleviate Aβ toxicity. We envision that the peptides we have identified, as well as other compounds selected using different Aβ forms, can be used to illuminate the role of protein aggregates in amyloid diseases.

Experimental Section

Library Construction. DNA cassettes encoding the library were generated by annealing degenerate codon-containing, complementary oligonucleotides (synthesized at UW-Madison DNA Synthesis Laboratory) with designed overhangs for ligation into the XhoI and XbaI restriction sites of the M13 phage display cloning vector mBax (a generous gift of Dr. Brian Kay, Argonne National Laboratory). The oligonucleotides were named for the library for which they encode (either PoPo or LA; Chart 1C) and for the strand of the duplex upon annealing (forward (For) or reverse (Rev) for the forward and reverse strands, respectively). "PoPoFor": 5′-TCG AGT CAG GGT TCT GGT VRS VRS VRS AAG CTG GTA TTC TTC VRS VRS VRS VRS GGC TCC GGC AAC T-3′. "PoPo Rev": 5′-CT AGA GTT GCC GGA GCC SYB SYB SYB SYB GAA GAA TAC CAG CTT SYB SYB ACC AGA ACC CTG AC-3′. "LAFor": 5′-TCG AGT CAG GGT TCT GGT NNS NNS NNS AAG CYS CYS TNS TNS VRS VRS VRS VRS GGC TCC GGC AAC T-3′. "LARev": 5′-CT AGA GTT GCC GGA GCC SYB SYB SYB SYB SNA SNA SRB SRB CTT SNN SNN SNN ACC AGA ACC CTG AC-3′. Where N = G, A, T, or C; S = C or G; V = A, C, or G; R = A or G; Y = C or T.[41] Additionally, the oligonucleotides (For and Rev) for each library (PoPo and LA) were designed such that, upon translation, they afford SSQGSG and GSGN flexible linkers at the N- and C- termini, respectively. In these linker regions, nonredundant codons for Ser and Gly were employed to ensure specific annealing.

The libraries were cloned using the procedure of Lowman and Wells.[42] Briefly, each oligonucleotide strand of the library was phosphorylated with T4 polynucleotide kinase (Promega), after which the samples were combined and heated to 65 °C for 15 min and then cooled slowly to anneal the strands. The resulting mixture was treated with mBax vector (generous gift of Brian Kay, Argonne National Laboratory) and T4 ligase (Promega). The ethanol-precipitated ligation reactions were transfected into electro-competent JS5 cells ~100 times to give 3.6×10^8 and 1.3×10^5 total transformants for the PoPo and LA libraries, respectively. The libraries were amplified and characterized by sequencing.[33]

Phage Display Affinity Screens. For the screen against aggregated Aβ, Aβ (1 mg, Aβ_{1-40}, CalBiochem) was dissolved in 111 μL 0.1% trifluoroacetic acid (TFA), 10% dimethyl sulfoxide (DMSO), diluted to 46 μM in phosphate buffered saline (PBS, 2.67 mM KCl, 1.5 mM KH_2PO_4, 137.9 mM NaCl, 8.1 mM Na_2HPO_4, pH 7.2), and allowed to aggregate in polystyrene microtiter wells (37 °C, 24 h). The wells were subsequently washed twice with Tris buffered saline with detergent (TBST, 50 mM Tris, 150 mM NaCl, 0.1% Tween-20, pH 7.0). The same procedure was followed for the Zn^{2+} and ethylenediaminetetraacetate (EDTA) screens (Chart 1); however, the aggregation buffer was supplemented with either 500 μM $ZnCl_2$ or 3 mM EDTA, respectively. For the screen against monomeric Aβ, we employed a procedure analogous to that used to produce monomeric Aβ immobilized on surfaces for SPR.[24] Briefly, the previously described Aβ stock solution was diluted (46 μM in 10% DMSO in PBS) and exposed to maleic anhydride-conjugated polystyrene microtiter wells (37 °C, 1.5 h). The resulting surface was washed twice with guanidinium chloride (GnHCl, 4 M in PBS) and twice with TBST.

A typical screen consisted of incubating 10^{10} pfu/mL of phage library on empty polystyrene microtiter wells (37 °C, 1.5 h) to remove plastic binders. The unbound phage were then incubated in the Aβ wells (37 °C, 2 h). To remove phage with low affinity to Aβ, the wells were washed four times with TBST. Bound phage were eluted in GnHCl, immediately diluted into PBS, and amplified. Although the presence of the denaturant in the elution step could be problematic, this elution procedure is compatible with phage stability as the phage titer under these conditions was constant during test experiments.

Convergent clones were identified by sequencing,[33] and peptides corresponding to the encoded sequences were synthesized by standard solid-phase peptide synthesis with N-terminal Fmoc protection. The synthetic peptides were generated as the C-terminal amide and with acetylated N-termini. All peptides were purified to homogeneity by reverse-phase HPLC and characterized by MALDI-TOF mass spectroscopy.[33]

SPR-Based Affinity Assay with Immobilized Aβ and Aβ Truncations. The peptides (Aβ_{1-10}: Ac-DAEFRHDSGYGGSGC−COOH; Aβ_{11-20}: Ac-EVHHQKLVFFGGSGC−COOH; Aβ_{16-25}: Ac-KLVFFAEDVGGGSGC−COOH; Aβ_{21-30}: Ac-AEDVGSNKGAGGSGC−COOH; Aβ_{31-40}: Ac-IIGLMVGGVVGGSGV−COOH; Aβ_{10-35}: NH_2-YEVHHQKLVFFAEDVGSNKGAIIGLM-Aha-C−COOH) used for immobilization to SPR chips were synthesized by the UW Peptide Synthesis Facility by standard solid-phase peptide synthesis. All peptides were purified to homogeneity by reverse-phase HPLC and characterized by MALDI-TOF mass spectrometry.[33]

To evaluate the ability of the peptides to bind to Aβ, we employed the SPR assay described by Cairo et al.[24] All flow cells of a carboxylic acid-presenting B1 chip (Biacore) were activated by injecting (70 μL) an aqueous solution of *N*-hydroxysuccinimide (6 mg/mL) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (40 mg/mL) at a flow rate of 5 μL/min using 4-(2-hydroxylethyl)piperazine-1-ethanesulfonic acid (HEPES)-buffered saline (HBS, 10 mM HEPES, 150 mM NaCl, pH 7.4) as the running buffer. Ethylenediamine (67 μL/ml in water, pH 8.8) was injected (70 μL) to convert the carboxylic acid surface to an amine-presenting one. Immediately following, ethanolamine (1 M in water, pH 8.5) was injected (70 μL) to quench any unreacted succinimide esters. Individually, each flow cell was then injected (70 μL) with an aqueous solution of 3-maleimidobenzoyl-*N*-hydroxysulfosuccinimide ester (Sulfo-MBS, 1 mg/mL, Pierce) to attach thiol-reactive functionality to the amine surface. Immediately, either cysteine (70 μL injection of 100 mM Cys, 10 mM NaOAc, pH 5.0), for the control flow cell, or peptide (140 μL injection, initially dissolved 5 mg/mL in10% DMSO in HBS and diluted 100 fold in HBS immediately before injection) was immobilized to the flow cells one at a time. Peptide immobilization was followed by an injection (70 μL) of Cys (100 mM Cys, 10 mM NaOAc, pH 5.0) to cap unreacted maleimide. To remove noncovalently bound peptide, all flow cells were then washed (5 pulses of 10 μL) individually with guanidinium chloride (GnHCl, 4 M in HBS). After washing and equilibration in HBS, the final signal (in response units (RU)) after activation was determined as follows. Aβ_{1-10}: 163 RU; Aβ_{11-20}: 263 RU; Aβ_{16-25}: 216 RU; Aβ_{21-30}: 79 RU; Aβ_{31-40}: 122, RU; Aβ_{10-35}: 1177 RU.

Solutions of peptides corresponding to the sequences identified from phage display at various concentrations were injected (150 μL) through all flow cells, samples were allowed to equilibrate, and the signal from the channel capped with Cys was subtracted from the response. The corrected response values were plotted versus concentration and fit to a 1:1 binding isotherm to obtain disassociation constants.[24] The surfaces were regenerated by injection of GnHCl (5 pulses of 10 μL).

Thioflavin T Aggregation Assay. The thioflavin T (ThT) assay was performed as previously described.[43] Briefly, $A\beta_{1-40}$ was dissolved in 0.1% TFA at a concentration of 10 mg/mL, and the solution was incubated at 37 °C for 4 h to break up potential aggregation "seeds." This stock (or vehicle) was combined with phage display-derived peptides (or vehicle) (1:1, 115 μM in PBS) and incubated (37 °C, 7 d). The solution was then diluted (5.75 μM Aβ) with a ThT solution (4 μM final concentration in PBS) and fluorescence intensity was read (Ex = 450 nm, Em = 485 nm).

Laser Light Scattering. Phosphate-buffered saline with azide (PBSA, 0.01 M Na_2HPO_4/NaH_2PO_4, 0.15 M NaCl, 0.02% (w/v) NaN_3, pH = 7.4) was double filtered through 0.22 μm filters. Urea (8 M) was prepared in glycine−NaOH buffer (10 mM, pH 10) then double filtered through 0.22 μm filters. Lyophilized Aβ(1−40) (AnaSpec, San Jose, CA) was dissolved (2.8 mM) in urea (8 M). After approximately 15 min dissolution to break up any aggregates and to remove any residual secondary structure, samples were diluted to 140 μM Aβ into filtered PBSA or PBSA containing the test peptide. Samples were rapidly filtered through 0.45 mm filters directly into light-scattering cuvettes. Cuvettes were placed in a bath of the index-matching solvent decahydronaphthalene, which was controlled at 25 °C. Dynamic light scattering data at a 90° scattering angle were taken using a Coherent (Santa Clara, CA) argon ion laser operated at 488 nm and a Malvern 4700c system (Southborough, MA), as described in more detail elsewhere. Both total intensity (counts per second) and autocorrelation data were collected over a 24 h period. Autocorrelation functions were analyzed using the method of cumulants to determine an average hydrodynamic diameter.

Electron Microscopy. $A\beta_{1-40}$ was dissolved in 0.1% TFA at a concentration of 10 mg/mL, and the solution was incubated at 37 °C for 4 h to break up potential aggregation "seeds." This stock solution was diluted to 0.5 mg/mL (115 μM) in PBS with or without an equimolar concentration of phage-display derived peptide sequence. Aggregation was allowed to progress at 37 °C with mixing by gentle inversion for 18−24 h. Electron micrographs were performed using a JEOL 100CX transmission electron microscope with uranyl acetate negative staining.

Acknowledgment. We thank Dr. B. Kay for the mBax phage display vector. This research was supported by the NIH (NS37728). SPR data were obtained at the University of Wisconsin-Madison Biophysics Instrumentation Facility, which has been supported by the NSF (BIR-9512577) and the NIH (RR13790). B.P.O. thanks the NIH (AG19550) for a postdoctoral fellowship. We thank D. S. Peal, M. W. Peczuh, E. S. Underbakke, T. Gibson, D. J. Austin, and C. Cairo for helpful discussions.

Supporting Information Available: Detailed characterization of the libraries and synthesized peptides, and additional screening results, laser light scattering data, electron micrographs, and correlations. This material is available free of charge via the Internet at http://pubs.acs.org.

JA0619861