Selection of D-Amino-Acid Peptides That Bind to Alzheimer's Disease Amyloid Peptide $A\beta_{1-42}$ by Mirror Image Phage Display

Katja Wiesehan,[a, b] Katrin Buder,[b] Reinhold P. Linke,[c] Stephan Patt,[d] Matthias Stoldt,[a, b, e] Eberhard Unger,[b] Bettina Schmitt,[b] Enrico Bucci,[b, f] and Dieter Willbold*[a, b, e]

A mirror image phage display approach was used to identify novel and highly specific ligands for Alzheimer's disease amyloid peptide Aβ(1–42). A randomized 12-mer peptide library presented on M13 phages was screened for peptides with binding affinity for the mirror image of Aβ(1–42). After four rounds of selection and amplification the peptides were enriched with a dominating consensus sequence. The mirror image of the most representative peptide (D-pep) was shown to bind Aβ(1–42) with a dissociation constant in the submicromolar range. Furthermore, in brain tissue sections derived from patients that suffered from Alzheimer's disease, amyloid plaques and leptomeningeal vessels containing Aβ amyloid were stained specifically with a fluorescence-labeled derivative of D-pep. Fibrillar deposits derived from other amyloidosis were not labeled by D-pep. Possible applications of this novel and highly specific Aβ ligand in diagnosis and therapy of Alzheimer's disease are discussed.

KEYWORDS:

Alzheimer's disease · amyloid peptide · enantiomers · ligand design · phage display

Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder that is characterized by memory loss, confusion, and a variety of cognitive disabilities.[1] The only reliable diagnosis to date of a patient with AD is through the post mortem identification of amyloid plaques and neurofibrillary tangles in the respective brain. A major component of the amyloid plaques is the Aβ peptide, which consists of 39–43 amino acid residues. Aβ exists in a soluble overall helical structure[2] and an aggregated overall extended (β-sheet) structure.[3] Such a β-sheet conformation is found in the fibrils that build up amyloid plaques. Therefore, these plaques are stainable with chromophores, such as Congo red and thioflavin. These substances show high affinity for β-sheet structures irrespective of the identity of the peptides contained therein. The fact that no reliable method for pre mortem detection of plaques is available is not only unsatisfactory for people directly or indirectly affected by AD, but also hinders the search for therapeutic approaches because neither the time course of the disease nor any treatment success can be monitored in vivo and online. This applies to humans and animal models alike.

Thus, a lot of effort is currently being invested in the development of novel diagnostic tools that enable online monitoring of amyloid plaque load in living brains. In particular, imaging techniques such as magnetic resonance tomography (MRT)[4] and positron emission tomography (PET) are under investigation for their use in AD diagnosis. Probes for use in any of these detection techniques are often based on Aβ-binding substances like Congo red and thioflavin-T.[5–7] Very recently, novel substances have been invented that have been shown to cross the blood–brain barrier.[8, 9] Even Aβ itself has been used as a radio-labeled probe.[10] Still, a reliable diagnostic tool for AD does not exist.

During the last two decades, methods were developed that allow the screening of very large randomized peptide libraries

[a] *Prof. Dr. D. Willbold, Dr. K. Wiesehan, Dr. M. Stoldt*
Forschungszentrum Jülich
IBI-2, 52425 Jülich (Germany)
Fax: (+49) 2461-612023
E-mail: dieter.willbold@uni-duesseldorf.de

[b] *Prof. Dr. D. Willbold, Dr. K. Wiesehan, K. Buder, Dr. M. Stoldt, Prof. Dr. E. Unger, Dr. B. Schmitt, Dr. E. Bucci*
Institut für Molekulare Biotechnologie
Beutenbergstrasse 11, 07745 Jena (Germany)

[c] *Prof. Dr. R. P. Linke*
Max-Planck-Institut für Biochemie
Am Klopferspitz 18, 82152 Martinsried (Germany)

[d] *Prof. Dr. S. Patt*
Friedrich-Schiller-Universität
Institut für Neuropathologie, 07743 Jena (Germany)

[e] *Prof. Dr. D. Willbold, Dr. M. Stoldt*
Institut für Physikalische Biologie
Heinrich-Heine-Universität, 40225 Düsseldorf (Germany)

[f] *Dr. E. Bucci*
Istituto di Biostrutture e Bioimmagini
Via Mezzocannone 6/8, 80134 Naples (Italy)

 © 2003 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim DOI: 10.1002/cbic.200300631

for peptides that bind virtually any given target. Peptide phage display,[11] polysome display,[12] and ribosome display[13] are some examples. Phage display is a method used to present a huge library of different peptides on the surface of bacteriophages to be screened for variants that bind to a certain target molecule. The library may contain up to 10^{12} different peptides which, of course, consist exclusively of proteinogenous L-amino acids. Unfortunately, such peptides are sensitive to proteases and often have immunogenic potential, which renders them unsuitable for multiple application directly to higher animal models, or use in human treatment.

A recently developed method, called mirror image phage display,[14] allows the use of phage display to ultimately identify peptides that bind specifically to a given target and consist solely of D-amino acids. D-Amino-acid peptides are known to be less protease sensitive,[15, 16] more resistant to degradation in animals,[17, 18] and less or even not at all immunogenic,[18] as compared with L-amino-acid peptides.[14, 19–21] In order to obtain the exact mirror image of a given protein (L-protein), it is necessary to synthesize a protein with the same amino acid sequence but composed exclusively of D-amino acid residues (D-protein). Such a D-protein can be used as a target for phage display selection like any other target. In the study described herein, we used the D-enantiomer of Aβ (D-Aβ) as the target and a 12-mer randomized peptide library displayed on the surface of M13 bacteriophages to be screened for D-Aβ-binding peptides. For reasons of symmetry, the D-enantiomeric form of the selected 12-mer peptide should interact with the Aβ protein consisting of L-amino acids (L-Aβ). Examples of successful applications of mirror image phage display have been reported recently.[21, 22]

For diagnosis and potential therapy, compounds that directly address or bind to native Aβ amyloid are still highly desired. By employing mirror image phage display with the D-amino-acid enantiomer of Aβ(1 – 42) as a target, we identified D-amino-acid peptides that bind to the L-Aβ peptide with high specificity. The properties of the derived D-amino-acid peptide were investigated and potential applications are discussed below.

Results

Characterization of the Aβ(1 – 42) D-enantiomer

To carry out a mirror image phage display experiment, it is crucial to use the exact mirror image of the target molecule. Therefore, to confirm the identity of the biotinylated (bio) L- and D-Aβ(1 – 42) peptides, circular dichroism (CD) and nuclear magnetic resonance (NMR) spectroscopy were used in addition to mass spectrometry. The complete NMR assignment was deposited in the BioMegResBank (BMRB) data bank under accession number 5400. The CD spectra of bio-L-Aβ(1 – 42) and bio-D-Aβ(1 – 42) in hexafluoroisopropanol are identical except that they have opposite signs, exactly as is to be expected from the peptide and its mirror image (Figure 1 A).

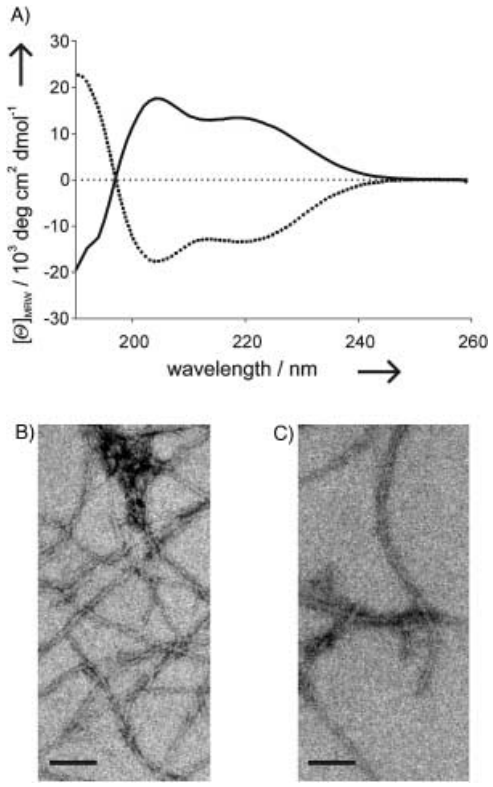

Figure 1. A) CD analysis of bio-L-Aβ(1 – 42) (dotted line) and bio-D-Aβ(1 – 42) (solid line). Spectra were recorded from 260 to 190 nm at 10 nm min^{-1} on a Jasco J710 CD spectrometer. Samples contained 54.2 or 41.2 μM peptide in hexafluoroisopropanol. Fibrils formed by bio-L-Aβ(1 – 42) (B) and bio-D-Aβ(1 – 42) (C) were negatively stained by uranyl acetate and examined by electron microscopy. The bars each indicate a length of 200 nm.

Fibril-forming properties of synthetic biotinylated Aβ(1 – 42)

For phage display selection experiments, synthetic bio-D-Aβ(1 – 42) peptide was used as the target. To verify whether bio-D-Aβ(1 – 42) is still able to form typical fibrils, we prepared electron micrographs (EM). EM pictures clearly show that bio-L- and bio-D-Aβ(1 – 42) peptides form fibrils with the typical thickness of 6.5 – 10.5 nm (Figure 1 B, C), which is very similar to the reported values of 7 – 10 nm for fibrils derived from AD brains.[23]

Mirror image phage display selection results

A commercially available phage-displayed peptide library (PhD12, New England Biolabs) was screened for peptide variants with affinity for synthetic bio-D-Aβ(1 – 42) immobilized on the surface of streptavidin-coated tubes. After four rounds of selection and amplification, 39 randomly picked phages were subjected to DNA sequence analysis of the 5′-end of the gene coding for the minor coat protein. Upon translation into amino acid sequences, 23 of the resulting peptides showed strong sequence similarity (Table 1). One of the sequences occurred 20 times. Three sequences have only minor differences compared to the dominating sequence. In addition, various peptide sequences were obtained that did not show any similarity to

 © 2003 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

Table 1. Peptide sequences obtained after selection from the library for binding to D-Aβ(1 – 42).

Peptide sequences	Number of identical sequences
Q S H Y R H I S P A Q V	20
Q S H Y R H I S P D Q V	1
Q S H Y R H I S P A R V	1
K S H Y R H I S P A KV	1
H H G H S P N V S Q V R	1
G S F S T Q V G S L H R	1
H T G T Q S Y V P R L R[a]	1
T L A Y A R A Y M V A P	2
A T P Q N D L K T F P H	1
T Q P E T D L L R V Q F	1
C I T W P P T G L T Y P	1
T F L E T G P I Y A D G	1
L V P P T H R H W P V T	1
A P P G N W R N Y L M P	1
D N Y S N Y V P G T K P	1
S V S V G M K P S P R P	1
S L P N P F S V S S H G	1
Y V H N P Y H L P N P P	1
C R R L H T Y L G P V T	1

[a] Sequence contains an amber stop codon (TAG) on the DNA level. For the production of phages we used *E. coli* strain ER2738, which suppresses TAG by introducing a glutamine residue on the amino acid level.

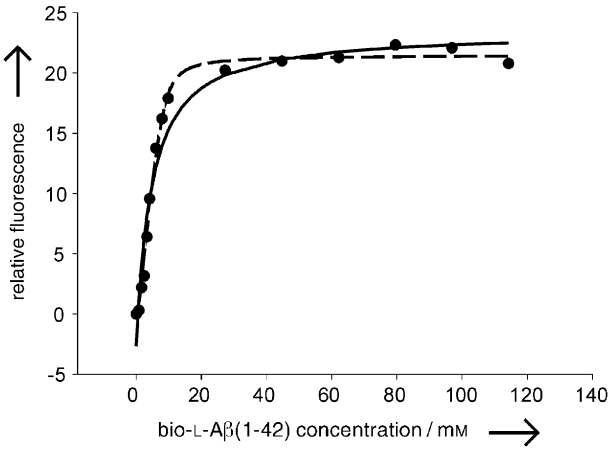

Figure 2. Binding of bio-L-Aβ(1 – 42) to 0.8 μM fluo-D-Pep as a function of bio-L-Aβ concentration. The values plotted result from the difference in fluorescence between hexafluoroisopropanol (HFP) solvated L-Aβ and HFP without bio-L-Aβ. Assuming a simple bimolecular reaction between bio-L-Aβ and D-Pep, analysis by nonlinear curve fitting (solid line) yielded a dissociation constant, K_D, of 4 μM. An additional fitting of the experimental data using a model for binding between D-pep and bio-L-Aβ with an undetermined number of equally affine and independent Aβ binding sites on D-pep yielded a stochiometry of one D-pep to about eleven L-Aβ(1 – 42) molecules, and a K_D value of 0.4 μM (dashed line).

each other. We concluded that the dominating amino acid sequence describes a peptide that binds to D-Aβ with significant affinity.

Direct binding of the D-enantiomer of the phage-displayed peptide to synthetic L-Aβ(1 – 42)

Based on mirror image conversion rules, a peptide with the same sequence as L-pep but synthesized solely from D-enantiomers of the respective amino acids (herein called, "D-pep") is anticipated to bind naturally occurring L-Aβ(1 – 42). To check whether D-pep is able to bind L-Aβ we used chemically synthesized D-pep with a fluorescein label at its carboxy-terminal end (fluo-D-pep) for fluorescence titration studies. Figure 2 shows the fluorescence intensity of fluo-D-pep as a function of L-Aβ(1 – 42) concentration. To rule out fluorescein-mediated binding of fluo-D-pep to L-Aβ(1 – 42), fluorescein was titrated with L-Aβ. No L-Aβ concentration dependence of the fluorescein fluorescence signal was observed. A simple bimolecular reaction between L-Aβ(1 – 42) and D-pep was assumed and analysis by nonlinear curve fitting[24] yielded a K_D value of 4 μM. Fluorescein was used as a control and did not show any fluorescence dependence on L-Aβ(1 – 42) concentration. To avoid the assumption of a bimolecular binding model, we carried out an additional evaluation of the experimental data by using a model for binding between D-pep and L-Aβ with an undetermined ratio of D-pep and L-Aβ molecules within the complex. This ratio was treated as an additional parameter to be fitted during the fitting procedure, which then yielded a stoichiometry of one D-pep molecule to eleven L-Aβ(1 – 42) molecules, as well as a K_D value of 0.4 μM (Figure 2).

Staining of AD brain tissue sections with the selected D-peptide

To investigate whether D-pep is able to bind to natural Aβ amyloid fibrils, brain tissue sections derived from patients who suffered from AD were stained with fluo-D-pep and with a commercially available anti-Aβ(1 – 16) antibody detected with a Cy3-labeled secondary antibody as a control (Figure 3). In

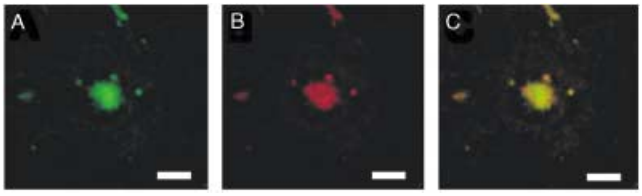

Figure 3. Fluorescence microscopy of a brain tissue section derived from a patient who suffered from AD. Co-staining was carried out with fluo-D-pep and anti-Aβ(1 – 16) antibody detected with a Cy3-labeled secondary antibody. Pictures were taken under two different filter conditions (A, fluorescein, excitation 450 – 490 nm, emission 515 – 565 nm; B, Cy3, excitation 510 – 560 nm, emission starting at 590 nm; C, superposition of the two pictures). The white bars indicate a length of 20 μm.

principle, both probes stained identical structures that can easily be recognized as typical amyloid plaques. In brain tissue sections of a patient without clinical and histologic signs of AD (used as a control), no plaque-like structures could be stained by any of the probes used (data not shown).

Control staining of tissue sections from other amyloidoses

Further controls were carried out to clarify whether D-pep unspecifically binds to any other amyloid. Tissue sections derived

 © 2003 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

from patients that suffered from amyloidosis other than AD were stained with fluo-D-pep and, in addition, with Congo red as a positive control (Figure 4). Congo red is known to bind to any amyloid and to amyloid-like fibrils, regardless of their chemical identity.[25, 26] A comparison of the micrographs from tissue sections obtained from Aβ amyloid plaques, ALλ amyloid deposits, ALκ deposits, and AA deposits revealed that, while Congo red bound to all amyloids, the fluo-D-pep only stained Aβ amyloid (Figure 4, first row). Some weak unspecific background staining by fluo-D-pep was observed in the case of AA amyloid affected tissue. In all other tissues, no fluo-D-pep-induced staining was detectable. Use of the fluorescein-optimized excitation and emission filters made some structures visible even without fluo-D-pep staining (Figure 4, first column). This effect may be due to the presence of autofluorescent substances such as lipofuscin which are frequently found in tissues.[27]

Staining of precipitates from cerebral congophilic angiopathy

Cerebral congophilic angiopathy (CAA) is characterized by Aβ amyloid deposits in leptomeningeal vessels.[28] To clarify whether D-pep binds specifically to Aβ amyloids of AD and CAA, we stained brain tissue sections of a patient who suffered from CAA. Indeed, vessel walls containing Aβ amyloid were stained with fluo-D-pep and with the anti-Aβ antibody used as a control (Figure 5).

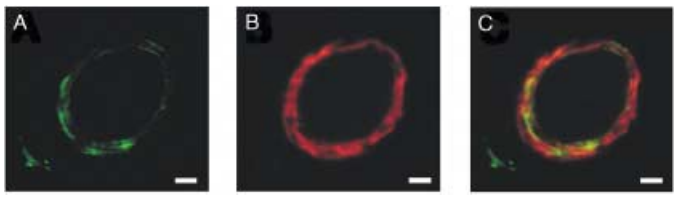

Figure 5. Fluorescence microscopic pictures of Aβ deposits in a brain tissue section derived from a person that suffered from CAA. Costaining was carried out with anti-Aβ(1 – 16) antibody detected with Cy3-labeled secondary antibody, and with fluo-D-pep. The pictures were taken under two different filter conditions (A, fluorecein, excitation 450 – 490 nm, emission 515 – 565 nm; B, Cy3, excitation 510 – 560 nm, emission starting at 590 nm; C, superposition of (A) and (B). The white bars in the pictures indicate a length of 50 μm.

Discussion

A phage-displayed peptide library was screened for 12-mer peptides that bind to the mirror image of Alzheimer's disease

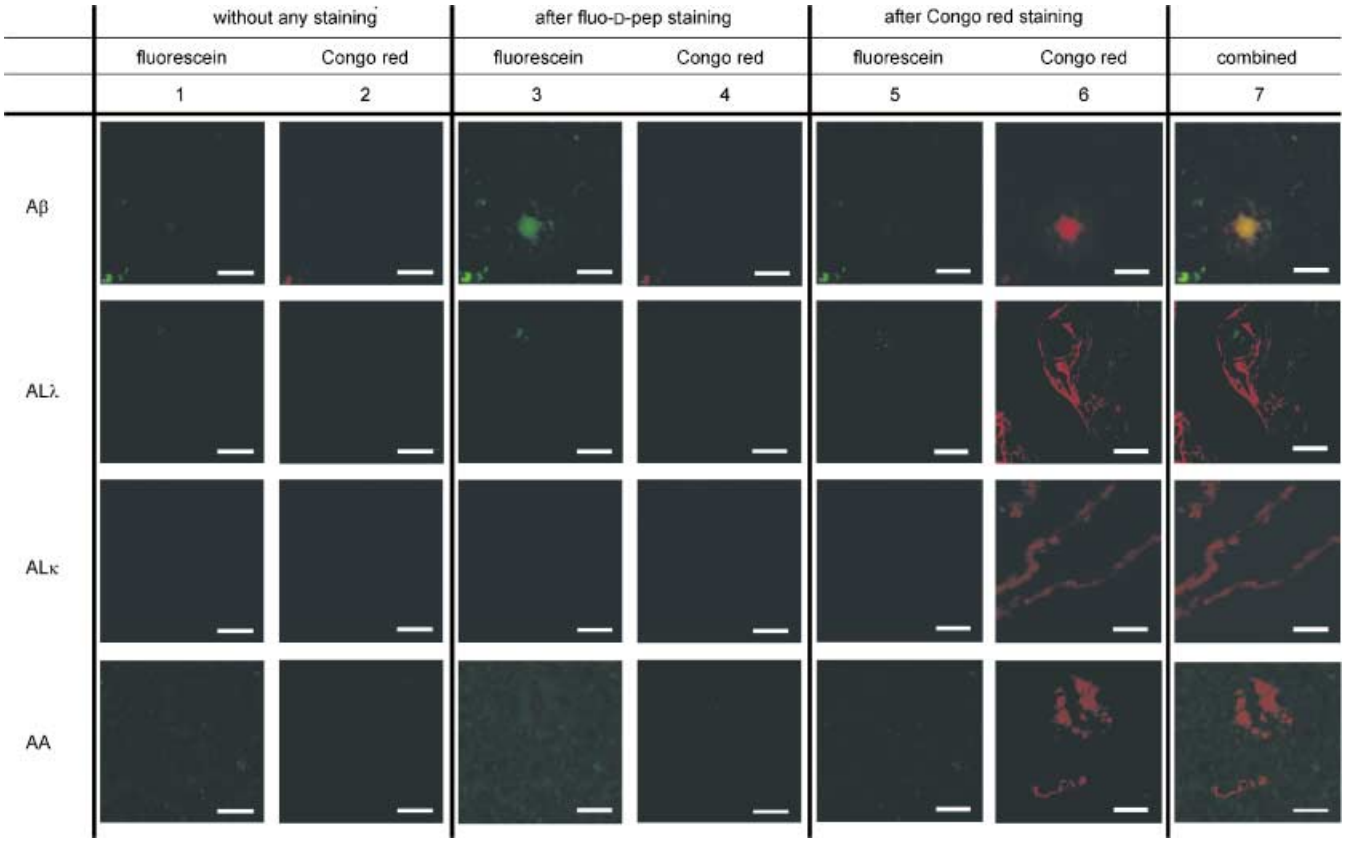

Figure 4. Double staining of amyloid peptides from various tissues: Aβ from an Alzheimer's disease affected brain, ALλ from the pancreas, ALκ from the tongue, and AA from the kidney (see the Experimental Section for details). Microscopic pictures were taken of tissue samples related to the disease peptides given on the left both before (columns 1 and 2) and after staining with fluo-D-pep (columns 3 and 4) and Congo red (columns 5 and 6). Each picture was taken under two different filter conditions (fluorescein, excitation 450 – 490 nm, emission 515 – 565 nm, columns 1, 3 and 5; Congo red, excitation 510 – 560 nm, emission starting at 590 nm, columns 2, 4, and 6). Column 7 shows a superposition of the pictures in columns 3 and 6 to enable comparison of the extent of staining after treatment with fluo-D-pep to that after treatment with Congo red. The white bars in the pictures indicate a length of 20 μm in the case of Aβ amyloid plaques, and 50 μm in all other cases.

 © 2003 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

amyloid peptide Aβ(1 – 42). By employing NMR and CD spectroscopy, we confirmed that the bait (D-Aβ) was the exact mirror image of naturally occurring L-Aβ(1 – 42).

Screening of the peptide library yielded several 12-mer peptides consisting of L-amino acids that were selected during the selection process based on D-Aβ(1 – 42) binding. Among the various amino acid sequences identified (Table 1), one sequence was clearly dominant. We chose to use this dominating sequence to synthesize a peptide exclusively consisting of D-amino acids (D-pep), which indeed bound directly to L-Aβ(1 – 42) (Figure 2). On first sight, an approximately one-to-eleven binding stochiometry between D-pep and L-Aβ may be hard to imagine. One has to keep in mind, however, that only a small fraction of the Aβ molecules within an amyloid fibril may be accessible to D-pep. Further, D-pep possibly binds to sites, that are each built up by several neighbouring Aβ molecules.

In brain tissue sections derived from patients that suffered from AD, plaques were stained similarly by fluo-D-pep, Congo red, and anti-Aβ(1 – 16) antibody. Amyloid deposits in sections from three different types of non-AD amyloidosis, Muckle – Wells syndrome, IgGκ multiple myeloma, and severe cardiomyopathy, were not stained by fluo-D-pep. This result clearly indicates that D-pep binds specifically to Aβ deposits, in contrast to dyes like Congo red. Thus, D-pep may be suitable to be used as a specific ex vivo probe for the detection of Aβ-containing plaques. The peptide D-pep exhibits Aβ-binding properties virtually indistinguishable from an anti-Aβ antibody. Compared to an antibody, however, D-pep is of much lower molecular weight and fully synthetic, which renders it amenable to any desired modification or labeling.

Taking all data together, we identified a peptide (D-pep) consisting of D-amino acids that is able to specifically bind to Aβ fibrils contained in plaques and leptomeningeal vessels. Since such D-peptides are considered to be rather protease resistant[15–18] and mostly nonimmunogenic,[14, 18–21] D-pep may be very suitable for use as a probe for the detection of amyloid plaques in living humans or animal models used to search for compounds that are suitable for AD therapy. D-pep may be synthetically fused to any substance known to cross the blood – brain barrier, and can be combined with any marker employed for physiologic detection or medical imaging techniques. So far, it has not been possible to unambiguously detect Aβ plaques in living humans or in animals. Any AD therapeutic approach directed either against amyloid fibril formation or against β- or γ-secretase activities, will have to rely on correct monitoring of the cerebral and cerebrovascular amyloid load. It is the aim of researchers to assess quantitatively the increase of Aβ amyloid during the natural course of AD and related diseases and record exactly the alterations of this course induced by a succesful therapeutic intervention. Thus, there is a huge demand for nontoxic and noninvasive probes for online and in vivo detection of Aβ in brains and D-pep could represent a suitable candidate.

Experimental Section

Peptides (L-Aβ, DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA, all amino acids as L-enantiomers; bio-L-Aβ, same sequence as above with a biotin residue at the amino-terminal end; bio-D-Aβ and D-Aβ, same sequence as bio-L-Aβ and L-Aβ, respectively, but all amino acids as D-enantiomers; D-Pep, QSHYRHISPAQV, all amino acids as D-enantiomers; fluo-D-pep, D-pep with an additional fluoresceinyl-lysin residue at the carboxy-terminal end) were purchased as reversed phase high performance liquid chromatography purified products (Jerini Biotools, Berlin, Germany). The identities of the peptides were confirmed by matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS), as well as by NMR and CD spectroscopy.

NMR spectroscopy: NMR spectra of bio-L- and bio-D-Aβ were recorded on a VARIAN UNITY INOVA750 spectrometer at 298 K in deuterated dimethylsulfoxide (DMSO-d6). Double quantum coherence filtered correlation (DQF-COSY), total correlation (clean-TOCSY; mixing time, 80 ms), and nuclear Overhauser enhanncement (NOESY; mixing time, 200 ms) spectra were employed for sequence-specific assignment of virtually all peptide proton resonances.

CD spectroscopy: Circular dichroism (CD) spectra of bio-L-Aβ(1 – 42) and bio-D-Aβ(1 – 42) were recorded from 260 – 190 nm at 10 nm min^{-1} on a Jasco J710 CD spectrometer. The samples contained 54.2 or 41.2 μM peptide in hexafluoroisopropanol. The sample volume was 2.7 mL. The reference sample contained hexafluoroisopropanol without peptide. Four scans were accumulated for each sample.

Electron microscopy: L-Aβ and D-Aβ were added to H_2O at a concentration of 350 μM. This mixture was shaken at 200 rpm and 37 °C for five days. An aliquot was placed on 400 mesh carbon-coated formvar copper grids. After 10 min, excess fluid was discarded and the samples were negativly stained with uranyl acetate (2% (w/w)) in water for 30 seconds. Finally, the specimens were examined and photographed in a Zeiss EM 902 A microscope at 80 kV.

DNA sequence analysis: DNA sequences were determined by using the Big Dye Terminator Cycle Sequencing Kit, PE Applied Biosystems (Warrington, Great Britain) with Ampli Taq DNA Polymerase.

Fluorescence titration measurement: Fluorescence measurements were carried out at 298 K on an SLM Aminco Fourier Transform Spectrofluorometer 48 000 MHF (SLM Instruments Inc., USA) in standard fluorescence cells of SUPRASIL quartz glass (10 × 10 mm, Hellma, Germany). Fluorescence was measured with permanent stirring in buffer (phosphate-buffered saline (PBS) with 1 mM sodiumdodecylsulfate) at excitation and emission wavelengths of 495 and 520 nm, respectively, with a D-Pep concentration of 0.8 μM. Appropriate amounts of L-Aβ were dissolved in HFP. As a control the same titrations were performed with HFP devoid of L-Aβ. As a further control, fluorescein was titrated with L-Aβ. No L-Aβ concentration dependence of the fluorescein fluorescence signal was observed.

Phage display: For phage display, a stock solution of bio-D-Aβ (2 mM) in HFP was prepared and further diluted to 250 μM in dimethyl sulfoxide. 1×10^{11} phages (Ph.D.-12 Phage Display Peptide Library Kit, New England Biolabs) in TBST (0.8 mL; 50 mM tris(hydroxymethyl)aminomethane (Tris)-HCl, pH 7.5, 150 mM NaCl, 0.1% (v/v) Tween-20) containing bovine serum albumine (BSA, 0.1% (w/w)) were mixed in a 1:1000 ratio with bio-D-Aβ (250 μM) in DMSO to yield a final concentration of bio-D-Aβ of 250 nM. After 10 min the phage – peptide suspension was transferred into a streptavidin-coated tube (Roche-Boehringer, Mannheim, Germany) and gently shaken for 15 min at room temperature. Nonbinding phages were discarded. To displace any streptavidin-binding phages biotin (0.1 mM) was added and the mixture was left for 5 min. Thereafter, the tube was washed ten times with TBST (1.5 mL). Phages were eluted by 10-min incubation with glycine-HCl (0.8 mL, 0.2 M, pH 2.2) and neutralized with Tris-HCl (0.2 mL, 1 M, pH 9.1). Eluted phages were amplified to

 © 2003 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

yield 1×10^{11} phages to be used as input for the next selection round, as described in the standard protocol (New England Biolabs).

Staining of brain tissue sections with fluo-D-Pep and a mouse anti-Aβ(1 – 16)/Cy3-labeled sheep antimouse antibody system: Formalin-fixed (4% (v/v), buffered formaldehyde) paraffin-embedded brain tissue sections with a thickness of 4 – 6 μm were used. Tissue sections were deparaffinized by twofold application of Rotihistol (Roth, Karlsruhe, Germany). The tissue sections were then treated with ethanol/water (95:5 (v/v)) and PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4). Subsequently, the tissue sections were incubated with alkaline sodium chloride solution[25] (0.4 mL, 1% (w/w), sodium hydroxide solution mixed with 40 mL sodium chloride in ethanol 80% (v/v)) for 20 min, washed four times for 5 min in PBS, and incubated with BSA (10 mg mL^{-1}) in PBS at room temperature for 20 min. The mouse anti-Aβ(1 – 16) antibody (Chemicon) was diluted 1:50 with fluo-D-pep in BSA (10 mg mL^{-1}) in PBS to give a final fluo-D-pep concentration of 5 μM, and applied to tissue sections for 1 h at room temperature. The sections were then washed twice with PBS. Cy3-labeled sheep antimouse IgG (whole molecule, Sigma) was used as a second antibody for the detection of mouse anti-Aβ(1 – 16) antibody and applied to the tissue sections as a 1:50 dilution in PBS with BSA (10 mg mL^{-1}) for 1 h at room temperature. Finally, the tissue sections were washed three times with PBS, covered with fluorescence-mounting medium (Dako), and viewed and photographed with an Axiovert 135 microcope (Zeiss, Jena) with a filter set for the detection of fluorescein (excitation 450 – 490 nm, emission 515 – 565 nm; Zeiss, Jena) and a filter set for the detection of Cy3 (excitation 510 – 560 nm, emission starting at 590; Zeiss, Jena). Pictures were taken with a CCD camera (Sony 3 CCD Color Video Camera).

Staining of tissue sections from four different types of amyloidosis: Formalin-fixed (4% (w/w) buffered formaldehyde) paraffin-embedded tissue sections of aproximately 4 – 6 μm thickness from four different types of amyloidosis, amyloid A (AA) amyloidosis caused by Muckle – Wells syndrome, ALκ amyloid (immunoglobulin kappa light chain derived) in IgG κ multiple myeloma, ALλ-amyloid (immunoglobulin lambda light chain derived) from severe cardiomyopathy, and cerebal amyloid found in AD patients, were examined. The patients from whom the samples came and the identification of the amyloid class has recently been summarized.[29] The sections were deparaffinized and rehydrated as described above. These tissue sections were incubated in alkaline sodium chloride solution for 20 min, washed four times with PBS, and incubated in PBS with BSA (10 mg mL^{-1}) at room temperature for 20 min. After further washing with PBS and H_2O, the sections were covered with glycerol (90% (v/v)) in PBS, viewed, and photographed. The glycerol was then removed by washing three times with PBS. The tissue sections were incubated in PBS with BSA (10 mg mL^{-1}) for 20 min and stained by treatment with fluo-D-pep (5 μM) in PBS with BSA (10 mg mL^{-1}) for 1 h at room temperature. After further washing with PBS and H_2O, the sections were covered with glycerol (90% (v/v)) in PBS, viewed, and photographed. The glycerol was then removed by washing three times with PBS. Staining with Congo red was carried out by treatment of the sections with sodium hydroxide solution (0.4 mL, 250 mM) and Congo red solution (40 mL; Congo red (0.2% (w/w)) in ethanol/water (80:20 (v/v); Sigma). The tissue sections were washed with H_2O, covered with fluorescence-mounting medium (Dako), viewed, and photographed.

We thank Matthias Gerdts (University of Bayreuth) for help with the data fitting.

[1] C. Soto, *Mol. Med. Today* **1999**, *5*, 343 – 350.
[2] H. Sticht, P. Bayer, D. Willbold, S. Dames, C. Hilbich, K. Beyreuther, R. W. Frank, P. Rösch, *Eur. J. Biochem.* **1995**, *233*, 293 – 298.
[3] L. C. Serpell, *Biochim. Biophys. Acta* **2000**, *1502*, 16 – 30.
[4] N. C. Fox, W. R. Crum, R. I. Scahill, J. M. Stevens, J. C. Janssen, M. N. Rossor, *Lancet* **2001**, *358*, 201 – 205.
[5] N. A. Dezutter, R. J. Dom, T. J. de Groot, G. M. Bormans, A. M. Verbruggen, *Eur. J. Nucl. Med.* **1999**, *26*, 1392 – 1399.
[6] W. E. Klunk, Y. Wang, G. F. Huang, M. L. Debnath, D. P. Holt, C. A. Mathis, *Life Sci.* **2001**, *69*, 1471 – 1484.
[7] D. M. Skovronsky, B. Zhang, M. P. Kung, H. F. Kung, J. Q. Trojanowski, V. M. Lee, *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 7609 – 7614.
[8] E. D. Agdeppa, V. Kepe, J. Liu, S. Flores-Torres, N. Satyamurthy, A. Petric, G. M. Cole, G. W. Small, S. C. Huang, J. R. Barrio, *J. Neurosci.* **2001**, *21*, RC189.
[9] K. Shoghi-Jadid, G. W. Small, E. D. Agdeppa, V. Kepe, L. M. Ercoli, P. Siddarth, S. Read, N. Satyamurthy, A. Petric, S. C. Huang, J. R. Barrio, *Am. J. Geriatr. Psychiatry* **2002**, *10*, 24 – 35.
[10] T. M. Wengenack, G. L. Curran, J. F. Poduslo, *Nat. Biotechnol.* **2000**, *18*, 868 – 872.
[11] G. P. Smith, *Science* **1985**, *228*, 1315 – 1317.
[12] L. C. Mattheakis, R. R. Bhatt, W. J. Dower, *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 9022 – 9026.
[13] J. Hanes, A. Plückthun, *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 4937 – 4942.
[14] T. N. Schumacher, L. M. Mayr, D. L. Minor, Jr., M. A. Milhollen, M. W. Burgess, P. S. Kim, *Science* **1996**, *271*, 1854 – 1857.
[15] L. E. Zawadzke, J. M. Berg, *J. Am. Chem. Soc.* **1992**, *114*, 4002 – 4003.
[16] J. F. Poduslo, G. L. Curran, A. Kumar, B. Frangione, C. Soto, *J. Neurobiol.* **1999**, *39*, 371 – 382.
[17] M. A. Findeis, G. M. Musso, C. C. Arico-Muendel, H. W. Benjamin, A. M. Hundal, J. J. Lee, J. Chin, M. Kelley, J. Wakefield, N. J. Hayward, S. M. Molineaux, *Biochemistry* **1999**, *38*, 6791 – 6800.
[18] H. M. Dintzis, D. E. Symer, R. Z. Dintzis, L. E. Zawadzke, J. M. Berg, *Proteins* **1993**, *16*, 306 – 308.
[19] M. Herve, B. Maillere, G. Mourier, C. Texier, S. Leroy, A. Menez, *Mol. Immunol.* **1997**, *34*, 157 – 163.
[20] S. C. Robson, V. K. Young, N. S. Cook, R. Metternich, W. Kasper Konig, B. A. Lesnikoski, R. N. Pierson, 3rd, W. W. Hancock, D. Candinas, D. J. White, F. H. Bach, *Transplantation* **1996**, *61*, 862 – 868.
[21] D. M. Eckert, V. N. Malashkevich, L. H. Hong, P. A. Carr, P. S. Kim, *Cell* **1999**, *99*, 103 – 115.
[22] I. A. Kozlov, S. Mao, Y. Xu, X. Huang, L. Lee, P. S. Sears, C. Gao, A. R. Coyle, K. D. Janda, C. H. Wong, *ChemBioChem* **2001**, *2*, 741 – 746.
[23] D. J. Selkoe, *Nature* **1999**, *399*, A23 – 31.
[24] B. Müller, T. Restle, J. Reinstein, R. S. Goody, *Biochemistry* **1991**, *30*, 3709 – 3715.
[25] H. Puchtler, F. Sweat, *J. Histochem. Cytochem.* **1965**, *13*, 693 – 694.
[26] W. E. Klunk, R. F. Jacob, R. P. Mason, *Anal. Biochem.* **1999**, *266*, 66 – 76.
[27] S. A. Schnell, W. A. Staines, M. W. Wessendorf, *J. Histochem. Cytochem.* **1999**, *47*, 719 – 730.
[28] G. G. Glenner, J. H. Henry, S. Fujihara, *Ann. Pathol.* **1981**, *1*, 120 – 129.
[29] R. P. Linke, *Virchows Arch.* **2000**, *436*, 439 – 448.

Received: April 22, 2003 [F 631]

 © 2003 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim