Directed selection of a conformational antibody domain that prevents mature amyloid fibril formation by stabilizing Aβ protofibrils

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The formation of amyloid fibrils is a common biochemical characteristic that occurs in Alzheimer’s disease and several other amyloidoses. The unifying structural feature of amyloid fibrils is their specific type of β-sheet conformation that differentiates these fibrils from the products of normal protein folding reactions. Here we describe the generation of an antibody domain, termed B10, that recognizes an amyloid-specific and conformationally defined epitope. This antibody domain was selected by phage-display from a combinatorial library of camelid antibody domains. Surface plasmon resonance, immunoblots, and immunohistochemistry show that this antibody domain distinguishes Aβ amyloid fibrils from disaggregated Aβ peptide as well as from specific Aβ oligomers. The antibody domain possesses functional activity in preventing the formation of mature amyloid fibrils by stabilizing Aβ protofibrils. These data suggest possible applications of B10 in the detection of amyloid fibrils or in the modulation of their formation.

Results
Conformational Specificity of B10. B10 was selected based on its affinity for mature Aβ(1–40) amyloid fibrils. Selection was carried out by immobilizing partially biotinylated fibrils at the surface of streptavidin-coated paramagnetic beads. Addition of a 10-fold molar excess of disaggregated Aβ(1–40) peptide to the supernatant ensured the removal of sequence-specific binders to the beads. The resulting supernatant was then evaluated for its ability to bind the synthetic immobilized Aβ fibrils. Binding of B10 to the Aβ fibrils was observed by surface plasmon resonance. Streptavidin-coated sensor chips were then conjugated to partially biotinylated Aβ(1–40) amyloid fibrils or disaggregated Aβ(1–40) peptide. SPR revealed that three of the four VH-domain libraries interact significantly with streptavidin (data not presented), whereas the fourth domain (B10) shows specificity for amyloid fibrils.

From SPR data, we calculated an apparent dissociation constant (Kd) of 475 ± 54 nM for fibril binding and a molar

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Amyloidoses are a group of conformational diseases where natural polypeptide chains do not fold up into their respective native states but form, instead, fibrillar aggregates termed “amyloid fibrils” (1, 2). These fibrils occur inside the body associated with aging or a number of diseases, such as Alzheimer’s, atherosclerosis, AA, and AL amyloidosis (2–4). All amyloid fibrils possess the same basic conformational arrangement, termed cross-β. This structure consists of β-sheets with β-strands oriented perpendicular to the main fibril axis (5). A considerably diverse number of polypeptide sequences can promote amyloid fibril formation, including even cases where normal protein folding would lead to an α-helical globular state, e.g., myoglobin (6).

The conformational difference between native proteins and amyloid fibrils raises possibilities to discriminate between these states with conformation-sensitive (“conformational”) antibodies. Such methods of detection are potentially important for clinical amyloid diagnosis, basic research, and therapy. Indeed, active or passive vaccination strategies are currently explored for the treatment of amyloid-related diseases (7, 8), including AL amyloid (9), Alzheimer’s (10), and prion diseases (11, 12). Vaccination was found to lead also to conformational antibodies (13–15). These or other vaccination-derived antibodies have been shown to prevent aggregation (10), clear preexisting tissue deposits (10), repress prion replication (16), or impair aggregate cytotoxicity (13, 17, 18).

Here we describe the generation of a conformation-sensitive and amyloid-specific antibody domain that was selected from a recombinant antibody domain library. Recombinant libraries represent a modern and cost-effective source for the generation of proteinaceous binders. Binders derived from such libraries can have very high affinity or specificity. In addition, they are readily available for genetic manipulations, large-scale expression and detailed functional analysis (19, 20). Selection can be carried out in the presence of two competing antigens, thereby eliminating unwanted cross reactivities. The library used in this study is a fully synthetic library of camelid VHH-domains. VHH-domains are the terminal Ig domains of heavy chain antibodies from camelidae (camels, dromedaries, etc.). These antibodies do not contain a light chain (21, 22), and all antigen specificity is encoded within a single polypeptide chain. This situation is of advantage for phage display. We have selected from this library the conformation-sensitive VHH-domain B10, which recognizes specifically amyloid fibrils from Alzheimer’s Aβ peptide. However, B10 binds only weakly to the freshly dissolved, disaggregated peptide or some Aβ oligomers.
stochiometry of Aβ-binding of 1:10 (B10:Aβ). This value is in good agreement with the molecular dimensions of the B10 homology model (Fig. 1a) and current structural data on Aβ(1–40) fibrils. The three complementarity determining regions that are responsible for the specificity of the VHH-domain span, in B10 homology model, a distance of 31 Å. X-ray diffraction measures, for Aβ(1–40) amyloid fibrils, a peptide repeat of 4.76 Å (23). Hence, one molecule of B10 covers approximately seven molecules of Aβ(1–40). The slight deviation from the experimental 1:10 ratio is consistent with unoccupied binding sites and imperfections in the B10 binding density. Genetic fusion of B10 to E. coli alkaline phosphatase, a homodimeric protein (24), leads to a fusion protein dimer that is termed B10AP. B10AP possesses divalent binding characteristics, along with an improved fibril affinity ($K_D$: 7.22 ± 0.97 nM) (Fig. 1b). Neither B10AP (Fig. 1b) nor B10 show significant interactions with disaggregated Aβ(1–40) peptide. They both differ from sequence-specific antibodies, such as 22C4 (Fig. 1b) or 4G8 (not presented), that interact with amyloid fibrils and disaggregated Aβ(1–40) peptide. The fibril affinity of B10AP is significantly higher ($K_D$ value = 7 nM) than that of conventional amyloid fibril ligands, such as serum amyloid P component (~1 μM) (3), Congo red (0.1–1.6 μM) (25, 26), or thioflavin T (ThT) (0.5–2 μM) (27).

The phosphatase moiety of B10AP facilitates detection in immunoblots or immunohistochemistry. Spotted onto nitrocellulose, B10AP detects Aβ(1–40) amyloid fibrils in quantities of >50 ng (Fig. 1c). B10AP staining of hippocampal sections from confirmed Alzheimer’s cases shows plaques with diameters >10 μm (Fig. 1d). Eleven of 12 confirmed Alzheimer’s cases show plaque staining with B10AP. None of the 10 age-matched controls produces this effect (Fig. 1e). B10AP detects large cell-culture derived Aβ plaques and immuno-doublistaining shows clear colocalization with a sequence-specific anti-Aβ antibody (Fig. 1f). Smaller Aβ assemblies stain more heterogeneously. We conclude that the B10-epitope is conformationally defined and specific for amyloid fibrils. The fact that B10AP recognizes amyloid fibrils formed in vitro or in vivo implies that Aβ amyloid fibrils of either origin share the same basic conformation and surface texture.

The B10-Epitope Is Common to Different Amyloid Fibrils. B10AP binds several different amyloid fibrils. It recognizes fibrils that were grown from Aβ(1–40) in vitro under different conditions of incubation, such as in Hepes buffer (pH 7.4) or sodium borate (pH 9.0). Furthermore, it binds to Aβ(1–42) amyloid fibrils (Fig. 2a) and tissue-extracted AA (derived from serum amyloid A protein) or AL amyloid fibrils (derived from Ig light chains). Both precursor sequences are not homologous to the Aβ sequence. Binding to non-Aβ fibrils can be demonstrated by immunoblot (Fig. 2a) or SPR [supporting information (SI) Fig. 5]. Although B10 binds obviously to all analyzed amyloid samples, the B10-epitope density and B10AP staining intensity varies for different fibrils (Fig. 2a). These data show that B10 recognizes an epitope that is common to different amyloid fibrils, if not generic. Binding to AL and AA amyloid can be competed by preincubation of B10AP with Aβ fibrils (Fig. 2b), indicating that all fibrils bind to the same structural region of B10.

The B10 epitope is localized on the fibril structure with transmission electron microscopy (TEM) and cerium phosphate...
staining. This method takes advantage of the ability of the B10AP phosphatase moiety to catalyze the formation of cerium phosphate, an electron-dense precipitate that accumulates in the vicinity of the active site (28). Electron micrographs of fibrils incubated with B10AP show cerium phosphate staining along the entire fibril length (Fig. 2c); i.e., the B10-epitope occurs at the fibril side and not only at the fibril ends. The cerium phosphate stain is spread evenly at abundant levels along all fibrils, indicating that substantial structural disruptions are absent in these fibrils. Because this analysis was carried out with a sample containing different fibril morphologies (SI Fig. 6), these data imply also that B10AP can bind to different fibril morphologies. No cerium phosphate staining is seen in B10AP-free control samples (Fig. 2d).

**B10 Binds Only Weakly to Aβ Oligomers.** Next we have checked as to whether B10AP interacts also with some nonfibrillar Aβ aggregates, sometimes termed also Aβ“oligomers.” These non-fibrillar aggregates show diameters from ~10 to 60 nm (Fig. 3a) and interactions with A11 antibody (data not presented). Although different oligomer preparations differ in their B10 affinity, oligomers generally stain less well with B10AP than fibrils (Fig. 3b). Analysis with far-UV circular dichroism (far-UV CD) reveals that oligomers possess a very high β-sheet content, similar to amyloid fibrils (Fig. 3c). These data show that B10 does not recognize all types of aggregates equally well. Moreover, presence of a β-sheet structure does not suffice to explain B10 binding. Consistent with this, several techniques demonstrate structural differences between amyloid fibrils and oligomers. Oligomers interact less well with amyloid-specific dyes, such as ThT and Congo red (CR) (Fig. 3e and f) and attenuated total reflectance Fourier-transform infrared (ATR-FTIR) spectroscopy produces a peaks at 1,693 cm⁻¹ and 1,628 cm⁻¹, consistent with the presence of an amyloid-like, aggregated β-sheet conformation (29), only oligomers show a clearly resolvable peak at 1,693 cm⁻¹. Such a peak is generally assumed to indicate antiparallel β-sheet structure (30). Aβ(1–40) amyloid fibrils are usually believed to encompass parallel β-sheets (31).

**B10 Prevents the Formation of Mature Amyloid Fibrils by Stabilizing Aβ Protofibrils.** Aβ(1–40) amyloid fibrils form through a nucleation-dependent reaction that involves the transient stabilization of several metastable intermediates (32, 33). These intermediates can have the shape of short linear protofibrils that possess smaller diameters than mature fibrils and a more curly appearance (34). The nucleation-dependence of amyloid fibril formation separates this process into two phases: the lag phase during which the nuclei are formed and the growth phase when the nuclei are extended into fibrils (32). Both phases are well resolvable for incubation of Aβ(1–40) peptide without B10AP (Fig. 4a). However, dot blot experiments with B10AP measure a shorter lag phase (5.9 h) than fluorescence measurements with ThT (7.3 h). These data demonstrate the formation of B10-epitopes at significant level before the formation of ThT-positive fibrils. TEM analysis demonstrates small amounts of nonfibrillar aggregates after 4 h, larger quantities of fibrils after 18 h, and both fibrils and nonfibrillar structures after 7 h (Fig. 4a). The apparent quantity of the nonfibrillar aggregates declines progressively upon incubation.
Addition of B10AP to Aβ(1–40) at 1:10 (B10:AB) molar ratio potently blocks the formation of ThT-positive fibrils (Fig. 4b) and leads, instead, to the stabilization of much more irregular, curvilinear fibrils, similar to what has been described as protofibrils (35, 36). The presently analyzed fibrils possess amyloid-like characteristics, evident from an ATR-FTIR peak at 1,624 cm⁻¹ and x-ray diffraction (Fig. 4d). Fully hydrated protofibril-B10AP complexes show reflections at 4.62 and 0.02 Å, the characteristic spacings of an amyloid-like core structure (5). Nevertheless, protofibril-B10AP complexes show only small interactions with amyloid-specific dyes, such as ThT or CR (Fig. 4e and f), consistent with previous reports about Aβ protofibrils (37). Control reactions show that their small ThT and CR affinities cannot be explained with a steric hindrance due to B10AP or with a short fibril length, because B10AP-decorated protofibrils but not when it is freshly dissolved and putatively monomeric. B10 recognizes amyloid fibrils derived from different polypeptide sequences, such as serum amyloid A protein and Ig light chains (Fig. 2a). A similar sequence-independence is described also for WO1 and WO2 antibodies (15). The observation that B10AP binds amyloid fibrils that were formed in vitro or in vivo testifies to the notion that amyloid fibrils of either origin share the same basic conformational arrangement.

Experiments in which B10AP is added to Aβ peptide under the conditions of fibril formation show that B10AP possesses functional activity in abrogating the formation of mature amyloid fibrils (Fig. 4). The ability of antibodies to interfere with amyloid formation or pathogenicity has been rationalized previously from some sequence-specific antibodies that were reported to induce the dissociation of preformed fibrils (38).

**Discussion**

We describe here the generation of the VHH-domain B10 that binds conformationally specific to amyloid fibrils. B10 does not recognize a certain polypeptide sequence and binds only to Aβ peptide when it is present in the form of amyloid fibrils or protofibrils but not when it is freshly dissolved and putatively monomeric. B10 recognizes amyloid fibrils derived from different polypeptide sequences, such as serum amyloid A protein and Ig light chains (Fig. 2a). A similar sequence-independence is described also for WO1 and WO2 antibodies (15). The observation that B10AP binds amyloid fibrils that were formed in vitro or in vivo testifies to the notion that amyloid fibrils of either origin share the same basic conformational arrangement.
viously with opsonization (9), a “peripheral sink” mechanism (10), the neutralization of cytotoxicity (13, 18), the interference with the cellular processing or the trafficking of the Aβ precursor protein (39), fibril destabilization, and the prevention of fibril formation (17, 38, 40). Evidence for fibril destabilization was provided mostly with binders that were sequence-specific or conformationally sensitive for the native state of a protein (17, 38, 40). By contrast, amyloid-specific antibodies may be assumed to rather stabilize these fibrils than to induce their disassembly. Consistent with this, we do not observe any significant dissociation of preformed amyloid fibrils in the presence of B10AP (Fig. 4). Instead, B10AP prevents selectively the formation of mature amyloid fibrils, whereas the formation of amyloid protofibrils is promoted.

Amyloid protofibrils have been defined as metastable aggregates that kinetically precede the thermodynamically more stable mature amyloid fibrils (35). The fact that B10AP binds either structural state implies that protofibrils and mature fibrils possess the same surface texture. Whether mature fibrils form by coalescence of protofibrils or by reassembly of the peptides after secondary protofibril dissociation is not finally established. Consistent with this, the present observations that B10AP impairs the transition into mature fibrils can be explained with an interference of B10AP with the association of protofibrils into mature fibrils or with an aborted protofibril dissociation upon B10AP binding. Given the potential cytotoxicity of amyloid protofibrils (33), it cannot be assumed that stabilizing protofibrils may be intrinsically more desirable than mature fibril formation. However, the higher susceptibility of the former toward proteolytic degradation suggests that B10AP-stabilized protofibrils may be dealt with more readily by natural clearance mechanisms than mature amyloid fibrils.

Finally, this work provides clear evidence that recombinant libraries can be used to directly select conformationally specific and proteinaceous binders of certain types of aggregates. This approach represents a fast and cost-effective way for the generation of such highly specific binders. Moreover, it avoids unnecessary animal experiments for antibody generation and allows, by application of competitive selection conditions (that are not available during animal vaccination), to fine-tune the experimental conditions so that unwanted cross-reactivities can be directly ruled out. Resulting binders can discriminate between different conformational forms of the same polypeptide chain. Potential fields of application of these binders include basic research and diagnosis. Selected binders can potentially dissect out discrete aggregate populations at the level of otherwise transient conformational ensembles. Alternatively, they could provide also efficient means of targeting specific pathologically relevant aggregate forms in the course of new therapeutic strategies.

Materials and Methods

Source of Amyloid Fibrils. Aβ(1–40) peptide was obtained from Jena BioScience and Bachem. Unless stated otherwise, fibrils were obtained by incubation of 1 mg/ml Aβ(1–40) peptide for 5 days in 50 mM Hepes with 50 mM NaCl (pH 7.4, 37°C) or 50 mM sodium borate (pH 9.0, room temperature). Aβ (1–42) (Bachem) fibrils were grown at 0.2 mg/ml concentration in borate buffer. AA and AL amyloid fibrils were purified according to Skinner (41), dissolved in H2O and sonicated for 1 min.

Aβ(1–40) Oligomer Preparation. Aβ(1–40) (2.5 mg/ml) was dissolved in 100% 1,1,1,3,3,3-hexafluoro-2-propanol. After incubation for 15 min at room temperature, the solution was diluted 10-fold with H2O. After further incubation for 15 min (room temperature) large aggregates were removed from the sample by spinning down at 14,000 × g for 15 min.

Selection and Purification of B10. Phage display was carried out according to standard procedures (42, 43), using a fully synthetic library of camellid VHH-domains generated in house and displayed on M13 phages (G.H. and U.H., unpublished data). Biotinylated Aβ(1–40) amyloid fibrils (SI Text) were immobilized on streptavidin-coated magnetic beads (Dynabead). Panning was carried out in 10 mM Hepes buffer (pH 7.4) containing 0.15 M NaCl and a 10-fold surplus of freshly dissolved Aβ(1–40) in the soluble phase. Blocking solutions contained additionally 2% BSA (albumine fraction V protease free) or skim milk. After three panning cycles, the DNA from several single phages was isolated and sequenced. The predominant sequences were subcloned into p416His (for expression as single VHH-domain) and pGH-AP (for expression as fusions with E. coli alkaline phophatase) and expressed in E. coli TG-1 (Stratagene). Purification of B10 and B10AP was carried out by nickel chelate chromatography, ion exchange chromatography and, for B10 only, reversed phase chromatography.

SPR. N-biotinylated, disaggregated Aβ(1–40) peptide or Aβ(1–40) fibrils (SI Text) were captured on different flow cells of a SA sensor chip (Biacore). 22C4 and B10AP were injected in 10 mM Hepes buffer (pH 7.4), 0.15 M NaCl, 3 mM ethylene-diamine-tetraacetic acid, 5 μM surfactant P20 at a flow rate of 10 μl/min. Regeneration was done with 10 μM glycine/HCl, 1 M NaCl (pH 1.5) for 1 min. Kd values were calculated from the concentration dependent steady-state binding of B10 or B10AP using the 1:1 steady-state affinity model. Molar stoichiometry of B10/Aβ(1–40) fibril complexes was estimated from the measured maximum binding capacity of the fibril coated sensor surface, the response level of immobilized Aβ fibrils, and the molecular mass of B10 (15725 Da) and Aβ(1–40) (4530 Da).

Immunoblots. If not stated otherwise, 1 μg of polypeptide was blotted onto 0.45 μm nitrocellulose membrane (GE Healthcare or Schleicher & Schuell) and blocked with 2% BSA in Tris-buffered saline (pH 7.4, TBS) at room temperature for 1 h. Equal loading was confirmed with control membranes using Ponceau red or Coomassie staining. Membranes were washed twice in H2O and once in TBST (TBS with 0.1% Tween 20) for 5 min before they were incubated for 1 h at room temperature with 0.5 μg/ml B10AP in TBS. Competition studies were performed by preincubating B10AP for 1 h with Aβ(1–40) fibrils at 1:0.5, 1:5, and 1:10 wt/wt ratios of AA or AL fibrils to Aβ. Membranes were washed twice with H2O and once in TBST for 5 min. Alternatively, washing and blocking may be performed in TBS containing 0.3 M NaCl. B10AP binding was visualized with NBT/BCIP (Pierce). Densitometric quantifications used TotalLab 100 software.

Electron Microscopy. For cerium phosphate staining, 8 μg from a 1 mg/ml fibril solution were incubated with 10-fold molar excess of B10AP (1 h, 4°C). Three microliters of the solution mix (32 μg/ml) were applied onto a nickel grid to adhere for 30 sec. The grid was rinsed five times to wash off unbound B10AP by rotating the nickel grid magnetically on the surface of a 1-ml drop of water/bruter. Cerium phosphate staining was performed by incubating three times for 10 min in 3.5 mM cerium(III) chloride, 1.5 mM p-nitrophenylphosphate, 25 mM tricine, 30% sucrose in 50 mM glycine-NaOH buffer (pH 9.0) and rinsed with 25 mM glycine-NaOH (pH 10.0) (28). Negative staining techniques are described in ref. 23.

CR Absorption Spectroscopy. All spectra were recorded at room temperature, using a Cary UV-VIS spectrophotometer. Samples contained 50 mM sodium borate (pH 9) or 50 mM Hepes buffer (pH 7.4), 50 mM NaCl, 10 μM CR with or without 5 μM B10AP. Aβ concentration was kept constant at 50 μM Aβ.
present either as fibrils, fragmented fibrils, or protofibrils (1:10 B10AP:Aβ).

**ThT Fluorescence.** ThT spectra of single samples were recorded at room temperature with a Shimadzu RF-5301PC fluorimeter using an excitation wavelength of 482 nm and a cuvette with 5-mm path length. Samples contained 50 mM sodium borate-buffered Hepes (pH 7.4), 50 mM NaCl, 20 μM ThT, 5 μM Aβ incorporated into oligomers, fibrils, fragmented fibrils, or fibrils decorated with 0.5 μM B10AP. Online ThT aggregation kinetics measurements (Fig. 4b) were carried out in a 96-well format at 37°C as described (44) but with modifications. Pitch of the data points: 30 min. Incubation conditions: 50 μM Aβ (1–40), 50 mM Hepes, (pH 7.4), 50 mM NaCl, 20 μM ThT and different B10AP concentrations.

**X-Ray Diffraction.** Exposures were carried out as described (23) using B10AP-protofibril complexes that were pelleted by ultracentrifugation and placed under buffer in a wax-sealed glass capillary.

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