BRICHOS domains efficiently delay fibrillation of amyloid β-peptide*

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Running head: BRICHOS delays Aβ fibril formation

Background: Alzheimers disease (AD) is associated with Aβ protein misfolding and aggregation into fibrils rich in β-sheet structure.

Results: BRICHOS domains prevent fibril formation of Aβ far below stoichiometric ratio.

Conclusion: Aβ is maintained as unstructured monomer in the presence of BRICHOS.

Significance: BRICHOS domain can have a natural protective role against Aβ aggregation, which may open new routes towards AD therapy.

SUMMARY
Amyloid diseases such as Alzheimers, Parkinsons and prion diseases are associated with a specific form of protein misfolding and aggregation into oligomers and fibrils rich in β-sheet structure. The BRICHOS domain of ~100 residues is found in membrane proteins associated with degenerative and proliferative disease, including lung fibrosis (surfactant protein C precursor; proSP-C) and familial dementia (Bri2). We find that recombinant BRICHOS domains from Bri2 and proSP-C prevent fibril formation of amyloid β-peptides (Aβ40 and Aβ42) far below stoichiometric ratio. Kinetic experiments show that a main effect of BRICHOS is to prolong the lag time in a concentration dependent, quantitative and reproducible manner. An ongoing aggregation process is retarded if BRICHOS is added at any time during the lag phase but it is too late to interfere at the end of the process. Results from circular dichroism and NMR spectroscopy, as well as analytical size exclusion chromatography imply that Aβ is maintained as unstructured monomer during the extended lag phase in the presence of BRICHOS. Electron microscopy shows that although the process is delayed, typical amyloid fibrils are eventually formed also when BRICHOS is present. Structural BRICHOS models display a conserved array of tyrosine rings on a five-stranded β-sheet, with inter-hydroxyl distances suited for hydrogen bonding peptides in extended β-conformation. Our data imply that the inhibitory mechanism is reliant on BRICHOS interfering with molecular events during the lag phase.
Misfolding of protein is the underlying cause of at least 27 amyloid diseases, e.g. Alzheimer’s (AD), Parkinson’s and prion diseases. These diseases are associated with a specific form of misfolding, where the protein aggregates into amyloid fibrils with β-strands running perpendicular to the fibril axis (1-3). AD is a progressive neurodegenerative disorder (4) and the senile plaques found in brain of AD patients contain amyloid fibrils of amyloid β–peptide (Aβ) (5,6). Aβ is derived from the transmembrane region of amyloid precursor protein (APP)1, which is processed in one amyloidogenic and one non-amyloidogenic pathway. Amyloidogenic Aβ, mainly consisting of 42 residues, is released by β- and γ-secretase (7). In a pure system, Aβ aggregation has the appearances of a nucleated polymerization reaction and a phase-transition (8). Fibrillar aggregates have low solubility but remain in dynamic equilibrium with free protein (8). In vivo, the aggregation process occurs in the presence of other proteins, membranes, metabolites, etc., each of which may affect both its rate and equilibrium. Many studies indicate that small soluble oligomers, are more potent than monomers or fibrils in causing neuronal dysfunction (9-11), suggesting that either oligomeric intermediates or the aggregation process are toxic.

In search for effective AD prevention and therapy, many studies have identified high or low molecular weight compounds that interfere with secretase activities or Aβ aggregation, or derived antibodies against Aβ monomer and oligomers (for review see (12)). A complementary approach is to identify and quantify the effects of intrinsic human components on Aβ aggregation, to allow future therapies to be directed toward activation of inbuilt defence routes. Molecular chaperones have evolved to counteract misfolding in the cell. Examples are the heat-shock proteins (Hsp)70, Hsp90, Hsp104 (13-16), and the small heat shock protein αA-crystallin, which affect fibril formation of Aβ in vitro (17). Hsp70 and Hsp104 also affect the aggregation of α-synuclein (18) and Sup35 (19). sHsp, Hsp20 and HspB8 co-localize with amyloid plaques in AD and other neurodegenerative disorders (20). This suggests that the cellular quality control machinery is activated in an attempt to prevent the accumulation of misfolded species while overload of chaperone capacity may impede the prevention (21); however, colocalization alone is not an indication of disease-related interactions. An extracellular quality control system includes clusterin (apolipoprotein J), haptoglobin and α2-macroglobulin (22-24), which are upregulated during stress, interact with pre-fibrillar species, inhibit amyloid formation, occur in amyloid deposits (25-29), and promote uptake of misfolded or aggregated proteins by cells for further degradation (30).

The BRICHOS domain of ~100 amino acids (Figure 1) was identified in the protein Bri2 related to familial British dementia, chondromodulin associated with chondrosarcoma, and lung surfactant protein C (SP-C/proSP-C) (31). The BRICHOS domain, found in twelve distantly related protein families with very similar predicted secondary structure, contains two strictly conserved cysteine residues, which form a disulphide bridge in proSP-C (32). ProSP-C BRICHOS exhibits a chaperone function during SP-C biosynthesis and prevents its transmembrane domain from misfolding (33,34). Mutations in the BRICHOS domain of proSP-C lead to proSP-C aggregation, amyloid formation and interstitial lung disease (35,36). ProSP-C BRICHOS has broad substrate specificity and binds unfolded or extended stretches of non-polar residues, but lacks general chaperone activity (37,38). Nerelius et al. found that proSP-C BRICHOS prevents fibril formation of Aβ40 and forms a complex with Aβ oligomers in vitro (37). Bri2 is expressed in neuronal tissue and has been shown to suppress Aβ deposition and influence APP processing in transgenic mice, suggesting an in vivo role for Bri2 in AD (39-41). The C-terminal region of Bri2 has high β-sheet propensity. In familial British and Danish dementia, mutant longer and more aggregation-prone peptides derived from this region form amyloid fibrils (referred to as
ABri or ADan) (42). The Bri2 BRICHOS domain binds the C-terminal region of Bri2 and Aβ40 in vitro. In the latter case, aggregation and fibril formation is inhibited. Bri2 BRICHOS binds segments of hydrophobic residues flanked by charged residues (43) but its exact substrate specificity is unknown.

A general function of BRICHOS domains may be to limit misfolding of aggregation-prone polypeptide regions. This function may be harnessed in future amyloid disease therapy requiring extensive investigations of the substrate specificity and concentration-dependence of the inhibitory effect. Therefore, we investigate here in detail the inhibition of aggregation of Aβ40 and the more disease relevant Aβ42 by recombinant forms of human proSP-C and Bri2 BRICHOS. Aggregation kinetics are studied using an approach that generates highly reproducible kinetic data (8), combined with structural investigations by circular dichroism (CD) and NMR spectroscopy, interaction studies by size exclusion chromatography, and morphological analyses by electron microscopy (EM). Our data reveal a very high potency of BRICHOS domains to delay Aβ amyloid formation and provide insights into the mechanism behind the anti-amyloidogenic function of this novel chaperone.

Experimental procedures

Aβ peptides. Aβ(M1-40) and Aβ(M1-42) were expressed in E. coli BL21 from synthetic genes and purified in batch format using ion exchange and size exclusion steps as described (44), which combined with highly pure monomeric peptide (amino acid sequences presented in Figure 1). Purified peptide was divided into 20-30 identical aliquots and stored at -20 °C. Monomer was then isolated by gel filtration of an aliquot of purified peptide just prior to setting up each of the experiments to remove traces of aggregate formed during freezing and thawing, and to exchange buffer to the one used in the respective experiments. The latter part of the monomer peak was collected in low-bind Eppendorf tubes (Axygene) on ice and the concentration was determined by absorbance and amino acid analysis after acid hydrolysis. The monomer was used as is or diluted to the desired concentration for the respective experiment.

Bri2 BRICHOS. The expression and purification of the Bri2 BRICHOS domain have been described previously (43). Briefly, the Bri2 BRICHOS construct was expressed in E. coli as a fusion protein with thioredoxin-His6 and S-tag. The protein was then purified using two rounds of Ni-NTA agarose column chromatography. Thrombin was used to remove the thioredoxin- and His6-tag. The eluted protein was analysed with SDS-PAGE and nondenaturing PAGE. The concentration was determined by amino acid analysis after acid hydrolysis. The amino acid sequence is presented in Figure 1.

ProSP-C BRICHOS. Cloning, expression and purification were performed as described in (45), with the following changes: The cells were lysed by lysozyme (1 mg/ml) for 30 minutes and incubated with DNase and 2 mM MgCl2 for 30 min on ice. The cell lysate was centrifuged at 6000x g for 20 min and the pellet was suspended in 2 M urea in 20 mM Tris, 0.1 M NaCl, pH 8 and sonicated for 5 min. After centrifugation at 6000x g for 30 min at 4 °C, the supernatant was filtered through a 5 μm filter and poured on a 2.5 ml Ni-Agarose column (Qiagen, Ltd.,West Sussex, UK). The column was washed with 100 ml 2 M urea in 20 mM Tris, 0.1 M NaCl, pH 8 and then with 100 ml 1 M urea in 20 mM Tris, 0.1 M NaCl, pH 8, and finally with 100 ml 20 mM Tris, 0.1 M NaCl, 20 mM imidazole, pH 8. The protein was eluted with 200 mM imidazole in 20 mM Tris, 0.1 M NaCl, pH 8, and dialyzed against 20 mM Tris, 0.05 M NaCl, pH 8, and cleaved by thrombin for 16 h at 4 °C (enzyme/substrate weight ratio of 0.002) to remove the thioredoxin- and His6-tag, and then reapplied to a Ni2+ column to remove the released tag. The protein was then further purified using ion exchange chromatography as previously described (45). The concentration was determined by amino acid analysis after acid hydrolysis. The amino acid sequence is presented in Figure 1.
Anti-thrombin. Human anti-thrombin was purchased from Baxter (Vienna, Austria).

Cystatin C. Chicken cystatin C was purified from egg white, as described by Lindahl et al (46).

Monellin. Single-chain monellin with net charge -2 (scMN-2; obtained through mutagenesis to incorporate the five substitutions C41S, Q13E, N14D, Q28E, and N50D) was expressed in E. coli from a synthetic gene and purified using ion exchange and size exclusion chromatography as described (47).

Size exclusion chromatography. Size exclusion chromatography on a Superdex75 column (GE Healthcare, Uppsala, Sweden) was performed using a BioLogic HR FPLC system (Biorad). The column was equilibrated and operated in degassed buffer (20 mM Na-phosphate, 200 µM EDTA, 0.02% NaN₃ at pH 7.4 or pH 8.0 to prepare samples for aggregation studies, and 10 mM Na-phosphate, 40 mM NaF, pH 7.4, to prepare samples for CD studies). Samples were injected from a 1 mL loop and chromatograms recorded by monitoring the absorbance at 280 nm. To monitor protein interactions, mixtures of Aβ and BRICHOS domains were injected directly after mixing, or after 2 and 20 hours incubation at 37ºC in 20 mM Na-phosphate, 200 µM EDTA, 0.02% NaN₃ at pH 7.4 or pH 8.0. Fractions (0.3-0.7 ml) were collected during the chromatogram, lyophilized and analyzed by SDS PAGE in a 10-20% gradient gel.

Aggregation kinetics. Aggregation kinetics were studied by recording the ThT fluorescence intensity as a function of time in a plate reader (FluoStar Omega from BMG Labtech, Offenberg, Germany). The fluorescence was recorded using bottom optics in half-area 96-well PEG-coated black polystyrene plates with clear bottom (Corning 3881) using a 440 nm excitation filter and a 480 nm emission filter. Aβ monomer was isolated by gel filtration as above in 20 mM Na-phosphate, 200 µM EDTA, 0.02% NaN₃ (at pH 7.4 in the case of Aβ(M1-40) and at pH 8.0 for Aβ(M1-42)) and diluted to 6 or 8 µM in the case of Aβ(M1-40) and 3 or 6 µM in the case of Aβ(M1-42) in the same buffer and supplemented with 20 µM ThT from a 2 mM stock. To each well in the 96-well plate was first added either 10 µl buffer (20 mM Tris/HCl pH 7.4) or 10 µl of BRICHOS protein or control protein at ten times the desired final concentration in 20 mM Tris/HCl pH 7.4. To each well was then added 90 µl of the ice-cold Aβ monomer solution and the plate was immediately placed in the plate reader at 37 ºC, with fluorescence read every 6 minutes with continuous shaking at 100 rpm between readings. Aβ(M1-40) was studied alone or with proSP-C BRICHOS at concentrations ranging from 17 pM to 17 µM or Br2 BRICHOS at concentration ranging from 60 pM to 6 µM. Aβ(M1-42) was studied alone or with proSP-C BRICHOS at concentrations ranging from 60 pM to 17 µM or Br2 BRICHOS at concentration ranging from 12 nM to 6 µM. The concentrations of Aβ and BRICHOS proteins were determined by amino acid analysis after acid hydrolysis.

The half time t₁/₂ was obtained by fitting a sigmoidal function to each kinetic trace
\[
y = y_0 + \left( y_{max} - y_0 \right) / \left( 1 + \exp\left( -k(t-t_{1/2}) \right) \right)
\]
and the lag time, tₗₐ₉ was defined as
\[
t_{lag} = t_{1/2} - 2/k
\]

CD spectroscopy. CD spectra were recorded in a 10 mm quartz cuvette using a JASCO J-815 spectropolarimeter. Far-UV spectra were recorded at 1 nm intervals between 185 and 250 nm using a scan rate of 20 nm/min, with response time 8 s, and band pass 1 nm. Aβ(M1-40) monomer was isolated by gel filtration in 5 mM sodium phosphate buffer, pH 7.4 with 40 mM NaF and 200 µM EDTA, collected on ice and divided into three samples which were supplemented with buffer, proSP-C or Br2 BRICHOS to final
concentrations of 8 µM Aβ(M1-40) and no addition or 0.8 µM proSP-C BRICHOS or 0.8 µM Bri2 BRICHOS. The samples were heated to 37 °C and studied directly or after different times of incubation at 37 °C with 100 rpm shaking, up to 18 hours. A spectrum of the buffer was recorded separately in the same cuvette and subtracted from the Aβ spectra. Spectra of 0.8 µM proSP-C or Bri2 BRICHOS were recorded separately and subtracted from the spectra of Aβ and BRICHOS.

NMR spectroscopy. NMR data were collected at 4 °C using a Bruker Avance 400 MHz spectrometer. The protein concentrations were 110 µM 15N labeled Aβ40 and 140 µM proSP-C or Bri2 BRICHOS in 20 mM sodium phosphate buffer at pH 6.8, 10 mM EDTA and 0.02% NaN3. All samples contained 90% H2O and 10% (v/v) D2O. [15N,1H]-HSQC experiments were performed with 1024 points × 128 increments and referenced according to the water resonance frequency. NMR data were processed using NMRPipe (48) and analyzed using SPARKY (T.D. Goddard and D.G. Kneller, SPARKY 3, University of California, San Francisco).

Electron microscopy (EM). Aliquots of 2 µl were taken at different time points, during the aggregation kinetic experiments, for Aβ40 or Aβ42 in the absence or presence of proSP-C or Bri2 BRICHOS protein. A low concentration of ThT, 0.5 µM, was used to enable samples to be picked at time point representing specific levels of reaction progression. The samples were adsorbed on copper grids, stained with 2.5% uranyl acetate in 50% ethanol, and examined and photographed with a Hitachi H7100 microscope operated at 75 kV.

Homology modeling. A homology model for Bri2 BRICHOS was constructed using coordinates for the human proSP-C BRICHOS domain (pdb code 2yad. (36)). The sequences of Bri2 and proSP-C BRICHOS were aligned using ClustalW (49) (Fig 1). The insertion in the Bri2 sequence was manually adjusted in order to align the strictly conserved cysteine residues at positions 164/121 and 223/189 in Bri2 and proSP-C, respectively. A model for Bri2 BRICHOS was generated using the software SOD (50) and the molecular graphics program O (51). Side chain rotamers were selected to correspond as closely as possible to those in the experimental model except when a different rotamer had to be chosen to avoid steric clashes.

Results

Aggregation kinetics. Thioflavin T (ThT) was used as a reporter on fibril formation in kinetic experiments for Aβ(M1-40) or Aβ(M1-42), herein referred to as Aβ40 and Aβ42, respectively, alone or with different concentrations of the BRICHOS proteins ranging from 0.00001 to 0.6 molar equivalents. Examples of aggregation kinetics for Aβ40 alone and with 0.018 to 0.18 molar equivalents of proSP-C BRICHOS are shown in Figure 2A, and with 0.006 to 0.061 molar equivalents of Bri2 BRICHOS in Figure 2B. The mid-point of the aggregation process, t1/2, and the lag time were obtained by fitting a sigmoidal function to each kinetic trace and is plotted versus molar ratio of BRICHOS:Aβ40 in Figure 2C. Clearly, the lag time for Aβ40 aggregation has increased extensively in the presence of proSP-C or Bri2 BRICHOS, while the elongation rate is largely unaffected. Very large effects on the lag time are observed far below equimolar concentration of proSP-C or Bri2 BRICHOS relative to Aβ40. A doubling of the lag time for Aβ40 aggregation requires ca. 0.01 molar equivalents of proSP-C BRICHOS, and a 10-fold increase in lag time is seen around 0.01 equivalents of Bri2 BRICHOS or 0.03 equivalents of proSP-C BRICHOS. The retarding effect increases with increasing BRICHOS concentration, and the lag time exceeds one week and becomes practically difficult to quantify above 0.025 (1 Bri2 BRICHOS per 40 Aβ40) or 0.06 molar ratio (1 proSP-C BRICHOS per 16 Aβ molecules). Thus both BRICHOS proteins are very potent inhibitors of Aβ40 aggregation,
with the strongest effects observed for Bri2 BRICHOS.

Both proSP-C and Bri2 BRICHOS retard also the aggregation of Aβ_{42} and only sub-stoichiometric amounts of the BRICHOS proteins are required (Figure 2D-F). At 0.06-0.1 molar ratio (1 BRICHOS protein per 10-16 Aβ_{42}), both the lag time and half time are doubled compared to the uninhibited case, and the elongation rate is not affected. A ten-fold increase in lag time is seen at ca. 0.6 molar equivalents of the BRICHOS proteins, under which conditions the elongation rate is found to be significantly reduced. Although strong effects are seen on Aβ_{42} aggregation kinetics, it is clear that higher concentrations of the BRICHOS proteins are needed to exert the same effect as on Aβ_{40} aggregation, and the retarding effects of proSP-C and Bri2 BRICHOS are quantitatively more similar in the case of Aβ_{42}.

The BRICHOS domain contains two strictly conserved Cys, which have been shown to form a disulphide in proSP-C BRICHOS (32). We performed fibrillation kinetics experiments for Aβ_{40} and Aβ_{42} in the absence and presence of Bri2 or pro-SPC BRICHOS that had been reduced with 1 mM DTT prior to the ThT measurements. Both BRICHOS proteins were found to be significantly less efficient in hindering Aβ from forming fibrils in their reduced forms (Supplemental Fig. 1). A Bri2 BRICHOS construct lacking the N-terminal S-tag shows virtually identical effects on Aβ_{42} aggregation, and the retarding effects of proSP-C and Bri2 BRICHOS are quantitatively more similar in the case of Aβ_{42}.

CD and NMR spectroscopy. Structural transitions during the aggregation process were studied using CD spectroscopy (Figure 3A). The data for Aβ_{40} alone agrees with other reports (54), and shows a continuous progression from a spectrum typical for random-coil peptide towards a spectrum indicative of β-sheet structure. The structural transition starts to develop while the aggregation process as observed by ThT fluorescence is still in the lag phase and thus reports on the appearance of intermediates with β-sheet structure. In the presence of 0.1 molar equivalents of proSP-C BRICHOS or Bri2 BRICHOS, the structural transition appears to be delayed; spectra at 300 minutes and 15 hours report on mainly random coil structure. Thus, the presence of BRICHOS reduces the concentration of intermediates with β-sheet structure and keeps Aβ in a mainly unstructured state during the extended lag phase.

To further investigate structural conversions of Aβ_{40} when interacting with BRICHOS, 1H-15N HSQC experiments of 15N labeled Aβ_{40} with and without proSP-C BRICHOS were performed. The 1H-15N HSQC spectra for Aβ_{40} show typical random coil resonances as previously reported (55) (Figure 3B, red spectrum). With a 30% excess
of proSP-C BRICHOS added, $^1$H-$^{15}$N HSQC spectra show no difference in resonances compared to Aβ$_{40}$ alone (Figure 3B, blue spectrum). This argues that the interaction between Aβ and proSP-C BRICHOS does not induce structural conversion of the NMR visible Aβ$_{40}$ peptide. After 48h at room temperature a new spectrum was collected without any change in resonances (data not shown). After 2 weeks at room temperature the spectrum of Aβ$_{40}$ in the presence of BRICHOS still has the same resonances as the spectrum of newly dissolved Aβ$_{40}$ alone (Figure 3B, black spectrum), although a decrease in the intensity of the resonance signals, suggests that some aggregation had occurred over time. The same results were obtained with Bri2 BRICHOS (Supplemental Figure 3). The sample with only Aβ$_{40}$ had, after 2 weeks at room temperature, aggregated and no resonances were visible.

**Size exclusion chromatography.** The interaction between the BRICHOS proteins and Aβ was studied using gel filtration. Samples of 8 μM Aβ$_{40}$ and 0.8 μM proSP-C BRICHOS, or 8 μM Aβ$_{40}$ and 0.8 μM Bri2 BRICHOS, were incubated for 20 hours at 37°C followed by gel filtration on a Superdex75 column, and SDS PAGE of collected fractions (Figure 4 and Supplemental Figure 4). At 20 h, Aβ alone has fibrillated and reached the equilibrium plateau, whereas samples containing 0.1 molar equivalents of proSP-C or Bri2 BRICHOS are still in the lag phase (Figure 2). The data in Figure 4 show clearly that in the mixed samples almost all of Aβ$_{40}$ is monomeric after 20 h, while a minor fraction (< 1%) elutes at 10-12 ml together with proSP-C BRICHOS (Supplemental Figure 4). Similar results are observed for 8 μM Aβ$_{40}$ and 0.8 μM Bri2 BRICHOS; in which case only monomer was detected during the extended lag phase (Supplemental Figure 4). Data for 8 μM Aβ$_{42}$ and 0.8 μM Bri2 BRICHOS or proSP-C BRICHOS at 0 and 2 h incubation (Supplemental Figure 4) agree with these findings. During the extended lag phase in the presence of BRICOS Aβ$_{42}$ is mainly monomeric with none or trace amounts eluting together with BRICHOS.

**Stopping experiments.** To monitor the effect of BRICHOS addition during an ongoing aggregation process, samples with 8 μM Aβ$_{40}$ were monitored by recording the ThT fluorescence intensity as a function of time. 800 nM Bri2 BRICHOS was added from a concentrated stock before start or at time points ranging from 0.3 to 11.2 hours. A similar experiment was performed for 3 μM Aβ$_{42}$ with 1.8 μM Bri2 BRICHOS added before start or at time points ranging from 6 to 109 minutes. As shown in Figure 5 and Supplemental Figure 5, the aggregation process is delayed if BRICHOS is added anywhere during the lag time. If BRICHOS protein is added during the early part of the elongation phase, the process appears to halt with no further growth of ThT positive aggregates. When added close to the mid-point of the transition, the BRICHOS protein seems to halt the process from further progression or cause the process to reduce its speed and progress at lower rate. When added at the end of the transition, no effect is seen.

**EM analyses.** Samples were taken for EM analyses at different time points of the fibril formation process, which was followed by ThT fluorescence (Figure 6). At the end of the lag phase, Aβ$_{42}$ on its own has formed typical fibrils (panel d), while such structures are rare in the presence of proSP-C and absent in the presence of Bri2 BRICHOS at the equivalent time point (panels e and f). When Aβ$_{42}$ alone has reached equilibrium, fibrils are clearly present in this sample (panel g). At the same time samples with BRICHOS are in the elongation phase and fibrils have started to appear (panels h,i). At later time-points, when the ThT fluorescence has reached the plateau level also for the samples with BRICHOS, EM analyses show the presence of larger amounts of amyloid fibrils (panels k,j). Similar results were observed for Aβ$_{40}$ with fibrils detected at the end of the lag phase in the absence and presence of BRICHOS, and
larger amounts of fibrils when the ThT plateau is reached (Supplemental Figure 6).

Discussion
Stimulation of chaperone activity may be explored as a route towards prevention or treatment of amyloid diseases (30,56) requiring detailed molecular understanding. Broad substrate specificity makes it difficult to target chaperones to specific proteins, and some chaperones seem to promote amyloid formation (57). Therefore, it will be essential to identify chaperones with some level of specificity for Aβ and/or natural colocalization with APP/Aβ, and to know at what stage of APP processing and/or Aβ aggregation a specific chaperone might interfere.

Here we investigate the mechanism by which the BRICHOS domains affect Aβ fibrillation kinetics, structure, and complex formation between Aβ and BRICHOS. We find that BRICHOS domains from two human proteins, Bri2 and proSP-C, delay Aβ fibril formation in a concentration dependent manner. The aggregation of the more disease-relevant Aβ42 is retarded at sub-stoichiometric BRICHOS:Aβ42 ratios with a doubling of the aggregation lag time at 1 BRICHOS domain per 10 Aβ42. This is an important result, which may be harnessed in design of future AD therapy. Even lower BRICHOS concentration is needed to inhibit aggregation of Aβ40. As little as 1 Bri2 BRICHOS per 400 Aβ40 (or 1 proSP-C BRICHOS per 160 Aβ40 molecules) is needed for doubling of the lag time. Above 1 BRICHOS per 40 or 10 Aβ40, the aggregation process is so much retarded that it does not occur within one-week compared to a few hours for Aβ40 alone.

For Aβ40, the lag phase is prolonged in the presence of BRICHOS, while the elongation rate is essentially unaffected. The results of the stopping experiments show that an ongoing aggregation process can be strongly delayed as long as BRICHOS domains are added during the lag phase. The process is only temporarily halted if BRICHOS is added at t½, the mid-point of the process, after which it is too late to interfere. This is well in line with the previous observation that addition of proSP-C BRICHOS to already formed fibrils resulted in no apparent effect (37). The present results imply that BRICHOS domains interfere with molecular events that occur during the lag phase. For Aβ42 in the presence of BRICHOS the effect on the lag phase is less pronounced, and the apparent elongation rate is affected. This suggests that the BRICHOS mechanism of action may differ for Aβ40 and Aβ42. Further detailed kinetic analyses are required to allow a description of the mechanisms of action for BRICHOS on Aβ40 and Aβ42.

Another potent inhibitor, the small engineered protein Z(Abeta3) was recently reported to inhibit aggregation of Aβ by monomer binding and monomer depletion from solution (58,59). The extensive delay of fibrillation by very low concentrations of BRICHOS proteins, far below equimolar amounts, implies that its mechanism of action is not to bind and reduce the concentration of free monomer. The lag time for aggregation of pure Aβ42 displays a strong concentration dependence (8), from which we can estimate that the removal of 0.1 molar equivalents by monomer capture would cause only a 17 % increase in lag time, compared to the 100% increase seen with 0.1 molar equivalents of proSP-C or Bri2 BRICHOS. At the same molar ratio, Aβ40 aggregation is retarded beyond detection.

The mechanism of action of the BRICHOS domain must therefore clearly be something else than monomer binding to BRICHOS. CD and NMR spectroscopy as well as gel filtration experiments were employed to study structural transitions and protein-protein interactions during the lag phase. The CD and NMR analysis implies that Aβ40 is kept in a mainly unstructured state during the extended lag phase in the presence of BRICHOS. In addition, size exclusion chromatography identifies only a minor fraction of Aβ eluting together with the BRICHOS proteins, while the majority of the peptide elutes as monomer. The results from the EM studies indicate that the presence of BRICHOS delays the formation of fibrillar
assemblies, which start to appear at the end of the lag phase for Aβ alone, and at the end of the extended lag phase for samples containing BRICHOS. Eventually similar amounts of amyloid fibrils are formed both in the absence and presence of BRICHOS. This suggests that BRICHOS interferes with nucleation processes. Since no binding of Aβ monomer to BRICHOS was detected, it is likely that BRICHOS instead binds to assemblies formed early in the aggregation processes. The chaperones Hsp104, Hsp70 and Hsp40, are active at sub-stoichiometric amounts and preferentially interact with intermediates formed in the fibril formation process (13,14). In over-expressing cell lines, Hsp70 protects against Aβ42-induced neuronal death (60), while αB-crystallin, Hsp27, Hsp20, and HspB2/B3 reduce Aβ cell toxicity (61). The extracellular chaperones clusterin, α2-microglobulin and haptoglobin inhibit aggregation by binding to prefibrillar Aβ42 species and clusterin decreases Aβ42 toxicity (26,28,29).

The present results indicate that the proSP-C and Bri2 BRICHOS domains interfere with Aβ fibril formation in mechanistically similar ways although they are evolutionarily distant, with sequence identity less than 25% (Fig. 1). We have speculated that the BRICHOS domains bind to regions within their respective precursor proteins that show high propensity to form β-sheet structures, preventing them from aggregation during biosynthesis (43,62,63). The Aβ central hydrophobic segment flanked by charged residues (H14HKLVFFAED23) is suggested as a target for both BRICHOS proteins (34,38,43).

To gain further insight into potential binding determinants in BRICHOS, a homology model for Bri2 BRICHOS was built using the X-ray structure of proSP-C BRICHOS (36) as a template (Figure 7A). The structure consists of a central five stranded β-sheet with one helix on each side. The β-sheet is highly conserved throughout BRICHOS sequences; in particular, a number of highly conserved tyrosine residues are found centrally located on face A of the sheet (Figure 7B). This exposed array of phenol rings is a striking structural feature with resemblance to polyphenolic compounds found to retard Aβ aggregation (64). Common to other amyloid retarding compounds, for example N-methylated peptides (65), and polymeric nanoparticles with amide units in the backbone (66,67) is the hydrogen bonding capacity, which may allow these compounds to interact with edge strands of growing aggregates to prevent further propagation. The hydroxyl groups of the conserved BRICHOS tyrosine array (maybe together with adjacent His/Asp/Glu/Lys) may be able to form hydrogen bonds with peptide in extended β-structure, thereby preventing further growth of aggregated species. Indeed, in our structural model, the distances between the hydroxyl groups in the conserved array of Y113, Y122 and Y195 match the distances between the carbonyls of residues i, i+2 and i+4 of a peptide in extended β-conformation. Mapping sequence differences to the structure reveals a number of changes from hydrophobic residues in proSP-C BRICHOS to charged residues in Bri2 BRICHOS on face A of β-sheet (Fig 7B), suggesting that this surface may be important for substrate specificity, as previously hypothesized (37,38,43).

There are clear pathological and clinical similarities between familial British or Danish dementia on the one hand and AD on the other, and it has been shown that Bri2 interacts with Aβ and regulates APP processing (68). Bri2 suppresses Aβ deposition in APP transgenic mice by influencing APP processing (39). Together with our results it is tempting to speculate that the Bri2 BRICHOS domain indeed has a natural protective role against Aβ aggregation and AD. Our results may open new routes towards AD therapy based on this natural human chaperone.

**Concluding remark.** Both Aβ40 and Aβ42 aggregate significantly slower in the presence of BRICHOS domains from two human proteins, proSP-C and Bri2. The main effect is on the lag time for aggregation. The inhibitory mechanism involves interference with nucleation processes, such that the main pool
of the peptide is kept monomeric and unstructured for extended time.

**Footnotes**

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1 Abbreviations: AD, Alzheimer’s Disease; Aβ, amyloid β-peptide, Aβ40, Aβ(M1–40), methionine followed by residues 1-40 of Aβ; Aβ42, Aβ(M1–42), methionine followed by residues 1-42 of Aβ; proSP-C, surfactant protein C precursor; ThT, Thioflavin T.

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Figure Captions

Figure 1. Amino acid sequences of the Aβ peptides, proSP-C BRICHOS and Bri2 BRICHOS. The alignment of proSP-C and Bri2 BRICHOS domains is made with Clustal W and corresponds to the proSP-C domain as derived from the x-ray structure, pdb code 2yad (36). Asterisks and double dots mark identical residues and conservative replacements, respectively.

Figure 2. Aggregation kinetics as monitored by ThT fluorescence. Examples of raw data for A) 6 µM Aβ40 at pH 7.4 in the absence (black) or presence of 0.018 (blue, 108 nM), 0.06 (green, 360 nM) and 0.18 (red, 1.08 µM) molar equivalents of proSP-C BRICHOS, B) 6 µM Aβ40 at pH 7.4 in the absence (black) or presence of 0.006 (blue, 36 nM), 0.017 (green, 102 nM) and 0.061 (red, 366 nM) equivalents of Bri2 BRICHOS, D) 6 µM Aβ42 at pH 8.0 in the absence (black) or presence of 0.10 (green, 600 nM) and 0.62 (red, 3.7 µM) equivalents of proSP-C BRICHOS. E) 6 µM Aβ42 at pH 8.0 in the absence (black) or presence of 0.1 (green, 600 nM) and 0.61 (red, 3.7 µM) equivalents of Bri2 BRICHOS. C,F) Relative half time of fibrillation versus molar ratio BRICHOS:Aβ for proSP-C (black dots) and Bri2 BRICHOS (open squares) for C) Aβ40 and F) Aβ42. Each data point is the average of 6-8 measurements with the standard deviations shown as error bars, and the data are plotted relative to the half time obtained in the absence of BRICHOS protein.

Figure 3. Far-UV CD spectroscopy and NMR spectroscopy of BRICHOS effect on Aβ. A) CD Spectra were recorded in 5 mM Na-phosphate, 40 mM NaF, 200 µM EDTA, pH 7.4, 37 °C at start (black line) and after 100 minutes (blue), 200 minutes (cyan), 300 minutes (green), 400
minutes (yellow) and 15 hours (red) incubation of 8 μM Aβ40 (left), 8 μM Aβ40 + 0.8 μM proSP-C (middle), or 8 μM Aβ40 + 0.8 μM Bri2 BRICHOS (right).

B) 1H-15N HSQC spectra of 110 μM 15N-Aβ40 with or without 140 μM proSP-C BRICHOS recorded in 20 mM Na-phosphate, 10 mM EDTA, 0.02% NaN₃, pH 6.8, 4 °C. Only Aβ40 (red lines), Aβ40 plus proSP-C BRICHOS directly after mixing (blue lines) and after 2 weeks at room temperature (black lines).

**Figure 4. Gel filtration of Aβ-BRICHOS mixtures.** Mixture of 8 μM Aβ40 and 0.8 μM proSP-C BRICHOS, immediately after mixing, ie 0 hours (top chromatogram) and after 20 hours incubation (middle chromatogram). A chromatogram of 8 μM Aβ40 alone at 0 h is also shown (bottom chromatogram), scaled by a factor of 0.5 because twice the volume was injected.

**Figure 5. Investigating the effect of BRICHOS on Aβ by stopping experiments.** Aggregation of 8 μM Aβ40 was monitored by recording the ThT fluorescence intensity as a function of time. 800 nM Bri2-BRICHOS was added from a concentrated stock before start (top traces) or at different time points ranging from 2.1 to 7.7 hours as indicated at the vertical arrows.

**Figure 6. EM of Aβ assemblies at different time points during fibril formation.** Aliquots from aggregation kinetics experiments with Aβ42 and BRICHOS proteins were adsorbed on copper grids, stained with uranyl acetate and analyzed by EM. Each sample is taken at a specific time-point during the aggregation process as followed by ThT fluorescence, which is shown schematically for Aβ42 alone (black line) or Aβ42 co-incubated with BRICHOS protein (dotted line). Electron micrographs of only proSP-C or Bri2 BRICHOS incubated for the same time periods showed no assemblies (Supplemental Figure 6).

**Figure 7. Bri2 BRICHOS structure model.** A) Ribbon diagram of the molecular model of Bri2 BRICHOS, based on the recent crystal structure of proSP-C BRICHOS, pdb code 2yad (36). The secondary structure elements are labeled from the N-terminal strand, β1 to the last and fifth strand, β5. The β-sheet is enclosed by two helices α1 and α2. All residues that are shown as stick representation are situated on face A, which is the putative binding site. B) Sequence comparison between Bri2/proSP-C in the β-sheet face A. Identical residues in proSP-C and Bri2 BRICHOS are boxed in green, and conservative replacements are in open boxes.
Figure 1.
Figure 2
Figure 3
Figure 4
Figure 5.
Figure 7.
BRICHOS domains efficiently delay fibrillation of amyloid β-peptide
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