Sulfated Polysaccharides Promote the Assembly of Amyloid β1–42 Peptide into Stable Fibrils of Reduced Cytotoxicity*

The histopathological hallmarks of Alzheimer disease are the self-aggregation of the amyloid β peptide (Aβ) in extracellular amyloid fibrils and the formation of intraneuronal Tau filaments, but a convincing mechanism connecting both processes has yet to be provided. Here we show that the endogenous polysaccharide chondroitin sulfate B (CSB) promotes the formation of fibrillar structures of the 42-residue fragment, Aβ1–42. Atomic force microscopy visualization, thioflavin T fluorescence, CD measurements, and cell viability assays indicate that CSB-induced fibrils are highly stable entities with abundant β-sheet structure that have little toxicity for neuroblastoma cells. We propose a wedged cylinder model for Aβ1–42 fibrils that is consistent with the majority of available data, it is an energetically favorable assembly that minimizes the exposure of hydrophobic areas, and it explains why fibrils do not grow in thickness. Fluorescence measurements of the effect of different Aβ1–42 species on Ca2+ homeostasis show that weakly structured nodular fibrils, but not CSB-induced smooth fibrils, trigger a rise in cytosolic Ca2+ that depends on the presence of both extracellular and intracellular stocks. In vitro assays indicate that such transient, local Ca2+ increases can have a direct effect in promoting the formation of Tau filaments similar to those isolated from Alzheimer disease brains.

Pathogenesis in Alzheimer disease (AD)3 is linked to the accumulation of the highly amyloidogenic self-aggregating amyloid β peptide (Aβ). The amyloid cascade hypothesis postulates that AD pathology is initiated by an extracellular accumulation of Aβ that in turn triggers a transmembrane signal having as ultimate effect the formation of neurofibrillary tangles by the microtubule-associated protein Tau (1–3), followed by collapse of the microtubular cytoskeleton. Some of the mechanisms that have been proposed to explain how extracellular Aβ exerts its cytotoxic effects include the promotion of oxidative stress (4), disruption of Ca2+ homeostasis (5, 6), the targeting and functional disruption of particular synapses by Aβ oligomers (7), and the stimulation of synthesis and release of toxic molecules such as nitric oxide (8). It is generally accepted that the aggregative state of Aβ is a paramount issue in determining its degree of neurotoxicity. Existing data have shown that soluble Aβ oligomers may be the key effectors of cytotoxicity in AD (1, 7, 9–11). Other results, however, indicate that the neurotoxic activity of Aβ requires its aggregation in fibrillar form (12, 13), yet it has also been suggested that the aggregates may be a mechanism of defense acting by concealing and immobilizing neurotoxic soluble Aβ (14). Among the different forms of Aβ, the 42-residue fragment (Aβ1–42) readily self-associates and forms nucleation centers from where fibrils can quickly grow. In a previous work, we characterized the different species that appear during Aβ1–42 fibrillogenesis in vitro, from oligomers through protofibrillar forms to mature fibrils (15). Although active debate is centered on the issue of which is the pathogenic species of the peptide that ultimately causes the synaptic loss and dementia associated with AD (16), comparatively less effort is being devoted to the study of endogenous factors that can promote or inhibit the formation of the candidate neurotoxic forms.

There are evidences indicating that the appearance of insoluble fibrillar structures enriched in β-sheets is facilitated by diverse environmental factors (17). The amounts of Aβ in vivo are generally much smaller than the concentrations required to induce fibril formation in vitro. It is therefore likely that other molecules exist that play an important role in the formation and deposition of amyloid fibrils and plaques. Carbohydrates are ubiquitous components of plasma, like glucose, that is found in brain tissue in millimolar amounts (18), and are also present in long-lasting structures of the extracellular matrix (ECM) such as proteoglycans. Proteoglycans are highly glycosylated proteins often carrying multiple negatively charged polysaccharides termed glycosaminoglycans (GAGs), which are among the most abundant components in the ECM of many tissues,

* This work was supported in part by the Generalitat de Catalunya, Spain (Grant 2005-SGR00037) and the Ministerio de Ciencia e Innovación (MCI), Spain (Grants BIO2002-00128, BIO2005-01591, and CSD2006-00012), which included Fondo Europeo de Desarrollo Regional funds. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked ‘advertisement’ in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 Supported by the Juan de la Cierva Programme (MCI).

2 To whom correspondence should be addressed. Tel.: 34-93-403-7180; Fax: 34-93-403-7181; E-mail: xfernandez_busquets@ub.edu.

3 The abbreviations used are: AD, Alzheimer disease; Aβ, amyloid β peptide; AFM, atomic force microscopy; CFP, cyan fluorescent protein; CSB, chondroitin sulfate B; ECM, extracellular matrix; GAG, glycosaminoglycan; PB, phosphate buffer; PBS, phosphate-buffered saline; TEM, transmission electron microscope; ThT, thioflavin T; YC, yellow chameleon; YFP, yellow fluorescent protein; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; HOPG, highly oriented pyrolytic graphite.
Non-toxic Aβ Fibris Induced by Dermatan Sulfate

including the brain (19, 20). From a chemical point of view, GAGs are unbranched polymers of repeated disaccharide units, usually containing an amino sugar and a uronic acid. Except in the case of hyaluronan, GAGs have varying degrees of sulfation.

Proteoglycans and GAGs are found associated with all types of amyloid deposits (21–23) and have been implicated in the nucleation of fibrils (24). They can also stabilize mature fibrils against dissociation (25) and proteolytic degradation (26), and they have been shown to facilitate the formation of fibrils of amylin (27), apo-serum amyloid A (28), α-synuclein (29), prion protein (30), Tau (31), and Aβ (32). It has been proposed that GAGs may have a scaffolding role, promoting fibrillogenesis-prone conformations of the amyloid precursor proteins (21). Aβ1−42 and other aggregating peptides like prion protein peptide 106–126 and Tau peptide 317–335 share cationic motifs that may be involved in binding to the negative charges of sulfated GAGs (33, 34). Other linear anionic polymers such as nucleic acids have also been described to bind prions with high affinity (35, 36), suggesting that polymeric molecules with repeating anionic and hydrophobic surfaces might be efficient inducers of amyloid structure. Chondroitin sulfate proteoglycans have been found to be associated with the lesions of AD (37), and heparan and chondroitin sulfate GAGs attenuate the neurotoxic effect of Aβ in primary neuronal cultures (38) and in neuron-like cell lines (39). Because GAGs can bind Aβ, it is conceivable that GAG-mediated neuroprotection is due to the sequestering of Aβ, in agreement with the hypothesis that the generation of senile plaques in AD would be a partially protective response aimed at reducing Aβ neurotoxicity. Supportive of this view is the finding that low susceptibility of neurons and cortical areas to neurofibrillary deposition corresponds with high proportions of aggregating chondroitin sulfate proteoglycans in the neuronal microenvironment (40). Despite this clearly established relationship between GAGs, Aβ, and AD onset and progression described above, little is known about the type of Aβ-containing structures induced by carbohydrates in general and by GAGs in particular.

Much attention has focused on Aβ as causative agent for AD, but accumulating evidence points to disruptions in neuronal Ca2+ signaling as a consistent progenitor for the disease, occurring prior to the development of the histopathological markers and cognitive decline (41). The Ca2+ hypothesis of AD proposes that sustained and accumulated alterations in Ca2+ homeostasis are a proximal cause in neurodegenerative diseases (42). Thus, alterations in the permeability to Ca2+ of the cell membrane could be one of the events linking Aβ and Tau aggregation, the two processes central to the amyloid cascade theory. It is likely that if Aβ has a role in permeabilizing the cell membrane to Ca2+, this effect will depend on its aggregated state, i.e. globular oligomers and fibrils might elicit different responses. In turn, because Aβ aggregation is influenced by ECM components such as GAGs, these might be able to modulate a putative Aβ-induced rise in cytosolic Ca2+. To tackle these issues, we have exposed Aβ1−42 to carbohydrates, studying the structures formed, their cytotoxicity, their ability to alter the permeability to Ca2+ of culture cells, and the effect of Aβ-induced Ca2+ dyshomeostasis on Tau aggregation.

EXPERIMENTAL PROCEDURES

Preparation of Aβ1−42 and Tau—Chemicals and reagents were purchased from Sigma, except where otherwise indicated. Aβ1−42 synthesized by Peptide Institute, Inc. (Japan) was pur chased lyophilized in glass vials and stored at −80 °C immediately upon arrival. As a rule, lyophilized Aβ1−42 was dissolved to 10 μM in 10 mM phosphate buffer, pH 7.4 (PB). When complete disaggregation of Aβ1−42 was required, we followed a variant of Zagorski’s protocol (43) as described (15). Samples were incubated in the dark for the times indicated in 1.5-mL Eppendorf tubes at 37 °C with gentle rocking. For the production of control oligomeric or fibrillar preparations of Aβ1−42 we followed established protocols (9). Briefly, oligomers were prepared by diluting 5 mM Aβ1−42 in dimethyl sulfoxide (DMSO) to 200 μM in phenol red-free Ham’s F12 cell culture medium, immediately vortexing for 30 s, and incubating at 4 °C for 24 h. Fibris were prepared by diluting 5 mM Aβ1−42 in DMSO to 200 μM in 10 mM HCl, immediately vortexing for 30 s, and incubating at 37 °C for 24 h. When carbohydrates had to be present they were included in the solutions prior to adding them to the peptide. A stock solution of recombinant Tau protein was prepared by dissolving the lyophilized protein to 0.5 mg/ml in 30 mM TBS, pH 7.4, and stored at −20 °C. For assays, Tau aliquots were diluted to the required concentrations in PB and incubated in the dark at 37 °C.

Electrophoresis and Immunoblots—Polyacrylamide gel electrophoresis was performed as described previously (15), in Tricine gels containing 0.1% SDS. Immunoblots were transferred to (and dot blots spotted on) a polyvinylidene difluoride membrane (Immobilon, Millipore) with a Mini Trans-Blot Cell (Bio-Rad), blocked in 0.1 M Tris-HCl, pH 7.5, 0.5% Tween 20, 1% Triton X-100, 3% bovine serum albumin, and incubated in the presence of rabbit anti-Aβ1−40, mouse anti-chondroitin-4-sulfate (Acris Antibodies), or mouse anti-Tau (Zymed Laboratories Inc.), diluted 1:2000 in blocking solution. The enhanced chemiluminescence Western blotting detection system (Amer sham Biosciences Corp.) was used to visualize the decorated bands or dots.

Atomic Force Microscopy—Images were obtained with a commercial MultiMode atomic force microscope controlled by a Nanoscope IV electronics (Digital Instruments, Santa Barbara, CA) equipped with either a 12-μm scanner (E-scanner) or a 120-μm scanner (J-scanner), or with a commercial MFP-3D (Asylum Research, Santa Barbara, CA). Oxide-sharpened pyramidal Si3N4 tips mounted on triangular 100-μm long cantilevers (k = 0.08 N/m) were purchased from Olympus (Tokyo, Japan). Except where otherwise indicated, images were taken in liquid using a tapping mode cell without the O-ring seal, following established protocols (15). After the indicated incubation times and immediately before imaging, 10 μl of the sample were allowed to adsorb for about 5–10 min at room temperature on freshly cleaved muscovite mica (Asheville-Schoonmaker Mica Co.) or highly ordered pyrolytic graphite (Nt-MDT Co., Zele nograd, Moscow, Russia), and finally overlaid with ~100 μl of the corresponding incubation buffer. Mica-supported dipalmi tolylphosphatidylcholine lipid bilayers were prepared as described (44). For images taken in air, the surface was carefully
rinsed with deionized water and gently dried under a N2 stream. Except where otherwise indicated, z scale for amplitude images was 0.2 V.

**Cell Cultures and Ca2+ Imaging**—Cell culture media and other reagents were obtained from Biological Industries, unless specified otherwise. SH-SY5Y human neuroblastoma cells (CRL-2266, ATCC) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum (PAA), glutamine (2 mM), penicillin (50 units/ml), and streptomycin (50 μg/ml). Cells were maintained at 37 °C in the presence of 5% CO2, and the medium was replaced every 2 days. Cells were plated at a density of ~5000 cells/dish in 35-mm glass-bottom culture dishes (MatTek Corp.) for at least 24 h before transfection. One μg of plasmid encoding cameleon YC3.60 (45) (kindly provided by Dr. Atsushi Miyawaki, RIKEN Brain Science Institute, Saitama, Japan) was mixed with 3 μl FuGENE-6 transfection reagent (Roche Diagnostics Corporation, Basel, Switzerland) in 1.5 ml Opti-MEM serum-free medium (Invitrogen), and added to the cells. After 6 h, the medium was removed and cells were incubated in Ham’s F-12 containing 15% fetal calf serum for 4–6 days. One day before the measurements, the culture medium was changed to Ham’s F12 containing 0.5% fetal calf serum. In the experiments where extracellular Ca2+ was depleted, cells were rinsed twice with Hanks’ balanced salt solution without Ca2+ and Mg2+, and medium was changed to Hanks’ balanced salt solution without Ca2+/Mg2+ containing 2 mM EGTA before application of the amyloids. Thapsigargin, Saitama, Japan) was mixed with 3 μl Opti-MEM serum-free medium (Invitrogen), and added to the cells. After 6 h, the medium was removed and cells were incubated in Ham’s F-12 containing 15% fetal calf serum for 4–6 days. One day before the measurements, the culture medium was changed to Ham’s F12 containing 0.5% fetal calf serum. In the experiments where extracellular Ca2+ was depleted, cells were rinsed twice with Hanks’ balanced salt solution without Ca2+ and Mg2+, and medium was changed to Hanks’ balanced salt solution without Ca2+/Mg2+ containing 2 mM EGTA before application of the amyloids. Thapsigargin (Molecular Probes, Eugene, OR), a specific blocker of sarcoplasmic/endoplasmic reticulum calcium ATPase Ca2+ pumps, was added to a final concentration of 2 μM in Ham’s F-12 containing 0.5% fetal calf serum. Amyloids were applied by pipetting a fixed aliquot (100 μl) of the preincubated solution into the temperature controlled recording chamber containing the cells in 900 μl of medium (for a 1–ml final volume). Controls included the incubation of cells in HCl-containing medium, which in the absence of Aβ1–42 did not induce any Ca2+ response. The Ca2+ ionophore ionomycin (Calbiochem) was used as a control at the end of each experiment to saturate YC3.60. Cells were visualized with a confocal laser scanning microscope Leica TCS SP2 (Leica Lasertechnik GmbH, Mannheim, Germany) adapted to an inverted Leitz DMRBE microscope with a CO2- and temperature-controlled atmosphere (Life Imaging Services, Reinsch, Switzerland). Cameleon YC3.60 was excited with a 458 nm line of an argon ion laser. Cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) signals were collected by using a 458/514 double dichroic mirror and 510/590 (YFP) and 465/530 (CFP) emission ranges. Images (512 x 512 pixels) were acquired every 5 s at a 400-Hz scan rate with a 40× Leitz Pla-Apochromat objective (numerical aperture 1.25, oil). To quantitatively analyze the changes in fluorescence resonance energy transfer, raw CFP and YFP fluorescence intensity were quantified in Leica LCS software and the data obtained were exported to Microsoft Excel for further analysis. Fluorescence intensity was calculated as the average within the regions drawn around individual cells, and a suitable threshold was set to avoid background signal. Signals are expressed as normalized fluorescence ratio (R/Rb), where R is the fluorescence ratio at any time calculated as F_{YFP}/F_{CFP} and Rb is the basal fluorescence ratio, which is obtained by averaging 20 scans before amyloid stimulation.

**Immunocytochemistry**—SH-SY5Y cells were cultured on coverslips in Petri dishes as described above. After incubation for the specified times in the absence or presence of Aβ1–42 HCl fibrils, cells were rinsed with PBS and fixed for 15 min with a solution of 3% paraformaldehyde in PBS containing 60 mM saccharose. Fixed cells were washed with PBS containing 20 mM glycine and permeabilized for 10 min with a solution of 0.05% Triton X-100 and 20 mM glycine in PBS. To block nonspecific antibody binding, coverslips were incubated for 20 min at room temperature in a blocking PBS solution containing 1% bovine serum albumin and 20 mM glycine. Cells were then incubated at 37 °C in a humidified chamber for 1 h with monoclonal antibody anti-Tau or anti-α-tubulin diluted in blocking solution at 20 μg/ml. After a washing step, cells were incubated for 1 h at room temperature with secondary antibody goat anti-mouse conjugated to Alexa Fluor 546 (Molecular Probes). Confocal images were obtained with an Olympus Fluoview 500 confocal scanning laser microscope adapted to an inverted Olympus IX-70 inverted microscope and a 60× Plan Apochromatic objective (numerical aperture 1.4, oil). Alexa Fluor 546 was excited at 543 nm with a He-Ne laser, and image size was 1024 × 1024 pixels. Three-dimensional maximum projection images were obtained from 12 serial optical sections (z-step = 500 nm) at the confocal microscope normal rate.

**Fluorescence Spectroscopy and CD Assays**—A stock solution of 10 μM thioflavin T (ThT) prepared in PB was filtered and diluted in PB to a final concentration of 20 μM. Except where otherwise indicated, samples containing 20 μM Aβ1–42 and/or 2 mg/ml carbohydrate were mixed with an equal volume of 20 μM ThT. Fluorescence was measured in 348-well plates (Nunc) using a BioTek FL600 spectrofluorometer with excitation and emission wavelengths of 440 and 485 nm, respectively. The reported values have been corrected by subtracting the buffer fluorescence in the absence of amyloid and/or carbohydrate. Samples were prepared in triplicate for each experiment.

CD measurements were performed with a UV-visible Jasco 715 spectropolarimeter equipped with a Peltier temperature control system using a 1-mm path length silica quartz cuvette (Hellma). Samples were measured at wavelengths between 190 and 250 nm with a 1 nm step resolution and an integration time of 2 s. Spectra recorded at 25 °C were an average of 20 scans baseline-corrected from where the buffer spectrum was subtracted. The contribution of CSB to the CD signal in the Aβ/CSB mixture was removed by subtracting the corresponding spectrum.

**Cell Viability Assays**—SH-SY5Y were plated at 20,000 cells well in 96-well plates in 100 μl of Ham’s F-12 medium with 15% fetal calf serum. After 24 h at 37 °C in 5% CO2 atmosphere the medium was substituted by 100 μl of peptide-containing solution prepared by dissolving 10 μl of concentrated Aβ1–42 in 90 μl of Ham’s F-12 with 0.5% fetal calf serum, to reach the desired final Aβ concentration. Incubation was resumed for the times indicated. 10 μl of 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate labeling reagent (WST-1, Roche Diagnostics GmbH) was added to each well, and the plate was incubated in the same conditions for 4 h. After thor-
Non-toxic Aβ Fibrils Induced by Dermatan Sulfate

oughly mixing for 1 min on a shaker, the absorbance of the samples was measured at 450 nm. WST-1 in the absence of cells was used as blank. Samples were prepared in triplicate for each experiment.

Transmission Electron Microscopy—Aβ fibrils were fixed with 2% paraformaldehyde and 2.5% glutaraldehyde in PB and kept in the fixative for 3 h at 4 °C. Fixed fibrils were washed and postfixed at 4 °C with 1% osmium tetroxide in the same buffer containing 0.8% potassium ferricyanide. Then, the sample was dehydrated in acetone, with addition of 5% uranyl acetate in the 70% acetone step, infiltrated with Lowicryl HM20 resin during 2 days, embedded in the same resin, and polymerized at 60 °C for 48 h. Ultrathin sections were obtained with a Leica Ultracut UCT ultramicrotome and mounted on Formvar-coated copper grids. Sections were finally stained with 2% uranyl acetate in water and 0.8% lead citrate. For Tau samples, a 5- to 10-μl aliquot was placed on a Formvar-coated copper grid, allowed to stand for 1 min, washed with distilled water, and finally stained with 2% uranyl acetate for 1 min. All samples were observed in a JEM-1010 electron microscope (jeol, Japan) operated at 80 kV.

RESULTS

Protofibrillar and Oligomeric Aβ1-42 Can Form Different Types of Associations—Atomic force microscope (AFM) visualization on highly oriented pyrolytic graphite (HOPG) provides a good resolution of molecular species that have affinity for hydrophobic surfaces. During the first stages of aggregation, Aβ1-42 can adopt either a globular or a fibrillar structure depending on the method used to prepare the sample (Fig. 1). Aβ1-42 directly dissolved in phosphate buffer or PBS quickly forms protofibrils up to 100 nm long (Fig. 1, A–D). Protofibrils have a characteristic ribbon-like shape ~1.5 nm high and ~5.5 nm wide (15) and can associate in elongated fibril-like clusters (Fig. 1, A and B). AFM images taken minutes apart have revealed rearrangements of individual protofibrils (Fig. 1, C and D) that include shuffling and growth. Pretreatment of Aβ1-42 with trifluoroacetic acid reduces the formation of protofibril nucleating centers, and globular structures with a mean height of ~5 nm deposit on a hydrophilic mica substrate as the major initial species (15). When this sample is imaged on HOPG, globular structures can be observed forming patches, or “rafts” (Fig. 1E), that tend to be confined between HOPG steps.

The association of protofibrils and/or of globular oligomers generates ~1.5 nm-wide fibrils of the nodular type (Fig. 1F), formed by the stacking of ~100 nm-long protofibril bundles (15). Nodular fibrils may evolve further to yield mature smooth fibrils, the highest order of Aβ1-42 fibrillogenesis (15). High resolution AFM images of the globular structures deposited on HOPG (Fig. 1G) show that they have a diameter similar to that of fibrils, but their height varies in apparently discrete steps of ~2 nm (Fig. 1H), suggesting that the higher structures are stacks of several individual units.

Sulfated Polysaccharides Promote the Formation of Amyloid Structure—ThT fluorescence analysis showed that sulfated polysaccharides induced a >100% increment in the fluorescence of Aβ1-42-containing solutions (Fig. 2A), an indication of increasing ordered amyloid β-sheet structure. This effect was not exhibited by control carbohydrate samples including monosaccharides, the chondroitin sulfate disaccharide unit α-ΔUA-[1→3]-GalNAc-4S, and the positively charged polysaccharide chitosan. According to the available literature,
Non-toxic Aβ Fibrils Induced by Dermatan Sulfate

the concentration of Aβ_{1–42} in the brain ranges from ~10 ng/ml as found in brain extracts or cerebrospinal fluid (46) to >1 g/ml inside tightly packed amyloid fibrils, indicating that locally the peptide:carbohydrate ratios will span a wide range of values, including the ones used in this work. Although at the concentrations used monosaccharides like glucose do not promote the formation of amyloid structure as sulfated polysaccharides like CSB do, Aβ_{1–42} incubated in the presence of either has significantly less cytotoxicity than the peptide alone 48 h after addition to SH-SY5Y cells (Fig. 2B). Thus, to further characterize the effect of carbohydrates on Aβ_{1–42} fibrillogenesis and cell viability we selected CSB and glucose, two abundant endogenous components of the brain ECM.

For comparative AFM studies of fibril formation where high resolution was not required we have used a mica substrate, which is less likely than HOPG to affect fibril deposition (47). Aβ_{1–42} incubated at 37°C in PB forms fibrils and globular aggregates (Fig. 2, C and F), but in the presence of 2 mM glucose the sample is abundantly populated by globular structures with a height of ~2–5 nm and a width similar to that of fibrils (Fig. 2D). In the presence of CSB there is a remarkable enrichment in the number and length of fibrillar structures (Fig. 2F). These CSB-induced fibrils are very persistent and can be stored at 4°C for up to 40 days without apparent major alterations (Fig. 2H).

Incubation of CSB in the same conditions but in the absence of Aβ_{1–42} did not yield any detectable structures by AFM imaging on graphite or mica.

The globular and fibrillar structures formed by Aβ_{1–42} during its aggregation do not enter the SDS gels used for Western blot analyses (Fig. 2F). However, in the presence of CSB, smaller species are formed that do enter the upper part of the gel. From their size, ranging from 200 to ~1000 kDa, these entities correspond to protofibrils. Protofibrils are highly hydrophobic and do not settle on mica efficiently (15), which explains why they are not observed in the CSB-containing samples shown in Fig. 2 (F and H). The less aggregative peptide Aβ_{1–40} is not much affected by the presence of CSB (Fig. 2I).

Aβ_{1–42} fibrils formed in the presence of CSB were centrifuged down and washed with PB, and the resulting pellet was examined in dot blot assays for the presence of CSB. Whereas Aβ_{1–42} was abundant in the fibril precipitate, CSB was not detected in dots containing up to 100 μg of Aβ_{1–42} (data not shown). Anti-CSB antibody controls were able to detect down to 1 μg of the GAG.

CSB and Glucose Reduce the Cytotoxicity of Aβ_{1–42} Oligomers and Fibrils—As a reference to study glucose- and CSB-induced oligomers and fibrils, we followed previously established protocols for the generation of both types of structures (9). Essentially, fibrils were obtained by incubation in 10 mM structures formed by Aβ_{1–42} in the presence of glucose and CSB. A, ThT assay of 20 μM Aβ_{1–42} incubated for 30 days in the presence of 2 mg/ml of different carbohydrates. Control samples containing carbohydrates in the absence of Aβ induced levels of ThT fluorescence ~5% of the value measured for Aβ_{25} (data not shown). B, cell viability assay of SH-SY5Y cells exposed for 48 h to 10 μM Aβ_{1–42} that had been preincubated for 24 h either alone or in the presence of 1 mM glucose or 0.5 mg/ml CSB. Survival of control cells not exposed to Aβ was assigned 100% viability. C–H, AFM analysis of the
Non-toxic Aβ Fibrils Induced by Dermatan Sulfate

FIGURE 3. Cytotoxicity of fibrillar and oligomeric Aβ1–42 structures. A, cell viability analysis of SH-SY5Y cells treated for 24 h with freshly PB-diluted Aβ1–42 (Aβ), HCl fibrils, or Ham’s-oligos. AFM qualitative control images on mica of fibril and oligomer samples are shown (z scale = 40 nm; bar = 200 nm). B, effect of glucose and CSB on the electrophoretic analysis of HCl fibrils and Ham’s-oligos. Aβ1–42 was preincubated for 24 h in fibril- or oligomer-forming conditions; in the absence and in the presence of 10 mM glucose or 8 mg of CSB/ml, and the resulting samples were added to SH-SY5Y neuroblastoma cultures. Final concentrations of Aβ1–42 glucose, and CSB were 10 μM, 1 mM, and 0.8 mg/ml, respectively. The aggregation state of Aβ1–42 was determined by Western blot analysis of 20-μl aliquots removed from the culture supernatants after 6 and 24 h of incubation. C, cell viability analysis 6 h after addition of the indicated peptide-containing solutions, and of control Aβ1–42 samples preincubated for 24 h as described for Fig. 2B.

HCl (HCl fibrils), whereas oligomers were prepared by incubation in Ham’s culture medium (Ham’s-oligos). Both Ham’s-oligos and HCl fibrils showed concentration-dependent cytotoxicity on the neuroblastoma cell line SH-SY5Y (Fig. 3A).

To study the effect of carbohydrates on the toxicity of oligomers and fibrils, Aβ1–42 was incubated in the same conditions described above for the formation of both types of structures, including in this case glucose or CSB at the start of the process. After addition of the Aβ-containing samples to cells, aliquots were removed from the culture medium at 6 and 24 h of incubation and analyzed by Western blot (Fig. 3B). After 6 h the medium treated with Ham’s-oligos showed a dominant band corresponding to a ~55-kDa species, whereas the medium treated with HCl fibrils contained aggregated structures that did not enter the gel, consistent with the presence of large fibril-like forms. The presence of glucose and CSB stimulated aggregation in oligomer-forming conditions and slowed down aggregation in fibril-forming conditions. Beyond 6 h of incubation with the cells, oligomeric species were significantly reduced, probably as a consequence of their progressive incorporation into fibrillar or aggregated forms. Based on these results, we explored the effect of the presence of glucose and CSB in oligomer- and fibril-forming conditions on the viability of cells 6 h after addition of the samples to the cultures. The results indicate that preincubation in the presence of both carbohydrates significantly reduces the toxicity of oligomers and fibrils, as it does for untreated Aβ1–42 (Fig. 3C). This effect is particularly clear for CSB, which is able to restore 100% cell viability.

Oligomeric and Fibrillar Aβ1–42 Forms Have Different Effects on Cytosolic Ca2⁺ Levels—We have used fluorescence resonance energy transfer technology to study if the several oligomeric and fibrillar forms of Aβ1–42 had a differential effect on the levels of cytosolic Ca2⁺ in culture cells. SH-SY5Y cells that had been transfected to express the Ca²⁺-sensitive indicator yellow chameleon YC3.60, composed of cyan and yellow fluorescent proteins (45), were treated with Aβ1–42 incubated in a variety of conditions, and the corresponding variations in cytosolic Ca²⁺ were analyzed by confocal fluorescence microscopy (Fig. 4A). Of all samples tested, only HCl fibrils had a dramatic effect in triggering a pulse of intracellular Ca²⁺ increase followed by a drop in cytosolic fluorescence below resting conditions. Ionomycin applied after HCl fibril exposure did not result in the expected rise in cytosolic fluorescence. The presence of CSB in HCl fibril-forming conditions did not significantly affect the initial Ca²⁺ pulse, but eliminated the subsequent fluorescence drop. We examined the possibility that the effect induced by HCl fibrils could result from the influx of extracellular Ca²⁺ or from Ca²⁺ liberated from intracellular stores. To do so, cells were either placed in Ca²⁺-free medium or treated with thapsigargin, respectively. The absence of extracellular Ca²⁺ in the presence of intracellular Ca²⁺ completely abolished the normal response to HCl fibrils. Following pretreatment with thapsigargin, which induces the leakage of Ca²⁺ from the ER into the cytosol, but in the presence of extracellular Ca²⁺, HCl fibrils did not trigger the cytosolic Ca²⁺ pulse, although the fluorescence drop phase was maintained. Controls done in the presence of 10 mM nickel, a voltage-gated calcium channel blocker, did not influence the HCl fibril effect (data not shown).

The different response of cytosolic Ca²⁺ levels to stimulation with HCl fibrils and with CSB fibrils suggests that both types of fibrillar species may have significant differences in structure. Indeed, high resolution AFM images of HCl fibrils resemble immature nodular fibrils, short and bent (Fig. 4B), whereas CSB fibrils are clearly of the smooth type, long and straight (Fig. 2, F and H). Consecutive AFM scans of HCl fibrils show increasing fibril disruption characteristic of weakly structured entities (Fig. 4, B and C), whereas fibrils formed in the presence of CSB
Aβ₁₋₄₂ fibrils are not altered after repeated scanning by the AFM tip (Fig. 4, D and E), suggesting that they have a much more stable architecture. ThT assays reveal that samples containing CSB fibrils have a fluorescence signal severalfold that of HCl fibril samples (Fig. 4F), indicating that the former have a much more ordered β-sheet structure. The presence of CSB in HCl fibril-forming conditions also induces a severalfold increase in ThT fluorescence. The high signal obtained reflects the concentration dependence of Aβ fibrillogenesis. Because only a small volume could be added to cell cultures, Aβ₁₋₄₂ concentration was very high during the preincubation step (200 μM versus 20 μM in the standard ThT assay of Fig. 2A). Repeated pipetting of HCl fibrils results in a sample with abundant protofibrils (Fig. 4G). Artificial membranes overlaid with such protofibril-enriched preparations show protuberances consistent with the insertion of protofibrils into the lipid bilayer (Fig. 4, H and I).

Aβ₁₋₄₂ HCl Fibrils Induce Intracellular Tau Aggregation—After 24 h of continued exposure to Aβ₁₋₄₂ HCl fibrils, the distribution of endogenous Tau in SH-SY5Y cells was examined by confocal fluorescence immunocytochemistry. Tubulin controls did not reveal significant changes in the microtubular network (Fig. 5, A and B). In untreated SH-SY5Y cells, Tau is

Non-toxic Aβ Fibrils Induced by Dermatan Sulfate

**FIGURE 4.** Effect of different oligomeric and fibrillar Aβ₁₋₄₂ forms on cytosolic Ca²⁺ levels. A, Ca²⁺-triggered fluorescence of YC3.60-expressing SH-SY5Y cells treated with Aβ₁₋₄₂ (arrow) that had been incubated in different conditions for the generation of oligomers and fibrils. The final concentrations of Aβ₁₋₄₂, glucose, and CSB were 20 μM, 1 mM, and 0.8 mg/ml, respectively. Ca²⁺ saturation was induced with addition of 2 μM ionomycin (arrowheads). Each curve represents the fluorescence in a region of interest corresponding to a single cell. Ca²⁺ response is expressed as ΔR/R₀ of five cells in one representative experiment out of three independent replications. B–E, consecutive height AFM scans of Aβ₁₋₄₂ fibrils generated in the presence of HCl (B and C) and in the presence of CSB (D and E). z scale = 12 nm (B and C) and 35 nm (D and E). F, ThT assay of the Aβ₁₋₄₂ preparations from A at the moment of addition to the cells. G, amplitude AFM image on graphite of an HCl fibril sample subjected to repeated pipetting. H and I, amplitude AFM images of mica-supported lipid bilayers overlaid in PBS with the sample from G. Arrows indicate membrane swellings of dimensions consistent with the presence of underlying protofibrils. z scale = 0.04 V (G and I) and 0.03 V (H).

**FIGURE 5.** Immunocytochemical detection of tubulin and Tau in SH-SY5Y cells treated with Aβ₁₋₄₂ HCl fibrils. Cell cultures were incubated for 24 h in the absence (A and C) or in the presence of 20 μM Aβ₁₋₄₂ that had been preincubated in HCl fibril-forming conditions (B and D). The samples were examined by confocal microscopy after immunocytochemical fluorescent staining of endogenous tubulin (A and B) and Tau (C and D).
homogeneously distributed throughout the cytosol (Fig. 5C), whereas after 24 h in the continued presence of Aβ1--42 HCl fibrils, Tau-containing fibril-like structures can be observed inside the cells (Fig. 5D). Western blot analyses of Tau in cell extracts did not reveal any SDS-resistant aggregated structures (data not shown).

**Calcium and Pre-aggregated Tau Trigger Tau Aggregation**—To explore if Tau could be aggregated by Ca\(^{2+}\) *in vitro* we exposed recombinant Tau to a Ca\(^{2+}\) concentration of 1.25 mM (Fig. 6A), that can be achieved locally *in vivo* in the vicinity of permeabilized membrane regions. In the absence of Ca\(^{2+}\), Tau shows a tendency to form ~200-kDa species that might correspond to a trimer or tetramer of the protein. As previously described (48, 49), GAGs such as heparan sulfate and especially CSB promote the formation of larger Tau aggregates that do not enter SDS-polyacrylamide gels. In the presence of 1.25 mM Ca\(^{2+}\) Tau becomes aggregated into large structures excluded from the gels. Mg\(^{2+}\) has no effect on Tau even at the very high concentration of 10 mM. In addition, in the absence of Ca\(^{2+}\) Tau can be aggregated by pre-aggregated Tau (Fig. 6B).

AFM imaging revealed that the large aggregated species formed by Tau incubated in the presence of 1.25 mM Ca\(^{2+}\) were long fibrils (Fig. 7A), similar to those formed in the presence of CSB (50). In the absence of the aggregation triggers/enhancers Ca\(^{2+}\) and CSB, Tau did not form any identifiable structures (data not shown), in agreement with existing data (51). In the vicinity of the fibrils, the surface was covered by a regular arrangement of parallel thin filaments with a mean width of 4.0 ± 0.6 nm and a height of 0.9 ± 0.2 nm (Fig. 7B). These dimensions are consistent with those of β-sheets existing in Tau fibrils that associate to form paired helical filaments (52, 53). Transmission electron microscope (TEM) analysis permitted a better resolution of fibrils and filaments, whose growth and relative amounts are affected by incubation time and Ca\(^{2+}\) and CSB concentration. Fig. 7C shows a group of small fibrils besides a grid-like expanse of short filaments. Higher resolution images indicate that the grid-forming structures (Fig. 7E) have a mean width of 5.1 ± 0.7 nm. Fig. 7D shows characteristic larger fibrils with an internal structure made of long filaments that assemble into bundles. These fibrils can be several microns in length, and their constituent filaments have a mean thickness of 4.8 ± 0.7 nm (Fig. 7F).

**CSB Promotes the Formation of Highly Structured Aβ1--42 Fibrils**—CD analysis indicates that CSB promotes the formation of Aβ1--42 species enriched in β structure, according to the 9-fold increase in the minimum at 220 nm in the far-UV spectra.
Non-toxic Aβ Fibrils Induced by Dermatan Sulfate

Aβ is cleaved from its precursor protein in the membrane interface, and its cytotoxic effect is likely related to the amyloid-lipid interaction (54). AFM imaging has revealed that certain oligomeric Aβ₁₋₄₂ species can form associations or rafts that have a high affinity for the amphipathic graphite/water interface. Because Aβ oligomers are basically hydrophilic and do not settle well on graphite (15), we conclude that Aβ rafts are formed by a previously undescribed hydrophobic oligomeric species. TEM and AFM studies performed with artificial membranes have shown that, upon interaction with the membrane lipids, Aβ in fibrillar form reverts to globular peptide oligomers that associate into disordered domains (55). Thus, raft-forming oligomers could be part of a physiologically relevant pathway leading to the incorporation of soluble Aβ into cell membranes.

Although glucose and CSB restore the viability of cells treated with otherwise toxic Aβ₁₋₄₂ concentrations, the clear difference between both carbohydrates in their ability to promote fibrillogenesis suggests distinct mechanisms. In the case of glucose the cytoprotective effect might be partially related to its role as an energy source that the cell metabolism can use to overcome the amyloid insult. Electrophoretic analysis indicates that the promotion of fibril formation mediated by CSB involves protofibrils rather than oligomers. This observation is in agreement with data indicating that oligomers are not an obligatory intermediate in the process of fibril formation, as suggested by the finding that oligomerization is inhibited at concentrations of urea that have no effect in fibril formation (56). Both Aβ oligomers and fibrils have been described to be key agents in the Alzheimer-related cytotoxicity. However, different oligomeric and fibrillar types exist, and carbohydrates seem to promote more innocuous forms of both. A possible mechanism to explain functional differences between structurally similar species could be related to the formation of cross-linked structures by advanced glycation end-products (57), which might lock oligomers in fixed conformations preventing dynamic events otherwise leading to cytotoxicity. CSB, on the other hand, could act by speeding up the pathway toward highly stable smooth fibrils, which likely consist of densely packed β-sheet stacks.

Side-by-side association of long protofibrillar rods has been proposed in models derived from high resolution AFM images of soluble Aβ₁₋₄₂ oligomers (58). However, a stacked protofila-
model for Aβ fibril structure has an important flaw: in such an assembly, Aβ fibrils could experience an unlimited growth in thickness by lateral addition of more protofibrils or protofilaments. Instead, Aβ fibrils have a well-defined cross-

\[ 4 \text{ J. Ponce, T. Martianyey, A. Gella, R. Bravo, J. Cladera, X. Fernández-Busquets, and N. Durany, manuscript in preparation.} \]

(FIG. 8A). High resolution AFM images show that smooth Aβ₁₋₄₂ fibrils are straight rods containing internal parallel structures (Fig. 8B). Cross-section analysis of smooth fibrils (made 45° relative to the perpendicular for a better resolution) reveals 6 protofibril subunits and a fibril height of 11.5 nm (Fig. 8C). This image is consistent with a cylindrical structure composed of 12 subunits, of which 6 can be observed in an upper view such as that provided by AFM scans. AFM height measurements are very accurate, but lateral distances can be greatly distorted by the shape of the cantilever tip. In structures like the fibril shown in Fig. 8B the most reliable width dimensions are those obtained for the internal structures (15). The measured width for the topmost subunit is 3 nm, whereas the apparent width for the other subunits is increasingly magnified by their location closer to the vertical sides of the cylinder.

TEM images of ultrathin sections of CSB fibril pellets contain abundant smooth fibrils cut at different angles. Both transversal (Fig. 8D) and longitudinal sections (Fig. 8E) show that smooth fibrils have an electron dense outer shell surrounding a hollow inner core. The diameter of fibrils in TEM images is ~10 nm, slightly below the 11.5 nm measured in liquid medium with the AFM. To investigate the possible presence of CSB in the fibril structure, we prepared fibrils using CSB labeled with 2 nm gold beads bound to sulfate groups. Although some labeling could be detected by TEM in CSB fibrils, it was not significantly different from controls done with gold beads alone or conjugated to sulfated monosaccharides (data not shown).

DISCUSSION

(FIG. 8A). High resolution AFM images show that smooth Aβ₁₋₄₂ fibrils are straight rods containing internal parallel structures (Fig. 8B). Cross-section analysis of smooth fibrils (made 45° relative to the perpendicular for a better resolution) reveals 6 protofibril subunits and a fibril height of 11.5 nm (Fig. 8C). This image is consistent with a cylindrical structure composed of 12 subunits, of which 6 can be observed in an upper view such as that provided by AFM scans. AFM height measurements are very accurate, but lateral distances can be greatly distorted by the shape of the cantilever tip. In structures like the fibril shown in Fig. 8B the most reliable width dimensions are those obtained for the internal structures (15). The measured width for the topmost subunit is 3 nm, whereas the apparent width for the other subunits is increasingly magnified by their location closer to the vertical sides of the cylinder.

TEM images of ultrathin sections of CSB fibril pellets contain abundant smooth fibrils cut at different angles. Both transversal (Fig. 8D) and longitudinal sections (Fig. 8E) show that smooth fibrils have an electron dense outer shell surrounding a hollow inner core. The diameter of fibrils in TEM images is ~10 nm, slightly below the 11.5 nm measured in liquid medium with the AFM. To investigate the possible presence of CSB in the fibril structure, we prepared fibrils using CSB labeled with 2 nm gold beads bound to sulfate groups. Although some labeling could be detected by TEM in CSB fibrils, it was not significantly different from controls done with gold beads alone or conjugated to sulfated monosaccharides (data not shown).

DISCUSSION

(FIG. 8A). High resolution AFM images show that smooth Aβ₁₋₄₂ fibrils are straight rods containing internal parallel structures (Fig. 8B). Cross-section analysis of smooth fibrils (made 45° relative to the perpendicular for a better resolution) reveals 6 protofibril subunits and a fibril height of 11.5 nm (Fig. 8C). This image is consistent with a cylindrical structure composed of 12 subunits, of which 6 can be observed in an upper view such as that provided by AFM scans. AFM height measurements are very accurate, but lateral distances can be greatly distorted by the shape of the cantilever tip. In structures like the fibril shown in Fig. 8B the most reliable width dimensions are those obtained for the internal structures (15). The measured width for the topmost subunit is 3 nm, whereas the apparent width for the other subunits is increasingly magnified by their location closer to the vertical sides of the cylinder.

TEM images of ultrathin sections of CSB fibril pellets contain abundant smooth fibrils cut at different angles. Both transversal (Fig. 8D) and longitudinal sections (Fig. 8E) show that smooth fibrils have an electron dense outer shell surrounding a hollow inner core. The diameter of fibrils in TEM images is ~10 nm, slightly below the 11.5 nm measured in liquid medium with the AFM. To investigate the possible presence of CSB in the fibril structure, we prepared fibrils using CSB labeled with 2 nm gold beads bound to sulfate groups. Although some labeling could be detected by TEM in CSB fibrils, it was not significantly different from controls done with gold beads alone or conjugated to sulfated monosaccharides (data not shown).

DISCUSSION

Aβ is cleaved from its precursor protein in the membrane interface, and its cytotoxic effect is likely related to the amyloid-lipid interaction (54). AFM imaging has revealed that certain oligomeric Aβ₁₋₄₂ species can form associations or rafts that have a high affinity for the amphipathic graphite/water interface. Because Aβ oligomers are basically hydrophilic and do not settle well on graphite (15), we conclude that Aβ rafts are formed by a previously undescribed hydrophobic oligomeric species. TEM and AFM studies performed with artificial membranes have shown that, upon interaction with the membrane lipids, Aβ in fibrillar form reverts to globular peptide oligomers that associate into disordered domains (55). Thus, raft-forming oligomers could be part of a physiologically relevant pathway leading to the incorporation of soluble Aβ into cell membranes.

Although glucose and CSB restore the viability of cells treated with otherwise toxic Aβ₁₋₄₂ concentrations, the clear difference between both carbohydrates in their ability to promote fibrillogenesis suggests distinct mechanisms. In the case of glucose the cytoprotective effect might be partially related to its role as an energy source that the cell metabolism can use to overcome the amyloid insult. Electrophoretic analysis indicates that the promotion of fibril formation mediated by CSB involves protofibrils rather than oligomers. This observation is in agreement with data indicating that oligomers are not an obligatory intermediate in the process of fibril formation, as suggested by the finding that oligomerization is inhibited at concentrations of urea that have no effect in fibril formation (56). Both Aβ oligomers and fibrils have been described to be key agents in the Alzheimer-related cytotoxicity. However, different oligomeric and fibrillar types exist, and carbohydrates seem to promote more innocuous forms of both. A possible mechanism to explain functional differences between structurally similar species could be related to the formation of cross-linked structures by advanced glycation end-products (57), which might lock oligomers in fixed conformations preventing dynamic events otherwise leading to cytotoxicity. CSB, on the other hand, could act by speeding up the pathway toward highly stable smooth fibrils, which likely consist of densely packed β-sheet stacks.

Side-by-side association of long protofibrillar rods has been proposed in models derived from high resolution AFM images of soluble Aβ₁₋₄₂ oligomers (58). However, a stacked protofila-
model for Aβ fibril structure has an important flaw: in such an assembly, Aβ fibrils could experience an unlimited growth in thickness by lateral addition of more protofibrils or protofilaments. Instead, Aβ fibrils have a well-defined cross-

\[ 4 \text{ J. Ponce, T. Martianyey, A. Gella, R. Bravo, J. Cladera, X. Fernández-Busquets, and N. Durany, manuscript in preparation.} \]
section of ~11.5 nm (15), and any higher order structures result from the association of fibrils, not from individual fibril enlargement. A model that can explain this characteristic of Aβ fibrils is the wedged cylinder structure (Fig. 9A). In Aβ_{1–42}, residues 1–17 are disordered, whereas residues 18–42 form a β-strand-turn-β-strand motif formed by residues 18–26 (β₁) and 31–42 (β₄) (59). Aβ₁₋₄₂ stacking ultimately leads to the formation of hydrophobic β-sheets 5.5 nm wide and 1.5 nm high termed protofilaments, which can intertwine to form helical structures found at the core of the nodular type of fibrils (15). Nodular fibrils likely undergo internal rearrangements to yield smooth fibrils, the final stage in Aβ₁₋₄₂ fibrillogenesis. This internal remodeling seems to consist of switching from a helical to a completely parallel protofilament alignment. We propose that this change results in the transition from a rectangular to a wedged conformation, where the loose 1–17 residues form the narrow end. In this model, wedged protofilaments ~3 × 5.5 nm fill the same volume as rectangular-section protofilaments 1.5 × 5.5 nm. Finally, wedged protofilament association could assemble a cylindrical rod containing 12 protofilament subunits. Tightly packed β-sheets would form the electron-dense outer shell observed in TEM images, whereas the unstructured 1–17 region would occupy the relatively hollow core. A cylindrical structure ~11.5 nm in diameter has a circumference of ~36 nm that can accommodate precisely 12 wedged subunits with an external arc of ~3 nm each. In this model, the hydrophobic interactions driving Aβ self-association will promote the formation of a structure energetically more stable than a stacked protofilament assembly, where a much larger hydrophobic surface remains exposed to the aqueous external milieu. Once the cylindrical structure is closed it cannot grow further in width, thus precluding the formation of thicker fibrils. A multimeric array of protofilaments ~3 nm across organized in a tubular configuration was already described for Aβ₁₋₄₀ fibrils with an average diameter of ~7 nm (60). A hollow core has been also deduced from electron micrograph image reconstruction of transthyretin fibrils (61), indicating that this can be a general feature of amyloids.

Aβ has a cluster of basic amino acids at the N terminus that are considered critical for GAG binding (34) (Fig. 9B). In agreement with this view we have shown that sulfated polymeric GAGs are efficient promoters of Aβ fibril formation. Other studies (62) have shown that CSB-derived monosaccharides represent the minimal GAG subunit required for Aβ binding and that lateral aggregation between Aβ fibrils or the transition of protofilaments into mature amyloid fibrils requires at least a sulfated GAG disaccharide, with the disulfated derivative being the most effective. These results suggest that subtle changes in the GAG backbone and distribution of sulfation have significant effects on the ability of chondroitin sulfate to organize Aβ into amyloid fibrils. However, the observation that, unlike Aβ₁₋₄₀, Aβ₁₋₄₂ does not increase significantly its tendency to aggregate in the presence of CSB (24) indicates that the main factor behind amyloid fibril formation is the intrinsic peptide propensity toward fibrillogenesis. Sulfated polysaccharides could act as a template, interacting with the 1–17 tail of Aβ₁₋₄₂ in nascent protofibrils (Fig. 9B), stabilizing them and facilitating their growth. Our failure to detect CSB in the final structure

FIGURE 9. Proposed model for Aβ₁₋₄₂ smooth fibrils. A, wedged cylinder versus protofilament stacking models. Hydrophobic boundaries are drawn as red lines. B, Aβ₁₋₄₀ sequence showing charged residues in the 1–17 tail, and schematic depicting the possible role of sulfated polysaccharides in promoting the assembly of Aβ₁₋₄₂ fibrils. C, scheme of the effect on cell viability and on Ca²⁺ influx of different Aβ₁₋₄₂ species.
suggests that the GAG is shed from the fibrils during their growth, but we cannot rule out a small relative CSB content below the detection limit of current methods. Other techniques such as solid-state NMR or x-ray diffraction analysis might provide answers, although the insolubility and polydispersity of Aβ fibril preparations are serious obstacles.

A wedged cylinder is consistent with the majority of data available for protofibril and protofilament structure and explains why protofibrils 5.5 nm wide give rise to fibrils precisely twice that width (15, 63). This model is compatible with the existence of certain oligomeric Aβ species like the so-called doughnuts (11, 64) and Aβ*56, a recently described Aβ assembly in vivo (65). Aβ*56 is an apparent dodecamer detected in the brains of a transgenic mouse line expressing a human Aβ precursor protein variant linked to AD. Whether Aβ*56 represents a stable assembly in vivo is currently unknown. The cross-section of the wedged cylinder model contains an Aβ dodecamer, which might exist as an independent soluble association of 12 Aβ subunits arranged in a circle (Fig. 9A). Doughnuts and Aβ*56 could actually be one and the same entity: a dodecamer whose center would contain the dangling 1–17 regions that confer it a less structured conformation (the hole in the doughnut). Our high resolution AFM images on HOPG of raft-forming globular structures have dimensions that closely fit those predicted for such disc-shaped dodecamers that can stack their hydrophobic faces to form cylinders. A ~55-kDa species consistently appears in Western blots transferred from gels run in the presence of SDS, indicating the existence of a very stable species in solution that could correspond to Aβ*56.

Resistance of different cell types to Aβ-induced toxicity appeared in part related to the ability of cells to counteract alterations of intracellular free Ca2+, suggesting that membrane destabilization and the subsequent early derangement of ion balance is a key event leading to amyloid-induced cell death (66). Several mechanisms have been proposed to account for the Ca2+-mobilizing actions of amyloids in their oligomeric and fibrillar aggregation states. These include a direct interaction with membrane components to destabilize the membrane structure (67), insertion into the membrane to form a cation-conducting pore (10, 68, 69), activation of cell surface receptors coupled to Ca2+ influx (70, 71), and oxidative stress leading to dysregulation of mitochondrial Ca2+ homeostasis (72).

Exposure of neuroblastoma cells to various aggregative states of Aβ1–42 elicits different Ca2+ responses. Unstructured fibril-induced cytosolic Ca2+ elevation requires the presence of both extracellular and intracellular Ca2+. A second effect of unstructured fibrils is a rapid leakage of fluorescence from the cells that suggests a massive plasma membrane permeabilization. In agreement with this observation, Aβ1–42 has been described to induce a strong membrane destabilization in giant unilamellar vesicles (73). Our observation that protofibrils derived from weakly structured fibrils can insert into lipid bilayers indicates a role for protofibrils in the membrane perturbations induced by Aβ. The presence of CSB in unstructured fibril forming conditions might accelerate the incorporation of protofibrils into stable fibrils, an effect that can contribute to the reduction of cytosolic calcium variations and the recovery of cell viability. Resting cytosolic free Ca2+ is normally maintained at very low levels despite enormous transmembrane concentration gradients so that even a brief increase might disrupt important cell processes. Indeed, Ca2+ dyshomeostasis is implicated in both necrotic and apoptotic cell death (74), and amyloid-induced Ca2+ changes are directly correlated with cell viability (75). An intriguing possibility in neurological diseases is that an enhanced plasma membrane ion conductance may cause a sustained depolarization and increased electrical excitability, leading to excitotoxic injury (5). These considerations are in agreement with the hypothesis that the toxic effects of Aβ on neurons are various, both in their mechanism and possibly also in their temporal component (76). For instance, Aβ monomers, oligomers, and fibrils are cytotoxic after a few hours according to cell viability assays (9), whereas soluble oligomers have been also associated to synaptic dysfunction in AD brain (77), which suggests a longer time of action.

AD pathophysiology has been related to sustained increased intracellular Ca2+ levels (78). Here, we propose that pernicious, long term cumulative effects might result from brief local Ca2+ influxes that over time perturb intracellular mechanisms, thus leading to cell collapse. In vitro, Ca2+ promotes the formation of Tau filaments with a structure very similar to that observed in brain-derived paired helical filaments (79), suggesting that increments in the ion concentration may trigger the deposition of nucleating Tau aggregates in vivo (80). If, as we have shown, aggregated Tau can capture soluble Tau in the absence of Ca2+, such small Tau nuclei can act as seeds for the growth of paired helical filaments (52), even if Ca2+ levels have been restored back to normal conditions. The results presented here are consistent with data suggesting that Aβ1−42 induces a rise in resting Ca2+ levels that is associated with an ER Ca2+ leak ultimately leading to the pathological accumulation of Tau (81). Ca2+-activated proteases such as calpain have been related to Tau neurofibrillary pathology (82).

Our data suggest that sulfated GAG-mediated neuroprotection is due to the sequestering of Aβ. Thus, GAGs of the extracellular matrix and cell surface could act as key modulators of amyloid homeostasis influencing which tissues or cell types are harmfully affected by the process of amyloidogenesis. Interestingly, chondroitin sulfate content has been shown to be inversely correlated with the amount of hyperphosphorylated Tau in cortical areas of patients with AD (40), a good marker for Aβ-induced neuronal dysfunction (1, 4). Weakly structured Aβ fibrils could be leaking neurotoxic soluble peptide into the surrounding tissue, thus functioning as reservoirs of the bioactive oligomers (Fig. 9C). This observation conciliates apparently contradictory data that point at both fibrils and soluble oligomers as the culprits in AD pathogenesis. On the other hand, our results support the hypothesis that fibrillar deposits formed in the presence of sulfated GAGs are very stable and constitute thermodynamic sinks from where the incorporated peptides cannot easily escape.

Acknowledgments—We thank Miriam Funes, Nieves Hernández, Gema Martinez, Elisenda Coli, and Carmen López from the Scientific and Technical Services of the University of Barcelona for technical assistance.
Non-toxic Aβ Fibrils Induced by Dermatan Sulfate

REFERENCES

1. King, M. E., Kan, H. M., Baas, P. W., Erisir, A., Glabe, C. G., and Bloom, G. S. (2006) J. Cell Biol. 175, 541–546
2. Wolfe, M. S. (2006) Sci. Am. 294, 60–67
3. Avila, J., Lucas, J. J., Pérez, M., and Hernández, F. (2004) Physiol. Rev. 84, 361–384
4. Yankner, B. A. (1996) Neuron 16, 921–932
5. Demuano, A., Mina, E., Kayed, R., Milton, S. C., Parker, I., and Glabe, C. G. (2005) J. Biol. Chem. 280, 17294–17300
6. Quist, A., Doudeski, I., Lin, H., Azimova, R., Ng, D., Frangione, B., Kagan, B., Ghiso, J., and Lar, R. (2005) Proc. Natl. Acad. Sci. U.S.A. 102, 10427–10432
7. Lacor, P. N., Buniel, M. C., Chang, L., Fernandez, S. J., Gong, Y., Viola, Teague, J., Bruce, V. C., Kahn, S. E., and Wight, T. N. (2003)
8. Hu, J., Akama, K. T., Krafft, G. A., Chromy, B. A., and Van Eldik, L. J. (1998) Brain Res. 878, 195–206
9. Dahlgren, K. N., Manelli, A. M., Stine, W. B., Jr., Baker, L. K., Krafft, G. A., and LaDu, M. J. (2002) J. Biol. Chem. 277, 32046–32053
10. Kayed, R., Sokolov, Y., Edmonds, B., Mcintire, T. M., Milton, S. C., Hall, W. I., and Ingram, V. M. (2004) Proc. Natl. Acad. Sci. U.S.A. 101, 10554–10559
11. Haass, C., and Selkoe, D. J. (2007) Neuron 53, 132–140
12. Serpell, L. C. (2000) Biochim. Biophys. Acta 1502, 16–30
13. Walsh, D. M., Hartley, D. M., Kusumoto, Y., Fezoui, Z., Condron, M. M., Shulman, G. I., Shulman, R. G., and Tamborlane, W. V. (1992) J. Biol. Chem. 267, 1101–1110
14. Arimon, M., Díez-Pérez, I., Kogan, M. J., Durany, N., Giralt, E., Sanz, F., and Fernández-Busquets, X. (2005) FASEB J. 19, 1344–1346
15. Mandavilli, A. (2006) Nat. Med. 12, 747–751
16. Alexanderrescu, A. T. (2005) Protein Sci. 14, 1–12
17. Meyer-Puttlitz, B., Milev, P., Junker, E., Zimmer, I., Margolis, R. U., and Margolis, R. K. (1995) J. Neurochem. 65, 2327–2337
18. Rossalhti, E. (1995) Glycobiology 6, 489–492
19. Ancsin, J. B., and Kisilevsky, R. (1999) Proc. Natl. Acad. Sci. U.S.A. 96, 877–887
20. Franzén, B., Lindkvist, B., Mok, L.-W., Asberg, M., and Englund, M. (1994) J. Biol. Chem. 269, 803–810
21. Heikkinen, K., Honkanen, M., Mattila, S., Vaheri, A., and Hakala, H. (2003) J. Proteome Res. 2, 196–207
22. Kurniawan, T., and Lim, M. H. (2006) J. Biol. Chem. 281, 1812–1826
23. van Horssen, J., Wesseling, P., van den Heuvel, L. P., de Waal, R. M., and Teplow, D. B. (1999) J. Biol. Chem. 274, 25945–25952
24. Rigacci, S., Bucciantini, M., and Stefani, M. (2005) J. Cell Sci. 118, 1337–1346
25. Loach, D., Loach, D., and Asberg, M. (1994) J. Biol. Chem. 269, 320–321
26. Margittai, M., and Langen, R. (2004) Proc. Natl. Acad. Sci. U.S.A. 101, 10278–10283
27. von Bergen, M., Barghorn, S., Biernat, J., Mandelkow, E. M., and Mandelkow, E. (2005) Biochim. Biophys. Acta 1739, 158–166
28. Naito, A., and Kawamura, I. (2007) Biochim. Biophys. Acta 1768, 1900–1912
29. Widbrenden, M. J., Rajadas, J., Sutardja, C., and Fuller, G. G. (2006) Biochim. Phys. 1, 9057–9080
30. Chen, Y. R., and Glabe, C. G. (2006) J. Biol. Chem. 281, 24414–24422
31. DeGroot, J. (2004) Curr. Opin. Pharmacol. 4, 301–305
32. Mastrangelo, I. A., Ahmad, M., Sato, T., Liu, W., Wang, C., Hough, P., and Smith, S. O. (2006) J. Mol. Biol. 358, 106–119
33. Lahrs, T., Ritter, C., Adrian, M., Riek-Loher, D., Bohrmann, B., Dobeli, H., Schubert, D., and Riek, R. (2005) Proc. Natl. Acad. Sci. U.S.A. 102, 17342–17347
34. Malinchik, S. B., Inouye, H., Szumowski, K. E., and Kirschner, D. A. (1998) Biophysics J. 74, 537–545
35. Serpell, L. C., Sunde, M., Fraser, P. E., Luther, P. K., Morris, E. P., Sangren, O., Lundgren, E., and Blake, C. C. (1995) J. Biol. Chem. 270, 1323–1332
36. Garcia-Manyes, S., Oncins, G., and Sanz, F. (2005) J. Mol. Biol. 348, 133–140
Non-toxic Aβ Fibrils Induced by Dermatan Sulfate

72. Mattson, M. P. (2004) Nature 430, 631–639
73. Ambroggio, E. E., Kim, D. H., Separovic, F., Barrow, C. J., Barnham, K. J., Bagatolli, L. A., and Fidelio, G. D. (2005) Biophys. J. 88, 2706–2713
74. Berridge, M. J., Bootman, M. D., and Lipp, P. (1998) Nature 395, 645–648
75. Bucciantini, M., Calloni, G., Chiti, F., Formigli, L., Nosi, D., Dobson, C. M., and Stefani, M. (2004) J. Biol. Chem. 279, 31374–31382
76. Deshpande, A., Mina, E., Glabe, C., and Busciglio, J. (2006) J. Neurosci. 26, 6011–6018
77. Kokubo, H., Kayed, R., Glabe, C. G., and Yamaguchi, H. (2005) Brain Res. 1031, 222–228
78. Stutzmann, G. E. (2007) Neuroscientist 13, 546–559
79. Ruben, G. C., Wang, J. Z., Iqbal, K., and Grundke-Iqbal, I. (2005) Microsc. Res. Tech. 67, 175–195
80. Yang, L. S., and Ksiezak-Reding, H. (1999) J. Neurosci. Res. 55, 36–43
81. Christensen, R. A., Shtifman, A., Allen, P. D., Lopez, J. R., and Querfurth, H. W. (2004) J. Biol. Chem. 279, 53524–53532
82. Adamec, E., Mohan, P., Vonsattel, J. P., and Nixon, R. A. (2002) Acta Neuropathol. (Berl.) 104, 92–104