Amyloid-β Interactions with Chondroitin Sulfate-derived Monosaccharides and Disaccharides

IMPLICATIONS FOR DRUG DEVELOPMENT*

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In Alzheimer’s disease, the major pathological features are diffuse and senile plaques that are primarily composed of the amyloid-β (Aβ) peptide. It has been proposed that proteoglycans and glycosaminoglycans (GAG) facilitate amyloid fibril formation and/or stabilize the plaque aggregates. To develop effective therapeutics based on Aβ-GAG interactions, understanding the Aβ binding motif on the GAG chain is imperative. Using electron microscopy, fluorescence spectroscopy, and competitive inhibition ELISAs, we have evaluated the ability of chondroitin sulfate-derived monosaccharides and disaccharides to induce the structural changes in Aβ that are associated with GAG interactions. Our results demonstrate that the disaccharides GalNAc-4-sulfate(4S), ΔUA-GalNAc-6-sulfate(6S), and ΔUA-GalNAc-4,6-sulfate(4S,6S), the iduronic acid-2-sulfate analogues, and the monosaccharides β-GalNAc-4S, β-GalNAc-6S, and β-GalNAc-4S,6S, but not δ-GalNAc, β-GlcNAc, or ΔUA-GalNAc, induce the fibrillar features of Aβ-GAG interactions. The binding affinities of all chondroitin sulfate-derived saccharides mimic those of the intact GAG chains. The sulfated monosaccharides and disaccharides compete with the intact chondroitin sulfate and heparin GAGs for Aβ binding, as illustrated by competitive inhibition ELISAs. Therefore, the development of therapeutics based on the model of Aβ-chondroitin sulfate binding may lead to effective inhibitors of the GAG-induced amyloid formation that is observed in vitro.

Alzheimer’s disease is characterized neuropathologically by amyloid deposits, neurofibrillary tangles, and selective neuronal loss. The major component of the amyloid deposits is a 39–43-residue peptide, amyloid-β (Aβ).† Aβ fibrillogenesis in vitro is a nucleation-dependent process consisting of a slow lag phase for nucleation followed by faster propagation of fibrils (1–3). However, in vivo fibrillogenesis is likely a complex pathway involving many factors that modulate the aggregation of Aβ. Two mechanisms have been proposed for the nucleation of Aβ fibrils. The first involves the self-assembly of Aβ monomers, which undergo a conformational change to become the fibril nucleus. The second involves an alternative pathway of heterogeneous nucleation, which results from outgrowth of fibrils from non-Aβ seeds (1).

Many proteins are associated with amyloid plaques; their presence may result in heterogeneous nucleation of Aβ (4–11). Although heparan sulfate proteoglycans have been extensively correlated with plaque formation, in Alzheimer’s disease at least four types of proteoglycans are associated with amyloid plaques (12–17). Aβ-proteoglycan interactions are mediated predominantly through Aβ-glycosaminoglycan (GAG) binding with GAGs acting as a scaffold for the assembly of the fibrils. The scaffold may function by enhancing the structural features that favor a β-sheet conformation thereby increasing the number of nucleation seeds, as demonstrated by a virtually instantaneous structural transition in Aβ upon addition of GAGs (18).

In the later stages of the amyloid pathway, GAGs also act by enhancing lateral aggregation of small fibrils to confer insolubility and protection from proteolysis (18–21). A structure-activity relationship for Aβ-GAG interactions is slowly emerging based on the affinities of various GAGs. In vitro studies have shown that the chondroitin sulfates are more effective at both nucleation and lateral aggregation of Aβ fibrils than the heparin GAGs (18). Chondroitin sulfates are sulfated on a single face of the polymer and may represent an ideal distribution of charge for Aβ interactions. Therefore, these GAGs were used as the prototype to determine Aβ binding and potentially to develop compounds that could compete with all identified proteoglycans associated with plaques.

The GAG binding site on the Aβ peptide has been investigated using amino acid substitution of the Aβ1–28 peptide and as a function of aggregation state (22, 23). These studies have demonstrated that although electrostatic interactions through basic amino acids contribute to GAG binding, nonionic interactions, such as hydrogen bonding and van der Waals packing, play a role in GAG-induced Aβ folding and aggregation (22). Furthermore, GAG-Aβ interactions are more sensitive to the conformation and aggregation state of Aβ rather than the primary sequence (22, 23). Together these results suggested that inhibition of Aβ-GAG interactions through targeting of the GAG binding site on Aβ may not provide a viable therapeutic. Alternatively, the Aβ-GAG interaction may be mediated by a unique binding site on the GAG backbone that could serve as a target for inhibition of amyloid formation. This therapeutic strategy is supported by in vitro studies, which demonstrated

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2 This paper is available on line at http://www.jbc.org
that polysulfated compounds could inhibit binding of heparan sulfate to Aβ (24). Also using an in vivo model of splenic amyloidosis, small sulfonated or sulfated molecules have been shown to be active inhibitors of amyloid deposition (25). Alternatively, small GAG-derived saccharides may have the alternate effect of enhancing Aβ precipitation into nontoxic plaques and thereby decreasing the presence of toxic Aβ species. To develop more specific therapies directed toward Aβ fibrillogenesis, we determined the minimum GAG unit necessary for Aβ binding, fibrillogenesis, and lateral aggregation. Here, we examine the interaction of chondroitin sulfate-derived monosaccharides and disaccharides with Aβ40 and Aβ42. Fluorescence spectroscopy and electron microscopy demonstrate a potent effect of both monosaccharides and disaccharides on the formation and structure of Aβ fibrils. In addition, competitive inhibition ELISAs demonstrate that the binding of both monosaccharides and disaccharides to Aβ inhibits interaction with the polymeric chondroitin sulfate and heparan sulfate GAGs.

MATERIALS AND METHODS

Aβ Peptides—Aβ40 and Aβ42 were synthesized by solid phase Fmoc (9-fluorenylmethoxycarbonyl) chemistry by the Hospital for Sick Children’s Biotechnology Center (Toronto, ON). Peptides were isolated by reverse phase high pressure liquid chromatography on a C18 µBondapak column, and purity was determined using mass spectrometry and amino acid analyses. Peptides were initially dissolved in 0.5 ml of 100% trifluoroacetic acid (Aldrich), diluted in distilled H₂O, and immediately lyophilized (28). Peptides were then dissolved in 40% trifluoroethanol (Aldrich) in H₂O and stored at −20 °C until use. Alternatively, the lyophilized peptides were dissolved in distilled H₂O at 2.5 mM concentration and used immediately.

Glycosaminoglycans and Subunits—Chondroitin-4-sulfate (bovine trachea, 8000 Da), dermatan sulfate (bovine mucosa, 16,000 Da), chondroitin-6-sulfate (shark cartilage), heparan (porcine intestinal mucosa), heparan sulfate (bovine kidney), and keratan sulfate (bovine cornea) were purchased from Sigma. Chondroitin sulfate-derived disaccharides ΔUA-GalNAc, ΔUA-GalNAc-4S, ΔUA-GalNAc-6S, and ΔUA-GalNAc-4S,6S were purchased from Dextra Laboratories (Reading, United Kingdom), and ΔUA-2S-GalNAc, ΔUA-2S-GalNAc-4S, ΔUA-2S-GalNAc-6S, and ΔUA-2S-GalNAc-4S,6S were purchased from Calbiochem. Monosaccharides D-GalNAc, D-GalNAc-4S,6S, and D-GalNAc-6S were purchased from Sigma. All GAGs and saccharides were dissolved in distilled H₂O at 10 mg/ml and stored at −20 °C until use.

Tyrosine Fluorescence Assay—Tyrosine emission spectra from 290 to 340 nm were collected (excitation wavelength 281 nm, 0.5 nm/scan, band pass = 4 nm). A cuvette with a 1-cm path length was used. For the centrifugation studies, 1 µM Aβ40 or Aβ42 was incubated in the presence of one chondroitin sulfate subunit at a 1:1 ratio for 24 h. Samples were centrifuged for 30 min at 15,600 × g to sediment aggregates and fibrils as described previously (27, 28). The relative amount of tyrosine in the supernatant was then determined. The fluorescence of the noncentrifuged sample was used as a measure of the total tyrosine fluorescence.

Electron Microscopy—For negative staining, carbon-coated pioform grids were floated on aqueous solutions of peptides (100 µg/ml). After grids were blotted and air-dried, the samples were stained with 1% (w/v) phosphotungstic acid, pH 7.0. The peptide assemblies were observed in a Hitachi H-7000 operated with an accelerating voltage of 75 kV (28).

Amyloid Staining—Thioflavin T fluorescence assay of Aβ in the presence and absence of GAGs (29, 30) and GAG-derived disaccharides and monosaccharides was used to evaluate the similarity between Aβ-GAG fibrils and classical amyloid fibrils. Samples were incubated at a 1:1 ratio by weight with a final Aβ concentration of 200 µM for 3 days. Samples were vortexed and 40-µl aliquots were added to 960 µl of 10 µM Thioflavin T in phosphate-buffered saline, pH 6.0. Steady state fluorescence was measured at 20 °C using a Photon Technology International QM-1 fluorescence spectrophotometer equipped with excitation intensity correction and magnetic stirrer. Thioflavin T emission spectra from 475 to 495 nm were collected (excitation wavelength 437 nm, 0.5 nm/scan, band pass = 4 nm). A cuvette with a 1-cm path length was used.

Competitive Inhibition ELISAs—Nunc Immunosorp plates were coated with 100 µl of GAGs (5 µg/ml) and incubated overnight, 4 °C. Simultaneously, Aβ40 and Aβ42 were incubated with chondroitin sulfate-derived monosaccharides and disaccharides at 1:1 or 1:10 ratio by weight. The plates were rinsed twice with water and blocked with 100 µl of 1% bovine serum albumin in phosphate buffered saline. After incubation for 1 h at room temperature, the plates were washed 3 times with 0.05% Tween 20/phosphate-buffered saline and twice with phosphate-buffered saline. Aβ was then added to the plates and incubated for 2 h at room temperature with shaking. Plates were washed as above before the addition of monoclonal antibody against Aβ, 6F3D5 ( Dako), 6E10, or 4G8 (Senetek, Carpinteria, CA). The reaction with 50 µl of horseradish peroxidase-conjugated goat anti-mouse IgG 1:2000 was performed at room temperature for 1 h. Color development was achieved with 100 µl of 2,2’-azino-di-[3-ethyl-benzthiazoline-6-sulfonic acid] in 0.1 M acetate buffer, pH 4.2. The absorbance was monitored at 415 nm on a Bio-Rad Benchmark microtiter plate reader.

RESULTS AND DISCUSSION

Induction and Morphology of Aβ Fibril in the Presence of Chondroitin Sulfate-derived Disaccharides—We have previously shown that the chondroitin sulfate GAGs are the most efficient at inducing a β-structural transition in Aβ and subsequent fibrillogenesis (18). Therefore, we have used the chondroitin sulfate-derived monosaccharides and disaccharides (Fig. 1) to elucidate the minimum sugar moiety necessary for Aβ binding and fibrillogenesis. Chondroitin sulfate subunits are derived by enzymatic cleavage of the GAG chain by chondroitinases ABC, AC-I, -B, or -C. The saccharides used in this study represent repeat disaccharides present in chondroitin-4-sulfate, chondroitin-6-sulfate, and dermatan sulfate, which retain the charge distribution present in the intact GAG (Fig. 1). The monosaccharides are generated by removal of uronic acid from and desulfation of the disaccharides.

To investigate the effect of chondroitin sulfate-derived saccharides on Aβ nucleation, the intrinsic tyrosine fluorescence of Aβ40 and Aβ42 was used to monitor the amount of soluble peptide after incubation in the presence and absence of the disaccharides. After 24 h of incubation, soluble Aβ was separated from aggregated and fibrillar peptide by centrifugation (23, 24). Different chondroitin sulfate-derived saccharides had variable effects on the amount of pelletable aggregates detected with greater effects seen for Aβ42 (Fig. 2) over Aβ40 (data not shown). At 24 h, the disaccharides ΔUA-GalNAc-4S, ΔUA-GalNAc-6S, and ΔUA-GalNAc-4S,6S' significantly increased the amount of aggregated Aβ40/42 with ΔUA-GalNAc-4S,6S being the most effective (Fig. 2C). It is interesting to note that all disaccharides and in particular ΔUA-GalNAc-4S,6S induced the same extent of fluorescence loss as the intact GAG, dermatan sulfate (Fig. 2D). These results demonstrate that the presence of chondroitin sulfate-derived disaccharides can corre-
lated with an increased amount of aggregated Aβ.

The characteristics of Aβ40 and Aβ42 fibrils in the presence and absence of chondroitin sulfate-derived saccharides were examined by electron microscopy. Previous investigations indicated that GAG promoted morphological changes in the fibrous structures formed by Aβ40 and Aβ42 (18). Unseeded samples of both Aβ40 and Aβ42 were incubated in the presence of chondroitin sulfate-derived disaccharides, intact chondroitin sulfate GAGs, and alone for up to 96 h. Negative stain electron microscopy demonstrated that Aβ42 fibrils were 50–70 Å in diameter with an average length of 750 Å (Fig. 3A). These were indistinguishable from those of Aβ42 in the presence of the desulfated DUA-GalNAc (Fig. 3B) or DUA-GlcNAc (data not shown). The monosulfated disaccharides, DUA-GalNAc-6S and DUA-GalNAc-4S, induced fibrils of similar size but with increased lateral aggregation as compared with control (Fig. 3C). The bundles of fibers were similar to those seen in the presence of polymeric chondroitin sulfate GAGs (18). In the presence of DUA-GalNAc-4S,6S, Aβ42 formed many fibers displaying extensive lateral aggregation as illustrated by the heavily stained clusters of fibrils (Fig. 3D). These results demonstrate that the sulfate group on the second position of the iduronic acid does not effect the morphology of Aβ fibrils formed in the presence of sulfated GalNAc disaccharides. Furthermore, these data suggest that using a disaccharide with a sulfated iduronic acid does not contribute to the minimal unit necessary for Aβ binding and enhanced fibrillogenesis.

GAGs also laterally aggregate preformed Aβ fibrils into large masses characteristic of insoluble plaques. To evaluate this phenomenon as induced by chondroitin sulfate-derived disaccharides, we incubated the saccharides with preformed Aβ40 fibrils and examined the morphology by negative stain electron microscopy.
microscopy. Preincubated Aβ40 forms long fibers that are non-aggregated (Fig. 4A). In contrast, dermatan sulfate induced a consistent lateral aggregation of Aβ40 fibrils with apparent helical twisting (Fig. 4B). The fiber bundles had a cumulative diameter of up to 200 Å, but the average length of the fibers remained unaffected. In the presence of chondroitin sulfate-derived disaccharides, Aβ40 lateral aggregation was similar to that of an extended GAG polymer, with the disulfated ΔUA-GalNAc-4S,6S being the most effective (Fig. 4C). The fibers were aligned in large bundles rather than a haphazard distribution across the grid. These data indicate that the binding of chondroitin sulfate-derived disaccharides to Aβ fibrils is sufficient to stabilize the macromolecular structure of pre-existing fibers by lateral aggregation and represent the smallest unit responsible for interfiber stabilization. Similar to polymeric GAGs, all data taken together demonstrate that the extent of...
sulfation on the disaccharide backbone defines the extent to which the disaccharide interacts with and stabilizes Aβ. Furthermore, these small GAG-derived saccharides demonstrate the potential use of these molecules to decrease the soluble pool of Aβ in situ by enhancing the precipitation of nontoxic fibers.

**Effect of Chondroitin Sulfate-derived Monosaccharides on Aβ Fibrillogenesis**—The effect of chondroitin sulfate-derived monosaccharides on the induction of Aβ fibrillogenesis was investigated. To investigate the effect of chondroitin sulfate-derived saccharides on Aβ nucleation, the intrinsic tyrosine fluorescence of Aβ40 and Aβ42 was used to monitor the amount of soluble peptide after incubation in the presence and absence of the monosaccharides. At 24 h, no significant difference could be detected in the amount of Aβ40/42 pelleted in the presence and absence of the monosaccharides D-GalNAc, D-GalNAc-4S, and D-GalNAc-6S, whereas a slight increase in the amount of Aβ42 pelleted in the presence of D-GalNAc-4S,6S could be detected (Fig. 2B).

The morphology of Aβ fibrils in the presence of chondroitin sulfate-derived monosaccharides was investigated using negative stain electron microscopy. Similar to Aβ42 alone (Fig. 5A), the presence of nonsulfated D-GalNAc and D-GlcNAc induced

**Fig. 4.** Negative stain electron microscopy of seeded Aβ40 was examined in the presence and absence of chondroitin sulfate GAGs. Aβ40 formed long fibers (A) and upon incubation with dermatan sulfate appeared to be thicker as a result of lateral aggregation with a 150 nm periodic twist (B). In the presence of ΔUA-GalNAc-4S,6S, Aβ40 fibrils were organized into thick bundles (C, arrows delineate bundles) characteristic of the intact dermatan sulfate. Scale bar is 50 nm.

**Fig. 5.** Negative stain electron microscopy of Aβ42 in the presence of chondroitin sulfate-derived monosaccharides. Aβ42 incubated in buffer alone (A) demonstrates many thin fibers. When incubated in the presence of D-GalNAc (B), a similar structure of the fibrils to Aβ42 alone could be detected. Alternatively, lateral aggregation was apparent in the fibrils formed in the presence of D-GalNAc-6S (C). In the presence of D-GalNAc-4S,6S, only protofibrils of Aβ42 were detected (D). Scale bar is 50 nm.
fibrils similar to mature amyloid fibers (Fig. 5B). In the presence of D-GalNAc-4S or D-GalNAc-6S (Fig. 5C), the fibers were less abundant and of varying lengths but exhibited some aggregation as demonstrated by the uneven, heavily stained distribution of fibers across the grid. Alternatively, in the presence of D-GalNAc-4S or D-GalNAc-6S (Fig. 5B), the Thioflavin T fluorescence increased in the presence of D-GalNAc, D-GalNAc-6S but to a lesser extent than chondroitin-6-sulfate. When incubated with D-galacturonic acid or D-GalNAc-4S,6S, Aβ42 demonstrated a morphology similar to protofibrils and exhibited a Thioflavin T fluorescence greater than Aβ42 alone. These results demonstrate that the protofibrils induced by chondroitin sulfate-derived monosaccharides have characteristics similar to typical amyloid fibers. In summary, our data demonstrate that chondroitin sulfate-derived monosaccharides

![Graph showing Thioflavin T assay of chondroitin sulfate-Aβ fibrils.](image)

**Fig. 6. Thioflavin T assay of chondroitin sulfate-Aβ fibrils.** Thioflavin T binding to chondroitin sulfate-Aβ complexes resulted in both an increase in fluorescence and a shift in the emission spectra. Thioflavin T fluorescence as a result of Aβ binding was similar in the presence or absence of D-GalNAc. D-GalNAc-6S induced a modest increase in the Thioflavin T fluorescence in comparison to Aβ alone, indicating a slight increase in aggregation. Chondroitin-6-sulfate and dermatan sulfate increased the extent of fluorescence indicating enhanced fibrillogenesis. Similarly, D-galacturonic acid enhanced the fluorescence to approximately the same extent. D-GalNAc-4S,6S binding to Aβ42 resulted in the most intense fluorescence indicating that it is qualitatively more effective at enhancing Aβ fibrillogenesis. Values for Thioflavin T fluorescence of Aβ alone were set at 100%, and the Thioflavin T fluorescence of all samples treated with GAGs is reported relative to this value. Values are reported as the mean ± S.D. of three experiments.

### Table I

| Competitive inhibition ELISAs for Aβ42 binding |
|-----------------------------------------------|
| Chondroitin-4-sulfate | Chondroitin-6-sulfate | Dermatan sulfate |
|------------------------|------------------------|------------------|
| **Aβ42**               | **Aβ42**               | **Aβ42**         |
| 1:1                    | 108 ± 10.3             | 102 ± 7.4        | 100 ± 4.6 |
| 1:10                   | 55.6 ± 30.2*           | 39.8 ± 7.8*      | 37.6 ± 0.3* |
| D-GlcNAc               | 99 ± 9.8               | 81.7 ± 7.2       | 60 ± 21.7 |
| 1:1                    | 121 ± 12.5             | 93.5 ± 5.6       | 84 ± 9.7 |
| 1:10                   | 100 ± 12.9             | 96.9 ± 3.9       | 102 ± 2.1 |
| D-GalNAc               | 98 ± 19.9              | 95.9 ± 7.7       | 97.6 ± 3.5 |
| 1:1                    | 111 ± 9.0              | 108 ± 15.6       | 91.4 ± 17.8 |
| 1:10                   | 112 ± 10.1             | 105 ± 5.6        | 90.8 ± 24.7 |
| UA-GalNAc              | 98.9 ± 8.9             | 89.9 ± 8.6       | 99.3 ± 9.5 |
| 1:1                    | 100 ± 13.5             | 104 ± 5.8        | 91.1 ± 9.2 |
| 1:10                   | 89.5 ± 18.5            | 95.8 ± 7.8       | 94.9 ± 9.6 |
| D-GalNAc-4S            | 95.9 ± 10.8            | 94.5 ± 6.7       | 95.3 ± 5.8 |
| 1:1                    | 115 ± 2.8              | 112 ± 7.8        | 98.4 ± 43 |
| 1:10                   | 55 ± 10.4*             | 83.7 ± 19.7      | 63.5 ± 15.8 |
| UA-GalNAc-4S           | 87.6 ± 6.2             | 83 ± 5.7         | 92 ± 2.6 |
| 1:1                    | 99.4 ± 2.9             | 55 ± 8.8*        | 84.6 ± 4.3 |
| 1:10                   | 85.1 ± 18.3            | 85.6 ± 16.3      | 77.8 ± 2.6* |
| D-GalNAc-6S            | 50 ± 1.7*              | 55.9 ± 3.6*      | 60 ± 7.9* |
| 1:1                    | 105 ± 9.5              | 124 ± 21.9       | 87.0 ± 4.4 |
| 1:10                   | 92.6 ± 2.4             | 106 ± 6.4        | 76.3 ± 35 |
| UA-GalNAc-6S           | 94.5 ± 4.1             | 129 ± 10.2       | 63.9 ± 4.7 |
| 1:1                    | 131 ± 17.0             | 83.5 ± 3.9       | 59.6 ± 11.3* |
| 1:10                   | 99 ± 19.8              | 93.6 ± 2.9       | 97.3 ± 2.7 |
| D-GalNAc-4S,6S         | 41 ± 3.5*              | 62.2 ± 37.1      | 41.9 ± 5.2* |
| 1:1                    | 102.9 ± 11.9           | 120 ± 11.5       | 72.2 ± 23 |
| 1:10                   | 88.3 ± 5.7             | 105 ± 3.5        | 63.4 ± 18 |
| UA-GalNAc-4S,6S        | 113 ± 9.5              | 111 ± 8.6        | 110 ± 6.8 |
| 1:1                    | 86.5 ± 9.2             | 94.7 ± 10.2      | 95.2 ± 12.2 |

* p < 0.001 when compared with Aβ42 binding alone.
bind Aβ and induce fibrillogenesis without lateral aggregation.

**Characterization of the Aβ-Saccharide Binding Site**—To compare the specificity of chondroitin sulfate-derived saccharide binding, we used competitive inhibition ELISAs to determine whether the chondroitin sulfate-derived saccharides could compete with intact GAG chains for Aβ40 and Aβ42 binding (Table I). Concentration-dependent studies were used to determine both the specificity of competition and the relative binding strengths of each component. The chondroitin sulfate-derived monosaccharides and disaccharides were preincubated with Aβ40 and Aβ42 before incubation with chondroitin-4-sulfate, chondroitin-6-sulfate, or dermatan sulfate. The amount of Aβ bound to the intact GAG chain on the microtiter plate was determined using the anti-Aβ antibodies 6E10, 4G8, or 6F3D. All antibodies demonstrated similar concentration-dependent inhibition profiles indicating that the detection antibody was not a determining factor. D-GalNAc and ΔUA-GalNAc were unable to compete with the chondroitin sulfate GAGs for Aβ binding, which is in agreement with our electron microscopy data in which Aβ40/42 fibrils were indistinguishable in the presence and absence of D-GalNAc and ΔUA-GalNAc. The monosaccharides d-galacturonic acid, D-GalNAc-6S, and D-GalNAc-4S,6S were all effective at competing with all chondroitin sulfate GAGs for Aβ binding at a 1:1 ratio (by weight). These results suggest that the alterations in fibrous structure detected by electron microscopy and fluorescence studies can be attributed to the binding of these monosaccharides to the GAG binding site on Aβ40 and Aβ42. These observations further suggest that the monosaccharides d-galacturonic acid, D-GalNAc-6S, and D-GalNAc-4S,6S are sufficient to induce Aβ binding and structural transitions associated with Aβ-GAG interactions. It was not surprising to find that D-GalNAc-4S competed poorly with all GAGs for Aβ binding because the intact chondroitin-4-sulfate is the least effective of all the chondroitin sulfate GAGs at inducing the structural transitions necessary for fibril formation and aggregation (18).

The chondroitin sulfate-derived disaccharides had variable abilities to compete for Aβ binding with ΔUA-Gal-4S,6S being the most effective (Table I). The differences in binding of the disaccharides reflect the varying abilities of the chondroitin sulfate GAGs to bind, induce a structural change in Aβ, and enhance lateral aggregation. One corollary to our results is that we cannot rule out the possibility that the disaccharides induced a conformational change in Aβ that allowed the intact GAG chain to elicit binding between Aβ fibrils as has been previously suggested for GAG binding to preformed fibers (18). The chondroitin sulfate-derived monosaccharides and disaccharides binding strengths, as determined by the extent of competition, may reflect the fluctuation of Aβ binding to surfaces with slight variations in charge distribution. These characteristics have been reported previously for myo-inositol and its phosphorylated analogues as well as alterations in the distribution and surface charge of the antibiotic rifampicin (28, 31).

**Chondroitin Sulfate-derived Saccharides Inhibit Heparan Sulfate GAG Binding to Aβ**—The similarities in GAG structure between chondroitin sulfate and heparan sulfate GAGs previously have stimulated the suggestion that proteins that bind to chondroitin sulfate should interact with heparan sulfate and vice versa (32–34). The basic fibroblast growth factor of the heparan sulfate-binding proteins, platelet-derived factor 4, and fibronectin react weakly with dermatan sulfate, whereas heparin cofactor II and hepatocyte growth factor have a comparable high affinity for both heparan sulfate and dermatan sulfate (35–39). To determine whether the chondroitin sulfate-derived monosaccharides could compete with other GAGs for Aβ binding, we repeated the competitive inhibition ELISAs using heparan (Table II). It is speculated that polymeric GAGs bind to the same region or structural motif in Aβ (40); therefore it was not unexpected to find that the monosaccharide D-GalNAc-4S,6S could compete to the same extent with heparan as was seen for dermatan sulfate. Our results for heparin competition illustrate that the competition detected between heparin and the chondroitin sulfate-derived saccharides is independent of the detection antibody, as both the Aβ-specific antibodies, 6E10 and 4G8, detect a similar concentration-dependent inhibition (Table II). These results suggest that development of an inhibitor for GAG binding to Aβ could represent an agent to block Aβ-proteoglycan interactions.

Further investigation into the ability of chondroitin sulfate-derived saccharides to inhibit heparan sulfate binding to Aβ demonstrated similar results to those of both chondroitin and dermatan sulfates. For example, the disaccharide D-GalNAc-4S,6S was preincubated with the chondroitin sulfate-derived monosaccharides or disaccharides before co-incubation with intact GAGs. The amount of Aβ42 that bound each intact GAG was set at 100% binding, and all other values were calculated with respect to this value. Values are reported as mean ± S.D. for at least three separate ELISA assays. Paired t test indicates that p < 0.05 when compared with Aβ42 binding alone.

### Table II

| Antibody for detection | Aβ40 | Aβ42 |
|------------------------|------|------|
| 6E10                   | 0    | 0    |
| 1:1                    | 100  | 100  |
| 1:5                    | 86 ± 2.3 | 38 ± 7.6 |
| 1:10                   | 43 ± 2.9 | 30 ± 5.5 |
| 6G8                    | 0    | 0    |
| 1:1                    | 86 ± 9.4 | 122 ± 4.3 |
| 1:5                    | 79 ± 9.4 | 46 ± 2.8 |
| 1:10                   | 33 ± 23.3 | ND |

*p < 0.1 (%) when compared with Aβ binding alone.

**ND,** not determined.

### Table III

| Antibody for detection | Aβ40 | Aβ42 |
|------------------------|------|------|
| 6E10                   | 0    | 0    |
| 1:1                    | 100  | 100  |
| 1:5                    | 86 ± 2.3 | 38 ± 7.6 |
| 1:10                   | 43 ± 2.9 | 30 ± 5.5 |
| 6G8                    | 0    | 0    |
| 1:1                    | 86 ± 9.4 | 122 ± 4.3 |
| 1:5                    | 79 ± 9.4 | 46 ± 2.8 |
| 1:10                   | 33 ± 23.3 | ND |

*pND,** not determined.
dermatan sulfate (Table III). None of the nonsulfated monosaccharides or disaccharides could inhibit Aβ binding to both heparan sulfate and keratan sulfate; these results are similar to those for dermatan sulfate competition studies. The iduronic acid-2-sulfated disaccharides were unable to compete for heparan sulfate binding, whereas ΔUA-2S-GalNAc-4S and ΔUA-2S-GalNAc-6S were able to compete for keratan sulfate binding. These results suggest that subtle changes in the GAG backbone and distribution of sulfation have significant effects on the ability of chondroitin sulfate-derived saccharides to compete for GAG binding sites. The disaccharides ΔUA-GalNAc-4S, ΔUA-GalNAc-6S, and ΔUA-GalNAc-4S,6S all competed with heparan sulfate for Aβ binding with the disulfated derivative being the most effective (Table III). As was seen for competition for chondroitin sulfate GAGs, the monosaccharide D-GalNAc-6S were able to compete for keratan sulfate binding. These results suggest that a therapeutic approach based on this structural motif may inhibit Aβ binding to all GAGs present in the central nervous system.

Cumulatively, our results demonstrate that chondroitin sulfate-derived monosaccharides represent the minimal GAG subunit required for Aβ binding and that lateral aggregation between Aβ fibers or the transition of protofilaments into mature amyloid fibers requires a sulfated GAG disaccharide. These results suggest that the size constraints of the monosaccharide are insufficient to facilitate the association of fibers but are sufficient to bind Aβ. Development of drugs based on these monosaccharide compounds will have to take into account the potential for stabilization of toxic Aβ intermediates. We have previously shown that Aβ42 is stabilized in a nontoxic oligomer in the presence of inositol stereoisomers; this illustrates the potential for drug design based on the present methodology (28, 31, 41). Alternatively, GAG-derived disaccharides may represent a template in which to develop drugs that will decrease available monomer in situ by accelerating precipitation of Aβ fibers. These studies further emphasize the importance of investigations into the design of GAG mimetics as potential amyloid therapeutics.

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