Islet amyloid, a pathologic feature of type 2 diabetes, contains the islet β-cell peptide islet amyloid polypeptide (IAPP) as its unique amyloidogenic component. Islet amyloid also contains heparan sulfate proteoglycans (HSPGs) that may contribute to amyloid formation by binding IAPP via their heparan sulfate (HS) chains. We hypothesized that β-cells produce HS that bind IAPP via regions of highly sulfated disaccharides. Unexpectedly, HS from the β-cell line β-TC3 contained fewer regions of highly sulfated disaccharides compared with control normal murine mammary gland (NMuMG) cells. The proportion of HS that bound IAPP was similar in both cell lines (~65%). The sulfation pattern of IAPP-bound versus non-bound HS from β-TC3 cells was similar. In contrast, IAPP-bound HS from NMuMG cells contained frequent highly sulfated regions, whereas the non-bound material demonstrated fewer sulfated regions. Fibril formation from IAPP was stimulated equally by IAPP-bound β-TC3 HS, non-bound β-TC3 HS, and non-bound NMuMG HS but was stimulated to a greater extent by the highly sulfated IAPP-bound NMuMG HS. Desulfation of HS decreased the ability of both β-TC3 and NMuMG HS to stimulate IAPP maximal fibril formation, but desulfated HS from both cell types still accelerated fibril formation relative to IAPP alone. In summary, neither binding to nor acceleration of fibril formation from the amyloidogenic peptide IAPP is dependent on overall sulfation in HS synthesized by β-TC3 cells. This information will be important in determining approaches to reduce HS-IAPP interactions and ultimately prevent islet amyloid formation and its toxic effects in type 2 diabetes.

Islet amyloid deposition is a pathological hallmark of the pancreatic islet in type 2 diabetes (1). Aggregation of islet amyloid polypeptide (IAPP), a normal peptide product of the islet β-cell, underlies the deposition of islet amyloid, a process that contributes to the decreased β-cell volume that characterizes type 2 diabetes (2–4). The mechanism(s) that govern the aggregation of this normally soluble polypeptide are poorly understood. In addition to amyloidogenic IAPP, several other components of islet amyloid have been identified, including apolipoprotein E (5, 6), serum amyloid P component (7), and the heparan sulfate proteoglycan perlecain (6, 8).

Heparan sulfate proteoglycans (HSPGs) are a heterogeneous population of proteoglycans involved in a diverse range of cellular processes ranging from vascular development to cell signaling (9). HSPGs are components of amyloid deposits that form in a number of diseases, including type 2 diabetes (6, 8) and Alzheimer disease (10). HSPGs may play a role in stimulating amyloid deposition via a direct interaction with amyloidogenic peptides. We and others have shown that HSPGs are capable of binding the amyloidogenic peptide IAPP (11, 12), its precursor pro-IAPP (13) and other amyloidogenic peptides, including amyloid-β (14), and serum amyloid A (15), the unique amyloidogenic peptides from Alzheimer disease-related amyloid and inflammation-associated AA amyloidosis, respectively. Upon binding amyloidogenic peptides, HSPGs or their oligosaccharide heparan sulfate (HS) glycosaminoglycan...
chains have been shown to induce structural changes in the peptide to the β-sheet structure required for amyloid fibril formation (16) and to increase fibril formation from the amyloidogenic peptides (11, 14). These findings suggest that HSs are important for amyloid formation.

Heparan sulfate undergoes a complex set of modifications following its synthesis in the endoplasmic reticulum. Many of these modifications are sulfation reactions, catalyzed by a diverse array of enzymes including N-deacetylase/N-sulfotransferase and 2-, 3-, or 6-O-sulfotransferases (17). These modifications typically result in the formation of domains containing either non-sulfated (acetylated) or highly sulfated oligosaccharides, with the size, arrangement, and composition of these sulfated domains determining the ability of HS to bind a diverse set of protein ligands (18). The composition of HS attached to a given core protein has been shown to vary widely among tissue types, suggesting a complex level of tissue specificity in HS synthesis and modification (17). For example, HS among tissue types, showing that the immortalized murine pancreatic islet cell line β-T3 synthesizes and secretes several proteoglycans, predominantly HSPGs (12, 21). Furthermore, we also demonstrated that these β-T3 cell proteoglycans are capable of binding amyloidogenic human IAPP (12), suggesting that these locally produced proteoglycans may be important in IAPP fibril formation and the deposition of islet amyloid in type 2 diabetes. We therefore hypothesized that the degree of sulfation of β-T3 cell HS is an important determinant of IAPP binding and thereby IAPP fibril formation. Here, we report the composition, IAPP-binding, and fibril-enhancing ability of HS synthesized by β-T3 cells.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Metabolic Labeling**—The murine insulinoma cell line, β-T3 (passage 55–57), was utilized for this study, with normal murine mammary gland epithelial (NMuMG, passage 4–7) cells as a control cell line. The latter were obtained from ATCC. Cells were seeded at 1 × 10^5 cells per ml and cultured for up to 4 days in DMEM containing 10% fetal bovine serum and 5.5 mM or 25 mM glucose for 48 h in DMEM containing 10% FBS and 16.7 or 25 mM glucose. NMuMG cells, respectively, followed by metabolic labeling for 16 h at 37 °C to digest core proteins and then subjected to DEAE ion exchange chromatography with the DEAE equilibrated in PBS containing 0.15 M NaCl, pH 7.2, and samples eluted in PBS containing 3 M NaCl. Glycosaminoglycans were liberated from residual core protein fragments by alkaline elimination and borohydride reduction; samples were then desalted by precipitation (three times) in 95% ethanol/1.3% CH₃CO₂K followed by resuspension in deionized water. Chondroitin/dermatan sulfate glycosaminoglycans were removed by digestion with chondroitinase ABC (0.5 units/ml, Seikagaku, Tokyo, Japan; 16 h at 37 °C). Samples were then dialyzed over 24–48 h against at least four changes of deionized water, and the presence of HS/lack of contaminating chondroitin or dermatan sulfate in the sample was verified by size exclusion chromatography ± heparinase I, II, and III digestion.

**IAPP Affinity Column**—Human IAPP (200 μg; Bachem, Torrance, CA) was covalently linked to AminoLink Plus Coupling Gel (Pierce, Thermo Fisher Scientific, Rockford, IL) according to the manufacturer’s instructions. Human IAPP was used for this and subsequent experiments as this form of IAPP is amyloidogenic, whereas mouse and rat IAPP are not (22). Furthermore, we have shown that β-T3 cell proteoglycans bind human but not rodent IAPP (12). Samples were applied to the IAPP affinity column equilibrated in 50 mM Tris-HCl, 0.15 M NaCl, pH 7.4 (Tris-buffered saline, TBS). For intact ^[35]S)sulfate-labeled proteoglycans, 200,000 dpm were loaded, eluted over a 0.15–3 M NaCl gradient in TBS, and finally washed in 8 M urea buffer containing 3 M NaCl. For double-labeled HS samples 100,000 dpm (based on ^[3]H incorporation to yield equal quantities of HS) were loaded, the column washed in TBS containing 0.15 M NaCl and bound material eluted in TBS containing 3 M NaCl, followed by a final wash in 8 M urea buffer containing 3 M NaCl. Eluted material was analyzed by SDS-PAGE (for intact proteoglycans; 20,000 dpm per lane was loaded) or was subjected to heparinase I or heparinase III enzyme digestion (for HS samples; see details below) followed by Bio-gel P10 size exclusion chromatography.
**β-TC3 Heparan Sulfate and IAPP Binding**

Solid Phase IAPP Binding Assay—β-TC3 proteoglycans were subjected to Sepharose CL-4B size exclusion chromatography and subdivided into three fractions based on molecular weight. IAPP binding of proteoglycans by solid phase assay was assessed in fractions M1 and M2 only, as these fractions but not M3 were shown to bind amyloidogenic human IAPP by affinity chromatography. Proteoglycans were concentrated by precipitation in 95% ethanol containing 1.3% potassium acetate, reconstituted in transfer buffer (12.5 mM Tris, 96 mM glycerine, 10% (v/v) methanol), and 2,000 dpm per sample was applied to a prewet nitrocellulose membrane via a Bio-Dot microfiltration system (Bio-Rad). Heparan sulfate or chondroitin/dermatan sulfate glycosaminoglycans were selectively removed from the immobilized proteoglycans by heparinase digestion (2.6 units/ml heparinase I, 1.3 units/ml heparinase II + 2.6 units/ml heparinase III, Sigma) or chondroitin ABC lyase digestion (0.1 units/ml, respectively, for 2 h at 37 °C. Control (undigested) samples were incubated in the absence of enzyme and negative (no proteoglycan) controls consisted of digestion buffer (0.1 M Tris, 10 mM calcium acetate, 18 mM sodium acetate, pH 7.0) alone. Following digestion, proteoglycans were incubated with 4% (w/v) nonfat dry milk in TBS (100 mM Tris, pH 7.5, 150 mM NaCl, 0.02% NaN₃) containing 0.1% (v/v) Tween 20) for 3 h at room temperature followed by overnight incubation at 4 °C with human IAPP (10 μg/ml in TBS + 0.1% (v/v) Tween 20). Bound IAPP was detected using the monoclonal anti-IAPP antibody F055 (kind gift from Amylin Corp., San Diego, CA; 1:100,000) and visualized using alkaline phosphatase-conjugated antibody (Bio-Rad). Heparan sulfate or chondroitin/dermatan sulfate glycosaminoglycans were selectively removed from the immobilized proteoglycans by heparinase digestion (2.6 units/ml heparinase I, 1.3 units/ml heparinase II + 2.6 units/ml heparinase III, Sigma) or chondroitin ABC lyase digestion (0.1 units/ml, respectively, for 2 h at 37 °C. Control (undigested) samples were incubated in the absence of enzyme and negative (no proteoglycan) controls consisted of digestion buffer (0.1 M Tris, 10 mM calcium acetate, 18 mM sodium acetate, pH 7.0) alone. Following digestion, proteoglycans were incubated with 4% (w/v) nonfat dry milk in TBS (100 mM Tris, pH 7.5, 150 mM NaCl, 0.02% NaN₃) containing 0.1% (v/v) Tween 20) for 3 h at room temperature followed by overnight incubation at 4 °C with human IAPP (10 μg/ml in TBS + 0.1% (v/v) Tween 20). Bound IAPP was detected using the monoclonal anti-IAPP antibody F055 (kind gift from Amylin Corp., San Diego, CA; 1:100,000) and visualized using alkaline phosphatase-conjugated anti-mouse immunoglobulins and enhanced chemiluminescence. Presence of residual proteoglycans on the nitrocellulose membrane following heparinase digestion was achieved using the heparan sulfate stubs antibody 3G10 (Seikagaku; 1:1000) and visualized as for anti-IAPP antibody.

**Compositional Analyses of HS**—Heparan sulfate from β-TC3 and NMuMG were analyzed in total HS preparations and in IAPP-bound and non-bound fractions following human IAPP affinity chromatography. Samples (10,000 dpm) were subjected to no treatment (intact HS), low pH (1.5) nitrous acid treatment (23), and heparinase I or heparinase III digestion to assess the proportion of N-sulfated highly sulfated or poorly sulfated heparan sulfate disaccharides, respectively (2.6 units/ml for each enzyme, digested for 2 h at 37 °C). Reaction products were separated by size exclusion chromatography on a Bio-gel P10 column equilibrated in 0.5 mM NH₄HCO₃ and/or Sepharose CL-6B equilibrated in TBS, pH 7.0, and the content of [³H]glucosamine and [³⁵S]sulfate-labeled HS per fraction was determined by liquid scintillation counting. Undigested HS elutes in the void volume (V₅) of the Bio-gel P10 column (24) (data not shown) and elutes in the included volume of the Sepharose CL-6B column (12).

**Disaccharide Analysis**—Non-radiolabeled HS was quantified by 1,9-dimethylmethylene blue assay, with glycosaminoglycans of known concentration as standards (25). Analysis of heparan sulfate disaccharide abundance was performed by the Glycotechnology Core Resource at the University of California, San Diego. The limit of detection for heparan sulfate disaccharides is 0.5 pmol/liter.

**Chemical Desulfation**—Pyridine salts of HS samples (10 μg for each) were generated by ion exchange on Dowex 50 (H⁺) under acidic conditions followed by neutralization with pyridine. These samples were then incubated in 10% (v/v) methanol in dimethyl sulfoxide for 18 h at 80 °C, adjusted to pH 9 with NaOH, dialyzed against several changes of deionized water and lyophilized (26, 27).

**Human IAPP Fibril Formation Assay**—For these studies, IAPP was obtained commercially (Bachem) or was kindly provided by Dr. Daniel Raleigh (State University of New York, Stony Brook, NY). IAPP fibril formation alone and in the presence of HS samples did not differ by source of IAPP. For the end point fibril formation assay, HS samples (12.5 μl in duplicate) in water were added to an equal volume of synthetic human IAPP peptide to give final concentrations of 25 μg/ml for HS and 100 μg/ml (25 μM) IAPP, yielding a 1:4 (wt/wt) HS:IAPP ratio. For the kinetic fibril formation assay, this HS:IAPP ratio was titrated to ensure good resolution of lag phases and maximal fibril formation within the 132-h time frame of the assay; a 1:32 HS:IAPP ratio was used for these studies. IAPP stock solution (1.6 mM) was filtered (0.2 μm), lyophilized, and reconstituted (final concentration 48 μg/ml) immediately prior to use to ensure no aggregates were present at baseline. Sulfated or desulfated HS samples used at a final concentration of 1.5 μg/ml. For both studies, control samples were IAPP alone (positive control for fibril formation) and HS samples with no IAPP (negative controls). Thioflavin T (4 μM) was added to samples, and fibril formation assessed by fluorescence at 480 nm/530 nm. For the end point assay, fibril formation was assessed over a period of 6 h, and data are reported after 30 min, which is representative of the whole time course. For the kinetics assay, data were collected every 10 min over 132 h, and data are shown for the whole period.

**Data Analysis**—Data shown are representative for the following sample sizes. For intact proteoglycan studies, IAPP affinity chromatography was performed twice, and solid phase binding assay was performed at least three times on two independent proteoglycan preparations. For HS analyses, IAPP affinity columns for radiolabeled samples were performed on three independent preparations; radiolabeled composition studies (total and after IAPP column) were performed on the same preparations, with each analysis being performed at least twice. Disaccharide analysis was performed on two independent HS samples. IAPP affinity columns for non-radiolabeled HS samples were performed on two independent preparations, one of which was used for disaccharide analysis, and both of which were used for fibril formation assays. Chemical desulfation was performed on two independent preparations of HS, both of which were used to verify desulfation by disaccharide analysis, and one of which was used for fibril formation assay.

**RESULTS**

**Binding of β-Cell Proteoglycans to Amyloidogenic Human IAPP Requires HS**—We have previously shown that β-TC3 proteoglycans from medium and cell layer bind human IAPP equivalently (12); therefore, we only analyzed the binding ability of proteoglycans from medium for the first portion of the study. [³⁵S]sulfate-labeled total medium proteoglycans were
applied to an IAPP affinity column and eluted over a 0.25–3 M NaCl gradient (Fig. 1A). The six fractions demarcated in Fig. 1A were collected, and the nature of the proteoglycans in these fractions was then analyzed by SDS-PAGE (Fig. 1B). Proteoglycans in the low molecular weight M3 pool (eluted in fraction 1), which we have previously shown to be comprised of chondroitin/dermatan-free glycosaminoglycans (12), did not bind IAPP, demonstrated by elution at low NaCl. Material from pool M2 contains proteoglycans that bound IAPP with intermediate (fractions 2–4) and high affinity (fractions 5 and 6), whereas proteoglycans from M1 bound IAPP with high affinity (eluted in fractions 5 and 6).

IAPP binding of the proteoglycans in pools M1 and M2 was further investigated by solid phase binding assay. As expected, proteoglycans from both M1 and M2 bound IAPP (Fig. 1C, upper row). Binding of proteoglycans from both M1 and M2 was markedly reduced following treatment with heparinas I, II, and III (Fig. 1C, middle row), suggesting that the binding of IAPP by β-TC3 proteoglycans requires HS. Chondroitinase ABC treatment was not associated with a reduction in IAPP binding (Fig. 1C, lower row). If anything, chondroitinase treatment resulted in an increase in IAPP binding, suggesting both that chondroitin/dermatan sulfate are not important for the binding of IAPP and may even impede the binding of IAPP by β-TC3 HSPGs. Thus, HS from β-TC3 proteoglycans in both pools M1 and M2 appeared to mediate IAPP binding.

**Composition of HS from β-TC3 and NMuMG Cells**—For compositional analysis of HS, we used total HS pooled from both medium and cell layer. As described above, we have previously shown that glycosaminoglycans from β-TC3 medium versus cell layer are very similar with respect to molecular weight, heparinase/chondroitinase sensitivity, and IAPP binding capacity (12). Furthermore, β-TC3 HS from both M1 and M2 fractions were capable of binding human IAPP (Fig. 1). Finally, HS from fractions M1 and M2 had very similar composition based on sensitivity to nitrous acid at pH 1.5, and to heparinase I or heparinase III (data not shown). HS from NMuMG cells served as a control for all subsequent analyses.

First, the proportion of N-sulfated disaccharides was assessed by cleavage with nitrous acid at pH 1.5. Surprisingly, β-TC3 cell HS was relatively insensitive to nitrous acid at pH 1.5. Surprisingly, β-TC3 cell HS was relatively insensitive to nitrous acid treatment as demonstrated by the persistence of larger molecular weight [3H]glucosamine-labeled oligosaccharides for β-TC3 (Fig. 2A, solid circles) compared with NMuMG HS (Fig. 2B, solid circles). As expected, almost all [35S]sulfate-labeled material (open circles) for both β-TC3 and NMuMG cells eluted at the total column permeation volume (Vt) following nitrous acid treatment (pH 1.5), consistent with liberation of N-sulfates.
β-TC3 Heparan Sulfate and IAPP Binding

FIGURE 2. Bio-gel P-10 size exclusion chromatography of HS from β-TC3 (A) and NMuMG cells (B), labeled with [3H]glucosamine (closed circles) and [35S]sulfate (open circles), following deaminative cleavage with nitrous acid. Nitros acid treatment resulted in the liberation of free sulfate from both β-TC3 and NMuMG HS, whereas [3H]glucosamine labeling showed the persistence of significantly larger oligosaccharides in β-TC3 compared with NMuMG HS. This size distribution suggests a low level of N-sulfation of HS from β-TC3 cells.

from glucosamine residues as free sulfate (Fig. 2, A and B). Of note, however, a small peak (~10% of total counts) of sulfated material remained at the $V_o$ in β-TC3 cell HS following nitrous acid treatment, suggesting that β-TC3 HS contained a minor component of O-sulfated residues in the absence of N-sulfation in the same or neighboring disaccharide. Second, to determine the presence of highly sulfated disaccharides, β-TC3 and NMuMG HS were digested with heparinase I. β-TC3 HS was relatively insensitive to heparinase I treatment (Fig. 3A), with only 29% of labeled HS showing heparinase I sensitivity, compared with NMuMG HS where virtually all HS was sensitive to heparinase I (Fig. 3B). In addition, this analysis using Sepharose CL-6B showed that HS from NMuMG cells was larger than that from β-TC3 cells. Third, to determine the proportion of poorly sulfated disaccharides, β-TC3 and NMuMG HS were digested with heparinase III. In this case, β-TC3 HS was more sensitive to heparinase III digestion, demonstrating higher overall sulfation in this HS preparation.

Composition of IAPP-bound and Non-bound HS—Given our unexpected finding that β-TC3 HS contains relatively few regions of highly sulfated disaccharides compared with NMuMG HS.

The findings for the radiolabeled enzyme digestion composition studies were confirmed by disaccharide analysis (Table 1). The proportion of N-acetylated disaccharides was higher in β-TC3 HS than in NMuMG HS. Conversely, the proportion of N-sulfated disaccharides was ~60% lower in β-TC3 than NMuMG HS. Other sulfated disaccharides were more frequent in NMuMG HS, especially the trisulfated UA-2S-(1,4)-GlcNS-6S that was three times more abundant in NMuMG HS than in β-TC3 HS.

Compartmental analysis of radiolabeled HS from β-TC3 and NMuMG cells showed significant differences in the proportion of heparan sulfate that was capable of binding IAPP and whether the sulfation of each bound and non-bound fraction differed. Surprisingly, the proportion of heparan sulfate glucosaminoglycans that bound human IAPP was similar between β-TC3 cells and NMuMG cells (63 ± 15% and 68 ± 19%, respectively, data are mean ± S.D.). The sulfation patterns of IAPP-bound and non-bound HS from each cell type was assessed by heparinase I or III digestion. IAPP bound HS from β-TC3 cells was relatively insensitive to heparinase I (Fig. 5A;
that NMuMG-derived HS is heterogeneous and that two pop-
to bind IAPP. Furthermore, it raises the interesting observation
rinase I sensitive material eluting in or near the Bio-gel P10
cells had a very different enzyme sensitivity profile, with hepa-
In marked contrast, non-bound heparan sulfate from NMuMG

To rule out the possibility that the non-bound fraction had not

Disaccharide analysis of total HS from β-TC3 and NMuMG cells

| Disaccharide                  | β-TC3 HS | NMuMG HS |
|------------------------------|----------|----------|
| UA-(1,4)-GlcNAc              | 82 ± 14  | 49 ± 3   |
| UA-(1,4)-GlcNS               | 8 ± 8    | 21 ± 3   |
| UA-(1,4)-GlcNAc-6S           | 4 ± 1    | 13 ± 9   |
| UA-2S-(1,4)-GlcNAc           | Not detected | 3 ± 0.6 |
| UA-(1,4)-GlcNS-6S            | 2 ± 2    | 4 ± 1    |
| UA-2S-(1,4)-GlcNS            | 2 ± 2    | 4 ± 1    |
| UA-2S-(1,4)-GlcNS-6S         | 2 ± 2    | 6 ± 1    |
| Total                        | 100      | 100      |

About 20% of [35S]sulfate-labeled material eluted in included
volume) and sensitive to heparinase III (Fig. 5B), similar to that
seen in the whole preparation (Figs. 3A and 4A). Non-bound
material from β-TC3 cells had similar sensitivity to heparinase
I (Fig. 5C; 25% of material eluted in the included volume) and
heparinase III (Fig. 5D) compared with IAPP-bound material.
To rule out the possibility that the non-bound fraction had not
bound IAPP on the affinity column due to saturation of binding
sites, non-bound β-TC3 HS was collected and reapplied to the
IAPP affinity column. Only 9.0 ± 0.8% of previously non-bound
material bound to the IAPP affinity column (compared with
63% of the total HS sample). Thus, the non-bound material is
truly incapable of binding IAPP, despite the fact that its com-
position does not appear to differ from that of the human IAPP-
bond β-TC3 HS.

In contrast to the findings for β-TC3 cell HS, IAPP-bound
HS from NMuMG cells was extremely sensitive to heparinase I
digestion (Fig. 6A) with heparinase III digestion yielding mainly
large fragments eluting in or near the Bio-gel P10 V0 (Fig. 6B).
In marked contrast, non-bound heparan sulfate from NMuMG
cells had a very different enzyme sensitivity profile, with hepa-
rinase I sensitive material eluting in or near the Bio-gel P10 V0
(Fig. 6C), but showing greater sensitivity to heparinase III diges-
tion (Fig. 6D). This demonstrates that regions of highly sulfated
HS are important in determining the capability of NMuMG HS
to bind IAPP. Furthermore, it raises the interesting observation
that NMuMG-derived HS is heterogeneous and that two pop-
ulations of HS that have different arrangement of highly sul-
fated disaccharides can be separated from the same pool on the
basis of IAPP binding ability.

Disaccharide analysis confirmed that the composition of
IAPP-bound and non-bound HS from β-TC3 cells was similar
(Table 2), consistent with the size exclusion profiles shown in
Fig. 5. Disaccharide composition of NMuMG HS also did not
differ between IAPP-bound and non-bound material. This
finding was unexpected, given the marked difference in enzyme
sensitivity between the bound and non-bound fractions. How-
ever, these data are consistent with differences in the sequential
arrangement of the variously sulfated disaccharides between
the bound and non-bound fractions rather than a difference in
disaccharide composition per se. These data suggest that
regions of highly sulfated disaccharides occur more frequently
in the IAPP-bound NMuMG HS, generating regions of hepari-
nase I-sensitive material, whereas in the non-bound samples,
these highly sulfated disaccharides may be distributed more
evenly along the HS chain. HS size and the hypothesized
arrangement of sulfated and non-sulfated regions for each sam-
ple is illustrated in Fig. 7.

Ability of HS Preparations from β-TC3 and NMuMG Cells to
Stimulate IAPP Fibril Formation—To determine whether HS
from β-TC3 or NMuMG cells that bound or did not bind IAPP
had a differential effect to stimulate fibril formation, we per-
formed thioflavin T assays. As demonstrated in Fig. 8, IAPP
alone resulted in fibril formation as expected, and all HS sam-
ple had some effect to increase maximal fibril formation, as
demonstrated by increased thioflavin T fluorescence. HS from
β-TC3 cells had a moderate effect to stimulate IAPP fibril for-
mation, and this effect was similar in HS populations regardless
of whether or not they bound IAPP in the affinity column exper-
iments. In contrast, the highly sulfated population of
NMuMG HS that bound IAPP in the affinity column experi-
ment was more effective at stimulating IAPP fibril formation
compared with both the more poorly sulfated non-bound
NMuMG HS and to either IAPP-bound or non-bound β-TC3
HS.

Finally, the requirement of HS sulfation for stimulation of
fibril formation was tested. Chemical desulphation resulted in at
least an 80% decrease in abundance of sulfated disaccharides in β-TC3 and NMuMG HS, respectively. Intact HS from both β-TC3 and NMuMG cells markedly accelerated fibril formation (Fig. 9), resulting in a decrease in lag time from 72 h with IAPP alone to essentially zero. (Fibrils were already detectable at the first measurement.) Intact HS from both cell types also increased maximal fibril formation, with NMuMG HS having a greater effect to do so. In contrast, desulfated HS from β-TC3

FIGURE 5. Double-labeled (closed circles, [3H]glucosamine; open circles, [35S]sulfate) β-TC3 HS following application to and elution from an IAPP affinity column, and following heparinase I or heparinase III digestion. Reaction products were separated by Bio-gel P-10 size exclusion chromatography. IAPP-bound HS is shown in A (heparinase I digested) and B (heparinase III digested), whereas non-bound HS is shown in C (heparinase I digested) and D (heparinase III digested). β-TC3 HS showed similar composition regardless of IAPP binding ability, with both bound and non-bound material being relatively insensitive to heparinase I treatment but sensitive to heparinase III treatment.

FIGURE 6. Double-labeled (closed circles, [3H]glucosamine; open circles, [35S]sulfate) NMuMG HS following application to and elution from an IAPP affinity column, following heparinase I or heparinase III digestion. Reaction products were separated by Bio-gel P-10 size exclusion chromatography. IAPP-bound HS is shown in A (heparinase I digested) and B (heparinase III digested), whereas non-bound HS is shown in C (heparinase I digested) and D (heparinase III digested). NMuMG HS that bound IAPP has a markedly different composition from non-bound material. IAPP-bound HS from NMuMG cells was extremely sensitive to heparinase I digestion, but resistant to heparinase III digestion, suggesting the frequent presence of regions of highly sulfated disaccharides. In contrast, non-bound HS from NMuMG cells was relatively resistant to heparinase I digestion but was sensitive to heparinase III digestion, suggesting that this population of HS contained fewer regions of highly sulfated disaccharides.
DISCUSSION

We have shown that β-TC3 cells synthesize HS with few regions of highly sulfated disaccharides, a pattern that differs from the more conventional arrangement of sulfated domains seen in the control NMuMG cells. Fig. 7 summarizes our data, presenting a likely arrangement of sulfated regions. Both the ratio of sulfated to non-sulfated disaccharides, and their arrangement is similar for human IAPP-bound or non-bound HS from β-TC3 cells. It is likely, however, that a small difference exists between bound and non-bound material with the sequence or clustering of sulfated disaccharides creating an additional highly sulfated region (demonstrated by third black bar) in IAPP-bound β-TC3 HS. β-TC3 HS is similar to non-bound HS from NMuMG cells, except for the difference in molecular weight. The overall ratio of sulfated to non-sulfated disaccharides in NMuMG HS is similar between IAPP-bound and non-bound material, but the arrangement is different, such that larger regions of highly sulfated disaccharides are present in the IAPP-bound material.

and NMuMG cells did not enhance maximal fibril formation over that seen with IAPP alone. However, desulfated HS from both cell types still accelerated fibril formation, albeit less effectively than intact HS, with lag times at ~30 h for IAPP + desulfated β-TC3 HS and <20 h for IAPP + desulfated NMuMG HS.

resulted in marked differences in modification of a bacterial HS-like substrate (28). Specifically, in the absence of phosphoadenosine 5-phosphosulfate, N-deacetylation/N-sulfotransferase activity was uncoupled, leading to N-deacetylation at random intervals, even if phosphoadenosine 5-phosphosulfate was reintroduced. This contrasted with the coupled N-deacetylation/N-sulfotransferase activity in a sequential manner creating regions of consecutively sulfated disaccharides (28). Of note, these two different patterns of modification are similar to those we observed in the present study in β-TC3 and NMuMG HS samples, respectively.

Despite the low overall sulfation, HS from β-TC3 cells was capable of binding amyloidogenic human IAPP to the same extent as the much more highly sulfated HS synthesized by the control NMuMG cells. Interestingly, the composition of β-TC3 HS was very similar regardless of its ability to bind IAPP. Because this was an unexpected finding, we confirmed that the non-bound fraction was really incapable of binding IAPP by reapplying it to the IAPP column and noting that only 10% of the material bound. These data raise the possibility that there is
NMuMG were still capable of accelerating fibril formation relative to IAPP different HSPGs (possible that these different HS populations are derived from we used total HS from NMuMG cells for these analyses, it is sulfated domains, are synthesized by this cell type. Because carbohydrate composition but that differ in the arrangement of gests that two distinct populations of HS, with similar disaccharides into regions that are susceptible to heparinase I digestion resulted in a greater effect to enhance IAPP fibril formation. Despite the low overall sulfation of β-TC3 HS, it was capable of increasing IAPP fibril formation, and this effect was similar regardless of whether or not β-TC3 HS bound IAPP during affinity chromatography experiments. HS from NMuMG cells that did not bind IAPP was also equally effective in stimulating fibril formation compared with both β-TC3 samples. However, highly sulfated HS (IAPP-bound material from NMuMG cells) had the greatest effect to stimulate maximal IAPP fibril formation. We directly assessed the importance of HS sulfation to the stimulation of IAPP fibril formation for each cell type by comparing the effect of untreated versus chemically desulfated HS in a fibril formation kinetics assay. Desulfation of HS from both cell types ablated their ability to enhance maximal fibril formation relative to IAPP alone, suggesting that the degree of sulfation is critical in determining the extent to which HS can enhance total fibril formation. This observation is consistent with the previous report by Castillo et al. (11) that sulfation of HS determines fibril formation from IAPP and that perlecan or perlecan-derived HS from Engelbreth-Holm-Swarm sarcoma cells were effective at stimulating IAPP fibril formation, an effect diminished upon chemical desulfation of HS. However, our data show that desulfated HS was still able to accelerate IAPP fibril formation, although to a lesser degree relative to fully sulfated HS, with desulfated NMuMG HS having a greater effect to accelerate fibril formation than desulfated β-TC3 HS. An additional explanation for the more effective acceleration in fibril formation with NMuMG HS even after desulfation may be its larger size relative to β-TC3 HS. Thus, while confirming the previously published requirement for the presence of highly sulfated HS to markedly stimulate IAPP fibril formation, we have made the novel and interesting observation that highly sulfated HS is not required for IAPP binding or acceleration of fibril formation per se.

The low overall HS sulfation in β-TC3 cells could be a result of the use of this isolated cell line, which is derived from a transgenic mouse with β-cell expression of SV40 and is therefore transformed. However, our previous studies suggest that the degree of sulfate incorporation into HS in primary mouse islets is similar to that of β-TC3 cells (21). The analysis of HS composition in primary islets is complicated, however, by the multiple cell types present in islets. Although insulin and IAPP-producing β cells are the predominant cell type in islets, the islet has a complex cellular composition, making β-cell lines such as β-TC3 a more suitable model for analyses of β-cell HS such as those performed here.

3 R. L. Hull, M. J. Peters, S. Potter-Perigo, C. K. Chan, T. N. Wight, and M. G. Kinsella, unpublished observations.
The effect of β-TC3 HS to increase maximal IAPP fibril formation is relatively small (∼1.5-fold). However, it is important to note that increases in amyloid formation of similar magnitude (1.5-fold or less) in cultured islets from transgenic mice expressing amyloidogenic human IAPP (a model of islet amyloid deposition) result in significant increases in β-cell apoptosis (33, 34). Furthermore, because islet amyloid deposition is a cumulative process, with the extent of fibril formation being associated with increased β-cell loss and/or cell death in islet culture models (35), animal models (36, 37), and in humans with type 2 diabetes (4), even a small acceleration and/or increase in maximal IAPP fibril formation will likely have a dramatic impact on β-cell function and viability over the long term.

In summary, we have determined that β-cells synthesize poorly sulfated HS that is capable of both binding human IAPP and stimulating fibril formation. Although the stimulation of fibril formation occurs to a lesser degree than the more highly sulfated HS synthesized by NMuMG cells, this effect does not rely solely on sulfation. Our data raise several possibilities; that sulfated HS synthesized by NMuMG cells, this effect does not stimulate fibril formation to a lesser degree than the more highly and stimulating fibril formation. Although the stimulation of poorly sulfated HS that is capable of both binding human IAPP term.

References to reduce HS-IAPP interactions and ultimately prevent islet amyloid formation and its toxic effects in type 2 diabetes.

Acknowledgments—We thank John Miller for help developing the solid phase binding assay and members of the Wight laboratory and the Metabolism group at VA Puget Sound Health Care System for helpful discussions during performance of these studies.

REFERENCES
1. Hull, R. L., Westermark, G. T., Westermark, P., and Kahn, S. E. (2004) Islet amyloid: a critical entity in the pathogenesis of type 2 diabetes. J. Clin. Endocrinol. Metab. 89, 3629–3643
2. Westermark, P., and Wilander, E. (1978) The influence of amyloid deposits on the islet volume in maturity onset diabetes mellitus. Diabetologia 15, 417–421
3. Clark, A., Wells, C. A., Buley, I. D., Cruickshank, J. K., Vanhegan, R. I., Matthews, D. R., Cooper, G. J., Holman, R. R., and Turner, R. C. (1988) Islet amyloid, increased A-cells, reduced B-cells, and exocrine fibrosis: quantitative changes in the pancreas in type 2 diabetes. Diabetes Res. 9, 151–159
4. Jurgens, C. A., Toukatly, M. N., Fligner, C. L., Udayasankar, J., Subramanian, S. L., Zraika, S., Aston-Mourney, K., Carr, D. B., Westermark, P., Westermark, G. T., Kahn, S. E., and Hull, R. L. (2011) β-cell loss and β-cell apoptosis in human type 2 diabetes are related to islet amyloid deposition. Am. J. Pathol. 178, 2632–2640
5. Chargé, S. B., Esiri, M. M., Bethune, C. A., Hansen, B. C., and Clark, A. (1996) Apolipoprotein E is associated with islet amyloid and other amyloidoses: implications for Alzheimer’s disease. J. Pathol. 179, 443–447
6. Kahn, S. E., Andrikopoulos, S., and Verchere, C. B. (1999) Islet amyloid: a long-recognized but underappreciated pathological feature of type 2 diabetes. Diabetes 48, 241–253
7. Ohsawa, H., Kanatsuka, A., Tokuyama, Y., Yamaguchi, T., Makino, H., Yoshida, S., Horie, H., Mikata, A., and Kohen, Y. (1991) Amyloid protein in somatostatinoma differs from human islet amyloid polypeptide. Acta Endocrinol. 124, 45–53
8. Young, I. D., Ailles, L., Narindrasorasak, S., Tan, R., and Kisilevsky, R. (1992) Localization of the basement membrane heparan sulfate proteoglycan in islet amyloid deposits in type II diabetes mellitus. Arch. Path. Lab. Med. 116, 951–954
9. Wight, T. N. (1991) in Cell Biology of Extracellular Matrix (Hay, E., ed) pp. 45–78, Plenum Press, New York
10. Tomlinson, B. E., Blessed, G., and Roth, M. (1970) Observations on the brains of demented old people. J Neurol Sci 11, 205–242
11. Castillo, G. M., Cummings, J. A., Yang, W., Judge, M. E., Sheardoon, M. J., Rimvall, K., Hansen, J. B., and Snow, A. D. (1998) Heparan sulfate and specific glycosaminoglycan backbone of perlecan are critical for perlecan’s enhancement of islet amyloid polypeptide (amylin) fibril formation. Diabetes 47, 612–620
12. Potter-Perigo, S., Hull, R. L., Tsoi, C., Braun, K. R., Andrikopoulos, S., Teague, J., Bruce Vercrhe, C., Kahn, S. E., and Wight, T. N. (2003) Proteoglycans synthesized and secreted by pancreatic islet β-cells bind amylin. Arch. Biochem. Biophys. 413, 182–190
13. Park, K., and Vercrhe, C. B. (2001) Identification of a heparin binding domain in the N-terminal cleavage site of pro-islet amyloid polypeptide. Implications for islet amyloid formation. J. Biol. Chem. 276, 16611–16616
14. Castillo, G. M., Ngo, C., Cummings, J., Wight, T. N., and Snow, A. D. (1997) Perlecan binds to the β-amyloid proteins (Aβ) of Alzheimer disease, accelerates Aβ fibril formation, and maintains Aβ fibril stability. J. Neurochem. 69, 2452–2465
15. Ancsin, J. B., and Kisilevsky, R. (1999) The heparin/heparan sulfate-binding site on aposerum amyloid A. Implications for the therapeutic intervention of amyloidosis. J. Biol. Chem. 274, 7172–7181
16. McCubbin, W. D., Kay, C. M., Narindrasorasak, S., and Kisilevsky, R. (1988) Circular dichroism studies on two murine serum amyloid A proteins. Biochem. J. 256, 775–783
17. Esko, J. D., and Lindahl, U. (2001) Molecular diversity of heparan sulfate. J. Clin. Invest. 108, 169–173
18. Kreuger, J., Spillmann, D., Li, J. P., and Lindahl, U. (2006) Interactions between heparan sulfate and proteins: the concept of specificity. J. Cell Biol. 174, 323–327
19. Knox, S., Merry, C., Stringer, S., Melrose, J., and Whitehead, J. (2002) Not all perlecans are created equal: interactions with fibroblast growth factor (FGF) 2 and FGF receptors. J. Biol. Chem. 277, 14657–14665
20. Kato, M., Wang, H., Bernfield, M., Gallagher, J. T., and Turnbull, J. E. (1994) Cell surface syndecan-1 on distinct cell types differs in fine structure and ligand binding of its heparan sulfate chains. J. Biol. Chem. 269, 18881–18890
21. Hull, R. L., Zraika, S., Udayasankar, J., Kisilevsky, R., Szarek, W. A., Wight, T. N., and Kahn, S. E. (2007) Inhibition of glycosaminoglycan synthesis and protein glycosylation with WAS-406 and azaserine result in reduced islet amyloid formation in vitro. Am. J. Physiol. Cell Physiol. 293, C1586–C1593
22. Westermark, P., Engström, U., Johnson, K. H., Westermark, G. T., and Betsholtz, C. (1990) Islet amyloid polypeptide: pinpointing amino acid residues linked to amyloid fibril formation. Proc. Natl. Acad. Sci. U.S.A. 87, 5036–5040
23. Lindahl, U., Bäckström, G., Jansson, L., and Hallén, A. (1973) Biosynthesis of heparin. II. Formation of sulfamino groups. J. Biol. Chem. 248, 7234–7241
24. Kinsella, M. G., and Wight, T. N. (1988) Structural characterization of heparan sulfate proteoglycan subclasses isolated from bovine aortic endothelial cell cultures. Biochemistry 27, 2136–2144
25. Farnsdale, R. W., Buttle, D. J., and Barrett, A. J. (1986) Improved quantitation and discrimination of sulphated glycosaminoglycans by use of dimethylmethylene blue. Biochem. Biophys. Acta 883, 173–177
26. Nagasawa, K., Inoue, Y., and Kamata, T. (1977) Solvolytic desulfation of glycosaminoglyconuran sulfates with dimethyl sulfoxide containing water
or methanol. **Carbohydr. Res.** 58, 47–55

27. Kazatchkine, M. D., Fearon, D. T., Metcalfe, D. D., Rosenberg, R. D., and Austen, K. F. (1981) Structural determinants of the capacity of heparin to inhibit the formation of the human amplification C3 convertase. *J. Clin. Invest.* 67, 223–228

28. Carlsson, P., Presto, J., Spillmann, D., Lindahl, U., and Kjellén, L. (2008) Heparin/heparan sulfate biosynthesis: processive formation of N-sulfated domains. *J. Biol. Chem.* 283, 20008–20014

29. Rosenberg, R. D., Shworak, N. W., Liu, J., Schwartz, J. J., and Zhang, L. (1997) Heparan sulfate proteoglycans of the cardiovascular system: Specific structures emerge but how is synthesis regulated? *J. Clin. Invest.* 99, 2062–2070

30. Maccarana, M., Casu, B., and Lindahl, U. (1993) Minimal sequence in heparin/heparan sulfate required for binding of basic fibroblast growth factor. *J. Biol. Chem.* 268, 23898–23905

31. Schönherr, E., Järveläinen, H. T., Sandell, L. J., and Wight, T. N. (1991) Effects of platelet-derived growth factor and transforming growth factor-β 1 on the synthesis of a large versican-like chondroitin sulfate proteoglycan by arterial smooth muscle cells. *J. Biol. Chem.* 266, 17640–17647

32. Schönherr, E., Järveläinen, H. T., Kinsella, M. G., Sandell, L. J., and Wight, T. N. (1993) Platelet-derived growth factor and transforming growth factor-β 1 differentially affect the synthesis of biglycan and decorin by mononuclear arterial smooth muscle cells. *Arterioscler. Thromb.* 13, 1026–1036

33. Zraika, S., Aston-Mourney, K., Marek, P., Hull, R. L., Green, P. S., Udayasankar, J., Subramanian, S. L., Raleigh, D. P., and Kahn, S. E. (2010) Neprilysin impedes islet amyloid formation by inhibition of fibril formation rather than peptide degradation. *J. Biol. Chem.* 285, 18177–18183

34. Aston-Mourney, K., Hull, R. L., Zraika, S., Udayasankar, J., Subramanian, S. L., and Kahn, S. E. (2011) Exendin-4 increases islet amyloid deposition but offsets the resultant β-cell toxicity in human islet amyloid polypeptide transgenic mouse islets. *Diabetologia* 54, 1756–1765

35. Zraika, S., Hull, R. L., Udayasankar, J., Utzschneider, K. M., Tong, J., Gerchman, F., and Kahn, S. E. (2007) Glucose and time dependence of islet amyloid formation in vitro. *Biochem. Biophys. Res. Commun.* 354, 234–239

36. Hull, R. L., Andrikopoulos, S., Verchere, C. B., Vidal, J., Wang, F., Cnop, M., Prigeon, R. L., and Kahn, S. E. (2003) Increased dietary fat promotes islet amyloid formation and β-cell secretory dysfunction in a transgenic mouse model of islet amyloid. *Diabetes* 52, 372–379

37. Butler, A. E., Janson, J., Soeller, W. C., and Butler, P. C. (2003) Increased β-cell apoptosis prevents adaptive increase in β-cell mass in mouse model of type 2 diabetes: evidence for role of islet amyloid formation rather than direct action of amyloid. *Diabetes* 52, 2304–2314

38. Lindahl, B., and Lindahl, U. (1997) Amyloid-specific heparan sulfate from human liver and spleen. *J. Biol. Chem.* 272, 26091–26094