We characterized the interaction of amylin with heparin fragments of defined length, which model the glycosaminoglycan chains associated with amyloid deposits found in type 2 diabetes. Binding of heparin fragments to the positively charged N-terminal half of monomeric amylin depends on the concentration of negatively charged saccharides but is independent of oligosaccharide length. By contrast, amylin fibrillogenesis has a sigmoidal dependence on heparin fragment length, with an enhancement observed for oligosaccharides longer than four residues. Binding of heparin fragments to the positively charged primary sequence of amylin accelerates the fibrillization of the incompletely processed amylin pro-hormone considered herein, GAGs were also reported to accelerate amylin fibrillation catalytically. Short heparin fragments containing two- or eight-saccharide monomers protect against amylin cytotoxicity toward a MIN6 mouse cell model of pancreatic β-cells.

Type 2 diabetes accounts for ~90% of adult diabetes. The disease currently affects 200 million people worldwide, and its incidence is projected to rise to 300 million by 2025 (1). Like many complex diseases, type 2 diabetes has multifactorial origins (1–4). The disease is characterized by insulin resistance, which causes the pancreas to synthesize more of the hormones insulin and amylin (5). The late stages of the disease are associated with β-cell dysfunction and a loss of β-cell mass (3, 5).

Amylin (also known as islet amyloid polypeptide) is a 37-residue (4-KDa) endocrine hormone that is synthesized by pancreatic β-cells and co-secreted with insulin. In its normal state, amylin works together with insulin to control blood sugar (5). Additional amylin functions include suppressing appetite, slowing down the emptying of the stomach, and inhibiting glucagon secretion (5, 6). Like some 30 other polypeptides associated with human amyloid pathologies (6), amylin can undergo aggregative misfolding into fibrils with a cross-β structure (5, 7, 8). Although it is still uncertain whether fibrils or soluble oligomeric precursors are responsible for adverse effects (8–12), extracellular amylin aggregates have been implicated in the destruction of pancreatic β-cells during the progression of type 2 diabetes (5, 9, 13). Amylin is the main component of fibrillar amyloid deposits found in the interstitial fluid between pancreatic islet cells of type 2 diabetes patients tested post-mortem (3, 5, 14), and extracellular aggregates of amylin are toxic when added to cultures of β-cells (9, 13). Overexpression of human amylin has been found to correlate with β-cell apoptosis and diabetes-like symptoms in several transgenic mouse and rat models whose endogenous amylin does not fibrillize (15). Genetic evidence that amylin is involved in pathology comes from the familial S20G mutation, which leads to early onset of type 2 diabetes (16) and produces an amylin variant that aggregates more readily (17).

Although there has been much progress in understanding the biophysical and structural basis of amyloid diseases from studies on purified amyloidogenic polypeptides (6), the amyloid deposits found in situ often interact or are associated with a variety of cofactors (18). In the case of amylin, accessory molecules that could play a role in modulating pathology include insulin (19, 20), membrane lipids (20, 21), metals such as zinc (22), and glycosaminoglycans (GAGs) (23–26). GAGs are a class of heterogeneous negatively charged oligosaccharides composed of seven different types of repeating disaccharide units (27). GAGs and proteoglycans, particularly those composed of dermatan and heparan sulfate, are found ubiquitously in amyloid deposits formed in situ (28), and the heparan sulfate proteoglycan (HSPG) perlecan has been shown to co-localize with pancreatic amylin deposits isolated from patients with type 2 diabetes (26). GAGs and HSPGs enhance the rate of amylin fibrillation in vitro (24, 25, 29, 30) and stabilize fibrils once formed (18). In addition to the mature form of the hormone considered herein, GAGs were also reported to accelerate the fibrillation of the incompletely processed amylin pro-peptide (23, 31).

Here, we investigate the interactions of the GAG heparin with amylin fibrils. Although heparin is found primarily in the cytoplasmic granules of mast cells, its extensive characterization and similarity to heparan sulfate make it a suitable model for the GAG chains of HSPGs (23, 25, 29, 31, 32). The availability of fluorescein-tagged heparin made it possible to examine whether GAGs associate with amylin fibrils. The availability of

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* The on-line version of this article (available at http://www.jbc.org) contains supplemental Table S1 and Figs. S1–S3.
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2 The abbreviations used are: GAG, glycosaminoglycan; HSPG, heparan sulfate proteoglycan; HMWH, high molecular weight fraction of heparin; FH, fluorescein-labeled heparin; ThT, thioflavin T; TIRFM, total internal reflection fluorescence microscopy; dp, degree of polymerization; HSQC, heteronuclear single quantum correlation; DMSO, dimethyl sulfoxide.
a series of heparin fragments of defined length enabled us to look at how oligosaccharide size and charge content affect binding to amylin monomers, fibrillation kinetics, and amylin cytotoxicity.

**EXPERIMENTAL PROCEDURES**

**Materials**—Synthetically prepared human amylin with an amidated C terminus and a Cys-2–Cys-7 disulfide bond was from AnaSpec (Freemont, CA). Mature human amylin is naturally amidated at its C terminus, and amidation is required for hormone activity (5). Except for NMR experiments to look at the interactions of amylin monomers with heparin fragments, all experiments were done with the C-terminally amidated peptide, which is the physiological form of the hormone (5). For the NMR experiments, we used 15N-labeled recombinant amylin expressed in *Escherichia coli*, which was purchased from rPeptide (Bogart, GA). This peptide has a free α-carboxyl group at the C terminus, but we can conclude that the lack of C-terminal amidation has no bearing on results because the NMR signals from the C-terminal half of the peptide are unaffected by the oligosaccharides. Heparin-dp2 was from V-LABS (Covington, LA), whereas the heparin fragments dp4 through dp20 were from Iduron (Manchester, UK). The low molecular weight fraction of heparin (LMWH) (catalog number A16198) was from Alfa Aesar (Ward Hill, MA), and the high molecular weight fraction of heparin (HMWH) (catalog number H3393) was from Sigma. Thioflavin T (ThT) was from Sigma or AnaSpec (ultrapure grade). Fluorescein-labeled heparin (FH) with an average molecular mass of 18 kDa and Alamar Blue were from Invitrogen. All other chemicals for solutions and buffers were from Fisher.

**NMR Spectroscopy**—Lyophilized aliquots of 0.1 mg of 15N-labeled amylin were suspended in 500 μL of 20 mM sodium phosphate buffer, pH 6.0, in 90% H2O, 10% D2O to give a 50 μM peptide concentration. A temperature of 10 °C was used for NMR experiments to avoid exchange of amylin amide protons with solvent. The binding of heparin fragments was monitored using two-dimensional 1H-15N HSQC spectra collected with 1H × 15N spectral windows of 6000 × 1200 Hz and matrix sizes of 2048 × 32 complex points. The experiments were done on a 600-MHz Varian Inova spectrometer equipped with a cryogenic probe. Assignments for amylin 1H-15N HSQC spectra were from the literature (33).

**ThT Assays of Fibrillation Kinetics**—Stock solutions of amylin were prepared in 100% DMSO, which disaggregates amylin (34), and stored at −80 °C when not in use. Starting from the 100% DMSO stock solutions, samples for fibrillation reactions were prepared to contain final concentrations of 5 μM amylin and 10 μM ThT in 20 mM sodium phosphate buffer, pH 7.4, with 4% (v/v) DMSO. To reduce the dead time for the reactions, amylin was the final component added to the solutions. The reaction mixtures of 200 μL were placed into 96-well clear-bottom plates from Corning (Corning, NY) and sealed with clear polyester sealing tape from Fisher to prevent evaporation. The plate was incubated at 25 °C without agitation. Fluorescence intensity was recorded at 1-min intervals with excitation at 440 nm and emission at 490 nm on a Fluoroskan Ascent 2.5 fluorescence plate reader.

Fibrillization lag times were obtained from the x axis value at the intersection of the lag phase baseline slope and the slope of the steepest part of the growth phase (35). Fibrillation rates were calculated by omitting the time points for the lag phase and fitting the initial part of the growth curve to the exponential function $y = A + B \times \exp(-kt)$ (35) where $k$ is the rate constant, $B$ is the initial amplitude, and $A$ is the plateau. ThT fluorescence plateau values were obtained from steady-state fluorescence intensities at 300 min.

**Fluorescence Spectroscopy**—Proximity between amyloid-bound ThT and FH was analyzed by fluorescence resonance energy transfer (FRET) (36) using a Fluorolog 3-22 spectrophotometer. Samples containing 5 μM amylin, 10 μM ThT, and 0.28 μM FH were loaded into a 4 × 4-mm quartz cuvette, and emission spectra (460–570 nm) were recorded at 6-min intervals for 3 h using excitation at 440 nm with a 4-nm band-pass filter.

To assess the amount of heparin incorporated into amylin fibrils (37), fibrils grown in the presence of 0.28 μM FH were run through an Amicon Ultra 100-kDa cut-off centrifugal filter at 13,000 rpm for 15 min. The filtrand containing fibrils was washed with deionized water, and the suspension was filtered a second time. Fluorescence spectra of the filtrates and filtrands were used to determine the fraction of FH tightly associated with fibrils.

**Fluorescence Microscopy**—Total internal reflection fluorescence microscopy (TIRFM) experiments were done on an Andor Revolution XD spinning disk confocal instrument with a Nikon TiE inverted microscope and an iXon EMCCD camera. ThT was excited with a 405 nm laser, and emission was filtered through a 483-nm band-pass filter. FH excitation was achieved with a 488 nm laser, and emission was filtered through a 523-nm band-pass filter.

**Transmission Electron Microscopy**—Aliquots from the stationary phases of fibrillation reactions containing either amylin or amylin and heparin fragments were blotted for 1 min onto a carbon-coated 400-mesh Maitaxform copper/rhodium grid from Ted Pella Inc. (Redding, CA) followed by negative staining with 1% uranyl acetate. EM images were recorded on an FEI Tecnai G2 BioTWIN transmission electron microscope equipped with an AMT XR-40 camera.

**Cytotoxicity Assays**—Cytotoxicity was measured using the mouse insulinoma MIN6 cell line model of β-pancreatic cells (38), which was a gift from Dr. Anil Rustgi (University of Pennsylvania). Cells were seeded to a density of 20,000/100-μL well in a black clear-bottom 96-well plate from BD Falcon containing DMEM medium from Invitrogen with 15% fetal bovine serum, 25 mM glucose, 2 mM L-glutamine, 500 mM sodium pyruvate, 5 μM β-mercaptoethanol, 1000 units/ml penicillin, and 100 μg/ml streptomycin. After 24 h of incubation at 37 °C, in a humidified incubator with 5% CO2, the culture medium was removed and replaced with fresh medium containing amylin or controls. The cells were incubated for another 24 h followed by the addition of 10% (v/v) Alamar Blue. Fluorescence, due to the reduction of the Alamar Blue dye by viable cells for 3 h, was measured using excitation and emission wavelengths of 544 and 590 nm, respectively (8). Cell viability (in percentage) was calculated from the ratio of Alamar Blue fluorescence in treated cells to cells seeded in DMEM media alone. Uncertainties were
Heparin Enhancement of Amylin Fibrillization

calculated from the standard errors of measurements done in triplicate.

Amylin was prepared by dissolving lyophilized peptide in 100% DMSO to a 1.1 mM concentration for fresh amylin and 4 mM for experiments with mature amylin fibrils. The stock solutions were sonicated continuously for 5 min at 75% amplitude before use. To look at the concentration dependence of cytotoxicity with fresh amylin, the amylin stock solution (1.1 mM) was diluted with reaction buffer and culture medium to final concentrations of 1, 5, 10, 40, and 80 μM. For studies with mature amylin fibrils, fibrils were formed for 48 h at 25 °C using amylin monomer concentrations of 10, 50, 100, 400, and 800 μM. Aliquots of the fibril suspensions were then added to the culture medium to achieve final amylin concentrations equivalent to those used with the freshly prepared peptide (1, 5, 10, 40 and 80 μM). For these experiments, we assumed that the higher amylin concentrations used to prepare mature fibrils would affect fibrillation rates but not the cytotoxicity of the fibrils.

To look at the effects of heparin fragments on cytotoxicity with fresh amylin, cells were incubated in the presence of 40 μM amylin and 80 μM oligosaccharides (polymer concentration). For mature fibrils, aliquots of a fibril suspension grown from 400 μM amylin and 800 μM oligosaccharides over 48 h were diluted 10-fold to obtain equivalent final concentrations expressed in amylin monomer equivalents (39).

RESULTS

Heparin has previously been shown to enhance amylin fibril formation (25, 29). To explore the interaction between the glycosaminoglycan and the polypeptide, we looked at a series of heparin fragments of defined length ranging from a 2-mer to a 20-mer. The fragments are products of the digestion of porcine high-grade heparin by bacterial heparinase and were purified by high-resolution gel filtration to a uniform fragment length or “degree of polymerization (dp)” (40): dp2 (0.66 kDa), dp4 (1.33 kDa), dp8 (2.66 kDa), dp12 (4.00 kDa), dp16 (5.32 kDa), and dp20 (6.66 kDa). The main building block of the fragments (75%) is the 1S disaccharide IdUA,2S-GlcNS,6S, which has four negatively charged groups: three sulfates and one carboxylate. Because sulfation of heparin is done by sulfotransferase enzymes after the polymer is synthesized, there is some variation in the sulfate content of the disaccharide building blocks, with about 75% in the trisulfated form and 25% in the monosulfated forms (40). For the molecular masses quoted above, we assumed full sulfation of the parent disaccharides, although in practice, the average sulfation of the heparin parent fragment is 2.5 (29), leading to a 40-dalton lower molecular mass per disaccharide as compared with the fully sulfated form. In addition to the fragments of defined length, we also did experiments on an LMWH with an average molecular mass of 5 kDa (4 – 6 kDa) and an HMWH that contains fragments from 6 to 30 kDa with most chains in the 17–19-kDa range.

When considering the heparin fragments, it is important to note that both the molecular mass and the number of charges increase with increasing fragment length. Unless otherwise stated, the concentrations of the heparin fragments quoted in this work are polymer concentrations (e.g. [dp20] or [dp16]).

For experiments in which we want to vary the chain length but keep the ionic strength constant, it is more useful to work with concentrations expressed in terms of saccharide monomers (e.g. [dp20]/20, [dp16]/16), and we explicitly note in those cases that we are reporting the saccharide monomer concentration.

Heparin Binds to the Positively Charged N-terminal Half of Amylin Monomers—Fig. 1A shows a two-dimensional 1H-15N HSQC spectrum of 50 μM amylin at pH 6 and a temperature of 10 °C. Under these conditions, we see a single set of resonances corresponding to amylin monomers. Any fibrils formed during the 30-min time used to acquire the spectrum would be too large to be seen by NMR. We detected 1H-15N correlations for all 37 residues in the peptide except Lys-1–Asn-3, which are lost due to fast hydrogen exchange with solvent. In the presence of an equimolar concentration of the dp2 heparin fragment, the 1H-15N HSQC spectrum of amylin is unaffected (Fig. 1B). By contrast, in the presence of an equimolar concentration of the longer dp16 fragment (50 μM), resonances from residues 1–20 are broadened beyond detection, whereas resonances from residues 21–37 persist (Fig. 1C). The cross-peaks that remain in the presence of dp16 fragment show chemical shift changes that become progressively smaller, going from residue 21 to residue 37. The effects on the NMR spectrum of amylin could be due to the longer size of the dp16 fragment or to the presence of eight times as many negatively charged saccharide monomers as compared with dp2.

To distinguish between these possibilities, we looked at the NMR spectra of amylin in the presence of an excess of the dp2

![Figure 1](image-url)
fragment. At a 2:1 molar ratio of dp2:amylin, the NMR spectrum of amylin is largely unperturbed except for selective partial broadening of cross-peaks from residues 4–6, 10–12, and 18–21. These segments are close to the only four charged groups in the amylin sequence: the α-amino group, Lys-1, Arg-11, and His-18. Of these, the cross-peak for His-18 shows the most pronounced decrease in intensity. At molar ratios of 8:1 dp2:amylin or above, the effects on the amylin NMR spectrum were similar to those obtained at 50 μM dp16, with residues 1–20 broadened beyond detection (Fig. 1D and supplemental Fig. S1). Thus when the dp2 concentration is increased 8-fold to give the same 400 μM charged saccharide monomer concentration as with an equimolar ratio of amylin:dp16, we see analogous effects on the NMR spectrum of the amylin monomer. This indicates that binding of heparin to amylin in its monomeric state depends on the amount of charged saccharide monomers rather than on the length or conformational properties of heparin. The NMR line broadening suggests intermediate exchange on the chemical shift timescale. The range of concentrations over which the effects are observed points to a $K_d$ of ~200 μM for the binding of disaccharides to monomeric amylin.

**Fibrillization Enhancement Depends on Heparin Oligosaccharide Length**—In contrast to the binding of heparin to amylin monomers, enhancement of amylin fibrillization depends on heparin fragment length. Amylin fibrillation kinetics were followed using the ThT assay; the marked changes in the fluorescence properties of this dye when it binds amyloid cross-β sheet structures make it a specific probe for fibrils (34, 37, 41–43). We first looked at the effects on amylin fibrillization of heparin fragments ranging from 2 to 20 saccharide monomers included in the reactions at a constant polymer concentration of 10 μM (Fig. 2A). With increasing length, heparin shortens the lag times for fibrillation, increases fibril growth rates, and increases the value of the steady-state ThT fluorescence plateau at the end of the reactions. In addition to the dp2–dp20 series, we also looked at the LMWH and HMWH fractions, using average molecular mass of 5 and 18 kDa to calculate a 10 μM concentration (Fig. 2A). Although the effects of the heparin fragments of defined length level off beyond dp12, the heterogeneous heparin fractions enhance amylin fibrillation more than any of the dp2–dp20 fragments. The effects of LMWH and HMWH are difficult to interpret because they contain a heterogeneous mixture of heparin fragments with a poorly defined length distribution. The data for the series of defined-length fragments (dp2–dp20) shows that fibrillation is accelerated with increasing oligosaccharide size. In a mixture of short and large heparin fragments, the effects of the longest would be expected to predominate so that the enhancement of fibrillation would not scale with the average molecular weight. Consequently we did not include LMWH and HMWH in our analysis of amylin fibrillation kinetics.

The trend of increasing fibrillation rates with larger heparin fragments in Fig. 2A could reflect the increasing length of the oligosaccharide chains or could be due to an increase in the concentration of charged saccharide monomers as seen for the NMR experiments monitoring binding of heparin fragments to amylin monomers (Fig. 1). To distinguish between these possibilities, we carried out fibrillation reactions varying the concentration of the smallest dp2 fragment between 50 and 500 μM (Fig. 2B). The fibrillation kinetics showed only small variations and no systematic trend with increasing concentration of dp2 (Fig. 2B). This indicates that the enhancement of amylin

![FIGURE 2. Effects of heparin fragments on amylin fibrillization. A, fibrillization at a constant heparin polymer concentration of 10 μM. B, fibrillation over a 10-fold range of dp2 concentrations from 50 to 500 μM. C, fibrillation over a range of fragment concentrations selected to keep the saccharide monomer concentration constant at 200 μM. The control sample in each series is amylin without heparin. The data are presented as averages of duplicate experiments. Representative standard errors are shown for the LMWH sample in panel A.](image-url)
fibrillization depends primarily on oligosaccharide length rather than on the amount of charged saccharide monomers. Finally, we carried out fibrillization reactions in the presence of heparin fragments of different lengths, varying the polymer concentration to maintain a fixed saccharide monomer concentration of 200 μM (Fig. 2C). With the charged saccharide monomer concentration fixed, we once again saw an increase in amylin fibrillization that depended only on the lengths of the heparin oligosaccharides.

Fig. 3 summarizes how the three parameters that characterize the kinetics of the amylin fibrillation depend on heparin fragment length. We focus our analysis on the reactions where the heparin fragment length varies but the charged saccharide monomer concentration is fixed (Fig. 2C). With increasing oligosaccharide length, the lag time for fibrillization decreases (Fig. 3A), whereas the elongation rates (Fig. 3B) and steady-state fluorescence plateaus (Fig. 3C) increase. All three parameters show a sigmoidal dependence on oligosaccharide length, starting at dp4, reaching a mid-point near dp6, and leveling off beyond dp12. Fibrillation reactions in the presence of a constant oligosaccharide polymer concentration show a similar but less well defined sigmoidal dependence on heparin fragment length (supplemental Fig. S2).

To demonstrate that fibrils are formed in the presence of heparin fragments, we obtained electron microscopy (EM) data on aliquots taken from the stationary phases of the amylin fibrillization reactions of the control, dp2, dp8, and dp16 samples shown in Fig. 2A (Fig. 4). The images for amylin fibrils grown in the presence of heparin fragments are similar to those obtained for the control sample containing amylin alone. Although EM does not lend itself to quantifying the amounts of fibril formed, we saw a qualitative increase in the number of fibrils in the presence of the longer heparin fragments. The amylin fibrils grown in the presence of dp8 and dp16 fragments also appeared to be longer than those from the dp2 or control samples (Fig. 4).

Co-localization of Heparin with Amylin Fibrils by FRET—Immuno-histochemical studies have previously shown that the HSPG perlecan is associated with plaques of amylin isolated from patients with type 2 diabetes (26). Given the complex composition of the amyloid plaques, which can include a variety of proteins and cofactors besides HSPGs (18, 28), we wanted to ascertain more directly whether GAGs are associated with amyloid fibrils using heparin as a model. To this end, we used FH with an average molecular mass of 17 kDa that has one fluorescein conjugated per heparin irrespective of the oligosaccharide size (44).

Fig. 5A compares amylin fibrillization kinetics in the presence of ThT alone, FH alone, and both dyes. The data in the presence of ThT alone are typical of a fibrillization reaction. With FH alone, the fluorescence is nearly constant as amyloid starts to form. Fluorescein does not undergo the large changes in fluorescence properties that are a hallmark of the binding of ThT dye to amyloid fibrils (42). Moreover, the spectral parameters were selected for the detection of amyloid-bound ThT (excitation 440 nm, emission 490 nm) rather than fluorescein (excitation 488 nm, emission 518 nm). In the presence of both ThT and FH, we observed an ~50% reduction in the quantum yield of the amyloid-bound ThT donor due to FRET to the FH acceptor fluorophore (Fig. 5A). Additional control experiments were done to rule out that the hydrophobic fluorescein dye could be driving the interaction with amylin fibrils. FRET was only observed when fluorescein was conjugated to heparin to form FH, and the effect was reduced when FH was competed with unlabeled heparin (supplemental Fig. S3).

To further characterize FRET between FH and amyloid-bound ThT, we collected emission spectra on a fluorescence spectrophotometer using excitation at 440 nm (Fig. 5B). As amylin fibrils started to form in the reaction, between 10 and 158 min, we saw an increase in fluorescence at 478 nm due to
fibril-bound ThT (42) and at 508 nm due to FRET between the ThT donor and FH acceptor. The increases in fluorescence are accompanied by a blue shift in the FH emission maximum from 513 to 508 nm (Fig. 5B) as amylin fibrils start to grow. The increase in fluorescence at 508 nm was about half that at 478 nm (Fig. 5C), consistent with the previously observed 50% FRET from ThT to FH (Fig. 5A). The experiments indicate that the two fluorophores come within a 1–10-nm FRET distance (36) of each other in the presence of amylin fibrils.

Co-localization of Heparin with Amylin Fibrils by TIRFM—We next examined the association of FH with amylin fibrils using TIRFM. The TIRFM technique has been previously used to image a variety of amyloid fibrils through the fluorescence of the non-covalently bound dye ThT (41, 43, 45). In the first experiment, we started with amylin fibrils grown in the presence of ThT, which were imaged using a 483-nm emission filter after excitation with a 405 nm laser. After imaging the fibrils using ThT fluorescence, the solution containing ThT was removed from the coverslip and replaced with a solution containing FH. The same area was used to image fibrils using FH fluorescence, detected using a 523-nm emission filter after excitation with a 488 nm laser. As compared with the ThT image, the FH image is more diffuse, but the same fibrils are observed (Fig. 6A).

ThT may remain associated to the fibrils after the addition of FH in the experiment shown in Fig. 6A. The spectral parameters used to obtain the image after the addition of FH (488 nm excitation, 513 nm emission) should be selective for FH, although there is a chance we could still be detecting ThT fluorescence because the emission maxima of ThT and FH are separated by only ~25 nm. To rule out that the fibrils were detected through ThT fluorescence, we did a second experiment in which we grew fibrils in the presence of only FH (Fig. 6B). We once again obtained images of species with a fibrillar morphology detected using FH fluorescence. The solution containing FH was replaced by a ThT solution followed by imaging of the same section of the coverslip using ThT fluorescence filters. As compared with the image obtained using ThT fluorescence, the image obtained using FH fluorescence shows additional globular species, which are no longer seen when the FH solution is replaced with the ThT solution. Presumably these objects were too weakly attached to the glass coverslip to withstand dilution when the FH solution was replaced with the ThT solution. A number of species with fibrillar morphology (indicated by arrows) are seen in both images, however, demonstrating that FH and ThT bind to the same individual fibrils. In this regard, it is interesting to note that a very recent confocal microscopy study reported co-localization of FH with amyloid β fibrils (46).
In a final experiment, we grew amylin fibrils in the absence of fluorophores and then added FH. Within 5 min of adding FH, we saw species with a fibrillar morphology (Fig. 6C). The experiment demonstrated that like ThT (41), FH can bind rapidly to fibrils after they are formed.

Amylin Fibrils Contain Weakly and Tightly Bound Heparin—To determine whether heparin is incorporated into amylin fibrils, we carried out a fibrillization experiment using 5 μM amylin in the presence of 0.28 μM FH. After the fibrillation reaction was complete, the sample was run through a 100-kDa cutoff centrifugal filter. A 36% fraction of the fluorescence was retained in the filter, whereas 63% passed through the filter corresponding to unbound FH or FH bound to amylin species with a molecular mass smaller than 100 kDa. The fibrils and FH retained in the filter were washed with deionized water and run through the 100-kDa filter a second time. After the second filtration, 30% of the initial fluorescence was retained in the filter, corresponding to an FH that is tightly bound to the fibrils (supplemental Table S1). The results of the experiment with amylin are similar to those previously reported for α-synuclein fibrils grown in the presence of FH (37), where 15% of FH was incorporated into the fibrils, 12% was weakly associated, and the remainder of the FH was not bound (37).

Effect of Heparin Fragments on Amylin Cytotoxicity—We next wanted to see whether heparin fragments had an impact on amylin cytotoxicity to a MIN6 mouse cell line model of pancreatic β-cells (38). There are conflicting reports regarding whether amylin is cytotoxic when added as a freshly dissolved peptide or in its fibrillar form (9, 13, 15, 47). To address this, we first looked at the dependence of cytotoxicity on amylin concentration using either freshly dissolved amylin or mature amylin fibrils formed over 2 days (48 h). With freshly dissolved amylin, we saw an apparent concentration mid-point at ~25 μM amylin, with cell viability reaching an ~50% asymptotic minimum at amylin concentrations ~40 μM (Fig. 7A, solid line). By contrast, with mature amylin fibrils, cytotoxicity appeared to be independent of peptide concentration within experimental error (Fig. 7A, dashed line), giving an average cell viability of 71 ± 0.6% over the five points sampled in the 1–80 μM amylin concentration range. The lowered cytotoxicity with mature fibrils as compared with freshly dissolved amylin is consistent with previous reports (13, 15, 47). The lack of a concentration dependence for mature fibrils is puzzling and could signify an equilibrium between non-toxic fibrils and a toxic species. The latter toxic species could be formed simultaneously with the fibrils or could dissociate from them when they are transferred to cell cultures. The lack of an apparent concentration dependence could be due to a limiting factor for the toxic species such as its solubility. Although with the mature fibrils the reaction has reached a steady state, with fresh amylin, the reaction is ongoing so that the toxic species could be replenished as the reaction proceeds in the presence of the cells. A possible precedent for such a mechanism has been recently reported for mouse prion, where the levels of a distinct toxic species are uncoupled from the titer of infective prion (48).

Fig. 7B summarizes the effects of heparin on cytotoxicity obtained with either fresh amylin or amylin fibrils. The first
column shows untreated cells. The Alamar Blue fluorescence of all other experiments was normalized to the untreated cells, which were assigned a cell viability of 100%. The next five experiments show controls. The 1% DMSO vehicle present in all experiments except the untreated cells had no effect on cell viability. The dp2, dp8, and dp20 heparin fragments by themselves also had little effect on cell viability. The bee venom toxin melittin, used as a positive control, reduced cell viability to 45% at a 5 μM concentration. The next series of five experiments (striped columns) looks at the effects of freshly dissolved amylin. Amylin alone at a 40 μM concentration reduced cell viability to 61%. Amylin together with an 80 μM concentration of the longest dp20 heparin fragment had a similar cell viability of 68%. Inclusion of the short dp8 and dp2 oligosaccharides res-

FIGURE 6. TIRFM images of fibrils formed from a 100 μM amylin sample detected with ThT and FH fluorophores. A, fibrils grown for 210 min in the presence of 15 μM ThT followed by the addition of FH to a final concentration of 0.3 μM. B, fibrils grown for 210 min in the presence of 0.3 μM FH followed by the addition of ThT to a final concentration of 15 μM. Arrows indicate examples of species with a fibrillar morphology that are observed in both images. C, fibrils grown for 240 min without dye followed by the addition of FH to a final concentration of 1 μM. The images on the right-hand side were obtained within 5 min of adding the respective fluorescence probes.
Heparin Enhancement of Amylin Fibrillization

FIGURE 7. Amylin cytotoxicity toward mouse MIN6 cells (38). The data are presented as means ± S.E. from experiments done in triplicate. A, concentration dependence of cytotoxicity for freshly dissolved amylin and amylin fibrillized for 2 days. B, effects of heparin fragments on amylin cytotoxicity. The first five experiments (white bars) are controls: untreated MIN6 cells; 1% DMSO, which was present in all experiments except the first; dp2, dp8, and dp20 oligosaccharides at 80 μM concentrations in the absence of amylin; and a 5 μM bee venom melittin positive control. The next five experiments (striped bars) are for freshly dissolved amylin alone or in the presence of oligosaccharides. The next five experiments (gray bars) are for amylin fibrils formed for 2 days in the absence or presence of the indicated oligosaccharides.

cued cell viabilities to levels of 93 and 96%, respectively. We also looked at the effects of adding a mixture of 80 μM dp2 and 80 μM dp20 to 40 μM amylin, which gave a cell viability of 90%. This value is greater than the average for the data for the individual dp2 and dp20 oligosaccharides (82%), indicating that the dp2 disaccharide has a protective effect even in the presence of the longer dp20 heparin fragment. The last set of five experiments looks at amylin fibrils (Fig. 7B, gray columns). The trends are similar, although less clear, because the fibril samples had less of an effect than freshly dissolved amylin. Amylin fibrils grown in the presence of the dp20 and dp8 oligosaccharides showed reduced cell viabilities of 66 and 73%, respectively. Fibrils grown in the presence of dp2 or of a mixture of the dp2 and dp20 fragments gave cell viabilities approaching those of the untreated cells.

DISCUSSION

HSPGs and GAGs are ubiquitous components of the extracellular matrix that are found in association with amyloid deposits formed by a variety of disease-linked proteins (18, 28) including amylin (26). GAGs have been shown to enhance the fibrillization of amylin in vitro (24, 25, 29). The interaction of heparin with proamylin has also been investigated because GAGs could play a role in amyloidosis of the incompletely processed hormone (23, 31). Finally, heparin is given as an anticoagulant to diabetic patients receiving pancreas transplants and could contribute to graft dysfunction by stimulating amylin fibrillization (49). To better understand how GAGs affect amylin dysfunction, we characterized the interaction of heparin with amylin and looked at the effects of heparin fragments on amylin cytotoxicity.

NMR experiments show that heparin binds to the positively charged N-terminal half of amylin (Fig. 1). Binding does not depend on the length of heparin fragments but on the concentration of negatively charged saccharide monomers, in as much as the broadening effects on the amylin NMR spectrum with dp16 can be replicated with an 8-fold excess of the shorter dp2 fragment. Neutralization of the positive charges on amylin by the negative charges on heparin would render the peptide more hydrophobic, thus facilitating aggregation and fibrillization. In this regard, it is worth noting that the loss of a single charge on His-18 at high pH dramatically increases the rate of amylin fibrillization (22, 34).

By contrast, the enhancement of amylin fibrillization by heparin depends on oligosaccharide length rather than the amount of charged monosaccharides (Fig. 2). The oligosaccharide length dependence is sigmoidal (Fig. 3), with an enhancement of fibrillogenesis starting at four monomers (dp4) and leveling off beyond 12 monomers (dp12). Heparin adopts a left-handed helix structure with a 21 symmetry axis and four sugar residues per turn, based on solution NMR studies (50, 51). The length dependences of the kinetic parameters in Fig. 3 suggest that a minimum of one turn of heparin helix is needed to favor amylin fibrillization and that enhancement of fibrillation begins to level off beyond three turns of the heparin helix. Heparin is the most negatively charged naturally occurring biopolymer known, and its binding to proteins is mediated through electrostatic interactions with basic groups in proteins (52). We have proposed that electrostatic repulsion between like charges, replicated by the symmetry of the cross-β structure along the length of fibrils, could be one of the main forces opposing fibrillogenesis (53). The heparin polyanion could aid fibrillogenesis by providing a suitable scaffold to complement the positive charges displayed on the surfaces of amylin fibrils. Indeed, it has been shown that the enhancement of amylin fibrillization drops by 86%, going from heparin to completely desulfated heparin, emphasizing the importance of charge-coupling in the interactions between the two polymers (29).

Heparin is composed of the disaccharide unit iduronic acid (IdoUA)-glucosamine (GlcN). In the heparin structure determined by NMR (PDB code: 1HPN), the 6S sulfate of residue GlcN (i-1), the 2S sulfate of residue IdoUA (i), the NS sulfate of residue GlcN (i+1), and the carbamyl C’ of residue IdoUA (i+2) form an array of negative charges on one side of the heparin helix. The distances between the charged groups vary between 6.1 Å for the 2S(IdoUA) to NS(GlcN) contact and 7.1 Å for the NS(GlcN) to C’(IdoUA) contact. After a 7.3 Å contact between C’(IdoUA) and 6S(GlcN), the pattern is repeated on the same
side of the helix for the next four saccharide monomers. In
the structure of amylin fibrils based on solid-state NMR (7),
the average spacing between positively charged groups on alternate
amylin monomers laying along the fibril axis is 5.8 Å for His-18
(Cy atoms) and 6.7 Å for Arg-11 (Cz atoms). Thus the spacing
of negative charges in the heparin helix is complimentary to the
spacing of positive charges from alternating amylin monomers
running along the fibril axis. Because heparin has a flexible
structure that can kink to achieve optimal binding to proteins
(52, 54) and because side chains can adjust in both polymers,
the match may be even better than suggested by considering
individual structures. Our conclusions about the structural
basis of interactions between amylin fibrils and heparin are sup-
ported by recent molecular dynamics modeling of the interac-
tions of amyloid β with GAGs (46).

Although amylin fibrils are formed in the presence of heparin
fragments of various lengths (Fig. 4), the effects of the oligosac-
charides on fibrillization could be catalytic or could involve
association with the fibrils. Our FRET (Fig. 5) and TIRFM (Fig.
6) data indicate that heparin associates with amylin fibrils dur-
ing fibrillogenesis. TIRFM also indicates that heparin can bind
to amylin fibrils after they are formed (Fig. 6C). Filtration ex-
periments suggest that of the fluorescein-tagged heparin bound to
amylin fibrils, a fraction of 83% is tightly associated, whereas
another 17% is weakly associated.

Because heparin affects fibrillization kinetics and associates
with fibrils, we wanted to see whether there is an effect on the
biological activity of amylin. GAGs could increase cytotoxicity
simply by enhancing fibrillogenesis. Alternatively, fibrils could
disrupt normal function by sequestering important compo-
nents of the extracellular matrix (55). In contrast to these
hypotheses, a number of recent reports indicate that heparin
(56–58) as well as other GAGs (59) reduce the cytotoxicity of
the Alzheimer amyloid β peptide and of the aortic amyloid
peptide medin (60). In the present work, we found that the
cytotoxicity of amylin toward a mouse model of β-pancreatic
cells depends on heparin fragment length (Fig. 7). Amylin in the
presence of the longest dp20 heparin fragment has a cytotoxic-
ity comparable with amylin alone. By contrast, the shortest dp2
fragment protects against cytotoxicity with both freshly dis-
solved and fibrillar amylin samples.

The protective effects of the dp2 fragment are intriguing
but will need further study to establish a mechanistic basis.
Based on the current data, we can conclude that the effects of
the heparin fragments on cytotoxicity are probably unre-
lated to their effects on fibrillization kinetics. The dp2 frag-
ment has negligible effects on amylin fibrillization kinetics
(Figs. 2B and 3) yet protects against cytotoxicity (Fig. 7B). By
contrast, the dp20 fragment enhances amylin fibrillization
(Figs. 2B and 3), yet amylin cytotoxicity is similar in the
presence and absence of dp20 (Fig. 7B). One possibility is
that the heparin fragments exert their effects on soluble
amylin oligomers or some other species and that these rather
than the fibrils are cytotoxic (13, 15, 47, 58). The roles of
putative intermediates in cytotoxicity remain poorly charac-
terized and controversial not only for amylin but for other
amyloidogenic proteins (8, 11). An alternative possibility is
that the structure or surface properties of the fibrils differ in
the presence of heparin fragments of different lengths in a
way that affects their interactions with β-cells (59). Finally,
heparin fragments could affect cofactor molecules (57) that
mediate the interactions of amylin with β-cells.

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Heparin Enhancement of Amylin Fibrillization

JULY 1, 2011 • VOLUME 286 • NUMBER 26

JOURNAL OF BIOLOGICAL CHEMISTRY 22903
Heparin Enhancement of Amylin Fibrillization

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