Fibrillin-1 Interactions with Heparin
IMPLICATIONS FOR MICROFIBRIL AND ELASTIC FIBER ASSEMBLY*

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Fibrillin-1 assembly into microfibrils and elastic fiber formation involves interactions with glycosaminoglycans. We have used BIAcore technology to investigate fibrillin-1 interactions with heparin and with heparin saccharides that are analogous to S-domains of heparan sulfate. We have identified four high affinity heparin-binding sites on fibrillin-1, localized three of these sites, and defined their binding kinetics. Heparin binding to the fibrillin-1 N terminus has particularly rapid kinetics. Hyaluronan and chondroitin sulfate did not interact significantly with fibrillin-1. Heparin saccharides with more than 12 monosaccharide units bound strongly to all four fibrillin-1 sites. Heparin did not inhibit fibrillin-1 N- and C-terminal interactions or RGD-dependent cell attachment, but heparin and MAGP-1 competed for binding to the fibrillin-1 N terminus, and heparin and tropoelastin competed for binding to a central fibrillin-1 sequence. By regulating these key interactions, heparin can profoundly influence microfibril and elastic fiber assembly.

Elastic fibers are insoluble extracellular assemblies in elastic tissues such as aorta, lung, and skin (1, 2). Their major role is to endow vascular and other dynamic connective tissues with elasticity and resilience. They morphologically comprise an elastin core and an outer mantle of fibrillin-rich microfibrils. During elastic fiber formation, tropoelastin is deposited on preformed microfibrillar templates. Deposits of elastin first appear within microfibril bundles and gradually coalesce, whereas microfibrils are gradually displaced to the periphery or subsumed into the elastin core.

Elastin is synthesized as the soluble precursor tropoelastin (2). It has a molecular mass of ~70 kDa, and its alternating hydrophobic and cross-linking domains are critical for assembly and cross-linking by lysyl oxidases. Fibrillins, the principal structural molecules of microfibrils, are large glycoproteins (~350 kDa) with multiple calcium-binding epidermal growth factor (EGF)-like domain arrays interspersed with eight-cys-
sugars, e.g. fibroblast growth factor-2 (19). In other cases, saccharide requirements for ligand binding reflect the length and preference for C-6 sulfation over C-2 or C-sulfation (22). For other heparan sulfate-binding proteins, especially multifunctional cytokines such as interleukin-8, the region of heparan sulfate that interacts with the ligand is more extended, encompassing typically two S-domains, including the intervening regions of lower sulfation (23).

In vitro binding assays have revealed various other interactions between elastic fiber-associated molecules. N- and C-terminal fibrillin-1 interactions probably drive microfibril assembly (24, 25). MAGP-1 (microfibril-associated glycoprotein-1), decorin, biglycan, and fibrillin-5 interact with tropoelastin (7, 8, 10, 11, 26–28). Ternary complexes of fibrillin-1, decorin, and MAGP-1 are important in elastic fiber formation (10, 11). The fibrillin-1 N terminus is particularly multifunctional because it can bind to itself and to the fibrillin-1 C terminus (24, 25), MAGP-1 (10, 28, 29), LTBP-1 (30), and versican (12). Tropoelastin binds two internal fibrillin-1 sites and can be transglutaminase cross-linked to fibrillin-1 (29).

In this study, we have used BIACore technology to investigate fibrillin-1 interactions with glycosaminoglycans. We have mapped four high affinity heparan sulfate-binding sites on fibrillin-1 and defined their kinetics, established the binding efficiencies of heparin fragments of defined lengths, and showed how heparin binding to fibrillin-1 affects interactions with tropoelastin and MAGP-1 but not the homotypic fibrillin-1 interactions. We have also demonstrated that hyaluronan and chondroitin sulfate do not interact significantly with fibrillin-1. These data contribute significantly to our understanding of how heparin, and by analogy its chemical analogue heparan sulfate, regulates microfibril and elastic fiber assembly.

**MATERIALS AND METHODS**

**Recombinant Fibrillin-1 Fragments and MAGP-1—**Recombinant human fibrillin-1 fragments PF1 encoded by exons 1–11 (residues 1–489), PF2 encoded by exons 9–17 (residues 330–722), PF5 encoded by exons 18–25 (residues 723–1069), PF7 encoded by exons 24–30 (residues 952–1279), PF8 encoded by exons 30–38 (residues 1238–1605), PF9 encoded by exons 37–43 (residues 1525–2186), PF10 encoded by exons 41–52 (residues 1688–2165), PF11 encoded by exons 37–52 (residues 1528–2165), PF12 encoded by exons 50–58 (residues 2055–2443), PF13 encoded by exons 58–65 (residues 2402–2871), PF14 encoded by exons 33–40 (residues 1363–1688), and PF14 encoded by exons 33–40 (residues 1363–1688) (3) were expressed and purified in milligram amounts by using a mammalian episomal expression system and 293-EBNA cells, as described previously (25, 29, 31) (Fig. 1, A and B). Fragment PF14 is described in this paper for the first time. Fibrillin-1 fragments expressed in a similar system are correctly folded (9, 24). PF1 fragments incorporating three separate Marfan syndrome-causing point mutations, R62C, T101A, and V449I, were also generated. Full-length human MAGP-1 was expressed in same mammalian expression system, purified under denaturing conditions using 8 M urea, and refolded as described previously (29). Recombinant tropoelastin was generated as described previously (33, 34).

**Heparin and Heparin Saccharides—** Heparin was the sodium salt from porcine intestinal mucosa, with a molecular mass of ~3000 Da (Sigma, catalog number H3400). The heparin saccharides were not obtained from a commercial source and were prepared by digestion of heparin with bacterial heparinase, as reported previously (35). Three defined size heparin saccharides were used, dp12, dp16, and dp24, which contain 12, 16, and 24 monosaccharides, respectively. These fragments are chemical analogues of the sulfated domains (S-domains) of heparan sulfate; the domain structure of heparan sulfate is shown in Fig. 2. The chondroitin 6-sulfate sodium salt used was from shark cartilage (Fluka, UK, catalog number 27045). The biotinylated hyaluronan was purchased from Sigma (catalog number B1557).

**Heparin Binding to Fibrillin-1**—Two methods of biotinylation were used in order to confirm the heparin saccharide interactions with fibrillin. The first approach was via oxidized disulphide bonds (Fig. 2) (36). Chondroitin sulfate, heparin, and heparin species dp12, dp16, and dp24, in 0.1 M solutions, were made up in 0.1 M NaOAc, pH 5.5 (coupling buffer). To a volume of 100 μl was added the same volume of 20 mM sodium metaperiodate in coupling buffer. After 30 min on ice in the dark, 20 μl of 150 mM glycerol was added to stop the oxidation, for 5 min on ice in the dark. The samples were dialyzed into coupling buffer before the addition of biotin-LC-hydrazide (Pierce) at a final concentration of 0.5 mM. After incubation for 2 h at room temperature, the nonreacted biotin was removed by extensive dialysis into coupling buffer.

The second biotinylation approach involved coupling via the heparin reducing end in two stages (36, 37). First, reductive amination with ammonia and then biotin was coupled to the free amines (Fig. 2). Heparin species dp12, dp16, and dp24, (0.1 mg) were dissolved in 2 M NH,HCl, in a volume of 100 μl. 2 mg of NaCNBH, was added, and the mixture was heated at 70 °C for 2 days. After cooling, the mixture was dialyzed extensively into Dulbecco’s phosphate-buffered saline (Cambrex, UK). 10 μl of 3 mg/ml sulfo-NHS-LC-biotin (Pierce) was added and incubated overnight at 4 °C. The nonreacted biotin was then removed by further dialysis into 0.1 M NaOAc, pH 5.5.

**BIACore 3000 Binding Analysis**—For kinetic binding studies of heparin, heparin oligosaccharides, chondroitin sulfate, and hyaluronan with fibrillin-1 by surface plasmon resonance, a BIACore biosensor was used (BIACore 3000, BIACore AB, Sweden). The heparin fragments, chondroitin sulfate, and hyaluronan oligosaccharides were immobilized onto commercially prepared SA sensor chips, which have preimmobilized streptavidin, to allow biotin capture. By using heparin concentrations of 1 μM, typically 150–200 RU of biotinylated heparin samples was immobilized, which was at a saturation level. Samples were applied to the sensor chip surface in 0.1 M NaOAc, pH 5.5. All subsequent binding experiments were performed in 10 mM HEPES, pH 7.4, 0.1 M NaCl, 1 mM CaCl2, and 0.005% surfactant P20 (designated HBS-Ca).
Heparin, heparin saccharides, hyaluronan, and chondroitin sulfate binding analyses were performed using a panel of fibrillin-1 protein fragments encompassing full-length fibrillin-1, as analytes, at 100 nM and a flow rate of 30 μl/min for 3 min. A high flow rate of 30 μl/min was used to minimize mass transport effects. After 2.5 min dissociation, the chip was then stabilized for 20 min using HBS-Ca, before the next injection was carried out. The analyte was simultaneously passed over a blank flow cell, and this base line was subtracted from the experimental flow cell. None of the analytes was found to interact with the blank flow cell; however, this control allowed bulk shifts caused by subtle differences in solvents to be eliminated. The maximum relative response value for each injection was calculated using the binding assay result wizard (BIAcore control software 3.2).

BIAcore 3000 Kinetic Analysis of Molecular Interactions—Kinetic analysis was performed using heparin and the heparin saccharides dp12, dp16, and dp24 immobilized to SA sensor chips and fibrillin-1 fragments PF1, PF7, PF11, and PF13 that were shown in analyte screening to bind heparin and HS. Protein fragments were injected at concentrations ranging from 0.25 to 5 μg/ml at a flow rate of 30 μl/min. Samples were injected for 3 min, dissociated for 10 min, regenerated for 1 min using 50 mM NaOH, 1 mM NaCl, followed by 1 min using HBS-Ca containing 800 mM NaCl, and then stabilized for 10 min before the next injection. After subtraction of each response value from the blank cell, association and dissociation rate constants were determined by global data analysis. All curves were fitted using 1:1 Langmuir association/dissociation model (BIAevaluation 4.1, BIAcore AB). This model was found to fit the data for three fragments (PF7, PF11, and PF13) very well, with low χ2 values. χ2 values are a standard statistical measure of the closeness of fit (mean square of the signal noise).

Two alternative methods were used for PF1 binding to heparin. PF1 binding to heparin was calculated independently by using equilibrium analysis. The injection time was increased to 15 min and the dissociation time to 30 min. The equilibrium response was plotted against concentration, and nonlinear regression using the equation for one site binding was used to calculate K_d values. To calculate association and dissociation constants for PF1, the observed rate constant k_{obs} was calculated from the association phase using the 1:1 Langmuir association (k_{obs}) model (BIAevaluation 4.1, BIAcore AB). A secondary plot of k_{obs} against concentration (C) allows k_a and k_d to be calculated using linear regression, using the equation k_{obs} = k_a C + k_d.

The binding of PF1 mutants R62C, T101A, and V449I was also analyzed against immobilized dp24, using the same methods. To investigate the kinetics of interactions between the PF1 mutants and MAGP-1, MAGP-1 was immobilized on the surface of a CM5 sensor chip via amine coupling, as described previously (29). PF1 and PF1 mutants R62C, T101A, and V449I were then injected at concentration ranges of 0.25–5 μg/ml at a flow rate 30 μl/min. Kinetic analysis was also performed on immobilized hyaluronan using the versican G1 domain (kindly provided by Dr. A. Day, MRC Immunochemistry Unit, Oxford, UK). Versican was injected at concentrations ranging from 1 to 5 μg/ml at a flow rate 30 μl/min. For a potential positive control for chondroitin 6-sulfate, binding of fibroblast growth factor-1 and -2 was tested, but no significant binding was detected. However, chondroitin ABC lyase lowered the response level of the chondroitin sulfate immobilized flow cell, indicating cleavage may have taken place.

Inhibition Assays Using BIAcore—To investigate the effects of MAGP-1 on binding of PF1 to dp24, kinetic analysis was performed using 1 μg/ml PF1 preincubated with 0.4–1200 nm MAGP-1 in HBS-Ca. For MAGP-1 only, 0.4–1200 nm was separately passed over the sensor chip, as a control. These responses were then subtracted from the corresponding PF1/MAGP-1 data. Analytes were injected over the sensor chip in the same manner as described for the kinetic studies. An inhibition curve was then plotted using the response value of each normalized curve, at the end of the association period. To investigate the effects of dp24 on the interaction of PF1 to MAGP-1, MAGP-1 was immobilized, using amine coupling, as described previously (29). Kinetic analysis was carried out using 1 and 5 μg/ml of PF1 preincubated with increasing concentrations of un-biotinylated dp24 0.4–4000 nm; dp24 0.4–4000 nm was separately passed over the sensor chip and then subtracted from the PF1/dp24 plots. An inhibition curve was plotted, as described above.

To investigate the effects of dp24 on the interaction of PF7 to tropoelastin, tropoelastin was immobilized, using amine coupling as described previously (29). Kinetic analysis was carried out using 1 and 5 μg/ml of PF7 preincubated with increasing concentrations of un-biotinylated dp24 0.4–4000 nm or with dp24 only (0.4–4000 nm). An inhibition curve was plotted, as described above.

Calcium dependence for fibrillin-1/heparin interactions was determined by kinetic analysis, monitoring immobilized biotinylated dp24 binding to 5 μg/ml PF1, PF7, or PF11, in the presence of increasing concentrations of EDTA (0.01–10 mM). EDTA only sensorgrams were also plotted and subtracted from the FBN-1/EDTA sensorgrams. An inhibition curve was plotted, as described above.

Inhibition Using Solid Phase Binding Assays—Competition binding assays were also conducted using both nonbiotinylated and biotinylated soluble proteins; biotinylation was carried out as described previously (29). Flat-bottomed microwell plates were coated with PF1 and PF13, at 100 nM in 50 mM Tris, pH 7.4, 0.1 mM NaCl, 1 mM CaCl2 (TBSa/CaCl2), overnight at 4 °C. Nonspecific binding sites were then blocked with TBSa/CaCl2 containing 4% BSA at room temperature for at least 2 h. The plates were washed three times with TBSa, CaCl2, 0.1% BSA and incubated with either 100 nM of biotinylated PF1 or PF13, which had been preincubated with heparin oligosaccharide dp24 (4–12000 nm) in TBSa/CaCl2, overnight at 4 °C. After a further three washes, plates were incubated with 1:200 dilution of extravidin peroxidase conjugate at room temperature for 15 min. Bound protein was quantified after four more washes by a colorimetric assay using 40 nm 2,2'-azino-bis(3-
number control wells were fixed with the addition of 10.

numbers of cells were added to unblocked wells on the plate. Known cell

was aspirated, and excess stain was removed by four washes with 400

0.1% (w/v) crystal violet in 0.2M MES, pH 5, for 1 h. The crystal violet

ments and MAGP-1.

rin saccharides with fibrillin-1 frag-

ation of polymeric heparin and hepa-

100 nM were injected over cis-diol biotiny-

MAGP-1 fragments at a concentration of

100

by aspiration, and then adherent cells were fixed with the addition of

glutaraldehyde. Nonadherent cells were removed from the other wells

incubated for 30 min at 37 °C, 5% CO2. To estimate cell number, known

dp24) at double the desired concentration were added to the wells and

was aspirated, and 50

mg/ml heat-denatured BSA for 1 h. Human dermal fibroblasts suspen-

and absorbed onto wells of a 96-well microtiter plate for 1 h, and

washed four times, and the color was developed. Any nonspecific fibril-

was added to the wells and

acid, and the absorbance was measured at 570 nm. The absorbance

l of distilled H2O. The dye was solubilized in 100

l of 5% glutaraldehyde for 20 min. The wells were washed three

times with 200 l of distilled H2O, and cells were stained with 100 l of

0.1% (w/v) crystal violet in 0.2 M MES, pH 5, for 1 h. The crystal violet

was aspirated, and excess stain was removed by four washes with 400

l of 10% (v/v) acetic acid, and the absorbance was measured at 570 nm. The absorbance

from the known cell number wells was used to express data as percent-

age cell attachment.

Cell Attachment Assay—Cell attachment assays were performed as
described previously (31). Recombinant fibrillin-1 fragments PF14,
PF9, and PF10 were diluted to 10

mg/ml in phosphate-buffered saline and absorbed onto wells of a 96-well microtiter plate for 1 h, and unbound ligand was aspirated. Nonspecific binding was blocked with 10

mg/ml heat-denatured BSA for 1 h. Human dermal fibroblasts suspensions

were prepared, counted, and adjusted to 1 × 106 cells/ml. The BSA

aspirated, and 50 μl of cell suspensions and 50 μl of heparin sulfate

(dp24) at double the desired concentration were added to the wells and

incubated for 30 min at 37 °C, 5% CO2. To estimate cell number, known

numbers of cells were added to unblocked wells on the plate. Known cell

number control wells were fixed with the addition of 10 μl of 50%

glutaraldehyde. Nonadherent cells were removed from the other wells

by aspiration, and then adherent cells were fixed with the addition of

100 μl of 5% glutaraldehyde for 20 min. The wells were washed three

times with 200 μl of distilled H2O, and cells were stained with 100 μl of

0.1% (w/v) crystal violet in 0.2 M MES, pH 5, for 1 h. The crystal violet

was aspirated, and excess stain was removed by four washes with 400

μl of distilled H2O. The dye was solubilized in 100 μl of 10% (v/v) acetic

acid, and the absorbance was measured at 570 nm. The absorbance

from the known cell number wells was used to express data as percent-

age cell attachment.

Modeling of Fibrillin-1 TB5/Heparin Interaction—The cbEGF-TB4-

cbEGF structure was taken from the Protein Data Bank (code 1UZJ)

(38); the file was edited to remove the first cbEGF domain, and the

remaining TB4-cbEGF domains were used as the negative (non-hepa-

ran sulfate binding) control. The TB4-cbEGF structure was also used as

a starting model to generate a TB5-cbEGF homology model. Modeling

was performed in Quanta2000 (Accelrys) on a Silicon Graphics O2

R12,000 using the protein modeling modules. Partial charges were

assigned in the ADT program (AutoDock tools) (39).

The structures of two heparin pentasaccharides that differ in the

conformation of the iduronate residues (1C or 2S) were generated from

the Protein Data Bank (code 1HPN) (40). Partial charges were assigned
to atoms according to the PIM force field (41) and allowed torsionable
bonds were assigned in ADT.

Docking of the pentasaccharides was performed using the AutoDock

3.0 program (39). The pentasaccharides were placed arbitrarily at two

different positions on either side of the TB-cbEGF structure, 10 Å from

the protein surface. Grids of probe atom interaction energies and electrostatic

potential were generated with a spacing of 0.375 Å, using the AutoGrid

program within the AutoDock suite. For each pentasaccharide, one sim-

ulation of 100 Lamarckian Genetic Algorithm runs was performed using

200 individuals and an energy evaluation number of 3 × 10⁵. Each run

produced one binding solution, and cluster analysis was performed at

the end of each simulation. Solutions that were within 2 Å root mean square
deviation of each other belonged to the same cluster, and clusters were

ranked according to energy. Docking runs were performed with either the

TB5-cbEGF model or the TB4-cbEGF structure.

RESULTS

Defining Glycosaminoglycan Interactions with Fibrillin-1

Fibrillin-1 Binding to Heparin, Specificity of GAG Interac-
tions—BIAcore 3000 analysis was used to map the fibrillin-1

molecular regions that interact with heparin. Biotinylated poly-

meric heparin and heparin dp24 were immobilized on the

surface of SA sensor chips. Fibrillin-1 fragments covering the

entire molecule (Fig. 1) were then each passed over the sensor

chip surface at a concentration of 100 nM. Heparin and dp24

gave identical profiles (Fig. 3, A and B). Four nonoverlapping

fibrillin-1 fragments were found to bind to heparin and dp24.
The N-terminal fibrillin-1 fragment PF1 (encoded by exons 1–11) exhibited by far the highest binding response (≈5000 response units (RU) for dp24). Internal fragment PF7 (encoded by exons 24–30) exhibited a response of over 200 RU that did not greatly depreciate after 2.5 min of dissociation. Because overlapping fibrillin-1 fragments PF5 and PF8 did not bind heparin, this binding site must be within the four cbEGF-like domains encoded by exons 26–29. Fragments PF2, PF12, and PF14 did not interact with heparin. Of the four overlapping fibrillin-1 fragments, PF9, PF10, PF11, and PF14, all except PF14 bound polymeric heparin and dp24. Thus, this heparin-binding site is located in the overlap between PF9 and PF11 (TB5 and the downstream cbEGF-like domain are encoded by exons 41–43). The C-terminal fragment PF13 also interacted with both heparin and dp24 (≈100 RU). These data show that there are four heparin-binding sites on fibrillin-1 and further localize the first three of these sites.

When the fibrillin-1 fragments were passed over chips coated with hyaluronan or chondroitin 6-sulfate, no interactions with fibrillin-1 were detected. However, when versican G1 domain, a positive control, was passed over the hyaluronan sensor chip, an interaction was detected with a dissociation constant ($K_D$) of 11.0 nM. Thus, hyaluronan had been immobilized in a suitable manner to allow interactions with proteins. A direct positive control for chondroitin 6-sulfate binding could not be found; however, cleavage of immobilized chondroitin sulfate chains by

![FIG. 4. BIACore analysis of interactions of heparin with fibrillin-1 fragments. Fibrillin-1 protein fragments PF9 (A), PF10 (B), PF11 (C), PF14 (D), PF1 (E), PF7 (F), and PF13 (G) were injected over a cis-diol biotinylated heparin-immobilized surface. Each sensorgram shows analyte concentrations ranging from 0.25 to 5 µg/ml, with duplicate concentrations included in every run. One representative experiment is shown. The response difference (Resp. Diff.) is the difference between experimental and control flow cells in RU. Time is shown in seconds. The residual deviations from the 1:1 binding model are indicated as insets for all binding fibrillin-1 fragments, apart from PF1. The equilibrium binding curve for the calculation of PF1 $K_D$ is shown in E.](image-url)
Analysis of interactions between heparin and fibrillin-1 protein fragments by surface plasmon resonance.

Surface plasmon resonance binding was performed as described under “Experimental Procedures.” The kinetic parameters were derived from typical sensorgrams shown in Fig. 4. Evaluation was done according to a 1:1 binding model. Values are mean ± S.E. of at least two separate experiments. NA indicates not allowed; NB indicates no significant binding detected. All χ² values were below 0.25 apart from PF1, which did not follow the 1:1 binding model, so for this fragment, $k_D$ was calculated by global fitting, although of the same order. To calculate the observed rate constant $k_{a_C}$, values were calculated using the equation $k_{obs} = k_a = k_c C + k_a$ as described in the text.

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<td>2.37 ± 0.74</td>
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<td>5.2 ± 0.49</td>
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<td>5.0 ± 0.48</td>
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<tr>
<td>PF10</td>
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<td>3.79 ± 0.18</td>
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<td>PF13</td>
<td>29 ± 7.1</td>
<td>0.43 ± 0.12</td>
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Heparin Binding to Fibrillin-1

TABLE II

Analysis of interactions between heparin saccharides and fibrillin-1 protein fragments by surface plasmon resonance.

Surface plasmon resonance binding was performed as described under “Experimental Procedures.” The sensorgrams for dp24-red are shown in Fig. 5. Evaluation was done according to a 1:1 binding model. Values are mean ± S.E. of at least two separate experiments. NB indicates no significant binding detected. All χ² values were <10. c/d indicates biotinylation using cis-diol oxidation; red indicates biotinylation via reducing end.

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<td>PF1</td>
<td>5.8 ± 3.5</td>
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<td>PF7</td>
<td>3.3 ± 0.9</td>
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<td>PF11</td>
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<tr>
<td>dp16-red</td>
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Impact of Heparin on Fibrillin-1 Assembly and Interactions with MAGP-1 and Tropoelastin

Effects of Heparin on Fibrillin-1 Assembly and Interactions with MAGP-1 and Tropoelastin—To investigate whether homotypic fibrillin-1 interactions were affected by heparin, solid phase inhibition assays were performed. In a recent study, we used solid phase binding assays to show that PF1 and PF13 both interact strongly in a homotypic manner and with each other (25). Here heparin dp24 (4–12000 nM) was incubated with PF1 or PF13 (100 nM), before addition to either PF1 or PF13 preincubated with MAGP-1 (0.4–12000 nM). Reduced binding of MAGP-1 was seen even at high concentrations (Fig. 7C). Reduced binding at EDTA concentrations that exceed buffer Ca²⁺ concentration indicates that these interactions between heparin and fibrillin-1 are all directly or indirectly calcium-dependent. Fragment PF1 contains 2 chEGF-like domains; PF7 contains 6 chEGF-like domains, and PF11 contains 10 chEGF-like domains.

Effects of Heparin dp24 Binding to PF1 on N- and C-terminal Fibrillin-1 Interactions—To determine whether homotypic fibrillin-1 interactions were affected by heparin, solid phase inhibition assays were performed. In a recent study, we used solid phase binding assays to show that PF1 and PF13 both interact strongly in a homotypic manner and with each other (25). Here heparin dp24 (4–12000 nM) was incubated with PF1 or PF13 (100 nM), before addition to either PF1 or PF13 preincubated with MAGP-1 (0.4–12000 nM). Reduced binding of MAGP-1 was seen even at high concentrations (Fig. 7C). Reduced binding at EDTA concentrations that exceed buffer Ca²⁺ concentration indicates that these interactions between heparin and fibrillin-1 are all directly or indirectly calcium-dependent. Fragment PF1 contains 2 chEGF-like domains; PF7 contains 6 chEGF-like domains, and PF11 contains 10 chEGF-like domains.

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nM concentration range) and passed over the dp24 sensor chip surface. The binding response between PF1 and dp24 decreased with increasing concentrations of MAGP-1. The inhibition curve is shown in Fig. 7A. The EC\textsubscript{50} value was calculated using GraphPad Prism 2.0 (nonlinear regression, sigmoidal dose response) and was found to be 57.1 nM. Similar experiments were conducted to determine whether heparin dp24 could inhibit the interaction of PF1 and MAGP-1. Heparin dp24 was found not to interact with MAGP-1 at concentrations up to 4000 nM. Heparin (0.4–4000 nM) was then preincubated with PF1 at 1 \mu g/ml (20 nM), prior to analysis of binding to MAGP-1. Increasing concentrations of dp24 inhibited the response of PF1 with MAGP-1 (Fig. 7A), and the EC\textsubscript{50} value was calculated to be 59.9 nM. These data show that the heparin- and MAGP-1-binding sites on PF1 are adjacent or overlapping and that heparin strongly regulates MAGP-1 interactions with N-terminal fibrillin-1.

To gain further insights into the location of the fibrillin-1 PF1 heparin-binding site, the potential effects of three classical Marfan syndrome disease-causing mutations\textsuperscript{2} on PF1 binding to biotinylated dp24 were investigated (Table III and Fig. 8). The binding kinetics of PF1 mutants R62C (mutation within domain encoded by exon 2) and V449I (mutation within domain encoded by exon 11) were similar to wild-type PF1. However, PF1 mutant T101A (mutation within domain encoded by exon 3) bound dp24 with significantly lower affinity. This result implies that heparin binding involves the PF1 EGF-like domain encoded by exon 3.

![FIG. 5. BIACore analysis of interactions of heparin dp24 with fibrillin-1 fragments.](image)

![FIG. 6. Summary of kinetic data of fibrillin-1 binding to heparin and heparin oligosaccharides.](image)
Wild-type PF1 and the three PF1 mutants were also passed over immobilized MAGP-1 (Fig. 8). These experiments showed that wild-type PF1 and the three mutants all bound MAGP-1 with similar affinities. Thus, although the PF1-binding sites for heparin and MAGP-1 are adjacent or overlapping, they are not identical.

Effects of Heparin dp24 Binding to Fibrillin-1 PF7 on Tropoelastin Binding—We showed previously (29) that PF7 interacts with high affinity with tropoelastin. Here we investigated whether dp24 competes with tropoelastin for binding to PF7. For these experiments, tropoelastin was immobilized onto a CM5 sensor chip using amine coupling (29). Initially, dp24 passed over the tropoelastin interacted strongly, with a dissociation constant of 1.0 nM (Fig. 7B). The heparin dp24 fragment (0.4–4000 nM) was then preincubated with PF7 at 1 μg/ml (20 nM), prior to analysis of PF7 interactions with immobilized tropoelastin. When the dp24/tropoelastin control binding curves were subtracted from the preincubated PF7/dp24 binding to tropoelastin sensorgrams, it was found that the response decreased with increasing concentrations of dp24. The inhibition curves were plotted (Fig. 7B), and the EC50 value was calculated to be 5.3 nM. These experiments show that heparin regulates tropoelastin deposition on a fibrillin-1 template.

**TABLE III**

Analysis of interactions between heparin saccharides and MAGP-1 with fibrillin-1 PF1 mutants by surface plasmon resonance

Surface plasmon resonance binding was performed as described under “Experimental Procedures.” Evaluation was done according to a 1:1 binding model. Values are mean ± S.E. of at least two separate experiments. NB indicates no significant binding detected. All χ2 values were <10.

<table>
<thead>
<tr>
<th>HS dp24</th>
<th>MAGP-1, KD (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ka × 10^3 s^-1</td>
<td>k_d × 10^-3 s^-1</td>
</tr>
<tr>
<td>PF1-WT 2221 ± 1089</td>
<td>0.77 ± 0.29</td>
</tr>
<tr>
<td>PF1-R62C 1280 ± 340</td>
<td>0.30 ± 0.07</td>
</tr>
<tr>
<td>PF1-T101A 154 ± 102</td>
<td>1.20 ± 0.23</td>
</tr>
<tr>
<td>PF1-V449I 1688 ± 827</td>
<td>0.86 ± 0.01</td>
</tr>
</tbody>
</table>

Wild-type PF1 and the three PF1 mutants were also passed over immobilized MAGP-1 (Fig. 8). These experiments showed that wild-type PF1 and the three mutants all bound MAGP-1 with similar affinities. Thus, although the PF1-binding sites for heparin and MAGP-1 are adjacent or overlapping, they are not identical.
Effects of Heparin dp24 on RGD-mediated Cell Adhesion to Fibrillin-1—Cell attachment assays were performed to study the effects of heparin on RGD-mediated cell adhesion. Cell binding to fibrillin-1 fragments PF14 (contains RGD but no heparin binding), PF9 (contains RGD and binds heparin), and PF10 (no RGD and binds heparin) was not affected by the presence of 125 nM or 1.25 μM heparin sulfate dp24 (data not shown).

Modeling of Heparin Docking to TB5 and Downstream cbEGF-like Domain—Modeling of the two domains that comprise the overlap region of PF9 to PF11, which contains a high affinity heparin-binding site, was conducted to investigate its potential docking mechanism. Two heparin pentasaccharides that differ in their conformation of the idurionate residues were used for docking to the model of fibrillin-1 TB5-cbEGF (Fig. 9), which corresponds to the overlap within fragments PF9 to PF11 that binds heparin, and the non-heparin-binding fibrillin-1 TB4-cbEGF structure (38) as a control. The pentasaccharides were placed at two different starting sites near the protein surface, and docking runs were performed followed by cluster analysis that revealed two potential heparin-docking sites. Clusters with multiple members and negative docking energy are described. For TB5-cbEGF position 1, no clusters were found with the $^{2}S_{0}$ conformation, but clusters of 5 and 4 members were found with the $^{1}C_{4}$ conformation which with further inspection were found to bind to the same site. Docking in position 2 yielded a cluster of 10 members for the $^{1}C_{4}$ conformation and a cluster of 12 members for the $^{2}S_{0}$ conformation, and both of these clusters bound to the same site. For the TB4-cbEGF control, no negative energy clusters were found for either heparin conformation.

All clusters were visualized using Quanta2000, and hydrogen bonds were calculated. The clusters with 5 and 4 members docked to the same site on TB5-cbEGF and bound in the interface between TB5 and the following cbEGF (site 1). The residues forming the pentasaccharide-binding site were Arg$^{1697}$, Asn$^{1724}$, Arg$^{1727}$, and Arg$^{1762}$ from the TB5 domain and Ser$^{1768}$ and Arg$^{1780}$ from the cbEGF domain (Fig. 9A). The clusters with 10 and 12 members bound toward the N-terminal region of the TB5 domain (site 2). The residues forming the pentasaccharide-binding site were Arg$^{1694}$, Arg$^{1692}$, Asn$^{1713}$, Asn$^{1730}$, Lys$^{1731}$, and Tyr$^{1766}$ all from the TB5 domain (Fig. 9B). For both heparin-binding sites, the surface charge distribution was viewed using Grasp (42) (Fig. 9, C and D). The N-terminal site (site 2) is distinct from the TB5-cbEGF interface site (site 1) and is actually on the opposite face of the TB5 domain (Fig. 9E).

DISCUSSION

We have investigated the nature of the specific fibrillin-1 interactions with heparin in order to determine how these glycosaminoglycans influence microfibril and elastic fiber assembly, and to obtain insights into the molecular basis of physiological heparan sulfate interactions with fibrillin-1. In these studies, we have used polymeric heparin and defined heparin saccharides that mimic the binding properties of the S-domains of heparan sulfate. We have shown that there are four distinct high affinity heparin-binding sites on fibrillin-1, and we have localized three of these sites to the N terminus, the “neonatal Marfan” region, and to a site downstream from the Arg-Gly-Asp (RGD) cell attachment motif. A fibrillin-1 C-terminal heparin-binding site was mapped previously adjacent to the furin-cleavage site (13). Heparin binding affinity increases with increasing saccharide length, for all four fibrillin-1 sites. Although occupancy of the heparin-binding site of the N terminus of fibrillin 1 (PF1) did not inhibit its propensity for homophilic interaction, its capacity to interact with MAGP-1 was significantly reduced; likewise, MAGP-1 bound to PF1-reduced subsequent heparin binding to PF1. Heparin bound to the central fibrillin-1 sequence inhibited tropoelastin binding. Thus, in vitro, it is likely that the S-domains of heparan sulfate do not influence linear fibrillin-1 assembly but regulate the association of MAGP-1 with microfibrils and elastic fiber assembly. In future studies, it will be interesting to investigate the interactions of the four heparin binding regions of fibrillin-1 with authentic S-domains of heparan sulfate isolated from connective tissues to define the sulfation requirements for saccharide recognition at each site. Previous studies have suggested that small chondroitin sulfate proteoglycans may interact with microfibrils (10, 43); however, neither chondroitin sulfate nor hyaluronan binds directly to fibrillin-1.

The predominant disaccharide unit (~80% of total disaccharides) of heparin used in this study was the trisulfated unit GlcNS,6S-IdoA,2S-; this unit is found exclusively in the S-domains of heparan sulfate, although it is very rare to find S-domains composed entirely of trisulfated disaccharide sequences (18). Fibrillin-1 fragment affinity for heparin increased with saccharide chain length. Comparison of two biotinylation methods confirmed similar profiles of heparin saccharide binding to four fibrillin-1 fragments, and thus the specificity of these interactions.

Binding of heparin to the N-terminal fibrillin-1 fragment PF1 exhibited high affinity and rapid binding kinetics. The importance of protein conformation in this interaction is suggested because a specific Marfan N-terminal mutation (T101A, within the first EGF-like domain) markedly reduces the affinity of PF1 for heparin despite not involving a positively charged residue. The distinctive kinetics may depend, in part, on protein folding involving residues within or adjacent to this domain to create the glycosaminoglycan-binding site. The eight Arg and Lys residues that occur in the mutated domain and its flanking domains are not clustered, and because the structure of PF1 is not known, the exact site of heparin binding is unclear, although it is likely to be conformational rather than linear. Because the T101A mutation does not involve a charged residue, it is possible that Thr$^{103}$ is critical for the correct folding of the EGF-like domain encoded by exon 3. In addition, the N-terminal region of fibrillin-1 has a tendency to form disulfide-bonded aggregates that could be im-

![Fig. 8. Summary of kinetic data of fibrillin-1 PF1 mutants binding to heparin and MAGP-1. Kinetic analysis using BIAcore was carried out on fibrillin-1 PF1 mutants against HS dp24 biotinylated using cis-diol oxidation (c/d) and MAGP-1. The dissociation constant $K_D$ is shown on a log scale of fibrillin-1 protein fragments PF1 (black), PF1 R62C (light gray), PF1 T101A (dark gray), and PF1 V449I (white). The values are shown in Table III.](http://www.jbc.org/content/7/100/30534)

**Heparin Binding to Fibrillin-1**

Influence of Heparin on RGD-mediated Cell Adhesion to PF9/PF11

The predominant disaccharide unit (~80% of total disaccharides) of heparin used in this study was the trisulfated unit GlcNS,6S-IdoA,2S-; this unit is found exclusively in the S-domains of heparan sulfate, although it is very rare to find S-domains composed entirely of trisulfated disaccharide sequences (18). Fibrillin-1 fragment affinity for heparin increased with saccharide chain length.
important in lateral assembly (44); heparin binding may be enhanced by such multimerization.

By using overlapping fibrillin-1 fragments (PF5, PF7, and PF8), we localized a second heparin-binding site to four cbEGF-like domains encoded by exons 26–29 (residues 1070–1237), which are within the neonatal Marfan region (5).2 We can exclude a previously predicted HS-binding site (GKKGKT; residues 1313–1318) (9). There are nine Arg and Lys residues within these four cbEGF-like domains, seven of which are in domains encoded by exons 26 and 27. Four of these residues are on one face and three on the other (45) (Fig. 9F), and could form heparin-binding site(s).

The third heparin-binding site was localized using overlapping fibrillin-1 fragments (PF9, PF10, PF11, and PF14) to the domains encoded by exons 41–43 (residues 1689–1807; TB5 and following cbEGF-like domain). This site is two domains downstream from the RGD-containing TB4 motif; however, bound heparin did not disrupt RGD-dependent cell attachment. Modeling revealed two possible heparin-docking sites; site-directed mutagenesis is now warranted to determine the usage of these sites.

The C-terminal HS-binding site was previously localized to the last 17 residues of mature processed fibrillin-1, which ends with the furin cleavage sequence RKRR (13). We have shown that the presence or absence of the post-furin cleavage sequence has no effect on the ability of heparin to bind this fragment, but heparan sulfate may, in vivo, regulate the furin cleavage event.

![Model of heparin/fibrillin-1 interaction](Figure 9)

**Figure 9.** Model of heparin/fibrillin-1 interaction. Docking prediction of heparin to fibrillin-1 domains TB5-cbEGF. A and B show close-up views of the two sites where heparin pentasaccharide is predicted to bind; the amino acids involved are labeled. At site 1 (A), heparin (green) is predicted to bind in the interface between the TB5 (gray) and the following cbEGF (orange). Site 2 (B) heparin (yellow) is predicted to bind at the N terminus of TB5. C and D show the grasp surface charge representation at these sites. E shows the distance between sites 1 and 2 and indicates it is unlikely that a longer HS chain could straddle the two sites. F shows by schematic representation a pair of cbEGFs encoded by exons 26 and 27, as determined previously (45). The positively charged residues are shown in blue and are found to lie on either side of the molecule.
Analysis of the kinetics of heparin binding to fibrillin-1 revealed that the N-terminal and central sites all bind heparin very strongly with low nanomolar $K_d$ values, so these interactions are likely to be physiologically very significant. The different kinetic parameters between the N-terminal PF1 fragment and the other peptides suggest different physiological roles. The rapid association of heparin with PF1 suggests that this is likely to be a more dynamic site, whereas interactions with PF7 and PF11 are “lazy” in that heparin both loads on and dissociates slowly. The C-terminal interaction is also likely to play an important role in fibrillin-1 biology. Implications of these kinetic studies for assembly and function are further discussed below.

A previous study indicated that 5 mM EDTA (heparin affinity chromatography) or 10 mM EDTA (solid phase assays) did not significantly affect heparin binding to the fibrillin-1 N terminus, although it did influence an internal fibrillin-1 binding region that includes the PF7 sequence. Here BiACore analysis has revealed that increasing EDTA concentrations (1–10 mM) have complex effects but correlate with progressive reduction in heparin binding to N-terminal PF1, as well as the two internal sites identified here (PF7 and PF11). Thus, we conclude that calcium plays a role, directly or indirectly through conformation changes, in all three interactions.

The N terminus of fibrillin-1 is highly interactive. We and others have shown that it strongly binds to itself and to the fibrillin-1 C terminus (24, 25) and to MAGP-1 (28, 29). Here we have shown that heparin does not inhibit its ability to bind homotypically or with the C terminus, so heparin is unlikely to regulate linear fibrillin-1 assembly. Because heparin and MAGP-1 compete in a 1:1 manner for binding to the fibrillin-1 N-terminal fragment PF1, their binding sites must be adjacent or overlapping. However, the Marfan mutation T101A, which reduces heparin affinity for PF1, does not affect MAGP-1 binding, so MAGP-1 and heparin-binding sites are not identical. The ability of tropoelastin to bind fibrillin-1 (PF7) was also compromised by heparin; however, previous binding assays using overlapping fibrillin-1 fragments indicated that this tropoelastin-binding site was within the domains encoded by exons 24 and 25 (29). Thus, it is likely that tropoelastin and heparan sulfate sites are adjacent but not overlapping. These studies have shown that heparan sulfate may profoundly influence microfibril and elastic fiber assembly.

In vivo, heparan sulfate occurs in two main sites, as syndecan and glycan cell surface receptors (46), and as perlecan in basement membranes. Fibrillin-1 interactions with heparan sulfate may be important at both sites. Interactions with cell surface receptors may profoundly influence microfibril and elastic fiber assembly. Fibrillin-1 binds integrin receptors α5β1 and αvβ3 (31, 47, 48), whereas tropoelastin binds integrin αvβ3 and the elastin-binding protein (49, 50). It is possible that syndecan heparan sulfate chains may interact with newly secreted fibrillin-1 and influence molecular alignment and multimerization. Heparan sulfate proteoglycans with several heparan sulfate chains in close proximity on the core proteins might act as a template for assembly of fibrillin monomers using the N-domain as a docking site with fast association/dissociation from heparan sulfate chains. Previous studies have revealed that heparan sulfate, and sulfation, also play a key role in elastic fiber formation (14, 15). Heparan sulfate can interact strongly with tropoelastin, which contains numerous charged Lys residues, thereby influencing its potential to interact with fibrillin-1 and to form lysyl-derived cross-links. A recurring theme is the colocalization of fibrillin-1 microfibrils with basement membranes. Our previous structural studies of microfibrils allowed us to map the alignment of fibrillin-1 within mature beaded microfibrils (32). The central fibrillin-1 heparan sulfate-binding sites map within the “interbed” region of microfibrils and are likely to be available for interactions with heparan sulfate chains. However, N- and C-terminal binding sites may be within the bead structure and unavailable for interactions.

In summary, this study has provided important new insights into the molecular basis of heparan/heparan sulfate interactions with fibrillin-1, shown how these glycosaminoglycans regulate fibrillin-1 interactions with MAGP-1 and tropoelastin, and demonstrate that they do not influence RGD-dependent cell attachment to fibrillin-1.

REFERENCES
Heparin Binding to Fibrillin-1

30537


