Heparan Sulfate Regulates Fibrillin-1 N- and C-terminal Interactions

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Fibrillin-1 N- and C-terminal heparin binding sites have been characterized. An unprocessed monomeric N-terminal fragment (PF1) induced a very high heparin binding response, indicating heparin-mediated multimerization. Using PF1 deletion and short fragments, a heparin binding site was localized within the domain encoded by exon 7 after the first hybrid domain. Rodent embryonic fibroblasts adhered to PF1 and deletion fragments, and, when cells were plated on fibrillin-1 or fibronectin Arg-Gly-Asp cell-binding fragments, cells showed heparin-dependent spreading and focal contact formation in response to soluble PF1. Within domains encoded by exons 59–62 near the fibrillin-1 C terminus are novel conformation-dependent high affinity heparin and tropoelastin binding sites. Heparin disrupted tropoelastin binding but did not disrupt N- and C-terminal fibrillin-1 interactions. Thus, fibrillin-1 N-terminal interactions with heparin/heparan sulfate directly influence cell behavior, whereas C-terminal interactions with heparin/heparan sulfate regulate elastin deposition. These data highlight how heparin/heparan sulfate controls fibrillin-1 interactions.

Assembled microfibrils form a template for tropoelastin deposition during pericellular elastic fiber formation (2, 5).

It is increasingly clear that fibrillin-1 is a major extracellular heparin/heparan sulfate-binding molecule (6–8). We identified four high affinity heparin-binding regions on fibrillin-1 and partially localized three of these sites (6). Fibrillin-1 is likely to interact with heparan sulfate in the form of the cell surface heparan sulfate proteoglycans syndecans or glypicans (9). Basement membrane perlecans also interacts with fibrillin-1, mainly through protein-protein interactions with a central fibrillin-1 region but also through lower affinity interactions with perlecans and heparan sulfate chains (10).

The importance of heparan sulfate interactions for microfibril assembly has been suggested by cell culture experiments. Supplementation of cultures with exogenous heparin or heparan sulfate, blocking heparan sulfate attachment to core proteins with β-D-xylosides, or blocking glycosaminoglycan sulfation all disrupted microfibril deposition (7, 8). It remains unclear how heparin influences microfibril assembly. Binding of MAGP-1 (microfibril-associated glycoprotein-1) to an N-terminal fibrillin-1 fragment (PF1) was inhibited by heparin, but heparin did not block interactions between fibrillin-1 N-terminal (PF1) and C-terminal (PF13) fragments (6).

In this study, we have mapped an N-terminal fibrillin-1 terminal heparin binding site that induces heparin-inhibitable cellular responses. We have also identified a novel high affinity C-terminal heparin binding site that competes with tropoelastin. These data highlight the importance of heparan sulfate for fibrillin-1 interactions with cells and elastic fiber molecules.

EXPERIMENTAL PROCEDURES

Recombinant Fibrillin-1 and MAGP-1—The cloning, expression, and purification of recombinant fibrillin-1 fragments PF1, PF2, PF4, PF8, PF12, and PF13 (Fig. 1), using the mammalian expression vector pCEP-pu/AC7 and 293-EBNA cells, has been described (6, 11, 12).

In this study, the following recombinant human fibrillin-1 fragments were generated (Fig. 1): PF1 encoded by exons 1–11 (residues 31–489), Ex1F-11 encoded by residues 45–489,

The abbreviations used are: PF, protein fragment; TB, transforming growth factor-β-binding protein-like domain; EGF, epidermal growth factor-like; cbEGF, calcium-binding epidermal growth factor-like; REF, rat embryonic fibroblast; MEF, mouse embryonic fibroblast; BSA, bovine serum albumin; FN, fibronectin; RGD, Arg-Gly-Asp.

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Ex3–11 encoded by exons 3–11 (residues 81–489), Ex4–11 encoded by exons 4–11 (residues 115–489), Ex5–11 encoded by exons 5–11 (residues 147–489), Ex6–11 encoded by exons 6–11 (residues 179–489), Ex7–11 encoded by exons 7–11 (residues 246–489), Ex5–7 encoded by exons 5–7 (residues 147–287), PF2 encoded by exons 9–17 (residues 330–722), PF4 encoded by exons 1–8 (residues 1–329), PF8 encoded by exons 30–38 (residues 1238–1605), and PF16 encoded by exons 50–65 (residues 2055–2871), which encompasses overlapping PF12 and PF13 fragments. In all cases, cDNA sequencing and mass spectrometry confirmed the DNA and protein sequences, respectively (not shown).

Gel filtration chromatography was used to ensure that only monomeric recombinant fragments were used in all experiments. Recombinant fragments were concentrated after elution from nickel affinity chromatography, using Vivaspin columns (Sartorius), prior to separation using Superdex 200 10/300 GL (GE Healthcare), using an AKTA Purifier system. Monomeric protein was pooled for further experimentation. All recombinant fragments had the correct molecular mass as determined by SDS-PAGE. Fragments with N-glycosylation sites were N-glycosylated, as determined by treatment with PNGase F (New England Biolabs). In some experiments, PF12, PF13, and PF16 were pretreated with trypsin for 10–120 min before SDS-PAGE analysis.

Our initial PF16-expressing 293-EBNA cultures produced two bands (see Fig. 3A), which were shown by mass spectrometry to correspond to intact PF16 plus a large cleavage fragment lacking some C-terminal sequence (designated PF16trunc). PF16 was separated from PF16trunc using S200 size fractionation. Some transfected 293-EBNA cultures expressed recombinant PF16trunc, only, as judged by SDS-PAGE, immunoblotting, and mass spectrometry. Both PF16trunc preparations gave similar results in binding assays and solution studies.

Full-length human MAGP-1 was expressed in the same mammalian expression system, purified, and refolded, as described (12). Recombinant tropoelastin was a gift from Dr. A. S. Weiss (Sydney, Australia) (13, 14).

**Analysis of PF16 by Analytical Ultracentrifugation**—Sedimentation velocity experiments were performed, as reported (15), on PF12 (4.5 μM), PF13 (3.4 μM), and PF16 (9.1 μM) in 0.15 M NaCl, 10 mM HEPES, 1 mM CaCl₂, pH 7.4, and wavelength 230 nm, using an Optima XL-A analytical ultracentrifuge (Beckman Coulter, Inc.). Analyses of the raw data were performed using the distribution of Lamm equation solutions software Sedfit version 9.4 (16). The correction of s_{20,w} from standard conditions along with an estimation of the frictional ratio (f/f₀) and hydrodynamic radius (R_h) was performed using Sednterp with a partial specific volume of 0.71 ml/g, as published previously (17, 18). Hydropro (19) was used to derive shell-based bead models around the coordinates obtained from ab initio modeling from small angle x-ray scattering (20). This approach gave theoretical sedimentation coefficients and hydrodynamic radius measurements that were compared with those derived from analytical ultracentrifugation.

**Analysis of Fibrillin-1 Fragments by Multiangle Laser Light Scattering**—Monomeric recombinant fibrillin-1 fragments were isolated by Superdex 200 gel filtration (GE Healthcare). Samples eluting from the column passed through a Wyatt EOS 18-angle light scattering detector fitted with a 688-nm laser and an Optilab r-EX refractometer. The solute M_w and R_h values were determined using in-line multiangle laser light scattering attached to a (quasielastic) QELS and a differential refractometer (Wyatt Technology Corp.).

Analysis of the relationship between R_h and solute molecular mass provided details of the size and shape of C-terminal fibrillin-1 and how these parameters are affected by fragment mass. For a single molecule, a rodlike conformation gives an approximated α value of 1, whereas a compact symmetrical structure gives an approximated α value of 0.33. For each fibrillin-1 mass value, the slope α was calculated from the coordinates of the tangent fitted by regression analysis of the 10 surrounding mass values.

**Modeling**—Fibrillin-1 oligomers were represented as an assembly of spheres using the equation set out by Bloomfield et al. (21), which allows the calculation of theoretical friction values (f) for molecules of known shape. Other molecules modeled with good correlation in this way include phycocyanin and fibrinogen (21).

Modeling was performed using the assembly of 2-nm diameter spheres, and the theoretical S values were calculated for measurements performed in 0.1 M NaCl, 50 mM Tris, 1 mM CaCl₂, pH 8.0. We used 0.71 ml/g for the partial specific volume of fibrillin-1 (17, 18). Fibrillin-1 dimensions used as constraints in our modeling were as follows: (i) fibrillin-1 domain widths of ~2 nm based on solution NMR and crystallography, electron microscopy, and previous modeling (17, 22, 23); (ii) 90-nm solution length of fibrillin-1 determined by small angle x-ray scattering (20); (iii) the 150–180-nm extended length of fibrillin-1 as seen on mica (24); and (iv) the 50–60-nm periodicity of isolated and tissue microfibrils (18, 22, 24, 25). Calculated S values from the model were compared with experimental S values; the sphere arrangement in the model was modified accordingly and in all cases was limited by the above constraints and available structural information.

PF12 and PF13 were modeled using shapes provided by small angle x-ray scattering (20), as described (17). The calculated S values from the model were compared with experimental S values. Then PF12 and PF13 models were combined, taking into account their 3-nm overlap, which approximates to one TB domain and one cbEGF domain.

**Biotinylation of Heparin Saccharide**—The heparin used was a defined sized heparin saccharide dp24, which contains 24 monosaccharides (6) (kindly provided by Prof. J. Gallagher, University of Manchester). This fragment is a chemical analogue of the sulfated domains (S domains) of heparan sulfate. Biotinylation involved coupling via the heparin reducing end, in two stages (6, 26, 27). First, reductive amination with ammonia and then biotin was coupled to the free amines. Heparin species dp24 (0.1 mg) were dissolved in 2 M NH₄Cl, in a volume of 100 μl. 2 mg of NaCNBH₃ 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). 10 μl of 3 mg/ml sulfo succinimidyl-6-(biotinamido) hexanoate (Sulfo-NHS-LC-Biotin) (Pierce), was added and incubated...
overnight at 4 °C. The unreacted biotin was then removed by further dialysis into 0.1 M sodium acetate, pH 5.5.

**BIAcore Analysis of Heparin Interactions with Fibrillin-1 Fragments**—For kinetic binding studies of dp24 heparin saccharides with fibrillin-1 by surface plasmon resonance, a BIAcore biosensor was used (Biacore 3000; GE Healthcare). Biotinylated heparin fragments were immobilized onto commercially prepared SA sensor chips, which have preimmobilized streptavidin, to allow biotin capture. Using heparin commercially prepared SA sensor chips, which have preimmobilized streptavidin, to allow biotin capture. Using heparin concentrations of 1 μM, typically 150–200 response units of biotinylated heparin samples were immobilized, which was at a saturation level. Samples were applied to the sensor chip surface in 0.1 M sodium acetate, 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). Protein fragments were injected at concentrations ranging from 1 to 20 μg/ml at a flow rate of 12 μl/min. Samples were injected for 2 min and dissociated for 10 min, before the chip was regenerated using 5 mM NaOH, 1 M NaCl twice for 1 min each and then stabilized for 10 min using HBS-Ca, 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. Initially, all curves were fitted using a 1:1 Langmuir association/dissociation model (BIAevaluation 4.1; GE Healthcare). This model was found to fit the data for all of the protein fragments very well, apart from PF1 and PF4, with low χ² values. χ² values are a standard statistical measure of the closeness of fit (mean square of the signal noise).

Because of very high binding responses, PF1 and PF4 binding to heparin was calculated independently using equilibrium analysis. The equilibrium response was plotted against concentration, and nonlinear regression using the equation for one-site binding used to calculate Kᵋ.

**FIGURE 1.** Schematic diagram of recombinant fibrillin-1 protein fragments. Domain structures of N- and C-terminal fibrillin-1 fragments are shown, with a he of the different domains, N-glycosilation sites and N- and C-terminal proprotein convertase cleavage sites. All fibrillin-1 protein fragments are color-coded, and those used in this study are shown.
serum (BioWhittaker), 1 mM sodium pyruvate, nonessential amino acids (100×), minimum Eagle’s medium vitamins (100×), 2 mM L-glutamine, 10 μg/ml penicillin, 5 μg/ml streptomycin sulfate (Invitrogen) at 37 °C, 5% CO2 up to passage 6. Wild-type rat embryonic fibroblasts (REFs), mouse embryonic fibroblasts (MEFs; wild-type, syndecan-4 null), and CHOOK1 and CHO761 cells were available in the Couchman laboratory.

Cell Attachment Assays—Cell attachment assays were performed using 24-well plates coated with recombinant fibrillin-1 PF1 fragment, as previously reported (9, 28). Bound cells were quantified by incubating with 15 μM the fluorescent dye Calcein (Invitrogen) and then measuring in a Fluostar Galaxy plate reader (BMG Labtechnologies) at 485-nm excitation and 520-nm emission. Relative cell attachment was determined by comparison with known numbers of cells added to uncoated, unblocked wells. In all experiments, triplicate wells were used. Data were statistically analyzed using unpaired Student’s t tests (GraphPad Prism 2.0). Error bars represent S.D. values of the three experiments. Results were statistically significant when p was <0.05 (*, p < 0.05; **, p < 0.001; ***, p < 0.0001).

Immunofluorescence Microscopy—Spread cells were fixed after 2 h or 2 h 30 min in 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. Cells to be stained for focal adhesion kinase were fixed and permeabilized in ice cold methanol. Primary antibodies used were a monoclonal paxillin antibody Z035 (Zymed Laboratories Inc.), monoclonal anti-Tyr(P) antibody 4G10 (Upstate Biotechnology), monoclonal vinculin antibody hVin1 (Sigma), and polyclonal anti-focal adhesion kinase antibody BC3 (Upstate Biotechnology). Double fluorescence microscopy was performed using the described antibodies, followed by incubation with appropriate fluorochrome-conjugated secondary antibodies and phalloidin (Alexa Fluor 488/568; Molecular Probes, Inc., Eugene, OR). Samples were analyzed on an Olympus AX70 fluorescence microscope (Olympus; objectives, UPlanApo ×40, 1.0 numerical aperture oil iris, UPlanApo ×60, 1.4 numerical aperture oil iris).
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**RESULTS**

*Fibrillin-1 Terminal Heparin Binding Sites*

**N-terminal Fibrillin Binding Sites**

We previously reported that heparin binds with high affinity to the N-terminal fibrillin-1 fragment PF1 (6). Here, a series of PF1 deletion fragments has been generated to localize the heparin binding site(s) and define the binding kinetics (Fig. 1).

**Heparin Binding to PF1 and Deletion Fragments**—The association ($k_a$) and dissociation ($k_d$) rate constants of the molecular interactions between a polymeric heparin saccharide (dp24) and the fibrillin-1 fragments and their dissociation constants ($K_D$) were determined by surface plasmon resonance (Fig. 2, A and B, and Table 1). Heparin strongly bound to PF1 with a $K_D$ of 27.3 ± 0.9 nM. It also interacted with all the PF1 deletion fragments, but some of these interactions had markedly lower affinities for heparin than PF1. The affinities of the deletion PF1 fragments for heparin ranged from 116 ± 4.5 nM for the Ex1F-11 fragment to 248 ± 32 nM for Ex5–11. A short form of PF1, designated PF4 (11), which lacks the proline-rich region and flanking domains, bound heparin with an affinity of 104 ± 12 nM. Fragment PF2 overlaps PF1 at the proline-rich region and flanking domains but did not bind heparin.

Although binding kinetics for intact PF1 and PF4 could be calculated using global fitting and the 1:1 binding equation, the fitting for intact PF1 and PF4 could not be calculated using a 1:1 binding model. For these fragments, $K_D$ was calculated using equilibrium data.

### Table 1

| Fragments | $k_a$ ($\times 10^9$ M$^{-1}$ s$^{-1}$) | $k_d$ ($\times 10^{-1}$ s$^{-1}$) | $K_D$ (nM) |
|-----------|-----------------------------------|---------------------------------|------------|
| PF1       | 2.3 ± 0.1                         | 1.7 ± 0.2                       | 104        |
| Ex1F-11   | 2.8 ± 0.6                         | 2.1 ± 0.3                       | 116 ± 4.5  |
| Ex3–11    | 6.02 ± 0.8                        | 0.90 ± 0.08                     | 126 ± 6.6  |
| Ex4–11    | 1.30 ± 0.5                        | 0.21 ± 0.03                     | 144 ± 23   |
| Ex5–11    | 4.33 ± 1.3                        | 1.04 ± 0.29                     | 178 ± 41   |
| Ex6–11    | 6.20 ± 1.8                        | 0.92 ± 0.14                     | 248 ± 32   |
| Ex7–11    | 6.82 ± 0.9                        | 0.57 ± 0.13                     | 155 ± 23   |
| Ex5–7     | 57.8 ± 1.5                        | 0.35 ± 0.11                     | 144 ± 36   |
| Ex3–7     | 57.0 ± 1.5                        | 0.32 ± 0.11                     | 6 ± 1.8    |
| PF2       | 104 ± 12                          | 3.8 ± 2.3                       | 6 ± 1.8    |

### Table 2

| Fragments | $K_D$ (nM) | $R_{max}$ (S.E. of at least two separate experiments) |
|-----------|------------|--------------------------------------------------|
| PF1       | 36.5 ± 3.2 | 319 ± 66                                         |
| Ex1F-11   | 43.4 ± 5.9 | 284 ± 75                                         |
| Ex3–11    | NB         | NB                                               |
| Ex4–11    | NB         | NB                                               |

*PF1 and PF4 did not follow the 1:1 binding model, so, for these fragments, $K_D$ was calculated using equilibrium data.

The relative binding responses of all N-terminal fibrillin-1 fragments (PF1, PF1 deletion fragments, PF2, and PF4) to heparin were also plotted, for an analyte concentration of 10 µg/ml, after 12 min of association (Fig. 2B). Both PF1 and PF4 had a highly elevated response compared with all of the other fragments, with response units of 5315 and 4250 compared with the PF1 deletion fragments, which had response values ranging from 624 for Ex1F-11 to 44 for Ex5–11.

The enhanced binding response of PF1 and PF4 to immobilized heparin was not due to PF1 being aggregated prior to Biacore analysis. Multiscan laser light scattering and analytical ultracentrifugation results confirmed that the PF1 fragments were monomeric (not shown). PF1 differs from Ex1F-11 only by the additional 17-amino acid N-terminal sequence that precedes the proprotein convertase cleavage site (AGNVKET-RASRAKR), which thus contains a heparin-mediated fibrillin-1 multimerization signal.

We also mapped MAGP-1 binding to the same 17-amino acid precursor site sequence site (Fig. 2C), which accords well with the MAGP-1 binding sites.
with reported MAGP-1 binding and competition with heparin (12, 31). Only PF1 and Ex1F-11 interacted with MAGP-1 (KD of 36 ± 3.2 and 43 ± 5.9 nM, respectively; Table 2). N-terminal heparan sulfate binding is thus likely to be a critical determinant of microfibril assembly and of MAGP-1 binding during subsequent elastic fiber assembly.

**Heparin Binds a Three-domain N-terminal Region**

—Heparin binding to the PF1 deletion fragments and to PF4 indicated that a heparin binding site is present within the cbEGF domains encoded by exons 7 and 8. To confirm this localization, a recombinant fragment comprising the hybrid domain within PF1 and flanking cbEGF domains (see Fig. 1) was tested for heparin binding by BIAcore analysis (Fig. 2). Ex5–7 was found to bind with a high affinity of 6.0 ± 1.8 nM (Table 1). Together, these data localize this heparin binding site within the cbEGF domain encoded by exon 7.

**C-terminal Heparin Binding Sites**

Heparin has been shown to bind to the last 17 residues of the C-terminally furin-processed fibrillin-1, which is encoded by exon 64 and ends with RKR (7). Here, we have identified a novel upstream high affinity fibrillin-1 heparin binding site within a new recombinant fibrillin-1 fragment, designated PF16, which is encoded by exons 50–65 and comprises overlapping PF12 and PF13 fragments (6) (Fig. 1).

SDS-PAGE analysis of recombinant PF16 fragment revealed two bands with molecular mass values of 89 and 63 kDa (Fig. 3A). The lower mass band, designated PF16trunc, had 26 kDa less mass than PF16. Mass spectrometry analysis of PF16 and PF16trunc confirmed that the 89-kDa fragment corresponded to furin-processed PF16, since the last detected peptide was INGYPKR, which immediately precedes the RKRR furin cleavage site (3). The PF16trunc fragment was C-terminally truncated, and the last detected PF16trunc tryptic peptide was GFSLDQTGSSCED-VDECEGNHR, which is within the cbEGF encoded by exon 7.

**TABLE 3**

|          | Troponin | Heparin |
|----------|----------|----------|
| k_{a}    | \(10^3\) | \(10^3\) |
| PF16     | 93.7 ± 1.0 | 0.1 ± 0.0 |
| PF16_{trunc} | 88.3 ± 4.2 | 0.2 ± 0.0 |
| k_{d}    | \(10^{-3}\) | \(10^{-3}\) |
| PF16     | 1.5 ± 0.4 | 1.7 ± 0.3 |
| PF16_{trunc} | 0.9 ± 0.4 | 2.0 ± 0.1 |
| k_{d}    | \(10^3\) | \(10^3\) |
| PF16     | 0.0 ± 0.0 | 0.0 ± 0.0 |
| PF16_{trunc} | 0.2 ± 0.0 | 0.0 ± 0.0 |

**FIGURE 3. BIAcore analysis of interactions of heparin with C-terminal fibrillin-1 fragments.** A, SDS-PAGE showing recombinant PF16 and PF16_{trunc} fragments run in reducing conditions and corresponding tryptic peptides identified by mass spectrometry. B, C-terminal fibrillin-1 protein fragments PF16 and PF16_{trunc} were injected over a heparin oligosaccharide-immobilized surface. Each sensorgram shows analyte concentrations ranging from 1 to 20 \(\mu\)g/ml, with duplicate concentrations included in every run. One representative experiment is shown. Response difference (Resp. Diff.) is the difference between experimental and control flow cells, in response units (RU). Time is shown in seconds (s).

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Biocore analysis showed that PF16 and PF16_{trunc} both interacted very strongly with heparin (5.6 ± 0.2 and 8.1 ± 2.1
FIGURE 4. Attachment and spreading of REFs on PF1. A, REFs attached to PF1 in a heparin-inhibitable but not chondroitin sulfate-inhibitable manner. B, REFs spread poorly on PF1 (10 μg/ml) and Ex3–11 (and on Ex5–11 and Ex6–11; not shown) compared with REFs on FN (10 μg/ml). Actin filaments are fluorescently stained with phalloidin, and vinculin is detected by immunofluorescence staining. C, CHOK1 or CHO761 cells on FN or PF8 or on PF8 in the presence of soluble PF1 (1 μg/ml) or hepll (1 μg/ml). The CHOK1 cells spread and formed focal plaques on FN or on PF8 in the presence of either PF1 or hepll. However, the CHO761 cells failed to form focal adhesions on PF8 in the presence or absence of PF1 or hepll.
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Heparin Regulates Fibrillin-1 Interactions with Cells and Elastic Fiber Molecules

Heparin-inhibitable PF1-mediated Cell Attachment and Spreading

Although PF1 does not contain known integrin adhesion motifs, REFs adhered to PF1 (Fig. 4A) and to all the PF1 deletion fragments (not shown). When REFs were plated on PF1, attachment was significantly inhibited by heparin (1–100 µg/ml). Chondroitin sulfate had no effect on adhesion. REFs spread poorly on all of the PF1 fragments (Fig. 4B).

We have reported that the fibrillin-1 RGD-containing fragment PF8 supports cell adhesion and spreading, mainly through α5β1 integrin, and may activate syndecan-4 (9). REFs plated on PF8 had some stress fibers but no focal plaques (Fig. 5A). Soluble fibronectin heparin-binding fragment hepII, which activates syndecan-4 (not shown), or PF1 or the PF1 deletion fragments all induced stress fibers and some focal plaques (Fig. S2). Exogenous heparin (1–100 µg/ml) similarly disrupted focal plaque induction in REFs plated either on PF8 when incubated with soluble PF1 or on the 110-kDa fibronectin fragment (designated 110FN; comprises repeats III3–11 (includes the RGD and synergy region) when incubated with hepII (Figs. 5A and S3).

When plated on PF8, CHO761 mutant cells, which do not produce glycosaminoglycans, did not induce focal plaques in response to PF1 or hepII, unlike control CHOK1 cells (Fig. 4C). However, when we analyzed syndecan-4 null MEFs, it was evident that although syndecan-4 null MEFs on PF8 had poorly organized cytoskeletons, the addition of PF1 still induced some organized plaques comparable with wild-type MEFs (58 and 44%, respectively; Fig. S1). Interestingly, when syndecan-4 null MEFs were plated on PF8 and soluble hepII was added, the cells could hardly form any plaques (8%). Thus, although PF1-induced focal plaques on PF8 are heparin/heparan sulfate-inhibitable, perhaps more than one syndecan is involved in this process.

REFs plated on PF8 (Fig. 5A; Fig. S2) or on 110FN (Fig. 5B) and incubated with PF1 deletion mutants were compared for focal plaques. Abundant plaques were detected on 110FN in the presence of all PF1 deletion fragments. For REFs on PF8, fewer

![FIGURE 5. Spreading of REFs on PF8 or FN in the presence of soluble PF1.](image)

A, REFs on FN spread and induced focal plaques. REFs on PF8 spread but only induced focal plaques in the presence of soluble PF1 (1 µg/ml), which was

nm, respectively) (Fig. 3B and Table 3), although PF16 trunc lacks the previously reported 17-amino acid C-terminal heparin binding site that precedes the convertase cleavage site (7). We confirmed that C-terminal fragment PF12 did not bind heparin, whereas PF13 bound strongly (15–20 nm) (6). Taken with the PF16 trunc data, this heparin binding site is further localized within three fibrillin-1 cbEGFs encoded by exons 59–62.
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FIGURE 6. Binding of C-terminal fibrillin-1 recombinant protein fragments to tropoelastin. A, Biaffore analysis of the interactions of PF16 and PF16trunc with tropoelastin. Fibrillin-1 fragments at a concentration of 1–5 μg/ml were injected over tropoelastin-immobilized sensor chip surfaces. One representative experiment is shown. Response difference (Resp. Diff.) is the difference between experimental and control flow cells, in response units (RU). Time is shown in seconds (s). B, using solid-phase binding assays, tropoelastin (12.5–200 nM) was plated at 4 °C prior to blocking with BSA and subsequent binding of biotinylated C-terminal fibrillin-1 fragments, as described under "Experimental Procedures." Nonspecific binding to BSA was subtracted from all data points. Results shown are the means ± S.E. of triplicate values of a single experiment, with each experiment repeated at least twice. Binding of each fibrillin-1 fragment to tropoelastin (relative to BSA control) was determined biophysically (Table 4). PF12 and PF13 have similar affinity binding of tropoelastin (12); PF16trunc also bound immobilized tropoelastin with very high affinity (Kd = 1.7 ± 0.3 nM). Solid phase binding assays allowed comparison of tropoelastin interactions with PF16, PF16trunc, PF12, or PF13, using wells coated with tropoelastin (12.5–200 nM). PF16 interacted most strongly with tropoelastin, PF16trunc, and PF13 showed similar strong levels of binding, but PF12 showed little binding (Fig. 6B). We also showed that REFs on PF18 in the presence of soluble PF13 formed focal plaques (Fig. 5D). These data extend our previous report that PF13 (but not PF12) binds tropoelastin (12); PF16trunc allows us to localize the site within domains encoded by exons 59–62 and also indicates that high affinity binding of tropoelastin is conformation-dependent.

Biotinylated PF16 (100 nM) pretreated for 30 min at room temperature with or without 100 nM heparin (Fig. 6C) was incubated in wells precoated with tropoelastin (12.5–200 nM). At equimolar concentrations, preincubation of PF16 with heparin significantly inhibited PF16 binding to tropoelastin. Thus, tropoelastin and heparin binding sites on PF16 may overlap.

The mass and shape of PF12, PF13, and PF16 were also determined biophysically (Table 4). PF12 and PF13 have similar Rh/Rh values (3.7 and 3.4 nm, respectively), whereas PF16 has a higher

Sequence that induces a high binding response (see Fig. 2B) (Table 1). Focal plaques induced for cells on PF8 by PF1 appeared less mature than fibronectin-mediated adhesions, since they contained vinculin (Fig. 5, A and B), paxillin (not shown), and some phospho-Tyr but little focal adhesion kinase (Fig. 5C).

Together, these experiments show that PF1 induces heparin/heparan sulfate-inhibitable focal plaques, probably through the heparin binding site in cbEGF encoded by exon 7.

Heparin Inhibits C-terminal Interactions with Tropoelastin

Heparin binds to fibrillin-1 and induces a high binding response (Fig. 2B) (Table 1). Focal plaques induced for cells on PF8 by PF1 appeared less mature than fibronectin-mediated adhesions, since they contained vinculin (Fig. 5, A and B), paxillin (not shown), and some phospho-Tyr but little focal adhesion kinase (Fig. 5C).

Together, these experiments show that PF1 induces heparin/heparan sulfate-inhibitable focal plaques, probably through the heparin binding site in cbEGF encoded by exon 7.

Heparin Inhibits C-terminal Interactions with Tropoelastin

BIAcore analysis of tropoelastin binding to fibrillin-1 revealed that PF16 bound tropoelastin very strongly, with a Kd of 1.5 ± 0.4 nM (Fig. 6A and Table 3). PF16trunc also bound immobilized tropoelastin with very high affinity (Kd = 1.7 ± 0.3 nM). Solid phase binding assays allowed comparison of tropoelastin interactions with PF16, PF16trunc, PF12, or PF13, using wells coated with tropoelastin (12.5–200 nM). PF16 interacted most strongly with tropoelastin, PF16trunc, and PF13 showed similar strong levels of binding, but PF12 showed little binding (Fig. 6B). We also showed that REFs on PF18 in the presence of soluble PF13 formed focal plaques (Fig. 5D). These data extend our previous report that PF13 (but not PF12) binds tropoelastin (12); PF16trunc allows us to localize the site within domains encoded by exons 59–62 and also indicates that high affinity binding of tropoelastin is conformation-dependent.

Biotinylated PF16 (100 nM) preincubated for 30 min at room temperature with or without 100 nM heparin (Fig. 6C) was incubated in wells precoated with tropoelastin (12.5–200 nM). At equimolar concentrations, preincubation of PF16 with heparin significantly inhibited PF16 binding to tropoelastin. Thus, tropoelastin and heparin binding sites on PF16 may overlap.

The mass and shape of PF12, PF13, and PF16 were also determined biophysically (Table 4). PF12 and PF13 have similar Rh/Rh values (3.7 and 3.4 nm, respectively), whereas PF16 has a higher

Sequence that induces a high binding response (see Fig. 2B) (Table 1). Focal plaques induced for cells on PF8 by PF1 appeared less mature than fibronectin-mediated adhesions, since they contained vinculin (Fig. 5, A and B), paxillin (not shown), and some phospho-Tyr but little focal adhesion kinase (Fig. 5C).

Together, these experiments show that PF1 induces heparin/heparan sulfate-inhibitable focal plaques, probably through the heparin binding site in cbEGF encoded by exon 7.
Rh value of 5.1 nm. Furthermore, the frictional ratios \( (f/f_0) \) of PF12 and PF13 are 1.6 and 1.5, respectively, whereas the frictional ratio of PF16 is 1.8. Spherical structures have a frictional ratio of 1, and the value rises as the molecule elongates. PF12 and PF13 are relatively elongated in solution, but PF16 is even more elongated (Fig. 7). Bead modeling (21) predicts that PF16 has an S-shaped structure, with the binding region for heparin and tropoelastin at a loop adjacent to the PF12-PF13 overlap (Fig. 7). This result accords well with small angle x-ray scattering analysis, which showed nonlinear solution shapes for the smaller fragments, PF12 and PF13 (20).

### DISCUSSION

Although it is now clear that fibrillin-1 is a major extracellular heparin/heparan sulfate binding molecule, the consequences of heparin/heparan sulfate binding are less apparent. Heparan sulfate may regulate fibrillin-1 multimerization and microfibril assembly, interactions with cell surfaces through syndecan or glypican receptors, and fibrillin-1 interactions with other elastic fiber molecules during elastic fibrillogenesis. Here, we analyzed heparin interactions with fibrillin-1 N- and C-terminal regions, which are considered to regulate linear assembly. We have defined novel N-terminal heparin binding sites at both termini, shown that heparin may support N-terminal multimerization, demonstrated that the PF1 fragment directs heparin-inhibitable cell spreading and focal plaque formation, and identified a novel high affinity, conformation-dependent tropoelastin binding site close to the C terminus that is inhibited by heparin.

Three regions of fibrillin-1 were originally shown to interact with heparin/heparan sulfate (7, 8). Our previous studies identified four binding sites (6). Data in this study, combined with previous data, highlight that fibrillin-1 contains at least five high affinity heparin binding sites, confirming the critical role of heparan sulfate in fibrillin-1 biology.

Mapping of N-terminal heparin binding sites revealed that the sequence preceding this convertase cleavage site plays a critical role in heparin binding. When present, monomeric N-terminal fragments exhibit a huge binding response to heparin-coated chips, indicating that this short sequence controls N-terminal fibrillin-1 multimerization. Thus, heparin-induced N-terminal multimerization and subsequent furin cleavage (which occurs extracellularly (4); the sequence preceding the cleavage site was not detected in mass spectroscopy of tissue microfibrils (30)) may control microfibril assembly. Interestingly, we mapped MAGP-1 binding to the adjacent unique region immediately after the cleavage site and preceding the first EGF-like domain (see Fig. 1), which accords well with reported MAGP-1 binding and competition with heparin (12,
N-terminal heparan sulfate binding is thus likely to be a critical determinant of fibrillin-1 polymerization during microfibril assembly and of MAGP-1 binding during elastic fiber assembly.

Using deletion, overlapping, and short fragments, we have mapped a heparin binding site to the cbEGF encoded by exon 7, which contains three lysines. This site may bind cell surface heparan sulfate and induce a cellular response. Within assembled microfibrils, this N-terminal region may be at the beads (20). The ability of this N-terminal fibrillin-1 sequence to induce heparin-inhibitable focal plaques implies that it can associate with cell surface heparan sulfate proteoglycans. However, the efficient formation of N-terminal fibrillin-1-induced plaques in syndecan-4 null cells indicates the involvement of other syndecans or glypicans.

High affinity heparin binding to a C-terminal site was localized within cbEGFs encoded by exons 59–62, a region that contains seven lysines and two arginines. A high affinity C-terminal tropoelastin binding site identified within the same cbEGFs may be critical for elastic fiber formation. Since heparin competes with tropoelastin, these sites are probably overlapping. Heparan sulfate may thus regulate elastin deposition onto microfibrils.

This C-terminal tropoelastin binding site may be conformation-dependent, since the affinity of tropoelastin for PF16 is much higher than for PF13 (12). Bead modeling based on biophysical solution measurements predicted that the longer PF16 fragment has an S-shaped structure with a loop at the beginning of the shorter fragment PF13 that forms the tropoelastin and heparin binding sites. Perhaps newly secreted fibrillin-1 initially binds cell surface heparan sulfate through its C terminus, and subsequently tropoelastin binds this region following microfibril assembly. Within an assembled microfibril, this C-terminal region is likely to be adjacent to the bead (20).

Heparan sulfate is a critical determinant of microfibril assembly, since exogenous heparin, inhibition of sulfation, and inhibition of attachment of heparan sulfate to protein cores all block assembly in culture (7, 8). Heparin does not impede fibrillin-1 secretion (8) but may compete with cell surface heparan sulfate to interact with fibrillin-1.

There are few reports of heparin binding to cbEGFs in extracellular matrix molecules. Heparin binding to fibrillin-1 through cbEGFs raises the intriguing possibility that this unusual feature may influence cell behavior through epidermal growth factor or related receptors in a manner similar to heparan-binding EGF-like growth factor (HB-EGF), which regulates cell signaling in cancer, wound healing, and other pathologies (32). In summary, we have provided new insights into novel heparin binding sites on fibrillin-1 and into the biological consequences of these strong interactions.

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