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Heparin/heparan sulfate controls fibrillin-1, -2 and -3 self-interactions in microfibril assembly

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ABSTRACT

Fibrillins form multifunctional microfibrils in most connective tissues. Deficiencies in fibrillin assembly can result in fibrillinopathies, such as Marfan syndrome. We demonstrate the presence of heparin/heparan sulfate binding sites in fibrillin-2 and -3. Multimerization of all three fibrillins drastically increased the apparent affinity of their interaction with heparin/heparan sulfate. Surprisingly, contrary to other reports heparin/heparan sulfate strongly inhibited homo- and heterotypic N-to-C-terminal fibrillin interactions. These data suggest that heparin/heparan sulfate controls the formation of microfibrils at the bead interaction stage.

Structured summary of protein interaction:
rFBN1-N binds to rFBN1-C by solid phase assay (View interaction)
rFBN1-N binds to rFBN2-C by solid phase assay (View interaction)
rFBN2-N binds to rFBN1-C by solid phase assay (View interaction)
rFBN2-N binds to rFBN2-C by solid phase assay (View interaction)
Fibronectin binds to rFBN2-C by solid phase assay (View interaction)
Fibronectin binds to rFBN2-N by solid phase assay (View interaction)
Fibronectin binds to rFBN1-N by solid phase assay (View interaction)
Fibronectin binds to rFBN1-C by solid phase assay (View interaction)
Fibronectin binds to rFBN3-C by solid phase assay (View interaction)

1. Introduction

Three extracellular glycoproteins, fibrillin-1, -2 and -3 constitute the fibrillin family. Each member of this family is characterized by a modular organization composed primarily of calcium-binding epidermal growth factor-like (cbEGF) domains and transforming growth factor (TGF)-β binding domains (TB) [1]. Fibrillins are the main integral components of multi-component assemblies, termed microfibrils [2]. Extracted microfibrils display a characteristic "bead-on-a-string" structure [3]. Microfibrils fulfill a number of crucial physiological functions in the cardiovascular system, bones, eyes, skin and other tissues [4]. They act as a scaffold in elastic fiber formation, as stress-bearing entities, and as reservoirs for growth factors of the TGF-β superfamily [5–7]. Deficiencies in microfibrils have devastating consequences on tissue function and integrity resulting in severe connective tissue disorders [8]. Fibrillin-1 mutations result for example in Marfan syndrome, autosomal dominant Weill–Marchesani syndrome and stiff skin syndrome, whereas fibrillin-2 mutations cause congenital contractural arachnodactyly [9–12].

Despite recent advances, the complete mechanism of fibrillin assembly into microfibrils is still poorly defined. We previously demonstrated that the recombinant C-terminal half of fibrillin-1 multimerizes in a cell-associated fashion [13]. The multimers have a characteristic bead shape with 8–12 peripheral arms, closely

Abbreviations: BSA, bovine serum albumin; cbEGF, calcium-binding epidermal growth factor-like domain; MAGP-1, microfibril-associated glycoprotein-1; TB, transforming growth factor-β binding domain; TBS, Tris-buffered saline; TBST, TBS/Tween-20

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resembling the beads in microfibrils. We also showed that multimerization of the fibrillin-1 C-terminus increases the apparent affinity to its N-terminus [13]. Elongation occurs through fibrillin N-to-C terminal interactions in a polarized manner [14,15]. Several molecules have been implicated in microfibril assembly including heparan sulfate and fibronectin [16–19].

Proteoglycans and glycosaminoglycans have been localized to microfibrils and some have been implicated in microfibril assembly. The dermatan sulfate-containing biglycan interacts with microfibrils, whereas decorin can form a ternary complex with fibrillin-1 and microfibril-associated glycoprotein-1 (MAGP-1) [20]. The heparan sulfate-containing proteoglycan perlecan in basement membranes directly interacts with fibrillin-1 and colocalizes with microfibrils at basement membrane zones with potential implications on microfibril assembly in these regions [21]. Kielty et al. have demonstrated that microfibril integrity is disrupted by treatment with chrondroitinase-4,6-sulfate lyases [22]. The most studied glycosaminoglycan in microfibril assembly is heparin/heparan sulfate. Heparan sulfate consists of repeats of sulfated disaccharide units of glucuronic acid and N-acetylglucosamine, and each unit can be modified by N- and O-sulfation as well as by uronate epimerization [23]. These modifications often occur in clusters resulting in sulfated domains. The degree of modification regulates protein interaction to heparan sulfate. For example, fibrillin-1 only interacts with highly sulfated heparan sulfate [17]. Heparan sulfate is not found as a free glycosaminoglycan in tissues. In its physiological state it is covalently linked to a number of core proteins to form various proteoglycans including cell surface located syndecans and glypicans or matrix located perlecan, agrin, collagen type XV or type XVIII [23]. Heparin is structurally very similar to heparan sulfate and is frequently used experimentally as a cost-effective substitute for heparan sulfate [23].

Fibrillin-1 interaction domains with heparin/heparan sulfate have been described in seven regions of the protein (see Fig. 1A) [17,19,24–26]. A heparin/heparan sulfate interaction site in fibrillin-2 has been localized to cbEGF7-TB3 [19]. The fibrillin-1 heparin/heparan sulfate interaction sites were shown to be specific for heparin/heparan sulfate and could not interact with other glycosaminoglycans [17]. Knowledge of heparin/heparan sulfate interactions with fibrillin-2 is still rudimentary, and it is not known if fibrillin-3 also interacts with this glycosaminoglycan. Heparin/heparan sulfate may regulate the composition of microfibrils as it inhibits in vitro the interactions of tropoelastin or MAGP-1 with

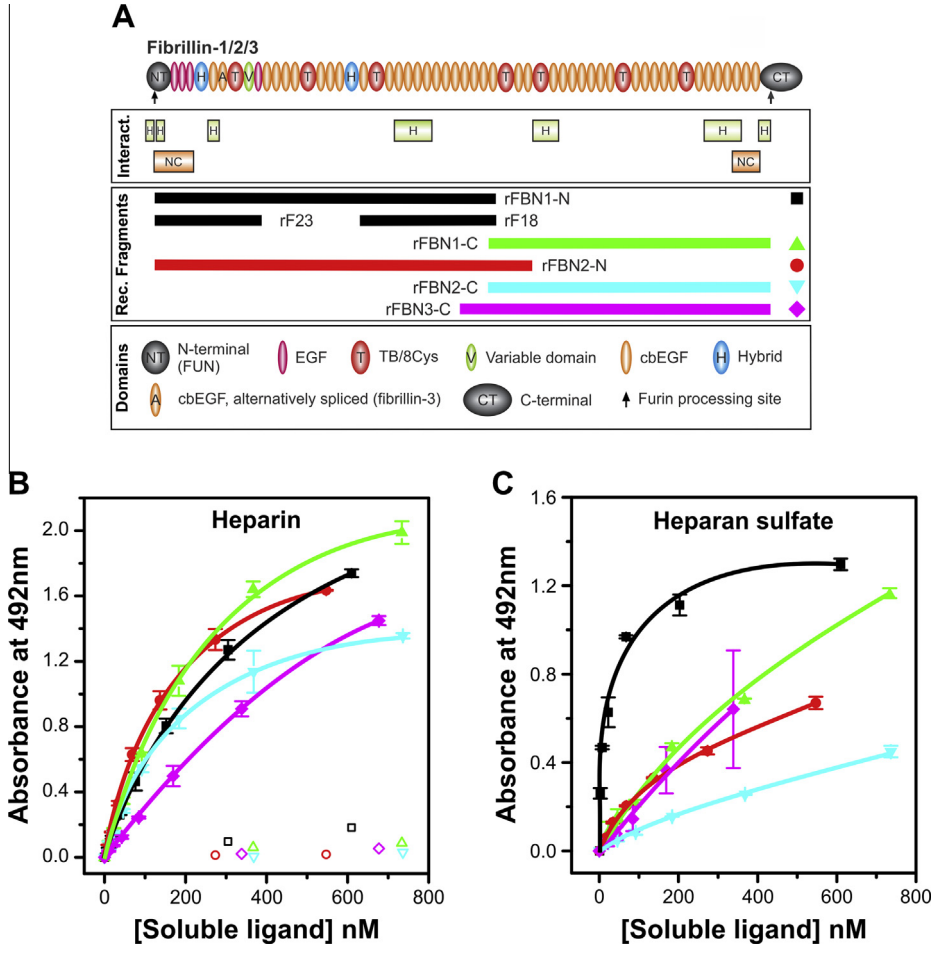

Fig. 1. Fibrillin interactions with heparin and heparan sulfate. (A) A schematic drawing of recombinant fibrillin-1, -2 and -3 is shown on top. The "Interactions" panel indicates previously identified heparin-binding sites ("H") as well as N-to-C-terminal ("NC") fibrillin interaction sites in fibrillin-1. The "Recombinant Fragments" panel illustrates the fibrillin fragments used in this study. The color and symbol of each fragment corresponds to the respective fragment binding profile in B and C. Note that the first heparin-binding site located in the N-terminal propeptide of fibrillin-1 is not included in the recombinant fragments. The "Domains" panel indicates the names of the domains corresponding to the schematic fibrillin drawing on top. (B) Shown is a representation of a typical solid phase binding assay. Heparin-BSA (closed symbols) and BSA (open symbols) used as a control, were immobilized. Serial dilutions of recombinant fibrillin fragments were added as soluble ligands at the indicated concentrations. (C) Soluble recombinant fibrillin fragments were added to immobilized heparan sulfate-BSA. For B and C, all C-terminal fragment preparations consisted of a mixture of fibrillin assembly states and were not gel-filtrated. Data sets represent means of duplicates; standard deviations are indicated. Non-specific interaction with BSA (OD_{492nm} = 0.1–0.2) was subtracted from all values.

fibrillin-1 [24]. Heparin/heparan sulfate interactions with fibrillins play a critical role in fibrillin network assembly as it inhibits fibrillin-1 fiber formation when added exogenously to mesenchymal cell cultures [17,19]. However, the exact role of heparin/heparan sulfate in the biogenesis of microfibrils is not well defined.

2. Materials and methods

2.1. Antibodies

The generation of rabbit polyclonal antibodies against the N- and C-terminal halves of fibrillin-1 (anti-rFBN1-N, anti-rFBN1-C) [17,21], fibrillin-2 (anti-rFBN2-N, anti-rFBN2-C) [15], and fibrillin-3 (anti-rFBN3-C) [16] was described previously. The monoclonal anti-fibronectin antibody (anti-FN clone 15) was purchased (Sigma; #F7387). Secondary antibodies labeled with fluorescent dyes were purchased from Jackson ImmunoResearch Laboratories.

2.2. Proteins and glycosaminoglycans

The production and purification of the N- and C-terminal halves of fibrillin-1 (rFBN1-N, rFBN1-C) [27], fibrillin-2 (rFBN2-N, rFBN2-C) [15], and fibrillin-3 (rFBN3-C) [16] was described previously. Purification of the N-terminal half of fibrillin-3 was intensively attempted, but remained unsuccessful due to extreme proteolytic susceptibility. Purification of the recombinant fibrillin-1 fragments rF18 and rF23 and of human plasma fibronectin was previously described [14,28,29]. Heparin from porcine intestinal mucosa (Sigma; #H3393) was resuspended in 50 mM Tris–HCl, pH 7.4, 150 mM NaCl Tris-buffered saline (TBS) and dialyzed against the same buffer. Heparin was also coupled to bovine serum albumin (heparin–BSA) (Thermo Fisher Scientific; #BP1605) as described [30]. The same procedure was used to couple heparan sulfate (Sigma; #H7640) to BSA.

2.3. Cell culture conditions and heparin inhibition

Human skin fibroblasts were isolated from foreskin after circumcision and cultivated under standard conditions in DMEM containing 10% fibronectin-depleted fetal calf serum. For inhibition, heparin (0.25–1 mg/mL) and heparin-BSA (3.1–25 μg/mL) was added at the time of cell seeding (7.5×10^4 cells/well) in eight-well chamber slides. Cells were fixed after 3 days and stained for fibronectin, fibrillin-1 and fibrillin-2 as described previously [16].

2.4. Solid phase and inhibition assay

Solid phase binding assays were performed as previously described [15]. In brief, 10 μg/mL TBS of heparin-BSA and heparan sulfate-BSA, fibronectin, or fibrillin were immobilized in 96-well plates (Maxisorp). Blocking was performed with 5% non-fat milk in TBS including 2 mM $CaCl_2$. All washing steps were performed with 0.05% Tween-20 in TBS (TBST). Recombinant fibrillin fragments were diluted in 2% non-fat milk in TBS including 2 mM $CaCl_2$ (binding buffer) and incubated with the immobilized protein for 2 h. For inhibition experiments, constant concentrations (50 μg/mL) of soluble ligands were added with various concentrations of heparin in binding buffer. Primary antibody (1:1000 diluted) was added for 1.5 h. Peroxidase-conjugated goat anti-rabbit antibody (1:800 diluted) was incubated for 1.5 h followed by a color reaction.

2.5. Surface plasmon resonance spectroscopy

Surface plasmon resonance spectroscopy was performed with a BiacoreX instrument (GE Healthcare). C1 sensor chips were used with heparin-BSA or fibronectin immobilized by amine coupling on one channel and BSA as a control immobilized on the second channel. Real-time association and dissociation were monitored in duplicates or triplicates by injecting proteins diluted at 0.1–100 μg/mL in TBS/2 mM $CaCl_2$, pH 7.4. Kinetic analyses were performed using the BIAevaluation software by fitting the association and dissociation curves with a 1:1 binding model.

2.6. Gel filtration chromatography

Multimers, intermediates and monomers of the recombinant C-terminal halves of fibrillin-1 (rFBN1-C), fibrillin-2 (rFBN2-C) and fibrillin-3 (rFBN3-C) were separated by Superose 6 gel filtration chromatography (GE Healthcare; 100 mL column) as described previously using ~2.7 mg of recombinant proteins [13,16].

3. Results

3.1. Fibrillin-1, -2 and -3 interactions with heparin and heparan sulfate

Previous studies have identified seven heparin/heparan sulfate binding sites in human fibrillin-1 and one in the central region of human fibrillin-2 [17,19,24,25]. To investigate if additional heparin interaction sites exist in human fibrillin-2 and whether fibrillin-3 also interacts with heparin, solid phase binding assays were performed with recombinant N- and C-terminal halves of fibrillin-1 and -2 and with the C-terminal half of fibrillin-3 (Fig. 1A and B). Fibrillin-2 and -3 fragments bound heparin-BSA similar as fibrillin-1 fragments. No significant interaction between the fibrillin fragments and the BSA control was observed. We conclude that one or more high affinity heparin-binding sites exist in the fibrillin-2 N- and C-terminal halves and in the fibrillin-3 C-terminal half.

Heparin and heparan sulfate are structurally very similar as both macromolecules are composed of the same disaccharide repeating unit [23]. Heparin is thus used here and in many other studies as a cost-effective substitute for heparan sulfate, which is found in tissues where fibrillins are expressed [23]. Therefore, we compared the interactions of the fibrillins with heparin and heparan sulfate (Fig. 1B and C). Generally, all tested fibrillin fragments interacted with heparan sulfate-BSA. Fragment rFBN1-N typically showed stronger interaction and rFBN2-C was characterized by lower binding profiles with heparan sulfate as compared to heparin.

3.2. Fibrillin multimerization regulates heparin interaction

We have shown that C-terminally mediated multimerization of fibrillin is important for the generation of high affinity interaction sites for fibrillin-1 N-to-C-terminal self-interactions, and for interactions with fibronectin [13,16]. To investigate if multimerization is required for the interaction with heparin, multimers, intermediates and monomers of rFBN1-C, rFBN2-C and rFBN3-C were separated by gel filtration chromatography and tested for interaction with heparin-BSA (Fig. 2A). Multimers interacted strongly with heparin-BSA, intermediates interacted moderately, and monomers showed virtually no interactions in solid phase interaction assays. As an important control, we verified that the antibodies used to detect the bound ligands did not show increased affinity for the multimeric versus monomeric form of each fibrillin (Fig. 2B). We then compared the heparin interaction of the multimeric C-terminal fibrillin-1 with previously identified heparin-binding sites in the N-terminus mediated by monomeric fragments rF18 and rF23 (Fig. 2C) [13,17,24,25]. Again, relative to multimeric

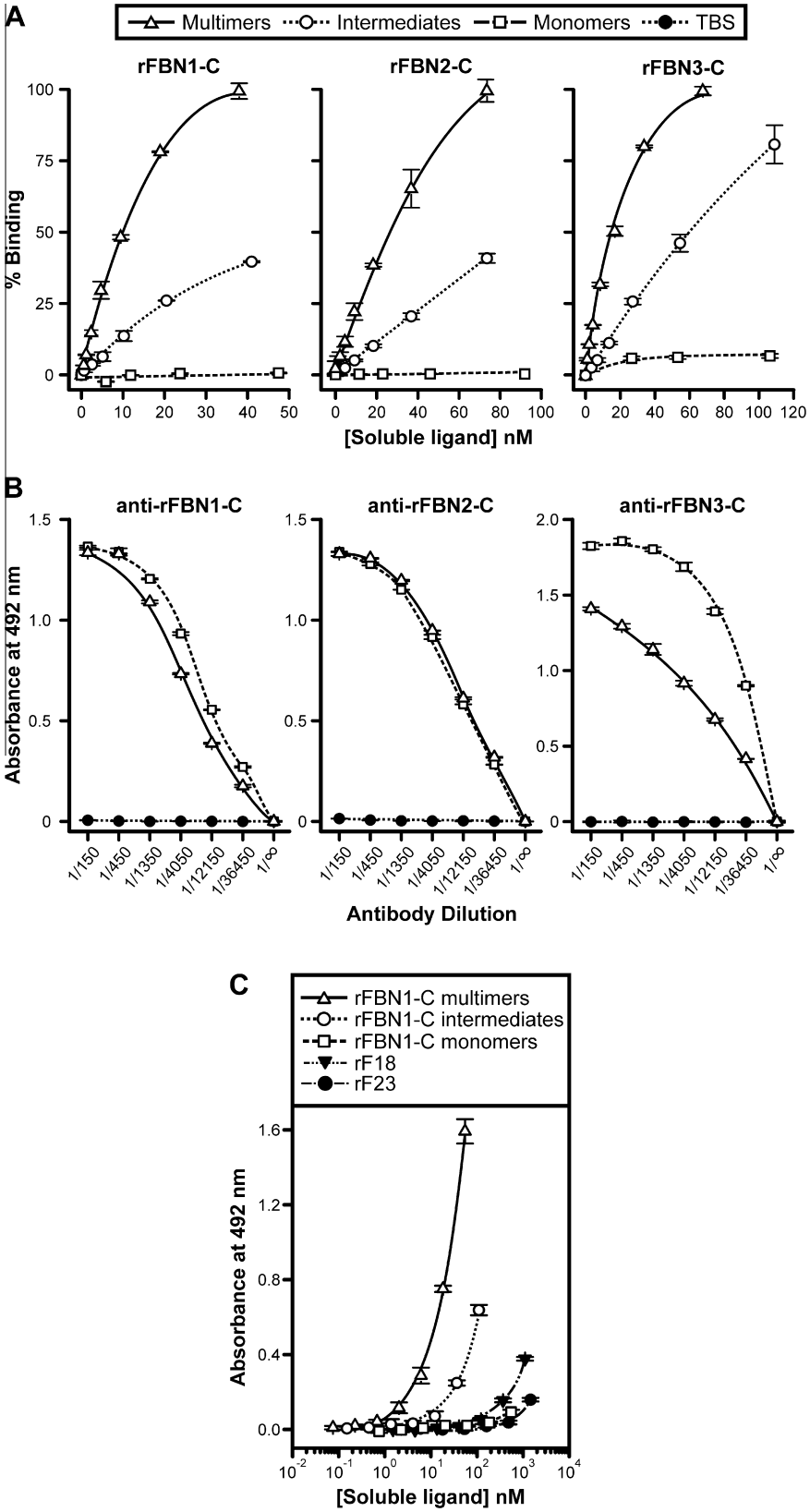

Fig. 2. Multimerization of the fibrillin C-terminal halves drastically increases the interaction with heparin. (A) Shown is a representative solid phase binding assay. Heparin-BSA was immobilized and incubated with serial dilutions of soluble rFBN1-C, rFBN2-C and rFBN3-C multimers (open triangles), intermediates (open circles) or monomers (open squares). Data sets represent means of duplicates. Standard deviations are indicated. (B) ELISA assay with immobilized rFBN1-C (left panel), rFBN2-C (middle panel), and rFBN3-C (right panel) monomers (open squares) and multimers (open triangles). TBS without immobilized protein (closed circles) was used as a control. The respective antisera were incubated with the ligands at the indicated dilutions. Error bars represent standard deviations of duplicate values. (C) Shown is a typical solid phase binding assay. Heparin-BSA was immobilized and incubated with soluble fibrillin-1 N-terminal monomeric fragments rF18 (closed triangles) and rF23 (closed circles). For comparison, multimers (open triangles), intermediates (open circles) and monomers (open squares) of rFBN1-C were also included. Means of duplicate data sets are indicated and background interaction with BSA (OD_{492nm} = 0.1–0.2) was subtracted. Error bars represent standard deviations.

Table 1
Binding parameters of soluble fibrillin C-terminal halves with immobilized heparin analyzed by surface plasmon resonance spectroscopy. Mixtures represent purified protein preparations including monomers, intermediates and multimers. Additional gel filtration of the protein preparations separated monomers, intermediates and multimers.

Fragment		K_D (nM)	χ^2	k_a ($M^{-1}s^{-1}$)	k_d (s^{-1})
rFBN1-C	Mixture	1.02 (±0.65)	4.19 (±1.65)	$7.36\ (\pm1.86) \times 10^5$	$7.50\ (\pm2.11) \times 10^{-4}$
	Monomers	16.63 (±5.92)	1.66 (±0.45)	$3.36\ (\pm0.96) \times 10^4$	$4.47\ (\pm0.64) \times 10^{-4}$
	Intermediates	2.68 (±0.42)	1.82 (±0.11)	$1.43\ (\pm0.14) \times 10^5$	$4.33\ (\pm0.98) \times 10^{-4}$
	Multimers	0.12 (±0.03)	2.43 (±0.26)	$4.35\ (\pm0.26) \times 10^5$	$5.35\ (\pm0.43) \times 10^{-5}$
rFBN2-C	Mixture	5.15 (±2.06)	4.82 (±2.55)	$2.33\ (\pm3.35) \times 10^5$	$1.20\ (\pm0.57) \times 10^{-3}$
	Monomers	34.39 (±10.31)	4.23 (±0.87)	$3.27\ (\pm0.25) \times 10^4$	$1.12\ (\pm0.29) \times 10^{-3}$
	Intermediates	19.71 (±6.02)	4.99 (±0.90)	$7.51\ (\pm1.47) \times 10^4$	$1.48\ (\pm0.21) \times 10^{-3}$
	Multimers	1.37 (±0.85)	5.48 (±2.16)	$2.27\ (\pm2.11) \times 10^5$	$3.11\ (\pm1.96) \times 10^{-4}$
rFBN3-C	Mixture	0.82 (±0.32)	3.74 (±1.85)	$6.69\ (\pm2.94) \times 10^5$	$5.48\ (\pm1.23) \times 10^{-4}$
	Monomers	38.78 (±15.50)	1.72 (±0.23)	$2.63\ (\pm0.60) \times 10^4$	$1.02\ (\pm0.30) \times 10^{-3}$
	Intermediates	0.35 (±0.10)	4.71 (±2.70)	$3.34\ (\pm0.50) \times 10^5$	$1.17\ (\pm0.41) \times 10^{-4}$
	Multimers	0.19 (±0.05)	3.34 (±1.59)	$3.38\ (\pm0.84) \times 10^6$	$6.42\ (\pm1.58) \times 10^{-4}$

rFBN1-C, the N-terminal monomeric rF18 and rF23 interacted at least two magnitudes weaker with heparin. It is important to mention that these binding experiments were optimized for the interaction of multimeric rFBN1-C with heparin. Extended assay times show heparin/heparan sulfate binding of rF18 and rF23 as reported previously, and as expected from other studies [17,24,25]. These experiments demonstrate that multimerization of all three fibrillins drastically increases their apparent affinity for heparin.

To quantify the affinities of multimeric, intermediate and monomeric fibrillin C-termini for heparin, we employed surface plasmon resonance spectroscopy (Table 1; Suppl. Fig. 1). Multimers of rFBN1-C, rFBN2-C and rFBN3-C showed dissociation constants in the low nanomolar and sub-nanomolar range, between K_D = 0.12–1.37 nM, demonstrating very high affinity for heparin. As expected from the solid phase assays, monomers showed significantly lower affinities for heparin with dissociation constants between 17 and 39 nM, and intermediates were characterized with dissociation constants between those of multimers and monomers. Differences in the monomer interaction with heparin between solid phase assays (virtually no interaction) and surface plasmon resonance spectroscopy likely originate from the different immobilization methods (adsorption versus amine coupling).

To compare these findings with the affinities of rFBN-C-fibronectin interactions, which are also dependent on fibrillin multimerization [16], we determined their dissociation constants by surface plasmon resonance spectroscopy (Table 2). As expected from the previous study, multimers of rFBN1-C, rFBN2-C and rFBN3-C interacted with fibronectin about 5–7 times more strongly ($K_D \sim$ 12–16 nM) than their respective monomers ($K_D \sim$ 55–96 nM). Compared to the interactions with heparin, the fibrillin–fibronectin interactions were characterized by lower affinities of 1–2 magnitudes.

Table 2
Dissociation constants of soluble monomeric and multimeric fibrillin C-terminal halves with immobilized fibronectin determined by surface plasmon resonance spectroscopy.

Fragment		K_D (nM)[a]
rFBN1-C	Monomers	55.34 (±1.82)
	Multimers	11.87 (±2.14)
rFBN2-C	Monomers	84.75 (±5.20)
	Multimers	16.28 (±3.76)
rFBN3-C	Monomers	95.51 (±9.67)
	Multimers	12.83 (±3.18)

[a] Closeness fit error parameter χ^2 is from 0.86 to 4.72.

3.3. Heparin inhibits fibrillin homo- and heterotypic interactions

Fibrillin-1 forms homotypic interactions between its N- and C-terminus and forms heterotypic interactions with fibrillin-2 [15]. Fibrillin-1 and -2 can co-polymerize in microfibrils [31]. To investigate whether heparin functionally interferes with the fibrillin N-to-C-terminal interactions, we tested its capacity to inhibit fibrillin homo- and heterotypic interactions (Fig. 3A). Heparin efficiently inhibited both, homotypic and heterotypic interactions of rFBN1-C and rFBN2-C with rFBN1-N and rFBN2-N, suggesting that heparin/heparan sulfate plays a regulatory role at the fibrillin self-assembly stage. Heparin did not inhibit the interaction between rFBN1-C, rFBN2-C, rFBN3-C, or rFBN1-N and rFBN2-N with fibronectin (Fig. 3B). As expected, heparin did inhibit the formation of fibrillin-1 and -2 networks in cell culture, but did not inhibit fibronectin network formation (Fig. 3C). Similar inhibition profiles were observed with heparin-BSA (data not shown).

4. Discussion

We have previously shown that fibrillin-1, -2 and -3 multimerize through domains in their C-terminal halves, generating bead-like structures that closely resemble the beads in microfibrils [13,16]. This multimerization is a critical step in fibrillin-1 assembly, as it increases the apparent affinity of the C-terminal region (cbEGF41-43) to an N-terminal region (FUN-EGF3) [13,15,26,32]. These findings led to the hypothesis that C-terminally multimerized and disulfide-bonded beads represent the initial microfibril assembly unit [13]. Other interesting, but unexplained, findings relevant to fibrillin assembly demonstrated that addition of heparin and heparan sulfate dose-dependently inhibited microfibril network formation in cell culture models [17,19]. Here, we show data providing mechanistic explanations for the role of heparin/heparan sulfate in microfibril assembly.

We determined that the C-terminal halves of fibrillin-2 and -3 interact with heparin and heparan sulfate with similar affinity as the respective fibrillin-1 region. Ritty et al. found that chemical multimerization of a synthetic peptide representing the last 17 residues of the processed fibrillin-1 protein (Cys^{2715}–Arg^{2731}) increases its affinity to heparin [19]. In the present study, we show with two independent methods that multimerization of the C-terminal halves of all three fibrillins dramatically increases the apparent affinity to heparin, compared to C-terminal monomers or monomeric fragments from the N-terminal half. Quantification of heparin-binding strengths by surface plasmon resonance spectroscopy clearly demonstrated that the apparent affinities significantly increase, up to two hundred-fold, from monomers to multimers of the C-terminal halves. The dissociation constants of multimers

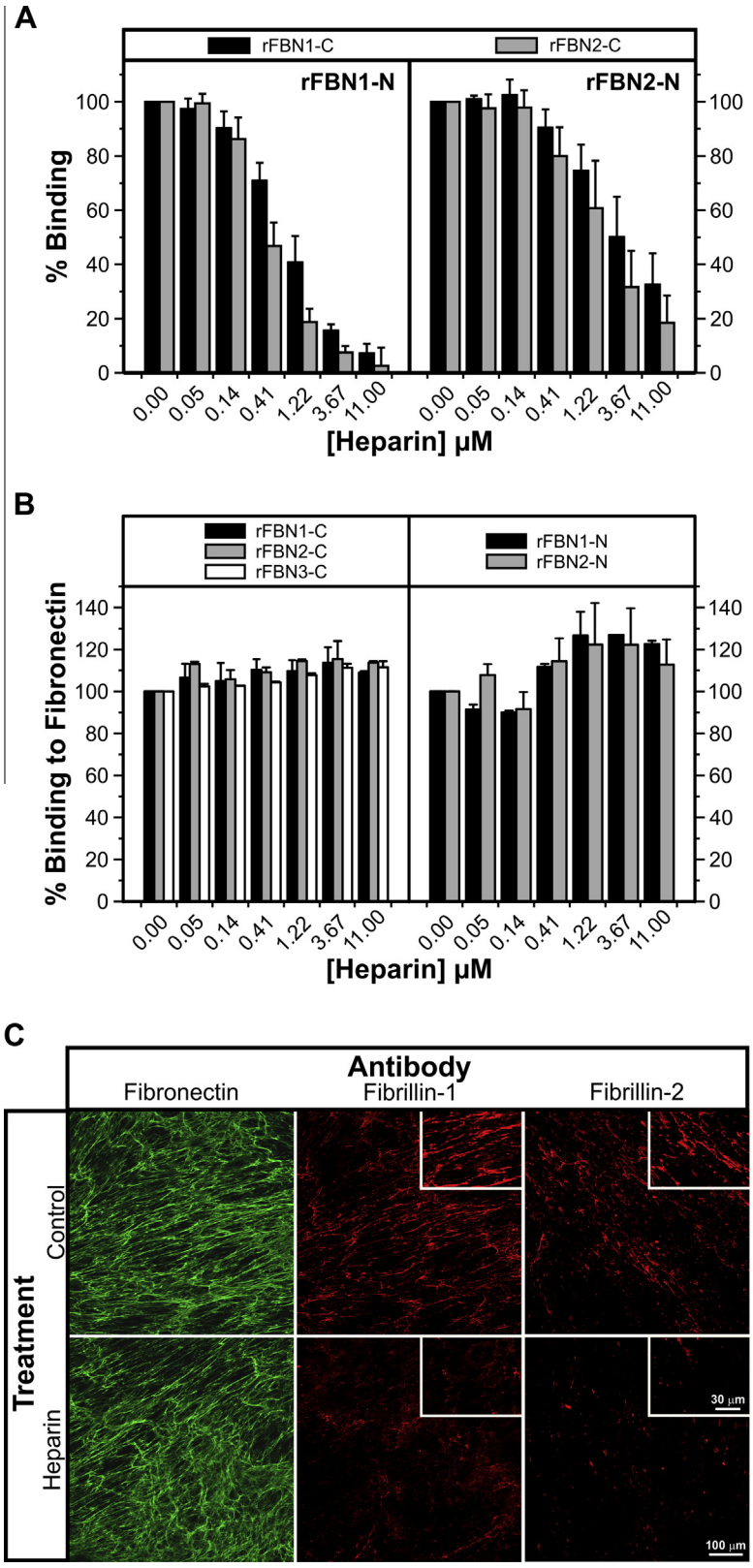

Fig. 3. Heparin inhibits fibrillin homo- and heterotypic interactions, but does not inhibit fibrillin interaction with fibronectin. (A and B) Shown are representative solid phase inhibition experiments. (A) rFBN1-N and rFBN2-N, as indicated, were immobilized and incubated with constant concentrations (50 µg/mL) of rFBN1-C (black bars) or rFBN2-C (grey bars) in the presence of increasing concentrations of heparin. The signal without heparin is set at 100%. Data sets represent means of quadruplicates for the rFBN1-N coated and sextuplicates for the rFBN2-N coated experiment. Standard deviations are indicated. (B) Fibronectin was immobilized and incubated with constant concentrations (50 µg/mL) of rFBN1-C (black bars), rFBN2-C (grey bars), or rFBN3-C (white bars) in the left panel, or rFBN1-N (black bars) or rFBN2-N (grey bars) in the right panel. Increasing concentrations of heparin as indicated were added simultaneously with the soluble ligands. The signal without heparin is set at 100%. Data sets represent means of duplicates. Standard deviations are indicated. (C) Indirect immunofluorescence staining for fibronectin (green, left panel), fibrillin-1 (red, middle panel) and fibrillin-2 (red, right panel) after fibroblasts were cultured for 3 days in the presence of a control volume of TBS (top row) or 0.25 mg/mL heparin (bottom row). Insets show higher magnifications of fibrillin-1 and -2 staining.

were in the picomolar range for fibrillin-1 and -3 and in the low nanomolar range for fibrillin-2. The K_D values observed here for the monomeric fractions of the C-terminal halves of all three fibrillins were typically higher (lower affinity) than the values observed for smaller monomeric recombinant C-terminal subfragments of fibrillin-1 used by Cain et al. (16.6–38.8 nM versus 2.9–17.1 nM) [25], whereas multimeric fragments displayed much lower K_D values (in the latter study, multimers were excluded by gel-filtration chromatography). The reason for the discrepancy with the monomeric fragments could stem from the different fragment sizes used. It is possible that binding sites are more cryptic in a larger protein context (e.g. C-terminal halves) versus in the smaller subfragments used by Cain et al. Different immobilization methods (heparin-BSA vs. biotinylated heparin) could also have contributed to these differences. Upon multimerization of the C-terminal halves, the drastic increase in the apparent affinity could occur through the exposure of such cryptic interaction sites and/or through the clustering of the individual binding sites (increase in avidity). Upon multimerization of the full-length fibrillin in cell culture or in vivo, it is possible that all heparin-binding sites in the analyzed monomeric fragments of the N-terminus will also have their apparent affinity for heparin/heparan sulfate raised. However, based on the shape of the C-terminal multimers with N-terminal "arms" loosely extending from the bead domain [13], we expect the increase in their apparent affinity to be much less pronounced than that of the densely packed C-terminal fibrillin multimers. The propensity to aggregate currently prevents the use of full-length fibrillin to study these questions [15].

It was previously reported by Cain et al. that heparin does not inhibit the interaction of an N-terminal fragment of fibrillin-1 (PF1, comprising the region between the N-terminus and EGF4) with a C-terminal fragment (PF13 spanning from TB7 to the C-terminus) [24,25]. Contrary to those results, in the present study we clearly demonstrate that heparin can strongly and effectively inhibit fibrillin-1 and -2 homo- as well as heterotypic N-to-C-terminal interactions. One obvious difference between the studies is that Cain et al. used monomeric fragments, whereas the preparations of the C-terminal halves contain significant amounts of ordered multimers [13]. Since two of the three heparin-binding sites in rFBN1-C are located adjacent to the N-to-C-terminal self-interaction site (see Fig. 1A), it is conceivable that high affinity heparin/heparan sulfate interaction with the multimers sterically interferes with this self-interaction at the bead stage. This provides a mechanistic explanation why exogenously added heparin or heparan sulfate to cell cultures prevents fibrillin network formation [17,19]. We excluded in cell culture an indirect inhibition through heparin-induced changes of the available fibronectin network, which has been previously shown to be critical for microfibril formation [16,18].

The assembly of fibrillins into microfibrils is a multistep and complex process. In Fig. 4, we attempt to combine the experiments presented here with previously published data into a refined model of microfibril assembly. After cleavage of the fibrillin propeptides (step 1), cell-associated multimerization into bead-like structures occurs through the C-terminal domain (step 2) [13,33]. Multimerization of fibrillins significantly increases the apparent affinity to (i) heparin/heparan sulfate (shown here), (ii) the N-terminus of fibrillin-1 [13], and (iii) fibronectin (shown in [16] and quantified here). Based on the much higher apparent affinities of the fibrillin beads to heparin/heparan sulfate compared to fibronectin, we propose that the beads first interact with cell surface-located heparan sulfate-containing proteoglycans (step 3). Based on the very high affinity of the densely packed fibrillin C-termini, it is more likely that this region interacts with cell surface-located heparin/heparan sulfate compared to the less densely packed N-termini, as others have suggested [26]. The positioning of beads to the cell surface is supported by a recent study demonstrating punctate cell surface-located fibrillin-1 deposits in early assembly [34]. Bax et al. observed that the heparin-binding fibrillin-1 fragments induced focal adhesions, suggesting that syndecans are involved in fibrillin-1-mediated focal adhesion formation [35]. Fibrillin-triggered focal adhesion formation and tension mediated through intracellular actin filaments potentially serves to position bead-like multimers in close vicinity (step 4). The high propensity of multimeric beads to interact in an N-to-C-terminal fashion likely generates the polarity of the fibrillin molecules in nascent microfibrils. Eventually, these nascent microfibrils are transferred onto

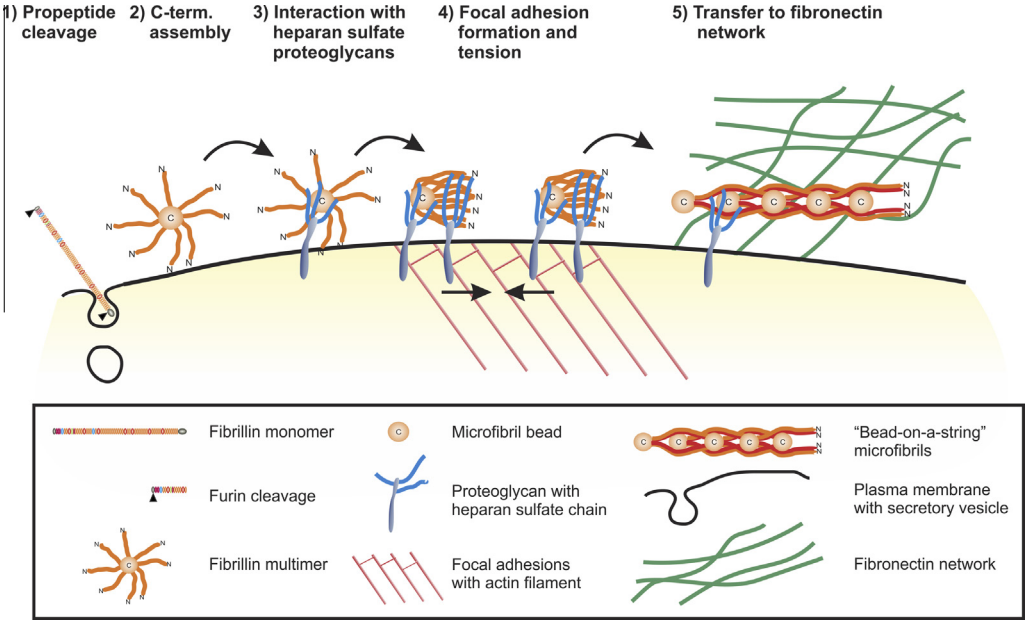

Fig. 4. Model of microfibril assembly. At the time of secretion, fibrillin propeptides are cleaved (Step 1), and the proteins assemble into multimers through their C-termini (Step 2). Multimers interact with heparan sulfate proteoglycans on the cell surface via C-terminal binding sites mediated by increased apparent affinities upon multimerization (Step 3). Induction of focal adhesion formation and tension hypothetically helps position the bead-like fibrillin multimers in close vicinity to promote N-to-C-terminal self-interaction of the beads (Step 4). Transfer of nascent microfibrils onto fibronectin fibers for further stabilization, elongation and interaction with other microfibril proteins (Step 5).

fibronectin fibers, which can act to stabilize growing microfibrils, or potentially provide a template for elongation and interaction with other microfibril proteins (step 5) [34,36].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2014.06.061.

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