Interactions of Fibrillin-1 with Heparin/Heparan Sulfate, Implications for Microfibrillar Assembly*

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Fibrillin-1 is a major constituent of the 10–12 nm extracellular microfibrils. Here we identify, characterize, and localize heparin/heparan sulfate-binding sites in fibrillin-1 and report on the role of such glycosaminoglycans in the assembly of fibrillin-1. By using different binding assays, we localize two calcium-independent heparin-binding sites to the N-terminal (Arg45–Thr450) and C-terminal (Asp1028–Arg1486) regions of fibrillin-1. A calcium-dependent-binding site was localized to the central (Asp1028–Thr1486) region of fibrillin-1. Heparin binding to these sites can be inhibited by a highly sulfated and iduronated form of heparan sulfate but not by chondroitin 4-sulfate, chondroitin 6-sulfate, and dermatan sulfate, demonstrating that the heparin binding regions represent binding domains for heparan sulfate. When heparin or heparan sulfate was added to cultures of skin fibroblasts, the assembly of fibrillin-1 into a microfibrillar network was significantly reduced. Western blot analysis demonstrated that this effect was not due to a reduced amount of fibrillin-1 secreted into the culture medium. Inhibition of the attachment of glycosaminoglycans to core proteins of proteoglycans by β-xylosides resulted in a significant reduction of the fibrillin-1 network. These studies suggest that binding of fibrillin-1 to proteoglycan-associated heparan sulfate chains is an important step in the assembly of microfibrils.

Microfibrils are supramolecular filaments, 10–12 nm in diameter, found in many extracellular matrices (1). They occur either as individual fibers, interconnected with basement membranes, or on the surface of elastic fibers (for review see Ref. 2). The major integral components of microfibrils are fibrillin-1 and fibrillin-2 (3, 4) and the microfibril-associated glycoprotein-1 (5). It has been suggested that other components such as microfibril-associated glycoprotein-2 (6), fibrillin-2 (7), latent transforming growth factor β-binding proteins-1 and -2 (8, 9), and chondroitin sulfate-containing proteoglycans (10–12) are associated with microfibrils. However, for most of these ligands direct interaction studies with fibrillins are lacking, and it is not known how these components contribute to structure and function of microfibrils in tissues.

The assembly of fibrillins into complex supramolecular tissue microfibrils is a multistep process. Several steps within the continuum of microfibrillar assembly have been described. One of the first steps in the assembly process is the oligomerization of fibrillin-1 into disulfide-bonded multimers, which occurs within a few hours after the secretion of fibrillin-1 from cells (13). It has not been demonstrated whether this process occurs in the extracellular space without the participation of cells or whether it is a cell-mediated process perhaps involving integrin receptors (14–16) or other cell-surface molecules. In cell culture, a fibrillin-containing network forms over a few days (17). However, it takes several weeks of culture for fibroblasts to produce the typical bead-on-a-string structures, discernible by electron microscopy (18). Tissue microfibrils do not appear as bead-on-a-string structures but as simple thread-like filaments (3). The molecular basis for the morphological differences during the formation of microfibrils is obscure. Controversial models have been proposed for the arrangement of fibrillin-1 within assembled microfibrils, based on cross-linking patterns (19), on the solution structure of epidermal growth factor (EGF)1 like modules (20), on the ultrastructural morphology of microfibrils (21), on mutation analysis of fibrillin-1 (22), on the localization of monoclonal antibody epitopes (23), or based on findings by atomic force microscopy (24) or electron tomography (25).

Many mutations in the fibrillin-1 protein lead to a heritable autosomal dominant disorder, the Marfan syndrome (Online Mendelian Inheritance in Man number 154700), with major complications in the skeletal, the cardiovascular, and the ocular system (26). The mutant fibrillin-1 is thought to exert a dominant negative effect on the structure or stability of microfibrils that precipitates the defects characteristic to Marfan syndrome, although the precise molecular pathogenetic pathway is not known. Central to these discussions are issues related to the effect of mutant fibrillin-1 molecules on the assembly of microfibrils. Are mutant fibrillin-1 molecules incorporated into microfibrils, where they may destabilize the microfibrils by proteolytic degradation (27)? Alternatively, mutant molecules may disrupt microfibril assembly. Pulse-chase experiments have revealed that many fibroblast strains isolated from patients with Marfan syndrome deposit reduced amounts of fibrillin into the extracellular matrix, suggesting that many mutations in fibrillin-1 impair the ability of the molecules to assemble (28, 29).

Here, we report on the occurrence, the identification, the characterization, and the localization of heparin/heparan sulfate-binding sites in fibrillin-1. The interaction of fibrillin-1 with heparin/heparan sulfate has an important role in the assembly of microfibrils.

* This work was supported by Deutsche Forschungsgemeinschaft Grant SFB365/A1 (to B. B.) and Grants Re1021/3 and Re1021/4 (to D. P. R.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: EGF, epidermal growth factor; BSA, bovine serum albumin; cb, calcium-binding; cbEGF, calcium-binding EGF; PBS, phosphate-buffered saline.
EXPERIMENTAL PROCEDURES

**Recombinant Proteins—**Recombinant subdomains of human fibrillin-1 rF6, rF18, rF6H, rF23, rF45, and rF47 have been described in detail previously. The expression plasmid for rF6 was designed to express the entire C-terminal half of fibrillin-1 (position 1487–2871; Ref. 23). However, it has been shown that the C-terminal unique domain of fibrillin-1 is proteolytically processed between position 2751 and 2771 by furin-type proteases (31). Consequently, rF6 spans amino acid residues 1487–2731 of fibrillin-1 and thus is almost identical to recombinant subdomain rF6H (position 1487–2725) except for a hexahistidine tag at the C-terminal end of rF6H. Similarly, the subdomain rF23 has been shown to be processed between positions 44 and 45, resulting in a truncated N-terminal end (7).

The recombinant subdomain rF51, spanning calcium binding (cb) EGF 6 to cbEGF 10 of fibrillin-1, human fibrillin-1 cDNA (32) was amplified by polymerase chain reaction with sense oligonucleotide 5′-CTGATGTAAGAGTACCTTCGTTG-3′ and antisense oligonucleotide 5′-ACCCGTGAGCTATTATGATGGTGA-TGGTGTAAGACAGATCCTTCGTGGC-3′ introducing a restriction site for Nhel at the 5′ end and a restriction site for Xhol plus a sequence for a hexahistidine tag and a stop codon at the 3′ end of the restriction site for Nhel into the Xhel-Xhol restricted plasmid pDNSP-F16 in frame to the sequence for a signal peptide (23). The correct sequence was verified by DNA sequencing. The resulting plasmid was termed pDNSP-rF51 and was used to recombinantly express the polyepitope rF51 with the amino acid residues 1487–2786 (Ala-Pro-Leu-Ala-Ala-Pro-Leu-Ala) resulting from the cloning strategy. The methods for transfection, selection of stable clones, and production of recombinant medium were described in detail previously (33). Purification of rF51 was performed as described for rF18 (23). Correct folding of rF51 was verified by binding to monomodal antibody 201 that is dependent on correct disulfide bonds.

**Polyclonal Antiserum and Monoclonal Antibodies—**A polyclonal antiserum (α-rF6H) was produced according to standard procedures in rabbit using the recombinant C-terminal half of fibrillin-1 rF6H as antigen. The specificity of this antiserum was verified by immunoblotting and enzyme-linked immunosorbent assays with rF6H, fibrillin-1 from cell culture medium, and other matrix proteins. The polyclonal antiserum B9543 was generated against an N-terminal half of fibrillin-1 and was characterized previously (34). The B9543 antiserum as well as monoclonal antibodies 201 and 84 were a generous gift from Prof. Lynn Y. Sakai, Shriners Hospitals for Children, Portland, OR.

**Protein-Ligand Binding and Inhibition Assays—**Affinity chromatography on heparin-Sepharose columns (HiTrap Heparin HP, 1 ml; Amersham Pharmacia Biotech) equilibrated in 20 mM Tris-HCl, pH 7.4, 50 mM NaCl, 2 mM CaCl₂, and 0.05% NaN₃ (buffer-M) was performed at room temperature (20 °C) with highly purified recombinant fibrillin-1 subdomains (100–200 μg) applied to the columns in equilibration buffer at a flow rate of 0.1 ml/min. After washing the columns with equilibration buffer, bound material was eluted with a linear NaCl gradient (0.05–1 M NaCl in 20 ml) in the same buffer at a flow rate of 0.5 ml generated by a Gradient Programmer GP-250 Plus and two P-500 pumps (Amersham Pharmacia Biotech). The flow-through and the eluted volume was continuously fractionated in 0.7-ml aliquots by a Frac-100 collector (Amersham Pharmacia Biotech), whereas the amount of protein in each fraction was monitored at 280 nm using a UV/vis spectrophotometer (Amersham Pharmacia Biotech). The NaCl concentrations in each individual fraction were determined by measurement of the conductivity and comparison to standards using a Microprocessor Conductivity Meter (LF3000, WTW, Germany). The correlation of the amount of individual recombinant fibrillin-1 subdomains with the absorbance recorded at 280 nm was verified by SDS-gel electrophoresis and Coomasie Blue staining.

**Solid phase binding assays of fibrillin-1 subdomains to heparin were performed at room temperature (20 °C) with highly purified recombinant fibrillin-1 subdomains (100–200 μg).** The first four steps were performed at room temperature (20 °C) with highly purified recombinant fibrillin-1 subdomains (100–200 μg) applied to the columns in equilibration buffer at a flow rate of 0.1 ml/min. After washing the columns with equilibration buffer, bound material was eluted with a linear NaCl gradient (0.05–1 M NaCl in 20 ml) in the same buffer at a flow rate of 0.5 ml generated by a Gradient Programmer GP-250 Plus and two P-500 pumps (Amersham Pharmacia Biotech). The flow-through and the eluted volume was continuously fractionated in 0.7-ml aliquots by a Frac-100 collector (Amersham Pharmacia Biotech), whereas the amount of protein in each fraction was monitored at 280 nm using a UV/vis spectrophotometer (Amersham Pharmacia Biotech). The NaCl concentrations in each individual fraction were determined by measurement of the conductivity and comparison to standards using a Microprocessor Conductivity Meter (LF3000, WTW, Germany). The correlation of the amount of individual recombinant fibrillin-1 subdomains with the absorbance recorded at 280 nm was verified by SDS-gel electrophoresis and Coomasie Blue staining.

**Detection of Secreted Fibrillin-1 and Fibronectin in Cell Culture—**Normal human skin fibroblasts were seeded at 5 × 10⁵ cells/3.8-cm² plate (12-well plates; Naخلف Nunc International) in the presence of 0–4 mg/ml heparin. The cells were grown confluent for 1 day, washed three times with PBS, and incubated with 2 ml serum-free culture medium including 0–4 μg/ml heparin for 3 days. In the presence and absence of heparin, no differences in the morphologies of the cells were observed. After harvesting and filtrating (0.22 μm pore size) the conditioned medium, the proteins were precipitated from 1-ml aliquots with 10% TCA and 10 mg/ml trichloroacetic acid. The non-reduced samples were analyzed by a standard Western blotting procedure using monoclonal antibodies (α-rF6H) to detect fibrillin-1 and 84 to detect fibronectin.
Results

Binding of Fibrillin-1 to Heparin and Identification of Binding Sites—Previously, it has been shown by others (15) that denatured (6 M guanidine HCl) and reduced fibrillin, isolated from fetal bovine ligamentum nuchae, bound to heparin-Sepharose. Since it was not clear whether this binding represented an authentic binding interaction or whether it was conferred by the reduced and denatured state of fibrillin, binding of native authentic fibrillin-1 to heparin was tested. Conditioned medium produced by skin fibroblasts was incubated with immobilized heparin. A specific antiserum was used to detect fibrillin-1, which bound in a dose-dependent manner to heparin (Fig. 1). This result clearly established that authentic binding interaction or whether it was conferred by heparin-Sepharose was above the physiological ionic strength indicating that these interactions potentially occur in tissues.

A solid phase binding assay was used to further confirm these binding data, whereby various recombinant fibrillin-1 subdomains were used in the soluble phase with immobilized heparin (Fig. 4). Saturable binding profiles in the presence of calcium were observed for rF6H, rF18, and rF23, whereas rF45 and rF51 showed no or only very minor binding (Fig. 4A). When the binding tests were performed in the presence of EDTA, the binding of rF18 to heparin was significantly reduced, whereas binding of rF23 and rF6H did remain on similar levels (Fig. 4B).

Taking the overlapping regions of the recombinant polypeptides into account, three regions with heparin-binding affinity within the fibrillin-1 molecule have been identified as follows: one calcium-dependent binding region in the center of the molecule (position 1028–1486) and two calcium-independent binding regions, one at the N-terminal end (position 45–489) and one within the C-terminal half (position 1528–2731).

Inhibition Studies with Glycosaminoglycans—Although heparin is not a component of extracellular matrices, it initially was used in this study because of its structural similarity with other glycosaminoglycans that can be found in the extracellular matrix. Subsequently, it was tested whether glycosaminoglycans such as various forms of chondroitin sulfates and heparan sulfates can compete with heparin binding of the fibrillin-1 subdomains rF6H, rF18, and rF23 (Fig. 5). Heparin itself inhibited the interaction between all three subdomains with immobilized heparin (Fig. 5A). These data demonstrate that the observed interactions are specific. The amounts of heparin that resulted in 50% inhibition (IC50 value) were in a range of 770–1730 μg/ml (51–115 μM). Chondroitin-4-sulfate (Fig. 5B), dermatan sulfate (Fig. 5C), and chondroitin 6-sulfate (Fig. 5D) had none or very minor inhibitory effects on the interaction of all three recombinant fibrillin-1 subdomains with immobilized heparin. On the other hand, inhibition with the highly sulfated and iduronated heparan sulfate 6 resulted in inhibition of the heparin interaction with all three fibrillin-1 subdomains (Fig. 5E). The IC50 values for heparan sulfate 6 were in a range between 235 and 720 μg/ml (12–36 μM), slightly lower than what has been observed with heparin as inhibitor. The high sulfation and/or iduronation pattern of heparan sulfate 6 was
necessary for binding since the lower sulfated and iduronated heparan sulfate 2 did not have any inhibitory activity (Fig. 5F).

These experiments demonstrate that the identified heparin binding regions in fibrillin-1 represent unique binding sites for a highly sulfated form of heparan sulfate but not for various forms of chondroitin sulfates. However, these results do not exclude the possibility that binding sites other than those for heparin/heparan sulfate exist in the fibrillin-1 molecule for binding to chondroitin sulfates.

It is known that clustering of binding sites within a narrow physical region can enhance binding strengths by several magnitudes (36). Glycosaminoglycans attached to the protein core of proteoglycans are almost always clustered. To mimic the clustered glycosaminoglycan pattern on proteoglycans, we used heparin coupled to bovine serum albumin (H10114–5 heparin chains per molecule albumin) in inhibition assays instead of soluble heparin (Fig. 6). For the interactions between the recombinant fragments rF6H, rF18, rF23 and heparin, the IC50 values of clustered heparin (BSA-heparin) were in the range of 0.07–0.77 μg/ml (2–26 nM heparin), which is 4400–25,500-fold less as compared with non-clustered soluble heparin (Fig. 5A and Fig. 6). These data demonstrate that the affinity of heparin significantly increases when the molecules are presented as clusters to the fibrillin-1 subdomains, as compared with non-clustered heparin molecules. It further suggests that potential binding of fibrillin-1 to glycosaminoglycan chains of proteoglycans are of high affinity.

Inhibition of Fibrillin-1 Assembly by Glycosaminoglycans—In order to study potential effects of glycosaminoglycans on fibrillin-1 assembly in cell culture, the culture medium of skin fibroblasts was supplemented with various concentrations of either soluble heparin (Fig. 7, A–D) or with heparan sulfate 6 (Fig. 7, E–H). After 5 days, fibrillin-1 assembly was evaluated by immunofluorescence with a specific fibrillin-1 antiserum. Addition of increasing concentrations of heparin (0–0.5 mg/ml) or heparan sulfate 6 (0–0.23 mg/ml) resulted in a dose-dependent reduction of the fibrillin-1 network. The network was re-
Ligands and Assembly of Fibrillin-1

Microfibrils, 10–12 nm in diameter, are supramolecular aggregates in the extracellular matrix with fibrillin-1 as a major backbone protein. The complete composition of microfibrils as well as the molecular mechanism of fibrillin-1 assembly from monomers into complex multimeric structures are not known. Here we identify heparin/heparan sulfate as a binding ligand of fibrillin-1 and demonstrate that this interaction plays an important role in the assembly of fibrillin-1.

Previously, it has been shown by others (15) that denatured and reduced fibrillin binds to heparin. It was not clear, however, whether this binding was conferred by the denatured state of the fibrillin molecules or whether it is a property of native fibrillin. In this study, we demonstrated that authentic fibrillin-1 produced by skin fibroblasts indeed binds to heparin. By using overlapping recombinant fibrillin-1 polypeptides, three heparin binding regions have been identified. Two calcium-independent binding regions were found at either the N-terminal half between position 45 and 450 or in the C-terminal half between position 1528 and 2731 of the fibrillin-1 molecule, whereas a third calcium-dependent binding region was identified in the longest stretch of chEFG modules in the center of the molecule between positions 1028 and 1486. The binding of heparin to proteins is often mediated through clusters of basic residues often composed by lysine or arginine residues (37). A cluster of basic amino acid residues (Gly-Lys-Lys-Gly-Lys-Thr) is located between positions 1313 and 1318 in the last loop of the chEFG module 17. By analogy to the threedimensional structure of chEFG module 32 (20), this sequence is expected to be prominently exposed on the surface of the molecule where it would be available for the interaction with heparin. Such a binding site could explain heparin binding to the central region of the fibrillin-1 molecule. Removal of calcium ions from the binding buffer resulted in a significant reduction of bound heparin to rF18. It has been demonstrated that the chEFG modules in fibrillin-1 (23, 38, 39) as well as heparin (40) bind calcium. Thus, it is possible that this interaction is mediated partially through calcium ions bound to both chEFG module(s) and heparin. Basic clusters can be composed by residues that are farther apart within the linear amino acid sequence of the polypeptide. For example heparin binding to the fibronectin module III-13 requires six basic discontinuous residues to form a cationic cradle (41). In the absence of three-dimensional structural information, it is thus often not feasible to predict the exact location of heparin-binding sites.
Binding of the recombinant fibrillin-1 fragments to heparin could be inhibited by heparan sulfate but not by chondroitin 4-sulfate, dermatan sulfate, or chondroitin 6-sulfate. These results clearly established that the heparin-binding sites in fibrillin-1 represent heparan sulfate-binding sites. The high sulfated and iduronated heparan sulfate 6 showed inhibitory activity, whereas the low sulfated and iduronated heparan sulfate 2 did not inhibit the fibrillin-1-heparin interaction. Heparan sulfate is composed of a repeated disaccharide structure (\(\Delta 4\)-glucuronic acid-\(\beta\)1–4 \(N\)-acetyl glucosamine-\(\alpha\)1–), which is modified to various degrees by \(N\)-deacetylation and \(N\)-sulfation of the hexosamine residue, by \(C\)5-epimerization of \(D\)-glucuronic acid to \(L\)-iduronic acid, and by additional \(O\)-sulfation on both sugars. It has been demonstrated that heparan sulfate 2 contains about 30% primarily non-sulfated iduronic acid, whereas heparan sulfate 6 contains about 65% almost completely 2-\(O\)-sulfated iduronic acid (35). Consequently, the heparan sulfate-binding sites in fibrillin-1 have a selective specificity for sulfated, \(L\)-iduronate-rich heparan sulfate. Typically, individual heparan sulfate chains are organized in clustered regions of low and high sulfation, whereby the precise patterns of those clusters are largely unknown (for review see Ref. 42). It may be possible that binding of fibrillin-1 molecules along heparan sulfate chains directs the molecules in a proper alignment for the formation of dimers and multimers, the first steps in fibrillin assembly (13) (see also below). It has been demonstrated by degradation experiments with chondroitin ABC lyase that chondroitin sulfate proteoglycans are associated with fibrillin and microfibrils (10, 11). Furthermore, immunoprecipitation studies suggested that the chondroitin sulfate containing proteoglycan decorin interacts with fibrillin-1 (12). It may be possible that interactions of such proteoglycans with fibrillin are mediated through chondroitin sulfate chains, which would require proper binding sites on the fibrillin-1 molecule. In the experimental set shown here, we did not analyze binding of fibrillin-1 to chondroitin 4-sulfate, dermatan

Fig. 7. Inhibition of fibrillin-1 assembly in cell culture by heparin and heparan sulfate. Skin fibroblasts were grown either without inhibitors in the culture medium (A and E), or in the presence of heparin at concentrations of 0.125 mg/ml (B), 0.25 mg/ml (C), and 0.5 mg/ml (D), or in the presence of the highly sulfated and iduronated heparan sulfate 6 at concentrations of 0.06 mg/ml (F), 0.11 mg/ml (G), and 0.23 mg/ml (H). The amount of fibrillin-1 network was visualized by indirect immunofluorescence with a specific antiserum against fibrillin-1. The bar represents 100 \(\mu\)m.

Fig. 8. Inhibition of fibrillin-1 assembly in cell culture by clustered heparin. Skin fibroblasts were grown either without (A) or in the presence of 3.1 \(\mu\)g/ml (B) or 12.5 \(\mu\)g/ml (C) BSA-heparin in the culture medium. The amount of the fibrillin-1 network was visualized by indirect immunofluorescence using a specific antiserum against fibrillin-1. The bar represents 100 \(\mu\)m.

Fig. 9. Secretion of fibrillin-1 and fibronectin by skin fibroblasts in the presence of heparin. Confluent layers of skin fibroblasts were incubated for 3 days with serum-free cell culture medium containing 0–4 mg/ml heparin as indicated on top of each lane. The amount of secreted fibrillin-1 (A, open triangle) or fibronectin (B, closed triangle) in the culture medium was determined by immunoblotting of equal aliquota (1 ml) using specific antibodies.

Fig. 10. Inhibition of fibrillin-1 assembly in cell culture by \(\beta\)-d-xylosides. Skin fibroblasts were grown either without (A) or in the presence (B) of 0.125 mM \(p\)-nitrophenyl-\(\beta\)-d-xylopyranoside and 0.125 mM 4-methylumbelliferyl-\(\beta\)-d-xylopyranoside in the culture medium. After 7 days the fibrillin-1 assembly was visualized by indirect immunofluorescence using specific antibodies against fibrillin-1. The bar represents 50 \(\mu\)m.
sulfate, or chondroitin 6-sulfate and therefore cannot exclude that these glycosaminoglycans have binding sites in fibrillin-1 different from those for heparin/heparan sulfate.

Based on the experiments described, we hypothesize that the observed interaction of fibrillin-1 with heparin/heparan sulfate reflects interactions of fibrillin-1 with heparan sulfate-associated proteoglycans in the extracellular matrix or on the surface of cells. Glycosaminoglycan chains on proteoglycans are frequently clustered in close proximity to each other. In order to mimic this structural property, we have used “clustered” heparin on albumin in inhibition experiments. Clustered heparin had a several thousand-fold higher inhibitory capacity on the fibrillin-1/heparin interaction indicating a much higher affinity as compared with soluble heparin. These data suggest that potential binding to clustered glycosaminoglycan chains on proteoglycans might be of high affinity. It has been demonstrated for other macromolecules that clustering or oligomerization of binding epitopes often increases binding strengths as compared with the monomers (36).

The glycosaminoglycans heparin and heparan sulfate 6 were used to test their influence on the ability of fibrillin-1 to assemble in cell culture. Both glycosaminoglycans completely inhibited the assembly at concentrations comparable to those used in the in vitro binding assays, whereas no effect was observed in the amount of fibrillin-1 secreted from the cells. With clustered heparin the same inhibitory effects were achieved at much lower concentrations, again correlating well with the inhibitory potency shown by in vitro experiments. These results can be interpreted in two ways. (i) The inhibiting glycosaminoglycans bind to sites on the fibrillin-1 molecules that are identical with or close to important assembly epitopes but are functionally not related to the assembly process. In this instance, the glycosaminoglycans would inhibit assembly by steric interference between ligands important for assembly. If this is true, then we can deduce important information from the presented data about the location of assembly epitopes. (ii) Binding of fibrillin-1 to heparan sulfate chains is a prerequisite for assembly. In this case, the glycosaminoglycans used in the inhibition experiments prevent binding of fibrillin-1 to these heparan sulfate chains, and the assembly process would not be initiated. Several lines of evidence exemplified below point to the second possibility. The assembly of fibrillin in cell culture can be disrupted by chlorate treatment which prevents sulfation of glycosaminoglycans and proteins (12). However, from these experiments it is not possible to distinguish whether sulfation of glycosaminoglycans or of proteins such as fibrillins and microfibril associated glycoprotein-1 are critical for the assembly. To shed light on this question, we have treated fibroblasts with β-d-xylidine derivatives, which reduce the amount of glycosaminoglycan chains in proteoglycans. Since β-d-xylidines are typically not completely specific in inhibition of just one type of glycosaminoglycan chain (43–45), a mixture of 4-methylumbelliferyl-β-d-xylopyranoside and p-nitrophenyl-β-d-xylopyranoside was used to obtain a maximum reduction of glycosaminoglycan chains on core proteins. Through this treatment, heparan sulfate and chondroitin sulfate are not biosynthesized on the protein cores of proteoglycans. The incorporation of fibrillin-1 into an extracellular network was significantly reduced in β-d-xylidines-treated fibroblasts, clearly indicating that glycosaminoglycans attached to the core protein of proteoglycans are involved in the microfibrillar assembly process. Certainly, detailed studies with more specific inhibition of glycosaminoglycans, for example by degradation with heparan sulfate-degrading enzymes, will be required. It is not clear at this stage whether proteoglycans secreted into the extracellular space or cell membrane-associated proteoglycans are necessary for microfibrillar assembly. Data are accumulating that the assembly of other extracellular proteins such as fibronectin, laminin, and thrombospondin are also dependent on the interaction with glycosaminoglycan chains of proteoglycans (43, 46). Candidate proteoglycans located on the cell surface are members of the syndecan or the glypican families (for review see Ref. 47). In fact, for fibronectin and laminin it has been shown that syndecan-2 plays an important function in the assembly process (48). Binding of fibrillin-1 to cell-surface proteoglycan(s) could promote several intriguing functions in its assembly process. (i) It is possible that the initiation of fibrillin-1 assembly requires a high local concentration, which would be achieved by binding to glycosaminoglycan chains of proteoglycans. (ii) Since fibrillin-1 only binds to highly sulfated and idurionate regions within a glycosaminoglycan chain, the patterns of high and low sulfated regions could determine a spatial arrangement of fibrillin-1 necessary to facilitate fibrillin-1 self-interactions or for disulfide bond formation, which is known as one of the initial steps in fibrillin-1 assembly (13). (iii) Binding of fibrillin-1 to glycosaminoglycans potentially confers conformational changes to the fibrillin-1 protein necessary to expose epitopes for assembly. Relatively fast on and off rates for protein binding to heparan sulfate chains are ideal to support the proposed functions (30). For example the glycosaminoglycan chains could provide surfaces upon which fibrillin-1 molecules quickly find each other in order to concentrate, to align in the proper register, and to change its conformation. Once the supported step in the assembly process has been “catalyzed,” the fibrillin-1 molecules or multimers could be released immediately into the extracellular matrix.

Acknowledgments—We thank Sille Heymann for excellent technical help. We also thank Prof. Lynn Y. Sakai, Shriners Hospitals for Children, Portland, OR, for providing high quality antibodies, and Prof. Anders Malmström, University of Lund, for providing excellent preparations of heparan sulfate 2 and heparan sulfate 6.

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