Homo- and Heterotypic Fibrillin-1 and -2 Interactions Constitute the Basis for the Assembly of Microfibrils*

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Fibrillin-1 and fibrillin-2 constitute the backbone of extracellular filaments, called microfibrils. Fibrillin assembly involves complex multistep mechanisms to result in a periodical head-to-tail alignment in microfibrils. Impaired assembly potentially plays a role in the molecular pathogenesis of genetic disorders caused by mutations in fibrillin-1 (Marfan syndrome) and fibrillin-2 (congenital contractual arachnodactyly). Presently, the basic molecular interactions involved in fibrillin assembly are obscure. Here, we have generated recombinant full-length human fibrillin-1, and two overlapping recombinant polypeptides spanning the entire human fibrillin-2 in a mammalian expression system. Characterization by gel electrophoresis, electron microscopy after rotary shadowing, and reactivity with antibodies demonstrated correct folding of these recombinant polypeptides. Analyses of homotypic and heterotypic interaction repertoires showed N- to C-terminal binding of fibrillin-1, and of fibrillin-1 with fibrillin-2. The interactions were of high affinity with dissociation constants in the low nanomolar range. However, the N- and C-terminal fibrillin-2 polypeptides did not interact with each other. These results demonstrate that fibrillins can directly interact in an N- to C-terminal fashion to form homotypic fibrillin-1 or heterotypic fibrillin-1/fibrillin-2 microfibrils. This conclusion was further strengthened by double immunofluorescence labeling of microfibrils. In addition, the binding epitopes as well as the entire fibrillin molecules displayed very stable properties.

Microfibrils are extracellular supramolecular aggregates found in many elastic and non-elastic tissues (1). Ultrastructurally, they appear as beaded filaments with a periodicity of 50–55 nm (2). The backbone of microfibrils is constituted by fibrillins, a family of large extended proteins (3, 4). Other proteins such as microfibril-associated glycoprotein (MAGP)1, and -2 (5, 6), fibulin-2 (7), versican (8), and latent transforming growth factor β-binding protein (LTBP)-1 and -2 (9, 10) were found associated with microfibrils. Although it is clear that one of the basic functions of fibrillins is the formation of the microfibrillar backbone through a complex multistep assembly mechanism, the functional importance for the associated proteins is presently obscure.

Fibrillins consist of characteristic extracellular repetitive domains such as the calcium-binding epidermal growth factor-like domains (cbEGF) also found in many other extracellular proteins, and the 8-cysteine-containing domains (8-CYS) found exclusively in fibrillins and LTBP (4, 11–13). Fibrillin-1 and fibrillin-2 share 100% homology on the domain level and ~68% homology on the overall amino acid residue level. Based on this homology, one would predict a similar mechanistic basis and architecture for the supramolecular assembly of both fibrillins. Whether fibrillin-1 and fibrillin-2 have intrinsic properties to self-assemble into homotypic or heterotypic microfibrils or both is not clear at present. Based on developmental studies, it is intelligible that adult tissues chiefly contain homotypic fibrillin-1 microfibrils, because fibrillin-2 generally is expressed early during mammalian embryogenesis and tends to disappear later (14). However, during development fibrillin-1 and fibrillin-2 often coincide in many tissues such as skin, lung, heart, aorta, central nervous system anlage, but are individually expressed in certain regions of kidney, liver, rib anlagen, and elastic cartilage (4, 15). In situations where both fibrillins are expressed simultaneously, it is theoretically possible that each fibrillin isoform forms separate homotypic fibrils or both isoforms together form heterotypic microfibrils. Different organization of fibrillins in tissues have different functional consequences that may be relevant in pathological situations.

Mutations in the genes for fibrillin-1 (FBN1) on chromosome 15 and fibrillin-2 (FBN2) on chromosome 5 are responsible for the genetic disorders Marfan syndrome (MFS, MIM no. 154700) and congenital contractual arachnodactyly (MIM no. 121050), respectively (for recent reviews, see Refs. 16 and 17). Although these autosomal dominant disorders share some skeletal complications such as arachnodactyly, scoliosis, and chest deformities, the cardiovascular and ocular features characteristic for MFS are typically absent in congenital contractual arachnodactyly. How mutant fibrillin-1 and -2 molecules exert dominant negative effects in these disorders is presently unknown. Certain lines of evidence point to the possibility that fibrillin assembly mechanisms are compromised. For instance, pulse-chase and immunofluorescence experiments have revealed reduced amounts of mutant fibrillin-1 deposited into the
extracellular matrix of many fibroblast strains obtained from individuals with MFS (18–21). These data suggest that many mutations in fibrillin-1 impair the ability to assemble into microfibrils. Similar analyses of fibrillin-2 mutations in cell culture are presently lacking. Aggregation of small N-terminal regions of fibrillins in recombinant systems has suggested that homotypic dimerization is involved in the multistep assembly mechanism of fibrillins (22–24). The static organization of fibrillin-1 in microfibrils has been examined by several groups and various techniques. Labeling of microfibrils with specific antibodies, high resolution structure of chEGF modules, and analysis of transglutaminase cross-links have led to various models of fibrillin alignment in microfibrils (25–29). Despite the unresolved controversy whether fibrillin molecules are arranged in a nonstaggered or in a staggered fashion, common to all models is a head-to-tail arrangement of fibrillin molecules originally proposed by Sakai and co-workers in 1991 (25). Mapping of monoclonal antibody epitopes in fibrillin-1 molecules and correlation with the epitopes in microfibrils revealed that the N- and the C-terminal ends of the fibrillin molecules are located in or close to the bead structures (25, 26). However, it is not clear whether microfibrillar backbone formation requires fibrillin-1 and fibrillin-2 to directly interact with themselves and with each other, or requires adapter molecules to connect fibrillin molecules in a head-to-tail fashion. Here, we have analyzed in detail the spectrum of homotypic and heterotypic molecular interactions of fibrillin-1 and fibrillin-2. The results demonstrate direct head-to-tail interactions of fibrillin-1 alone, and of fibrillin-1 with fibrillin-2. However, fibrillin-2 alone was not able to self-interact in a N- to C-terminal fashion, indicating a different assembly mechanism for fibrillin-2. The results presented suggest that fibrillins are able to form microfibrils in a direct head-to-tail fashion without the aid of adapter molecules.

EXPERIMENTAL PROCEDURES

Cloning of Human Fibrillin-2 cDNA

Human fibrillin-2 cDNA was synthesized by reverse transcription of total RNA isolated from MG-63 cells (American Type Culture Collection) using the antisense (s) primers specified below. The obtained cDNAs were used as templates for polymerase chain reactions (PCR) using the same antisense primers in combination with appropriate sense (s) primers (see below) and the high fidelity polymerase Pfx (Invitrogen). The 5' fragment of rFBN2-0s (nt 1–1782) was synthesized by PCR amplification using primers 0s (5'-ATTACCGCTAGCTACGGGAGGAGAACGGG-3') and 0as (5'-ACGCTTGCCATCATGGGGAGAAGACGGAGGC-3') and the high fidelity polymerase Pfx (Invitrogen). The 3' fragment of rFBN2-0s (nt 1–1782) was synthesized by PCR amplification using primers 1s (5'-CCTGAACTGAGCAGCAGGCTACTAAGAATTAA-3') and 1as (5'-CTGAACTGAGCAGCAGGCTACTAAGAATTAA-3'). The obtained PCR products were verified by DNA sequencing.

Construction of Expression Plasmids

Fibrillin-2 Constructs—To construct an expression plasmid for the N-terminal half of human fibrillin-2 (rFBN2-C, positions 1531–2479) and 2442), the entire sequence of the construct was obtained by PCR amplification using primers 1s (5'-CTGAACTGAGCAGCAGGCTACTAAGAATTAA-3') and the high fidelity polymerase Pfx (Invitrogen). The 3' fragment of rFBN2-0s (nt 1–1782) was synthesized by PCR amplification using primers 1s (5'-CTGAACTGAGCAGCAGGCTACTAAGAATTAA-3') and 1as (5'-CTGAACTGAGCAGCAGGCTACTAAGAATTAA-3'). The obtained PCR products were verified by DNA sequencing.
branes, Millipore). After dialysis against 20 mM phosphate, pH 7.2, 1 mM NaCl (equilibration buffer), the mixture was filtered on a High-Trap chelating column loaded with Co²⁺ (1-ml column size, Amersham Biosciences) equilibrated in the same buffer. After washing the column with equilibration buffer, bound proteins were eluted with a linear imidazole gradient in equilibration buffer (0–250 mM imidazole in 30 ml) and fractionated in 1-ml aliquots. The fractions were analyzed by gel electrophoresis and followed by Coomassie Blue staining and by Western blot analyses. Fractions containing the recombinant proteins were pooled and dialyzed against 50 mM Tris-HCl, pH 7.4, 150 mM NaCl (TBS).

**Quantification of Protein Concentrations**

Alliquots (50 μl) were supplemented with 450 μl of 6.67 mM guanidine HCl in TBS and incubated at room temperature for 30 min. The absorbance at 280 nm was determined on a Ultrospec 3000 spectrophotometer (Amersham Biosciences). Calculation of the molar extinction coefficient followed an established method (ε = n_{trp} × 5500 + n{trp} × 1490 + P_{cys-H-S-Cys} × 125 (m⁻¹ × cm⁻¹) (35)).

Alternatively, protein concentrations were determined by amino acid analyses. The proteins were hydrolyzed in 6 N HCl under N₂ for 24 h at 110 °C, and the amino acid composition was determined on a Biochrom 20 analyzer (Biochrom).**Circular Dichroism Measurements**

The purified recombinant proteins in TBS were dialuted to a concentration of 0.5 mg/ml. Spectra from 190 to 280 nm were recorded at 20 and 100 °C in a 1-mm quartz cuvette on a Jasco J-715 instrument.

**Electron Microscopy**

Purified recombinant proteins were dialyzed against 100 mM NH₄HCO₃ and adjusted to concentrations of 0.25 mg/ml. The samples were diluted with 0.05% (v/v) acetic acid to a final concentration of 60 μg/ml and mixed with glycerol to a final concentration of 50% (v/v) glycerol. The samples were sprayed onto freshly cleaved mica from a spray gun and dried at room temperature. The replicas were floated onto a very clean surface of distilled water and then supported using 400-mesh copper grids. The replicas were examined at 100 kV in a transmission electron microscope (Zeiss TEM 109).

**Antibodies**

Polyclonal antisera were produced in rabbits commercially (Biotrend, Cologne, Germany) against the recombinant polypeptides rFBN2-N, rFBN2-C, and an ~110-kDa N-terminal fragment of human fibrillin-2, rF37, described previously (30). The antisera were characterized by a standard ELISA technique. Generation and properties of anti-human fibrillin-1 polyclonal antisera B953 and α-rF6H, as well as the specificity of monoclonal anti-human fibrillin-2 antibody mAb 48 have been described elsewhere (36–38). B953 and mAb 48 were generous gifts from Dr. Lynn Y. Sakai (Shriners Hospital for Children, Portland, OR).

**Protein Interaction Assays**

**Solid Phase Binding Assay—Multiwell plates (96 wells, MaxiSorp, Nalge Nunc International) were coated overnight with purified recombinant proteins (10 μg/ml, 100 μl/well) in TBS at 4 °C. Non-specific binding sites were blocked for 1 h with 5% (w/v) nonfat milk in TBS. Each of the following incubations was performed in TBS/0.5% nonfat milk including either 5 mM CaCl₂ or 10 mM EDTA at room temperature (~20 °C) and was followed by three washes with TBS including 0.05% (v/v) Tween 20. Coated proteins were incubated with serial dilutions (1.3) of the soluble ligands (0.14–100 μg/ml) for 2 h. Incubation (1.5 h) with the primary polyclonal antibodies (1:200–1:1250 diluted) against the soluble ligands was followed by incubation with goat anti-rabbit IgG conjugated secondary goat anti-rabbit antibody (1:800 diluted; Bio-Rad) for 1.5 h. Color development was performed with 1 mM glucose-5-amino-salicylic acid (Sigma) in 20 mM phosphate buffer, pH 6.8, including 0.1% H₂O₂ for 3–5 min and stopped by adding 2 mM NaOH. Color yields were determined at 492 nm using a Microplate EL310 (Bio-Tek Instruments).**

To determine the stability of fibrillin interaction epitopes, the immobilized or the soluble binding ligands were heat-treated in discrete steps between 20 and 95 °C for 10 min and then used in the above described solid phase binding assay.

**Kinetic Binding Studies**

For kinetic binding studies of recombinant fibrillin-1 and fibrillin-2 fragments by surface plasmon resonance, a Biacore biosensor was used (Biacore 3000; Biacore AB). Purified recombinant fibrillin-1 and fibrillin-2 fragments were biotinylated using activated NHS-LC-biotin as instructed by the supplier (Pierce). Biotinylated fragments were coupled in TBS to a streptavidin sensor chip SA (Pioneer), which resulted in 1000–2500 response units. Binding studies were performed with soluble recombinant fibrillin-1 or fibrillin-2 fragments in concentrations of 12.5–1000 nM in TBS at flow rates of 10 μl/min. The binding sites of the immobilized ligands were regenerated by injection of a mixture of detergents (0.075% (v/v) each of CHAPS, Tweengent 3–12, Tween 80, Tween 20, Triton X-100) after each cycle. After subtraction of the blank curves, representing binding to bovine serum albumin, the association and dissociation rate constants were determined by separate kₐ/k₅ fitting all curves at once with the 1:1 Langmuir association/dissociation model (BIAevaluation software version 3.0, Biacore AB). Although this model produced the best fits, the observed interactions probably diverge somewhat from the 1:1 binding model because k₅ values slightly decreased with increasing concentrations of the soluble ligands. Mass transfer limitations were not apparent.

**Immunofluorescence**

Primary dermal fibroblasts from a 1-year-old individual and primary osteoblasts from a 42-year-old individual were cultivated in the first (fibroblasts) or fourth (osteoblasts) passage using culture conditions as described above for the recombinant cell clones. Confluent cells were trypanotized and seeded at densities of 7.5 × 10³ cells/well of a 6-well plate (Permanox; Nalge Nunc International). After 4 days, the cells were washed in PBS, and fixed in 70% (v/v) methanol, 30% (v/v) aceton for 5 min, and rehydrated in PBS. The nonspecific binding sites were blocked with 1:10 diluted normal goat serum for 30 min. The cells were incubated with monoclonal anti-fibrillin-2 antibody mAb 48 (1: 200) and the polyclonal anti-fibrillin-1 α-rF6H (1:400) in PBS for 1 h, followed by three washes with PBS. After incubation with 1:200 diluted goat anti-rabbit fluorescent conjugate (Jackson Immunoresearch) and Cy3-conjugated Affinipure goat anti-mouse IgG (Jackson Immunoresearch), the fibrillin-1 and fibrillin-2 networks were visualized by fluorescence microscopy with a Axioplan microscope (Zeiss). Digital images were recorded using a 3CCD color video camera (Sony) and AxioVision software version 3.0 (Zeiss).

**RESULTS**

To generate recombinant human fibrillin-2 fragments, the human fibrillin-2 cDNA was cloned from MG-63 cells. The cloned sequence was compared with the published sequence for human fibrillin-2 cDNA, and 19 differences have been identified (Table I, GenBank™ accession no. NM_0001999; Refs. 4 and 31). Based on sequence homology of the cloned and published sequence for human fibrillin-2 cDNA with the sequences for human fibrillin-1 (GenBank™ accession no. NM_000138; Refs. 11–13) and human fibrillin-3 (GenBank™ accession no. AB053450; Ref. 39), we conclude that all of the observed differences are correct in the sequence presented here. In some instances they likely represent polymorphisms (Table I). Some of the observed variations have been reported previously (30, 40).

To analyze the mechanisms of how fibrillins assemble into higher ordered structures, we have generated new recombinant fragments of fibrillin-2 as well as a recombinant full-length fibrillin-1 polypeptide. For fibrillin-2, we produced the N-terminal half rFBN2-N (position 1–1732) and the C-terminal half rFBN2-C (position 1531–2771) in human 293 cells (Fig. 1). rFBN2-N and rFBN2-C span the entire fibrillin-2 amino acid sequence except part of the C-terminal unique domain (position 2772–2911), which in analogy to fibrillin-1 processing presumably is cleaved by furin-type proteases (41, 42). Similarly, the recombinant fibrillin-1 full-length construct rFBN1 comprises the entire fibrillin-1 sequence except the processed C-terminal domain (position 2726–2871; Fig. 1). Additionally, two recombinant halves of fibrillin-1, rF16 and rF6H, described previously were used in this study (33). For clarity, these fragments...
have been renamed for this study to rFBN1-N and rFBN1-C (Fig. 1).

The recombinant fragments rFBN2-N and rFBN2-C were synthesized and secreted into the culture medium of recombinant cell clones at concentrations of ~5 μg/ml/day. Full-length rFBN1 was produced in significantly lower amounts of ~0.5 μg/ml/day. All recombinant polypeptides were purified to homogeneity by chelating chromatography (Fig. 2). The molecular masses for rFBN2-N (~210 kDa nonreduced and ~225 kDa reduced), rFBN2-C (~175 kDa nonreduced and ~185 kDa reduced), and rFBN1 (~322 kDa nonreduced and ~345 kDa reduced) corresponded well with the expected masses for these polypeptides. Freshly prepared and purified rFBN1 resulted in single bands in Coomassie-stained gels (Fig. 2). However, rFBN1 tended to precipitate in solution after a few days, or upon repeated freezing/thawing cycles (data not shown). The recombinant halves of fibrillin-1 and fibrillin-2 were soluble and did not precipitate in solution. All recombinant polypeptides reacted in Western blot analyses with specific monoclonal antibodies, which are dependent on correct disulfide bonds, indicating correct three-dimensional structures of the polypeptides (data not shown).

The new recombinant polypeptides were visualized by electron microscopy after rotary shadowing. rFBN2-N (Fig. 3A) and rFBN2-C (Fig. 3B) showed extended shapes similar to what was observed for the corresponding recombinant fibrillin-1 polypeptides (26). Occasionally, kinks and bends have been observed in both molecules. The length of the molecules were 74.9 ± 4.1 nm (rFBN2-N, n = 62) and 68.2 ± 4.8 nm (rFBN2-C, n = 30). The shape of the rFBN1 molecules was also threadlike and extended (Fig. 4). Again, kinks and bends could be observed within the molecules (Fig. 4). Length measurements of these molecules showed that 43.1% were in the range of monomers between 100 and 180 nm (139 ± 24 nm), 13.8% in the range of dimers between 240 and 320 nm (274 ± 21 nm), and 3.4% in the range of trimers between 380 and 460 nm (416 ± 27 nm). The remaining particles likely represented proteolytically truncated products of monomers, dimers, and trimers. These
data suggest that monomeric fibrillin-1 can associate to multimers in TBS buffer. Interestingly, the molecules appeared to connect at their ends with each other, because no significant overlap between two molecules have been observed in dimers and trimers (Fig. 4). Occasionally, kinks or globules were detected in the region where two molecules are in contact with each other. These results suggested that fibrillin-1 molecules can interact with each other without the support of other molecules.

To further analyze the self-interaction properties of fibrillin-1 and fibrillin-2, as well as the ability of fibrillin-1 to interact with fibrillin-2, binding activities of various combinations of the recombinant halves of fibrillin-1 and fibrillin-2 have been analyzed by solid phase binding assays (Fig. 5). Strong self-interactions were observed between the N- and the C-terminal halves of fibrillin-1 (Fig. 5A). Surprisingly, the corresponding constructs of fibrillin-2 did not show significant self-interaction properties (Fig. 5B). Fibrillin-1 also interacted strongly with fibrillin-2. The N-terminal half of fibrillin-1 clearly showed dose-dependent binding to the C-terminal half of fibrillin-2 (Fig. 5C), and the N-terminal half of fibrillin-2 interacted with the C-terminal half of fibrillin-1 (Fig. 5D). All of the observed interactions were dependent on calcium (Fig. 5).

The association ($k_a$) and dissociation rate ($k_d$) constants of the homotypic and heterotypic fibrillin binding interactions, and the dissociation constants ($K_D$), have been determined by surface plasmon resonance (Fig. 6). The kinetic data obtained from such experiments are summarized in Table II. In these experiments, high affinity self-interaction of fibrillin-1 has been observed between the rFBN1-N and -C (Fig. 6A; $K_D = 3.8–25.6$ nM), whereas the corresponding fragments of fibrillin-2 did not significantly interact (Fig. 6B). Heterotypic interaction between fibrillin-1 and fibrillin-2 was observed for combinations of rFBN1-N with rFBN2-C (Fig. 6C; $K_D = 4.3–20.5$ nM), and rFBN2-N with rFBN1-C (Fig. 6D; $K_D = 23.1–82.0$ nM). These results correlated well with the data obtained by solid phase binding assays and demonstrated that the homotypic interaction of fibrillin-1 and the heterotypic interaction of fibrillin-1 with fibrillin-2 are of high affinity.

The stabilities of the interacting epitopes have been studied by heat inactivation experiments (Fig. 7). The recombinant fibrillin polypeptides were incubated at increasing tempera-
Concentration of soluble ligand (μM)

**FIG. 5. Homotypic and heterotypic fibrillin-1 and -2 interaction properties.** Fibrillin-1 (A) and fibrillin-2 (B) N- to C-terminal homotypic self-interaction and N- to C-terminal heterotypic interaction between fibrillin-1 and fibrillin-2 (C and D) have been analyzed by solid phase binding assays in the presence of 5 mM CaCl2 (squares) or 10 mM EDTA (circles). The reaction partners were rFBN1-N and rFBN1-C (A), rFBN2-N and rFBN2-C (B), rFBN1-N and rFBN2-C (C), and rFBN2-N and rFBN1-C (D). The C-terminal fragments of both fibrillins have been used as soluble ligands and the N-terminal fragments as immobilized ligands. Data points are averages of duplicates. Standard deviations are indicated.

**FIG. 6. Quantification of homo- and heterotypic fibrillin-1 and -2 binding strengths by surface plasmon resonance.** Association and dissociation curves for the homotypic N- to C-terminal interaction of fibrillin-1 (A), and fibrillin-2 (B), and of N- to C-terminal heterotypic interactions between fibrillin-1 and fibrillin-2 (C and D) have been analyzed by real time kinetic studies using a Biacore 3000 instrument. The reaction partners were rFBN1-N and rFBN1-C (A), rFBN2-N and rFBN2-C (B), rFBN1-N and rFBN2-C (C), and rFBN2-N and rFBN1-C (D). Association time was 300 s, and dissociation time was 500 s. Concentrations of soluble ligands used are indicated in nM. RU, response units.
clearly correspond with the *in vitro* binding interaction of fibrillins described above.

**DISCUSSION**

Microfibrils 10–12 nm in diameter are supramolecular aggregates in the extracellular matrix consisting of fibrillins and other matrix proteins. Fibrillins are repetitively aligned within microfibrils and constitute their structural backbone. At present, the mechanistic basis for formation of the fibrillin backbone is unknown, as is whether fibrillins exclusively form homotypic microfibrils consisting of only one isoform or heterotypic microfibrils containing both isoforms. Because impaired assembly mechanisms may precipitate the pathogenetic pathways of genetic disorders caused by mutations in fibrillin-1 and fibrillin-2, it is important to answer these questions. In this study, we have analyzed in detail homotypic and heterotypic interaction repertoires and stabilities of fibrillin-1 and fibrillin-2.

Fibrillins cannot be extracted from tissues in their native form because they are heavily cross-linked by reducible and nonreducible cross-links (28, 44). A feasible alternative to obtain fibrillin for mechanistic studies is its recombinant expression in mammalian cells. Previously, we have recombinantly produced two halves of fibrillin-1 and demonstrated correct folding and functional properties (7, 26). Here, we have produced two corresponding halves of fibrillin-2 spanning the entire processed fibrillin-2 molecule. These constructs resembled the corresponding counterparts of fibrillin-1, as judged by gel electrophoretic analysis and electron microscopy after rotary shadowing. The extended shapes together with the reactivity with antibodies requiring native epitopes, stabilized by disul-

**Table II**

Surface plasmon resonance affinity measurements between various fibrillin fragments

| Interaction                | $k_a$ ($s^{-1} \times 10^3$) | $k_d$ ($s^{-1} \times 10^{-3}$) | $K_D$ (nm) | Ligand concentration (nM) | $\chi^2$ |
|----------------------------|-----------------------------|-----------------------------|-------------|---------------------------|-----------|
| rFBN1-N/rFBN1-C            | 75.1                        | 0.285                      | 3.79        | 50                        |           |
|                            | 34.6                        | 0.288                      | 3.32        | 100                       |           |
|                            | 20.5                        | 0.234                      | 10.9        | 200                       | 0.0505    |
|                            | 12.6                        | 0.242                      | 19.2        | 300                       |           |
|                            | 11.4                        | 0.292                      | 25.6        | 400                       |           |
|                            | 96.8                        | 1.020                      | 10.5        | 12.5                      |           |
|                            | 52.0                        | 0.223                      | 4.29        | 25                        |           |
| rFBN1-N/rFBN2-C            | 54.7                        | 0.283                      | 5.36        | 50                        | 0.1030    |
|                            | 41.9                        | 0.259                      | 6.18        | 100                       |           |
|                            | 20.0                        | 0.410                      | 20.5        | 300                       |           |
|                            | 4.15                        | 0.096                      | 23.1        | 100                       |           |
| rFBN2-N/rFBN1-C            | 5.33                        | 0.257                      | 48.2        | 200                       | 0.0685    |
|                            | 4.28                        | 0.351                      | 82.0        | 400                       |           |
|                            | 4.25                        | 0.316                      | 74.4        | 800                       |           |
| rFBN2-N/rFBN2-C            | ND                          | ND                         | ND          | 100–1000                   | ND        |

**Fig. 7.** Temperature stability of homo- and heterotypic fibrillin interaction epitopes. Fibrillin fragments have been incubated for 10 min at the temperatures indicated, and then used in homo- and heterotypic fibrillin solid phase interaction assays. Heat-treated or nontreated immobilized rFBN1-N (A) or rFBN2-N (B) were analyzed with soluble heat-treated or nontreated rFBN1-C or rFBN2-C. The combination of ligands for each analysis are indicated on top with shaded areas representing heat-treated proteins. The soluble ligands were used in serial dilutions (1:3) starting at 100 μg/ml. The entire binding curves for each temperature were numerically integrated using the trapezoidal rule, and the numerical integral for each analysis at 20 °C was set to 100% binding.

**Fig. 8.** Stability of recombinant fibrillin polypeptides. rFBN1-N (A), rFBN2-N (B), rFBN1-C (C), and rFBN2-C (D) were analyzed by far UV circular dichroism at 20 °C (solid lines) and at 100 °C (dotted lines). Molar ellipticities are plotted against the wavelengths.
within the same microfibrils. The bar
Superimposition of both labels are shown in
panels D
open circles
open squares
/H9251
). The
), and rFBN2-N (E–G–proposed (27–29). For further clarity, only 3 molecules are shown be-
tween each globular domain. They represent 6–8 fibrillin monomers in
cross-sectional diameter (29, 46). Globular bead regions are shown as
gray circles. In B it is not clear whether fibrillin-1 and fibrillin-2 are
regularly alternating in the microfibrils or whether they are arranged in clusters.

Fig. 9. Double immunofluorescence of fibrillin-1 and fibril-
lin-2 in cell cultures. A, the specificity of the polyclonal anti fibrillin-1
antiserum α-rF6H was analyzed by ELISA. The recombinant polypep-
tides were rFBN1-C (solid squares), rFBN1-N (solid circles), rFBN2-C
(open squares), and rFBN2-N (open circles). The α-rF6H antiserum was
diluted in 1:2 serial dilutions. Note that cross-reactivities of the anti-
serum with fibrillin-2 are negligible at dilutions of 1:400 and higher.
The specificity of mAb 48 for fibrillin-2 has been described elsewhere
(38). Dermal fibroblasts from a 1-year-old donor (B–D) or osteoblasts
from a 42-year-old donor (E–G) were simultaneously labeled with
α-rF6H (green signal, B and E) and mAb 48 (red signal, C and F).
Superimposition of both labels are shown in panels D and G. Note that
the yellow signal in D indicates the presence of fibrillin-1 and fibrillin-2
within the same microfibrils. The bar represents 25 μm.

Fig. 10. Co-organization of fibrillins in microfibrils. Based on
the results presented in this study, fibrillin-1 (red arrows) alone (A), or
fibrillin-1 together with fibrillin-2 (green arrows) (B) can assemble via
direct N-terminal (arrowhead) to C-terminal (arrowtail) interactions
into microfibrils. For clarity, only the previously proposed parallel non-
staggered alignment model is shown (26). However, the interaction
mechanisms described are also applicable to all other alignment models
proposed (27–29). For further clarity, only 3 molecules are shown be-
tween each globular domain. They represent 6–8 fibrillin monomers in
cross-sectional diameter (29, 46). Globular bead regions are shown as
gray circles. In B it is not clear whether fibrillin-1 and fibrillin-2 are
regularly alternating in the microfibrils or whether they are arranged in clusters.

head-to-tail manner. It has been observed by immunogold lo-
calization that MAGP-1 is located at or close to the bead struc-
tures, and thus it potentially could function as a fibrillin bridg-
ing molecule in microfibrils (5). However, it has been
demonstrated that MAGP-1 does only interact with fibrillin-1
via an epitope located at the N-terminal region of fibrillin-1,
whereas the C-terminal region of fibrillin-1 does not harbor a
binding site for MAGP-1 (33). Therefore, MAGP-1 alone cannot
function as an adapter to connect fibrillin molecules in the bead
regions of microfibrils. Other fibrillin-binding ligands such as
fibulin-2 (7), LTBP-1 (45), or versican (8) have been described,
but they are present only on some but not all microfibrils and
they are typically not periodically aligned along microfibrils.

In this study, we have found for fibrillin-1 very strong inter-
actions between the N- terminal and the C-terminal halves
with dissociation constants in the low nanomolar range. Sur-
prisingly, for fibrillin-2, this type of interaction could not be
observed, although various protein interaction assays have
been employed. Self-interaction properties in a head-to-tail
fashion were also observed for the recombinant full-length
fibrillin-1 polypeptide, which represented the C-terminally pro-
cessed form of fibrillin-1, because it lacks the small C-terminal
portion usually processed by furin-type proteases (41, 42). This
polypeptide clearly showed the tendency to precipitate in phys-
iological buffer solutions, indicating self-interaction properties.
When rFBN1 molecules were analyzed ultrastructurally by
electron microscopy after rotary shadowing, monomers,
dimers, trimers, and, very occasionally, tetramers were ob-
served. Because the analyzed samples originated from the
cleared supernatant of a rFBN1 preparation, we suspect that
the precipitate of this preparation is formed by even higher
molecular aggregates. The multimers of rFBN1 found among
the population of molecules appeared often as continuous
strings without obvious overlapping regions of the molecules
(Fig. 4). In some instances globules or kinks indicated the end
of one and the beginning of the next molecule. These results
suggest that the interaction epitopes are located relatively
close to the processed ends of the molecules. We have found
that recombinantly expressed fibrillin proteins representing
N-terminal parts of the molecules are not straight as suggested
from the domain structure, but often display a curved or glob-
ular shape (data not shown). Thus, the globules occasionally
found between two rFBN1 molecules potentially could repre-
sent the N-terminal region. In this light, it is possible that the N-terminal interaction epitope is not located strictly at the N terminus of the fibrillin-1 molecule but somewhat further C-terminal, for instance, in the region of the proline-rich domain.

To further understand the mechanism of fibrillin-2 assembly, which appears not to involve homotypic self-interaction, we determined the ability of fibrillin-2 and fibrillin-1 to interact heterotypically with each other. Interestingly, we have found strong interaction properties between fibrillin-1 and fibrillin-2 with dissociation constants again in the low nanomolar range. The N-terminal half of fibrillin-2 interacted with the C-terminal half of fibrillin-1, and vice versa, the C-terminal half of fibrillin-2 interacted with the N-terminal half of fibrillin-1. Because the recombinant halves of the fibrillins used in these assays are relatively large (185–225 kDa), we cannot draw a conclusion as to which domains are responsible for mediating the fibrillin homo- and heterotypic interactions. Interestingly, when smaller and well established recombinant fragments of fibrillin-1 were utilized to narrow down the binding epitopes for fibrillin-2, no binding activity could be observed (data not shown). One interpretation of such results is that the binding epitopes are stabilized by regions of the molecules that are not in the immediate vicinity of the binding epitope, e.g., by stabilizing long range structural effects. Previously, long range structural effects have been reported in fibrillin-1 (43). Another interpretation is that binding epitopes are located in regions at the ends of the recombinant subfragments used and thus are potentially truncated.

In addition, we have used a functional assay to assess the stability of the fibrillin interaction epitopes. Heat denaturation of recombinant proteins prior to the established interaction assay showed that the interaction epitopes are very stable of recombinant proteins prior to the established interaction stability of the fibrillin interaction epitopes. Heat denaturation determined the ability of fibrillin-2 and fibrillin-1 to interact stably, which appears not to involve homotypic self-interaction, we predicted that fibrillin-2 alone, in the absence of fibrillin-1, cannot form homotypic microfibrils by corresponding N- to C-interacting mechanisms. It is clear that fibrillin-2 is expressed in some tissues during development where fibrillin-1 is not expressed (4, 15). In these situations, fibrillin-2 may assemble into homotypic microfibrils by alternative mechanisms. Such mechanisms may require molecular adapters, or a new member of the fibrillin family, fibrillin-3, may play a critical role (39). Alternatively, fibrillin-2 assembles into another, not yet recognized form of supramolecular aggregates.

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