Fibrillin-1 Interactions with Fibulins Depend on the First Hybrid Domain and Provide an Adaptor Function to Tropoelastin*

Received for publication, August 25, 2006, and in revised form, December 13, 2006 Published, JBC Papers in Press, January 25, 2007, DOI 10.1074/jbc.M608204200

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Fibrillin-containing microfibrils in elastic and nonelastic extracellular matrices play important structural and functional roles in various tissues, including blood vessels, lung, skin, and bone. Microfibrils are supramolecular aggregates of several protein and nonprotein components. Recently, a large region in the N-terminal portion of fibrillin-1 was characterized as a multifunctional protein interaction site, including binding sites for fibulin-2 and -5 among others. Using a panel of recombinant fibrillin-1 swapped domain and deletion fragments, we demonstrate here that the conserved first hybrid domain in fibrillin-1 is essential for binding to fibulin-2, -4, and -5. Fibulin-3 and various isoforms of fibrillin-1 did not interact with fibrillin-1. Although the first hybrid domain in fibrillin-1 is located in close vicinity to the self-assembly epitope, binding of fibulin-2, -4, and -5 did not interfere with self-assembly. However, these fibulins can associate with microfibrils at various levels of maturity. Formation of ternary complexes between fibrillin-1, fibulins, and tropoelastin demonstrated that fibulin-2 and -5 but much less fibulin-4, are able to act as molecular adaptors between fibrillin-1 and tropoelastin.

The microfibril/elastic fiber system provides tissues, such as lung, blood vessels, and skin, with elastic properties. Microfibrils with a diameter of 10–12 nm are typically located on the outer surface of elastic fibers and are thought to play an essential role in elastogenesis (1). Whereas elastic fibers are always associated with microfibrils, microfibrils themselves can occur in the absence of elastin in certain tissues such as ocular ciliary zonules, the kidney, or in close proximity to various basement membranes. The microfibril/elastic fiber system is a multicomponent assembly in the extracellular matrix, and for both, the microfibrils and the elastic fibers, a number of constituents have been described (for a review, see Ref 2). For most of the associated molecules, the exact relationship in terms of physical interaction with microfibrils and/or elastic fibers, and their functional relevance is not clear.

The best described components of the microfibrils are a family of proteins consisting of three highly homologous members, fibrillin-1, -2, and -3 (3–9). Fibrillins, like many other extracellular glycoproteins, are characterized by a number of tandemly arranged domains. The most prominent domain is an epidermal growth factor-like domain (EGF), which occurs 46–47 times in fibrillins. These domains are stabilized by three intramolecular disulfide bonds, and the majority (42–43 domains) contain a consensus sequence for calcium binding (cbEGF) (10–12). The tandemly arranged EGF and cbEGF domains are interspersed by two other types of domains, the transforming growth factor β-binding protein (TB) or 8-Cys domains and the hybrid domains. The seven TB/8-Cys domains are characterized by four intramolecular disulfide bonds, and a similar arrangement is predicted for the two hybrid domains, although no structural data are available for this domain (12, 13). Sequence data have shown that the first hybrid domain in all fibrillins contains nine cysteine residues as compared with eight in the second hybrid domain. This 9-cysteine pattern is highly conserved in all species, ranging from invertebrates to humans. Previously, we have determined that cysteine 204 in human fibrillin-1 and cysteine 233 in human fibrillin-2 is unpaired and available for intermolecular disulfide bonding, which is essential for initial fibrillin assembly (14).

Genetic mutations in fibrillins cause a number of related connective tissue disorders, including Marfan syndrome (fibrillin-1), Beals-Hecht syndrome (fibrillin-2), Weill-Marchesani syndrome (fibrillin-1 and potentially fibrillin-3), and others (for a review, see Ref. 15). The clinical symptoms that characterize these connective tissue disorders exemplify the important roles for fibrillins in development and homeostasis of the cardiovascular, skeletal, and ocular systems. More specifically, for the cardiovascular system, it has been demonstrated by murine...
gene deletion experiments that fibrillin-1 and -2 are important for proper elastogenesis (16).

Fibulins constitute another family of extracellular matrix proteins that contain clusters of various domains (for a review, see Refs. 17–19). They are characterized by common homologous C-terminal domains of 120–140 amino acid residues preceded by an array of cbEGF domains. Fibulin-1 and -2 are larger in size as compared with other fibulins, with anaphylatoxin domains N-terminal of the tandem cbEGF stretches (20, 21). Fibulin-2 contains an additional large N-terminal domain, which is absent from all other fibulins. A second subgroup is formed by the relatively small (~50–70 kDa) fibulin-3, -4, and -5 (22, 28), which each contain five cbEGF domains preceded by a variably modified cbEGF domain at the N terminus.

In respect to the relationship of fibulins with the microfibril/elastic fiber system, it has been shown that several fibulins are associated with this system. Immunohistochemical experiments at the light and electron microscopic level revealed that (i) fibulin-1 is associated with the amorphous elastic core of elastic fibers (29, 30), (ii) fibulin-2 is colocalized with microfibrils at the interface with elastic fibers as well as with microfibrils in the absence of elastin (31, 32), and (iii) fibulin-5 is found on the internal aortic elastic lamina and elastic fibers produced by dermal fibroblasts in vivo and in vitro (33–35). Additional evidence for a close functional relationship of fibulins with components of the microfibril/elastic fiber system comes from in vitro protein–protein interaction studies. It has been demonstrated that fibulin-1, -2, -4, and -5 can interact with tropoelastin (30, 33, 36, 37). In addition, fibulin-2 and -5 were shown to interact with fibrillin-1, and the binding epitopes were mapped to a relatively large N-terminal region of fibrillin-1 (31, 37).

Gene targeting experiments in mice have shown that fibulin-4 and -5 are both essential for the biogenesis of elastic fibers (33, 34, 36). Mutations in some of the fibulins lead to a number of human genetic disorders, including Malattia Leventinese and Doyne honeycomb retinal dystrophy (fibulin-3), age-related macular degeneration (fibulin-5), and cutis laxa (fibulin-4 and -5) (38–42).

Here, we have characterized the molecular interactions of fibulins with fibrillin-1. Fibulin-2, -4, and -5 show similar binding to the N-terminal region of fibrillin-1, whereas other fibulins did not interact. A panel of swapped domain constructs established that the first hybrid domain in fibrillin-1 is essential but not sufficient for binding to fibrillin-2, -4, and -5. Whereas these fibulins are able to associate with microfibrils, they do not interfere with the ability of fibrillin-1 to self-interact. However, fibulin-2 and -5 can promote ternary complexes between fibrillin-1 and tropoelastin, indicating that they act as molecular adaptors between microfibrils and elastic fibers.

**EXPERIMENTAL PROCEDURES**

**Recombinant Proteins**—In order to map fibrin binding sites on fibrillin-1, wild-type and swapped domain recombinant fibrillin-1 fragments were generated. Production and characterization of recombinant fibrillin-1 fragments rF6H and rF16 (43) and rF51 and rF1F (44) have been described in detail previously. The generation of N- and C-terminal deletion constructs rF16N and rF16NEH will be described elsewhere.

The plasmids HFBN23, HFBN25, pBS-HFBN8-1–4, and pCDRTSGP-rF1A used as templates in PCRs have been described in detail in other studies (7, 44, 45). All fibrillin-1 expression plasmids were designed with a sequence for a signal peptide from the BM40 protein to ensure secretion into the culture medium and a sequence for a C-terminal hexahistidine tag to facilitate protein purification. Due to the cloning strategy, the recombinant proteins are expressed with an additional Ala-Pro-Leu-Ala sequence at their N terminus. All expression plasmids described below were analyzed and verified for correct insertion and orientation by DNA sequencing (Agowa, Berlin, Germany). The recombinant proteins expressed by the expression plasmids are schematically shown in Fig. 1.

Fragment rF1F is a wild-type fibrillin-1 fragment spanning the region between the N terminus and the second TB/8-Cys domain (Ser19–Gly714) (44). The plasmid for rF1G has been designed to express the identical region compared with rF1F but with swapped domains cbEGF 8 and 9 replacing cbEGF 1 and 2. For the cloning strategy, it was necessary to modify the polylinier region of the cloning plasmid pBluescript II SK+ (Stratagene) to introduce additional ClaI and AgeI restriction sites. For this goal, the pBluescript plasmid was restricted with Xhol and SacI, and the 2870-bp fragment was ligated with complementary oligonucleotides pBS3-S (5′-TCGAGTATC-GATTGACGTTCTACCGGTAGCTC-3′) and pBS3-AS (5′-CACCCTAGACCTACATGTCATTAC-3′), resulting in a plasmid termed pBS3. A 923 bp Clal-AgeI fragment from pDNSP-rF1F (44) was ligated with the Clal-AgeI-restricted pBS3 plasmid to yield plasmid pBS3-rF1F. To obtain the sequence for domains cbEGF 8 and 9 of fibrillin-1 and to introduce additional BtgI and NsiI cloning sites, template HFBN23 (7) was amplified by PCR using oligonucleotides rF1G-5′-ATTACCGTGGCTTCATTCCAAATATCCGGACTT-GTCAAGATATTAATGAACTGACTACAGC-3′ and rF1G-AS (5′-CTGAGTACATGTTTGTGGAATTCC-3′), resulting in a 293-bp product. The amplified DNA was ligated with the pCR4Blunt-TOPO vector (Invitrogen), and the 283-bp BtgI-NsiI fragment isolated form this plasmid was subcloned into the pCR4Blunt-TOPO vector, resulting in plasmid pDNSP-rF1G. Finally, the 917-bp Clal-AgeI fragment from pBS3-rF1G was ligated into the Clal-AgeI-restricted plasmid pDNSP-rF1F. The resulting expression plasmid was named pDNSP-rF1G.

The fibrillin-1 construct rF1H is identical to rF1F except that the first hybrid domain of fibrillin-1 was replaced by the second hybrid domain. To generate the expression plasmid for this construct, template pCDRTSGP-rF1A (44) was amplified using oligonucleotides rF1H-5′-ATGTGCATGCACTTACGGA-3′ and rF1H-AS (5′-AGAGCCCGGGATGGCCTGACATTACATC-3′), resulting in a 263-bp product, which was subcloned into the pCR4Blunt-TOPO vector. This plasmid was...

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restricted with SphI and Xmal, and the 245-bp fragment was ligated with the SphI-Xmal-restricted pBSS3-rF1F, resulting in plasmid pB5-rF1F. A 911-bp Clal-Agel fragment isolated from pB5-rF1H was then ligated into the Clal-Agel-restricted pDNSP-rF1H, and the new expression plasmid was termed pDNSP-rF1H.

The rF16H construct is identical to wild-type rF16 coding for the region in fibrillin-1 between the N terminus and cbEGF22 (43), except that the first hybrid domain was replaced with the second hybrid domain. A 1355-bp Nhel-Agel fragment from plasmid pDNSP-rF1H was subcloned into the Nhel-Agel-restricted pDNSP-rF16 (43), and the new plasmid was designated pDNSP-rF16H.

The wild-type fragment rF51 (44) was modified to replace the second hybrid domain with the first hybrid domain (rF51H). Template pDNSP-rF1F (44) was amplified using oligonucleotides rF51H-S 5′-CTTTTGATCCACAAAAACCACTG-CATAAGAGATTACAGGACAGGGCC-3′ and rF51H-AS (5′-GTGTTAACAACAGGCATTATTACACCTCTG-GGAACACTCTACATTCTATCTTGAAAGCTC-CCTGCGG-3′), resulting in a 289-bp product, which was ligated with the pCR-Blunt II-TOPO plasmid (Invitrogen). The 279-bp BamHI-HpaI-restricted fragment from this plasmid was ligated into the BamHI-Hpal-restricted fragment from plasmid pDNSP-rF16H, resulting in the expression plasmid pDNSP-rF15H.

Generation of stable recombinant cell clones using human embryonic kidney cells 293, production of recombinant medium, and purification of the histidine-tagged proteins by chelating chromatography was performed as described previously for other fibrillin-1 and -2 fragments with minor modifications (46).

Recombinant fibrillin-1C, -1D, -2, and -4 were prepared as described previously (21, 25, 47, 48). Expression and characterization of fibrillin-3 and -5 will be described in detail elsewhere. Briefly, cDNA coding for each fibrillin was inserted into the pCPE-Pu or pCPE-Pu/AC7 vector, and 293-EBNA cells (Invitrogen) were transfected by standard methods. Fibulins were purified from serum-free culture medium as described previously with minor modifications (25). Recombinant tropoelastin was kindly provided by Dr. Anthony S. Weiss (49).

Soluble Phase Microwell Assays—For protein-protein interaction assays, multiwell plates (Maxisorp, 96 wells; Nalge Nunc International) were coated with purified proteins (10–20 μg/ml; 50–100 μl/well) in 50 mM Tris-HCl, 150 mM NaCl, pH 7.4 (TBS), at 4 °C overnight. All subsequent steps were performed at 20 °C. Nonspecific binding sites were blocked for 1–2 h with 100 μl of TBS containing 2 mM CaCl₂ and 5% (w/v) nonfat milk (binding buffer). The wells were washed three times with TBS, including 2 mM CaCl₂ and 0.05% (v/v) Tween 20 (washing buffer). The coated proteins were typically incubated with either a single or with a duplicate serial dilution of the soluble ligands starting at 100–150 μg/ml for 2 h. In some cases, a second protein (fibulins or fibrillin-1 fragments) was added at constant concentrations in order to either test inhibitory effects of fibrillin self-assembly or enhancing effects on the fibrillin-1-tropoelastin interactions. After ligand incubation, the wells were washed three times with washing buffer and incubated for 2 h with 100 μl of the primary antibodies against the respective soluble ligands (diluted 1:500–1:1000 in binding buffer). After washing, the wells were incubated with 100 μl of horseradish peroxidase-conjugated secondary goat anti-rabbit antibodies (1:800 diluted in binding buffer) for 1.5 h. Color development was performed with 1 mg/ml 5-aminosalicylic acid in 20 mM phosphate buffer, pH 6.8, including 0.045% (v/v) H₂O₂ (100 μl/well) for 3–5 min and stopped by adding 100 μl of 2 M NaOH to each well. Color yields were determined at 490 nm using a Microplate EL310 autoreader (Bio-Tek Instruments). All solid phase interaction assays were repeated 3–7 times, resulting in similar binding profiles each time. Nonspecific binding of the soluble ligands to either the blocking reagents or the plastic surface was subtracted from binding profiles.

Blot Overlay Assay—Extraction of authentic fibrillin-2 followed an established procedure (31). Briefly, confluent layers of human skin fibroblasts were first washed twice with TBS, including protease inhibitors (2 mM phenylmethylsulfonyl fluoride, 5 mM N-ethylmaleimide), and the extracellular layer was then extracted with 0.1 ml/cm² TBS, including protease inhibitors and 10 mM EDTA for 10 min at 20 °C. Proteins in 1-ml aliquots of the EDTA extracts were precipitated with 10% (w/v) trichloroacetic acid, dissolved in nonreducing SDS sample buffer, and separated by SDS gel electrophoresis (5% (w/v) acrylamide). The proteins were transferred onto nitrocellulose membrane (Bio-Rad) in 10 mM sodium borate, pH 9.2, at a constant current of 0.4 A for 45 min at 4 °C. All of the following incubations were performed at 20 °C. The membranes were first incubated for 1 h with TBS containing 5% (w/v) nonfat milk to block nonspecific binding sites and then with 100 μg/ml recombinant fibrillin-1 fragments as soluble ligands for 3 h in TBS containing 5% (w/v) nonfat milk and 2 mM CaCl₂ (binding buffer). Incubation with binding buffer alone served as a negative control. After washing three times with TBS, including 0.05% (v/v) Tween 20 and 2 mM CaCl₂, the membranes were incubated for 2 h with monoclonal antibody 26 (~5 μg/ml in binding buffer) against the soluble ligands. The membranes were incubated for 1.5 h with horseradish peroxidase-conjugated goat-anti-mouse antibodies (1:800 diluted in binding buffer). The color was developed in TBS, 17% methanol, including 0.02% (v/v) H₂O₂ and 0.5 mg/ml 4-chloro-1-naphthol (Bio-Rad).

Antibodies—The following polyclonal and monoclonal antibodies have been generated and characterized previously. Polyclonal antisera anti-rF16 and anti-rF6H were raised in rabbits against the recombinant N- and C-terminal halves of human fibrillin-1, respectively (50, 51). Polyclonal anti-fibulin-2 antisem (1035+) was raised against human fibulin-2 (48). Monoclonal antibodies 26 and 201 against human fibrillin-1 were a generous gift from Dr. Lynn Y. Sakai (45). A monoclonal antibody against human plasma fibronectin was purchased from Sigma (product F7387). New polyclonal antisera have been raised in rabbits against full-length recombinant fibrillin-4 and -5. Potential cross-reactivities with other fibulins or fibrillin-1 have been tested by enzyme-linked immunosorbent assays (see

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supplemental data, S1). The antisera were used at concentrations excluding or minimizing cross-reactivity with other proteins.

Immunohistochemical Labeling of Cells—For indirect immunofluorescence double labeling experiments, human dermal fibroblasts were grown in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 2 mM glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, and 10% (v/v) fetal calf serum at 37 °C in a 5% CO2 atmosphere. Cells were trypsinized and seeded at 7.5 × 104 cells/well in 8-well chamber slides (Permanox; Nalge Nunc International). Either the cells were grown for 72 h without the addition of proteins, or fibulin-4 or -5 was added to the culture medium at a concentration of 50 µg/ml 24 h after seeding, followed by 48 h of incubation. Cells were washed with phosphate-buffered saline, fixed with 70% methanol in acetone, rehydrated, and blocked with 10% goat serum in phosphate-buffered saline for 30 min. The cells were incubated for 1 h with primary monoclonal mouse antibody 201 (anti-fibrillin-1; ~1 µg/ml), together with 1:1000 diluted polyclonal rabbit anti-fibrillin-2, anti-fibrillin-4, or anti-fibrillin-5 antibodies. Detection of bound antibodies was performed with goat anti-mouse fluorescein conjugate and goat anti-rabbit cyanine Cy3 conjugate (diluted 1:200 in phosphate-buffered saline; Jackson ImmunoResearch Laboratories Inc.). For visualization of the fluorescent signals, an Axiocam microscope was used with AxioVision software version 3.1.2.1 (Zeiss).

Extraction of Microfibrils and Identification of Associated Ligands—The experimental design for extraction and purification of microfibrils followed described procedures with some modifications (51, 52). Briefly, primary human dermal skin fibroblasts were grown for 6 weeks on a total culture area of ~1000 cm2 in Dulbecco’s modified Eagle’s medium as described above. The cells were washed two times with 50 mM Tris-HCl, pH 7.4, 400 mM NaCl, scraped off the culture flask, and incubated for 4 h at 4 °C with 1 mg/ml crude collagenase (from Clostridium histolyticum; Sigma) in the same buffer including 2 mM phenylmethylsulfonyl fluoride and 5 mM N-ethylmaleimide. The cell extract was centrifuged at 7000 × g for 20 min, and the pellet was again digested with crude collagenase for 3 h at 20 °C in the buffer described above, including additionally 5 mM CaCl2. After centrifugation as above, the supernatant was fractionated on a Sephacryl S-500 HR column (120-ml column volume; GE Healthcare) equilibrated in 50 mM Tris-HCl, pH 7.4, 400 mM NaCl containing 5 mM CaCl2 at a flow rate of 0.5 ml/min. From the eluted fractions, 50-µl aliquots were spotted onto nitrocellulose for further analysis by immunoblotting. Fibrillin-1 and fibulin-2, -4, and -5 were detected using the specific antibodies described above (1:1000 diluted). In order to further purify microfibril-containing fractions, a fibrillin-1 antibody affinity column was generated by coupling 11 mg of anti-rF6H antibody to 2.5 ml of cyanogen bromide-activated Sepharose 4B (GE Healthcare) as instructed by the supplier. A control column was generated by coupling Tris-HCl to the same resin instead of the antibody. The microfibril-containing fractions eluted from the S-500 column (see above) were passed over the fibrillin-1 affinity column, and bound material was eluted in one step with 100 mM glycine, pH 2.5. 100-µl aliquots of the eluted fractions were analyzed by dot blotting as described above.

Protein Quantification Methods—Protein concentrations were either determined spectrophotometrically in TBS including 6 M guanidine-HCl at 280 nm following an established method (53) or with the commercially available BCA protein assay kit (Pierce). For amino acid analysis, the proteins were lyophilized and hydrolyzed overnight at 110 °C in 6 N HCl, and the amino acid composition was determined on a Biochrom 20 amino acid analyzer (Biochrom Ltd.) using postcolumn ninhydrin derivatization.

RESULTS

Generation of Fibrillin-1 Swapped Domain and Deletion Fragments—Previously, we have described molecular interactions of fibulin-2 with fibrillin-1 and mapped the binding site to a relatively large N-terminal region of fibrillin-1 (N terminus to proline-rich domain) (Fig. 1) (31). In our experience, precise mapping of ligand binding sites in fibrillin-1 to individual domains often cannot be achieved by generation of small recombinant or proteolytic fragments of fibrillin-1 due to the fact that binding activities are frequently lost in such fragments. This may be due to the requirement of several epitopes for one binding ligand spaced over a larger physical distance in the protein or, alternatively, due to structural alterations in the absence of the stabilizing protein “context.” Therefore, in order to precisely map the binding epitope for fibulin-2 and other fibrulins, we utilized a domain swapping strategy where individual candidate domains in the binding region have been replaced with homologous fibrillin-1 domains from nonbinding regions. This rationale is based on the concept that domains in fibrillin-1 are autonomously folding units (11, 13).

A recently produced fibrillin-1 fragment rF1F spanning the region between the N terminus and the second TB/8-Cys motif, thus completely containing the fibrillin-2 binding site, was chosen for generation of the following domain swapping constructs (Fig. 1) (44). Fibrillin-1 interaction with fibulin-2 is calcium-dependent (31), leading to the hypothesis that the first two cbEGF domains (cbEGF1–2) may be involved in the molecular interaction. Therefore, we engineered two consecutive cbEGF domains from a nonbinding region (cbEGF8–9) into the position of cbEGF1–2 (rF1G). In addition, the first hybrid domain from the interacting region was replaced with the homologous fibrillin-1 domains from nonbinding regions. This rationale is based on the concept that domains in fibrillin-1 are autonomously folding units (11, 13).

All recombinant fibrillin-1 fragments were produced in an established procedure using human embryonic kidney cells. This method was previously used to produce a number of other recombinant fibrillin-1 fragments and other cysteine-rich and glycosylated extracellular matrix proteins that were demonstrated to be correctly folded (14, 44−46). Structural integrity was also verified by reaction with monoclonal antibodies that recognize folded and disulfide-bonded fibrillin-1 but not...
Amino acid analyses demonstrated the expected composition of each fragment within the typical margins of error for this method. Purified swapped domain constructs were compared with their wild-type counterpart by SDS gel electrophoresis, demonstrating no obvious difference (Fig. 1, inset). This was expected, since the swapped domains are highly homologous and almost identical in their molecular masses. A more detailed description of structural and biochemical properties of previously uncharacterized fibrillin-1 fragments will be presented elsewhere.

Fibulin-2 Interactions with Fibrillin-1—In order to precisely localize the binding domain for fibulin-2 in fibrillin-1, wild-type, swapped domain, and deletion constructs were tested for binding with recombinant fibulin-2 in solid phase binding assays (Fig. 2A). As expected from previous data (31), the N-terminal half of fibrillin-1 represented by rF16 and the subfragment rF1F showed saturable binding profiles to fibulin-2 but not rF51. Although the fibulin-2 binding epitope is fully contained within rF1F (31), the signal obtained in solid phase assays was lower as compared with the larger fragment rF16. This difference is due to different antibody titers against the various fragments as determined by an enzyme-linked immunosorbent assay (Fig. 2B). Despite this difference, the anti-fibrillin-1 antiserum used had a high titer for all, even the smallest, recombinant fibrillin-1 fragments used and was thus suitable for detection of the various bound fibrillin-1 fragments. Deletion of the N-terminal domain of fibrillin-1 (rF16N) did not significantly affect the ability for fibulin-2 binding. However, deletion of the first five domains, including the first hybrid domain (rF16NEH), completely abolished the interaction between fibrillin-1 and fibulin-2. These data indicate that the fibulin-2 binding site resides within the first five N-terminal domains of fibrillin-1. The fragment containing cbEGF8–9 instead of cbEGF1–2 (rF1G) showed a very similar binding pattern as compared with the wild-type rF1F, clearly disproving one of the original hypotheses that the binding site is dependent on cbEGF1-2. When the first hybrid domain was replaced by the second hybrid domain (rF1H), binding was completely abolished. Identical results were obtained with the large N-terminal construct rF16H, which also contains hybrid domain 2 instead of hybrid domain 1. These data clearly localize an important determinant of the binding epitope for fibulin-2 to hybrid domain 1 in fibrillin-1. The fragment containing cbEGF8–9 instead of cbEGF1–2 (rF1G) showed a very similar binding pattern as compared with the wild-type rF1F, clearly disproving one of the original hypotheses that the binding site is dependent on cbEGF1-2. When the first hybrid domain was replaced by the second hybrid domain (rF1H), binding was completely abolished. Identical results were obtained with the large N-terminal construct rF16H, which also contains hybrid domain 2 instead of hybrid domain 1.

Figure 1. Recombinant fibrillin-1 fragments. The schematic overview shows the domain model for fibrillin-1. Consecutive numbers for each domain type are shown in the overview on top for the relevant N-terminal region. Domains in the recombinant fragments that are not numbered are identical to the domains in the wild type. For clarity, the hybrid domains are always numbered. Fragments rF6H, rF16, rF51, and rF1F were produced previously for other studies (14, 43, 44). The arrows indicate domains replaced by homologous domains from another region in fibrillin-1. The multifunctional protein interaction region, including the previously identified fibulin-2 binding region at the N terminus of fibrillin-1, is marked by the horizontal bar. Inset, reducing SDS gel electrophoresis of new recombinant fibrillin-1 swapped domain fragments and their wild-type counterparts. Characterization of new deletion constructs will be presented elsewhere (see Footnote 3). Molecular masses are indicated in kDa.
**FIGURE 2.** **Interactions of N-terminal fibrillin-1 swapped domain and deletion fragments with fibulin-2.**

A, solid phase interaction assay with immobilized fibulin-2 and soluble fibrillin-1 ligands (as indicated). B, antibody titers of the anti-rF16 antiserum for the N-terminal fibrillin-1 fragments used in this study were determined by a typical enzyme-linked immunosorbent assay with antiserum dilutions in the range between 1:50 and 1:36,450. Titers resulting in a 50% signal are indicated for each fragment. C, blot overlay assay of recombinant fibrillin-1 fragments and authentic human fibulin-2. EDTA extracts (1-ml aliquots) of the extracellular layer of human skin fibroblasts were separated under nonreducing conditions by SDS gel electrophoresis and then transferred onto nitrocellulose. Soluble fibrillin-1 fragments were used at a constant concentration of 100 μg/ml and detected with monoclonal antibody 26 (epitope: TB/B-Cys domain 1) (64). Detection with monoclonal antibody 201 (epitope: cbEGF domain 6) (44) resulted in identical data (not shown). As a control (second to last panel), the soluble ligand was omitted, and the absence of fibrillin-1 in the extracts is shown by the lack of signal with this antibody. The position of fibulin-2 was determined by reaction with a polyclonal antiserum specific for fibulin-2 (last panel). The **arrow** indicates the position of fibulin-2 interacting with rF16, rF16N, rF1F, and rF1G. This position correlates with the position of a fibulin-2 dimer (~400 kDa) under nonreducing conditions (65).
isoforms fibulin-1C and fibulin-1D showed very little interaction with the N-terminal half of fibrillin-1 compared with the strong interaction with fibulin-2 (31). Fibulin-3 also did not significantly interact with fibrillin-1 fragments. However, fibulin-4 and fibulin-5 strongly bound to the N-terminal half of fibrillin-1, independent of whether they were used as soluble ligands or as immobilized ligands in solid phase binding assays (Fig. 3, A and B). No significant binding properties were observed between the C-terminal half of fibrillin-1 and all fibulins tested (Fig. 3C).

As expected based on the data shown in Fig. 3, no binding was detected between fibulin-3 and the panel of fibrillin-1 deletion and swapped domain constructs (Fig. 4A). In contrast, similar binding profiles were observed for the interaction of fibulin-4 and fibulin-5 with the fibrillin-1 mutants (Fig. 4, B and C). Fragments rF16, rF16N, rF1F, and rF1G efficiently bound to fibulin-4 and -5, but rF16H, rF16NEH, and rF1H did not significantly interact. These results demonstrate that hybrid domain 1 is also essential for binding of fibrillin-1 to fibulin-4 and fibulin-5. Binding of fibrillin-1 (rF16) to fibulin-4 was strongly calcium-dependent, whereas rF16 binding to fibulin-5 was only marginally dependent on calcium (Fig. 4, B and C, insets).

Association of Fibulins with Microfibrils—In order to analyze whether fibulin-2, -4, and -5 can interact with assembled immature microfibrils in cell culture, confluent layers of dermal fibroblasts, which produce a microfibril network after a few days in culture (50), were co-immunolabeled with antibodies against fibrillin-1 and the respective fibulin antibodies (Fig. 5). As expected, based on the previous localization of fibulin-2 to microfibrils on the electron microscopic level (31), endogenously expressed fibulin-2 strongly co-localized with the fibrillin-1-containing microfibrils (Fig. 5A). Fibulin-4 and -5 proteins were not expressed or were only very weakly expressed by the fibroblasts within the time frame (3 days) of this experiment (Fig. 5, B and D). However, when fibulin-4 and -5 were exogenously added to the culture medium, significant, but not exclusive, co-labeling with microfibrils was observed, demonstrating their ability to interact with immature fibrillin-1-containing microfibrils (Fig. 5, C and E). In addition to microfibrillar colocalization, exogenously added fibulin-4 and -5 tended to form small aggregates on the fibroblasts cell layers. In order to exclude the possibility that fibulin-4 and -5 bound to fibronectin, direct interactions were excluded by solid phase binding assays (see supplemental Fig. S3).

Mature microfibrils with a typical bead-on-a-string appearance can be extracted from long term (several weeks) fibroblast cultures with established protocols using collagenase digestion and separation by gel filtration chromatography (51, 52, 55). High molecular weight microfibrils typically elute in the void volume of the gel filtration column, whereas smaller aggregates or protein monomers elute later. The presence of proteins in
interaction epitope is situated in close proximity to the interaction epitope for fibrillin-2, -4, and -5, we tested whether binding of these fibulins can interfere with self-interaction properties of fibrillin-1. In an established assay (46), the recombinant fibrillin-1 C-terminal half was incubated with the N-terminal half in the presence of increasing concentrations of fibulins (Fig. 7). The results demonstrate that the presence of even high concentrations (100 μg/ml) of fibulins did not interfere with the ability of the two fibrillin-1 fragments to interact. Therefore, the binding site for fibrillin-2, -4, and -5 and the self-interaction site in fibrillin-1 are located on different epitopes.

**Fibulins Can Act as Adaptors between Fibrillin-1 and Tropoelastin**—Genetic targeting experiments of fibrillin-4 and -5 demonstrated profound effects on the formation and function of elastic fibers, indicating that these fibulins play important roles in the development and homeostasis of the elastic fiber system (33, 34, 36). In order to determine the
molecular relationship between fibrillin, fibulins, and elastin, we performed sandwich binding assays to analyze whether fibulins-2, -4, and -5 are able to mediate the interaction between fibrillin-1 and tropoelastin (Fig. 8). In contrast to published work (56, 57), we consistently observed no direct binding or only minor direct binding between the N-terminal half of fibrillin-1 (rF16) and tropoelastin. However, when fibulin-2 or -5 was added to the binding assay, rF16 strongly interacted with tropoelastin, whereas fibulin-4 only marginally mediated the formation of this ternary complex. These data demonstrate that at least fibulin-2 and -5 can mediate binding of fibrillin-1 to tropoelastin.

**DISCUSSION**

The microfibril/elastic fiber system is a multiprotein bio-composite, and genetic alterations in individual components cause various connective tissue disorders. In order to appreciate the functional relationship of these microfibril/elastic fiber components and to better understand pathogenetic pathways, it is necessary to ultimately elucidate the microfibril/elastic fiber “interactome” (i.e. the complete interaction repertoire between individual microfibril/elastic fiber components and the respective interaction epitopes). To contribute to this goal, we focus here on the structural and functional analysis of bind-

**FIGURE 7. Interference of fibulin binding with homotypic self-interaction of fibrillin-1.** In an established self-interaction assay (46), rF6H strongly interacts with rF16 in a dose-dependent manner. Shown is a typical solid phase binding assay with immobilized rF16. Soluble ligands, fibulin-2, -4, and -5 (as indicated), were added at increasing concentrations (0–100 μg/ml; plotted as nanomolar concentrations) together with a constant concentration of 50 μg/ml rF6H. Ligand binding was monitored with specific antibodies against the respective fibulin (filled symbols) or rF6H (open symbols). The values are expressed as a percentage of total binding for each ligand.

**FIGURE 8. Fibulins can act as adaptor molecules between fibrillin-1 and tropoelastin.** In this solid phase interaction assay, tropoelastin was immobilized, and rF16 was used as a soluble ligand at increasing concentrations (0–150 μg/ml; plotted as nanomolar concentrations) either together with (squares, triangles), or without (circles) various fibulins as indicated at constant concentrations (50 μg/ml). Interaction of rF16 or fibulins with tropoelastin was monitored using specific anti-fibrillin-1 (circles, squares) or anti-fibulin (triangles) antibodies. For clarity, the key at the top indicates the ligands present in the assays and the ligand that was specifically detected (underlined). Note that only in the presence of fibulin-2 and -5 was significant binding between rF16 and tropoelastin observed. Plotted is the absorbance at 490 nm for binding of rF16 (left y axis) or the respective fibulin (right y axis) against the concentration of the rF16 fragment (x axis).
Fibrillin-1-Fibulin Interactions

We have previously described molecular interactions of fibrillin-2 with fibrillin-1 (31). In that study, the fibrillin-2 binding epitope was mapped to a relatively large N-terminal region of fibrillin-1. This region appears to be multifunctional, since binding epitopes for other ligands, such as LTBP-1, MAGP-1, heparan sulfate, and fibrillin-1 itself, have also been located on this segment (43, 50, 55, 58, 59). In order to understand how multiple protein binding occurs within the same region of the molecule and how the interaction repertoire is ultimately regulated, it is important to precisely localize the binding sites of individual ligands.

In the present study, we provide evidence that hybrid domain 1 in fibrillin-1 is an important determinant of the fibrillin-2 binding site. Although this hybrid domain is absolutely necessary to promote interaction with fibrillin-2, it is alone not sufficient for complete binding. We suggest that fibrillin-2 interacts with hybrid domain 1 to establish an initial contact between the proteins, which is then further stabilized by additional contacts mediated by regions in relatively close proximity to hybrid domain 1. The fact that rF1G effectively interacted with fibrillin-2 excludes cbEGF1–2 for such a synergistic role in fibrillin-2 binding. Since the N-terminal region upstream of hybrid domain 1 contains an important self-assembly site, and fibrillin-2 interaction with fibrillin-1 does not interfere with fibrillin-1 self-assembly, we speculate that the additional synergy site(s) are located downstream of cbEGF2.

The first hybrid domains in fibrillin-1, -2, and -3 are highly conserved in all species. In humans, the homology on the protein level is 80–90%, which is significantly higher than the average homology of 61–69% between the full-length fibrillin isoforms. Even distant species, such as Homo sapiens and Fugu rubripes (Japanese pufferfish), show an ~88% homology in their first hybrid domains. These sequence similarities suggest that the fibrillin-2 binding site is conserved between fibrillin isoforms and between species. Indeed, binding of fibrillin-1 is virtually identical with mouse and human fibrillin-2 (31). On the other hand, the homology between the first and the second hybrid domains in individual human fibrillins is relatively low (31–41%), explaining the fact that the second hybrid domain is not able to mediate interaction with fibrillin-2.

We extended our study to other fibulins and found that fibrillin-4 and -5 bound to fibrillin-1, whereas fibrillin-1C, -1D, and -3 did not interact with fibrillin-1 in the assays employed. Although binding of fibrillin-5 to fibrillin-1 has recently been reported (37), this is, to our knowledge, the first report of a fibrillin-4/fibulin-1 interaction. Mapping studies revealed a binding mechanism for fibrillin-4 and -5 with fibrillin-1 identical to that of fibrillin-2, with the first hybrid domain playing an essential role. These fibulins not only directly interacted with fibrillin-1, but they associated with microfibrils at different levels of maturity. Fibroblasts produce a microfibrillar network after a few days in culture, probably representing immature microfibril assemblies. Endogenously produced fibrillin-2 and exogenously added recombinant fibrillin-4 and -5 colocalized with these immature microfibrils as shown by indirect immunofluorescence. It takes several weeks until more mature microfibrils with a typical bead-on-the-string appearance can be extracted from fibroblast cultures (52). Again, all three fibulins were associated with these mature microfibrils. This is in contrast to properties of other proteins interacting with fibulins, such as perlecan and latent transforming growth factor-β-binding protein 1, which cannot be found associated with microfibrils in this assay (51, 55). These data suggest that fibrillin-2, -4, and -5 may play essential functional roles, such as regulating the biogenesis of microfibrils or elastic fibers.

One of the first steps in microfibril biogenesis appears to be a fibrillin-1 self-interaction of the amino terminus with the carboxyl terminus (46, 58). Since the domain essential for fibrillin-2, -4, and -5 binding is situated relatively close to the fibrillin-1 self-interaction site, we hypothesized that fibrillin binding may modify the self-interaction properties. Although fibrillin-2, -4, and -5 bound to fibrillin-1, the self-interaction properties were not affected, clearly disproving this hypothesis. For fibrillin-5, this is in concordance with recently published data (37). These results exemplify a permissive topology for simultaneous binding of relatively large proteins (fibulins, fibrillin-1) within a narrow binding region and further demonstrate that fibrillin-2, -4, and -5 do not have a role in fibrillin N- to C-terminal self-interaction. Whether or not these fibulins fulfill a role in subsequent steps of the microfibril biogenesis remains to be elucidated.

Recently, it became clear that fibrillin-4 and -5 play critical roles in formation of complete elastic fibers. Fibrillin-4 and -5 null mice exhibit disrupted and disorganized elastic fibers in different tissues throughout the body, indicating that both molecules play essential roles in the formation of elastic fibers (33, 34, 36). The fibrillin-4 null phenotype is characterized by an almost complete loss of elastic fibers in the vascular wall and perinatal lethality, whereas fibrillin-5 null mice survive to adulthood with disorganized and fragmented elastic fibers in all elastogenic tissues. How fibrillin-4 and -5, and potentially fibrillin-2, contribute to the elastic fiber biogenesis or homeostasis is presently unclear. For fibrillin-5, it has been proposed that it promotes elastic fiber formation by linking elastic fibers to cells, although this functional role has not been demonstrated in vivo (33, 34). Morphological observations of elastic fiber formation in the embryo suggested that fibrillin-containing microfibrils provide a scaffold that guides tropoelastin deposition (1). Although normal elastic fiber assembly occurred in fibrillin-1 and fibrillin-2 mutant mice, the absence of elastic fiber assembly in mice lacking both fibrillins showed that either fibrillin-1 or fibrillin-2 is absolutely required for the formation of elastic fibers (16, 60–63). Direct molecular interactions between fibrillin-1 and -2 with tropoelastin have been reported, and the binding sites have been mapped to domains TB2 and probably TB3, both located in the N-terminal half of fibrillin-1 (56, 57). These data were obtained with relatively small overlapping recombinant fragments of fibrillin-1, and interestingly, the tropoelastin binding site on TB2 was no longer available in the presence of additional N-terminal domains (57). In our study, no direct interaction of the entire N-terminal half of fibrillin-1 with tropoelastin was observed, despite the fact that this fragment contains both previously identified tropoelastin binding sites and has binding activities for all other ligands identified.
thus far (43, 50, 55, 58, 59). One potential interpretation of this observation is that the tropoelastin binding sites identified on fibrillin-1 are cryptic and normally not available in larger recombinant constructs or in full-length native fibrillin-1. Despite the lack of direct interactions with tropoelastin, we found that fibrillin-1 can bind to tropoelastin when mediated by fibrillin-2 and -5 but much less by fibrillin-4. These data suggest that fibrillin-2 and -5 can act as molecular adapters between fibrillin-1 and tropoelastin. Immunogold localization of fibrillin-2 to the microfibril-elastic fiber system as well as of pathogenetic mechanisms promote molecular interactions of fibrillin-1 with tropoelastin.

In conclusion, we have demonstrated that the first hybrid domain in fibrillin-1 is essential for binding to fibrillin-2, -4, and -5. These fibrilins can associate with microfibrils at different levels of maturity, and at least fibrillin-2 and -5 can mediate or promote molecular interactions of fibrillin-1 with tropoelastin. We anticipate that these data will contribute to the understanding of basic mechanisms involved in the biology of the microfibril/elastic fiber system as well as of pathogenetic mechanisms originating in mutations of individual components of this system.

Acknowledgments—We thank Martina Alexander and Christine Fagotto for excellent technical assistance. We are grateful to Dr. Lynn Y. Sakai for providing the monoclonal antibodies 26 and 201 and to Dr. Anthony S. Weiss for providing tropoelastin.

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