Fibrillin Assembly Requires Fibronectin

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Fibrillins constitute the major backbone of multifunctional microfibrils in elastic and nonelastic extracellular matrices. Proper assembly mechanisms are central to the formation and function of these microfibrils, and their properties are often compromised in pathological circumstances such as in Marfan syndrome and in other fibrillinopathies. Here, we have used human dermal fibroblasts to analyze the assembly of fibrillin-1 in dependence of other matrix-forming proteins. siRNA knockdown experiments demonstrated that the assembly of fibrillin-1 is strictly dependent on the presence of extracellular fibronectin fibrils. Immunolabeling performed at the light and electron microscopic level showed colocalization of fibrillin-1 with fibronectin fibrils at the early stages of the assembly process. Protein-binding assays demonstrated interactions of fibronectin with a C-terminal region of fibrillin-1, -2, and -3 and with an N-terminal region of fibrillin-1. The C-terminal half of fibrillin-2 and -3 had propensities to multimerize, as has been previously shown for fibrillin-1. The C-terminal of all three fibrillins interacted strongly with fibronectin as multimers, but not as monomers. Mapping studies revealed that the major binding interaction between fibrillins and fibronectin involves the collagen/gelatin-binding region between domains FNI₆ and FNI₇.

INTRODUCTION

Fibrillins are extracellular matrix components with important functions in elastic and nonelastic tissues including blood vessels, bone, and the eye. Fibrillins, together with the latent transforming growth factor- β (TGF- β)-binding proteins (LTBPs), constitute the fibrillin-LTBP family of proteins (Hubmacher *et al.*, 2006). Fibrillins are ~350 kDa in size, are disulfide-rich and glycosylated, and have a characteristic modular structure. The three human fibrillins—fibrillin-1, -2, and -3—are encoded by different genes and are conserved at the amino acid level both in relation to one another and among species. Fibrillins are mainly composed of tandem arrays of calcium-binding epidermal growth factor-like domains interspersed with TGF- β -binding protein domains (TB/8-Cys) and hybrid domains (Kielty *et al.*, 2005; Hubmacher *et al.*, 2006). Mutations in fibrillins give rise to the so-called fibrillinopathies, which include Marfan syndrome and autosomal dominant Weill-Marchesani syndrome, both caused by mutations in fibrillin-1, and Beal's syndrome, caused by mutations in fibrillin-2 (Robinson *et al.*, 2006).

High-molecular-weight, multiprotein assemblies called microfibrils are the functional units of fibrillins, which serve as a scaffold for the biogenesis of elastic fibers, confer structural integrity to individual organ systems, regulate growth factor signaling of TGF- β -bone morphogenic protein (BMP) superfamily members and provide limited elasticity to tissues (Kielty *et al.*, 2002; Charbonneau *et al.*, 2004; Ramirez and Dietz, 2007). Extracted microfibrils from cell culture or tissues display a typical bead-on-a-string ultrastructure, having a 50–55-nm periodicity when analyzed by electron microscopy after rotary shadowing (Keene *et al.*, 1991; Kielty *et al.*, 1991). Although many publications over recent years have addressed the spatial organization of fibrillins as it relates to the bead-on-a-string microfibrillar structure (Sakai *et al.*, 1991; Reinhardt *et al.*, 1996; Downing *et al.*, 1996; Liu *et al.*, 1996; Qian and Glanville, 1997; Baldock *et al.*, 2001, 2006; Lee *et al.*, 2004; Kuo *et al.*, 2007), little information is available about the dynamic processes and components involved in microfibril formation. The pathogenetic relevance of this is highlighted by the fact that microfibril assembly is frequently disturbed in patients with fibrillinopathies (Tiedemann *et al.*, 2004).

One of the early mechanisms in the multistep, cell-associated fibrillin assembly process involves N-to-C-terminal (N–C) self-interactions, whereby the interaction sites for fibrillin-1 have been mapped to the N- and C-terminal ends of the molecule (Lin *et al.*, 2002; Marson *et al.*, 2005; Hubmacher *et al.*, 2008). In this process, cell-associated multimerization of the fibrillin-1 C-terminus appears to be an important determinant in generating high-affinity binding sites for the fibrillin-1 N-terminus (Hubmacher *et al.*, 2008). In addition to linear N–C interactions, lateral homotypic interactions in different regions of the fibrillin-1 molecule may play a role in stabilizing initial multimers or the lateral associa-

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Abbreviations used: DTT, dithiothreitol; HSF, human skin fibroblasts; LTBP, latent transforming growth factor-binding protein; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; TGF- β , transforming growth factor- β .

tions of individual microfibrils (Trask *et al.*, 1999; Ashworth *et al.*, 1999; Marson *et al.*, 2005). Although glycosaminoglycans of the heparin/heparan sulfate family have an important, but as yet undefined role in microfibril formation (Tiedemann *et al.*, 2001; Ritty *et al.*, 2003), it is currently not known whether other components on the cell surface or in the extracellular matrix are involved in the multifaceted fibrillin assembly process.

Fibronectin, like the fibrillins, is also a modular protein, consisting of fibronectin types I, II, and III (FNI, FNII, and FNIII) domains that confer self-assembly and ligand-binding properties (Hynes, 1985; Pankov and Yamada, 2002). Fibronectin exists in two forms: cellular fibronectin present in tissues where it is assembled into a fibrous network, and soluble plasma fibronectin, which polymerizes upon blood vessel injury. Fibronectin is secreted from cells as disulfide-bonded, soluble inactive dimers that must be activated to assemble into fibrils (Mao and Schwarzbauer, 2005). Activation occurs primarily through interaction with the cell surface $\alpha 5 \beta 1$ integrin, but can be replaced by other integrins (Akiyama *et al.*, 1989; Fogerty *et al.*, 1990; Takahashi *et al.*, 2007). Tension generated by the intracellular actin cytoskeleton stretches fibronectin molecules through ligated integrin receptors, leading to the exposure of cryptic self-interaction sites and subsequently to the formation of an extracellular fibronectin network (Zhong *et al.*, 1998; Baneyx *et al.*, 2002). There are at least four self-interaction sites present on each fibronectin subunit. The most important epitope for assembly is located in the N-terminally located five FNI domains, and this region is indispensable for fibronectin assembly (Keown-Longo and Mosher, 1985; McDonald *et al.*, 1987; Sottile *et al.*, 1991). This portion of the molecule is directly followed by the binding site for collagen/gelatin, which is located between FNI₆ and FNI₉ (Engvall *et al.*, 1978; Balian *et al.*, 1979; Shimizu *et al.*, 1997). Although a plethora of reports have been published on the initial assembly mechanisms for fibronectin, virtually nothing is known about how individual fibronectin molecules are spatially oriented and organized in early and fully assembled fibronectin networks.

The assembly of a few extracellular matrix proteins have been demonstrated to be dependent on the presence of fibronectin, including collagen types I and III and thrombospondin-1 (McDonald *et al.*, 1982; Sottile and Hocking, 2002; Velling *et al.*, 2002; Li *et al.*, 2003), fibulin-1 (Roman and McDonald, 1993; Godyna *et al.*, 1995), fibrinogen (Pereira *et al.*, 2002), and LTBP-1 (Dallas *et al.*, 2005). Further studies established for collagen type I, thrombospondin-1, and LTBP-1 that a continuous assembly and supply of fibronectin is a prerequisite for matrix stability of these proteins (Sottile and Hocking, 2002; Dallas *et al.*, 2005). These data suggest that fibronectin is a "master organizer" for the biogenesis of the extracellular matrix (Dallas *et al.*, 2006). It is conceivable that this functional aspect of fibronectin contributes to the severe in vivo defects in mice lacking the fibronectin gene, which include mesodermal, vascular, and neural tube defects, resulting in death around embryonic day 8.5 (George *et al.*, 1993).

Our study aimed to define the role of fibronectin in the assembly of fibrillin-1 and to characterize the molecular interactions between these two proteins. We show that the presence of a fibronectin network is essential for the assembly of fibrillin-1 in cultures of human dermal fibroblasts. Various protein-binding experiments demonstrate direct and strong interactions between fibrillins and fibronectin at the molecular level.

MATERIALS AND METHODS

Antibodies

Generation of rabbit polyclonal anti-fibrillin-1 antibodies against the N-terminal half (α -rFBN1-N; synonym α -rF16) or against the C-terminal half (α -rFBN1-C; synonym α -rF6H) of human fibrillin-1 was described in detail previously (Tiedemann *et al.*, 2001, 2005). For some experiments, the α -rFBN1-C antiserum was further purified by standard affinity chromatography using the rFBN1-C antigen. Polyclonal rabbit antibodies against the N-terminal half (α -rFBN2-N) or against the C-terminal half (α -rFBN2-C) of human fibrillin-2 have been characterized previously (Lin *et al.*, 2002). A new polyclonal rabbit antiserum against the recombinant C-terminal half of human fibrillin-3 (rFBN3-C, see *Proteins*) has been produced and characterized by standard techniques. The mouse monoclonal anti-fibrillin-1 antibody (mAb15) was a generous gift from Dr. Lynn Sakai (Shriners Hospital for Children, Portland, OR). The mouse monoclonal anti-fibronectin antibody (FN-15) was commercially purchased (Sigma, St. Louis, MO; product F7387). FITC- and Cy3-conjugated AffiniPure goat anti-rabbit IgG (H+L), FITC-, and Cy3-conjugated AffiniPure goat anti-mouse IgG (H+L); 12-nm colloidal gold-AffiniPure donkey anti-mouse IgG (H+L); and 18-nm colloidal gold-AffiniPure donkey anti-rabbit IgG (H+L) were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA).

To exclude cross-reactivity between the anti-fibrillin-1 antisera α -rFBN1-N and α -rFBN1-C with the anti-fibronectin antibody FN-15 in colocalization experiments, the antibodies were tested by standard enzyme-linked immunosorbent assays (ELISAs). The antibodies were used at appropriate concentrations, as indicated, where cross-reactivity was minimal. Additionally, absence of cross-reactivity between α -rFBN1-N and α -rFBN1-C with FN-15 was shown by immunofluorescence after down-regulation of the fibrillin-1 expression by small interfering RNA (siRNA) gene silencing.

Proteins

Recombinant Fibrillin Fragments. Production of the N- and C-terminal fragments of human fibrillin-1 (rFBN1-N, rFBN1-C, respectively) and fibrillin-2 (rFBN2-N, rFBN2-C, respectively) has been described in detail previously (Jensen *et al.*, 2001; Lin *et al.*, 2002). To produce a new recombinant C-terminal half of human fibrillin-3 (rFBN3-C; D¹³²¹-R²⁶⁸⁴), the cDNA for human fibrillin-3 (clone KIAA1776, Nagase *et al.*, 2001) was obtained from the Kazusa DNA Research Institute (Chiba, Japan) and cloned into the pBluescript II SK(+) plasmid (Stratagene, La Jolla, CA). The resulting plasmid pBS-FBN3 was cut with NheI $\times$ SgrAI and the 7888-base pair fragment was ligated with the complementary oligonucleotides 5'-CTAGCAGACCTGGATGAATGAATG-CATCTCCAGGAGCA-3' and 5'-CCGGTGCTGGGAGATGCATTCATC-CAGGTCTG-3' generating a NheI restriction site at the 5' end (pBS-rFBN3C-1). A PCR reaction with pBS-FBN3 as a template was performed with the sense primer 5'-AAGAACCTCATCGGTACCTTCG-3' and the antisense primer 5'-CATGAGCGGCGCCATTATAGTGATGGTGATGGTGATGGTCCCGAGG-GGAGAGGCCATTGA-3', introducing a heptahistidine tag, two consecutive stop codons and a NotI restriction site at the 3' end. After subcloning the 1416-base pair PCR product in the pCRII-TOPO vector (Invitrogen, Carlsbad, CA), an 874-base pair SphI $\times$ NotI fragment from the resulting plasmid was ligated with the 5677-base pair SphI $\times$ NotI fragment from pBS-rFBN3C-1, resulting in pBS-rFBN3-C. This plasmid was digested with NotI $\times$ NheI, and the 4127-base pair fragment was ligated with the vector backbone of the pDNSP-rFIF plasmid (El-Hallous *et al.*, 2007). The final expression plasmid pDNSP-rFBN3-C encodes the BM40 signal peptide for secretion, generating an artificial APLA sequence, followed by the human fibrillin-3 amino acid sequence and a heptahistidine tag (APLAD¹³²¹-R²⁶⁸⁴HHHHHHH). Transfection of human embryonic kidney cells (HEK293 cells), selection of protein-producing clones and protein purification using metal-affinity chromatography have been described in detail elsewhere (Lin *et al.*, 2002).

Fibronectin and Recombinant Fibronectin Fragments. Purified human plasma full-length fibronectin was commercially purchased (Millipore, Bedford, MA; product FC010). Production of the recombinant fibronectin fragment FNIII1-C has been described in detail previously (Ensenberger *et al.*, 2004). The generation of a 40-kDa, gelatin-binding fragment from human plasma fibronectin followed an established procedure (Chernousov *et al.*, 1991). The new FNSuper70K construct spans the N-terminus through module FNIII₃ plus 17 residues (GNFFKKTLPLMSYQDCS) from the following intron and is the human homolog to a splice variant found in zebrafish (Liu *et al.*, 2003). It was produced recombinantly by modification of the pCOCO baculovirus expression vector encoding FN70K (Mosher *et al.*, 2002; Tomasini-Johansson *et al.*, 2006). DNA for encoding the 17-residue sequence was produced by annealing overlapping oligonucleotides, 5'-ACGCTCCGGAACTCTTCAAGAAGA-CACCTTCCTATGTTATC-3' and 5'-CCATCTGCAGAACAACTCCTGATAA-GATAACATAGGAAGTGCTTC-3', and extending with Taq polymerase. The resulting 67-base pair segment was digested with BspEI and PstI and used as follows. BspEI and PstI sites were added to the 3' end of DNA encoding FNIII₃ by PCR amplification of a portion of the fibronectin cDNA from 5' to a natural NcoI restriction site through FNIII₃ using the sense primer 5'-CACAGAACTATGATGCCGACCAG-3' and the antisense primer 5'-

GAGACTGCAGTGTTCCTCGGAGCGTGGGGTGCCAGTGG-3'. This DNA segment was digested with NcoI and PstI and cloned into the baculovirus expression vector pCOCO encoding FN70K, extending the insert to encode the N-terminus through FNIII₃. The modified pCOCO was digested with XmaI and PstI, and the insert was ligated into pGEM-4z digested with these same enzymes. The modified pGEM-4z was cleaved with BspEI and PstI and ligated to the DNA with the intron-encoding sequence, thus further extending the coding sequence to include the 17 residues. The further modified pGEM-4z was then digested with NcoI and PstI, and the insert was ligated into the baculovirus expression vector containing FN70K, resulting in the final FNSuper70K construct ending in a C-terminal affinity tag sequence (AGHH-HHHH).

Cell Culture

Human skin fibroblasts (HSFs) were isolated after a standard circumcision procedure from the foreskins of healthy individuals (2–5 years of age). The procedure was approved by the local ethics committee (PED-06-054), and informed consent was obtained from the parents of the tissue donors. HSFs were used for the experiments between passage 2 and 7 and were incubated at 37°C in a 5% CO₂ atmosphere with DMEM supplemented with 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 10% fetal calf serum (Wisent, St. Bruno, QC, Canada). For some experiments, as indicated, the fibronectin present in the FCS was depleted using Gelatin Sepharose 4B as instructed by the manufacturer (GE Healthcare, Waukesha, WI), and the efficiency of the depletion was validated by immunoblotting.

Gene Silencing Experiments

Duplexed siRNAs were purchased and included Hs_FN1_7 (target: human FN1; 5'-CCCCTGTTTATGACAATGGA-3') and Hs_FBN1_1 (target: human FBN1; 5'-ACCGGTTTACCCGTTGATATT-3'; Qiagen, Chatsworth, CA). The "AllStars Negative Control" siRNA was used as control in all siRNA gene-silencing experiments (Qiagen). This siRNA has no homology to any known mammalian gene and has been validated using the Affymetrix GeneChip (Qiagen). The siRNAs (20 nM) were transfected into HSFs in suspension (1 × 10⁵ cells/ml) using Lipofectamine 2000 (Invitrogen) in DMEM (with fibronectin-depleted FCS and without antibiotics) according to the supplier's instructions. Transfected and nontransfected control HSFs were seeded either at 3.75 × 10⁴ cells/well in an eight-well chamber slide (0.7-cm² growth area; BD Biosciences, San Jose, CA) for immunofluorescence experiments or at 5.0 × 10⁵ cells/well in six-well plates (9.6-cm² growth area; Corning Costar, Acton, MA) for production of 1) serum-free conditioned medium used for immunoblotting experiments, 2) fibronectin-depleted conditioned medium to test assembly of exogenous fibrillin-1, and 3) cells for total RNA extraction. The medium containing the transfection solution was replaced by DMEM (with fibronectin-depleted FCS) 24 h after cell seeding (300 µl DMEM/well for eight-well chamber slides; 2 ml DMEM/well for six-well plates). For immunofluorescence assays, cells were immunostained 2–4 d after seeding. For immunoblotting, the DMEM culture medium in six-well plates was replaced by serum-free DMEM (2 ml/well) 48 h after cell seeding and was harvested after an additional 48-h incubation period, and 0.33-ml aliquots were analyzed after precipitation with 10% trichloroacetic acid by standard Western blotting under nonreduced conditions. Cell layers were then washed with 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, and 1.47 mM KH₂PO₄, pH 7.4 (phosphate-buffered saline [PBS]), immediately after harvesting the serum-free medium and used for RNA extraction (see below). To test the efficiency of all siRNA gene-silencing experiments, the silencing effect was monitored up to 12 d after siRNA transfection by immunofluorescence and immunoblotting (not shown), establishing the full extent of gene silencing up to days 4–5 after transfection and an ~50% remaining efficiency at about day 7 after the siRNA transfection.

RNA Extraction, Reverse Transcription, and Real-Time PCR

Total RNA was extracted from HSFs in six-well plates 96 h after siRNA transfection (see *Gene Silencing Experiments*) using RNeasy Plus Mini Kit as instructed by the supplier (Qiagen). After spectrophotometric quantification, 200 ng total RNA from each sample was reverse-transcribed in a 20-µl volume with random hexamers and Superscript III using the manufacturer's protocol (Invitrogen). Subsequently, cDNA samples corresponding to 50 ng total RNA (5 µl of the RT-PCR reaction) were used in real-time PCR analysis with Taqman Gene Expression Assays according to the manufacturer's protocol (Applied Biosystems, Foster City, CA; 7500 Fast Real-Time PCR System). The Taqman probes used include Hs01549940_m1 FN1 and Hs00171191_m1 FBN1 corresponding to the human gene for human fibronectin (FN1) and human fibrillin-1 (FBN1), respectively. Taqman probe Hs99999905_m1 GAPDH corresponding to the human gene for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a control. Relative quantification was determined by setting the signals from the AllStars siRNA negative control divided by the signal for GAPDH to 100% (Applied Biosystems; 7500 System software v1.3.0). For each total RNA sample, reverse transcription and real-time PCR reactions were repeated four times.

Immunofluorescence Microscopy

For single or double immunofluorescence experiments in eight-well chamber slides, cells were either prepared as described under *Gene Silencing Experiments* using single or double siRNA transfected cells as indicated, or nontransfected cells were seeded at 7.5 × 10⁴ cells/well for various time periods (24–48 h). After two washes with PBS (general washing buffer), the cells were fixed in ice-cold 70% methanol/30% acetone. Cells were washed again, blocked for 30 min with PBS including 10% normal goat serum (PBS-G; Jackson ImmunoResearch Laboratories), and incubated with primary antibodies for 90 min diluted in PBS-G. The primary anti-fibrillin-1 antibodies were α-rFBN1-C (1:1000) or mAb15 (1:500) and anti-fibronectin antibody FN-15 (1:1000; see *Antibodies*). After washing, the cells were incubated for 60 min with either FITC- and/or Cy3-labeled secondary antibodies (1:200 diluted in PBS-G) and washed again. The cells were then incubated with 4'-6-diamidino-2-phenylindole (DAPI; 1 µg/ml in PBS; Invitrogen) for a nuclear counterstain, washed again, cover-slipped, and examined using an Axioskop 2 microscope (Zeiss, Thornwood, NY). For visualization of the fluorescent signals, an AxioCam camera was used with AxioVision software version 3.1.2.1 (Zeiss).

Peptide Inhibition and Protein Binding Monitored by Immunofluorescence

The established, functional upstream domain (FUD) of *Streptococcus pyogenes*, which efficiently inhibits fibronectin assembly (Tomasini-Johansson *et al.*, 2001; Ensenberger *et al.*, 2004), and its inactive mutant Del29 lacking amino acid residue I²⁹ (Zhou *et al.*, 2008), were added at 500 nM to HSFs at the time of seeding (7.5 × 10⁴ cells/well) into eight-well chamber slides. After incubation for 48 h, the cells were processed and stained with anti-fibrillin-1 and anti-fibronectin antibodies as described in *Immunofluorescence Microscopy*.

For binding assays of recombinant fibrillin fragments to protein networks in cell culture, rFBN1-N and rFBN1-C were covalently labeled with Cy3 Mono NHS-ester (GE Healthcare) according to the manufacturer's instructions. The proteins were then dialyzed extensively against TBS. HSFs were seeded at 7.5 × 10⁴ cells/well in eight-well chamber slides and grown for 24 h. The Cy3-labeled proteins were added to the culture medium for 24 h at 10 µg/ml, and after washing with PBS, the cells were stained for fibronectin and processed as described in *Immunofluorescence Microscopy*. Labeling with the Cy3-conjugated proteins was detected using the Cy3 filter set of the fluorescence microscope.

Immunogold Staining and Electron Microscopy

HSFs were prepared, grown, fixed, and stained with antibodies as described in *Immunofluorescence Microscopy* with the following alterations. PBS including 10% normal donkey serum (PBS-D; Jackson ImmunoResearch Laboratories) was used as blocking and antibody dilution buffer. The primary anti-fibrillin-1 antibodies were α-rFBN1-C (1:100) and anti-fibronectin antibody FN-15 (1:100) diluted in PBS-D (see *Antibodies*). Twelve- and 18-nm gold-conjugated secondary antibodies were used diluted at 1:20 in PBS-D. After antibody incubation, the cells were fixed again with 2% glutaraldehyde and 1% OsO₄ and embedded in EPON epoxy resin. Ultrathin sections were stained with 4% aqueous uranyl acetate and Reynold's lead citrate following an established procedure (Sasaki *et al.*, 1996). Sections were then examined with a FEI Tecnai 12, 120 kV electron microscope (Eindhoven, Hillsboro, OR) equipped with a Gatan 792 Bioscan 1k × 1k wideangle multiscan CCD camera (Pleasanton, CA).

Assembly of Exogenous Fibrillin-1

To produce conditioned cell culture media containing fibrillin-1 and fibronectin, HSFs were seeded in six-well plates (Corning Costar, Acton, MA) at a density of 5.0 × 10⁵ cells/well in DMEM. After 48 h, the medium was replaced by serum-free DMEM, and after an additional 48-h incubation period, the conditioned medium was harvested. The production of fibronectin-depleted (but fibrillin-1 containing) conditioned medium was achieved in a similar manner except that 1) the HSFs were transfected with siRNA Hs_FN1_7 as described in *Gene Silencing Experiments* and 2) the transfection medium was replaced by DMEM after 24 h. Serum-free medium production as described above was started after an additional 24 h. Both, the normal and the fibronectin-depleted conditioned media were concentrated up to 30-fold by ultrafiltration (Centriplus YM-30; Millipore). Aliquots of the concentrated media (12.5 µl) were analyzed by standard Western blotting under nonreducing conditions using 1:500 diluted mAb15 to detect fibrillin-1 and 1:1000 diluted FN-15 to detect fibronectin.

To analyze the assembly properties of exogenously added fibrillin-1 to HSFs, the background level of endogenously produced fibrillin-1 or fibronectin was reduced by gene silencing with siRNA Hs_FBN1_1 or Hs_FN1_7 in eight-well chamber slides as described in *Gene Silencing Experiments*. The transfection medium was replaced 24 h after transfection with DMEM containing 10% fibronectin-depleted FCS (300 µl/well), which was again replaced after additional 24 h with 300 µl/well of unconcentrated, 15- or 30-fold concentrated conditioned fibronectin-containing or fibronectin-depleted media. After 48 h of incubation with the conditioned media, the networks were visualized by immunofluorescence (see *Immunofluorescence Microscopy*) after

exposure to mAb15 (diluted 1:500) to detect fibrillin-1, and FN-15 (diluted 1:1000) to detect fibronectin. The analysis of colocalization of exogenously added fibrillin-1 and fibronectin (in the form of conditioned medium) was identical to this procedure except that both fibrillin-1 and fibronectin expression was reduced by siRNA gene silencing simultaneously with siRNA Hs_FBN1_1 and Hs_FN1_7 and the α -rFBN1-C antiserum (diluted 1:1000) was used for detection of fibrillin-1. For these experiments, the conditioned medium applied was 15-fold concentrated and contained both fibrillin-1 and fibronectin. In addition, a nonconditioned DMEM was used as a control.

Protein Interaction and Inhibition Assays

Solid-phase binding assays were performed as described previously in detail with minor modifications (Lin *et al.*, 2002). In brief, 10 μ g/ml (100 μ l) of either human plasma full-length fibronectin, proteolytic fragment FN40K, recombinant fibronectin fragments FNsuper70K, or FNIII1-C were immobilized in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl (TBS) for 16 h at 4°C in 96-well plates (Maxisorp; Nalge Nunc International, Naperville, IL) and blocked with 5% (wt/vol) nonfat milk in TBS including 2 mM CaCl₂. Washing after this and all subsequent steps was performed with TBS including 2 mM CaCl₂ and 0.05% Tween 20. Serial dilutions (1:2) of fibrillin fragments in TBS including 2 mM CaCl₂ and 2% (wt/vol) nonfat milk (binding buffer) were incubated for 2 h at 22°C with the immobilized proteins. To detect bound ligands, the wells were incubated for 90 min with polyclonal antisera (diluted 1:1000) for rFBN1-N, rFBN1-C, rFBN2-N, rFBN2-C, and rFBN3-C (see *Antibodies*), followed by a 90-min incubation with horseradish peroxidase-conjugated goat anti-rabbit antibody (diluted 1:800) followed by the color reaction. In experiments to determine calcium dependency, the blocking, binding, and washing buffers outlined above were modified to include either 5 mM CaCl₂ or 10 mM EDTA. For inhibition experiments, the above described interaction assay was modified as follows. After blocking nonspecific binding sites, serial dilutions (1:2; starting at 10 μ g/ml) of gelatin from porcine skin (Sigma; product G1890) in binding buffer were incubated for 2 h with the immobilized proteins, followed by an incubation of recombinant fibrillin fragments at constant concentrations (75 μ g/ml the fibrillin C-termini and 100 μ g/ml the fibrillin N-termini).

Size-Exclusion Chromatography and Analysis of the Fibrillin C-terminal Multimers

The multimerization of the fibrillin-2 (rFBN2-C) and fibrillin-3 (rFBN3-C) recombinant C-terminal halves were analyzed by Superose 6 gel filtration chromatography (100-ml column on a high-precision ÄktaPurifier 10; GE Healthcare) as described in detail for the fibrillin-1 C-terminal construct rFBN1-C (Hubmacher *et al.*, 2008). Recombinant protein, 2.7 mg, previously

purified by metal-chelating chromatography were loaded onto the column equilibrated with TBS, including 2 mM CaCl₂ at a flow rate of 0.5 ml/min. Starting at the void volume (37 ml), the eluted proteins were collected in 1-ml fractions. Aliquots of the fractions were separated by SDS gel electrophoresis, in the presence or absence of 10 mM dithiothreitol (DTT), on 4–20% acrylamide gradient gels that were then analyzed by silver staining. The molar concentrations of rFBN1-C, rFBN2-C, and rFBN3-C were calculated as described in detail previously with an estimated average of 10 molecules per multimer (Hubmacher *et al.*, 2008 in Supplemental Information).

RESULTS

Fibronectin Expression Is Necessary for Fibrillin Assembly

To analyze the role of fibronectin on the assembly of fibrillin, we have used human dermal skin fibroblasts known to produce a fibrillin-1 network (Hollister *et al.*, 1990). The fibroblasts were treated with specific siRNA oligonucleotides for human fibrillin-1 or fibronectin and the extracellular assembly and expression of these matrix proteins were analyzed by indirect immunofluorescence, real-time PCR, and Western blotting (Figure 1). The extracellular networks produced by the respective siRNA-treated fibroblasts were significantly reduced as demonstrated by indirect immunofluorescence (Figure 1A). Fibronectin was normally assembled by fibroblasts treated with siRNA specific for fibrillin-1, demonstrating that fibronectin assembly is not dependent on the expression and assembly of fibrillin-1. However, when fibronectin expression, and consequently extracellular fibronectin assembly, was abolished by siRNA treatment, then the assembly of fibrillin-1 was significantly suppressed. As controls, the expression levels of mRNA (Figure 1B) and the protein content of the conditioned medium (Figure 1C) were significantly reduced by each siRNA as demonstrated by real-time PCR and Western blotting, respectively. In summary, these data show that endogenous fibronectin must be

Figure 1. Expression and network formation of the fibril-forming proteins fibronectin and fibrillin-1 after siRNA treatment. Human dermal fibroblasts were treated with siRNA oligonucleotides for fibronectin (FN) or fibrillin-1 (FBN1) as described in *Materials and Methods* and grown for a predetermined optimum time for each application (4 d for RNA extraction; 2 d for FN staining; 4 d for FBN1 staining). No effect on fibronectin or fibrillin-1 assembly was observed with control siRNAs (Allstars). (A) Fibronectin and fibrillin-1 network formation was monitored by indirect immunofluorescence as indicated. All images had a similar cell density as controlled by nuclear DAPI staining. Bar, 100 μ m for all images. (B) The total RNA was extracted, reverse transcribed and analyzed by real-time PCR. The mRNA expression was normalized to the expression of GAPDH. Signals from the AllStars siRNA negative control were set to 100%. (C) Conditioned medium (0.33 ml) was analyzed by Western blotting using the specific antibodies indicated. All bands migrated at positions characteristic for each protein above the highest marker protein (250 kDa) used.

Figure 2. Network formation of fibrillin-1 in the presence of bacterially derived fibronectin-binding peptides. (A) Shown is an indirect immunofluorescence after seeding human dermal fibroblasts with either no treatment (–), or peptide FUD, which inhibits fibronectin network formation, or peptide Del29, which is an inactive mutant of FUD. The peptides were added at a concentration of 500 nM. Note that FUD completely blocks fibronectin assembly and also fibrillin-1 assembly, but the Del29 peptide had no influence on both. DAPI staining confirmed equal numbers of cells in all images. Bar, 50 μ m for all images. (B) As a control, fibroblasts were treated in the presence of the indicated peptides identically as in A, except that the medium was replaced by serum-free (peptide-containing) medium for the 24-h condition period. Shown is a Western blot analysis of 1 ml conditioned culture medium stained with specific antibodies against fibronectin and fibrillin-1 as indicated.

expressed by the fibroblasts as a prerequisite for the organization of fibrillin-1 into a fibrillar matrix.

Fibronectin Assembly Is a Prerequisite for Fibrillin Assembly

To test the hypothesis that fibronectin assembly (rather than only expression, as shown in Figure 1) is required for the assembly of fibrillin-1, established function-blocking peptides for fibronectin were used (Figure 2; Tomasini-Johansson *et al.*, 2001). Blocking formation of the fibronectin network by the peptide FUD resulted in complete inhibition of fibrillin-1 network formation, whereas the control peptide Del29 had no effect on either fibronectin or fibrillin-1 net-

work formation (Figure 2A). Control experiments demonstrated that the expression and secretion of fibronectin and fibrillin-1 were unaffected by the peptides used (Figure 2B). In summary, these data demonstrate that the presence of assembled fibronectin is a critical prerequisite for the assembly of fibrillin-1.

Assembly of Exogenous Fibrillin-1 Requires the Presence of Fibronectin

It is well established that exogenously added fibronectin is able to assemble into a structured network. To test whether exogenously added fibrillin-1 is able to assemble into a network in fibroblast cultures, conditioned culture media containing fibrillin-1, either in the presence or absence of fibronectin, have been produced (Figure 3A). These conditioned media were added in increasing concentrations (up to 30-fold concentrated) to cells treated either with siRNA specific for fibrillin-1 or for fibronectin (Figure 3B). In the FBN1 siRNA-treated cells, a dose-dependent formation of a fibrillin-1 network was observed, indicating that exogenously added fibrillin-1 is able to form a network in the absence of endogenously produced fibrillin-1 (Figure 3B, column 1). Samples of additional experiments are shown in Supplemental Figure S1. As expected, fibronectin in the conditioned medium assembled into fibrous structures when added to fibronectin siRNA-treated cells (Figure 3B, column 2). In this situation, exogenously added fibrillin-1 also formed a network (Figure 3B, column 3). However, when fibronectin was depleted from the conditioned medium (Figure 3B, column 4; see *Materials and Methods* for details), then exogenously offered fibrillin-1 did not assemble into an extended network (Figure 3B, column 5). In this situation, only very few fibronectin and fibrillin-1 fibrils were observed. These data show that network formation of exogenously added fibronectin promotes network formation of exogenous fibrillin-1.

Fibrillin-1 and Fibronectin Colocalize in the Early Extracellular Matrix

The experiments described above indicate that fibrillin-1 must be associated, at some point in the assembly process, either directly or indirectly with the fibronectin network. To test this hypothesis, we performed indirect immunofluorescence analyses of fibronectin and fibrillin-1 network assembly (Figure 4A). After analyzing endogenously produced fibronectin and fibrillin-1, both molecules showed significant colocalization at the light microscopic level, indicating their close physical proximity (Figure 4A, row 1). When fibrillin-1 and fibronectin were added exogenously in concentrated conditioned cell culture medium on a double FN/FBN1 siRNA suppressed background, then both proteins formed extensive networks that were clearly colocalized at this level of resolution (Figure 4A, row 2). Controls for the double FN/FBN1 siRNA knockdown showed, as expected, that no background network formed from endogenously produced fibronectin or fibrillin-1 (Figure 4A, row 3). The analysis of individual fibrillin-1 and fibronectin fibrils produced under experimental conditions used in Figure 3, (column 4 and 5), also showed significant colocalization at the light microscopic level (Supplemental Figure S2). The antibodies used to study colocalization demonstrated negligible levels of cross-reactivity for fibrillin-1 and fibronectin when characterized by ELISA (Figure 4B). To analyze further whether fibronectin and fibrillin-1 were colocalized, we performed double immunogold labeling and analysis at the ultrastructural level of extracellular fibrils produced by dermal fibroblasts (Figure 4C). Frequently, but not always,

Figure 3. Network formation by exogenous fibronectin promotes assembly of exogenous fibrillin-1. Conditioned fibroblast culture medium, with or without fibronectin, was produced and concentrated as described in *Materials and Methods*. (A) Western blot analysis of the conditioned medium with antibodies against fibronectin or fibrillin-1 shows the efficacy of the fibronectin depletion, whereas the level of fibrillin-1 was not reduced. (B) Fibroblasts were treated with siRNA to reduce the level of fibrillin-1 (FBN1) or fibronectin (FN) expression. The conditioned media with (+) or without (–) fibronectin as analyzed in A were added to the cultures for 48 h, and the formation of fibrillin-1 and fibronectin networks were visualized by indirect immunofluorescence using antibodies against these proteins. Similar results were obtained when the cell cultures were incubated for 20 h with the conditioned media. The presence of similar numbers of cells in all images was verified with DAPI staining of cell nuclei. Additional sets of images for column 1 are shown in Supplemental Figure S1. Bar, 100 μ m for all images.

fibrils were labeled with both 12-nm gold particles representing fibronectin and 18-nm gold particles representing fibrillin-1. In some cases, we observed fibrils that were either labeled only for fibronectin or only for fibrillin-1 (data not shown). These data are consistent with the colocalization studies at the light microscopic level, corroborating that fibronectin and fibrillin-1 are colocalized in the extracellular matrix produced by dermal fibroblasts, at least in this relatively early stage of matrix formation.

Fibrillins and Fibronectin Interact Directly

Direct protein interactions between fibronectin and fibrillins were tested in solid-phase binding assays using purified and recombinant fibronectin and fibrillin fragments. A schematic

overview of the recombinant fibrillin and fibronectin fragments used is shown in Figure 5. The soluble C-terminal half of fibrillin-1, -2, and -3 interacted strongly with immobilized human plasma fibronectin. This interaction was not dependent on the presence of calcium (Figure 6). Similar results were obtained with cellular fibronectin purified from human dermal fibroblasts (data not shown). These data demonstrate that a binding epitope for fibronectin is located in the C-terminal half of fibrillin-1, -2, and -3 and that this binding epitope is conserved between all fibrillin isoforms. The N-terminal half of fibrillin-1 interacted moderately with fibronectin in the presence of calcium, indicating one or more additional fibronectin interaction epitopes in the N-terminal region of fibrillin-1. Virtually no binding of the N-terminal

Figure 4. Fibrillin-1 and fibronectin colocalize in the matrix produced by dermal fibroblasts. (A) Shown is indirect immunofluorescence labeling of networks which are assembled either from endogenously produced (row 1) or from exogenously added (row 2) fibrillin-1 and fibronectin. The cells in row 1 were not treated with siRNA, allowing endogenously produced fibronectin and fibrillin-1 to assemble. Cells in rows 2 and 3 were treated with siRNAs for both FBN1 and FN to reduce simultaneously the endogenous expression of fibrillin-1 and fibronectin. Conditioned medium containing fibrillin-1 and fibronectin (15-fold concentrated), as characterized in Figure 3A, was added to cells in row 2 and nonconditioned control medium was added to cells in row 3 as described in *Materials and Methods*. DAPI staining of cell nuclei verified similar cell densities in each field. Bar, 100 μ m for all images. (B) Shown is an ELISA analysis to demonstrate that cross-reactivity of the antibodies for fibrillin-1 and fibronectin is negligible. The polyclonal antiserum α -rFBN1-C (red and blue symbols) and the mAb FN-15 (green, yellow, and white symbols) were tested with rFBN1-N (downward-pointing triangles) and rFBN1-C (circles and upward-pointing triangles) or full-length fibronectin (FN; diamonds and squares) as indicated. (C) Shown is a double-immunogold localization of extracellular fibrils produced by human dermal fibroblasts after 3 d in culture. Eighteen-nanometer gold particles represent fibrillin-1, and 12-nm gold particles represent fibronectin. Bar, 100 nm for all images.

half of fibrillin-2 to fibronectin was observed. Attempts to recombinantly express the N-terminal half of fibrillin-3 for these studies failed because of significant proteolytic degradation (data not shown). Analysis of the reverse ligand orientation using fibronectin as a soluble ligand and the fibrillin fragments as immobilized ligands did not result in significant binding (data not shown), indicating that the soluble fibronectin dimer must undergo conformational changes upon absorption to the plastic surface in order to enable the interaction with fibrillins. Conformational changes in fibronectin are well-known requirements for other molecular mechanisms such as the activation of fibronectin assembly (Mao and Schwarzbauer, 2005). Because the C-terminal half of fibrillins contain interaction epitopes for heparin/heparan sulfate (Tiedemann *et al.*, 2001), the potential to inhibit the fibrillin-fibronectin interaction by heparin was tested. Heparin did not inhibit this interaction in concentrations up to 200 μ g/ml ($\sim$ 13 μ M), indicating different binding epitopes (data not shown). Recently, we demonstrated that C-terminal fibrillin-1 fragments have the

propensity to multimerize into structures with similarity to the beads found in the bead-on-a-string-structured, extracted microfibrils (Hubmacher *et al.*, 2008). It was further shown that this multimerization significantly increases the avidity for the self-interaction of the fibrillin-1 C-terminus with the N-terminus. Gel filtration chromatography of C-terminal fibrillin-2 and -3 fragments demonstrated that they have similar propensities to multimerize into reducible high-molecular-weight assemblies similar to the fibrillin-1 C-terminus (Figure 7A). The multimers and monomers of each fibrillin C-terminal half were tested as soluble ligands in solid-phase binding assays with immobilized full-length plasma fibronectin (Figure 7B). The results demonstrate that only the C-terminal multimers interact strongly with fibronectin, whereas the monomers do not interact.

The Fibronectin Collagen/Gelatin-Binding Region Contains Binding Sites for Fibrillins

To map the fibrillin-binding epitopes on fibronectin, the overlapping recombinant proteins FNsuper70K and

Figure 5. Schematic overview of recombinant fibrillin and fibronectin fragments used in this study. The N-terminal half of fibrillin-3 was not available as a recombinant purified protein. The proteins have been analyzed under nonreducing (–) and reducing (+) conditions using DTT (insets). Molecular marker proteins are indicated in kDa.

FNIII1-C and the proteolytic gelatin-binding FN40K fragment (Figure 5) were used in solid-phase binding assays

Figure 6. Interactions of fibrillins with fibronectin. Shown is a representative solid-phase binding assay with coated human plasma fibronectin and the soluble halves of fibrillin-1, -2, and -3 in concentrations as indicated. Solid symbols represent binding in the presence of 5 mM CaCl_2 , and open symbols represent binding of calcium-free fibrillin fragments in the presence of 10 mM EDTA. Note that the C-terminal half of all fibrillins bound dose-dependently and calcium-independently to fibronectin. The N-terminal half of fibrillin-1 bound moderately to fibronectin in the presence of calcium. A control interaction of rFBN1-C with rFBN1-N, known to be calcium-dependent (Lin et al., 2002), showed full calcium dependency under identical assay conditions (not shown). Data represent means of duplicates; error bars, SDs.

(Figure 8). The C-terminal fragments of all fibrillins bound strongly to FNsuper70K and FN40K, but not to FNIII1-C, demonstrating that the binding site is located in the collagen/gelatin-binding region of fibronectin, i.e., in domains FN₆₋₉ (Figure 8A). The N-terminal half of fibrillin-1 interacted only moderately with full-length fibronectin (see Figure 6). However, in interaction assays with recombinant and proteolytic fibronectin constructs, stronger interactions of rFBN1-N were observed primarily with fragments FNsuper70K and FN40K, and to some extent with FNIII1-C, indicating at least one binding site for the N-terminal half of fibrillin-1 in the fibronectin collagen/gelatin-binding region and potentially another binding site in the C-terminal portion of fibronectin (Figure 8B). Control experiments showed that all fibronectin fragments were efficiently coated to the microtiter plates (Supplemental Figure S3). With these experiments, it becomes clear that although there are multiple binding interactions between fibrillins and fibronectin, the major fibronectin–fibrillin interaction site(s) is located in the collagen/gelatin-binding region of fibronectin. To investigate further the importance of the collagen/gelatin-binding site of fibronectin, gelatin was used as an inhibitor of the various fibrillin–fibronectin interactions (Figure 9). Both, the interaction of the C-terminal rFBN1-C, -2-C, and -3-C with full-length fibronectin, as well as the interaction of rFBN1-N with the FN40K and the FNsuper70K fibronectin fragments, were efficiently inhibited by gelatin.

Binding of the fibrillin-1 N- and C-terminal halves to the fibronectin network were examined using the HSF model

Figure 7. Multimerization of the fibrillin-1, -2, and -3 C-terminal half into large-molecular-weight assemblies and interaction of monomers and multimers with fibronectin. (A) The recombinant C-terminal half of fibrillin-1 (rFBN1-C) as an established control (Hubmacher *et al.*, 2008), fibrillin-2 (rFBN2-C), and fibrillin-3 (rFBN3-C) were subjected to gel filtration chromatography (top panel). Plotted are the elution volumes versus their absorbance at 280 nm (mAU units). Aliquots (20 μ l) from the elution volumes indicated were analyzed by silver staining after SDS gel electrophoresis under reducing (+) and nonreducing (-) conditions (DTT; bottom panel). Molecular marker proteins are indicated in kDa. The positions of multimeric (Mu), intermediate (In), and monomeric (Mo) fragments are indicated in each panel. Note that both, fibrillin-2 and -3 C-terminal fragments form reducible high-molecular-weight assemblies similar to fibrillin-1, albeit with some variations in the total amounts. The position of the reducible fibrillin monomers in the gels are indicated by arrowheads. (B) Solid-phase interaction assay with soluble rFBN1-C, rFBN2-C, and rFBN3-C (as indicated) in the monomeric (○) and multimeric (□) states with immobilized full-length plasma fibronectin. Data represent means of duplicates; error bars, SDs. Note that only the fibrillin multimers bind to fibronectin but not the monomers.

(Figure 10). Both recombinant fragments were covalently labeled with the fluorescent dye Cy3 and added to an existing fibronectin network produced by dermal fibroblasts. Significant colocalization of Cy3-labeled rFBN1-N and rFBN1-C with fibronectin indicates that the fibrillin fragments interacted with the fibronectin network produced by fibroblasts. Significant colocalization of Cy3-labeled rFBN1-N and rFBN1-C with fibronectin was also observed after reducing the fibrillin-1 network by siRNA gene silencing (Supplemental Figure S4).

DISCUSSION

The functional structures created in the extracellular matrix by fibrillins are high-molecular-weight microfibrils. Although many publications in recent years have addressed the spatial organization of fibrillins in microfibrils (Sakai *et al.*, 1991; Downing *et al.*, 1996; Liu *et al.*, 1996; Reinhardt *et al.*, 1996; Qian and Glanville, 1997; Baldock *et al.*, 2001; Lee *et al.*, 2004; Baldock *et al.*, 2006; Kuo *et al.*, 2007), relatively little information is available about mechanisms and components involved in microfibril formation. Revealing these mechanisms is ultimately critical for the understanding of the pathobiology of microfibrilopathies associated with mutations in fibrillins. In this study, we have 1) found that fibronectin is an essential component in the assembly of fibrillin-1 into microfibrils and 2) analyzed in detail the molecular interactions of fibrillin-1, -2, and -3 with fibronectin.

The assembly of fibrillins likely requires many steps leading from individual fibrillin molecules to the final macro-molecular assemblies in tissues—a process that includes self-assembly (Trask *et al.*, 1999; Lin *et al.*, 2002; Marson *et al.*, 2005; Hubmacher *et al.*, 2008), disulfide and transglutaminase cross-link formation (Qian and Glanville, 1997; Reinhardt *et al.*, 2000), fibril elongation and others mechanisms (for review see Tiedemann *et al.*, 2004). Fibrillins require the presence of cells to multimerize, and mesenchymal cells such as fibroblasts or smooth muscle cells, which can be readily manipulated, are an ideal system to analyze fibrillin assembly on the molecular level.

In a candidate approach using primary human dermal fibroblasts from penile foreskin, we reduced the expression levels of extracellular proteins and membrane receptors by siRNA gene silencing approaches and screened for effects on fibrillin assembly. Among the targets tested, only fibronectin down-regulation had a negative impact on fibrillin assembly. Down-regulation of the fibronectin mRNA expression and peptide inhibition of extracellular fibronectin assembly similarly resulted in a significant disruption of fibrillin-1 assembly, demonstrating that the extracellular assembly of fibronectin is an absolute requirement for fibrillin-1 assembly. Fibronectin has previously been demonstrated to be essential for the organization of some matrix components, including collagen types I and III and thrombospondin-1 (McDonald *et al.*, 1982; Sottile and Hocking, 2002; Velling *et al.*, 2002; Li *et al.*, 2003), fibulin-1 (Roman and McDonald,

Figure 8. Mapping of fibrillin binding sites on fibronectin. Shown are representative solid phase interaction assays. The recombinant fibrillin-1, -2, and -3 C-termini (A) or fibrillin-1 and -2 N-termini (B) were analyzed as soluble ligands with immobilized recombinant fibronectin fragments FNsuper70K (○) or FNIII1-C (□), or with proteolytic fragment FN40K (▽). Efficient immobilization of fibronectin fragments was verified by ELISA assays (see Supplemental Figure S3). Data points represent means of duplicates; error bars, SDs.

1993; Godyna *et al.*, 1995), fibrinogen (Pereira *et al.*, 2002), and LTBP-1 (Dallas *et al.*, 2005). With fibronectin having a key role in the assembly of another important extracellular

Figure 9. Inhibition of fibrillin-fibronectin interactions by gelatin. Gelatin was first bound in increasing concentrations (A) to immobilized full-length plasma fibronectin and (B) to the fibronectin fragments FNsuper70K and FN40K. In a second incubation step at constant ligand concentrations, fibrillin-1, -2, and -3 C-terminal halves (as indicated) were bound to the immobilized full-length fibronectin (A), or the fibrillin-1 N-terminal half was bound to the immobilized fibronectin fragments (B). The signal without gelatin was set to 100%. Data points represent means of duplicates; error bars, SDs.

Figure 10. Binding of fluorescence-labeled recombinant halves of fibrillin-1 to matrix produced by dermal fibroblasts. Fragments rFBN1-N and rFBN1-C were covalently labeled with the fluorescent dye Cy3 and then added at 10 µg/ml (60 or 70 nM, respectively) for 24 h to the culture medium of cells precultured for 24 h. The cells were then washed and labeled with an mAb against fibronectin followed by FITC-conjugated secondary antibody. Shown is the fluorescence microscopic analysis of the localization of the Cy3-labeled fibrillin fragments (red) and the fibronectin staining (green). Note that the Cy3-labeled fibrillin fragments colocalize with fibronectin labeled fibrils. DAPI staining shows the density and distribution of the cell nuclei in the image fields. Bar, 100 µm for all images.

fibril system, the fibrillin-containing microfibrils, it becomes increasingly evident that fibronectin is a master orchestrator for the organization of various matrix components. In the case of collagen type I, thrombospondin-1, and LTBP-1, it has been shown that fibronectin is required for both, as an initiator for assembly and as a key element in subsequent matrix stability (Sottile and Hocking, 2002; Dallas *et al.*, 2005). For fibrillin assembly, it is clear from our study that fibronectin is required to initiate fibrillin assembly. Whether or not fibronectin regulates downstream stability of assembled premature or mature microfibrils remains to be established.

We have now demonstrated that soluble fibrillin-1 from fibroblasts in conditioned medium assembles into typical networks when reapplied to fibroblast cultures. These results correlate with data from others showing that fibrillin-1 produced from epithelial cells can be assembled when added to fibroblast cultures (Dzamba *et al.*, 2001). Here, we show that assembly of exogenously added fibrillin-1 only occurs when either endogenously produced or exogenously added fibronectin was assembled into a network. We conclude that the crucial interactions for assembly between fibrillin-1 and fibronectin do not occur in the late secretory pathway or in cell invaginations that are not accessible to exogenously added fibrillin. We suggest that the critical interactions occur either on, or close to, the cell surface.

N-C self-interaction of fibrillins is considered one of the first events in fibrillin assembly (Lin *et al.*, 2002; Marson *et al.*, 2005). Recently, we have shown that multimerization of the fibrillin-1 C-terminus is a prerequisite for high-affinity binding to the fibrillin-1 N-terminus, whereby the C-terminal self-interaction epitope is located within the last three calcium-binding epidermal growth factor-like domains (Hubmacher *et al.*, 2008). Here, we demonstrate that recombinant C-terminal expression constructs of fibrillin-2 and -3 also have the intrinsic property of forming high-molecular-weight, disulfide-bonded multimers similar to those found previously for fibrillin-1. Interestingly, only the C-terminal

multimers of all three fibrillins, but not the monomers, interacted strongly with fibronectin. These findings, together with our previous report on the fibrillin-1 N-C self-interaction properties, suggest that low-affinity binding sites to either itself or to fibronectin, which are situated in the C-terminal region of all fibrillins, become high-affinity binding sites upon multimerization. This multimerization occurs early in the fibrillin assembly process and requires cells, although it is not clear whether it occurs during late secretory stages or directly on the cell surface (Hubmacher *et al.*, 2008 in Supplemental Material). Typically, the secretion and assembly of fibronectin by fibroblasts occurs much faster compared with the assembly process of fibrillins (Wartiovaara *et al.*, 1974; Hollister *et al.*, 1990), suggesting that a fully or partially assembled fibronectin network is already present outside the cells once fibrillin multimers appear, or are generated, on the cell surface. These fibrillin multimers might then be directly deposited onto the existing fibronectin fibrils through direct molecular interactions. This notion is supported by our colocalization studies performed at the light microscopic and the ultrastructural level, suggesting colocalization of fibrillin-1 with fibronectin in relatively early stages of fibrillin assembly. Speculatively, this interaction may align the premature fibrillin assemblies, which could then permit interaction with other fibrillin assemblies deposited onto the fibronectin scaffold, thus leading to the cognate directionality of fibrillins in microfibrils. Binding of fibrillin to fibronectin may also induce conformational changes within individual fibrillin molecules in order to proceed with the subsequent assembly process. In this regard, the intrinsic protein disulfide isomerase activity present in the C-terminus of fibronectin may help to form proper intermolecular disulfide bonds in early fibrillin assemblies (Langenbach and Sottile, 1999; Reinhardt *et al.*, 2000; Hubmacher *et al.*, 2008). Recently, it has been reported that LTBP-1 and -4 are initially deposited, independent of the presence of fibrillin-1, onto fibronectin fibrils and later switch to different fibril systems including fibrillin-containing microfibrils (Dallas *et al.*, 2005; Kantola *et al.*, 2008). In this light, binding of fibrillin to fibronectin may not only promote fibrillin assembly but may be a mechanism to load microfibrils with LTBP-1 and -4, which both bind to fibrillins (Isogai *et al.*, 2003).

In addition, to the strong interactions of the fibrillin C-terminal multimers with fibronectin, we observed additional interactions mediated through the fibrillin-1 N-terminus, which were not observed for the fibrillin-2 N-terminus; the fibrillin-3 N-terminal half was not available for our studies. This interaction was relatively weak with full-length fibronectin as a binding ligand, but was much more pronounced using recombinant or proteolytic fibronectin fragments. Nevertheless, similar labeling intensities of the fibronectin network were observed when fluorescently labeled recombinant N- and C-terminal fibrillin-1 fragments were added to fibroblast culture medium. These results indicate that a weak fibronectin binding site for the fibrillin-1 N-terminus becomes more readily available once fibronectin is assembled into a network. Potentially, the binding site becomes fully available only after mechanical stretching of the fibronectin dimer on the cell surface via its well-established interaction with integrin $\alpha 5 \beta 1$ (Zhong *et al.*, 1998; Baneyx *et al.*, 2002). It is also possible that the site is more exposed in recombinant or proteolytic fragments as compared with the full-length (nonpolymerized) fibronectin, potentially explaining the observed differences in the *in vitro* binding of the fibrillin-1 N-terminal fragment to full-length versus recombinant or proteolytic fibronectin frag-

ments. The fact that the corresponding fibrillin-2 N-terminal fragment did not bind, or bound only very little, to fibronectin might reflect differences in how fibrillin-1 and -2 require fibronectin for assembly. It has been shown that fibrillin-1 and -2 frequently coassemble into the same microfibrils (Charbonneau *et al.*, 2003). Given that the fibrillin-2 N-terminus also does not efficiently mediate self-interaction with its C-terminus (Lin *et al.*, 2002), the current results can be speculatively interpreted as indicating that fibrillin-1 may be a carrier for fibrillin-2 coassembly, when they are expressed in the same context.

With recombinant and proteolytic fibronectin fragments, we have localized the major binding site for the C-terminal binding epitope of all three fibrillins and an N-terminal binding epitope for fibrillin-1 to the region in fibronectin spanning domains FNI₆₋₉. One or more additional minor binding sites for the fibrillin-1 N-terminus may be localized further C-terminal. This major binding region also mediates collagen/gelatin binding (Engvall *et al.*, 1978; Balian *et al.*, 1979; Shimizu *et al.*, 1997). It has been shown that all six modules in the gelatin-binding region contribute either directly or indirectly, through domain stabilization effects, to gelatin binding, whereby FNI₆ is likely involved in indirect contributions rather than directly (Ingham *et al.*, 1989; Pickford *et al.*, 2001; Katagiri *et al.*, 2003; Millard *et al.*, 2005; Pagett *et al.*, 2005). In our study, we observed that the interactions of both the N- and the C-terminal fibrillin fragments with the gelatin-binding region of fibronectin or fibronectin fragments could be efficiently inhibited by gelatin. This positions the major fibrillin binding site between domains FNII₁ and FNI₆. Further studies are required to determine whether the fibrillin-binding region spans this entire region similar to gelatin binding or whether more discrete epitopes mediate this interaction. Along these lines it will be also important to characterize the relationship of the fibrillin binding site(s) in fibronectin to that of other matrix molecules including collagens that have also been shown to be organized by fibronectin (Sottile and Hocking, 2002).

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