Effects of Fibrillin-1 Degradation on Microfibril Ultrastructure*

Current models of the elastic properties and structural organization of fibrillin-containing microfibrils are based primarily on microscopic analyses of microfibrils liberated from connective tissues after digestion with crude collagenase. Results presented here demonstrate that this digestion resulted in the cleavage of fibrillin-1 and loss of specific immunoreactive epitopes. The proline-rich region and regions near the second 8-cysteine domain in fibrillin-1 were easily cleaved by crude collagenase. Other sites that may also be cleaved during microfibril digestion and extraction were identified. In contrast to collagenease-digested microfibrils, guanidine-extracted microfibrils contained all fibrillin-1 epitopes recognized by available antibodies. The ultrastructure of guanidine-extracted microfibrils differed markedly from that of collagenase-digested microfibrils. Fibrillin-1 filaments splayed out, extending beyond the width of the periodic globular beads. Both guanidine-extracted and collagenase-digested microfibrils were subjected to extensive digestion by crude collagenase. Collagenase digestion of guanidine-extracted microfibrils removed the outer filaments, revealing a core structure. In contrast to microfibrils extracted from tissues, cell culture microfibrils could be digested into short units containing just a few beads. These data suggest that additional cross-links stabilize the long beaded microfibrils in tissues. Based on the microfibril morphologies observed after these experiments, on the crude collagenase cleavage sites identified in fibrillin-1, and on known antibody binding sites in fibrillin-1, a model is proposed in which fibrillin-1 molecules are staggered in microfibrils. This model further suggests that the N-terminal half of fibrillin-1 is asymetrically exposed in the outer filaments, whereas the C-terminal half of fibrillin-1 is present in the interior of the microfibril.

Microfibrils have been extracted from a variety of tissues and visualized by electron microscopy as distinctive beaded string structures (1, 2). The molecular composition of these structures is assumed to be complex. However, based upon immunolocalization of fibrillin to these structures (2, 3) and the shape of fibrillin monomers (4, 5), fibrillins are thought to be the major backbone components of these extracted microfibrils. Fibrillin-containing microfibrils are ubiquitous in the connective tissue space (6), providing architectural support as well as information essential for appropriate signaling during morphogenesis (7–9). In human disorders associated with fibrillins, the structural integrity of fibrillin microfibrils is required for the proper function of certain connective tissues (10). Therefore, determination of the organization of fibrillin molecules within the microfibril is important basic information.

The organization of fibrillin molecules within microfibrils has been controversial. Both parallel (head-to-tail) (3, 4) and antiparallel (11) arrangements of fibrillin molecules within microfibrils have been proposed. Staggered (12–14) and unstaggered (3) arrangements have also been debated. Based upon the length (~150 nm) of the fibrillin monomer (4), the periodicity of fibrillin immunolocalization in tissue microfibrils (~50 nm) (3), and the variable periodicities of unstretched and stretched extracted microfibrils (2), long fibrillin molecules were required to be compressed within the microfibril, if fibrillin molecules are unstaggered (3). However, because fibrillin molecules are composed of long tandemly repeated calcium-binding epidermal growth factor-like (cbEGF) domain modules, whose structures are predicted to be rather rigidly linear, compression of fibrillin molecules within microfibrils was predicted to be unlikely, and so staggered models were preferred (12, 15).

The prevailing model of fibrillin fibril organization is called the intramolecular pleating model, and it is based on best fitting fibrillin molecules to microfibril structure as determined by high resolution electron microscopic approaches (16, 17). Details of the structure of microfibrils, revealed by automated electron tomography, were used to support a complex model of microfibril extensibility and elasticity. An initial head-to-tail alignment of fibrillin monomers, followed by several different folding events at the N- and C-terminal ends of the monomers, was proposed to result in microfibrils with fibrillin monomers arranged in parallel and one-third staggered (16). Various other staggered models of fibrillin molecules do not appear to fit the images of microfibrils as well as the intramolecular pleating model does (17). Difficulties with these attempts to fit fibrillin...
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molecules to microfibril ultrastructure have been related primarily to the unknown contributions of other microfibril components. In the studies described here, we investigated the additional possibility that analyses of microfibrils obtained from enzymatic digestion of tissues could also lead to difficulties in interpretation, if fibrillin molecules are degraded during the extraction process.

Several different methods have been utilized for extraction and isolation of fibrillin microfibrils. Microfibrils can be extracted from fetal tissues (18) or from adult eyes (1, 19) in physiological buffers. Large yields of microfibrils can be obtained from tissues that have been digested with collagenase, a method originated by Kielty and co-workers in 1991 (20) and extensively utilized (16, 21–24). In addition, denaturing extraction methods have been successfully employed (2).

In our recent studies, denaturing as well as collagenase digestion methods were utilized in order to obtain microfibrils that retained evidence of attached or bound ligands (7, 25). We found that digestion of tissues with highly purified bacterial collagenase failed to yield microfibrils, indicating that microfibrils are not retained in tissues by simple entrapment among collagen fibers (25). Guanidine extraction of tissues yielded small amounts of microfibrils, compared with digestion of tissues with crude collagenase, which resulted in large yields of microfibrils. We concluded that digestion of tissues with crude collagenase allowed the extraction of microfibrils because certain proteins (e.g. versican) that connect microfibrils to the surrounding matrix are degraded during the extraction procedures (25).

Studies reported here were undertaken to determine whether fibrillin-1 molecules are themselves degraded during extraction procedures and whether microfibril morphology is affected by the extraction procedures. These investigations are important because the currently accepted model of microfibril organization is based primarily upon microfibril morphology with the assumption that fibrillin-1 monomers are intact (16, 20–24, 26–27). Moreover, mutations in FBN1 found in the Marfan syndrome were suggested to disrupt the ultrastructural appearance of microfibrils extracted using these degradative procedures (22, 23). Our results demonstrate that fibrillin-1 is cleaved in microfibrils extracted from tissues using crude collagenase. Therefore, models based upon microfibrils extracted with crude collagenase must be reevaluated. Furthermore, our data demonstrate that the proline-rich region of fibrillin-1 is available in intact microfibrils for both antibody and enzyme recognition, locating this region to exposed surfaces of the microfibril. In addition, ultrastructural images of microfibrils with intact fibrillin-1 suggest a more elaborate model of microfibril architecture with more limited elastic properties.

EXPERIMENTAL PROCEDURES

Reagents—All chemicals, including collagenase (type 1A), were purchased from Sigma. Fibrillin-1 monoclonal antibodies (mAbs) 26, 201, 15, and 69 have been characterized previously (3, 4, 6, 28, 29). mAb 78 was produced (as we have described for other mAbs) from a fusion of splenocytes immunized with a pepsin fragment called PF3 (28) and has been characterized as an antibody specific for fibrillin-1.5 Polyclonal antibody 9543 has been demonstrated to be highly specific for fibrillin-1 (30). Recombinant fibrillin-1 polypeptides were produced using human embryonic kidney 293 cells, purified, and characterized as described previously (3). Peptides were synthesized by a Milligen 9050 peptide synthesizer using standard Fmoc (N-(9-fluorenylemethyl)carbonyl) chemistry and were purified using reverse-phase chromatography (Vydac C-18 column). Purified peptide fractions containing the correct sequence were identified by mass spectrometry and amino acid analysis. Escherichia coli BL21 (DE3) and pET11a were purchased from Stratagene (La Jolla, CA); DH5-α cells were from Invitrogen.

Extraction and Isolation of Microfibrils—Microfibrils from collagenase-digested human fetal membranes were prepared as described previously (25). These preparations are designated as “collagenase I” in Fig. 1C. In these preparations, 30 mg of human fetal membrane tissue suspension was digested with 0.66 mg/ml type 1A collagenase (Sigma). A lower concentration of collagenase was used for “collagenase II” microfibril preparations (shown in Fig. 1C). For the collagenase II digest, protocols utilized by Kielty and co-workers (16, 20–24) were followed. 2 g (wet weight) of fetal membranes were homogenized and washed in 10 ml of 50 mM Tris-HCl, pH 7.4, containing 1 mM NaCl, 0.01 M CaCl2, 2 mM phenylmethylsulfonyl fluoride, and 10 mM N-ethylmaleimide. After centrifugation, the tissue was resuspended in 10 ml of 0.2 mg/ml type 1A collagenase (Sigma) in the same buffer and stirred for 48 h at 4 °C. The EDTA was then added to 10 mM. The sample was centrifuged, and the supernatant was applied to a Sepharose CL-2B column, equilibrated in 50 mM Tris-HCl, pH 7.4, containing 0.4 mM NaCl. Microfibrils, determined by dot blotting with fibrillin-1 antibodies and by rotary shadowing electron microscopy, eluted in the void volume of the column. A similar procedure was used to extract microfibrils from the matrices of fibroblast cell cultures, except that 0.1 mg/ml of type 1A collagenase (Sigma) was used.

For guanidine extraction, a pool of homogenized and repeatedly washed fetal membranes (25) was used. 20 ml of human fetal membrane tissue suspension was centrifuged to remove the buffer (50 mM Tris-HCl, pH 7.5, containing 1 mM NaCl). Then 20 ml of 0.6 M guanidine, 50 mM Tris-HCl, pH 7.5, was added, and the tissue suspension was stirred for 48 h at 4 °C. After centrifugation at 5000 rpm for 10 min, the residue was extracted again for 24 h using 20 ml of the same buffer at 4 °C. Extracts were combined and centrifuged at 15,000 rpm for 30 min using a Sorvall SS 34 rotor. The supernatant was concentrated to 6 ml using Centrispool 30 (Amicon) with centrifugation. The extract was then fractionated using a Sepharose CL-2B molecular sieve column (Amersham Biosciences) (Ve = 90 ml), equilibrated in 4 mM guanidine HCl, 50 mM Tris-HCl, pH 7.5, at a flow rate of 0.1 ml/min. Fractions were collected every 2 ml. The fractions eluting at the void volume (see Fig. 1A, bar) contained fibrillin microfibrils. These fractions were pooled and used for some experiments. For visualization by electron microscopy, CsCl was added to the pooled microfibril fractions to the initial density of 1.32 g/ml. Then after dissociative isopyknic centrifugation using a Beck-
man Ti-80 rotor at 40,000 rpm at 10 °C for 48 h, six fractions were obtained, and microfibril-containing fractions were identified (Fig. 1B).

**Immunooassays and Binding Assays**—ELISA was performed using synthetic peptides as the coated substrate. Peptides were incubated in microtiter wells at concentrations of 25 nm in 15 mM Na₂CO₃, 35 mM NaHCO₃, pH 9.2, overnight at 4 °C. Wells were then washed three times with Tris-buffered saline (TBS) containing 0.025% Tween 20 (Bio-Rad). 5% nonfat dry milk in TBS was incubated for 1 h at room temperature to block nonspecific sites. Primary monoclonal antibody (1 mg/ml) was 3-fold serially diluted, beginning with an initial dilution of 1:90, in 2% nonfat dry milk in TBS, and incubated for 1 h at room temperature. After washing the wells three times with TBS/Tween 20, horseradish peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad), diluted 1:500 in 2% nonfat dry milk in TBS, and incubated for 1 h at room temperature. After washing again, color reaction was achieved using 1 mg/ml 5-aminosalicylic acid in 20 mM phosphate buffer, pH 6.8, containing 0.1% H₂O₂. The color reaction was quantified using a Multiskan photometer.

Dot blotting and Western blotting were performed as described previously (25, 29). ELISA binding assays were performed as described (31). Protein concentrations were determined by the “Micro BCA assay” (Pierce) using bovine serum albumin as a standard.

**Collagenase Digestion of Extracted Microfibrils**—For these experiments, microfibrils were extracted from a pool of washed and homogenized human amnion, using 6 M guanidine in 50 mM Tris-HCl, pH 7.5. However, DEAE-cellulose was added to the extract overnight at 4 °C, before the extracts were applied to a Sephrose CL-2B molecular sieve column. Microfibrils were not further purified using CsCl density gradient centrifugation for these experiments. In addition, cell layers from normal human dermal fibroblast cultures, grown for 5 weeks, were dialyzed and were similarly extracted. Microfibrils were also extracted from amnion using collagenase digestion, according to protocols utilized by Kielty and co-workers (16, 20–24).

100-μl aliquots of guanidine or collagenase-extracted microfibrils were treated with increasing concentrations (50, 100, 200, and 400 μg/ml and 1 mg/ml) of Sigma type 1A collagenase for 48 h at 4 °C in TBS, pH 7.4, containing 10 mM CaCl₂. Samples were then dialyzed into water and concentrated by a SpeedVac (AS160) spinning for rotary shadowing and electron microscopy.

**Electron Microscopy**—All microfibril-containing samples were dialyzed against H₂O. Then either 2 mM CaCl₂ or 10 mM EDTA was added to the samples, followed by rotary shadowing. Rotary shadowing electron microscopy and immunogold negative staining were performed as described (25).

**Enzyme Digestion of Full-length Fibrillin-1 and Recombinant Fibrillin-1 Polypeptides**—For metabolic labeling, normal skin fibroblasts were fed with serum-free medium containing 50 μCi/ml [³⁵S]cysteine (Amersham Biosciences) for 48 h. 1 ml of the labeled medium was incubated with mAb 69 and then with protein G-coupled Sepharose (Amersham Biosciences). The immunoabsorbent was collected by centrifugation and washed five times with 0.2% Triton X-100 containing TBS, followed by two washes with TBS containing 2 mM CaCl₂. The precipitate was then incubated with crude collagenase at 37 °C for 18 h. After adding SDS-PAGE sample buffer containing dithiothreitol, the eluted immunoprecipitate was separated using a 7.5% acrylamide gel and dried. The bands were visualized by exposing the dried gel to x-ray film.

Recombinant fibrillin-1 peptides, rF11, rF23, rF20, rF31, and rF6, and recombinant fibrillin-2 peptide, rF32, were produced and purified as described elsewhere (3, 29, 32). These recombinant peptides were treated with 0.2 mg/ml Sigma collagenase (type 1A) in 50 mM Tris-HCl, pH 7.4, containing 0.01 mM CaCl₂, 2 mM phenylmethylsulfonyl fluoride, and 10 mM N-ethylmaleimide for 48 h at 4 °C. The incubation was terminated by adding 1 volume of 4× SDS sample buffer to 3 volumes of sample and then heating the samples at 95 °C for 5 min.

After the digestion, aliquots were separated by 10% (rF20 and rF6) or 15% (rF23) SDS-PAGE. Samples were run without reducing agent, and gels were stained by Coomassie Blue R-250 or blotted onto Immobilon-P membranes (Millipore Corp) for immunoblot analysis. For N-terminal sequence analysis of proteolytic products, protein bands transferred onto membranes were excised and then analyzed by Edman degradation.

**Circular Dichroism**—Purified recombinant polypeptides were analyzed in the presence and absence of calcium. 10 mM EDTA or no EDTA was added to samples that were then dialyzed into 2 mM Tris-HCl, pH 7.4, containing either no CaCl₂ or 1 mM CaCl₂, respectively. Calcium-free buffers were treated with 5 g/100 ml of Chelex 100 (Bio-Rad). Dialysis buffers were changed three times, and then CD measurements were performed as described (5).

To test the effects of guanidine, samples treated either with 10 mM EDTA or no EDTA were dialyzed into 6 M guanidine HCl, 50 mM Tris-HCl, pH 7.5, containing either 2 mM EDTA or 2 mM CaCl₂, respectively. Samples were incubated in this denaturing buffer for 48 h at 4 °C. Then the samples were dialyzed into 2 mM Tris HCl, pH 7.5, containing either no CaCl₂ or 1 mM CaCl₂, respectively. Calcium-free buffers were treated with Chelex 100. Dialysis buffers were changed three times. Samples were analyzed in a 0.1-mm cell at 25 °C, with CD measurements taken from 260 to 180 nm using a Jasco J-500A instrument. High background noise from 180 to 190 nm was not included in the results.

**Production and Purification of BMP-7 Prodomain**—cDNA encoding human BMP-7 prodomain was amplified by PCR using the BMP-7-pcDNA3.1(+) vector containing full-length BMP-7 cDNA as a template and master clone (5). The 5’-primer introduced an Ndel restriction site, and a BamHI site and six histidine residues in tandem followed by a termination signal were added to the downstream primer (5’-primer, 5’-GGAATTCATATGGATTTACGCTGGCAACAGGAGTG-3’; 3’-primer, 5’-CATGGGATTCTTCCATTGGTATGCTGGTGATGTTG-3’). The product of the PCR amplification was cloned into an Ndel/BamHI-digested pET11a vector so that the final construct contained the entire human BMP-7 prodomain coding sequence starting from Asp³⁰, after the predicted endogenous signal peptide cleavage site, followed by a C-terminal His₆ tag and a stop codon. This vector was transformed into competent cells of E. coli DH5α,
and the insert structure was verified by restriction analysis and DNA sequencing. For overexpression of the BMP-7 prodomain, the recombinant vector was retransformed into competent E. coli BL21 (DE3) cells. The cells were grown in LB medium to an absorbance of 0.6 at 540 nm, induced with isopropyl 1-thio-β-D-galactopyranoside for 90 min, harvested by centrifugation, and suspended in B-PERTM bacterial protein extraction buffer (Pierce). The suspension was treated with DNase I (final concentration, 40 μg/ml; Sigma) and phenylmethylsulfonyl fluoride (final concentration, 2 μM), incubated for 30 min at room temperature, and centrifuged. The supernatant was aspirated, and the pellet was washed twice with 10% B-PERTM. Finally, the pellet was resuspended in denaturing buffer containing 50 mM sodium phosphate buffer, pH 7.2, 8 M urea, and 1 M NaCl. Testing aliquots of all extraction fractions by Western blotting using monoclonal anti His6 tag antibody (R & D Systems, Minneapolis, MN) and mAb2 (5) revealed that most of the His6-tagged BMP-7 prodomain was present in the extraction fractions containing 8 M urea. The crude denaturing fraction was filtered and loaded on a 1-ml Hi-Trap column (Amersham Biosciences) previously charged with CoCl2 and equilibrated with 50 mM sodium phosphate buffer, pH 7.2, 8 M urea, and 1 M NaCl (buffer A). The loaded column was washed with 5 column volumes of buffer A and subjected to a fast protein liquid chromatography (Amersham Biosciences) gradient run starting with buffer A containing 0–100% buffer B (buffer A with 250 mM imidazole). Fractions were analyzed by SDS-PAGE for their purity and molecular mass, and imidazole in these fractions was removed by dialysis against buffer A.

RESULTS

Immunoreactive Differences between Collagenase-digested and Guanidine-extracted Microfibrils—Microfibrils were solubilized from human fetal membranes using two methods as follows: crude collagenase digestion (using Sigma type 1A collagenase) or guanidine extraction. Fig. 1A shows the elution profile of a guanidine-extracted sample applied to a Sepharose CL-2B molecular sieve column, equilibrated in 4 M guanidine HCl. Similar elution profiles were obtained for collagenase digests after samples were applied to Sepharose CL-2B in physiological buffers (24). Fractions 15–23 (in Fig. 1A) contained microfibrils and other fibrous elements, as demonstrated by rotary shadowing (data not shown). To obtain a cleaner preparation of microfibrils, ultracentrifugation in cesium chloride was performed. DNA and proteoglycan went to the bottom of the gradient, whereas microfibrils that fractionated at a density of 1.28–1.36 g/ml (Fig. 1B) appeared to be pure. Yields of microfibrils from guanidine extracts were ~10-fold less than yields from crude collagenase-digested tissues, suggesting that most of the microfibrils in fetal membranes may be covalently linked to other connective tissue elements and may therefore not be soluble in guanidine.

Microfibrils prepared from the high concentration collagenase digests (collagenase I, previously described in Ref. 24) were analyzed by dot blot analysis using fibrillin-1-specific mAbs 15, 26, 69, 78, and 201. Although mAbs 26, 69, and 78 reacted positively with collagenase I-digested microfibrils, mAbs 15 and 201 were negative in these assays (Fig. 1C). mAb 15 also failed to react with microfibrils prepared from tissue digests using the low concentration of collagenase (collagenase II), but mAb 201 reacted positively in dot blots with these microfibrils (Fig. 1C). Results similar to the collagenase II results were obtained when microfibrils were extracted from the matrices of fibroblast cell cultures (data not shown). In contrast, microfibrils prepared from guanidine extracts reacted positively in dot blots with all mAbs (Fig. 1C).

Identification of the Epitope Recognized by mAb 15—To determine one of the collagenase-sensitive sites in fibrillin microfibrils, various recombinant fibrillin-1 polypeptides
(shown schematically in Fig. 2) were used as test substrates in ELISA and immunoblotting to precisely map the epitope recognized by mAb 15. Because mAb 15 reacted with rF11, rF23, and rF30, but did not bind to rF6 or rF20 (data not shown), the location of the epitope recognized by mAb 15 was narrowed down to the three domains contained in rF30. Within rF30, there are three methionine residues (Met356, Met376, and Met393). The first two methionine residues are within the 8-cysteine domain structure, whereas Met393 lies in the region of the 8-cysteine domain adjacent to the proline-rich region. CNBr cleavage of rF30 yielded a product whose estimated size on gels and N-terminal sequence (394VIP) indicated that it contained the full proline-rich region and the following epidermal growth factor-like (EGF-like) domain. mAb 15 reacted with this CNBr cleavage product in immunoblotting experiments (Fig. 3A).

To further narrow down the epitope, a series of synthetic peptides was tested in ELISA. mAb 15 reacted with a synthetic peptide of 49 residues that spans the proline-rich region (Glu400 to Asn448) of fibrillin-1 but did not react with peptide sequences (labeled A and B in Fig. 3B) representing the N-terminal (Glu400 to Pro426) and C-terminal portions (Val427 to Asn448) of the proline-rich region. However, a peptide (Pro417 to Tyr436) (labeled C in Fig. 3B) spanning the junction between peptide A and peptide B reacted positively with mAb 15 (Fig. 3B), demonstrating that this sequence of 20 amino acids contains the mAb 15 epitope.

Immunochromic data suggested that collagenase digestion, resulting in loss of the epitope recognized by mAb 15, specifically cleaves fibrillin-1 in the proline-rich region. To test this hypothesis, the small recombinant polypeptide rF30 was incubated with the enzyme and analyzed by SDS-PAGE, immunoblotting, and N-terminal amino acid sequence analysis. The N-terminal sequence (LYPSREP) of the resulting cleavage product and immunoreactivity of the peptide with anti-histidine, which detects the C-terminal histidine tag (data not shown), indicated that it was composed of the last 14 residues of the proline-rich region and the subsequent EGF-like domain (Fig. 3C). This cleavage product did not react with mAb 15 (data not shown). It is unlikely that cleavage of rF30 occurred at a single site, because the predicted N-terminal portion of rF30 (calculated to be ~11.3 kDa) was not apparent on Coomassie Blue R-250-stained gels (Fig. 3C) or after immunoblotting with mAb 26, which recognizes an epitope in the 8-cys domain (data not shown). However, additional cleavage sites could not be determined in this experiment. These data were consistent with mapping the epitope for mAb 15 to a region in the proline-rich domain (Pro417 to Tyr436) close to the cleavage site.
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**FIGURE 4. SDS-PAGE of fibrillin-1 monomers and recombinant polypeptides after digestion with crude collagenase.** A, fibrillin-1 monomers were immunoprecipitated from metabolically labeled fibroblast cell culture medium and treated with (+) or without (−) crude collagenase. Prominent cleavage products migrated on gels with an estimated molecular mass of 30–40 kDa. In addition, cleavage at the end(s) of fibrillin-1 molecules was suggested by the presence of prominent bands that migrated somewhat faster than full-length fibrillin-1. A faint band was seen that migrated with a molecular mass of around 80 kDa (arrow). Fibrillin-1 recombinant polypeptides, rF23, rF31, rF11, rF20, and rF6, were digested with crude collagenase. Untreated control samples are in lanes marked −. Samples treated with crude collagenase are in lanes marked +. B, Coomassie Blue R-250 (CBB)-stained gels are shown. Numbered arrows indicate bands that were sequenced (see Table 1). In C, immunoblots corresponding to the Coomassie Blue R-250-stained gels in B are shown. Monoclonal antibodies used are designated by number below each immunoblot. Immunoblotting of cleavage products with mAb15 demonstrated that this epitope is largely absent, although the presence of faintly reactive bands suggest that cleavage occurs around this epitope. The large cleavage product (band 9) of rF20 does not immunoblot with mAb201, locating this epitope in either cbEGF6 or 8cys2. Internal cleavage of cbEGF6 appears to destroy reactivity with mAb201. Samples were run without reducing agent.

(435LYPSREP) and the loss of this epitope after collagenase digestion of microfibrils.

**Determination of Sites in Fibrillin-1 Sensitive to Crude Collagenase**—To investigate the extent of sensitivity to crude collagenase, full-length monomers of fibrillin-1, radiolabeled and isolated from the medium of cultured fibroblasts, were subjected to digestion by the commercial enzyme preparation. Results from these experiments (Fig. 4A) suggested that cleavage sites were primarily at the end(s) of the fibrillin-1 molecule, because large stable products migrating slightly faster than uncleaved fibrillin-1 and a prominent smear of smaller products in the region of 30–40 kDa were obtained. In addition, a faint band (~80 kDa) was also seen.

To determine other cleavage sites, rF11 and rF6, the two large recombinate halves of fibrillin-1, as well as two fragments of rF11 (rF20 and rF23), were subjected to crude collagenase digestion, using the low concentration of collagenase. SDS-PAGE and immunoblotting as well as N-terminal sequence analyses revealed several sites that were consistently cleaved by crude collagenase. These results are shown in Fig. 4, B and C, and summarized in Table 1. Sizes of cleavage products predicted from the sequence and immunoblotting data were generally consistent with the estimated sizes of the N-terminal portion of fibrillin-1 up to the cleavage site defined between 8cys2 and cbEGF7 (721GSDINECALD) is 75 kDa. N-Linked glycosylation can be assumed to add ~10 kDa to the molecular mass of this region, because rF30 had an estimated molecular mass of 17 kDa compared with its calculated molecular mass of 6.3 kDa. Therefore, the faint band of around 80 kDa seen in Fig. 4A likely corresponds to peptides beginning with the N terminus or EGF2 and extending through the proline-rich region to the cleavage site at 721GSDINECALD.

**Treatment with Guanidine Does Not Irreversibly Denature Fibrillin-1**—Guanidine-extracted microfibrils were immunoreactive with all available fibrillin-1 monoclonal antibodies, indicating that these epitopes were not irreversibly denatured during the extraction procedure. However, to further test the suitability of using guanidine-extracted microfibrils for models of microfibril structure, additional investigations were performed.

Fibrillin-1 recombinant polypeptides rF23, rF18, and rF36 were selected for testing whether treatment with guanidine results in irreversible denaturation. The N-terminal recombinant polypeptide rF23 was selected because the arrangement and composition of domains in this region are unique. The molecular mass of the N-terminal portion of fibrillin-1 up to the cleavage site defined between 8cys2 and cbEGF7 (721GSDINECALD) is 75 kDa. N-Linked glycosylation can be assumed to add ~10 kDa to the molecular mass of this region, because rF30 had an estimated molecular mass of 17 kDa compared with its calculated molecular mass of 6.3 kDa. Therefore, the faint band of around 80 kDa seen in Fig. 4A likely corresponds to peptides beginning with the N terminus or EGF2 and extending through the proline-rich region to the cleavage site at 721GSDINECALD.
middle recombinant polypeptide, rF18, was selected because it contains the longest stretch of tandemly repeated calcium-binding EGF-like domains present in the molecule. It also contains a flanking 8-cysteine domain. The C-terminal polypeptide, rF36, was selected because it represents the pattern of calcium-binding EGF-like domains flanked on either end by an 8-cysteine domain characteristic of several regions of fibrillin. The domain structures of these recombinant polypeptides are represented in the schematic drawing in Fig. 2.

CD spectra were obtained for all three recombinant polypeptides in the presence and absence of calcium, and some differences in secondary structure were found between calcium-loaded and calcium-depleted polypeptides (Fig. 5). However, when the recombinant polypeptides were treated with guanidine for 48 h and were analyzed again after renaturation in calcium-containing or calcium-free buffers, only small differences in CD spectra were observed between the guanidine-treated/renatured samples and the untreated samples (Fig. 5). The overall shape of the spectra remained the same, and only the magnitude of the peaks changed slightly. Secondary structure calculations for the three polypeptides, which contain all domain structures present in fibrillin, were not significantly different after guanidine treatment (data not shown). Therefore, guanidine treatment did not irreversibly denature fibrillin-1.

Binding Sites Are Maintained in Guanidine-extracted Microfibrils—The prodomain of BMP-7 binds to fibrillin-1 recombinant polypeptides and targets BMP-7 to microfibrils in tissues (31). Therefore, we tested whether the prodomain of BMP-7 binds to guanidine-extracted microfibrils. For comparison, collagenase-digested microfibrils were also tested. Fig. 6 shows a result from an ELISA binding assay with polypeptides coated onto the wells and titrated BMP-7 prodomain ligand. Detection of bound ligand was accomplished using monoclonal antibodies specific for the BMP-7 prodomain (31). BMP-7 interacted with guanidine-extracted microfibrils in a concentration-dependent manner (Fig. 6A), demonstrating that the binding site for this ligand was not denatured by guanidine treatment. In contrast, BMP-7 prodomain failed to bind well to collagenase-digested microfibrils, suggesting that the binding site was cleaved during this extraction procedure (Fig. 6A). Control assays with polypeptides similarly coated onto wells and titrated antibodies to fibrillin-1 demonstrated that collagenase-digested microfibrils were present on the wells and available for binding to fibrillin-1 monoclonal antibodies 26 and 69 (Fig. 6B). Monoclonal antibody 15 did not react with the collagenase-digested microfibrils, confirming dot blot (Fig. 1) and immunolocalization (Fig. 7) data.

Ultrastructural Differences between Collagenase-digested and Guanidine-extracted Microfibrils—Microfibrils obtained from collagenase-digested and guanidine-extracted tissues were visualized after rotary shadowing and electron microscopy. The morphology of guanidine-extracted microfibrils was consistently different from that of the collagenase-digested microfibrils. The interbead strands in collagenase-digested microfibrils typically adopt close lateral alignments in the presence of calcium (Fig. 7A). In contrast, guanidine-extracted microfibrils were broader in width because of the projection of
the interbead strands beyond the width of the beads (Fig. 7, B and C). In addition, preparations of microfibrils from guanidine-extracted tissues often demonstrated linearly extended molecules that appeared to be attached to a beaded microfibril (Fig. 7B, arrows). Overlapping beaded microfibrils, perhaps
representing microfibril bundles, and beaded strings with occasional extra beads were also visualized in preparations from guanidine extracts (data not shown). The measured bead-to-bead periodicities of guanidine-extracted microfibrils were usually around 50 nm (Fig. 7B). Longer measurements of bead-to-bead periodicities of 70 to 80 nm were sometimes found, but these spanned only short stretches (Fig. 7C).

Preparations of microfibrils from guanidine extracts were labeled with mAbs 15, 26, 201, and 69 and were viewed after negative staining. In Fig. 7D, labeling of mAb 15 (gold particles) was clearly associated along the full-length of the guanidine-extracted microfibrils. Other antibodies also labeled these microfibrils (data not shown). In contrast, mAb 15 only occasionally labeled collagenase-digested microfibrils, leaving many bead-to-bead periods unlabeled (Fig. 7E). Compared with published images of negatively stained collagenase-digested microfibrils (3) and with microfibrils in Fig. 7E, clear periodic beads and interbead regions were not easily visualized in preparations of negatively stained guanidine-extracted microfibrils (Fig. 7D).

These results further demonstrate that the guanidine-extracted microfibrils, containing interbead filaments that extend beyond the surface of individual beads (Fig. 7, B and C), have a cylindrical three-dimensional structure different from the bead-interbead structure revealed by collagenase digestion.

CD spectra indicated that, even after guanidine treatment, fibrillin structure is sensitive to the presence of calcium (Fig. 5). Visualization of rotary-shadowed guanidine-extracted microfibrils in the presence (Fig. 8A) and absence (Fig. 8B) of calcium showed changes in microfibril ultrastructure. In the absence of calcium, interbead strands were more outwardly splayed than in the presence of calcium. These results are similar to previously reported ultrastructures of collagenase-digested microfibrils in the presence and absence of calcium (21). However, interbead filaments in guanidine-extracted microfibrils always extended beyond the width of the beads. In contrast, interbead filaments in collagenase-digested microfibrils did not extend beyond the width of the beads (Fig. 8C).

A Cross-linked Core Structure Stabilizes Microfibrils—Isolated guanidine-extracted microfibrils and collagenase-digested microfibrils were treated with increasing concentrations...
of Sigma type 1A collagenase. The epitopes recognized by mAbs 15 and 201 were readily abolished, whereas those of mAbs 26, 69, and 78 were resistant to collagenase digestion (data not shown). Resulting ultrastructures were quite similar. After treatment with collagenase, the outer extended filaments characteristic of guanidine-extracted microfibrils were lost, and an asymmetric dense region (Fig. 9, arrows) just to one side of the globular bead became apparent. Treatment with high concentrations of collagenase resulted in further loss of interbead structure, but microfibrils remained intact. These results demonstrated that a core ultrastructure is present in guanidine-extracted microfibrils and can be visualized by cleaving away the overlying filaments.

Similar experiments were conducted using microfibrils extracted from cell cultures with guanidine. Long microfibrils were isolated with an ultrastructural appearance similar to microfibrils extracted from tissues with guanidine (Fig. 10, A and B). When these microfibrils were digested with high concentrations of collagenase, microfibrils were degraded into irregular small groups of beads (Fig. 10, C and D). These results demonstrated that microfibrils isolated from cell cultures lack the necessary cross-links that stabilize and define the intact core microfibril structure found in tissue microfibrils.

DISCUSSION

The results presented in this study show that fibrillin-1 is cleaved during the crude collagenase digestions commonly used to extract microfibrils. Taken together, the data demonstrate that sites sensitive to proteolytic digestion are present in the proline-rich region, in EGF1, in cbEGF 6, in the junction between 8cys2 and cbEGF7, in cbEGF22, and in the junction between 8cys6 and cbEGF32. Other sites in EGF2, cbEGF7, and cbEGF8 may be predicted. The arrows in Fig. 2 designate these cleavage sites. Loss of mAb 15 and mAb 201 binding to microfibrils extracted by collagenase digestion of tissues results from cleavage in or near the epitopes recognized by these antibodies. Antibody epitopes are also marked in Fig. 2. These results confirm that at least two of the cleavage sites determined in in vitro assays can be identified in preparations of microfibrils digested with collagenase.

Other sites in fibrillin-1 may also be cleaved during microfibril preparation using crude collagenase digestion, as suggested by our in vitro enzyme digestions of monomers and by immunoblotting of guanidine-extracted microfibrils that were subsequently digested with collagenase. These immunoblotting results demonstrated numerous fibrillin-1 positive bands (data not shown). Cleavage at these other sites in fibrillin-1 could not be confirmed in collagenase-digested microfibrils, because antibodies specific for these regions are not yet available. However, based on the studies presented here, it is likely that these sites can also be cleaved during microfibril preparation using collagenase digestion.

In assembled microfibrils, the mAb 15 epitope in the proline-rich region of fibrillin-1 appears to be readily susceptible to proteolytic degradation. Loss of the mAb 15 epitope in collagenase-digested microfibrils, together with immunolocalization of mAb 15 to guanidine-extracted negatively stained microfibrils as well as to microfibrils in tissues, regardless of whether the microfibrils are stretched or unstretched, clearly demonstrate that the proline-rich region of fibrillin-1 is available to both enzymes and antibodies and is located on accessible surfaces of the microfibril.
A second site containing the epitope for mAb 201 (cbEGF6/8cys2) is also available for proteolytic degradation. This site is located midway in the pepsin fragment PF1. PF1 was shown to be a short linear peptide, identified by immunochemical analysis with mAb 201 (28). We reported that the N-terminal sequence of PF1, 434LYPSREPPRV, is within the proline-rich region of fibrillin-1 (33). It is likely that PF1 is generated from the interbead region, because it was purified as a homogeneous mono-meric peptide similar in appearance to a fragment of an interbead filament. Therefore, we propose that the distinct cleavage sites identified here in the proline-rich region and in the region around cbEGF6/8cys2 are located in the interbead region. This interpretation is an alternative to the proposal locating these domains within the cross-linked bead region of the microfibril (16, 26).

A proteomic analysis of collagenase-digested microfibrils compared with guanidine-extracted microfibrils identified tryptic peptides of fibrillin-1 in guanidine-extracted microfibrils that were not found in collagenase-digested microfibrils (34). Domains identified in guanidine-extracted microfibrils, but not in collagenase-digested microfibrils, included EGF1, EGF2, EGF4, cbEGF5, 8cys2, and cbEGF27/28. Collagenase cleavage sites were identified in our studies in EGF1, cbEGF6, between 8cys2 and cbEGF7, cbEGF22, and between 8cys6 and cbEGF32. Moreover, one of the collagenase susceptible sites that we identified was 116IQHCNIR, which was also identified as one of the tryptic peptides present in guanidine-extracted microfibrils (SIQHCNIR) (34). We conclude that this proteomics report (34) supports the presence of collagenase-susceptible regions in guanidine-extracted microfibrils that are close to the major sites identified in our study.

In contrast to collagenase-digested microfibrils, microfibrils prepared after guanidine extraction of tissues contained all fibrillin-1 epitopes examined in this study. By these immunological tests, “intact” fibrillin-1 appeared to be present in guanidine-extracted microfibrils. Moreover, retention of these epitopes after guanidine treatment suggests that this extraction method does not irreversibly denature fibrillin. In addition, the binding site for the prodomain of BMP-7 was present in guanidine-extracted microfibrils, suggesting that this binding site is also not irreversibly denatured by the guanidine used for extraction. In contrast, the BMP-7 prodomain did not bind well to collagenase-digested microfibrils, indicating that the binding site for BMP-7 may be composed of residues influenced by the cleavage of fibrillin-1 by crude collagenase digestion.

Other results presented in this study indicated that guanidine extraction does not irreversibly denature microfibrils. CD spectra showed that guanidine-treated fibrillin-1 retained the same overall secondary structure as untreated fibrillin-1. In addition, after guanidine treatment, the secondary structure of fibrillin-1 remained normally responsive to the presence or absence of calcium. Previously, we showed that velocity sedimentation of full-length fibrillin-1 was the same in denaturing, renaturing, and non-denaturing conditions and suggested that extensive intrachain disulfide bonding likely stabilizes the structure of fibrillin (4). Subsequent cloning and sequencing of fibrillin-1 and studies of recombinant protein confirmed that most of the domains present in fibrillin-1 are stabilized by intrachain disulfide bonds (33, 35–37). It is likely that the presence of extensive intramolecular disulfide cross-linking in almost all domains of fibrillin-1 protects fibrillin from irreversible denaturation during guanidine treatment.

From these studies, we conclude that the gross overall structure of fibrillin-1 is not perturbed in guanidine-extracted microfibrils. However, changes in fibrillin or microfibril structure, because of guanidine denaturation of unprobed regions, cannot be excluded. Partial denaturation of the structure of microfibrils could affect binding sites for important microfibril-associated proteins. In the future, it may be possible to compare guanidine-extracted microfibrils with microfibrils that are examined in their native state and that retain their associated networks of proteins. In the meantime, examination of guanidine-extracted microfibrils and comparison with collagenase-digested microfibrils have yielded new information about the structure of microfibrils.

The ultrastructural appearance of the guanidine-extracted microfibrils was very different from the appearance of collagenase-digested microfibrils. Instead of forming a linear interbead region equivalent to the width of each globular bead, filaments in guanidine-extracted microfibrils splayed outwardly away from and around each globular bead region, extending well beyond the width of each bead. These filaments appear to be mostly fibrillin. However, other linearly extended molecules may also be present in the guanidine-extracted microfibrils (see Fig. 7B, arrows). Previously, we showed that a stub of versican is present (25), whereas latent transforming growth factor β-binding protein-1 may not be present (7) in guanidine-extracted microfibrils.

Earlier studies by Mayne and co-workers (1, 19) described zonular microfibrils extracted in physiological buffers. Both “open” and “closed” morphologies were described. The open morphologies, which appeared similar to collagenase-digested microfibrils in EDTA, were associated with older preparations or incubation at 30 °C, whereas closed morphologies were observed only in freshly prepared materials (1). Images of freshly prepared zonular microfibrils demonstrated extensive bowing out of the filaments between each bead (19). These images of freshly prepared microfibrils are similar to our images of guanidine-extracted microfibrils. Our data presented here are consistent with these earlier studies, which suggested that degradation may underlie the open morphologies of extracted microfibrils.

Based on our ultrastructural images of guanidine-extracted microfibrils and of guanidine-extracted microfibrils cleaved with collagenase, on mapping of monoclonal antibodies bound to microfibrils (3), and on identification of collagenase cleavage sites in fibrillin-1, we propose that fibrillin-1 is staggered within the microfibril and that the N-terminal half of fibrillin molecules is on the outside of a core composed of C-terminal halves of fibrillin molecules (Fig. 11A). Collagenase digestion cleaves the outer filaments of fibrillin-1, exposing a core structure with an asymmetric electron density to the side of each bead (Fig. 11B). A potential cleavage site at the end of the 6th 8-cysteine domain (Fig. 11B, arrow) may be cleaved in the microfibrils, but the microfibril remains connected, likely due to the formation of intermolecular cross-links. These intermolecular cross-links...
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A

B

C

D

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are not completely established in cell cultures, because high concentrations of collagenase disrupts cell cultured microfibril connections, resulting in irregular clumps of beads (Fig. 11C).

We first proposed that fibrillin-1 is organized in parallel linear arrays within microfibrils, based upon asymmetric monoclonal antibody localization just to the side of each bead and the linear shape of fibrillin monomers (4). Additional mapping of monoclonal antibodies onto microfibrils led to two possible models as follows: an unstaggered model in which fibrillin-1 molecules 150 nm in length would have to be somehow compressed into a 50 nm period and a staggered model in which fibrillin-1 molecules could overlap, allowing N- and C-terminal epitopes to remain on either side of each bead (3). Fig. 11A depicts the antibody localization of epitopes mapped onto the microfibril (3). In 1996, we suggested that the unstaggered model should be preferred in order to accommodate the long periodicities that were sometimes observed in extracted microfibrils (3).

The currently accepted model of microfibril organization, the intramolecular pleating model (16, 17), is depicted in Fig. 11D. This model accommodates a long fibrillin monomer into a 50 nm microfibril period by folding the fibrillin molecules. Microfibrils are shown with both unextended and extended fibrillin-1 molecules, which are unstaggered but overlapping at the N and C termini. In contrast to the model proposed in this study, the intramolecular pleating model is not consistent with antibody mapping studies that positioned mAbs 26 and 69 to opposite sides of each bead and mAb 26 and mAb 201 on the same side of each bead (3).

High resolution imaging of microfibrils revealed details of the interbead region, including a double striation and holes (17). We suggest that the double striations correspond to the densities we visualized by rotary shadowing electron microscopy after extensive collagenase digestion of microfibrils. We also suggest that the high resolution images upon which the intramolecular pleating model is based represents the core structure of microfibrils (Fig. 11B). It is possible that the outer arms composed of the N-terminal halves of fibrillin-1 may be too flexible to resolve by these techniques.

A hallmark feature of collagenase-digested microfibrils is their extreme extensibility. Periodicities in collagenase-digested microfibrils can be stretched to measurements between 140 and 180 nm (16, 26). We suggest that the structure of guanidine-extracted microfibrils more closely resemble microfibrils within tissues, because fibrillin-1 does not appear to be degraded in the guanidine-extracted microfibrils. Average bead-to-bead periodicities of guanidine-extracted microfibrils were around 50 nm, consistent with the measurements of anti-body-induced periodicities of microfibrils in tissues (3, 6) and x-ray diffraction studies of dissected ocular zonules (27). Stretching experiments of tissues containing microfibrils have demonstrated reversible elastic changes in microfibril periodicities. Tissue extension (150%) of zonular microfibrils resulted in reversible periodicities up to 104 nm, and longer periodicities were not observed (27). In our studies, guanidine-extracted microfibrils extended only to 80 nm, but these microfibrils were not stretched. We suggest that collagenase-digested microfibrils are able to display more extended periodicities, some 75 nm greater than those observed in tissues, because fibrillin-1 molecules are cleaved. It may be that very long periodicities are due to cross-linked and cleaved fibrillin molecules that have become unraveled from the backbone structure of the microfibril. This unraveling likely occurs when the microfibril is stretched to periodicities greater than 80–100 nm, consistent with the conformational changes (bead unraveling) observed by others when collagenase-digested microfibrils are extended beyond these periodicities (16, 26).

In contrast to collagenase-digested microfibrils, the beads and interbead filaments in negatively stained images of guanidine-extracted microfibrils were not very well defined. Beads and interbead filaments in zonular microfibrils, viewed after quick-freeze deep-etch techniques, were also not apparent (38). In the latter study, intact native microfibrils were continuous and uniform, with a twisting beaded appearance. It is possible that the uniform appearance of intact native microfibrils may be due to associated proteins that obscure the core structure of the microfibril. However, guanidine-extracted microfibrils viewed by negative staining and rotary shadowing techniques in this study may be comparable to the microfibrils viewed by quick-freeze deep-etch techniques.

Other models of microfibrils in which fibrillin molecules are staggered have been proposed. A model with $\frac{1}{2}$ staggered fibrillin molecules was suggested, based on crystallographic and NMR studies of two or three fibrillin-1 domains (15). A model very similar to ours, in which fibrillin molecules are $\frac{1}{2}$ staggered, was proposed previously by investigators interested in the effects of mutations in fibrillin-1 on assembly of the microfibril (40). The staggered model of Liu et al. (40) positioned the “neonatal” region of fibrillin-1 (8cys3 through cbEGF18 (41)) in the region of the bead. Thus, mutations in this region of fibrillin-1 might be particularly severe (causing neonatal Marfan syndrome) because interactions important to the assembly or stability of the microfibril are mediated by the neonatal region. Our staggered model also positions the neonatal region close to each bead. Further studies are required to clarify the complex interactions between the neonatal region and the rest of the fibrillin molecule.
interactions between fibrillin monomers that mediate assembly of the microfibril.

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