Initial Steps in Assembly of Microfibrils
FORMATION OF DISULFIDE-CROSS-LINKED MULTIMERS CONTAINING FIBRILLIN-1*

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Fibrillins are the major constituents of extracellular microfibrils. How fibrillin molecules assemble into microfibrils is not known. Sequential extractions and pulse-chase labeling of organ cultures of embryonic chick aortae revealed rapid formation of disulfide-cross-linked aggregates containing fibrillin-1. These results demonstrated that intermolecular disulfide bond formation is an initial step in the assembly process. To identify free cysteine residues available for intermolecular cross-linking, small recombinant peptides of fibrillin-1 harboring candidate cysteine residues were analyzed. Results revealed that the first four cysteine residues in the unique N terminus form intramolecular disulfide bonds. One cysteine residue (Cys204) in the first hybrid domain of fibrillin-1 was found to occur as a free thiol and is therefore a good candidate for intermolecular disulfide bonding in initial steps of the assembly process. Furthermore, evidence indicated that the comparable cysteine residue in fibrillin-2 (Cys239) also occurs as a free thiol. These free cysteine residues in fibrillins are readily available for intermolecular disulfide bond formation, as determined by reaction with Ellman’s reagent. In addition to these major results, the cleavage site of the fibrillin-1 signal peptide and the N-terminal sequence of monomeric authentic fibrillin-1 from conditioned fibroblast medium were determined.

Fibrillins are ubiquitous extracellular matrix macromolecules that contribute to the structure of fibrous elements called “microfibrils” (1, 2). Single microfibrils can be extracted from connective tissues following digestion of the tissue with crude collagenase (3). However, microfibrils are difficult to solubilize into their molecular components, because they are heavily cross-linked. Therefore, our current understanding of how single molecules of fibrillin are organized in microfibril polymers is based primarily upon immunolocalization studies of microfibrils, using monoclonal antibodies specific for mapped regions in the fibrillin-1 molecule (4). According to this model, single molecules of fibrillin, which are extended molecules of ~150 nm in length (5), may be arranged periodically in a parallel unstaggered (4) or a parallel staggered (6) orientation along the length of the microfibril.

Initial investigations suggested that microfibrils are stabilized by disulfide bonds, because extraction of tissues with disulfide-bond reducing agents resulted in loss of the ultrastructural features of microfibrils (7). Fibrillin was first characterized as a cysteine-rich monomeric molecule secreted into the medium by fibroblasts in culture (1, 5). Cloning and sequencing of fibrillin demonstrated that most of the fibrillin molecule is composed of cysteine-rich domains (8–10), which are predicted to fold independently. The domain structure of fibrillin is depicted in Fig. 1. There are 47 epidermal growth factor-like domains, seven “8-cysteine”-containing domains, and two “hybrid” domains (9). If each of these domains is stabilized by intrachain disulfide bonds, as predicted (4, 6, 11), then cysteine residues present in the N terminus (four residues), the C terminus (two residues), or in the first hybrid domain (nine residues) may be free to participate in the formation of intermolecular disulfide bonds.

These investigations were carried out in order to obtain biochemical evidence for intermolecular disulfide bond formation by fibrillin and to determine potential free cysteine residues in the N terminus and in the first hybrid domain. Recombinant polypeptides of fibrillin utilized for these studies are depicted in Fig. 1.

More than 200 individual mutations in FBN1 are known to result in the Marfan syndrome, an autosomal dominant heritable disorder of connective tissue (12, 13). A dominant negative effect of the mutant fibrillin-1 on the structure or stability of microfibrils is thought to cause the skeletal, cardiovascular, and ocular symptoms of the disease. Central to these discussions are issues related to the effect of mutant fibrillin-1 molecules on assembly of microfibrils. Are mutant fibrillin-1 molecules incorporated into microfibrils, where they may destabilize the microfibrils by rendering them more susceptible to proteolytic attack (14, 15)? Alternatively, do mutant molecules disrupt microfibril assembly, resulting in short or fragmented microfibrils (15, 16)? It may be that different mutant fibrillin-1 molecules may demonstrate different fates, depending upon the location and type of mutation. In order to address these issues, molecular interactions that determine how fibrillin-1 assembles into microfibrils must be understood in more detail.

**Experimental Procedures**
Preparation of Native Monomeric Fibrillin-1—Serum-free medium from normal human skin fibroblasts was collected after a 48-h incubation period. After concentration by ultrafiltration (Amicon), the medium was applied to mAb1 26 antibody affinity chromatography, and fibrillin-1 was eluted from the column with 0.1 M glycine-HCl, pH 2.5. mAb

1 The abbreviations used are: mAb, monoclonal antibody; PBS, phosphate-buffered saline; DTE, dithioerythritol; DTNB, 5,5-dithiobis(2-nitrobenzoate); TBS, Tris-buffered saline; LTBP, latent transforming growth factor-β-binding protein; bp, base pair(s).
26 is specific for fibrillin-1 (17). Radioactive fibrillin-1 markers were prepared as described previously (1).

**Extraction of Chick Embryonic Tissue—**The embryos of White Leghorn chickens were subjected to 5 mg of β-aminopropionitrile in phosphate-buffered saline (PBS), pH 7.4, injected into the air sack on day 14. Embryos were sacrificed on day 17, and aortae were dissected and placed either in liquid N₂ or in organ culture medium (see below).

Aortae were extracted sequentially with the following reagents: (i) PBS, (ii) 1 M NaCl in 50 mM Tris-HCl, pH 7.5, (iii) 8 mM urea in 50 mM Tris-HCl, pH 7.5 (twice), and (iv) 50 mM dithioerythritol (DTE) in 8 M urea/50 mM Tris-HCl, pH 7.5. All extractions were performed at 4 °C with the following protease inhibitors present: 0.001 M N-ethylmaleimide, 0.004 mM EDTA, and 0.001 mM phenylmethylsulfonyl fluoride. Tissue was dissociated in extraction buffers with a Brinkmann polytron. Then, extracts were centrifuged at 4 °C for 1 h at 14,000 rpm in a Sorvall RC-5B centrifuge, and supernatants were stored at −90 °C. Pellets were then extracted with the next buffer, using the polytron, and centrifuged. The second 8 mM urea extraction was overnight at 4 °C. All chemicals were from Sigma.

**Organ Cultures of Chick Aortae—**Aortae from batches of 50–60 embryos at a time were dissected and incubated in Dulbecco’s modified Eagle’s medium, without cysteine (sometimes without methionine as well), in a shaking water bath at 37 °C. In preliminary experiments, they were radioactively labeled with medium containing 50 μCi/ml [³⁵S]cysteine (Amersham Pharmacia Biotech) and 50 μCi/ml [³⁵S]methionine (Amershams Pharmacia Biotech). In subsequent experiments, [³⁴C]cysteine (NEC Life Science Products) was used at a concentration of 1–5 μCi/ml of medium. The medium was also supplemented with 0.02 M HEPES and penicillin-streptomycin (50 μg/ml and 50 units/ml, respectively) (Life Technologies, Inc.). The aortae were then chased with medium containing cysteine (and methionine) and extracted as above. The medium usually contained 64 μg/ml β-aminopropionitrile, unless otherwise specified. For each experiment, equal numbers of aortae were removed at specified time points and immediately frozen in liquid N₂.

For immunoaffinity purification of fibrillin present in the 8 M urea extracts, the extracts were dialyzed against 50 mM Tris-HCl, pH 7.5, containing 0.5 M NaCl and 2 mM urea, at 4 °C overnight. Almost all of the material remained in solution after dialysis. Before application to mAb 201/Seapharose, the samples were diluted with cold PBS to a urea concentration of 0.5 M. After extensive washing of the column with PBS-0.05% Tween 20, the column was eluted with 0.1 M glycine-HCl, pH 2.5. Fractions were collected, and radioactivity was determined by scintillation counting.

**Expression of Recombinant Polypeptides of Fibrillin-1—**A recombinant fibrillin-1 expression construct rF24, coding for Met³¹ to Ile⁴⁸⁹ and an additional 60 bp 5’ to the proposed start codon, was prepared using expression construct rF23 (18). To replace the BM-40 signal peptide in rF24 with the putative fibrillin-1 signal peptide, a 1411-bp NheI-Xhol fragment from pCEPSP-rF25H was fused with the NheI-Xhol restricted pCEP4 vector (Invitrogen), which does not contain a sequence for a signal peptide. The resulting plasmid was designated pCEP-rF25H. To add the coding sequence for the putative fibrillin-1 signal peptide and 60 preceding bp, clone HFBN29 (9) was amplified using primer DRB2 (5’-ATAGTTTACGCCGCCTAGCCGCGACCGCACCAGGCGGGGG-3’), which introduced a NruI and an NheI restriction site at the 5’ end, and primer N911AS (5’-AATTTCCTCTCTGACAAGGCC-3’). A 134-bp NruI-NcoI fragment was subcloned into the NotI-NcoI restricted PBS-HFBN23/29 (4), and the 1478-bp NheI-AgeI fragment was then ligated with the NheI-AgeI restricted pCEP-rF32H. The resulting expression plasmid was designated pCEPSP-rF34H.

To make expression construct rF38, coding for Ser¹¹⁴ to Glu²⁸⁷, clone HFBN29 was amplified with sense primer DR90 (5’-CGTACGGTACCTCATCATAACACCTGCAAATTCG-3’), introducing a NheI restriction site at the 5’ end, and antisense primer DR91 (5’-ACCCGCTGAGGTACGATGTTGATGTCCTACATTTGGAGACTTCT-3’), introducing the sequence for 6 histidine residues, a stop codon, and a XhoI restriction site at the 3’ end. The 547-bp NheI-Xhol fragment was then subcloned in pCEP/S/HiII (19). The resulting plasmid was designated pCEPSP-rF38.

Transfection of 293/EBNA cells (Invitrogen) and selection of clones was performed as described previously (4). Purification of rF24 and rF38 by chelating chromatography was as described for construct rF20 (4). Alternatively, rF24 was purified by affinity chromatography with mAb 26 as described for recombinant fragment rF11 (4). Expression and purification of the fibrillin-1 recombinant fragment rF31 and the fibrillin-2 fragments rF33 and rF37 were described previously (17).

**Characterization of Recombinant Polypeptides—**Analysis of the secreted fraction of recombinant rF24 compared with the fraction retained within the cells was performed by Western blot analysis. 8 ml of serum-free medium (48-h incubation time) from a confluent 100-mm cell culture dish was harvested. The cell layer was then washed twice with PBS and solubilized in 0.2 ml of SDS sample buffer. 1 ml of TCA-precipitated culture medium and 0.03 ml of the cell fraction were then analyzed under nonreducing conditions with mAb 26 (1:10 μg/ml) and goat anti-mouse IgG conjugated to horseradish peroxidase (Bio-Rad; 1:800 dilution). Controls included nontransfected 293/EBNA cells and conditioned medium from these cells.

Electroblotted recombinant peptides were analyzed on an automated sequencer (Applied Biosystems) using Edman degradation.

**Analysis of Free Cysteine Residues—**To determine free cysteine residues in rF24 and rF38, the polypeptides were solubilized in 0.5 M Tris-HCl, pH 8.0, 6 M guanidine, 2 mM EDTA and derivatized with a 10-fold molar excess (over cysteine residues) of iodoacetamide under N₂ at 37 °C for 30 min in the dark. The polypeptides were desalted by reverse-phase chromatography (C₁₈, Vydac 218TP52), lyophilized, and reduced with a 10-fold molar excess (over cysteine residues) of dithiothreitol under N₂ at 37 °C for 2 h in the dark. The fragments were then derivatized with a 4-fold molar excess (over dithiothreitol) of 4-vinylpyridine at 25 °C for 90 min in the dark. The reduced and alkylated peptides were then again desalted by C₁₈ reverse-phase chromatography and either submitted directly to Edman degradation (rF24) or digested with endoproteinase Glu-C (Roche Molecular Biochemicals) (rF38) in 25 mM ammonium acetate, pH 4.0, at 25 °C for 24 h. Endo-Glu-C peptides of rF38 were separated by C₁₈ reverse-phase chromatography and then analyzed by Edman degradation. N-terminal sequences of rF24 or proteolytic peptides of rF35 were determined by automated protein sequencing (Hewlett Packard G1000S). Free cysteine residues were identified as amidomethyl cysteine, and disulfide-bonded cysteine residues were identified as pyridylethyl cysteines.

To determine whether free cysteines are located on the surface of molecules, recombinant polypeptides of fibrillin-1 (rF31 and rF38) and fibrillin-2 (rF33 and rF37) were derivatized with 5,5-dithiobis-(2-nitro-
FIG. 2. SDS-PAGE (3–5% polyacrylamide gradient) of [14C]fibrillin-1 polypeptides extracted from chick aorta that had been continuously pulsed for 17 h. Lane 1, fibrillin marker immunoisolated from the medium of human fibroblasts in culture; lane 2, PBS extract; lane 3, 1 M NaCl extract; lane 4, 8 M urea extract; lane 5, second 8 M urea extract; lane 6, DTE extract. Single arrow marks the top of the stacking gel; double arrow indicates the beginning of the running gel. Prior to electrophoresis, all samples were treated with reducing agent. Fb denotes the position of monomeric fibrillin-1.

benzamid) (DTNB) (Ellman’s reagent; Fluka). The reaction was carried out in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10 mM CaCl2, 9.3 μM (rF33), and 9.3 μM (rF33). Because the concentration of DTNB is much higher (130–200-fold) than the concentration of free cysteine residues, the reaction can be considered of pseudo-first order: protein-SH + DTNB → protein-S-D + TNB. The absorbance (at 412 nm) was measured over time (25 min). The concentration of free cysteine residues was equivalent to the concentration of TNB, calculated from the molar extinction coefficient of the TNB, τ = 13,600 M⁻¹cm⁻¹ (20). The following equation was used for curve fitting: [TNB] = [protein-SH]₀ × (1 − e⁻kt), where [TNB] = concentration of the TNB anion, [protein-SH]₀ = concentration (μM) of free thiols in the protein at time 0, τ = time (min), and k = rate constant of the reaction (min⁻¹).

RESULTS

Fibroblasts Secrete Fibrillin-1 Monomers into the Medium—Previous identification and characterization of native fibrillin-1 was accomplished using monoclonal antibodies specific for fibrillin-1 (1, 5, 17). Direct evidence that immunoisolated molecules synthesized and secreted by human fibroblasts are fibrillin-1 monomers has been obtained by N-terminal sequence analysis (data not shown). The authentic fibrillin-1 N terminus (RGGGG(H)DALKGPNVXG) begins after a furin-type cleavage site in profibrillin-1 (RARK), which was predicted to be utilized in studies employing recombinant fibrillin-1 polypeptides (4).

In Organ Cultures, Fibrillin-1 Is Rapidly Assembled into Disulfide-linked Multimers—In contrast to monomeric molecules, which can be obtained from the medium of cells in culture, the tissue form of fibrillin-1 is polymeric. After fibrillin-1 assembles into a microfibril, it is cross-linked and is very difficult to solubilize. In order to determine initial steps in the process by which fibrillin-1 becomes cross-linked and insoluble, pulse-chase and extraction studies were performed using radiolabeled organ cultures of aortae.²

FIG. 3. SDS-PAGE (3–5% polyacrylamide gradient) of [14C]fibrillin-1 polypeptides labeled (pulsed for 30 min) proteins extracted from equal volumes of chick aortae after different periods of chase. Equal volumes of extract were applied to each channel. Lanes 1–4, PBS extracts after 0 min (lane 1), 1 h (lane 2), 3 h (lane 3), and 19 h (lane 4) of chase. Lanes 5–8, 8 M urea extracts after 0 min (lane 5), 1 h (lane 6), 3 h (lane 7), and 19 h (lane 8) of chase. Lanes 9–12, second 8 M urea extracts after 0 min (lane 9), 1 h (lane 10), 3 h (lane 11), and 19 h (lane 12) of chase. Lanes 13–16, DTE extracts after 0 min (lane 13), 1 h (lane 14), 3 h (lane 15), and 19 h (lane 16) of chase. All samples were treated with reducing agent prior to electrophoresis. The position of fibrillin (Fb) is marked with an arrow.

² A version of these organ culture studies was previously published as an unreviewed proceedings of a meeting (21).
proteolytic processing sites in fibrillin-1. Recombinant fragment precipitated fibrillin from 8 M urea extracts of chick aortae pulsed for 1 h in kDa. indicated. The positions of globular marker proteins (here). Individual bands from the same fraction were analyzed by N-terminal sequencing after blotting onto membranes. The sequences are here). The purified material was subjected to SDS gel electrophoresis (shown in kDa). Lane 2, reduced (+) (with β-mercaptoethanol) fibrillin-1 immunoprecipitate. Lane 3, reduced (+) (with β-mercaptoethanol) fibrillin-1 immunoprecipitate. Lane 3, reduced (+) (with β-mercaptoethanol) fibrillin-1 markers from human fibroblast medium. b, 1.5% agarose gel of immunoprecipitated nonreduced fibrillin-1 (lane 1) shown in a, lane 1. The arrow indicates the position of the fibrillin-1 monomer. Lanes 2 and 3, nonreduced type IV collagen markers, with estimated molecular masses of 0.54 (monomer), 1.08 (dimer), 1.62 (trimer), and 2.16 (tetramer) million Da.

FIG. 4. a. SDS-PAGE (3–5% polyacrylamide gradient) of immunoprecipitated fibrillin from 8 M urea extracts of chick aortae pulsed for 1 h with 250 μCi/ml [35S]cysteine and chased for 5 h. Lane 1, nonreduced (−) (without β-mercaptoethanol) fibrillin-1 immunoprecipitate; unlabeled arrow indicates the presence of small amounts of monomeric fibrillin-1. Lane 2, reduced (+) (with β-mercaptoethanol) fibrillin-1 immunoprecipitate. Lane 3, reduced (+) (with β-mercaptoethanol) fibrillin-1 (Fb) and fibronectin (Fn) markers from human fibroblast medium. a, lane 1. The arrow indicates the position of the fibrillin-1 monomer. Lanes 2 and 3, nonreduced type IV collagen markers, with estimated molecular masses of 0.54 (monomer), 1.08 (dimer), 1.62 (trimer), and 2.16 (tetramer) million Da. 

FIG. 5. Identification of the signal peptide cleavage site and proteolytic processing sites in fibrillin-1. Recombinant fragment rF24 was purified by immunoadfinity chromatography using mAb 26. The purified material was subjected to SDS gel electrophoresis (shown here). Individual bands from the same fraction were analyzed by N-terminal sequencing after blotting onto membranes. The sequences are indicated. The positions of globular marker proteins (M) are indicated in kDa.

fibrillin (lane 4), and 8 M urea extracts contained smaller amounts of fibrillin (lanes 8 and 12) than at 1 and 3 h of chase. Most of the radioactively labeled fibrillin present at 19 h of chase was resistant to prior extractions and was solubilized by DTE (lane 16; compare this lane with lanes 4, 8, and 12). Because the 1 M NaCl extracts contained very little fibrillin, they are not shown here.

Electrophoresis and molecular sieve chromatography demonstrated that PBS extracts of aortae after 0–4 h of chase contained monomers, not multimers, whereas 8 M urea extracts of aortae after 4 h of chase contained multimers and small amounts of monomer (data not shown). The 8 M urea extract of pulse (1 h) and chased (4 h) aortae was diluted with PBS and applied to an antibody affinity column. Most of the fibrillin, eluted from the column, failed to enter the stacking gel when the sample was run without reducing agent (Fig. 4a, lane 1). A small amount of fibrillin monomer was present (arrow). When the sample was reduced (Fig. 4a, lane 2), the immunosolated material was composed primarily of fibrillin and additional species, which might represent nonreducibly cross-linked fibrillin molecules, degradation products of microfibrils, or contaminants of the immunoprecipitation (including potential fibrillin-binding proteins). When this immunosolated fibrillin aggregate was run on a 1.5% agarose gel and compared with mouse type IV procollagen markers, the minimum molecular mass calculated for the fibrillin aggregate was greater than 8 million Da (Fig. 4b).

Determination of Free Cysteine Residues—Fibrillin-1 is composed of 228 cysteine residues in 47 epidermal growth factor-like repeats, 56 cysteine residues in seven 8-cysteine repeats, 17 cysteine residues in two hybrid repeats, 4 cysteine residues in the N terminus, and 2 cysteine residues in the C terminus (9). Two recombinant polypeptides, rF24 and rF38 (Fig. 1), were produced in order to examine the cysteine residues present in the N terminus and in the first hybrid repeat, which contains nine cysteine residues. Sufficient quantities of a C-terminal recombinant polypeptide, rF8 (22), could not be produced for these studies.

For rF24, stably transfected 293/EBNA cells and serum-free medium were analyzed by Western blotting with mAb 26, specific for fibrillin-1 (17). The majority (>95%) of the 50-kDa rF24 polypeptide was secreted into the medium, demonstrating that the putative signal sequence starting at Met1 (10) is functional (data not shown). Immunoblotting and affinity chromatography with mAb 26 indicated correct folding of rF24, because binding of mAb 26 to fibrillin-1 relies on proper formation of disulfide bonds (4). rF24 was purified to homogeneity by affinity chromatography on mAb 26 (Fig. 5). Edman degradation of the major band resulted in two N-terminal sequences, 5ADANLEAGNVKE and 4RGGGGDALKGP, in approximately equal amounts. This demonstrates that the fibrillin-1 signal sequence is cleaved between Gly24 and Ala25 and that rF24 is also processed, like authentic fibrillin-1, after the furin-type cleavage sequence. Edman degradation of the minor band gave a single fibrillin-1 sequence, 5SIQHXNIRXRKMNG. This sequence begins after the first epidermal growth factor-like repeat in fibrillin-1, indicating a protease-sensitive site. rF24 was used to determine the free sulfhydryls in the unique N-termin-
nal end. To determine free sulfhydryls in the first hybrid motif, a smaller recombinant fragment, rF38, was used. SDS-PAGE of purified rF38 demonstrated the expected molecular mass of 20 kDa and a reducible dimer of 40 kDa (Fig. 6).

rF24 and rF38 were alkylated by iodoacetamide and then reduced and again alkylated with 4-vinylpyridine, resulting in amidomethyl cysteine residues for free sulfhydryl groups and pyridylethyl cysteine if the cysteine residue was originally involved in a disulfide bond. To promote accessibility to the alkylating agents, these reactions were performed in the presence of 6 M guanidine. All four cysteine residues of the unique N-terminal domain were modified with 4-vinylpyridine, demonstrating that they all were involved in disulfide bonds. Only the third cysteine (Cys204) in the first hybrid repeat was modified with iodoacetamide, whereas the other 8 cysteine residues involved in a disulfide bond. To promote accessibility to the intermolecular disulfide bond formation, the fibrillin-1 recombinant disulfide bonds coincides with progressive resistance of tissues, pulse-chase experiments demonstrate that intermolecular disulfide bond formation occurs within the first few hours after synthesis and secretion of the monomeric fibrillin molecule. Data presented here show that formation of intermolecular disulfide bonds coincides with progressive resistance of fibrillin to extraction. Selective final extraction with a reducing agent suggests that fibrillin is the only cysteine-rich high molecular weight microfibrillar component that is cross-linked and stabilized in this manner (Fig. 2).

In order to determine whether the free cysteine residue is located on the surface of molecules and readily available for intermolecular disulfide bond formation, the fibrillin-1 recombinant fragments rF31 and rF38 and the fibrillin-2 recombinant fragments rF33 (A and C) and rF31 (A and C) (a) or fibrillin-2 fragments rF37 (B and D) and rF33 (V and Y) (b) were reacted with Ellman’s reagent either in a physiological buffer, TBS (closed symbols), or in a denaturing buffer containing 6 M guanidine-HCl (open symbols). The amount of free thiols (mol/mol of protein) was calculated from the absorbance at 412 nm as described under “Experimental Procedures.”

**RESULTS**

Here, evidence is presented that indicates that intermolecular disulfide bond formation is the first step in fibrillin multimerization and microfibril assembly. In organ-cultured tissues, pulse-chase experiments demonstrate that intermolecular disulfide bond formation occurs within the first few hours after synthesis and secretion of the monomeric fibrillin molecule. Data presented here show that formation of intermolecular disulfide bonds coincides with progressive resistance of fibrillin to extraction. Selective final extraction with a reducing agent suggests that fibrillin is the only cysteine-rich high molecular weight microfibrillar component that is cross-linked and stabilized in this manner (Fig. 2).

Recombinant fibrillin polypeptides were produced in order to determine the location and number of free cysteine residues that could potentially be involved in the formation of intermolecular disulfide bonds. Using an approach that previously resulted in properly folded and well-characterized recombinant fibrillin polypeptides (4, 17, 18), N-terminal polypeptides rF24 and rF38 were produced and characterized here. Sequence analysis demonstrated that all four cysteine residues present in the fibrillin-1 N terminus were held in intrachain disulfide bonds. Because these four cysteine residues are conserved in the N-terminal region of fibrillin-2, it is likely that they also form intrachain disulfide bonds in fibrillin-2. A single cysteine residue was free in rF38 and was identified as the nonconserved cysteine residue (Cys204) present in the first hybrid domain of fibrillin-1. Evidence was presented to demonstrate...
that this free cysteine residue is available on the surface of the molecule for intermolecular disulfide bond formation.

In addition, reaction with Ellman’s reagent suggested that a large recombinant polypeptide of fibrillin-2, rF37, contained one free cysteine residue, whereas control fragments, rF31 (fibrillin-1) and rF33 (fibrillin-2), which are composed of all major types of domains present in fibrillins, did not react with DTNB. By comparison with results obtained for rF38 and with sequence homologies (Table I), it is likely that the free cysteine residue present in rF37 is the nonconserved cysteine residue present in the first hybrid domain.

The family of latent transforming growth factor-β-binding proteins (LTBP-1, -2, -3, and -4) (23–26) resembles fibrillins, except that the LTBP-1s have variable lengths and are smaller than the fibrillins. LTBP-1 has been immunolocalized to microfibrils (27–29). The LTBP-1s are composed of the same domains present in fibrillins, including one hybrid domain. However, the single hybrid domain in LTBP-1s, like the second hybrid domain in fibrillins, does not contain the extra cysteine residue (Table I).

Sequence analysis of rF24 allowed the determination of the fibrillin-1 signal peptide cleavage site, just before the sequence ADANLEAGNVKE. This finding agrees with recently published results using recombinant fibrillin-1 fragments expressed in an alternative expression system (30). In addition, utilization of a processing site after a consensus furin-type cleavage signal was demonstrated by N-terminal sequence analysis of rF24 as well as authentic fibrillin-1 from fibroblast medium.

In summary, the data presented suggest that the initial steps in the polymerization and stabilization of fibrillin-1 monomers into microfibrils involves the formation of intermolecular disulfide bonds. These intermolecular disulfide bonds may cross-link fibrillin monomers to other cross-linked fibrillin molecules or to high molecular weight undetermined species (Fig. 4a, lane 2). The nonconserved cysteine (Cys204) present in the first hybrid domain is an available site for the formation of these important intermolecular disulfide bonds.

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