Alignment of Fibrillin Molecules in Elastic Microfibrils Is Defined by Transglutaminase-Derived Cross-Links

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ABSTRACT: Microfibrils were extracted from human amnion in the form of a beaded filament and analyzed for the presence of transglutaminase-derived cross-links using acrylonitrile derivatization. The cross-link structure was isolated from protease hydrolysates of beaded filaments and identified as a phenylthiocarbamyl amino acid derivative by comparison to a standard. Acid hydrolysis of the isolated cross-link gave the expected lysine and glutamic acid in a 1:1 ratio. The beaded filaments were also treated with trypsin to produce a fraction that contained the bead structure and a fraction containing fragments of the interbead filaments. Cross-links were detected in the interbead filaments but not in the beads. A large tryptic peptide that contained a cross-link was isolated and sequenced. The two amino acid sequences obtained identified both of the cross-linked molecules as fibrillin-1 and enabled the approximate localization of the cross-link sites within the molecule. The locations of cross-link sites on two adjacent molecules fixed the relative positions of fibrillin monomers within the microfibrils, providing insight into the spatial organization of fibrillin within the elastic microfibrils.

Elastic microfibrils are associated with amorphous elastin in adult tissues and are thought to be the substrate onto which elastin is deposited during development (*1*, *2*). Two other populations of microfibrils in skin, called elaunin and oxytalan fibrils, do not contain amorphous elastin and can be distinguished by their histological staining properties (*3*, *4*). Other tissues such as ciliary zonules and placenta, which do not contain elastin fibers also contain elastic microfibrils. The major protein component of all of these microfibrils is fibrillin-1 (*5*). A second fibrillin molecule has been characterized and found to co-distribute with fibrillin-1, but it is more prevalent in the developing embryo and elastin-associated microfibrils (*6*). In addition to fibrillin, several other structural microfibrillar components have been characterized. MAGP1 and MAGP2 were isolated from bovine nuchal ligament (*7*−*9*), AMP from ciliary zonules (*10*), GP115/emilin from aorta (*11*, *12*) and MAP from porcine aorta (*13*). MFAP3 (*14*), MFAP4 (*15*), and LTBP-2 (*16*), genes that code for microfibrillar proteins, have been cloned but the corresponding proteins not yet isolated. Nothing is known about the organization of these proteins within the microfibrils. Other proteins are found associated with microfibrils under certain conditions or in specific tissues but are not considered to be structural components of microfibrils. These include fibronectin (*17*, *18*), amyloid P (*19*), vitronectin (*20*), lysyl oxidase (*21*), and type VIII collagen (*22*).

Fibrillin-1 is the best characterized of the elastic microfibril components and is of particular interest because defects in this protein result in Marfan syndrome. Marfan syndrome is an inherited, autosomal, dominant disorder which affects the skeletal, ocular, and cardiovascular systems (see ref 23 for a review). The fibrillin molecule is a single chain glycoprotein with an apparent molecular weight of approximately 350 kDa (*5*). It appears to be synthesized as a 375 kDa precursor (pro-fibrillin) which is processed to 350 kDa prior to secretion (*24*). Electron microscope images of rotary shadowed fibrillin molecules showed it to be a 140 nm long flexible rod with no obvious globular domains (*25*). A study of the biosynthesis of fibrillin in tissue culture showed that molecules were rapidly incorporated into a high molecular weight, nonreducible aggregate, supporting the notion that, extracellularly, fibrillin does not exist as single molecules (*24*). Attempts to extract components of the elastic microfibrillar system from mature tissues using reducing and/or denaturing conditions resulted in very small amounts being isolated, indicating again that the tissue form of fibrillin is insoluble (*26*, *27*). These observations suggested the presence of nonreducible cross-links in elastic microfibrils. Conceivably, the cross-links could be similar to the lysine-derived cross-links found in collagen or elastin or the transglutaminase-derived cross-links formed between lysine and glutamine residues. The presence of transglutaminase-derived cross-links in microfibrils extracted from the dermis of sea cucumber would suggest the latter (*28*). It has also been shown that fibrillin in the human dermis can be a substrate for transglutaminase (*29*).

In this report the extraction and purification of human elastic microfibrils and the detection of cross-links produced by transglutaminase in this structure are described. The cross-link structure was isolated and characterized, a tryptic peptide containing a cross-link isolated, and its amino terminal double sequence determined. Both sequences of the peptide were from fibrillin-1, which gave some insight into the alignment of the molecules within the microfibril.

EXPERIMENTAL PROCEDURES

Preparation of Fibrillin-Containing Beaded Filaments. Amniotic membranes were collected from approximately 60 full-term placenta. The amnion was stripped from the chorion, rinsed briefly with water and stored at 4 °C in water

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containing 2 mM NEM, 1 mM PMSF, and 5 mM benzamidine, until used. The amnions were homogenized into ice-cold water containing the above inhibitors and centrifuged, and the tissue pellet was washed once more in 6 L of the same solution. Finally the tissue was washed in 6 L of cold 10% NaCl solution containing the same inhibitors. Washed tissue (1 kg) was suspended in 3 L of 0.1 M Tris-HCl buffer, pH 7.5, containing inhibitors as above with 20 mM $CaCl_2$ and 0.5 M NaCl. Bacterial collagenase (12 500 units CLSPA, Worthington) was added to the tissue suspension which was stirred at 4 °C for 24 h and then at room temperature for a further 24 h. The digest was centrifuged and the pellet resuspended again in the same buffer and the digestion procedure repeated twice more. The final pellet was resuspended at 4 °C in 2 L 0.1 M Tris-HCl buffer, pH 8.5, containing 0.5 M NaCl, soybean trypsin inhibitor (2 mg, Sigma), and elastase (160 units, ESFF, Worthington) added. After 2 h the tissue digest was centrifuged and the tissue pellet extracted with 3 L of 0.1 M ammonium bicarbonate containing 0.5 M NaCl, overnight at 4 °C. The extract was centrifuged, the pellet was extracted a second time, and the supernatants were combined and precipitated with 20% ammonium sulfate.

The precipitate was dissolved in 3 L of 0.1 M ammonium bicarbonate containing 6 M urea and 0.05% Tween and centrifuged and the supernatant chromatographed on a molecular sieve column (4.8 × 120 cm) of Sephacryl S500HR (Pharmacia) at a flow rate of 120 mL/h with the same buffer. Fractions containing fibrillin filaments were pooled, dialyzed against 0.1 M ammonium bicarbonate, and precipitated with 20% ammonium sulfate. The precipitate was dissolved in 200 mL of 0.1 M Tris-HCl buffer, pH 7.8, containing 5 mM $CaCl_2$, 5 mM $MgCl_2$, 2 mM NEM, and 1 mM PMSF. DNAase I (2 mg, Sigma) was added and the digest incubated at 37 °C for 2 h, and then bacterial collagenase (1000 units, Boeringer-Mannheim) was added and the incubation continued at 4 °C overnight. The digestion was stopped by adding EDTA to 5 mM. The protein was again precipitated with 20% ammonium sulfate, the precipitate dissolved in 0.1 M ammonium bicarbonate containing 6 M urea and 0.05% Tween and rechromatographed on the Sephacryl S500HR column described above. Fractions containing fibrillin filaments were pooled and stirred for 4 h with QAE-52 cellulose (Whatman) also equilibrated with 0.1 M ammonium bicarbonate containing 6 M urea and 0.05% Tween at room temperature. The resin was removed by centrifugation (18000*g*, 1 h) and the sample dialyzed against 0.1 M ammonium bicarbonate and lyophilized. This procedure yielded about 90 mg of filaments per 1 kg of wet amnion.

The quality of the beaded filaments was checked by electron microscopy of rotary shadowed preparations (*30*), ELISA assay with mAb69 (*31*) and mAb201 (*5*), and 3−5% SDS-PAGE (*32*).

Cross-Link Analysis. Cyanoethylation of beaded microfibrils and their fragments was carried out according to the procedure of Pisano (*33*) with some modification. Proteins suspended in 0.5 mL of 0.1 M Tris-HCl, pH 8.0 buffer, and 30 μL of triethylamine were first dried under vacuum to remove traces of ammonia and then cyanoethylated with 0.3 mL of acrylonitrile and 30 μL of triethylamine in a sealed glass vessel at 42 °C for 96 h. After cyanoethylation the samples were evaporated to dryness. Cyanoethylated proteins were hydrolyzed in 0.2 mL of 6 M HCl at 115 °C for 24 h under nitrogen and analyzed on an amino acid analyzer for their lysine content.

Isolation of Cross-Link. Protein digestion was carried out by the sequential addition of pronase (5 mg/mL), leucine aminopeptidase (1 mg/mL), and carboxypeptidase A, B, and Y (0.4 mg/mL) as described (*34*). The digestion was terminated by the addition of cold TCA to a final concentration of 10%. Following a 30 min incubation at 4 °C, the precipitate was removed by centrifugation. The supernatant was extracted four times with ether to remove TCA, the pH adjusted to 7 with *N*-ethylmorpholine acetate buffer, and the extract then lyophilized. To purify the cross-link, the lyophilized proteolytic digest was dissolved in 0.2 M citric buffer, pH 2.5, (1 mL) and separated on a cation exchange resin column (Bio-Rad AG 50W-X4, 200−400 mesh, 50 × 0.9 cm), equilibrated with 0.2 M citric buffer, pH 3.31, according to the procedure Williams-Ashman et al. (*35*). A peak that eluted just between leucine and tyrosine was collected and analyzed by amino acid analysis and compared to a cross-link standard (Sigma).

Isolation of Cross-Linked Tryptic Peptide. The beaded string structure (200 mg) was suspended in 100 mL of 0.1 M Tris-HCl buffer, pH 8.0, containing 5 mM $CaCl_2$ and digested with TPCK-treated trypsin (2 mg) at 37 °C for 4 h, then TPCK−trypsin (2 mg) was added again to the digest and incubated overnight at 37 °C. The digest was clarified by centrifugation and fractionated on an ultrafiltration membrane (Amicon YM30).

After filtration the membrane retentate was washed twice with 0.1 M Tris-HCl buffer, pH 8.0 (5 mL), and then extracted with 10 mL of 0.1 M Tris-HAc buffer, pH 6.8, containing 0.1 M Na_2SO_4 and 8 M urea, to give a solution containing the bead structure (TF3). The beads were purified on a molecular sieve column (Bio-Rad TSK-4000). The fractions containing TF3 were pooled, dialyzed exhaustively against water, and lyophilized. For cross-link analysis the sample was finally purified using an affinity column prepared by coupling a fibrillin monoclonal antibody mAb-69 (*31*) to activated Sepharose.

The YM30 membrane filtrate, which contained tryptic peptides from the interbead filaments, was lyophylized, dissolved in 0.1% TFA, and chromatographed on a C18 reversed phase column (Vydac C18, 0.9 × 25 cm) at 50 °C. A 100 min gradient from 0% to 60% acetonitrile was used. Pools of every 10 fractions were made and analyzed for cross-link content. Each peak in the pool with the highest cross-link content was further purified on a molecular sieve column (BioRad, BioSil TSK-125, 600 × 7.5 mm) equilibrated at room temperature with TSK buffer. Each fraction was rechromatographed and desalted on a C18 reversed phase column (Vydac C18, 0.4 × 25 cm) at 50 °C. The amino acid composition and amino terminal amino acid sequence of each purified peptide was determined.

Capillary Electrophoresis. The purity of the sequenced cross-linked peptides was checked by capillary electrophoresis (P/ACE System 2000, Beckman) using a untreated capillary cartridge, 75 μm i.d. × 50 cm long (Beckman). The buffer used was 0.025 M sodium borate, pH 9.0. The applied voltage was 20 kV for 15 min and the column temperature 25×bcC. Eluted proteins were detected at 214 nm.

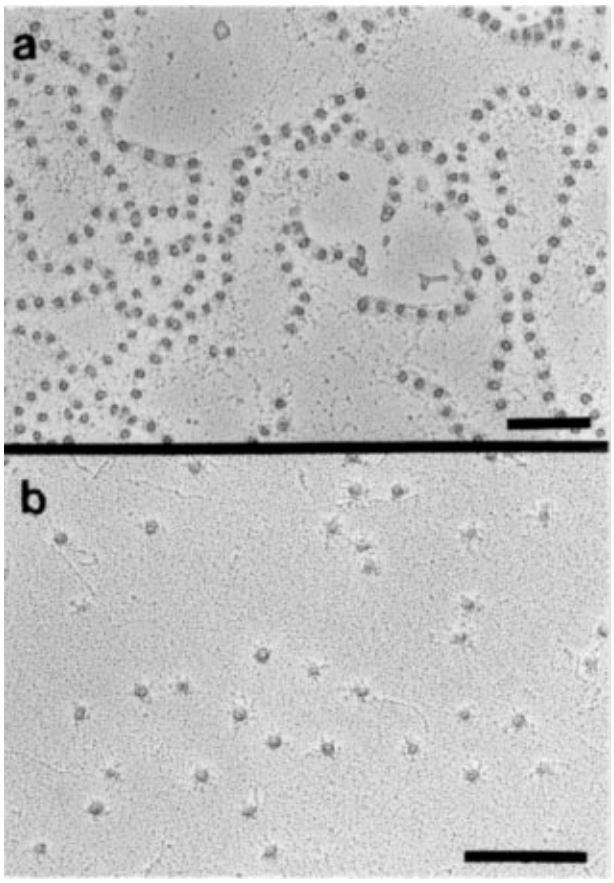

FIGURE 1: Electron micrographs of rotary shadowed preparations: (a) beaded filaments isolated from human amnion after bacterial collagenase and elastase treatment and (b) purified beaded structure isolated from a tryptic digest of the beaded filament. Bar = 200 nm.

Amino Acid Analysis and Sequencing. Amino acid analyses were carried out using phenylthiohydantoin-derivatives and the Pico Tag amino acid analyzer (Waters) with slight modifications (*36*). The amino acid sequence of the cross-linked tryptic peptide was determined using a gas-phase sequenator with standard operating programs (Applied Biosystems, Model 470A). The phenylthiohydantoin-derivatives were identified by an on-line analyzer (Applied Biosystems, Model 120A).

RESULTS

An electron micrograph of rotary shadowed beaded filaments used in these experiments is shown Figure 1a. Monoclonal antibodies mAb69 and mAb201 to fibrillin-1 and a polyclonal antibody to MAGP-1 react with this structure in ELISA. On SDS−PAGE no protein penetrates the separating gel, but on reduction several light bands are visible although the major part of the sample still cannot enter the gel. This indicates that the beaded string, which has a molecular weight of several million, is intact and held together by nonreducible covalent bonds. The appearance and properties of the beaded filament is as described previously (*30*). The scale of this procedure allowed the preparation of sufficient quantities of material for structural studies.

An indirect analysis was used to detect the presence of the ϵ-(γ-glutamyl)lysine cross-link structure. After blocking all primary amino groups in the sample with acrylonitrile,

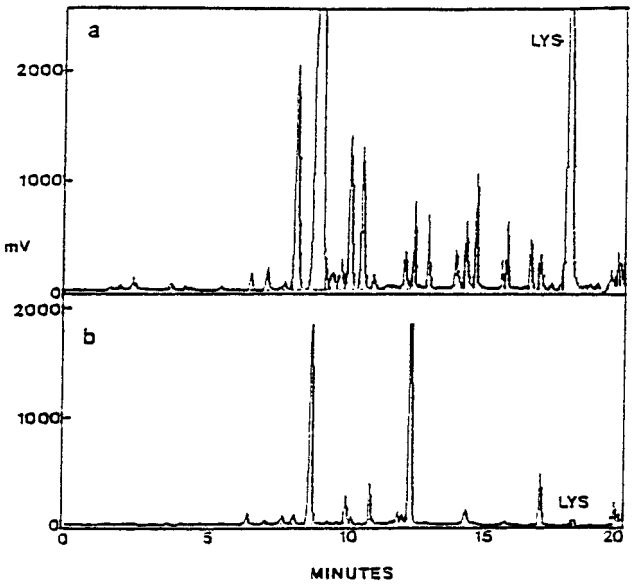

FIGURE 2: Cross-link analysis of the beaded filaments and bead structures shown in Figure 1. (a) Amino acid analysis of the basic amino acids from an acid hydrolysate of acrylonitrile treated beaded filaments. A prominent lysine peak indicated the presence of cross-links. (b) Amino acid analysis of the basic amino acids from an acid hydrolysate of acrylonitrile treated beads. No lysine was detected indicating the absence of cross-links.

the sample was hydrolyzed and the basic amino acids enriched and analyzed on an amino acid analyzer. The appearance of lysine, liberated by hydrolysis of the cross-link, indicated the presence of cross-links produced by transglutaminase in the beaded microfibril (Figure 2a). Because the beaded filament contains several proteins, at least one of which is a substrate for transglutaminase (*37*), it is not possible to calculate a molar cross-link content for the microfibrils or for fibrillin alone. Assuming that fibrillin-1 was the only component of the microfibril and using the arginine content calculated from the sequence of fibrillin-1 as a reference amino acid, it was calculated that there would be six cross-links per mole of fibrillin-1.

Initially it was thought that the cross-links would be in the beaded structure of the microfibril, so single beads were prepared by treating the microfibrils with trypsin. Two fractions were collected from the trypsin digest, one containing the beads (Figure 1b) and the second containing fragments of the rest of the interbead filamentous structure. The cross-link analyses of the single beads did not contain lysine, as shown in Figure 2b. Unexpectedly, the cross-links appeared to be in the interbead domains rather than in the beaded structure.

To isolate the cross-link structure, trypsin fragments of the interbead microfilaments were enzymatically hydrolyzed so that the cross-link structure remained intact and could be isolated. The elution position of the cross-link was known from calibrations using purchased ϵ-(γ-glutamyl)lysine. Fractions containing the cross-link were analyzed as the phenylthiocarbamyl amino acid derivative and migrated in the same position as the standard. As final proof, the isolated cross-link was hydrolyzed and analyzed on an amino acid analyzer. As expected for a genuine cross-link produced by transglutaminase, the fraction contained equimolar amounts of glutamic acid and lysine (not shown).

These analyses established the presence of cross-links produced by transglutaminase in elastic microfibrils. How-

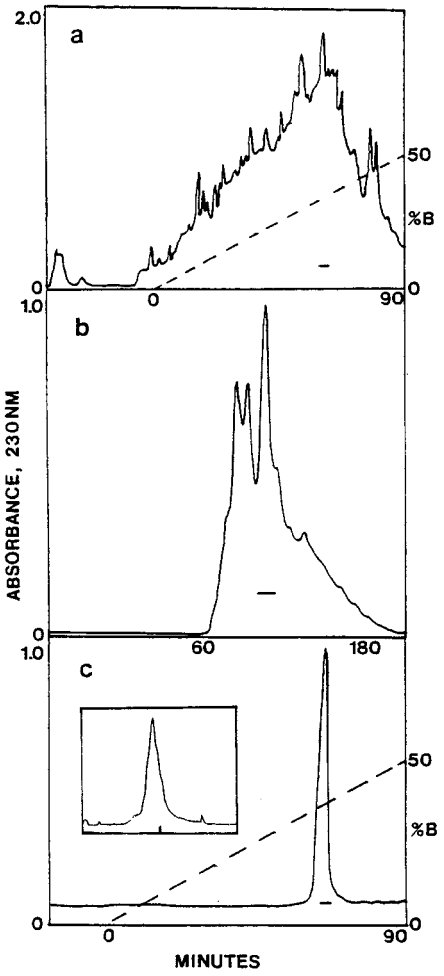

FIGURE 3: Chromatograms illustrating the purification of the cross-linked peptide. (a) Separation of a tryptic digest of the beaded structure on a C18 reverse phase HPLC column equilibrated with 0.1% TFA and eluted with 100% acetonitrile containing 0.08% TFA. (b) Separation of the pooled fractions from panel a indicated by a bar, on a TSK 2000 molecular sieve column equilibrated with 0.1 M ammonium acetate pH 6.8 (c) The pooled fractions from panel b were rechromatographed as in panel a and the indicated fractions pooled for sequence analysis. The inset shows the pool indicated in panel c analyzed by capillary electrophoresis.

ever, amino acid sequence analyses were required to determine the identity of the cross-linked proteins. A cross-linked peptide was isolated from a tryptic digest of the interbead microfilaments which had a molecular weight of 15−20 kDa (Figure 3b). All fractions of the initial reversed phase HPLC separation contained the cross-link, but the one from which the described peptide was isolated is marked (Figure 3a). After rechromatographing the fraction on a molecular sieve column (Figure 3b) and again on the reversed phase HPLC column, a symmetrical single peak was obtained (Figure 3c). The homogeneity of this fraction was further tested by capillary electrophoresis, which is a charge dependent separation. The insert in Figure 3c shows the chromatogram obtained. Although not perfectly symmetrical, it did indicate the presence of only one major component.

Sequence analysis showed that the peptide was composed of two sequences from fibrillin-1, as shown in Figure 4a. Recoveries of amino acids at positions 1, 6, and 10 are as follows: 172 pmol of N, 177 pmol of G, 213 pmol of G, 232 pmol of E; 132 pmol of N; 150 pmol of G. Other positions contain C, S, or T, which are known to have poor recoveries when sequencing. There was no other recognizable sequence present and the next largest peak in step 1 was 10% of N + G. The quantities of the amino acids in each step indicate a 1:1 ratio for the peptide chains. The locations of these sequences within the fibrillin molecule are shown in Figure 4b. Both sequences started in EGF-like domains at positions 580 and 2312. However, because of the size of the peptide, it is possible that the cross-link sites are located in the adjacent eight cysteine domains. It was not possible to sequence through the cross-link site due to the length of the peptides, and the quantity of peptide recovered was too small for further cleavages, so the exact residues involved in the formation of the cross-link could not be identified.

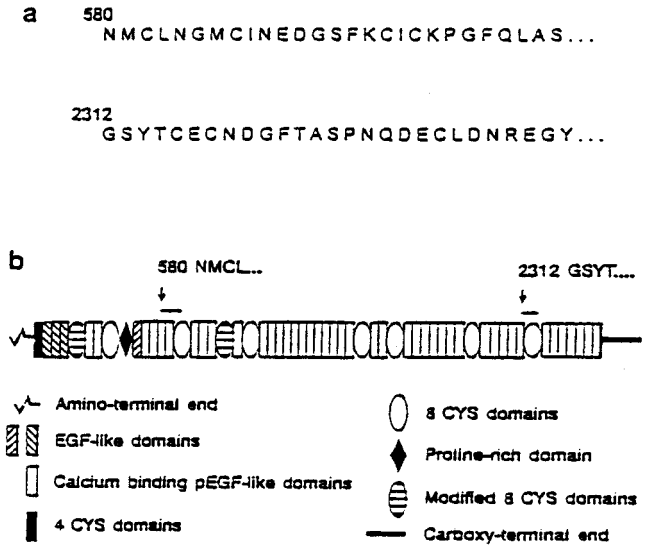

FIGURE 4: (a) Amino terminal 27 amino acids determined by sequencing the isolated cross-link peptide. Repetitive yields were 94% with a first step amount of approximately 175 pmol for each chain. Both sequences are from fibrillin-1 starting at positions 580 and 2312. (b) Schematic diagram showing the domain structure of fibrillin-1 and the approximate positions of the two determined sequences.

DISCUSSION

Transglutaminases (EC 2.3.2.13) catalyze a posttranslational modification of proteins which leads to the formation of a protease stable isopeptide bond within or between polypeptide chains. The Ca^{2+} dependent acyl-transfer reaction results in the formation of a new γ-amide bond between a γ-carboxyamide group of peptide-bound glutamine residues and a primary amine (*38*). When the primary amine is a peptide-bound lysine, an ϵ-(γ-glutamyl)lysine cross-link is formed. The specificity of transglutaminase for a particular glutamine in a protein is fairly high, but there is exceptionally low specificity for the lysine used. Nevertheless, it is still not possible to predict glutamine cross-linking sites from amino acid sequences. There are also several different types of transglutaminases with different glutaminyl substrate specificities (*39*). Stabilization of the extracellular matrices is thought to involve tissue transglutaminase which has been implicated in the cross-linking of fibrinogen (*40*), fibronectin (*41*), vitronectin (*42*), nidogen (*43*), type III collagen N-propeptide (*44*), osteonectin (*45*), and type VII collagen (*46*). Tissue transglutaminase is also involved in physiological processes such as bone growth (*47*), wound healing (*48*), and apoptosis (*49*).

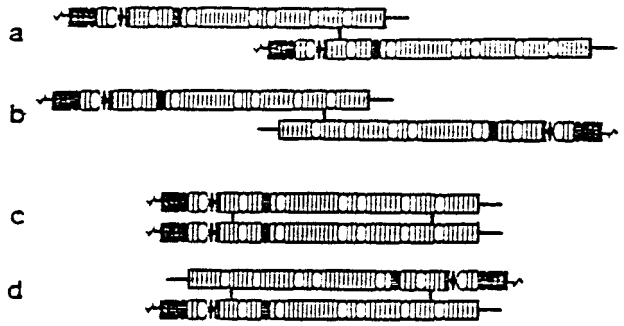

FIGURE 5: Schematic representation of the four possible alignments of two fibrillin-1 molecules. Parts a and b are staggered pairs, (a) with a parallel and (b) antiparallel arrangement. Parts c and d are aligned pairs (c) with a parallel and (d) antiparallel arrangement. Of the four possibilities only part a is consistent with both cross-link and immunological data.

The cross-link described here is certainly only one of several present in the elastic microfibril, as 10−15% of the lysine residues in the beaded filament are involved in cross-links. It connects fibrillin-1 molecules, as the determined amino acid sequences were sufficient to distinguish between fibrillin-1 and fibrillin-2. MAGP-1 contains glutamine residues that can serve as a substrate for transglutaminase (*37*), so cross-links between fibrillin and MAGP or other components are also likely. It appears therefore that fibrillin-1 is cross-linked to itself and perhaps to other elastic microfibrillar components by isopeptide bonds formed by transglutaminase. This is further supported by preliminary reports of fibrillin being modified by transglutaminase in culture dermal fibroblasts (*29*).

The distance between the beads in a relaxed structure is about 50 nm which represents about 33% of the length of the monomer. This agrees well with the overlap of 1000 amino acids dictated by the position of the cross-link described here. The number of fibrillin molecules associated with each bead is uncertain. The eight molecules illustrated here are based upon estimates of the maximum number of arms seen on beaded structures (fragment PF3) isolated from pepsin digests of amnion (*30*).

Although the exact identity of the lysine and glutamine residues involved in the cross-link were not identified, important information about the alignment of fibrillin in microfibrils could be deduced. Figure 5 shows possible alignments of pairs of fibrillin-1 monomers. The first two pairs are staggered, either in parallel with both C-termini to the right (a) or in an antiparallel fashion with a C and N terminus at each end (b). The characterized cross-linked peptide could only come from the parallel arrangement, as in the antiparallel pair the cross-linked peptides would be identical. There are also two possible arrangements for in register pairs, parallel and antiparallel, as shown in panels c and d, respectively. In this situation the antiparallel arrangement would enable the formation of the isolated cross-linked peptide, but the parallel alignment would not. Therefore both antiparallel in-register and parallel staggered alignments are consistent with the presence of the isolated cross-linked peptide. However, immunological data tell us that there is only one epitope for each of several monoclonal antibodies between the beads, so the microfibrils have a polarity because the beads in the beaded filaments are asymmetric (*30, 50*). This is caused by the N-termini of the fibrillin molecules all pointing in the same direction. The antiparallel in-register alignment is symmetrical with two epitopes between beads and therefore not consistent with observation, leaving the parallel staggered arrangement as the only possibility.

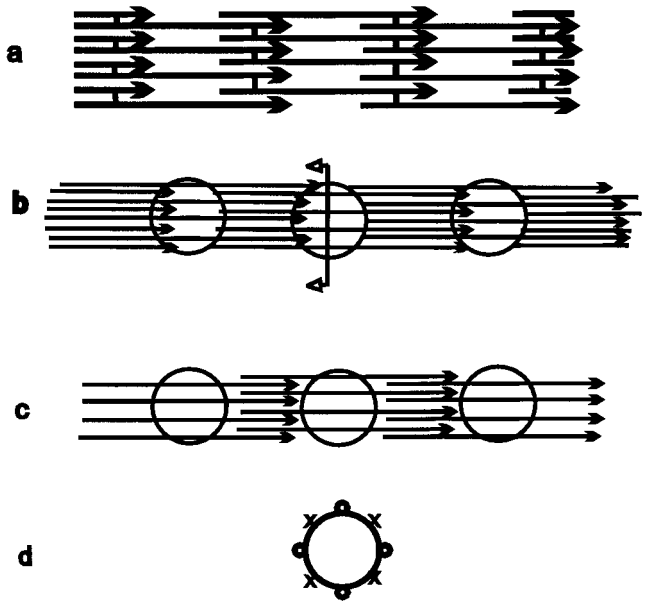

FIGURE 6: Schematic representation of the arrangement of the fibrillin-1 molecules within a beaded filament. A filament composed of bundles of four fibrillin molecules is shown (a), although the exact number present is unknown. The position of the identified cross-link in the overlap region is indicated. At least one other cross-link is required to connect all molecules. The beaded structure as visualized in Figure 1a is located between the overlapping regions of the fibrillin molecules as shown in panels b and c. In part b the ends of the molecules interact with the bead structure, whereas in part c they do not. A cross section of a filament through a bead at the position indicated in part b is shown in part d. The small circles indicate cut molecules while the crosses represent an end on view of the molecules.

Two models for the arrangement of fibrillin-1 molecules within elastic microfibrils have been previously proposed. One is based upon the position of monoclonal antibody epitopes as visualized in skin sections and extracted microfibrils using electron microscopy (*50*). It was concluded that the molecules are arranged in a head-to-tail fashion although nothing was said about the interactions of the ends of the molecules or the extent of overlap, if any. The second model is based upon the solution structure of five calcium binding EGF-like domains (*51*). The length of this unit was found to be 14.5 nm and based upon this, the length of the molecule was estimated as 130−140 nm, in good agreement with direct measurements of rotary shadowed molecules (*50*). It was reasoned that because the repeat distance of fibrillin epitopes for several monoclonal antibodies in tissue sections is about 50 nm, the fibrillin molecules in fibers, which are 140 nm long, must be overlapped by 50%. Both models rely on molecular lengths measured in tissue sections and from rotary shadowed images of extracted microfibrils and isolated molecules. As these lengths can vary depending upon the way the microfibrils or molecules are treated, and because of the inherent elasticity of the microfibrils, the figures are unreliable.

Figure 6a shows a schematic diagram of an arrangement of fibrillin molecules that is consistent with all available data and is derived independently of any length measurements. The molecules all have the same orientation and are staggered as in Figure 5a to accommodate cross-linking. Antibody

epitopes occur only once between each bead and are aligned across the microfibril, which is consistent with the banding patterns on elastic microfibrils in tissue sections stained with monoclonal antibodies (*50*). The overlap defined by the position of the cross-link is 1139 amino acids or approximately 35% of the molecule. A critical question that remains is, where are the bead structures located relative to this fibrillin framework? They could be either in the region of overlap between adjacent fibrillin monomers or between the overlap regions, but there are two observations which make the latter more likely. In a series of electron microscope studies of the vitreous humor of chicken and cow eyes, Mayne et al. were able to isolate the beaded filaments using mild extraction procedures, thus minimizing damage to the structure (*52*−*54*). When the microfibrils were rotary shadowed, they were able to see two cross-striations between each bead, and it was thought that they marked the position of a key structural element. In light of the present data, the cross-striations may mark cross-link sites and therefore indicate the presence of a second site which would enable all of the fibrillin molecules in a microfibril to be cross-linked to each other. Another observation supporting the proposed model was the "bowing out" of filaments in rotary shadowed images of beaded filaments such that a filament appeared to link every other bead (*53*). In a model in which the beads are located in the overlap regions, or if there are no staggered molecules, this would not be possible, as all molecules would have their ends associated with neighboring beads. Finally, data presented here indicate that the cross-link structures were in the tryptic fragments of the interbead filaments, which again suggests that the beads are located in the nonoverlapping region (Figure 6b,c).

There are a number of testable predictions that can be made on the basis of the proposed model. For the cross-links to form, the appropriate lysine and glutamine residues have to be aligned, suggesting that the overlapping regions of the fibrillin molecules have an affinity for one another. The central region of the fibrillin molecule should have an affinity for components of the bead. A cross-section through a bead at the position indicated in Figure 6b, is shown in Figure 6d and illustrates how the fibrillin molecules may be distributed around the bead. Irrespective of the actual number of fibrillin molecules involved, one would expect symmetry in the cross-section, which would suggest that the beaded structure is also symmetrical. The ends of the molecules are shown interacting with the bead structure in Figure 6b, although this is not necessary for the integrity of the model. It may be that one end or neither interacts as shown in Figure 6c. If the fibrillin molecules are only cross-linked in the interbead region and the center of the molecules are part of, or bind to the bead, the structure would be stable. Clearly, identifying the constituents of the bead structure is key to further elucidating the structure of the elastic microfibril.

Mutations that disrupt cross-link sites or misalign them are likely to have serious consequences for elastic microfibril formation and stability. In particular, severe forms of Marfan syndrome are caused by in-frame exon skipping, which leads to the deletion of EGF-like domains (*55*). Mutant molecules incorporated into microfibrils may have their cross-linking sites misaligned, thus preventing correct cross-link formation, leading to a weakened microfibril. Another group of Marfan patients have a severe neonatal form of the disease caused by mutations between exons 24 and 32 (*56*). This region of the molecule corresponds exactly to the nonoverlapping regions of fibrillin molecules in the proposed model, which is also the region that would interact with, or is part of, the bead structure. Mutations that disturb this domain may have drastic effects on the formation or stability of the microfibril. A knowledge of the exact locations of other cross-links will be important in determining the mechanism by which some mutations cause Marfan syndrome and also provide further insight into the three-dimensional architecture of the elastic microfibril.

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