FEBS 17034

FEBS Letters 386 (1996) 169–173

Fibrillin: evidence that chondroitin sulphate proteoglycans are components of microfibrils and associate with newly synthesised monomers

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Received 25 March 1996

Abstract We have investigated the potential association of proteoglycans with intact fibrillin-containing microfibrils from foetal bovine elastic tissues and with newly synthesised fibrillin in human and bovine cell cultures. Microfibril integrity was disrupted by chondroitinase ABC lyase and chondroitinase AC lyase, but not by keratanase or hyaluronidase. Following chondroitinase treatment, beads were disrupted but the underlying fibrillar scaffold appeared intact. Cuprolinic blue was prominently associated with beaded domains at a critical electrolyte concentration. Electron-dense rods were often associated with cuprolinic blue-treated microfibrils isolated from fixed tissues. Positive staining revealed charged foci at the beads. Newly synthesised fibrillin could be labelled with ^{35}S TransLabel, [^{3}H]glucosamine or $^{35}SO_4$ but its electrophoretic mobility was not influenced by treatment with chondroitinase ABC or AC lyase. A diffuse $^{35}SO_4$-labelled chondroitinase-sensitive component with a resistant band (M_r 35 000) co-immunoprecipitated with fibrillin. These experiments indicate that chondroitin sulphate proteoglycans associate with fibrillin and contribute to microfibril assembly. This association has major implications for microfibril function in health and disease.

Key words: Fibrillin; Microfibrils; Chondroitin sulfate proteoglycan

1. Introduction

Fibrillin-containing microfibrils are structural macromolecules which are widely distributed in elastic and non-elastic tissues [1,2]. Their critical contribution to connective tissue integrity and function was underlined by linkage of the fibrillin-1 gene (FBN1) on chromosome 15 to Marfan syndrome (MFS), a common heritable connective tissue disorder characterised by cardiovascular, skeletal and ocular defects [3-6], and its homologue (FBN2) on chromosome 5 to the related disorder congenital contractural arachnodactyly (CCA) [5,7]. Fibrillin-containing microfibrils are elastomeric polymers that appear to serve at least three functions. In elastic tissues, they act as a scaffold for elastin deposition and elastic fibre formation [8]. In non-elastic tissues, they provide structural anchorage, for example, the microfibril bundles of ciliary zonules which hold the lens in dynamic suspension [9]. Microfibrils may also directly influence cellular behaviour [10,11].

Rotary shadowing electron microscopy has highlighted the complex macromolecular organisation of the fibrillin-containing microfibrils [2,12-14]. Their morphological hallmark is pronounced beaded domains connected by interbead regions within which can sometimes be observed organised striations. Microfibrils have a diameter of 10–12 nm and average, but variable, axial periodicity of 50–55 nm. Biochemical, linkage, mutation and immunohistochemical approaches have confirmed that fibrillins are the principal structural components of microfibrils [2,6,15]. However, it is unclear whether microfibrils are simply fibrillin assemblies or complex multicomponent polymers, and the microfibril-matrix interface is also poorly defined. Distinct temporal and spatial expression patterns of fibrillin-1 and fibrillin-2 suggest microfibrillar heterogeneity in terms of fibrillin isomer content [16]. Immunohistochemical approaches have highlighted a number of potential microfibril-associated molecules; these include fibronectin, amyloid P, thrombospondin, vitronectin, and microfibril-associated glycoproteins (MAGPs, MFAPs, emilin) [2]. There are also reports of proteoglycan associations with mammalian ciliary zonules and oxytalan fibres, and with crinoid microfibrils [17-19].

We have used biochemical and ultrastructural approaches to investigate the potential relationship of fibrillin with proteoglycans, and its significance in terms of microfibril organisation. Our data, which show that chondroitin sulphate proteoglycans are components of assembled microfibrils, have major implications for microfibril organisation in health and disease.

2. Materials and methods

2.1. Materials

Bacterial collagenase (type 1A), phenylmethanesulphonyl fluoride (PMSF), *N*-ethylmaleimide (NEM), benzamidine, chondroitinase ABC lyase (EC 4.2.2.4, from *Proteus vulgaris*), chondroitinase AC lyase (EC 4.2.2.5, from *Arthrobacter aurescens*), keratanase (EC 3.2.1.103, from *Pseudomonas*) and hyaluronidase (type X, from leeches) were obtained from the Sigma Chemical Co., Poole, Dorset, UK. Sepharose CL-2B and CNBr-activated Sepharose CL-4B were supplied by Pharmacia-LKB, Milton Keynes, Bucks, UK. Cuprolinic blue was supplied by BDH Chemicals, Poole, Dorset, UK. Tissue culture medium and plastics were obtained from Gibco BRL, Paisley, UK. Mica sheets were supplied by Agar Scientific, Stansted, Essex, UK. Radioactive isotopes were from ICN, Thame, Oxon, UK. Bovine foetuses were obtained from the local abattoir within 1 h of maternal death. Two well-characterised anti-fibrillin antisera were used: one (5507) was raised to intact foetal bovine microfibrils [20], the other to a fibrillin-1 peptide [21].

2.2. Cells and cell culture

Cell cultures were established by explant. Bovine aortic smooth muscle cells were derived from second trimester foetuses and human smooth muscle cells from tissue removed during aortic root replacement surgery. Cells between passages 2 and 6 were used in labelling studies. Cells were grown in Dulbecco's minimum essential medium

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PII S0014-5793(96)00423-1

(DMEM) supplemented with 15% foetal calf serum, penicillin (400 units/ml), streptomycin 50 μg/ml), glutamine (200 μg/ml) and non-essential amino acids. Labelling was carried out in medium containing 1% foetal calf serum. Confluent cells were incubated for 16 h with 250 μCi ^{35}S-tranSlabel, 250 μCi $^{35}SO_4$ or [^{3}H]glucosamine. Cell layers were sequentially extracted in salt buffer (0.05 M Tris-HCl, pH 7.4 containing 0.4 M NaCl) and denaturing buffer (0.05 M Tris-HCl, pH 7.4 containing 8 M guanidine (HCl).

2.3. Immunoprecipitation and electrophoresis

Cell layer extracts were dialysed into phosphate-buffered saline. Prior to immunoprecipitation, 0.05% Nonidet P40 and protease inhibitors (2 mM PMSF, 5 mM NEM and 5 mM benzamidine) were added to medium and cell layer extracts. Fibronectin was removed by two incubations with gelatine-Sepharose. Fibrillin immunoprecipitations were carried out as previously described [20]. Immunoprecipitates were resolved on 8% SDS-PAGE gels under non-reducing conditions and visualised by fluorography.

2.4. Isolation of intact fibrillin-containing microfibrils

Intact fibrillin-containing microfibrils were isolated from foetal bovine nuchal ligament or aorta in the presence of protease inhibitors (2 mM PMSF, 5 mM NEM and 5 mM benzamidine) using bacterial collagenase digestion and Sepharose CL-2B size fractionation as previously described [14,20]. High-M_r material present in the excluded volume (V_o) was utilised in ultrastructural analyses.

2.5. Glycosaminoglycan (GAG) digestions

Intact microfibrils and imunoprecipitates were treated with chondroitinase ABC lyase, chondroitinase AC lyase, keratanase or leech hyaluronidase in 0.1 M Tris-HCl, pH 8.0, 0.05 M Tris-HCl, pH 7.4 containing 0.4 M NaCl and in 0.05 M sodium acetate, pH 6.0 containing 0.15 M NaCl. Microfibrils are soluble and conformationally stable in all these conditions. Protease inhibitors were added at the beginning of each enzyme incubation. Incubations were at 20 or 37°C for 4–24 h. The following enzyme: substrate ratios were used: 0.5 U of chondroitinase ABC lyase, 0.5 U chondroitinase AC lyase, 0.5 U keratanase and 5.0 U leech hyaluronidase with all immunoprecipitates and per 100 μl of V_o microfibril fraction (OD_{230}=0.150–0.250). Enzyme-treated microfibrils were also analysed by SDS-PAGE/Western blotting using anti-fibrillin antiserum.

2.6. Electron microscopy

Enzyme-treated and control V_o microfibril fractions were visualised by rotary shadowing electron microscopy using a modification of the mica sandwich technique [14,20]. For cuprolinic blue treatments, microfibrils were equilibrated in 0.025 M sodium acetate, pH 5.6 containing 0.3 M $MgCl_2$ and 0.02% cuprolinic blue for 4 h at 20°C [17,22,23] and visualised following adsorbtion onto carbon-coated grids. In some experiments, tissues were fixed for 16 h in 0.025 M sodium acetate, pH 5.6 containing 2.5% glutaraldehyde, 0.3 M $MgCl_2$ and 0.02% cuprolinic blue prior to homogenisation and rotary shadowing EM analysis of solubilised microfibrils.

3. Results

3.1. Microfibril treatments

Isolated microfibrils treated for 4–24 h with chondroitinase ABC lyase, chondroitinase AC lyase, keratanase or leech hyaluronidase in the presence of proteinase inhibitors were visualised by rotary shadowing electron microscopy (Fig. 1A–E). Chondroitinase ABC lyase and chondroitinase AC lyase treatments resulted in microfibrillar disruption and beaded morphology was progressively lost from the underlying fibrillar scaffold. Extended periodicity was a pronounced feature in some chondroitinase-treated preparations. Keratanase and hyaluronidase treatments had no visible effect on microfibril morphology. Cuprolinic blue was incorporated into beaded domains following incubation at a critical electrolyte concentration (Fig. 1F,G), and in some fields, microfibrils were surrounded by small precipitates (Fig. 1G). When cuprolinic blue-treated microfibrils were isolated following homogenisation from fixed tissues, precipitated proteoglycans were observed irregularly along the microfibrils (Fig. 1H,I); under these conditions, collagen fibrils were heavily decorated (Fig. 1J). Positive staining of isolated microfibrils with uranyl acetate (1% (w/v), pH 4.4) revealed charged foci at the beaded domains (Fig. 1K).

3.2. Immunoprecipitation of newly synthesised fibrillin

Newly synthesised fibrillin (M_r 300 000) were labelled with ^{35}S-tranSlabel, $^{35}SO_4$ or [^{3}H]glucosamine (Fig. 2, tracks 1–5). The $^{35}SO_4$ labelled immunoprecipitates also contained a strongly labelled diffuse band of M_r 110 000–160 000 (human adult SMC) (Fig. 2, track 1) or M_r 170 000–200 000 (foetal bovine SMC) (Fig. 2, track 3). The majority of ^{35}S-trans-labelled fibrillin but only 25–35% of the $^{35}SO_4$ labelled fibrillin-immunoreactive material, was present in the cell layer, predominantly in the guanidine fractions. Treatment of fibrillin immunoprecipitates with chondroitinase ABC lyase, chondroitinase AC lyase, keratanase or leech hyaluronidase did not alter the electrophoretic mobility of fibrillin (Fig. 2, tracks 7,8,10,11). However, chondroitinase ABC and AC lyase digestions degraded the diffuse $^{35}SO_4$-labelled band, yielding a resistant component of M_r 35 000 (Fig. 2, tracks 7,10,11). SDS-PAGE/Western blotting identified a pool of monomeric fibrillin solubilised by chondroitinase treatment of isolated microfibrils (Fig. 2, tracks 12–17).

4. Discussion

The fibrillin-containing microfibrils are structurally complex and their molecular composition and organisation is ill-defined despite intensive research efforts. Previous studies, using reducing and denaturing extraction conditions, have identified a number of microfibril-associated molecules [2]. However, many unanswered questions remain concerning microfibril composition and in particular, the relative contributions of the two fibrillin isomers, potential structural roles for microfibril-associated proteins, and the relationship of fibrillin and proteoglycans. In this investigation, we have used intact native microfibrils to address the question of whether proteoglycans are components of assembled native microfibrils.

The disruption of microfibrillar beaded organisation following removal of chondroitin sulphate without loss of the underlying fibrillar scaffold suggests that chondroitin sulphate is associated with the beads, and this is supported by beaded localisation of cuprolinic blue at a critical electrolyte concentration. The extended periodicity apparent in many chondroitinase-treated microfibrils suggests that chondroitin sulphate removal allows conformation changes in the fibrillin scaffold. Cuprolinic blue-precipitates irregularly associated with microfibrils from fixed tissues probably reflect the in vivo association of large proteoglycans with formed microfibrils. We and others have previously reported difficulties in resolving fibrillin monomers from tissue extracts. This is thought to reflect progressive formation of reducible and non-reducible cross-links through development [14,24]. The release of free fibrillin monomers following chondroitinase-digestion of foetal microfibrils confirms a chondroitin sulphate association with fibrillin in 'immature' tissue extracts.

Fibrillin interacted with a chondroitin sulphate-containing proteoglycan in cell culture medium and cell layers, but was

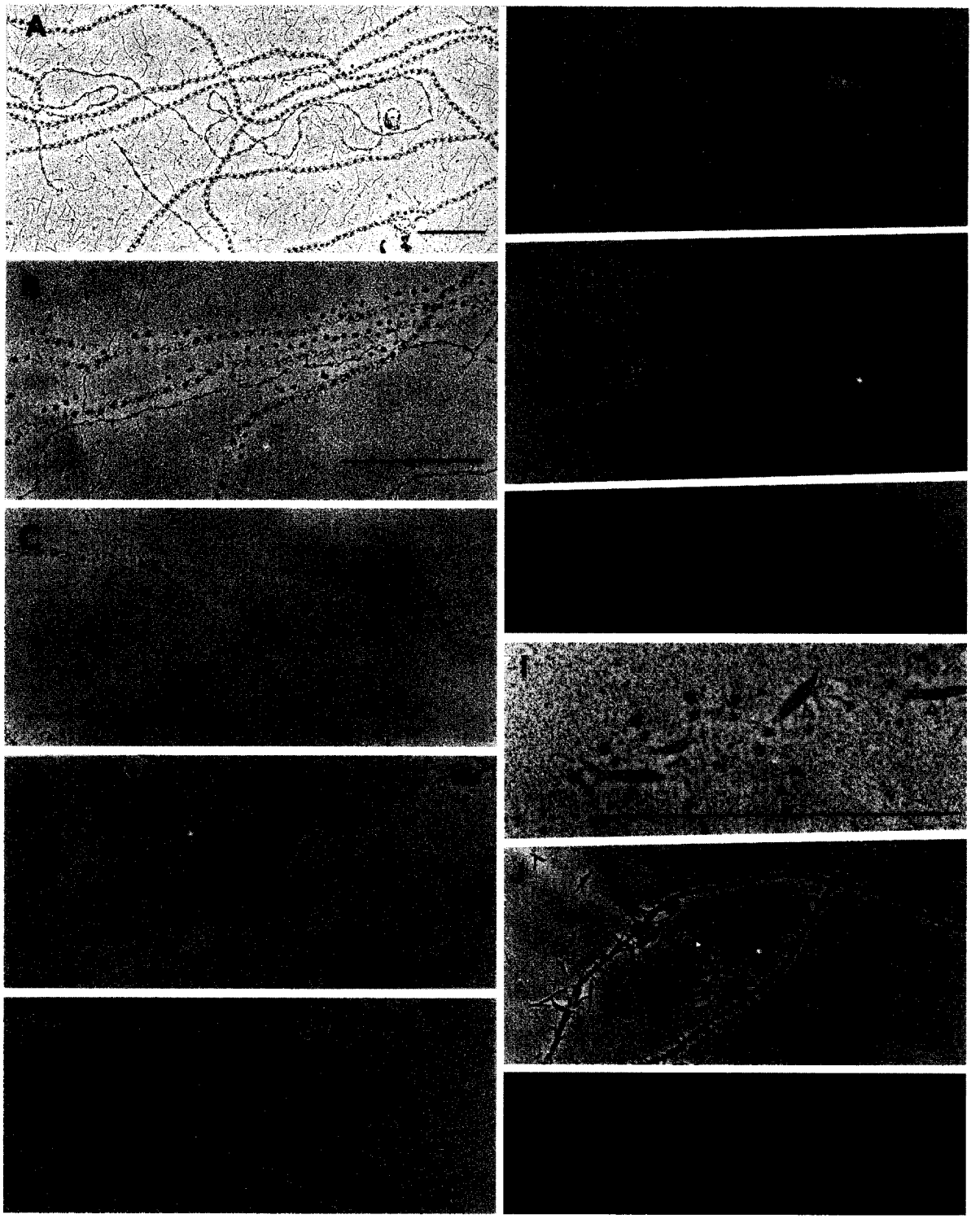

Fig. 1. Electron micrographs of fibrillin-containing microfibrils isolated from foetal bovine nuchal ligament. (A–E,I,J) Rotary shadowed at an angle of 4°; (F–H,K) unshadowed. (A–G,K) Isolated unfixed microfibrils; (H–J) from fixed tissues. (A) Untreated control microfibril preparation; (B) chondroitinase ABC lyase, 8 h, 37°C; (C) chondroitinase ABC lyase, 16 h, 37°C; (D) chondroitinase AC lyase, 8 h, 37°C; (E) chondroitinase AC lyase, 16 h, 20°C; (F,G) microfibrils incubated with cuprolinic blue at 0.3 M $MgCl_2$; (H) unshadowed cuprolinic blue-treated microfibrils from fixed tissue; (I) rotary shadowed cuprolinic blue-treated microfibril from fixed tissue; (J) rotary shadowed cuprolinic blue-treated collagen fibril from fixed tissue; (L) microfibrils positively stained with 1% (w/v) uranyl acetate pH 4.4. In (G–I), beaded domains are highlighted with solid triangles, and associated precipitates with open triangles. Chondroitinase treatment disrupted microfibrils with loss of beaded morphology. Cuprolinic blue was associated with beaded domains of unfixed microfibrils, and additional precipitated GAGs were identified in association with fixed microfibrils. Bars = 500 nm.

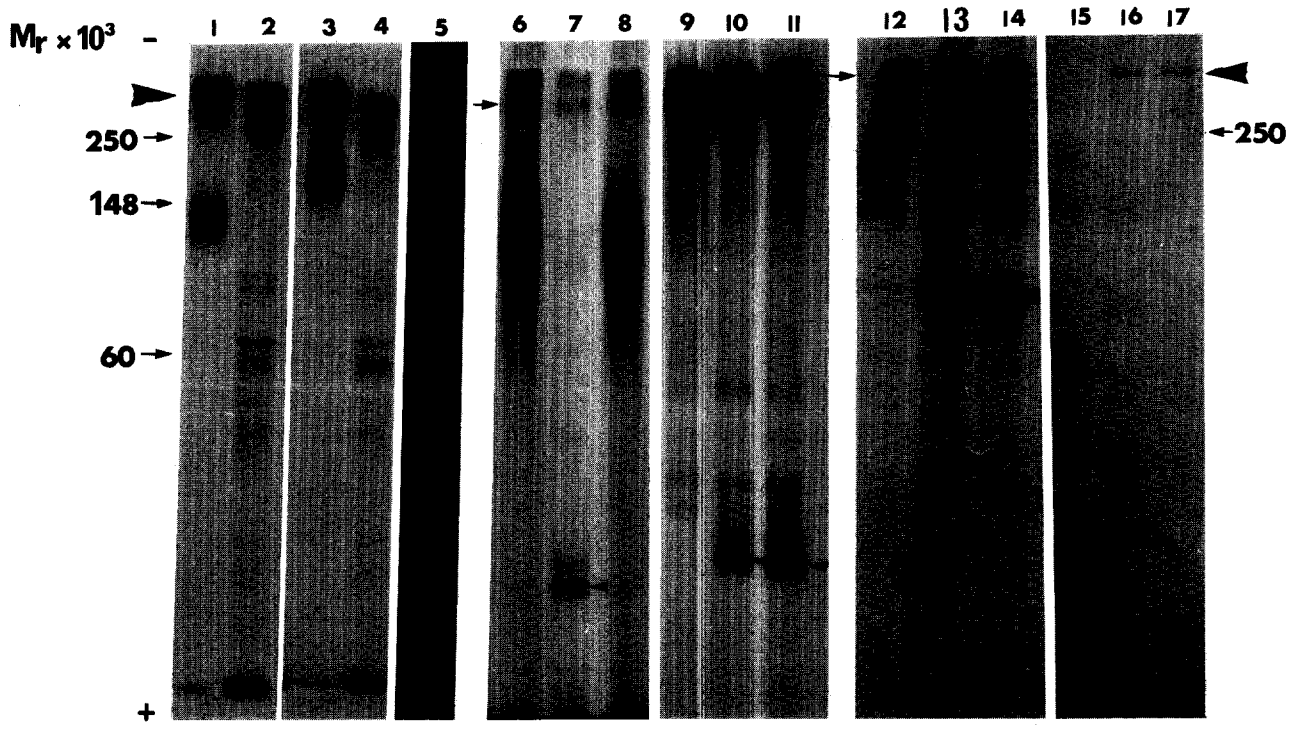

Fig. 2. Electrophoretic analysis of fibrillin immunoprecipitated from cell culture medium and extracted from foetal bovine nuchal ligament microfibrils. Tracks: (1–11) immunoprecipitates; (12–17) tissue-isolated microfibril extracts. Gels were run under non-reducing conditions. The electrophoretic mobilities of molecular weight markers myosin (M_r 250 000), phosphorylase *b* (M_r 148 000) and glutamate dehydrogenase (M_r 60 000) are indicated; (1,2,6–8) human smooth muscle cells; (3–5,9–11) foetal bovine smooth muscle cells; (12–17) microfibril extracts from foetal bovine nuchal ligament. Cells were labelled for 16 h; (1,3,6–8) cells labelled with $^{35}SO_4$; (2,4,9–11) cells labelled with ^{35}S-tranSlabel; (5) cells labelled with [^{3}H]glucosamine; (7,10) chondroitinase ABC lyase-treated; (11) chondroitinase AC lyase-treated; (8) leech hyaluronidase-treated. Fibrillin and a diffuse lower M_r chondroitinase-sensitive component were immunoprecipitated from $^{35}SO_4$-labelled cells; (12–14) Coomassie blue stained gel; (15–17) Western blotting using anti-fibrillin antiserum (5507) with detection by enhanced chemiluminescence; (12,15) control microfibril extracts; (13,16) chondroitinase ABC lyase-treated microfibrils; (14,17) chondroitinase AC lyase-treated microfibrils. Chondroitinases contain carrier protein (+).

not itself degraded by GAG-degrading enzyme treatments. This supports the ultrastructural observation that the fibrillin scaffold remains following GAG removal. The incorporation of *N*-glucosamine into newly synthesised fibrillin probably arises form the presence of N-linked oligosaccharides on fibrillin, whilst sulphation is more likely to reflect tyrosine modification than GAG attachment since there was no discernable change in fibrillin mobility on treatment with GAG-degrading enzymes.

The composition and organisation of the beaded domains highlighted by rotary shadowing EM has long been the subject of speculation and analysis. Previous studies by ourselves and others have shown that digestion with trypsin or pepsin generates beads containing fibrillin sequences together with fragmented cross-linked interbead-derived fibrillin [25,26]. The pronounced appearance of the beads following rotary shadowing may be due to attraction of metal ions to foci of charge arising largely from the presence of GAGs, although *N*- and *O*-linked oligosaccharides and fibrillin residues may also contribute.

In summary, we have shown that a chondroitin sulphate-containing proteoglycans associates with fibrillin and contributes fundamentally to microfibril organisation. Neither the nature of the proteoglycan nor whether it associates with fibrillin through its protein core or GAG chain(s) is known. Our biochemical data implicate the small proteoglycan family that includes decorin, biglycan and fibromodulin. Interestingly, there are reports of altered expression patterns of decorin in some cases of neonatal Marfan syndrome associated with microfibril disruption [27,28]. We speculate that microfibril formation proceeds through several stages including fibrillin alignment, cross-linking, association with proteoglycans and formation of beaded microfibrils. These insights into microfibril assembly have important implications for structure: function relationships in health and disease.

Acknowledgements: This work was funded by the Medical Research Council (UK).

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