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Fibrillin-rich microfibrils: elastic biopolymers of the extracellular matrix

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Abstract

Fibrillin-rich microfibrils are evolutionarily ancient macromolecular assemblies of the extracellular matrix. They have unique extensible properties that endow vascular and other tissues with long-range elasticity. Microfibril extensibility supports the low pressure closed circulations of lower organisms such as crustaceans. In higher vertebrates, microfibrils act as a template for elastin deposition and are components of mature elastic fibres. In man, the importance of microfibrils is highlighted by the linkage of mutations in their principal structural component, fibrillin-1, to the heritable disease Marfan syndrome which is characterised by severe cardiovascular, skeletal and ocular defects. When isolated from tissues, fibrillin-rich microfibrils have a complex ultrastructural organisation with a characteristic 'beads-on-a-strong' appearance. X-ray fibre diffraction studies and biomechanical testing have shown that microfibrils are reversibly extensible at tissue extensions of 100%. Ultrastructural analysis and 3D reconstructions of isolated microfibrils using automated electron tomography have revealed new details of how fibrillin molecules are aligned within microfibrils in untensioned and extended states, and delineated the role of calcium in regulating microfibril beaded periodicity, rest length and molecular organisation. The molecular basis of how fibrillin molecules assemble into microfibrils, the central role of cells in regulating this process, and the identity of other molecules that may coassemble into microfibrils are now being elucidated. This information will enhance our understanding of the elastic mechanism of these unique extracellular matrix polymers, and may lead to new microfibril-based strategies for repairing elastic tissues in ageing and disease.

Introduction

Tissue flexibility and elasticity have been essential requirements in the evolution of multicellular organisms. These properties allow transport of fluids, nutrients and oxygen, underpin movement, and endow an ability to respond to changing environment. Tissue elasticity is based on fibrillin-rich microfibrils and elastic fibres, insoluble extracellular matrix assemblies that permit long-range deformability and recoil. These properties are critical to the function of dynamic tissues such as blood vessels, lungs, skin and eyes. The biology of microfibrils has proved difficult to unravel because they are multi-component polymers with a hierarchical assembly and a complex molecular organisation that defines their unique elastomeric properties.

There is currently intense interest in the biology of the unique fibrillin-rich microfibrils of the ECM. This interest is driven by the need to understand the basis of tissue elasticity and genotype-to-phenotype correlations in heritable connective tissue diseases associated with mutations in fibrillin genes, and the possibility of exploiting their biomechanical properties in tissue engineering and wound repair strategies. Here we outline current knowledge of microfibril organisation, composition and elasticity, a molecular folding model of the elastic 'motor', and implications of loss of microfibril integrity in dynamic connective tissues in ageing and disease.

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Fibrillin-rich microfibrils

Isolated fibrillin-rich microfibrils have a unique and complex repeating beaded structure with an untensioned periodicity of ~56 nm (Sherratt *et al.*, 2001) (Figure 1A). Early indications that they have unique elastic properties were obtained from electron microscopy images that showed both untensioned and stretched regions (Keene *et al.*, 1991) (Figure 1B). Since then, studies of isolated microfibrils and microfibril bundles, and of hydrated tissues such as the ciliary zonules of the eye and crustacean aorta that are based on microfibril bundles, have confirmed and further delineated the elastic properties of microfibrils.

Fibrillins, the principal structural molecules, are encoded by three genes (Pereira *et al.*, 1993; Zhang *et al.*, 1994; Nagase *et al.*, 2001). They are large glycoproteins (~350 kDa) with calcium-binding epidermal growth factor-like (cbEGF) domain arrays that, in the presence of Ca^{2+}, adopt a rod-like arrangement (Downing *et al.*, 1996) (Figure 2A). These domains are interspersed with 8-cysteine motifs (also called 'TB' modules because of their homology to TGFβ binding modules in latent TGF-β binding proteins). Towards the fibrillin-1

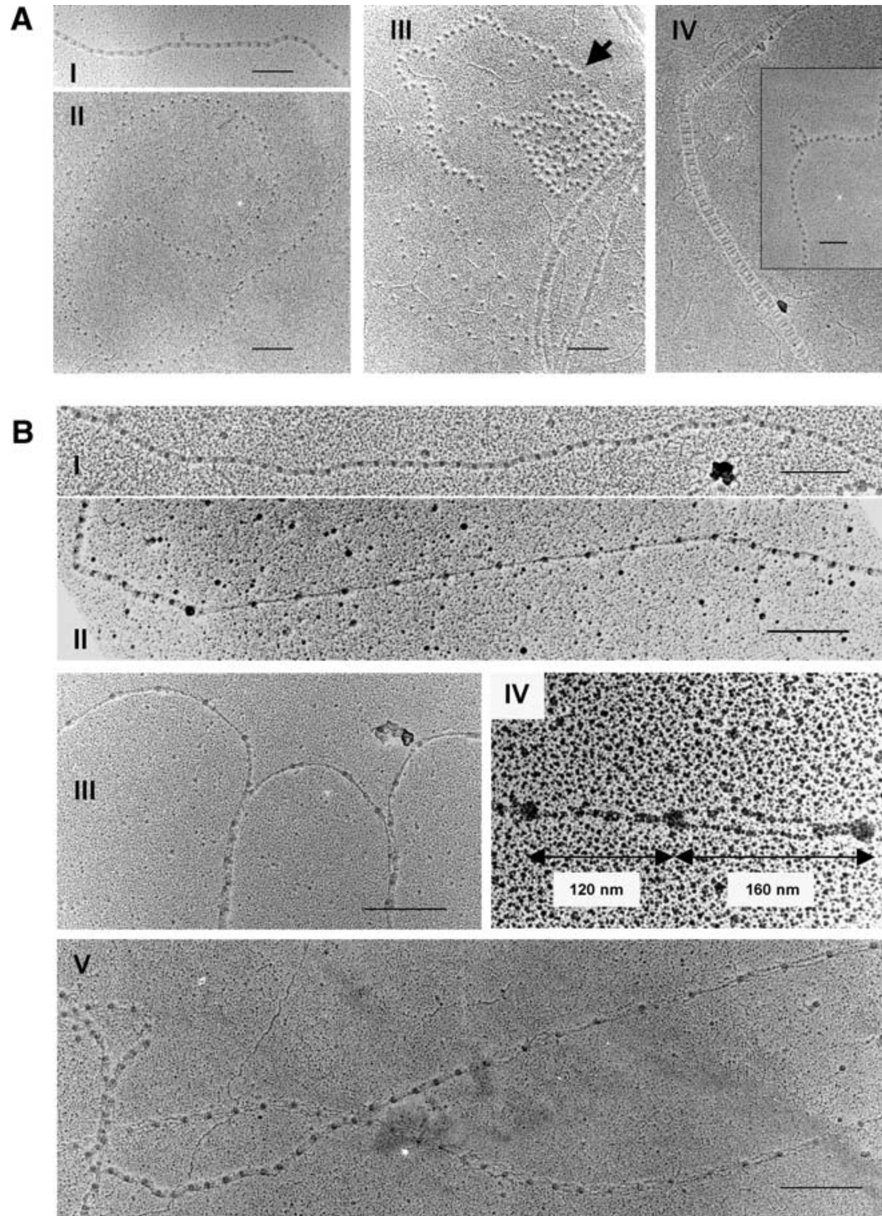

Fig. 1. Rotary shadowing electron micrographs of fibrillin-rich microfibrils isolated from foetal bovine skin or nuchal ligament, showing their characteristic repeating beaded structure. Panel A: (I) Microfibril with ~56 nm periodicity and 'closed' organisation. (II) Microfibril with ~56–60 nm periodicity and 'open' organisation. (III) After treatment with 5 mM EDTA, some microfibrils have reduced periodicity and increased flexibility (Wess *et al.*, 1998a). In some extracts, another population of beaded microfibrils is also apparent that has a more extended appearance. Collagen fibrils are also present. (IV) Invertebrate echinoderm dermis extract, showing a collagen fibril and (inset) a beaded fibrillin-rich microfibril. Panel B: (I) Microfibril with ~56 nm periodicity and 'closed' organisation. (II–V) Microfibrils with stretched regions (periodicities >100 nm). In (IV and V), highly stretched regions appear to comprise defined filaments.

N-terminus is a proline (pro)-rich sequence that may act as a 'hinge'; fibrillin-2 has a glycine (gly)-rich sequence, and fibrillin-3 a pro- and gly-rich sequence. Transglutaminase cross-links between fibrillin peptides have been identified that probably influence microfibril stability and elasticity (Qian and Glanville, 1997). Fibrillin-1 and fibrillin-2 have distinct, but overlapping expression patterns, with fibrillin-2 generally expressed earlier in development than fibrillin-1 (Zhang *et al.*, 1995). It is likely that microfibrils in some tissues may be assembled from different fibrillin isoforms. Determination of the molecular mechanism underlying the remarkable ability of fibrillin molecules, once assembled, to extend and retract is a major goal in microfibril biology (Figure 2B).

Fibrillin molecules and assembled microfibrils are remarkably well conserved throughout multicellular evolution from medusa jellyfish (*Podocoryne carnea* M. Sars), sea cucumber (*Cucumaria frondosa*) and other invertebrates, to man (Pereira *et al.*, 1993; Zhang *et al.*, 1994; Reber-Muller *et al.*, 1995; Thurmond and Trotter, 1996; Nagase *et al.*, 2001) (Figure 1A). In all these organisms, the tissue and developmental distributions of fibrillins correlate closely with the biomechanical needs

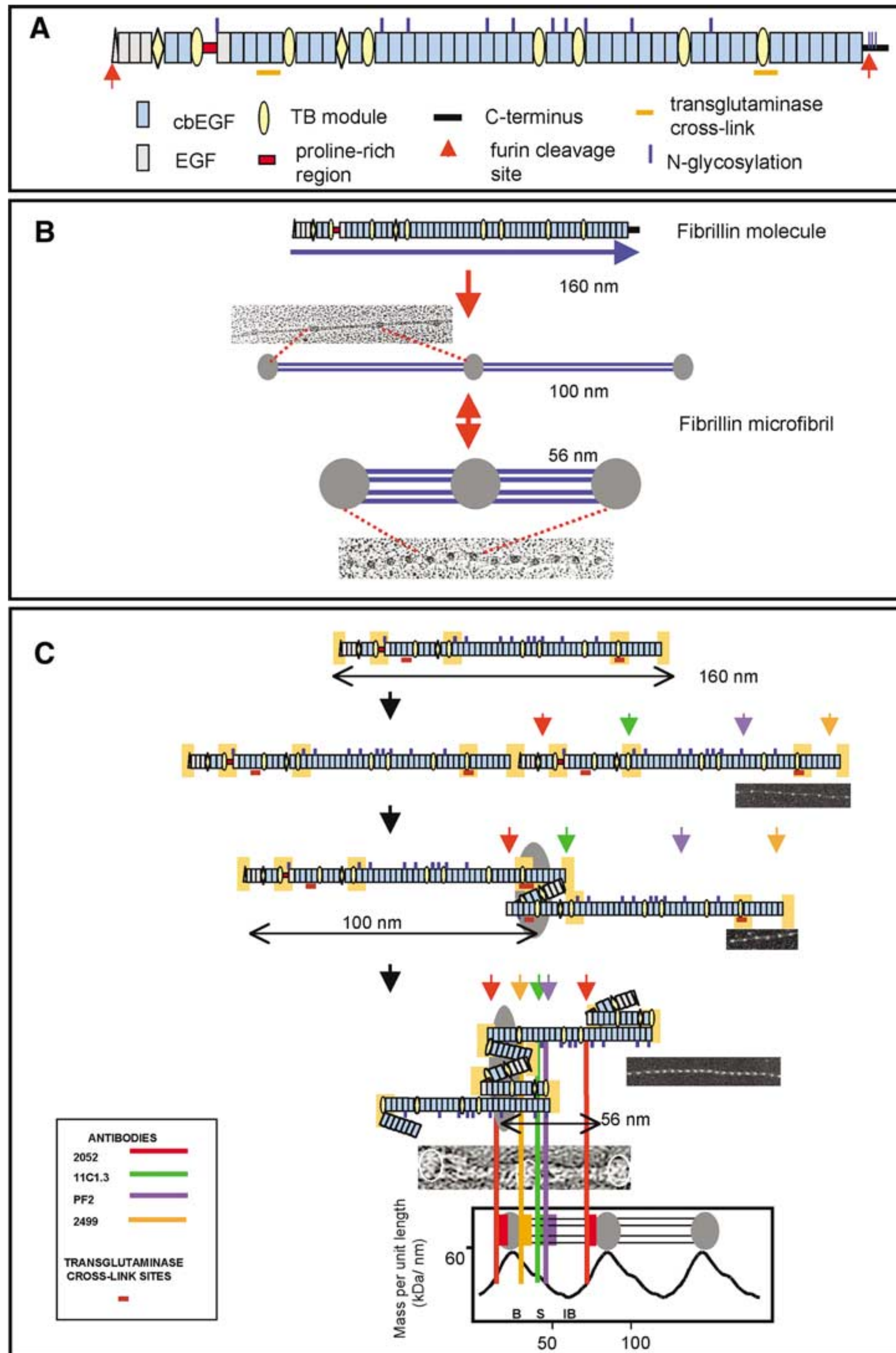

Fig. 2. Schematic representation of the fibrillin-1 molecule and its assembly into extensible microfibrils. Panel A: Domain structure of fibrillin-1, highlighting how contiguous arrays of cbEGF domains are interspersed with TB modules, and the locations of the proline-rich region, N-glycosylation consensus sequences and transglutaminase cross-link sites. Panel B: Schematic diagram outlining that fibrillin-1 molecules assemble to form beaded microfibrils with variable periodicity. Panel C: Schematic diagram depicting a possible folding arrangement of fibrillin molecules in a beaded microfibril (Baldock *et al*., 2001). The model predicts that two N and C terminally processed molecules associate head-to-tail to give ~160 nm periodicity. Subsequent molecular folding events could generate ~100 nm periodicity, then ~56 nm periodicity. The model is supported by AET, STEM data, antibody localisations, and microfibril extension studies. Whilst predicted fold sites are shown (orange boxes), it should be noted that the precise packing arrangement of folded segments contributing to the bead remains unresolved. Inset are STEM images of microfibrils with periodicities corresponding to those predicted. Observed antibody binding sites are overlaid on the ~56 nm periodic folding arrangement. The axial mass distribution of ~56 nm microfibrils (shown at the bottom) correlates well with the corresponding predicted molecular folding. This figure is reproduced, with minor modifications, from Kielty et al. (2002).

of the organism. Microfibrils, which are considerably more ancient than elastin (a vertebrate protein), have conferred long-range elasticity to connective tissues for at least 550 million years. Elastin evolved more recently

to reinforce high pressure closed circulatory systems in which interplay between highly resilient elastin and stiff fibrillar collagen is a critical feature (Faury, 2001). Elastic fibrillogenesis is a highly developmentally regulated process in which tropoelastin (the soluble precursor of mature elastin) is deposited on a preformed template of fibrillin-rich microfibrils (Mecham and Davis, 1994). Mature elastic fibres are thus a composite biomaterial comprising an outer microfibrillar mantle and an inner core of amorphous cross-linked elastin. The association of microfibrils with elastin undoubtedly modulates their biomechanical properties (Faury, 2001). In the low pressure circulatory system of the lobster (arterial pressure 3/1 to 16 mmHg), aortic elasticity is due almost entirely to microfibrils which reorientate and align at lower pressures then deform and elongate at higher pressures (McConnell *et al*., 1996). The distinct mechanical properties of aorta in the human high pressure closed circulation (120/80 mmHg) are due to the additional presence of elastin, a resilient rubber-like protein which, together with microfibrils, forms elastic fibres (elastin and microfibrils) that can keep their elastic properties up to 140% extension. Microfibrils also contribute important biomechanical properties to tissues that do not express elastin, e.g. the ciliary zonules that hold the lens in dynamic suspension (Ashworth *et al*., 2000), which emphasises their independent evolutionary function.

Involvement of microfibrils in ageing changes and connective disease diseases

Microfibrils and elastic fibres are designed to maintain elastic function for a lifetime. Loss of connective tissue elasticity is due, in large part, to degeneration of the microfibrillar and elastic fibre network. Degradation of these extracellular polymers is a major contributing factor in blood vessel ageing and development of aortic aneurysms, in lung emphysema, and in degenerative changes in sun-damaged skin. In ageing eyes, degeneration of microfibrils contributes to reduced elasticity of the ciliary zonules. Fibrillin molecules and microfibrils are susceptible to degradation by various matrix metalloproteinases (MMPs) and serine proteinases (Kielty *et al*., 1994; Ashworth *et al*., 1999a), and accumulative proteolytic damage is a major mechanism underlying degenerative changes in ageing. MMP treatment of isolated microfibrils can compromise the regular periodic repeat structure and extensible properties of microfibrils (Sherratt *et al*., 2001) (Figure 3). Some of the major fibrillin-1 proteolytic cleavage sites have been identified (Hindson *et al*., 1999), and they may provide clues to the elastic mechanism of microfibrils. The importance of microfibrils and elastic fibres is also highlighted by the severe heritable connective diseases that are caused by mutations in elastic fibre molecules (for recent reviews, see Milewicz *et al*., 2000; Robinson and Godfrey, 2000). Fibrillin-1 mutations cause Marfan syndrome which is associated with severe cardiovascular, ocular and skeletal defects, and fibrillin-2 mutations cause congenital contractural arachnodactyly with overlapping skeletal and ocular symptoms. Elastin mutations cause Williams syndrome, supravalvular stenosis, and cutis laxa (Tassabehji *et al*., 1998; Milewicz *et al*., 2000).

Microfibril organisation

Isolated untensioned microfibrils from mammalian tissues such as amnion, skin and ligament have a repeating

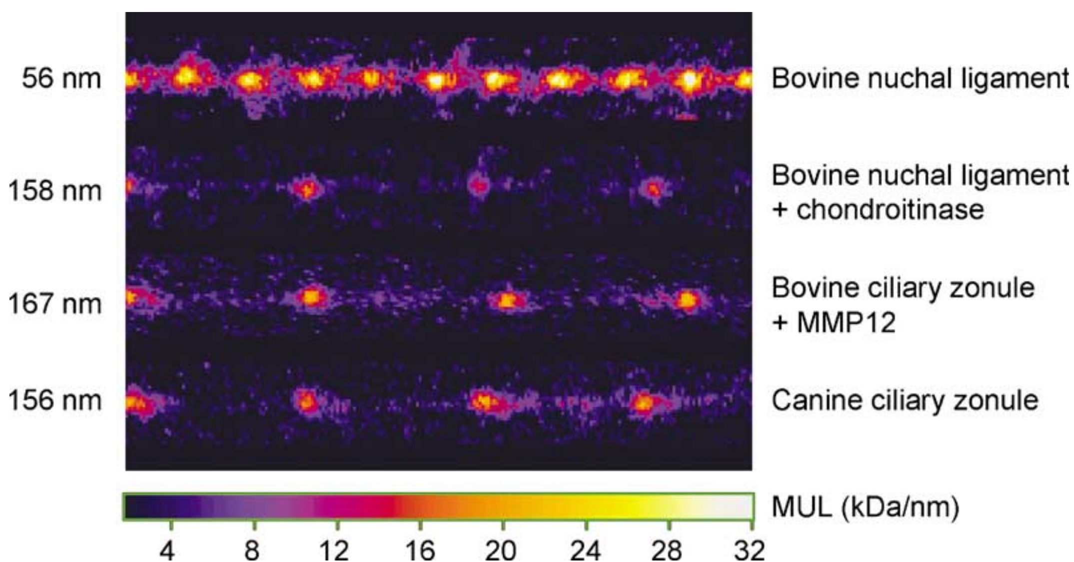

Fig. 3. STEM mass images of isolated fibrillin-rich microfibrils, showing some stretched areas. Bovine nuchal ligament microfibrils were either untreated or pre-incubated with chondroitinase ABC lyase. Bovine ciliary zonule microfibrils were pre-incubated with MMP-12. Zonular filament microfibrils were also examined from a canine ectopia lentis model. In many (but not all) regions of the treated and ectopia lentis microfibrils, highly stretched sections were apparent.

'beads-on-a-string' appearance with an average, but variable, periodicity of 56 nm (Sherratt *et al*., 2001) (Figure 1). In tissues, microfibrils are generally organised into loose, roughly parallel bundles (Figure 4A). X-ray fibre diffraction of hydrated zonular microfibril bundles has identified staggered 'junctions' that may modulate force transmission (Wess *et al*., 1998a). In elastic tissues, microfibril bundles form the template for

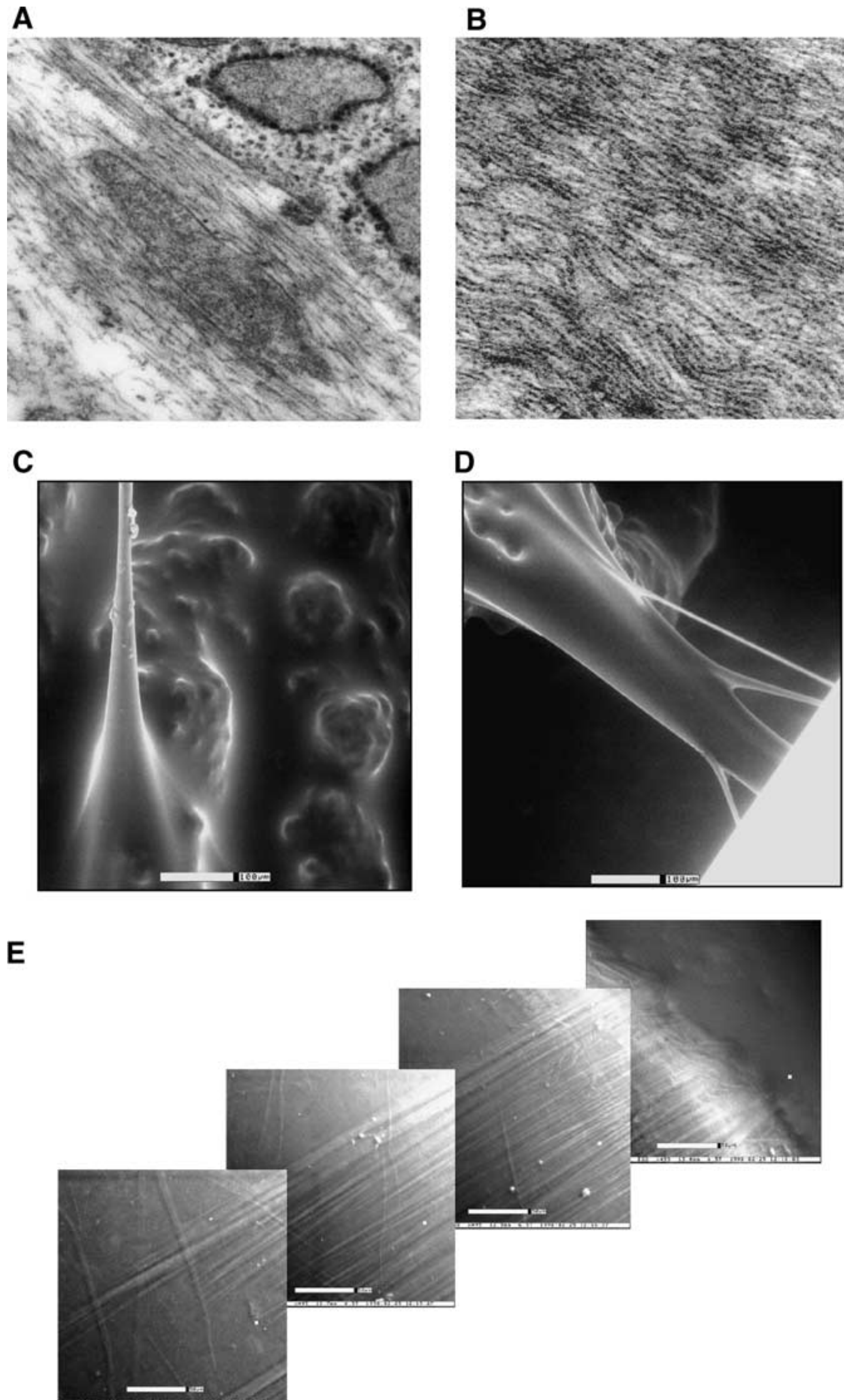

Fig. 4. Tissue microfibril organisation. Panel A: Microfibril bundles are laid down close to the surface of a bovine nuchal ligament fibroblast. Panel B: Microfibrillar array from lobster aorta. This figure is reproduced, with minor modification, from Kielty *et al*. (2002). Panels C and D: Environmental scanning electron microscopy (ESEM) of the posterior aspect of human zonules running from the ciliary process to the lens capsule (68 year old male). Panel C shows the origin of the zonular fibre from the ciliary process. Panel D shows branching of the zonular fibres as they approach the lens capsule. The scale bars = 100 μm. Panel E: Composite ESEM of human zonular filaments aligned on the surface of the lens capsule (68 year old male). The scale bars = 100 μm.

elastin deposition, and in this way influence the orientation and 3D structural architecture of the developing elastic fibre (Mecham and Davis, 1994). Distinct tissues can have profoundly different elastic fibre organisations that reflect specific functions. Thus, elastic fibres are arranged in concentric lamellae in elastic arteries, as networks in deformable auricular cartilage and in lung alveoli that provide elasticity during breathing, and in skin they impart elasticity through a continuous elastic fibre system from the microfibrillar bundles (oxytalan fibres) at the dermal–epidermal junction to the horizontal thick elastic fibres in the reticular dermis. In mature elastic fibres, microfibrils are apparent as an outer mantle, with some microfibrils embedded within the elastin core. The elastic properties of microfibrils may be modified both by inter-microfibrillar interactions within microfibril bundles and by association with elastin in elastic fibres.

Details of the alignment of fibrillin molecules within microfibrils have emerged from ultrastructural studies of isolated microfibrils. Rotary shadowing studies of antibody-binding sites on partially extended microfibrils indicated a parallel head-to-tail alignment of fibrillin molecules in microfibrils (Reinhardt *et al.*, 1996a, b). Automated electron tomography (AET) has provided further insights into the complex organisation of untensioned fibrillin-rich microfibrils, and the alignment of fibrillin molecules in the untensioned state (Baldock *et al.*, 2001). AET-generated 3D reconstructions at 18.6 Å resolution showed that twisting occurs within untensioned microfibrils and revealed new details of bead and interbead organisation. Localisation of antibody and gold-binding epitopes, and mapping of bead and interbead mass changes in untensioned and extended microfibrils provided compelling evidence that fibrillin molecules undergo significant intramolecular folding within each untensioned beaded repeat (Baldock *et al.*, 2001). In addition, STEM analysis defined microfibril mass and its axial distribution, and predicted up to eight fibrillin molecules in cross-section.

Microfibril extensibility

Invertebrate studies

Important insights into microfibril elasticity have emerged from invertebrate studies. Microfibril networks extracted from sea cucumber dermis using guanidine and bacterial collagenase were shown, by tensile testing, to be reversibly extensible up to approximately 300% of their initial length (Thurmond and Trotter, 1996). These networks behaved like viscoelastic solids, with a long-range elastic component as well as a time-dependent viscous component. The strength of the network appeared to be due to non-reducible cross-links, but its elasticity was dependent on disulphide bonds. The modulus of elasticity of lobster (*Homarus americanus*) aorta, a viscoelastic tissue based on microfibril bundles and smooth muscle cells, was found to be similar to that of the rubber-like protein elastin (0.1–1.2 MPa, commonly in range 0.4 MPa), (McConnell *et al.*, 1996). Lobster aorta elastic properties are non-linear; low stiffness allows arterial volume to increase, but then stiffer vessels correspond with arterial volume plateauing. Further assessment of this system revealed that microfibrils alone characterise the stress–strain behaviour of the vessel, when initial reorientation and subsequent deformation are accounted for (McConnell *et al.*, 1997).

X-ray fibre diffraction

Small-angle X-ray fibre diffraction has provided valuable insights into the organisation and elasticity of hydrated ciliary zonules (see Figure 4B for scanning image of zonules). A key advantage of these studies is that a statistically significant number of molecular conformations can be examined in hydrated intact tissues. This technique can thus reveal molecular features that cannot be readily examined by microscopic techniques, due to disruption of supramolecular organisation in sample preparation.

X-ray examination of hydrated bovine ciliary zonules revealed meridional diffraction peaks indexing on a fundamental periodicity of ~56 nm in the relaxed state (Wess *et al.*, 1997, 1998a, b). The strength of the third and sixth order Bragg reflections in the resting state suggested that a specific alignment or staggering of adjacent microfibrils gives rise to electron dense contrast at periodicities of one-third of the axial cell length. Remarkably similar results have now been obtained with zonular filaments from the eyes of pig, roe deer and sheep. The data from ovine eye are presented in Figure 5. All zonules exhibited strong third and sixth orders and comparable axial periodicity (pig (54.9 nm, data not shown), roe deer (56.2 nm, data not shown) and sheep (55.0 nm)) in the resting state, confirming a high degree of inter-species structural homology.

In bovine zonules, the application of a relatively low strain (up to 50% tissue extension) produced a minor reversible lengthening of periodicity by only 4%, as judged by alteration of the D spacing of the principal peaks (Wess *et al.*, 1997, 1998b). In contrast, further extension (to 100% of tissue rest length) produced a higher periodicity, indicating disruption of the supramolecular structure. The fibrillin diffraction image also contained an equatorial diffraction peak that was enhanced upon tissue extension. This peak estimated the nearest approach of microfibrils in the hydrated state under tension to be 28 nm.

Further X-ray diffraction studies have now investigated changes in the supramolecular packing of highly extended ovine zonular filaments (Figure 5). Samples were exposed to X-rays for 12 min in the native state and then in a fully extended state (150% length extension from resting state). Subsequently the tissue was relaxed back to the rest length and re-analysed. The results obtained in this experiment are comparable to

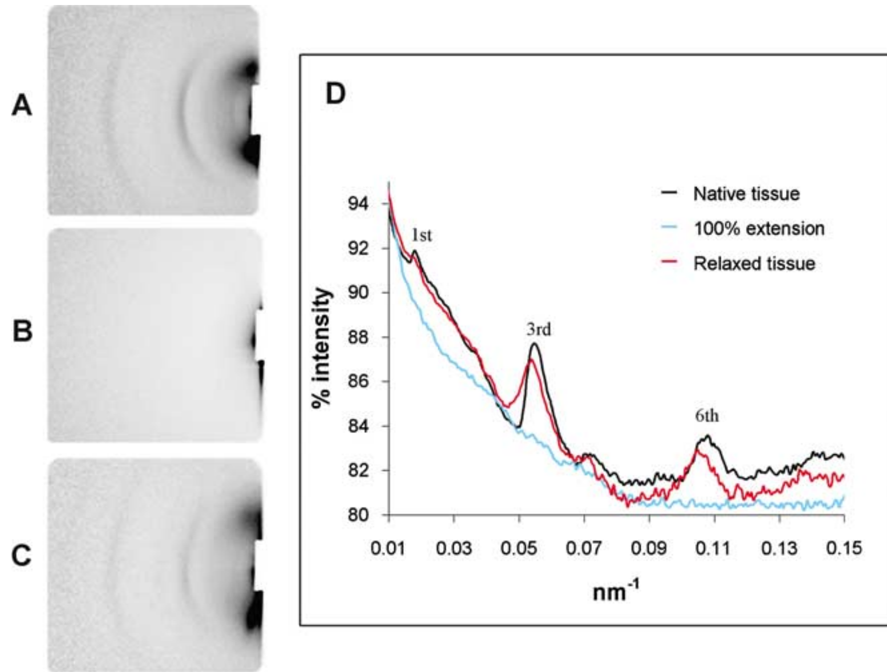

Fig. 5. X-ray fibre diffraction of ovine ciliary zonules before and after extension. X-ray fibre diffraction was carried out using an 8.25 m camera on beamline 2.1 at the CLRC Daresbury laboratory (UK), in order to investigate changes in the supramolecular packing of ovine zonular filaments under extension. Zonular filaments were dissected from ovine eye as previously described for bovine tissue (Wess *et al.*, 1997, 1998a). Briefly, eyes were obtained within 24 h post-mortem from a local abattoir. Dissection of the posterior chamber of the eye produced an intact preparation of ciliary body, lens and vitreous humor. The zonular filaments were mounted securely on small aluminium frames using cyanoacrylate. Samples were kept hydrated using Tris buffered saline (pH 7.2), and were analysed between thin mica sheets. Samples were exposed for 12 min in the native state and then in a fully extended state (150% length extension from resting state). Subsequently the tissue was relaxed back to the rest length and re-analysed. Panel A: native zonules before extension. Panel B: zonules with 100% extension. Panel C: relaxed zonules after extension. Panel D: linear profiles of the integrated small-angle meridional intensity region.

those seen with bovine zonules, with strong third and sixth orders and a weaker fourth clearly visible in the unstretched sample (Figure 5a). Upon stretching up to 50%, no real difference was observed in this diffraction pattern (data not shown). In contrast, tissue extensions of 150% produced a markedly different pattern, with a loss of coherence in the meridional diffraction series (Figure 5b). Relaxation of the tissue re-established the meridional series, indicating that the staggered alignment of adjacent microfibrils is recoverable (Figure 5c), although the relaxed tissue exhibited a slightly longer periodicity, as indicated by a reduction in the reciprocal distance of the third and sixth orders (Figure 5d). No observable difference was seen in the position of the first order, although changes in this peak are difficult to discriminate, even using an 8.25 m camera. Thus, although stretching of the zonular filaments produces an altered supramolecular structure of the component fibrillin-rich microfibrils, these X-ray diffraction studies suggest that the characteristic third order staggered array organisation is recoverable, even beyond what is probably the physiological range of microfibril elasticity (up to ~100% extension).

Mechanical testing of microfibril bundles

Tensile tests and stress-relaxation tests were used on whole ciliary zonular filaments to examine the relationship between their mechanical behaviour and the molecular organisation of the fibrillin-rich microfibrils upon which the tissue is based. Zonular filaments revealed a non-linear (J-shaped) stress–strain curve and appreciable stress-relaxation (Wright *et al.*, 1999), and it was proposed that this behaviour may reflect an initial strain-induced lateral alignment of untensioned microfibrils prior to elastic extension and recoil. A similar non-linear relationship between force and strain was observed for microfibril bundles of less than 1 μm in diameter (Eriksen *et al.*, 2001).

Isolated microfibrils

Early studies of isolated microfibrils revealed that, while most beaded repeats had a regular 56 nm periodicity characteristic of the untensioned state, there were often also seen some highly stretched beaded repeat regions with periodicities of up to ~160 nm (Keene *et al.*, 1991; Sherratt *et al.*, 2001). These highly stretched regions, which probably arose due to tangling in debris during preparation, appeared to be stable in solution, suggesting that they may have been irreversibly extended.

Stretching isolated microfibrils in solution under controlled conditions has proved a challenge, and reproducible approaches have yet to be established. Methods such as centrifugation and aspiring through fine-gauge needles did not reproducibly extend microfibrils

(Sherratt *et al.*, 2001). When microfibrils were adsorbed onto carbon-coated electron microscopy grids that had not been glow-discharged, surface forces were shown to extend beaded periodicity to ~100 nm and to exert a profound effect on their organisation (Baldock *et al.*, 2001). These forces would not have been sufficient to break covalent bonds, and thus the observed 56–100 nm periodic changes are likely to be due to non-covalent forces such as electrostatic or hydrophobic interactions. This observation is consistent with this periodic range being extensible. Incubation of isolated microfibrils with a monoclonal anti-fibrillin-1 antibody (11C1.3) generated extensive microfibril arrays with a double striated pattern corresponding to the beads and to the Mab interbead epitope which, in untensioned microfibrils, is ~41.1% of the bead-to-bead distance (Baldock *et al.*, 2001). However, after stretching these Mab-generated arrays with surface forces, the striation corresponding to the bound antibody was no longer detected except in microfibrils that were stretched only to ~70 nm periodicity. These data imply that the Mab epitope does not have to move until periodicity exceeds 70 nm (at that periodicity, the antibody epitope is 32% of bead-to-bead distance). At higher periodicities, the alignment of the epitope is lost, presumably due to a major structural rearrangement. Thus, microfibrils can stretch at least 10 nm before this epitope has to move.

Effects of calcium on elasticity

Velocity sedimentation and rotary shadowing have revealed a 20–25% decrease in the length of the rod-shaped fibrillin molecule in the presence of 5 mM EDTA (Reinhardt *et al.*, 1997). Bound calcium also strongly influences the organisation of isolated microfibrils (Kielty and Shuttleworth, 1993; Cardy and Handford, 1998; Wess *et al.*, 1998a). X-ray diffraction, dark field STEM and rotary shadowing all showed that chelation of calcium reduces beaded periodicity by 15–30% and produces a diffuse appearance with evidence for increased molecular flexibility. The observed decrease in fibrillin molecule and microfibril rest length is partly accounted for by an increase in the degrees of freedom of the linkages between consecutive cbEGF domains. NMR studies showed that, in the presence of bound calcium, a fibrillin-1 cbEGF domain pair is in a rigid, rod-like arrangement, stabilised by interdomain calcium binding and hydrophobic interactions (Downing *et al.*, 1996).

X-ray diffraction zonular stretching experiments have shown that bound calcium influences microfibril load deformation but is not necessary for high extensibility and elasticity (Wess *et al.*, 1998a, b). The influence of calcium has also been examined in mechanical tests of microfibril bundles using the micro-needle technique (Eriksen *et al.*, 2001). Calcium depletion resulted in a 50% decrease in rest length and a reduction in microfibril stiffness; this effect was reversible upon addition of calcium. However, at high strain, irreversible damage occurred irrespective of the presence or absence of calcium. Thus, it appears that microfibril elasticity is modified by, but not dependent on calcium-induced beaded periodic changes. These studies also showed that reversible extension occurs only within a specific periodic range and that irreversible transitions in the quaternary structure occur at high stretch.

STEM mass mapping

STEM analysis of microfibrils isolated from a canine ectopia lentis model that contained abundant examples of both untensioned and stably stretched regions revealed a sharp transition from 56 to >100 nm periodicities (Baldock *et al.*, 2001). Bead mass remained constant in the periodicity range 56–100 nm but interbead mass was markedly reduced. However, at periodicities >100 nm, bead mass was then rapidly reduced. These data showed that extensibility in the range 56–100 nm involved primarily rearrangements within the interbeads, and that more extended periodicities were accounted for by unravelling of the beads. These data suggested that interbead changes mainly define the reversible elastic range (~56–100 nm), and that bead changes at higher periodicities may be irreversible.

Atomic force microscopy

Air-dried fibrillin-containing microfibrils imaged by tapping mode atomic force microscopy (AFM) and non-contact mode AFM (Hanssen *et al.*, 1998; Sherratt *et al.*, 2001) exhibit the characteristic 56 nm beaded periodicity previously observed by TEM in negatively stained and rotary shadowed and by STEM in unstained microfibrils (Keene *et al.*, 1991; Kielty *et al.*, 2002). Using tapping mode AFM height measurements, we have now also shown that, within the periodic range 44–68 nm, interbead height is reduced at increasing periodicities but bead height remains constant along a 35 nm region (Figure 6). These investigations support the STEM observations and further narrow down the region involved in reversible elastic changes to the centre of the interbead.

AFM single molecule force spectroscopy has proved to be an invaluable tool in the investigation of molecular biomechanics. Using a stiff cantilever, Rief *et al.* (1997) tracked the unfolding of single domains within the giant muscle protein titin with a single amino acid resolution. The large mass (3–4 MDa) and long contour length (1 μm) make titin an ideal subject for single molecule biomechanical studies. The investigation of fibrillin biomechanics by single force spectroscopy AFM is currently underway using recombinantly expressed molecules.

Summary

The lobster aorta studies confirm that microfibrils are responsible for the elasticity of this invertebrate tissue.

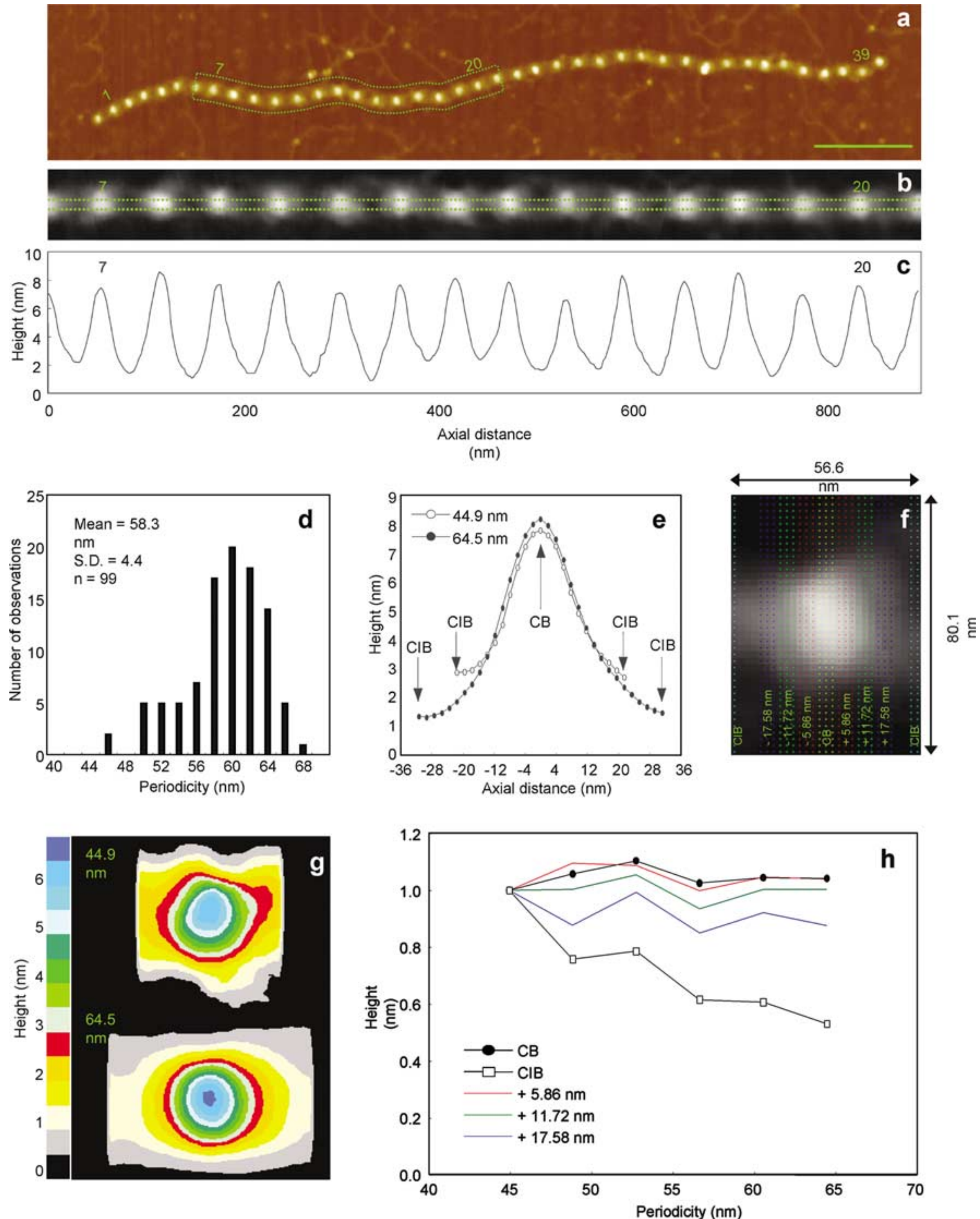

Fig. 6. AFM height measurements of isolated microfibrils. Panel A: AFM tapping mode visualisation of a microfibril isolated from bovine ciliary zonule. Scale bar = 200 nm. Panel B: Fourteen repeats from this microfibril, straightened using a plugin for ImageJ based on an algorithm described in Kocsis *et al.* (1991). Panel C: Mean axial height distributions were calculated at each axial position over 9.8 nm in the transverse direction. Panel D: Mean axial periodicity (determined for 99 repeats) = 58.3 nm. Panel E: Mean axial height distributions were calculated for microfibril repeats in the following range (23, 25, 27, 29, 31, 33 pixels, each ± one pixel, equivalent to 44.9, 48.8, 52.7, 56.6, 60.5, 64.5 ± 1.95 nm). The height of the centre of the bead (CB) is virtually unchanged at periodicities between 44.9 and 64.5 nm. However, the height of the centre of the interbead (CIB) does change. Panel F: In order to assess where height changes occur within the repeat, mean maximum heights were determined for transverse profiles (5.85 nm wide) at the centre of the bead (CB) and centre of the interbead (CIB) and at points 5.86, 11.72 and 17.58 nm to right (+) or left (−) of the bead centre (these correspond to lines on the graph shown in Panel H). Panel G: Mean 3-D height contour maps were determined for repeats in the range 44.9–64.5 nm. The shape and size of the central bead remains unchanged over this period range, but interbead height varies from 2–2.5 nm (44.9 nm) to 1–1.5 nm (64.5 nm). Panel H: Graph showing mean height as a proportion of height at 44.9 nm periodicity. The CB and regions to the plus side of the bead show little change with increasing periodicity. Only the interbead region changes. Over the periodicity range 44.9–68.5 nm, a narrow region at the centre of the interbead undergoes conformation change, but most of the repeat remains topographically unchanged.

The extracted microfibril bundles from sea cucumber infer key roles for transglutaminase cross-links in the provision of strength and disulphide bonds in elastic properties. Perturbing disulphide bonds will have the effect of profoundly altering the secondary and tertiary structure of the contiguous arrays of cbEGF domains and intercalating TB modules in fibrillins, all of which are stabilised by intradomain disulphide bonds, and will also disrupt any inter-molecular disulphide bridges within the microfibril. X-ray diffraction has shown that hydrated physiological microfibril bundles have a reversible range of elasticity (less than 100% stretch), and also that the predicted one-third staggered lateral alignment of microfibrils is recoverable after high stretch. Mechanical tests have shown that untensioned tissue microfibrils undergo initial alignment before extension with strain. The STEM and AFM data support the concept that individual microfibrils also have a reversible range of elasticity based largely on interbead reorganisation. The antibody binding data suggest that a major interbead conformation change occurs at ~70 nm periodicity. Bound calcium can modify microfibril elasticity, probably by altering molecular flexibility at strategic interdomain linkages.

Formation of microfibrils

The process of assembly of elastic fibrillin-rich microfibrils is a complex multistep process that remains poorly understood. Below is outlined our current knowledge of key phases in the assembly of these microfibrils and their deposition in the extracellular matrix.

Cellular regulation

Microfibril assembly is, in part, a cell-regulated process that proceeds independently of tropoelastin. Fibrillin-1 can undergo limited initial assembly in the secretory pathway (Ashworth *et al.*, 1999b; Trask *et al.*, 1999), and in this respect, is similar to other major ECM macromolecules such as collagens, laminins and proteoglycans. Chaperone interactions may be essential in regulating intracellular fibrillin assembly (Ashworth *et al.*, 1999c). Fibrillins have N- and C-terminal cleavage sites for furin convertase; extracellular deposition requires removal of the C-terminus (Raghunath *et al.*, 1999; Ritty *et al.*, 1999), a process influenced by N-glycosylation and calreticulin (Ashworth *et al.*, 1999c).

Cell surface assembly

Microfibrils assemble close to the cell surface, in a process that may require receptors, as shown for fibronectin (Sechler *et al.*, 2001). A role for heparan sulphate proteoglycans (HSPGs) in assembly has been proposed (Tiedemann *et al.*, 2001), and we have evidence that chondroitin sulphate proteoglycans (CSPGs) are involved. Sulphation is needed for microfibril assembly, since chlorate treatment ablates microfibril and elastic fibre formation (Robb *et al.*, 1999). This effect may reflect absence of a proteoglycan or under-sulphation of fibrillins or microfibril-associated glycoprotein (MAGP-1). Fibrillin RGD sequences (TB4 module) can also interact with several integrins (Pfaff *et al.*, 1996; Sakamoto *et al.*, 1996).

Extracellular maturation

Extracellular microfibril populations that may differ in macromolecular organisation, composition and stability have been tentatively identified, implying a major role for the extracellular environment in regulating microfibril fate. In human dermal fibroblast cultures, monoclonal antibody11C1.3 (binds beaded microfibrils) did not detect microfibrils until 2 weeks, but a polyclonal antibody (PF2) to a fibrillin-1 pepsin fragment detected abundant microfibrils within 3 days (Baldock *et al.*, 2001). The time-dependent appearance of 11C1.3-reactive microfibrils suggests 'maturation', perhaps due to conformational changes or unmasking of a cryptic epitope. It is not known how such maturation events might affect microfibril elasticity. In developing vascular tissues, 11C1.3 detected microfibrils associated with medial elastic fibres but another monoclonal antibody 12A5.18 (also binds beaded microfibrils, and at the same interbead site as antibody 11C1.3) only recognised microfibrils in collagen fibril rich tissues (Kogake *et al.*, 2002). Interactions with different ECM molecules may commit microfibrils to distinct extracellular fates in association with elastin or in collagen fibre rich tissues.

Microfibril-associated molecules and their interactions

We have described current understanding of microfibril elasticity and fibrillin assembly, implicitly in the context of fibrillin homopolymers. However, like other major supramolecular arrays, it is unlikely that microfibrils are totally independent homopolymer structures. For example, association of actin and myosin is required to generate the functional contractile unit of muscle, whilst collagen fibril formation in ECM requires terminal processing enzymes as well as regulation of fibril diameter and interactions with surrounding ECM by small leucine-rich proteoglycans (e.g. decorin, biglycan) (Danielson *et al.*, 1997; Xu *et al.*, 1998). Microfibrils are probably multimolecular assemblies and they certainly interact with other molecules during elastic fibre formation (Kielty *et al.*, 2002). In fact, a plethora of microfibril-associated molecules has been identified using immunohistochemical and biochemical approaches, and a major current challenge is to determine which molecules are actually structural components of microfibrils and how different associated molecules may modify microfibril elastic properties and integration in

the surrounding ECM. The observed time-dependent maturation of microfibrils may well reflect, in part, microfibril associations with other matrix molecules. Thus, it is necessary to consider in some detail the other known molecules of the microfibrillar system, and current understanding of how they may influence fibrillin assembly and microfibril elasticity.

MAGP-1 may also be an integral microfibril molecule (Gibson *et al.*, 1989; Trask *et al.*, 2000a). It is associated with virtually all microfibrils, and widely expressed in mesenchymal and connective tissue cells throughout development (Henderson *et al.*, 1996; Kielty and Shuttleworth, 1997). MAGP-2, the other member of this small microfibrillar protein family, is structurally related to MAGP-1 (Gibson *et al.*, 1998; Segade *et al.*, 2002), and localises to elastin-associated and elastin-free microfibrils in some tissues although it has a more restricted pattern of tissue localisation (Gibson *et al.*, 1998). MAGP-2 is an RGD-containing cell adhesion molecule and may have functions related to cell signalling during microfibril assembly and elastinogenesis (Gibson *et al.*, 1999) (Table 1).

Latent TGFβ binding proteins (LTBPs) are members of the fibrillin superfamily and also contain contiguous arrays of cbEGF domains interspersed with 8-cysteine motifs (also called TB modules due to TGFβ-binding properties) (Sinha *et al.*, 1998; Oklu and Hesketh, 2000). LTBPs form covalent large latent complexes with TGFβ intracellularly that are secreted, transglutaminase cross-linked within the ECM, then released by protolysis (Miyazono *et al.*, 1988; Sinha *et al.*, 1998). LTBP-1 colocalises with microfibrils in skin and in cell layers of cultured osteoblasts and in embryonic long bone, but not in cartilage (Taipale *et al.*, 1996; Raghunath *et al.*, 1998; Dallas *et al.*, 2000). LTBP-1 is unlikely to be an integral microfibril component, but its association implicates microfibrils in TGFβ targeting. LTBP-2 colocalises with fibrillin-rich microfibrils in elastic-fibre-rich tissues (Gibson *et al.*, 1995; Kitahama *et al.*, 2000; Sinha *et al.*, 2002), and is a good candidate for a microfibrillar molecule, although it does not bind TGFβ.

Several other microfibril-associated proteins have been identified immunohistochemically, but little is

Table 1. Reported microfibril and elastic fibre associated molecules

Molecule	Location	References
Fibrillin-1	Microfibrils	Pereira *et al.* (1993) Baldock *et al.* (2001)
Fibrillin-2	Microfibrils	Zhang *et al.* (1994)
Fibrillin-3	? microfibrils	Nagase *et al.* (2002)
MAGP-1	Microfibrils	Brown-Augsberger *et al.* (1996)
MAGP-2	Some microfibrils	Gibson *et al.* (1998)
LTBP-1	Some microfibrils	Miyazono *et al.* (1988) Sinha *et al.* (1998)
LTBP-2	Microfibrils/elastic fibres	Gibson *et al.* (1995) Bashir *et al.* (1996)
LTBP-3	?	Yin *et al.* (1995)
LTBP-4	?	Giltay *et al.* (1997)
Decorin	Microfibrils	Trask *et al.* (2000a)
Biglycan	Elastic fibre core	Reinboth *et al.* (2001)
Versican	Some microfibrils	Isogai *et al.* (2002)
MFAP-1	Some microfibrils	Liu *et al.* (1997)
MFAP-3	Some microfibrils	Abrams *et al.* (1995)
MFAP-4 (MAGP-36)	Some microfibrils	Lausen *et al.* (1999) Hirano *et al.* (2002)
Tropoelastin	Elastic fibre core	Mecham and Davis (1994)
Lysyl oxidase (LOX)	Microfibrils/ tropoelastin	Csiszar (2001)
LOXL	?	Borel *et al.* (2001)
LOXL2	?	Csiszar (2001)
LOXL3	?	Csiszar (2001)
BigH3 (keratoepithelin)	Elastic fibre/collagen interface	Gibson *et al.* (1997)
Fibulin-1	Elastic fibre core	Kostka *et al.* (2001)
Fibulin-2	Elastin–microfibril interface	Tsuda *et al.* (2001)
Fibulin-5	Elastic fibre–cell interface	Nakamura *et al.* (2002) Yanagisawa *et al.* (2002)
Emilin-1	Elastin–microfibril interface	Doliana *et al.* (1999)
Emilin-2	Elastin–microfibril interface	Doliana *et al.* (2001)
Elastin binding protein	Newly secreted tropoelastin	Prody *et al.* (1998)
Vitronectin	Some microfibrils	Dählback *et al.* (1990)
Amyloid	Some microfibrils	Dählback *et al.* (1990)
Collagen VIII (α1(VIII)	Some elastic fibres	Sadawa and Konomi (1991)
Collagen XVI (α1(XVI)	Dermal microfibrils	Grässel *et al.* (1999)
Endostatin (α1(XVIII)	Vascular elastic fibres	Miosge *et al.* (1999)
Collagen VI	Some microfibrils	Finnis and Gibson (1997)

known about whether they are essential microfibrillar components and how they may influence microfibril function. Microfibril-associated protein (MFAP)-1 (or AMP), MFAP-3 and MFAP-4 (or 'MAGP-36') colocalise with elastic fibres in skin and other tissues (Horrigan *et al.*, 1992; Abrams *et al.*, 1995; Liu *et al.*, 1997; Lausen *et al.*, 1999; Toyashima *et al.*, 1999; Hirano *et al.*, 2002). In some tissues, microfibrils associate with amyloid deposits and with adhesive glycoproteins such as vitronectin (Dählback *et al.*, 1990). MP78/70 (also known as beta ig-h3 or keratoepithelin) is another molecule that occasionally appears at elastic fibre interfaces. Originally identified in bovine tissue extracts designed to solubilise microfibrils (Gibson *et al.*, 1989), it localises to collagen fibres in ligament, aorta, lung and mature cornea, to reticular fibres in foetal spleen, and to capsule and tubule basement membranes in kidney (Gibson *et al.*, 1997; Schorderet *et al.*, 2000). In some elastic tissues, MP78/70 is present at the interface between collagen fibres and adjacent elastic fibre microfibrils, which suggests that it has a bridging function. Collagen XVI is expressed by human dermal fibroblasts and keratinocytes and is associated with the microfibrillar apparatus in the upper papillary dermis (Grässel *et al.*, 1999). The angiogenesis inhibitor endostatin is another potential component of elastic fibers in vessel walls (Miosge *et al.*, 1999).

Proteoglycans have important, but still poorly understood structural interactions with microfibrils, and they may contribute to their integration into ECM. Immunocytochemical studies have localised proteoglycans within normal elastic fibres (Baccarani-Contri *et al.*, 1990). Decorin and biglycan, members of the small leucine-rich PG family, have been identified within dermal elastic fibres; biglycan mapped to the elastin core and decorin to microfibrils. Ultrastructural approaches showed that CSPGs associate with microfibril beads (Kielty *et al.*, 1996). Versican, a large chondroitin sulphate PG (CSPG) colocalises with microfibrils in dermis and interacts directly with fibrillin (Zimmermann *et al.*, 1994; Isogai *et al.*, 2002).

Other molecules localise to the elastin–microfibril interface and to the cell surface–elastic fibre interface. These molecules may regulate tropoelastin deposition on microfibrils, and link microfibrils and elastic fibres to cell surfaces. Emilin, a 136 kDa glycoprotein, localises to the elastin microfibril interface (Bressan *et al.*, 1993; Doliana *et al.*, 1999). Four family members have now been identified, emilins-1, -2, -3, and multimerin (emilin-4), but it is not yet known which interact with microfibrils (Colombatti *et al.*, 2000; Doliana *et al.*, 2001). Collagen VIII has been localised to the vascular elastic fibre-cell interface (Sadawa and Konomi, 1991).

Three members of the fibulin family of cbEGF-like domain molecules are implicated in elastic fibre biology. Fibulin-1 is located within the amorphous core of elastic fibres (Kostka *et al.*, 2001). Fibulin-5 has a vascular pericellular localisation and has been shown, in mouse models to be essential for normal elastic fibre formation (Nakamura *et al.*, 2002; Yanagisawa *et al.*, 2002). Fibulin-2 localises preferentially at the interface between microfibrils and the elastin core. It colocalises with fibrillin-1 in skin (except adjacent to the dermal–epithelial junction), perichondrium, elastic intima of blood vessels, and kidney glomerulus, although is apparently not present in ciliary zonules, tendon, and surrounding lung alveoli and kidney tubules (Reinhardt *et al.*, 1996b; Utani *et al.*, 1997; Raghunath *et al.*, 1999; Tsuda *et al.*, 2001). Fibulin may not be needed for microfibril elasticity since it is absent from tissues subject to strong tensional forces (e.g., tendon, ciliary zonule).

Tropoelastin is synthesised as a soluble precursor with a molecular mass of ~70 kDa and alternating hydrophobic and cross-linking domains (Mecham and Davis, 1994; Brown-Augsberger *et al.*, 1995). The 67-kDa enzymatically inactive alternatively spliced variant of beta-galactosidase, which is identical to the elastin/laminin-binding protein, may influence elastin deposition on microfibrils (Prody *et al.*, 1998). Interactions between hydrophobic domains are important in assembly and essential for elasticity (Bellingham *et al.*, 2001; Toonkool *et al.*, 2001). The formation of covalent lysyl-derived desmosine cross-links by lysyl oxidase (Csiszar, 2001), stabilises the polymerised insoluble product (elastin). Five lysyl oxidase-like proteins have now been characterised (LOX, LOXL, LOXL2 [or WS9-14], LOXL3 and LOXC). However, only LOX and LOXL have so far been shown to be able to cross-link insoluble elastin (Borel *et al.*, 2001).

The identification of this plethora of microfibril-associated molecules has now led to an urgent need to define how they interact within functional elastic microfibrils and tissue microfibrillar bundles. Available information is derived from *in vitro* binding studies, so the temporal hierarchy and significance of these interactions in elastic microfibril formation will need to be established.

MAGP-1 interacts with the fibrillin-1 N-terminus (within exons 1–10) in a calcium-dependent manner (Jensen *et al.*, 2001). MAGP-1 and fibrillin-1 are both substrates for transglutaminase and, although only homotypic fibrillin-1 cross-links have been identified to date, MAGP-1 may be cross-linked within microfibrils (Brown-Augsberger *et al.*, 1996; Qian and Glanville, 1997). MAGP-1 and fibrillin-1 both interact with decorin, a sulphated CSPG (Trask *et al.*, 2000a). The fibrillin-1 interacting sequence is within or adjacent to the proline-rich region, and the interaction is with the decorin core protein. Decorin can interact with both fibrillin-1 and MAGP-1 individually, and together they form a ternary complex. However, fibrillin-2 appears not to interact with MAGP-1 or decorin. In a separate study, decorin and biglycan were shown not to bind MAGPs -1 and -2 in solid phase assays, although MAGP-1 in solution interacted with biglycan, but not with decorin (Reinboth *et al.*, 2001).

MAGP-1 also binds tropoelastin; the binding site in MAGP-1 was localised to a tyrosine-rich sequence within its positively charged N-terminal half, which may interact with a negatively charged pocket near the tropoelastin C-terminus (Brown-Augsberger *et al.*, 1996). MAGP-1 may interact first with fibrillin-1 and decorin during microfibril assembly, and then with tropoelastin during elastic fibre formation on the microfibrillar template. Sequences in fibrillins-1 and -2 (within exons 10–16) interact with tropoelastin, but only in solid-phase suggesting that exposure of a cryptic site is needed (Trask *et al.*, 2000b). Decorin and biglycan can both bind tropoelastin, biglycan more avidly than decorin, and the biglycan core protein more strongly than the intact PG (Reinboth *et al.*, 2001). The ability of biglycan to form a ternary complex with tropoelastin and MAGP-1 suggests a role in the elastinogenesis phase of elastic fibre formation.

Molecular folding model of extensible microfibrils

The AET, STEM, X-ray diffraction and immunoelectron microscopy findings described above have formed the basis of a model of fibrillin alignment in extensible microfibrils in which intramolecular folding would act as a molecular 'engine' driving recoil after extension in response to external stretch forces (Baldock *et al.*, 2001; Kielty *et al.*, 2002) (Figure 2C). The model predicts that maturation from initial parallel head-to-tail alignment ($\sim$160 nm) to a $\sim$one-third stagger ($\sim$100 nm) occurs by folding at the termini and the proline-rich region, which would align known fibrillin-1 transglutaminase cross-link sequences. Microfibril elasticity (in the range 56–100 nm) would require further intramolecular folding at flexible sites which could be links between TB modules and cbEGF domains. This molecular folding model is a novel concept in extracellular matrix biology. The model now needs rigorous, flexible sites within fibrillin molecules need to be determined, and the electrostatic and hydrophobic driving forces of this elastic motor defined. In addition, the multimolecular nature of microfibrils will need to be taken into account.

Future perspectives

We have outlined the evidence that microfibrils are elastic, and current understanding of the molecular mechanism underlying their assembly, and ability to extend and recoil. A priority now will be to assess the proposed molecular folding model, and to identify flexible regions within fibrillin molecules. The precise molecular composition of microfibrils in different tissues urgently needs to be determined using new analytical approaches, so that the implications for elasticity of the presence of other molecules can be evaluated. The molecular basis of microfibril maturation, and the effects of transglutaminase cross-linking on elasticity need to be established. In microfibril bundles, the nature of any inter-microfibrillar cross-links needs defining. This information will enhance our understanding of the elastic mechanism of the unique fibrillin-rich microfibrils of extracellular matrices, and may lead to new strategies for repairing elastic tissues and tissue engineering strategies.

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