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# The role of calcium in the organization of fibrillin microfibrils

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The microfibrillar glycoprotein fibrillin has a multidomain structure which contains forty-three epidermal growth factor-like motifs with calcium-binding consensus sequences. We have utilized intact microfibrils isolated from human dermal fibroblast cultures to investigate the putative influence of bound calcium on microfibrillar organization and integrity. Incubation with EDTA or EGTA rapidly resulted in gross disruption of microfibril morphology. The treatment induced disorganization of the interbead domains although the regular beaded arrangement was always apparent. These changes were readily reversible on replacing calcium, indicating that the treatment had not compromised microfibrillar integrity. The data localize calcium binding EGF-like repeats to the interbead domains and indicate that lateral packing of fibrillin monomers is calcium-dependent. This arrangement suggests how mutations in epidermal growth factor-like domains of fibrillin might cause the disruption in microfibril organization and interactions which underlies the clinical symptoms of some Marfan syndrome patients.

Fibrillin; Calcium; EGF motif; Microfibril

## 1. INTRODUCTION

The glycoprotein fibrillin is the principal structural component of a distinct class of extracellular matrix microfibrils which are key determinants of connective tissue architecture and integrity [1–3]. The importance of the fibrillin-containing microfibrils in connective tissues is underlined by the recent linkage of the fibrillin gene FBN1 on chromosome 15 to the Marfan syndrome, a heritable connective tissue disorder characterized by cardiovascular, skeletal and ocular abnormalities [4–12]. These microfibrils have a complex ultrastructure with a diameter of 10–14 nm and an average, but variable, beaded periodicity of 50–55 nm [13–16]. The organization of fibrillin monomers within assembled microfibrils and the molecular interactions involved in their polymerisation remain largely undefined.

Fibrillin has a complex multi-domain structure comprising multiple epidermal growth factor (EGF)-like motifs interspersed with 8-cysteine repeats with homology to the TGF-$\beta_1$ binding protein and several apparently unique cysteine-rich motifs [17,18]. Of forty-six EGF-like repeats, forty-three contain residues previously shown to constitute a calcium binding consensus sequence within EGF-like repeats occurring in other proteins [19–21]. A number of FBN1 mutations have now been identified in Marfan patients where single base changes give rise to amino acid substitutions in various EGF-like motifs [4–12]. Some of these mutations affect residues within the calcium-binding consensus sequence and in other cases cysteine residues are substituted with inevitable disruption of strategic intra-domain disulphide bond formation. These mutations are predicted to disrupt calcium-binding.

A large number of extracellular proteins with diverse biological functions possess EGF-like domains; these include matrix molecules such as thrombomodulin and BM-90 (fibulin), and the vitamin K-dependent blood coagulation factors VII, IX and X, protein S and protein C [22–29]. It has been proposed that these repeats may in some cases act as structural spacer elements [30–32]. The precise role of calcium in the EGF-like domain is unclear, but there is increasing evidence that occupation of these sites by calcium influences conformation and is essential in some cases for protein–protein interactions [21].

Despite fibrillin containing an abundance of EGF-like repeats with the potential to bind calcium, there has been no direct demonstration of the influence of calcium on fibrillin conformation and polymerisation, microfibril organization and/or functional interactions in the matrix. In the present study, we have examined ultrastructurally the effects of bound calcium on the organization of intact microfibrils isolated from human dermal fibroblast cultures. The results provide unequivocal evidence that calcium plays a central role in defining microfibril conformation but that the integrity of assembled microfibrils is not dependent on the presence of calcium. This is the first demonstration of the profound influence of bound calcium on microfibrillar organization. The data suggest a mechanism whereby individual mutations in fibrillin EGF-like domains in some patients with Marfan syndrome might influence microfibril structure/function relationships and cause the disease phenotype.

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## 2. MATERIALS AND METHODS

### 2.1. *Materials*

Bacterial collagenase (type 1A), phenylmethanesulphonyl fluoride (PMSF), *N*-ethylmaleimide (NEM), and benzyldimethylammonium chloride were obtained from the Sigma Chemical Co., Poole, Dorset, UK. Sepharose CL-2B was supplied by Pharmacia-LKB, Milton Keynes, Bucks, UK. Tissue culture media and plastics were obtained from Gibco BRL, Paisley, Scotland, UK. Mica sheets and 5 nm gold-protein A conjugate were supplied by TAAB Laboratory Equipment, Reading, Berks, UK. The Marfan line NB was supplied by Dr. Anne H. Child (St. George's Hospital, London, UK). These cells have a point mutation at nucleotide 3687 [33] causing a glutamate to aspartate amino acid substitution within an EGF-like repeat [11].

### 2.2. *Cells and cell culture*

Normal dermal fibroblasts derived from a skin biopsy of a 38-year-old woman and the Marfan dermal fibroblast line NB were grown in Dulbecco's minimum essential medium supplemented with 10% foetal calf serum, penicillin (400 units/ml), streptomycin (50 μg/ml) and glutamine (200 μg/ml). For microfibril extractions, cells were maintained at post-confluence for up to three weeks. Cell layers were washed in 0.05 M Tris-HCl, pH 7.4, containing 0.4 M NaCl, then incubated for 3 h at 20°C in 0.05 M Tris-HCl, pH 7.4, containing 0.4 M NaCl, 0.005 M $CaCl_2$, 0.1 mg/ml bacterial collagenase (type 1A), 2 mM PMSF and 5 mM NEM. Soluble extracts were clarified by centrifugation for 15 min at 7,500 × *g* on a bench microfuge prior to size fractionation by gel filtration chromatography.

### 2.3. *Size fractionation of fibrillin solubilized from cell layer extracts*

Post-confluent cell layer extracts solubilized by bacterial collagenase digestion were chromatographed directly without concentration under non-reducing, non-denaturing conditions on a Sepharose CL-2B column equilibrated and eluted at room temperature with 0.05 M Tris-HCl, pH 7.4, containing 0.4 M NaCl [15,34]. High-$M_r$ material present in the excluded volume ($V_0$) was utilized in calcium chelation and addition experiments and subsequent ultrastructural analyses.

### 2.4. *Calcium chelation and addition experiments*

Microfibril preparations were sequentially incubated with different combinations of EDTA, EGTA and $CaCl_2$. All incubations were for 15 min at 20°C. The final concentrations and combinations of additions were: (i) 1 mM EDTA, (ii) 1 mM EGTA, (iii) 1 mM EDTA then 1 mM $CaCl_2$, (iv) 1 mM EGTA then 1 mM $CaCl_2$, (v) 5 mM EDTA, (vi) 5 mM EGTA, (vii) 5 mM EDTA then 5 mM $CaCl_2$, (viii) 5 mM EGTA then 5 mM $CaCl_2$, (ix) 25 mM EDTA, (x) 25 mM EDTA then 25 mM $CaCl_2$, (xi) 25 mM $CaCl_2$, (xii) 25 mM $CaCl_2$ then 25 mM EDTA.

### 2.5. *Ultrastructural analysis*

Void volume fractions of cell layer extracts were visualized for their microfibril content by rotary shadowing using a modification of the mica sandwich technique [16].

## 3. RESULTS

### 3.1. *Isolation of intact microfibrils from post-confluent cell layers*

Intact fibrillin-containing microfibrils were isolated by size fractionation of bacterial collagenase-digested post-confluent cell layers on an analytical column of Sepharose CL-2B as previously described [15,16,34]. Examination of the $V_0$ fraction by rotary shadowing electron microscopy demonstrated the presence of extensive and abundant microfibrils which were normal in morphology and indistinguishable from those previously isolated from tissues [15,16] (Fig. 1A). The lengths of the isolated microfibrils were frequently in excess of 1 μM. These extensive assemblies were uniformly well defined with regular periodicities and diameters, and interbead striations were apparent.

### 3.2. *Effects of calcium on microfibril organization*

Incubation of microfibrils with EDTA or EGTA led to rapid and dramatic changes in their appearance. Treatment with 1 mM or 5 mM EDTA or EGTA effected the universal disruption of interbead domains, which appeared markedly frayed (Fig. 1B, C, F, G). Under these conditions, the beaded domains were apparently unaffected and normal regular beaded periodicity was retained. After incubation with 25 mM EDTA the interbead domains appeared more diffuse and individual frayed filaments were seldom clearly defined, although the integrity of the microfibrils was not affected (Fig. 1H). The length distributions of isolated microfibrils before and after all treatments with chelators were comparable.

When microfibril preparations preincubated with EDTA or EGTA were subsequently incubated with calcium (as $CaCl_2$), the effects of calcium chelation could rapidly be reversed. Microfibrils initially incubated with EDTA or EGTA and subsequently with an equimolar amount of calcium, appeared morphologically indistinguishable from untreated controls (Fig. 1D, I, J). Microfibrillar organization and diameter were restored and striations within the interbead domains were apparent. However, some regions within individual microfibrils treated initially with 25 mM EDTA and subsequently with 25 mM $CaCl_2$, remained disrupted (Fig. 1J).

Incubation of microfibrils with $CaCl_2$ without prior treatment with EDTA had no discernable influence on microfibrillar organization. Subsequent treatment with a 5 mM excess of EDTA led to similar morphological changes to those observed without pre-treatment with $CaCl_2$ (not shown).

Microfibrils extracted from post-confluent cell layers of the Marfan dermal fibroblast line NB exhibited diffuse interbead domains although the periodicity was retained (Fig. 1E). In this respect, they resembled normal microfibrils treated with 25 mM EDTA.

## 4. DISCUSSION

The potential of fibrillin to bind calcium is strongly suggested by the presence of an abundance of EGF-like motifs with calcium binding consensus sequences and has recently been demonstrated biochemically [17,35]. The identification in Marfan syndrome patients of point mutations within a number of these domains that are predicted to disrupt calcium binding has underlined the potential importance of calcium binding in terms of microfibrillar integrity and function. Despite these indications, bound calcium within assembled microfibrils

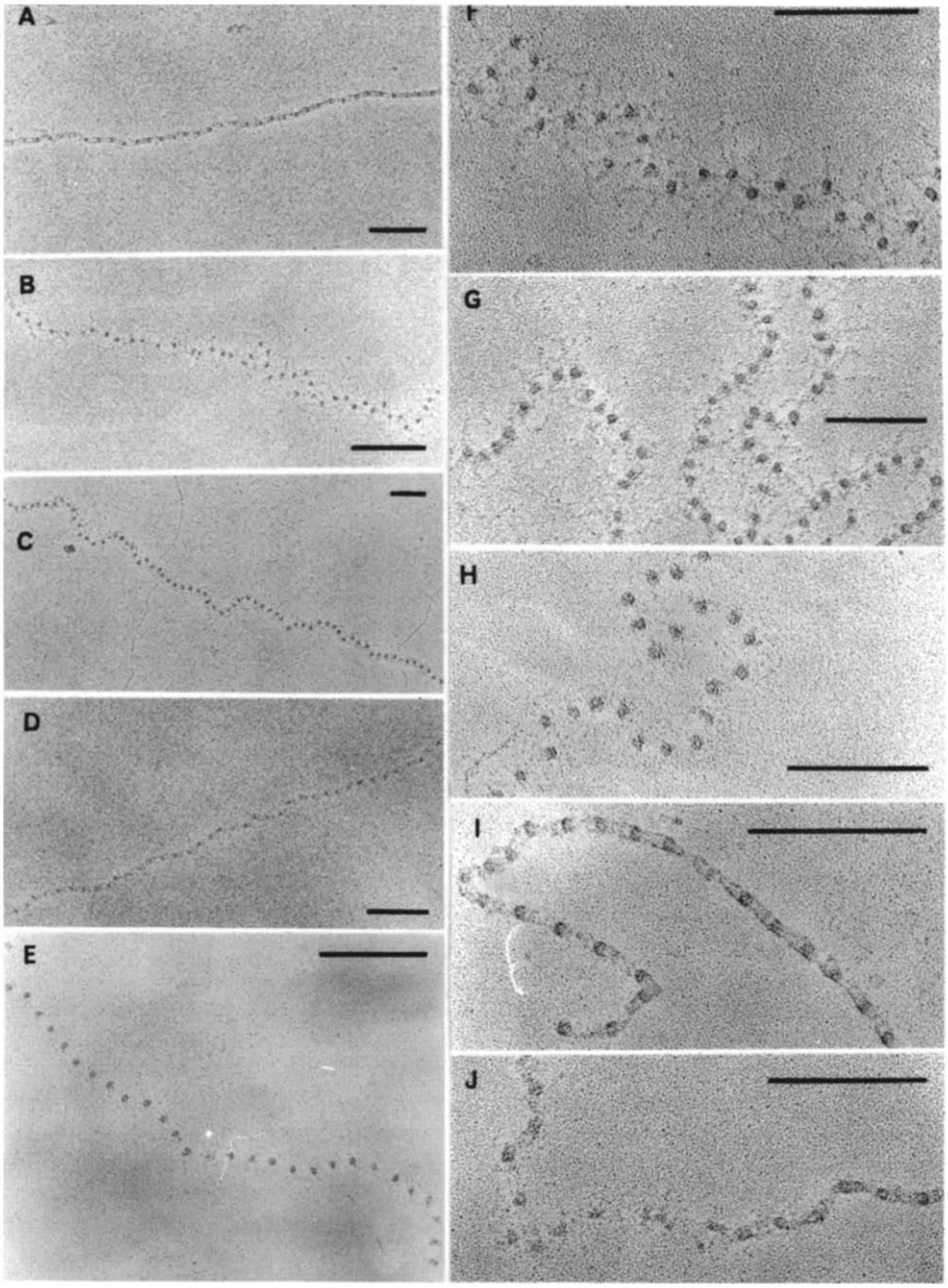


Fig. 1. Rotary shadowing electron micrographs of microfibrils isolated from post-confluent cell layers of normal human dermal fibroblasts, demonstrating calcium-dependent morphological changes. All incubations were for 15 min at 20°C. Microfibril preparations were treated with 1 mM, 5 mM or 25 mM EDTA, and in some experiments subsequently incubated in the presence of equimolar amounts of $CaCl_2$. Bars = 250 nm. (A) Untreated control. (B,F) 1 mM EDTA. (D) 1 mM EDTA and then 1 mM $CaCl_2$. (C,G) 5 mM EDTA. (I) 5 mM EDTA and then 5 mM $CaCl_2$. (H) 25 mM EDTA. (J) 25 mM EDTA and then 25 mM $CaCl_2$. Untreated microfibrils isolated from post-confluent cell layer of the Marfan dermal fibroblast line NB were also visualized (F).

and its influence on microfibril organization have not been demonstrated to date.

This study has shown that removal of calcium has a mjaor impact on the organization of the interbead domains although interestingly the regular beaded periodicity of the microfibrils and microfibrillar length appear unaffected by the treatment. The observations indicate that the long stretch of EGF-like domains within the C-terminal two-thirds of the fibrillin molecule constitutes the major structural element of the interbead domains. The local structural changes induced within individual EGF-like domains by calcium binding thus appear essential for lateral packing and alignment of adjacent fibrillin monomers within interbead domains. It is also possible that bound calcium may form bridges between EGF-like repeats.

A fascinating feature of the EDTA-treated microfibrils is that, despite gross disruption within the inter-

bead domains, the periodicity of the microfibrils is not compromised. The molecular basis of this regular beaded periodicity is not known, but the conformation changes induced both by calcium chelation and addition do not appear to alter beaded domain conformation. The stability of beaded domains may be underpinned by stable covalent and/or non-covalent interactions involving fibrillin, and possibly other molecules. One such candidate molecule is microfibril-associated glycoprotein (MAGP), which we have recently demonstrated in apparent covalent linkage with the beaded domains [36].

It has previously been proposed on the basis of immunohistochemical observations that fibrillin monomers are assembled in head-to-tail arrangement within microfibrils [3]. Our data neither confirm nor refute this possibility. However, the presence of frayed interbead domains within intact microfibrillar assemblies raises the alternative possibility that oppositely aligned C-termini stabilized by strong calcium-dependent non-covalent interactions between repeating EGF-like motifs interact directly within the interbead domains. The implication of such an arrangement would be that beaded domains contain fibrillin N-termini.

The observation that bound calcium has a major impact of microfibril organization has important implications for understanding how mutations within fibrillin EGF-like repeats give rise to the Marfan phenotype. Interestingly, we have now demonstrated abnormal interbead domains in microfibrils elaborated by a Marfan dermal fibroblast line that had been shown to contain a mutation in an EGF-like consensus sequence [11]. This single localised disruption apparently dramatically reduces the stability of the lateral packing arrangement of fibrillin monomers. The increasing number of mutations within EGF-like repeats that are being reported suggest that this class of microfibrillar abnormality may be a feature of Marfan syndrome. It is anticipated that analysis of further patients lines with similar mutations will provide major insights into genotype/phenotype relationships of Marfan syndrome and related diseases.

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