Fibrillin domain folding and calcium binding: significance to Marfan syndrome

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Background: Marfan syndrome is a heritable disorder of connective tissue which has been associated with mutations in a gene encoding fibrillin, a 350 kD glycoprotein found in microfibrils. This protein consists of ~60 domains, 47 of which have similarity to epidermal growth factor (EGF). The first mutations to be detected were found in two sporadic cases that had identical Arg to Pro changes within one EGF-like domain. Based on sequence features common to 43 of the EGF-like domains, it was proposed that these domains might bind calcium. Through the synthesis and characterization of wild-type and mutated single domain peptides, we examined the structural and calcium-binding properties of an isolated EGF-like domain from fibrillin and the effects of the Arg to Pro sequence change.

Results: A peptide corresponding to the thirteenth putative calcium-binding, EGF-like domain of fibrillin (the site of the first detected mutations) was synthesized. This peptide could be easily oxidized and refolded. The structure of this domain was probed using NMR methods, indicating features characteristic of the known structures of EGF-like domains. The domain bound to calcium with moderate affinity (K_d ~ 0.6 – 0.1 mM) with no major changes in structure induced upon calcium binding. A synthetic peptide containing the Arg to Pro mutation was found to be drastically impaired in its ability to fold *in vitro*.

Conclusions: As predicted, a fibrillin domain forms a calcium-binding, EGF-like module. As the putative calcium-binding sites are found at the amino terminal end of the modules, we propose that calcium ions may bind at the interfaces between domains, affecting the overal structure of the protein. The Arg to Pro mutation blocks domain folding *in vitro*, suggesting that lack of proper domain folding *in vivo* may contribute to the molecular defects responsible for Marfan syndrome.

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Introduction

Marfan syndrome is an autosomal dominant disorder of the connective tissue manifested by ocular, skeletal and cardiovascular abnormalities. Genetic linkage analyses and immunohistological studies have implicated mutations in a gene encoding fibrillin as the cause of Marfan syndrome [1–4]. Fibrillin is a glycoprotein component of extracellular microfibrils [5,6]. It contains a large number of cysteine-rich repeats, including 43 EGF-like domains that closely match the sequence D-X-D-E-C-X$_{4-7}$-C-X$_{3-6}$-C-X-N-X$_1$-(Y,F)-X-C-X-C-X$_{14-27}$-C-X [4,7]. Domains with analogous sequence [8] have been shown to bind calcium with moderate affinity in blood coagulation factors IX and X [9–13].

A number of different mutations have now been characterized in the fibrillin genes of individuals with Marfan syndrome [2,14–16]. Many of these occur in the proposed calcium-binding EGF-like domains. It has been proposed that different mutations in individual EGF-like domains of fibrillin may disrupt the normal intramolecular disulfide network, change local secondary structure, or adversely affect calcium binding activity. The first mutations to be identified involved identical changes from Arg to Pro at position 1137 of fibrillin in two unrelated individuals [2]. This residue is at position 25 of the 42 amino acids in the thirteenth putative calcium-binding, EGF-like domain of fibrillin. To test the hypothesis that these domains bind calcium and to gain insight into how this point mutation affects the structure and properties of this domain, we have synthesized and characterized a peptide corresponding to the thirteenth putative calcium binding EGF-like domain of fibrillin (Hfib13) and the corresponding Arg to Pro mutant peptide, Hfib13(R25P).

Results and discussion

Synthesis and folding of the wild-type domain (Hfib13)

A 42-residue peptide with the amino acid sequence corresponding to the thirteenth putative calcium binding EGF-like domain of fibrillin (Fig. 1) was synthesized by solid-phase methods. The peptide was purified to homogeneity in its reduced form by HPLC. The circular dichroism spectrum of this peptide revealed it to be in a random coil conformation (Fig. 2a). Folding of the peptide required the introduction of three disulfide bonds, which could be accomplished using a variety of oxidizing conditions such as air oxidation or various ratios of oxidized and reduced glutathione or cysteine. The HPLC analysis of the products from one such folding experiment is shown in Fig. 3a. A single major peptide product was generated. This material had the

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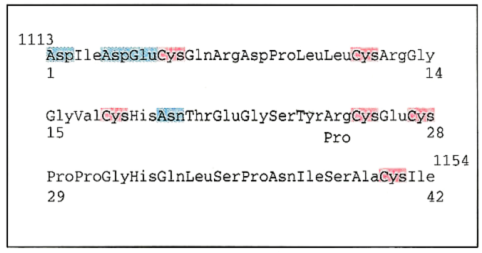

Fig. 1. The amino acid sequences of Hfib13 and Hfib13(R25P). The numbering scheme shown above the sequence corresponds to positions within intact fibrillin whereas the numbers shown below the sequence refer to the synthetic peptides. Hfib13(R25P) contains the single Arg to Pro mutation at position 25. Residues that may be involved in calcium binding are highlighted in blue. Cys residues are highlighted in pink.

expected molecular mass and was shown to contain three disulfide bonds and no free sulfhydryl groups [17]. The circular dichroism spectrum revealed significant structure as shown in Fig. 2a.

Structure and calcium binding of wild-type Hfib13

The one dimensional ^{1}H NMR spectrum of Hfib13 demonstrated strong evidence of a well-defined structure with well dispersed α proton and methyl group peaks (Fig. 4a). This spectrum was assigned using standard two-dimensional NMR methods [18]. The assignments of the amide and Cα proton shifts for Gly22 through Glu27are indicated in the fingerprint region of the nuclear Overhauser effect spectroscopy (NOESY) spectrum shown in Fig. 4b. Structural calculations were performed based on 481 distance and 18 dihedral angle constraints. Forty structures were generated, of which ten were found to have two or fewer constraint violations. The remaining structures had four or more violations. Fig. 4c shows the superposition of the ten structures which represent a single family and have a backbone

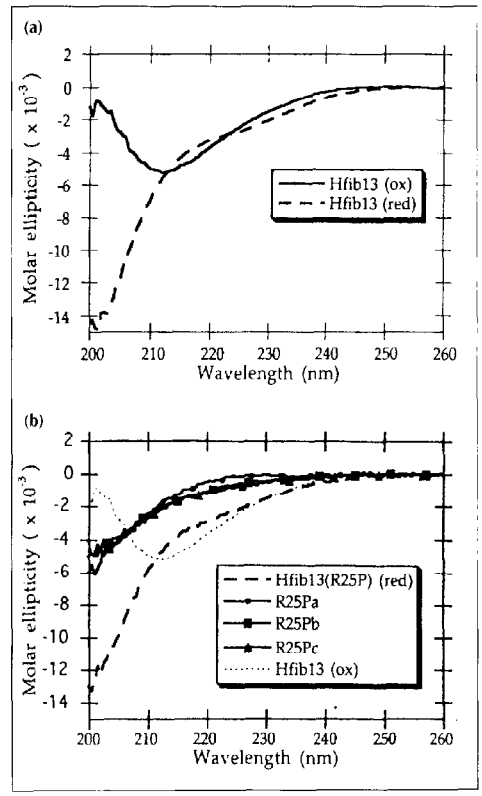

Fig. 2. Circular dichroism spectra of wild-type and mutated EGF-domain peptides. **(a)** The spectra of reduced and oxidized wild-type Hfib13. **(b)** The spectra of reduced Hfib13(R25P) and the three major oxidized products from the attempted folding reaction. The spectrum of oxidized Hfib13 is shown for comparison. All spectra were recorded at 21 °C.

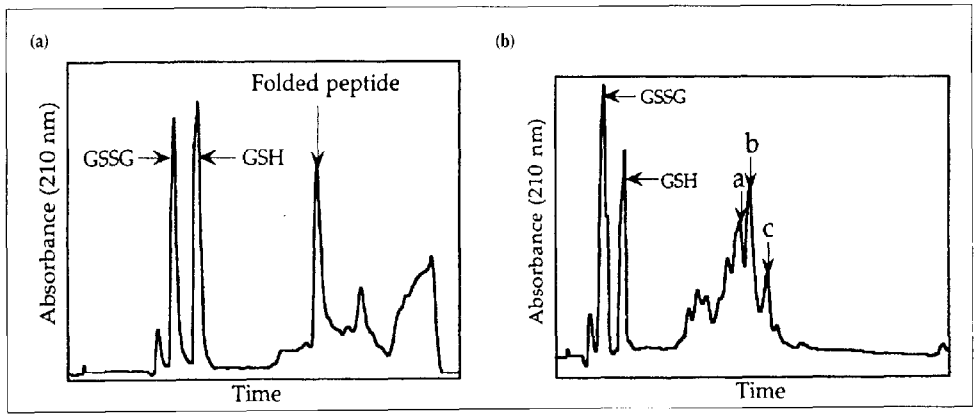

Fig. 3. HPLC analysis of folding reaction products. **(a)** The folding of wild-type Hfib13. GSSG and GSH indicate oxidized and reduced glutathione, respectively. **(b)** Attempted folding of Hfib(R25P). The three oxidized products with molecular masses corresponding to peptide monomers are indicated by a, b, and c.

root-mean-squared deviation of 1.36 Å relative to the average structure. A more detailed description of the structure determination will be published elsewhere. A ribbon diagram of the average structure of Hfib13 (Fig. 4d) shows a central antiparallel β-strand flanked by a small carboxy-terminal β-strand and a less-well-defined amino terminus. This resembles the structure of other EGF-like domains in terms of overall topology and disulfide connectivity [19].

Changes in the NMR spectrum of Hfib13 as a function of added calcium revealed that the isolated peptide binds calcium (Fig. 5a). Calcium binding was most easily monitored by observing the α proton resonance of Tyr24, which shifts upfield and sharpens with increasing calcium concentration. The chemical shift data for this proton as a function of added calcium concentration were fitted with a single-site binding equation as shown in Fig. 5b. The analysis revealed a dissociation constant of 0.6 ± 0.1 mM for the Hfib13–calcium complex. This binding is of the same order of magnitude as that observed for factors IX and X [9,10,12,13]. No significant changes were observed in the circular dichroism spectra of either the oxidized or the reduced forms upon addition of calcium (data not shown), suggesting that the structural changes that occur upon calcium

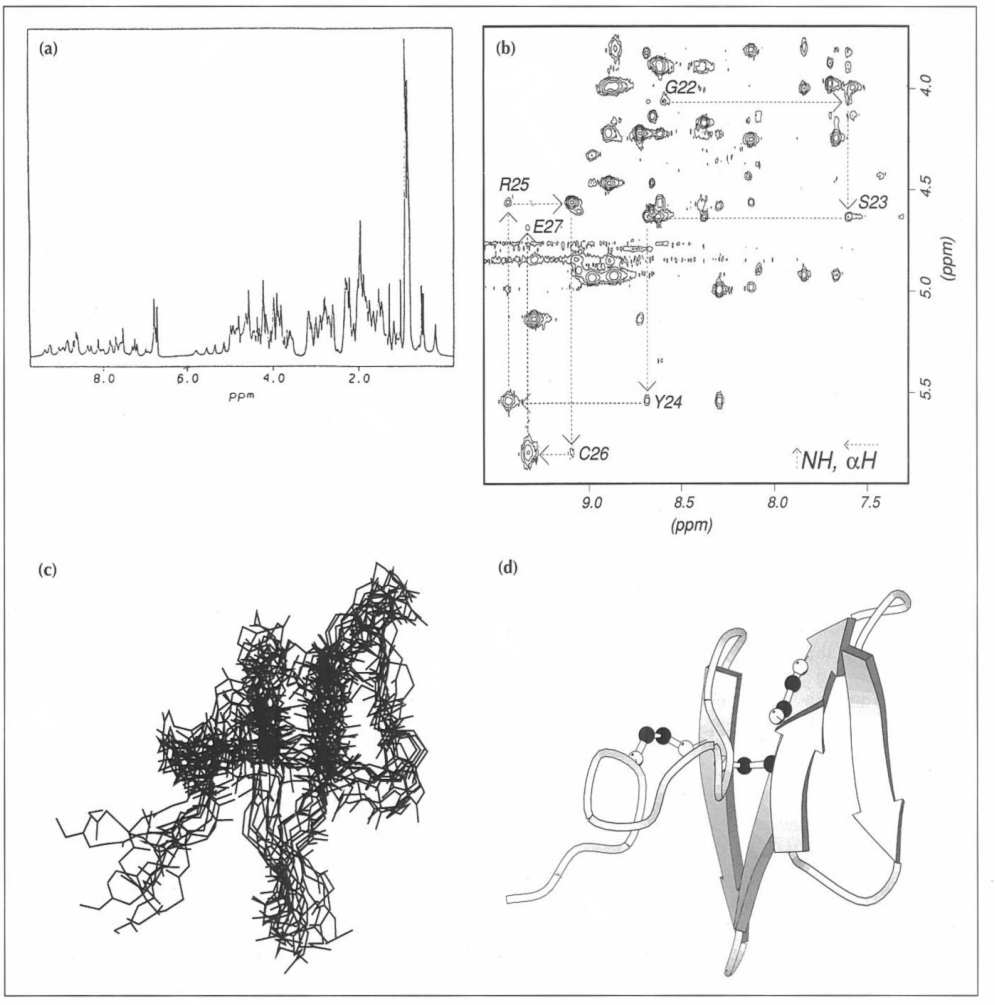

Fig. 4. A model of the structure of Hfib13 in the absence of bound calcium as probed using NMR methods. **(a)** The one-dimensional NMR spectrum of Hfib13 in H_2O. **(b)** Fingerprint region from a NOESY spectrum with a mixing time of 170 ms in H_2O at pH 6.5 and 25 °C. **(c)** Ten structures with two or fewer constraint violations generated by distance geometry/simulated annealing refinement. **(d)** A Molscript [25] diagram of the average structure from (c) showing the secondary structural elements, the disulfide bonds, and the overall fold.

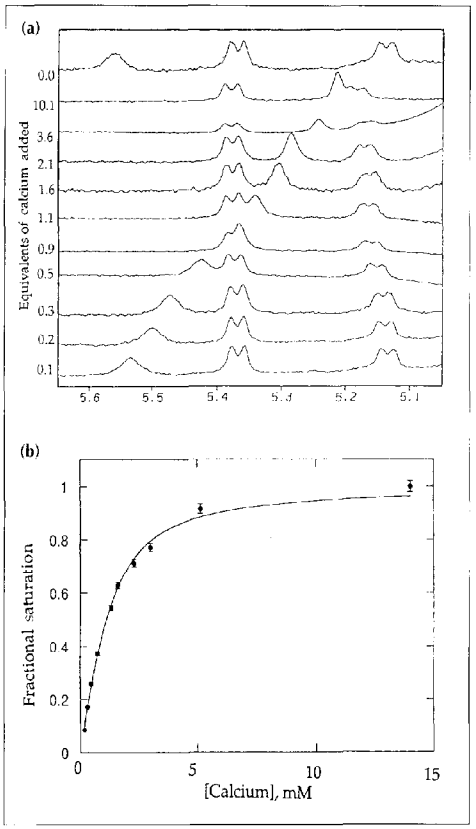

Fig. 5. Calcium binding by Hfib13. **(a)** One-dimensional NMR spectra of Hfib13 as a function of added calcium. **(b)** A fit of the chemical shift change for the alpha proton of Tyr24 as a function of added calcium concentration.

binding are relatively localized, and do not affect the overall folding of the peptide.

Synthesis and attempted folding of mutant Hfib13(R25P)

A 42-residue peptide containing a mutation at position 25, corresponding to the Arg to Pro mutation encoded in a fibrillin gene from two Marfan syndrome patients, was also studied. This peptide, Hfib13(R25P), was synthesized and purified to homogeneity in its reduced form. Folding of Hfib(R25P) was attempted using a variety of redox buffers and conditions, including those that were successful for the wild-type domain. Fig. 3b shows a typical result. A large number of products were generated, most of which were mixed disulfides involving glutathione. Three of the products (R25Pa, R25Pb, R25Pc) had the correct molecular mass. Further examination of these three products by circular dichroism spectroscopy (Fig. 2b) showed that none of these closely resembled the wild-type domain. Analysis of the disulfide bonds in these peptides [17] revealed that R25Pa appeared to have two disulfide bonds whereas R25Pb and R25Pc each have three. Further characterization of the most abundant product, R25Pb, by one- and two-dimensional NMR methods revealed spectra that were quite different from the wild-type peptide with somewhat broader lines. These results indicate that the mutated peptide does not fold efficiently to any form that resembles the wild-type domain in overall structure. Examination of the model of the wild-type structure reveals that Arg25 lies in the central β-sheet with two hydrogen bonds to His18 and is in an extended conformation. The $J_{NH\alpha}$ coupling constant of 10 Hz for Arg25 observed during NMR analysis confirms that this residue is in an extended conformation and suggests a value of ϕ near −120°. The substitution of Pro at this position would prevent the peptide from adopting an extended conformation at this position.

A model for calcium binding to intact fibrillin

We have demonstrated that a single EGF-like domain from fibrillin binds calcium with reasonable affinity. This fact strengthens the suggestion that the remaining 42 domains that share the calcium-binding consensus also bind calcium. Based on conserved sequence features and the calcium-binding properties of other EGF-like domains [11–13] it is likely that residues Asp1, Asp3, Glu4, and Asn19 of Hfib13 constitute part of the calcium-binding site. NMR structural studies of an EGF-like domain from factor X suggested that three of the side chains in these positions interact directly with calcium [11]. The structures generated for the factor X domain revealed at most five protein-derived ligands interacting with calcium. Calcium generally binds six or more ligands, suggesting that water molecules or ligands from other protein domains must also be part of the calcium coordination sphere. Modeling of this site within the context of a tandem array of directly abutted domains such as those present in fibrillin suggested that residues from the loop between the final pair of cysteines from the preceding domain could contribute one or more additional ligands.

Alignment of the amino acid sequences of all the EGF-like domains from fibrillin revealed the presence of a previously unnoted recurring Asp (or, more rarely, Asn) residue in the carboxy-terminal loops of these domains (Fig. 6a). These residues are present at the fourth and sixth residues prior to the last Cys in 19 and 11 of the 43 EGF-like domains, respectively. The occurrence of this residue appears to be unique to fibrillin as it is not found in other proteins with large tandem arrays of EGF-like modules such as Notch [20]. More detailed model building which explicitly included the interaction between calcium and the residue at the fourth position prior to Cys41 (Asn37 in the first domain) resulted in the model shown in Fig. 6b. The side chains of residue Asp1, Asp3, Glu4 and Asn19 from the second domain were also included as calcium ligands in the calculation.

The details of the model we propose are not crucial to our proposal that the calcium-binding sites occur at domain interfaces and may be important in stabilizing

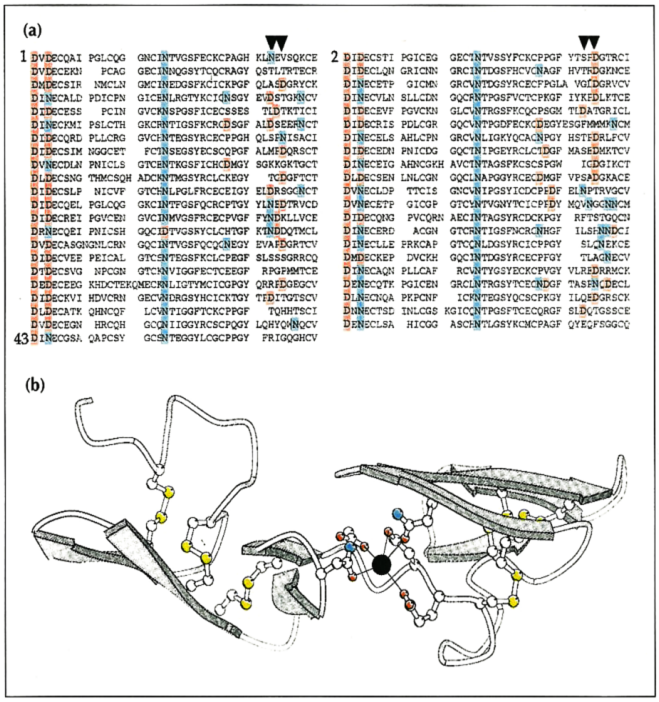

Fig. 6. A model for calcium binding to tandem arrays of EGF-like modules in fibrillin. **(a)** The sequences of the 43 calcium-binding EGF-like domains from fibrillin, illustrating the presence of a recurring potential calcium-ligating residue (Asp, red, or Asn, blue), indicated by the arrows in the region between the final pair of cysteines. **(b)** A Molscript [25] diagram of the refined model showing the calcium bridging between the two domains. The side chains for the proposed metal-binding residues and for the disulfide bonds are shown. See text for details.

interfaces. Such calcium-mediated interactions between EGF-like and other domains have been proposed in other systems although none has as yet been structurally characterized. The position of the binding sites in fibrillin between domains could allow calcium binding to contribute to the mechanical and dynamical properties of fibrillin. In addition, the inclusion of this interaction leads to the prediction that each domain is rotated by ~180° along the long axis of the array relative to the adjacent domains. A similar two-fold screw axis was predicted [21] and observed [22] for tandem type III fibronectin domains. In an interesting parallel to our proposal for fibrillin, a sodium ion was observed to lie at the domain interface coordinated by two ligands from each domain in the recently reported crystal structure of two tandem type III fibronectin domains from neuroglian [22].

Significance

Calcium binding is important in the structure of many proteins and in protein–protein interactions in a variety of systems. Studies of fibrillin purified from fibroblast cultures have demonstrated that it has an extended structure and that it binds calcium [6,7,23]. We have shown that a chemically synthesized peptide representing a single fibrillin domain folds into an EGF-like structure and binds calcium with reasonable affinity. When a mutation (Arg 1137 to Pro) found in two patients with the connective-tissue disorder Marfan syndrome is introduced into the peptide, the folding of the domain *in vitro* is disrupted. It is therefore possible that Marfan syndrome results from a misfolding of this domain, although direct proof that this also occurs *in vivo* remains to be obtained.

Since the report of the Arg1137Pro mutation, a number of additional mutations have been found that involve the substitution of cysteine residues presumed to be involved in intramolecular disulfide bonds [14]. Although no direct evidence is available, it seems likely that these mutations also cause global disruption of domain folding. Other mutations have been found that involve changes in the potential calcium-binding residues [15]. Based on our studies of individual domains, we have proposed a model for the role of calcium ions in which they help to form the interfaces in a tandem array of such domains. This model can be tested by preparing appropriate peptides comprising two adjacent domains.

The integrity of individual domains and relationships between adjacent domains is expected to be crucial to the assembly of fibrillin molecules into the oligomeric structures that form in microfibrils. Mutations that disrupt domain folding or relative domain orientation could adversely influence the intermolecular interactions that are required for proper microfibrillar assembly. It is

easy to imagine how such damaged molecules could poison the assembly of normal monomers resulting in the autosomal dominance that is characteristic of Marfan syndrome.

Materials and methods

Peptide synthesis

Peptides were synthesized on a Milligen/Biosearch 9050 peptide synthesizer using the solid-phase method with fluorenyl-9-methoxycarbonyl chemistry. Crude peptides were cleaved from the resin with a solution of 90:5:3:2 (v/v) trifluoroacetic acid : thioanisole : 1,2-ethanedithiol : anisole for 2 h, reduced with 110 mM dithiothreitol at 55 °C for 2 h and purified by reverse phase HPLC (Vydac C4 column, 0.1 % trifluoroacetic acid/acetonitrile gradient). Folding reactions were performed using a variety of conditions, including air oxidation and oxidation with several ratios of oxidized/reduced glutathione or cysteine. The procedure routinely performed for the wild-type peptide employed 3 mM oxidized glutathione, 3 mM reduced glutathione, 100 mM Tris, 1 mM EDTA, pH 8.8 under anaerobic conditions overnight. Folded products were purified by reverse phase HPLC, lyophilized, and stored at −20 °C.

Circular dichroism spectroscopy

Circular dichroism spectra were measured using an Aviv model 60DS spectropolarimeter. Spectra were recorded from 260 nm to 200 nm and averaged over six scans. Samples were in 5 mM Tris-HCl (pH 7.0) and peptide concentrations varied from 175 μg ml^{-1} to 370 μg ml^{-1}. The temperature was maintained at 21 °C using a water-jacketed cuvette holder and an external water bath.

Calcium binding studies

Peptide samples were dissolved in 99.9 % D_2O and lyophilized. The exchange and lyophilization procedure was repeated at least three times to exchange labile protons with deuterium. The dried sample was then dissolved in 99.96 % D_2O and the pH adjusted to 6.50 ± 0.05 (uncorrected for isotope effects) with aliquots of NaOD/DCl if necessary. Peptide concentrations ranged from 0.5 to 0.7 mM. Calcium chloride stock solutions were prepared in a similar manner to the peptide solutions. Aliquots of calcium stock solution were added to the peptide sample and the pH was measured and adjusted as described above. The titrations were continued until the calcium site was ~90 % saturated. All spectra were recorded on a Bruker AM600 MHz spectrometer at 298 K. Each spectrum contained 8192 points with 256 scans. The binding constant was calculated by iteratively fitting a one-site binding equation to the chemical shift change of the α proton resonance of Tyr24.

NMR studies

A sample of the wild-type peptide was dissolved in 90 % H_2O/10 % D_2O with 5 mM deuterated Tris to a concentration of ~5 mM and the pH was adjusted to 3.8 or 6.5. All two-dimensional NMR experiments were performed on a Bruker AM600 spectrometer. Double quantum filtered correlation spectroscopy (DQF-COSY) and NOESY spectra were recorded in the phase-sensitive manner using time proportional phase incrementation. The water signal was suppressed by presaturation for 1.5 to 2 s. DQF-COSY and homonuclear Hartmann–Hahn spectroscopy (t_m = 70 ms) data sets were collected at pH 3.8 and pH 6.5. In addition, a NOESY set was recorded with t_m = 170 ms at pH 3.8 and a NOESY build-up curve (t_m=25, 75, and 170 ms) was recorded at pH 6.5. Data were processed and analyzed with FELIX (Biosym Technologies, San Diego, CA).

Structural calculations

Structural calculations for Hfib13 (pH 6.5) were carried out using X-PLOR [24]. Calculations employed 481 NOE distance constraints determined from a 2D NOESY spectrum (t_m = 75 ms). 18 dihedral constraints were also included, as determined from JNHα coupling constants calculated from the COSY spectrum. The protocol involved distance geometry followed by simulated annealing and refinement with slow cooling. A total of 40 structures were generated and refined. Of these, 10 structures had two or fewer violations with NOE and dihehdral violations less than 0.8 Å and 6°, respectively.

A two-domain model was created employing CHAIN (Baylor College of Medicine, TX) by duplicating the Hfib13 structure and attaching the two domains head-to-tail. Refinement calculations for the two domain model were carried out similarly to those for Hfib13 with essentially the same constraints for each domain as for the single-domain calculations. However, no constraints taken from the single domain data were included for the last residue of the first domain or for the first five residues of the second domain in the two-domain model. Finally, for the two-domain model, a calcium ion was added with distances from the calcium to proposed calcium-binding ligands constrained to 2.5 Å.

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References

1. Hollister, D.W., Godfrey, M., Sakai, L.Y. & Pyeritz, R.E. (1990). Immunohistologic abnormalities of the microfibrillar-fiber system in the Marfan syndrome. *New Engl. J. Med.* **323**, 152–159.
2. Dietz, H.C., *et al.*, & Francomano, C.A. (1991). Marfan syndrome caused by a recurrent *de novo* missense mutation in the fibrillin gene. *Nature* **352**, 337–339.
3. Lee, B., *et al.*, & Hollister, D.W. (1991). Linkage of Marfan syndrome and a phenotypically related disorder to two different fibrillin genes. *Nature* **352**, 330–334.
4. Maslen, C.L., Corson, G.M., Maddox, B.K., Glanville, R.W. & Sakai, L.Y. (1991). Partial sequence of a candidate gene for the Marfan syndrome. *Nature* **352**, 334–337.
5. Sakai, L.Y., Keene, D.R. & Engvall, E. (1986). Fibrillin, a new 350 kD glycoprotein, is a component of extracellular microfibrils. *J. Cell Biol.* **103**, 2499–2509.
6. Sakai, L.Y., Keene, D.R., Glanville, R.W., Glanville, R.W. & Bächinger, H.P. (1991). Purification and partial characterization of fibrillin, a cysteine-rich structural component of connective tissue microfibrils. *J. Biol. Chem.* **266**, 14763–14770.
7. Corson, G.M., Chalberg, S.C., Dietz, H.C., Charbonneau, N.L. & Sakai, L.Y. (1993). Fibrillin binds calcium and is coded by cDNAs that reveal a multidomain structure and alternatively spliced exons at the 5' end. *Genomics* **17**, 476–484.
8. Handford, P.A., Mayhew, M. & Brownlee, G.G. (1991). Calcium binding to fibrillin? *Nature* **353**, 395.
9. Persson, E., Selander, M., Linse, S., Drakenberg, T., Öhlin, A.-K. & Stenflo, J. (1989). Calcium binding to the isolated β-hydroxyaspartic acid-containing epidermal growth factor-like domain of bovine factor X. *J. Biol. Chem.* **264**, 16897–16904.
10. Handford, P.A., *et al.*, & Campbell, I.D. (1990). The first EGF-like domain from human factor IX contains a high-affinity calcium

binding site. *EMBO J.* **9**, 475–480.
11. Selander-Sunnerhagen, M., Ullner, M., Persson, E., Teleman, O., Stenflo, J. & Drakenberg, T. (1992). How an epidermal growth factor (EGF)-like domain binds calcium: high resolution NMR structure of the calcium form of the NH_2-terminal EGF-like domain in coagulation factor X. *J. Biol. Chem.* **267**, 19642–19649.
12. Handford, P., Mayhew, M., Baron, M., Winship, P.R., Campbell, I.D. & Brownlee, G.G. (1991). Key residues involved in calcium-binding motifs in EGF-like domains. *Nature* **351**, 164–167.
13. Mayhew, M., Handford, P., Baron, M., Tse, A.G.D., Campbell, I.D. & Brownlee, G.G. (1992). Ligand requirements for Ca^{2+} binding to EGF-like domains. *Protein Eng.* **5**, 489–494.
14. Dietz, H.C., Saraiva, J.M., Pyeritz, R.E., Cutting, G.R. & Francomano, C.A. (1992). Clustering of fibrillin (FBN1) missense mutations in Marfan syndrome patients at cysteine residues in EGF-like domains. *Hum. Mutat.* **1**, 366–374.
15. Dietz, H.C., *et al.*, & Francomano, C.A. (1993). Four novel FBN1 mutations: significance for mutant transcript level and EGF-like domain calcium binding in the pathogenesis of Marfan syndrome. *Genomics* **17**, 468–475.
16. Hewett, D.R., Lynch, J.R., Smith, R. & Sykes, B.C. (1993). A novel fibrillin mutation in the Marfan syndrome which could disrupt calcium binding of the epidermal growth factor-like module. *Hum. Mol. Genet.* **2**, 475–477.
17. Thannhauser, T.W., Konishi, Y. & Scheraga, H.A. (1987). Analysis for disulfide bonds in peptides and proteins. *Methods Enzymol.* **143**, 115–119.
18. Wüthrich, K. (1986). *NMR of Proteins and Nucleic Acids.* Wiley-Interscience, New York.
19. Campbell, I.D. & Bork, P. (1993). Epidermal growth factor-like modules. *Curr. Opin. Struct. Biol.* **3**, 385–392.
20. Wharton, K.A., Johansen, K.M., Xu, T. & Artavanis-Tsakonas, S. (1985). Nucleotide sequence from the neurogenic locus *notch* implies a gene product that shares homology with proteins containing EGF-like repeats. *Cell* **43**, 567–581.
21. Leahy, D.J., Hendrickson, W.A., Aukhil, I. & Erickson, H.P. (1992). Structure of a fibronectin type III domain from tenascin phased by MAD analysis of the selenomethionyl protein. *Science* **258**, 987–991.
22. Huber, A.H., Wang, Y.-M.E., Bieber, A.J. & Bjorkman, P.J. (1994). Crystal structure of tandem type III fibronectin domains from *Droshophila* neuroglian at 2.0 Å. *Neuron* **12**, 717–731.
23. Keene, D.R., Maddox, B.K., Kuo, H.-J., Sakai, L.Y. & Glanville, R.W. (1991). Extraction of extendable beaded structures and their identification as fibrillin-containing extracellular matrix microfibrils. *J. Histochem. Cytochem.* **39**, 441–449.
24. Brünger, A.T. (1992). *X-PLOR: A System for X-ray Crystallography and NMR, Version 3.1.* Yale University Press, New Haven.
25. Kraulis, P. (1991). MOLSCRIPT: a program to produce both detailed and schematic plots of protein structures. *J. Appl. Crystallogr.* **24**, 946–950.

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