INTRODUCTION

Marfan syndrome (MFS), an autosomal dominant heritable disease of connective tissue, is characterized by defects in the ocular, skeletal and cardiovascular systems with an incidence of ∼1 in 5000 in the population (1,2). The defective protein is fibrillin-1, encoded by the human fibrillin-1 (FBN-1) gene which has been mapped to chromosome 15q21.1 (3). The FBN-1 gene is divided into 65 exons and codes for an ∼350 kDa extracellular glycoprotein profibrillin-1 which is secreted and proteolytically processed at the N- and C-termini (4–12). The processed ∼320 kDa fibrillin-1 is deposited into the extracellular matrix where it is the major component of a subclass of connective tissue microfibrils with a 10–12 nm diameter (13). The precise assembly mechanism of the fibrillin-1 monomers into the microfibrils is still unknown, but it is thought that MFS occurs via a dominant negative mechanism whereby the mutant protein disrupts the function of the normal fibrillin-1 protein during assembly (14,15).

Fibrillin-1 is comprised mainly of two repeated modules, the epidermal growth factor-like (EGF) domain and the transforming growth factor-β1 binding protein-like (TB) domain (Fig. 1) (16,17). There are 47 EGF domains, 43 of which are associated with calcium binding. The calcium binding epidermal growth factor-like (cbEGF) domains contain the calcium binding consensus residues (see above) constituting a calcium binding site (18–21). In addition each cbEGF contains six cysteine residues which disulphide bond in a 1–3, 2–4, 5–6 arrangement (22,23). The TB domain is characterized by eight cysteines predicted to form four intramolecular disulphide bonds (24). There are seven TB domains distributed throughout fibrillin-1 separated by variable numbers of tandemly repeated EGF domains, predominantly cbEGF domains. In addition there are two hybrid domains, a proline-rich region and termini which show homology to other matrix proteins (Fig. 1) (25). Over 150 mutations causing MFS and related disorders have been reported to date, spanning the whole length of the gene and with no clear genotype–phenotype relationship established (26,27). The one exception is a group of mutations which cluster in exons 24–32 associated with neonatal MFS, a severe form of the disease usually leading to death within the first year of life due to cardiovascular complications (28,29).

Mutations found so far include insertions, deletions and missense mutations. Those mutations specifically affecting calcium binding consensus residues (see above) constitute a significant proportion (∼10%) of the MFS-causing mutations found so far, highlighting the importance of understanding the structural implications of these mutations. Furthermore, similar mutations have been found to cause haemophilia B.
It has previously been shown that calcium plays a key role in the maintenance of microfibril architecture (34–36). At a molecular level, calcium has been shown to stabilize the rod-like conformation of a fibrillin-1 cbEGF domain pair and restrict conformational flexibility (37,38). As a consequence, calcium protects the fibrillin-1 molecule from proteolysis (39). Structural effects of calcium binding mutations might be expected therefore to cause changes in the proteolytic susceptibility of fibrillin-1. Previous calcium binding studies of an N2144S MFS-causing mutation in cbEGF32 suggested that the role of calcium in modulating structural interaction between TB6 and cbEGF32 may be different from cbEGF32–33. Covalent linkage of TB6 to cbEGF32 did not markedly alter the calcium binding properties of cbEGF32, in contrast to addition of an N-terminal cbEGF domain to an isolated cbEGF domain (40–42). If the structural effects of calcium binding mutations are quite different, then phenotype–genotype relationships in MFS patients with this type of mutation would be expected to be variable and thus complex to interpret.

In this study we have used proteases to investigate the structural consequences of calcium binding mutations in different contexts by: (i) probing wild-type interactions in TB6–cbEGF32 and cbEGF32–33 domain pairs in the presence and absence of calcium; and (ii) assessing the effects of an MFS N2144S and a protein engineered N2183S calcium binding mutation in these pairs. We show that calcium plays a variable role in pairwise interactions and that structural consequences of an N→S calcium binding mutation are markedly more severe when flanked at the N-terminus by cbEGF32 compared with TB6. Implications for understanding the phenotype–genotype relationship in MFS are discussed.

RESULTS

Expression and purification of wild-type and mutant domain pairs

The expression and purification of TB6–cbEGF32 and cbEGF32–33 wild-type and N2144S mutant domain pairs has been described previously (40,41). A calcium binding mutation structurally equivalent to N2144S was introduced into cbEGF33 (cbEGF32–33 N2183S) and the mutant domain pair expressed as a His-tag fusion protein. A well characterized in vitro refolding system was used to produce the native disulfide bonded structure. Native structure was confirmed by nuclear magnetic resonance (NMR) analysis by comparison with spectra obtained previously from cbEGF32–33 wild-type (data not shown) (37). The proteolytic susceptibility of the TB6–cbEGF32 and cbEGF32–33 wild-type domain pairs was examined with three different proteases: trypsin, elastase and plasmin.

Proteolysis of the TB6–cbEGF32 wild-type domain pair

Comparative digests of TB6–cbEGF32, performed in the presence and absence of calcium at constant ionic strength, revealed no calcium-dependent protection, irrespective of the protease used. Figure 2A shows the results of trypsin and elastase digestion of TB6–cbEGF32. In these experiments, it should be noted that tryptic digestion is enhanced in the presence of calcium since trypsin requires calcium to protect against autolytic cleavage.

N-terminal sequence analysis of the tryptic digests of the TB6–cbEGF32 wild-type pair identified identical cleavage sites whether or not calcium was present in the digest. A single cleavage site, 2108QICPYGS, was located in TB6 and a second site, 2108HGQCINT, was located in cbEGF32. The cleavage at 2108QICPYGS is in the last α-helix of the TB6 domain before the linker region joining TB6 and cbEGF32 (Fig. 3A). The cleavage at 2108HGQCINT is located between the second and third cysteines in the N-terminal region of the cbEGF32 domain (Fig. 3A). The proximity of these cleavage sites to the calcium binding site in cbEGF32 suggest that protection from digestion would be seen if calcium was restricting conformational flexibility between these domains, as is suggested to occur in tandem cbEGF domains. No calcium-dependent protection against proteolysis was observed after treatment with elastase (Fig. 2A) or plasmin (data not shown), despite the fact that these enzymes gave different cleavage patterns indicating an altered sequence specificity. Collectively these data are consistent with calcium independent domain–domain
interactions between TB6 and cbEGF32, despite the presence of a calcium binding site in cbEGF32.

**Proteolysis of the cbEGF32–33 wild-type domain pair**

In marked contrast to the TB6–cbEGF32 wild-type pair, increased susceptibility of the cbEGF32–33 pair to proteolysis by trypsin and elastase was seen in ethylene glycol-\(β\)-aminoethyl ether-\(N,N,N',N'\)-tetraacetic acid (EGTA) compared with calcium. Figure 2B shows comparative tryptic digests of cbEGF32–33 performed at constant ionic strength. Despite the fact that trypsin is susceptible to autolytic inactivation in the absence of calcium, more enzymatic cleavage is seen in EGTA than in calcium for this pair. This demonstrates calcium-dependent protection of this pair against proteolysis. Similar results were observed with elastase (Fig. 2B) and plasmin (data not shown).

Mapping of tryptic cleavage sites in EGTA for the cbEGF32–33 wild-type pair identified one cleavage site, \(^{2183}\)NVIGGFE, in cbEGF33. This site is located on the central anti-parallel β-sheet of cbEGF33 (Fig. 3B) and occurs adjacent to a predicted calcium binding ligand (N2183). The small amount of cleavage also observed in digests performed in parallel in calcium (Fig. 2B) indicated that calcium protection was not absolute under the experimental conditions used. However, quantitative assessment of cleavage after 120 min in parallel experiments identified approximately three times more cleavage in EGTA than calcium. These data, together with qualitative assessment of elastase (Fig. 2B) and plasmin (data not shown) digests are consistent with a calcium-dependent
TB6–cbEGF32 N2144S mutant. No difference in the proteolytic susceptibility of the TB6–cbEGF32 N2144S mutant in either EGTA or calcium was observed compared with the wild-type domain pair (Fig. 4A). The mutant therefore exhibited the same absence of calcium-dependent protection against proteolysis as seen in the wild-type. In addition, N-terminal sequences derived from the tryptic digests of wild-type and mutant TB6–cbEGF32 digested in EGTA or calcium were identical to those observed previously in the wild-type (Fig. 3A).

cbEGF32–33 N2183S mutant. In contrast to the cbEGF32–33 wild-type there was a marked enhancement in proteolysis of the cbEGF32–33 N2183S mutant in calcium. In EGTA both the wild-type and mutant pairs showed similar patterns of cleavage with a minor sequence-specific effect of the mutation on cleavage (Fig. 4B). In both 5 and 50 mM Ca²⁺ proteolysis of the cbEGF32–33 N2183S mutant was greatly enhanced compared with the cbEGF32–33 wild-type (Fig. 4B). This mutation thus abolishes calcium-dependent protection against proteolysis seen in the cbEGF32–33 wild-type and the cleavage pattern resembles that of the wild-type and mutant obtained in EGTA. N-terminal sequence analysis of the tryptic digest of the cbEGF32–33 N2183S mutant in EGTA and in calcium identified a single cleavage site, 2183SVIGGFE, which is the same site exposed in the cbEGF32–33 wild-type pair in EGTA (Fig. 3B).

cbEGF32–33 N2144S mutant. The cbEGF32–33 N2144S mutant showed no difference in proteolytic susceptibility to the cbEGF32–33 wild-type in either EGTA or calcium, hence exhibiting the same calcium-dependent protection against proteolysis as the wild-type (Fig. 4C). N-terminal sequence analysis of the tryptic cleavage products from the cbEGF32–33 N2144S mutant in EGTA and calcium gave the same results as for the cbEGF32–33 wild-type (the original N-terminus and 2183SVIGGFE in varying proportions) (Fig. 3B). Thus, reduced calcium binding of cbEGF32 does not affect the calcium-dependent interaction of cbEGF32–33. This is in agreement with previous calcium binding studies of this mutant using NMR methodology (41).

**DISCUSSION**

FBN-1 mutations which disrupt calcium binding in EGF domains by specific removal of a side chain ligand are a significant group of reported MFS-causing mutations (27). The grouping of such mutations implies a common underlying structural defect. However, in this study we have demonstrated two different structural effects of an N→S calcium binding mutation caused by the different domain context of the mutation. Comparative proteolytic digests of mutant and wild-type pairs revealed that an MFS-causing calcium binding mutation (N2144S) did not alter the proteolytic susceptibility of a TB6–cbEGF32 pair in the presence of calcium. This was in direct contrast to the effect of a structurally equivalent mutation (N2183S) expressed in a different domain pair (cbEGF32–33) which produced a marked increase in proteolysis. In each case the effect of the mutation was consistent with the observed proteolytic susceptibility of the wild-type domain pairs, which indicated that domain interactions between cbEGF32–33, but

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**Figures:**

**A** shows Schematic illustrations of (A) the TB6-cbEGF32 pair and (B) the cbEGF32–33 pair. The calcium ions are shown as spheres and the β-sheets depicted by arrows. The pairwise interaction of TB6-cbEGF32 is unknown so the TB6 domain and the cbEGF32 domain are not shown covalently linked. The positions of the cleavage sites and the N-termini from tryptic digests of the wild-type and mutant pairs are marked. This figure was rendered (50) from MOLSCRIPT (51) input.

**B** shows a diagram of a protein structure with the N-terminal sequence QHPFVGS and the cleavage site (NS)VIGGFE.

**Analysis of TB6–cbEGF32 and cbEGF32–33 N→S mutants**

An N2144S mutation was introduced previously into TB6-cbEGF32 and cbEGF32–33 (41). This MFS-causing mutation is predicted to remove one of the side chain ligands for calcium and is located on the central β-sheet of cbEGF32 (21,43). The high degree of structural homology observed between cbEGF32 and -33 (37) and other cbEGF domains (20,21) allowed the introduction of a structurally equivalent mutation N2183S into cbEGF33 of cbEGF32–33. Figure 4 shows schematic illustrations of the mutant domain pairs.

Comparative digests of mutant and wild-type pairs were performed under three conditions using trypsin, elastase and plasmin: (i) EGTA, to assess any sequence-specific effect of the introduced mutation on the rate of proteolysis; (ii) 5 mM Ca²⁺; and (iii) 50 mM Ca²⁺ to ensure saturation of each calcium binding site within the domain pairs during proteolysis (Fig. 2) (40,41). Protease digests performed in calcium were carried out at constant ionic strength (see Materials and Methods). N-terminal sequence analysis was carried out on tryptic digests performed in EGTA and calcium to identify any sites made susceptible to calcium-dependent proteolysis on introduction of the mutation.

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**Table:**

<table>
<thead>
<tr>
<th>Domain Pair</th>
<th>Calcium (mM)</th>
<th>Proteolytic Susceptibility</th>
</tr>
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<tbody>
<tr>
<td>TB6–cbEGF32</td>
<td>EGTA, 5 Ca²⁺</td>
<td>No difference</td>
</tr>
<tr>
<td></td>
<td>EGTA, 50 Ca²⁺</td>
<td>No difference</td>
</tr>
<tr>
<td></td>
<td>Calcium</td>
<td>Marked enhancement</td>
</tr>
<tr>
<td>cbEGF32–33</td>
<td>EGTA, 5 Ca²⁺</td>
<td>Similar pattern</td>
</tr>
<tr>
<td></td>
<td>EGTA, 50 Ca²⁺</td>
<td>Similar pattern</td>
</tr>
<tr>
<td></td>
<td>Calcium</td>
<td>Marked enhancement</td>
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**Graphs:**

- Figure 3: Schematic illustrations of the mutant domain pairs.
- Figure 4: Cleavage patterns of tryptic digests of wild-type and mutant pairs in varying calcium concentrations.
not TB6–cbEGF32, were calcium dependent (see Results). The significance of these data for identifying structure–function relationships in fibrillin-1 and MFS is that, although all calcium binding mutations may cause a similar structural defect within an individual domain, the interdomain effects of defective calcium binding may be quite different. It is now evident from this study, and previous genetic and biochemical studies, that structural consequences of calcium binding mutations may be influenced by the affinity, domain context and precise location of the cbEGF domain within fibrillin-1. This may explain why this group of mutations are associated with a variety of phenotypes (neonatal MFS, isolated ectopia lentis and classic MFS) (27).

Consequences of cbEGF–cbEGF domain interactions

In fibrillin-1, the cbEGF domain is most commonly preceded by either another cbEGF domain or a TB domain (Fig. 1). Figure 5 summarizes all the published calcium binding MFS mutations which occur in these two contexts (26,27). Other fibrillin cbEGF pairs are likely to adopt a calcium-dependent structure similar to cbEGF32–33 since they show sequence conservation with respect to those residues which maintain the rigid linkage between domains (i.e. calcium binding consensus residues, hydrophobic packing residues) and have the same number of residues between domains (37). The calcium-dependent resistance to proteolysis shown by cbEGF32–33 in this study is consistent with a key role for calcium in rigidifying the interdomain linkage, suggested by the NMR structure and recent dynamics analysis of this pair (37,38). The earlier prediction that reduction of calcium binding caused by specific mutation of a calcium ligand would destabilize the interdomain linkage of a cbEGF pair is supported by the increased proteolysis seen in the N2183S mutant pair (37,38).

It is interesting to note that in our study the amount of proteolytic cleavage at 2183SVIGGFE which occurred as a consequence of the N2183S mutation in cbEGF32–33 was similar to the cleavage observed in cbEGF32–33 wild-type in EGTA (Fig. 4B). Thus the removal of a single side chain calcium ligand on the central anti-parallel β-sheet of cbEGF33 had the same effect on structure (detected by increased proteolysis) as a complete absence of calcium binding. These data highlight the importance of this particular ligand for precise orientation of tandem fibrillin cbEGF domains and thus protection from proteolysis in our assay. It remains to be determined whether mutation of different side chain ligands for calcium and the nature of the amino acid substitution cause the same degree of structural change, both of which could modulate phenotypic severity if different effects were found.

The affinity of the calcium site itself has the potential to modulate the phenotypic expression of a calcium binding mutation. There is considerable variation in the $K_d$ value reported for the higher affinity calcium binding site in different
cbEGF pairs (Fig. 2B), the structural basis of which is not yet known (40,42,44). It has been noted previously that higher affinity of cbEGF13 in a cbEGF12–13 pair may be of functional significance (42). This is located in the region of fibrillin-1 where certain mutations cause neonatal MFS (28). Since this site would be predicted to be fully saturated under physiological conditions, a greater conformational change would be expected to result from a calcium binding mutation within this domain, than would occur at a site with lower affinity which was partially saturated under normal conditions.

Consequences of TB–cbEGF domain interactions

For TB6–cbEGF32, the absence of a detectable difference in proteolytic susceptibility between the mutant and wild-type forms suggests that the reduced calcium binding caused by the N2144S mutation causes a local conformational effect. This is in agreement with previous calcium binding data for these domain constructs (41). It has previously been suggested that this mutation may exert its effect either by increasing flexibility of a ‘mobile’ linkage or by altering the elastic properties of this region (41). Either one of these possibilities is consistent with our proteolytic data. In contrast to cbEGF pairs, the number of linker residues between TB and cbEGF domains (defined as the residues between the last cysteine in the TB domain and the first calcium binding ligand in the cbEGF) is variable, ranging from 11 to 19 residues. Therefore, although the observed structural effect of a calcium binding mutation in TB6–cbEGF32 is clearly different from the same mutation in cbEGF32–33, the structural effects of calcium binding mutations in other TB–cbEGF pairs cannot be predicted. Some evidence that these sites may be different has come from a study of a cbEGF22–TB4–cbEGF23 triple fragment which contains a potential RGD tripeptide integrin-binding motif. This had an average $K_d$ for calcium of 400 µM, compared with the $K_d$ of 1.6 mM in TB6–cbEGF32 (8,41). The increased affinity suggests a more important structural role for calcium at this domain junction than in TB6–cbEGF32 and thus a different function, possibly related to integrin binding (45,46).

Finally it should be noted that the precise location and associated function of a particular cbEGF domain may influence the phenotypic effect of a calcium binding mutation. Very little is known about higher order interactions between fibrillin-1 within the microfibril and other associated proteins, although specific cbEGF domains have been implicated in forming transglutaminase cross-links (47). It is possible that within the microfibril cross strand donation of a seventh protein ligand to the calcium binding site in specific cbEGF domains occurs, making these domains more important than others for stabilizing higher order interactions (37).

Implications for pathogenic mechanism in MFS

It has been suggested that proteolysis of mutant fibrillin-1 proteins may play a significant role in the pathogenic mechanism of MFS, since fibrillin-1 fragments are more rapidly degraded in the absence of calcium in vitro (39). In support of this, calcium binding mutations associated with MFS and related disorders were found to alter protease digestion profiles of recombinant fibrillin fragments in vitro (48,49). The use of proteases in our study also shows that susceptible sites may be introduced into fibrillin-1 by altering its calcium binding properties. However, it remains to be proven whether proteolysis, or the structural change itself (independent of proteolysis), is the cause of disease. It is possible that aberrant
assembly of microfibrils in the extracellular matrix, caused by the primary structural defect in fibrillin-1, results in the production of matrix-degrading proteases [such as the matrix metalloproteinases (MMPs)] and thus proteolysis has a secondary effect on disease pathogenesis. However, it is evident from analysis of TB6–cbEGF32 that there is no calcium-dependent protection from proteolysis even for the wild-type fragment under the experimental conditions used. Proteolysis does not therefore appear to be a cause of disease in the case of MFS caused by the N2144S calcium binding mutation, although we cannot formally exclude increased susceptibility to other types of protease such as MMPs. Development of in vitro models of microfibril assembly coupled with in vivo studies of Marfan pathology will be required to identify pathogenic mechanisms.

In summary, this study identifies the importance of domain context for modulating the structural effects of calcium binding mutations and suggests an explanation why MFS phenotypes associated with apparently similar mutations may be diverse.

MATERIALS AND METHODS

Cloning of wild-type and mutant domain pairs and purification of recombinant peptides

DNA fragments (nucleotides 6293–6628 and 6503–6748 of human fibrillin-1 cDNA) encoding the wild-type sequences of the domain pairs TB6–cbEGF32 and cbEGF32–33 (residues 2054–2165 and 2124–2205, respectively, numbered according to the published sequence) were amplified and cloned as previously described (41). The N2144S mutation was introduced into both plasmids, using PCR-based site-directed mutagenesis (41). The N2183S mutation was introduced into the plasmid containing the cDNA sequence for cbEGF32–33 by the same method and the clone sequenced to confirm introduction of the correct mutation. Protein production and purification of the wild-type and mutant domain pairs was carried out as described previously (40, 41). Mass spectrometry was performed on all samples (data not shown). NMR analysis was also performed to confirm correct folding of the cbEGF32–33 N2183S mutant. All other constructs were characterized as described previously (41).

Proteolytic degradation of recombinant domain pairs

Aliquots (50 µg) of the recombinant domain pairs peptides were supplemented with 50 mM EGTA, 5 or 50 mM CaCl₂ buffered at a final concentration of 50 mM Tris–HCl to pH 7.5 (trypsin, plasmin) or pH 8.0 (elastase) and adjusted to equivalent ionic strength with NaCl. The peptides were then incubated at 37°C with trypsin (bovine pancreatic, TPCK treated; Sigma, Poole, UK) at a final enzyme concentration of 10 µg/ml, elastase (porcine pancreatic; Sigma) at 70 µg/ml and plasmin (human; Sigma) at 405 µg/ml. A time course of digestion was performed and for each aliquot the reaction was stopped by the addition of 2 vol of 2-fold concentrated Laemmli’s sample buffer supplemented with 100 mM dithiotreitol, and immediately heating to 95°C for 5 min.

Analysis of proteolytic degradation

After proteolytic degradation, samples from digests were separated by sodium dodecyl sulphate (SDS) gel electrophoresis on homogeneous 16% acrylamide (v/v) gels. After electrophoresis the gels were stained in 0.125% (w/v) Coomassie brilliant blue R250 (Sigma) in 10% methanol, 10% acetic acid and 80% water.

For N-terminal sequencing of the tryptic digests, each reaction was stopped by acidification to pH 2 and purified under non-reducing conditions by reverse phase high pressure liquid chromatography (HPLC) using a 20–80% B gradient over 50 min (buffer A: 0.1% trifluoroacetic acid; buffer B: 80% acetonitrile, 0.1% trifluoroacetic acid). Aliquots of HPLC fractions were analysed by SDS gel electrophoresis and the remaining sample used directly for N-terminal sequencing on an Applied Biosystems 494A Procise sequencer (PE Biosystems, Warrington, UK). For quantitative analysis of digested products the amount of internally cleaved peptide (in pmol) was expressed as a percentage of the authentic N-terminus observed in the same sample. Cysteine residues reported in the N-terminal sequences are inferred from the published sequence (17).

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