A G1127S Change in Calcium-binding Epidermal Growth Factor-like Domain 13 of Human Fibrillin-1 Causes Short Range Conformational Effects*

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Human fibrillin-1, an extracellular matrix glycoprotein, has a modular organization that includes 43 calcium-binding epidermal growth factor-like (cbEGF) domains arranged as multiple tandem repeats. A missense mutation that changes a highly conserved glycine to serine (G1127S) has been identified in cbEGF13, which results in a variant of Marfan syndrome, a connective tissue disease. Previous experiments on isolated cbEGF13 and a cbEGF13-14 pair indicated that the G1127S mutation caused defective folding of cbEGF13 but not cbEGF14. We have used limited proteolysis methods and two-dimensional NMR spectroscopy to identify the structural consequences of this mutation in a covalently linked cbEGF12-13 pair and a cbEGF12-14 triple domain construct. Protease digestion studies of the cbEGF12-13 G1127S mutant pair indicated that both cbEGF12 and 13 retained similar calcium binding properties and thus a tertiary structure to the normal domain pair, because all identified cleavage sites showed calcium-dependent protection from proteolysis. However, small changes in the conformation of cbEGF13 G1127S, revealed by the presence of a new protease-sensitive site and comparative two-dimensional NOESY data, suggested that the fold of the mutant domain was not identical to the wild-type, but was native-like. Additional cleavage sites identified in cbEGF12-14 G1127S indicated further subtle changes within the mutant domain but not the flanking domains. We have concluded the following in this study. (i) Covalent linkage of cbEGF12 preserves the native-like fold of cbEGF13 G1127S and (ii) conformational effects introduced by G1127S are localized to cbEGF13. This study demonstrates that missense mutations in fibrillin-1 cbEGF domains can cause short range structural effects in addition to long range effects previously observed with a E1073K mutation in cbEGF12.

The epidermal growth factor-like (EGF) domain is a widely...
causing mutations are associated with extreme phenotypic diversity, suggests that structural investigations of this region may yield important insights into the mechanism of disease.

A previous study has shown that the G1127S mutation resulted in defective folding in vitro when introduced into the single cbEGF domain 13. When present in the covalently linked cbEGF13-14 domain pair, the C-terminal cbEGF14 domain adopted the native fold and retained calcium binding properties despite the fact that the adjacent domain 13 was misfolded. This suggested that the effects of the mutation were localized (21). Here the structural consequences of the G1127S mutation in the cbEGF12-13 domain pair and also in the triple domain fragment, cbEGF12-14, have been investigated, using a combination of protease digestion studies and two-dimensional NMR methods. The data indicate that covalent linkage of cbEGF12 moderates the effect of G1127S in cbEGF13 resulting in localized changes to the structure and calcium binding properties of domain 13, but not cbEGF12 or 14. The implications of such short range effects for the pathogenic mechanism of MFS are discussed.

**EXPERIMENTAL PROCEDURES**

Cloning of Wild-type and Mutant cbEGF Domain Constructs from Human Fibrillin-1—DNA fragments (nucleotides 3338–3595 and 3338–3721 of human fibrillin-1 cDNA) encoding the wild-type sequences of the cbEGF12-13 domain pair and the cbEGF12-14 triple construct (residues 1069–1154 and 1069–1196 respectively, numbering according to Ref. 22) were amplified by standard polymerase chain reaction techniques using Pfu polymerase (Stratagene). The forward primer in cbEGF12 and the reverse primers for cbEGF13 and cbEGF14, together with the cloning procedures used, were as described previously (21, 23).

The G1127S mutation was introduced into the pQE30 recombinant plasmids containing the cDNA sequences of the cbEGF12-13 double or cbEGF12-14 triple constructs by PCR-based site-directed mutagenesis. The plasmids were amplified using a forward primer: 5′-GAGTTAGT-GTTGATAC-3′ and a reverse primer: 5′-GGCATAGGAGGATC-3′. The amplified DNA was purified from a 0.7% agarose gel with GTTGCCATAAC-3′.

The plasmids were amplified using a forward primer: 5′-cbEGF12-14 triple constructs by PCR-based site-directed mutagenesis.

**TABLE I**

Characterization of the purified cbEGF domain pairs and triple constructs

The molecular masses were calibrated against horse heart myoglobin (16951.48 Da). The theoretical mass of each domain pair or triple construct is shown in parentheses. Reduced mass includes the His6 affinity tag used to facilitate purification and the Factor Xa cleavage site, which are cleaved after refolding.

| Peptide | Reduced mass | Oxidized mass |
|---------|--------------|---------------|
| cbEGF12–13 | 11512.44 ± 0.81 (11512.97) | 9645.89 ± 0.19 (9646.86) |
| cbEGF12–13 (G1127S) | 11541.57 ± 0.05 (11542.99) | 9677.48 ± 0.14 (9676.89) |
| cbEGF12–14 | not determined (14278.06) | 14279.04 ± 0.21 (14278.06) |
| cbEGF12–14 (G1127S) | 16179.22 ± 0.77 (16174.19) | 14308.00 ± 0.02 (14308.09) |

A previous study has shown that the G1127S mutation resulted in defective folding in vitro when introduced into the single cbEGF domain 13. When present in the covalently linked cbEGF13-14 domain pair, the C-terminal cbEGF14 domain adopted the native fold and retained calcium binding properties despite the fact that the adjacent domain 13 was misfolded. This suggested that the effects of the mutation were localized (21). Here the structural consequences of the G1127S mutation in the cbEGF12-13 domain pair and also in the triple domain fragment, cbEGF12-14, have been investigated, using a combination of protease digestion studies and two-dimensional NMR methods. The data indicate that covalent linkage of cbEGF12 moderates the effect of G1127S in cbEGF13 resulting in localized changes to the structure and calcium binding properties of domain 13, but not cbEGF12 or 14. The implications of such short range effects for the pathogenic mechanism of MFS are discussed.

**FIG. 1.** Schematic illustration of the domain organization of fibrillin-1. The positions of the neonatal region and the cbEGF domain constructs studied here are indicated. The G1127S mutation occurs in cbEGF13, highlighted by an asterisk.
Local Structural Effects of G1127S in Human Fibrillin-1

RESULTS

Expression and Purification of Wild-type and Mutant cbEGF Domain Constructs—The predicted structure of the cbEGF12-14 triple construct analyzed in this study, modeled on the coordinates of the cbEGF32–33 domain pair from human fibrillin-1, is shown in Fig. 2 (5, 23). This model was based on the coordinates of the solution structure of fibrillin-1 cbEGF32–33 determined in the presence of calcium (5, 23). The cbEGF12–13 domain pair and the cbEGF12–14 triple constructs are indicated. The position of G1127, the site of the mutation, is highlighted, and calcium atoms are shown as gray spheres.

1.93 mT, respectively. Added NaCl was not utilized in these investigations because high ionic strength may compromise spectral quality. Two-dimensional NOESY spectra (24, 25) were acquired for both samples at 0 mM CaCl₂ and 12.5 mM CaCl₂, to allow qualitative assessment of calcium binding. At 12.5 mM CaCl₂, the two calcium binding sites of the wild-type domain pair are saturated based on previous calcium binding studies of the cbEGF12–13 domain pair (26), and these calcium-loaded spectra were also used to assess the structural consequences of the G1127S mutation. All spectra were recorded with a mixing time of 150 ms at T = 33 °C. Water suppression was achieved using field gradients (27). Data were processed using Felix 2.3 (Biosym, Inc.). 1024 complex points were acquired in F₂ and F₃, for each experiment with a spectral width of 8000 Hz in each dimension. Spectra were referenced with respect to the H'HO resonance and were zero-filled to 8K in the F₂ dimension to yield a digital resolution of 0.98 Hz/pt.

Protease Digestion of the cbEGF12-14 Triple Domain Constructs—Trypsin digestion and subsequent SDS-PAGE analysis of both the wild-type and mutant cbEGF12-14 triple constructs in the presence of EGTA (10 mM) and Ca²⁺ (10 mM) again showed the protection against digestion afforded by calcium (Fig. 3B). The G1127S mutation did not appear to significantly affect the calcium binding properties of the construct, because the calcium-dependent Protection from proteolysis was indistinguishable from that of the wild-type on SDS-PAGE. Calcium protection was also evident on digestion by endoproteinase GluC (data not shown).

N-terminal sequence analysis of HPLC-purified digestion products obtained in the presence of EGTA or Ca²⁺ identified the cleavage sites shown in Table II. On endoproteinase GluC digestion, an extra site, 1134GSYRC, not present in the wild-type construct, was identified in the mutant. This site, located at the turn of the central two-stranded anti-parallel b-sheet, was the same site revealed in the cbEGF12-13 G1127S pair. In the case of trypsin, two additional sites, 1130GVSVCH and 1138CECPP, were seen upon digestion of the mutant construct. The additional site at 1126GSVCH is adjacent to the mutated residue whereas the other protease sensitive site, 1138CECPP, occurs distal to the mutation but still within cbEGF13 (Fig. 4). The comparison of the amount of each of these N termini identified on digestion in EGTA or calcium, and expressed relative to the authentic N-terminal sequence is shown in Table II. Calcium-dependent protection from proteolysis of all cleavage sites, including those in the mutant domain, was observed. All cleavage sites were represented in comparable amounts indicative of efficient cleavage of a single population of molecules.

Structural and Calcium Binding NMR Studies of the cbEGF12-13 Domain Pair—Comparative analysis of the calcium-saturated two-dimensional NOESY spectra for the wild-type and G1127S mutant cbEGF12-13 constructs indicated that, unlike the case for the cbEGF13-14 wild-type and mutant spectra (21), a significant number of peaks corresponding to domain 13 were unaffected or only mildly affected by the presence of the G1127S mutation. The fingerprint regions of these spectra are shown in Fig. 5. Most resonances appeared unaltered between the two spectra, and a subset of peaks were only slightly shifted. For example, of the two well resolved sets of triplet peaks in the lower left-hand corner of the spectra, the chemical shifts of the upper set of peaks were identical, whereas the positions of resonances in the lower triplet were slightly shifted. Because these peaks involve connectivities to

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2 R. S. Smallridge, P. Whiteman, J. M. Werner, P. A. Handford, and A. K. Downing, manuscript in preparation.
downfield-shifted CaH, which are typically involved in β-structure, the data suggest that domain 12 in the mutant cbEGF12–13 pair is unaffected by the presence of the G1127S mutation, and that the β-sheet region of domain 13 is only mildly affected.

To examine calcium binding properties of each construct, the aromatic regions of the two-dimensional NOESY spectra acquired at 0 and 12.5 mM CaCl2 were overlaid for both the wild-type and G1127S mutant cbEGF12–13 domain pair (Fig. 6). Chemical shift changes of the Hd* resonances of the consensus, aromatic, calcium binding residues for the N- and C-terminal domains of each pair, i.e. Phe1093 and Tyr1136, respectively, were used to assess calcium binding. The overlay of the wild-type NOESY spectra at zero and saturating calcium showed the expected, native binding properties previously observed when Kd values were determined for the two sites in this domain pair (26). Comparison of the wild-type spectral overlay with that of the G1127S mutant revealed that both domain 12 and 13 retained calcium binding properties in the mutant cbEGF12–13 pair. Domain 12 appeared to retain native calcium binding properties because the Phe1093 peak movement was identical to that seen in the wild-type spectrum. The peak movement seen for Tyr1136 in cbEGF13 was slightly altered, however, with a larger chemical shift change associated with binding by the mutant domain. Collectively, these comparative data indicate that when preceded by cbEGF12, the G1127S mutant cbEGF13 domain preserves a native-like but not identical fold.

**DISCUSSION**

In a previous study, it was shown that G1127S caused misfolding of isolated domain 13 with loss of calcium binding properties, whereas, in a cbEGF13–14 domain pair, cbEGF14 was unaffected. In this study, the effects of the G1127S mutation on the cbEGF12–13 domain pair have been assessed, and the results indicate that domain 12 is unaltered, and the con-
sequences of this mutation are again localized to domain 13. The demonstration that domain 13 in the 12-13 pair retains the ability to bind calcium is unlike the situation previously seen for both the single mutant cbEGF13 domain and the mutant cbEGF13-14 domain pair. As calcium binding by cbEGF domains is used as a probe for correct refolding, the fact that cbEGF13 continues to bind calcium in the mutant cbEGF12-13 pair suggests that this domain preserves a native-like fold. This hypothesis is also supported by a comparative analysis of the calcium-saturated wild-type and mutant two-dimensional NOESY spectra for the cbEGF12-13 pair, which show only minor differences (see Fig. 5).

These NMR studies indicate that the G1127S mutation has a less severe effect on folding when preceded by cbEGF12. Measurement of the calcium binding affinities of the two sites in the cbEGF12-13 wild-type pair by NMR and fluorescence spectroscopy has previously demonstrated that covalent linkage of an N-terminal cbEGF domain had a stabilizing effect on the adjacent C-terminal domain (26, 6). The observation in this study that cbEGF12 moderates the effect of the G1127S folding mutation also suggests an effect of N-terminal linkage, because structural features associated with cbEGF domains (β-sheet, calcium binding) were absent in cbEGF13 or cbEGF13-14 mutant constructs.

In parallel with NMR analyses, protease digestion studies have also been used to probe the structural and calcium binding properties of the wild-type and G1127S-containing fibrillin fragments. It has previously been shown that tandem repeats of wild-type cbEGF domains are susceptible to proteolytic cleavage at specific sites on removal of calcium by EGTA, whereas in the presence of calcium, protection from proteolysis is observed (7, 29). An increased susceptibility to proteolysis in
Local Structural Effects of G1127S in Human Fibrillin-1

The results of studies on the cbEGF13 domain, the cbEGF13-14 domain pair (21), the cbEGF12-13 domain pair, and the cbEGF12-14 triple construct (this study) are summarized. Calcium atoms are depicted as gray circles. The rectangle used to represent the state of cbEGF13 in the cbEGF12-13 and cbEGF12-14 G1127S mutant constructs highlights the difference between the native-like and native fold. A question mark is used to represent the state of cbEGF13 in the mutant triple construct, because protease sites not detectable in the cbEGF12-13 pair were observed in this construct (see text for details).

Fig. 7. Schematic diagram to summarize the calcium binding and structural consequences of the G1127S mutation. The results of studies on the cbEGF13 domain, the cbEGF13-14 domain pair (21), the cbEGF12-13 domain pair, and the cbEGF12-14 triple construct (this study) are summarized. Calcium atoms are depicted as gray circles. The rectangle used to represent the state of cbEGF13 in the cbEGF12-13 and cbEGF12-14 G1127S mutant constructs highlights the difference between the native-like and native fold. A question mark is used to represent the state of cbEGF13 in the mutant triple construct, because protease sites not detectable in the cbEGF12-13 pair were observed in this construct (see text for details).

Fig. 6. Comparison of the two-dimensional NOESY spectra of the cbEGF12-13 and cbEGF12-14 triple constructs were identical to those found in digests of a recombinantly expressed cbEGF10-22 construct containing a calcium binding mutation E1073K in cbEGF12 (30). E1073K is associated with neonatal MFS, the most severe form of the disease. In this case, the sites within mutant domain 12 showed enhanced susceptibility to proteolysis compared with the wild-type, in contrast to the present study in which the sites in mutant domain 13 containing the G1127S mutation were protected by calcium. In addition a cleavage site was also revealed N-terminal to cbEGF11, indicating a longer range structural effect of the calcium binding mutation. Collectively these data indicate that MFS-causing mutations in this region cause variable intramolecular effects on fibrillin-1 structure.

Because the effect of the G1127S mutation is confined to domain 13 and this domain retains a native-like fold, fibrillin-1 monomers containing this mutation are likely to be secreted by the cell and the effect of the mutation exerted either on or after incorporation into the microfibril. The small localized changes in structure and calcium binding in domain 13 could disrupt protein binding sites involved in the assembly process or the properties of the assembled microfibril. The protection afforded by calcium against proteolytic degradation of tandem repeats of cbEGF domains in fibrillin-1 (7, 29) suggests that missense mutations may result in increased proteolysis in vivo. Although cbEGF13 containing the G1127S mutation appears to be more susceptible to proteolysis than the wild-type in vitro, calcium-
dependent protection was observed for all the additional protease sites revealed in the domain (Table II), therefore increased proteolytic susceptibility seems less likely to be involved in the pathogenic mechanism than for the E1073K mutation.

In summary, interdisciplinary studies utilizing both high and low resolution methods have proved effective in identifying the structural consequences of the G1127S mutation. Further studies will now focus on identifying the functional effects of this and related mutations on fibrillin assembly.

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