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Cysteine Substitutions in Epidermal Growth Factor–Like Domains of Fibrillin-1: Distinct Effects on Biochemical and Clinical Phenotypes

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Summary

Fibrillin-1 (FBN1) contains 47 epidermal growth factor (EGF)–like domains characterized by six conserved cysteine residues. Cysteine substitutions that disrupt one of the three disulfide bonds are frequent causes of Marfan syndrome (MFS). We identified 19 new substitutions involving cysteine residues in each of the six positions of EGF-like domains. Allele-specific mRNA assays revealed equal abundance of mutant and normal FBN1 transcripts in all 10 individuals studied. Quantitative pulse-chase analysis of fibrillin protein was performed on 25 mutant fibroblast strains with substitutions of 22 different cysteine residues in 18 different EGF-like domains spanning the entire gene. Normal synthesis and stability of mutant fibrillin molecules was seen in 20/25 individuals, 11 of whom showed delayed intracellular processing and/or secretion. In the remaining five cases, the mutant protein was apparently unstable. In four of these five cases, the second or third disulfide bond of EGF-like domains immediately preceding an 8-cysteine or hybrid domain was affected. All but two mutations caused severe reduction of matrix deposition, which was attributed to a dominant-negative effect of mutant molecules. For genotype/phenotype comparisons, clinical data on 25 probands and 19 mutation-positive family members were analyzed. Ocular manifestations were among the most consistent features (ectopia lentis in 86%, myopia in 80%). Nine mutations encoded by exons 26–32 resulted in early-onset classic MFS and, in one case, neonatal-lethal MFS. Mutations outside this region were associated with variable clinical phenotypes, including individuals with fibrillinopathies not meeting diagnostic criteria for MFS.

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Introduction

One of the most prevalent disorders of connective tissue, Marfan syndrome (MFS [MIM 154700]) affects an estimated 1/5,000–10,000 individuals, without gender bias or ethnic predisposition. The autosomal dominant syndrome is characterized by a combination of cardiovascular, musculoskeletal, and ophthalmological features, including aortic dilatation and dissection, dolichostenomelia, chest deformity, scoliosis, lens subluxation, high myopia, and striae distensae (Pyeritz and McKusick 1979). The criteria for the diagnosis of MFS were recently revised, and it was proposed to include the presence of a disease-causing mutation as a diagnostic criterion (de Paepe et al. 1996).

MFS is caused by mutations in the fibrillin-1 gene (FBN1 [MIM 134757]) in band 15q21.1. The gene is subdivided into 65 exons. The 9.7-kb transcript codes for fibrillin-1, a 320-kD glycoprotein that is the primary constituent of unique microfibrils in the connective-tissue matrix of many organ systems (Sakai et al. 1986; Corson et al. 1993; Pereira et al. 1993). Forty-seven exons encode a complete epidermal growth factor (EGF)–like protein domain, and 43 of these include the consensus sequence for calcium binding Asp/Asn-*x*-Asp/Asn-Glu/Gln-*xm*-Asp/Asn*-*xn*-Tyr/Phe, where "*x*" represents any amino acid, "*" indicates possible β-hydroxylation of this residue, and "*m*" and "*n*" can be variable (Downing et al. 1996). Calcium plays a pivotal role in conformational stability of the domain and confers increased resistance to proteolytic degradation. In addition, it may facilitate interdomain as well as intermolecular interactions (Kielty and Shuttleworth 1993; Downing et al. 1996; Reinhardt et al. 1997*a*).

Each of the EGF-like domains contains six highly conserved cysteine residues that form three disulfide bridges—between C1 and C3, between C2 and C4, and between C5 and C6—creating an antiparallel β-pleated sheet conformation that enhances calcium binding (Campbell and Bork 1993; Kielty and Shuttleworth 1993; Dietz and Pyeritz 1995; Reinhardt et al. 1997*b*). Mutations in EGF-like domains in other genes encoding extracellular proteins cause diseases—for example, those of cholesterol regulation (Varret et al. 1998) and blood

Table 1

Primers and Conditions to Assess Allele-Specific Transcript Levels for Seven Different Cysteine Substitutions

Primer Pair			Annealing Temperature	Size	Restriction	
Designation	Sequence[a] (5′→3′)	Position	(°C)	(bp)	Site[b]	Proband
471	TATCCATCTCGGGAGCCACCA	1306	56	210	*Nci*I (+)[c]	9
2647	AATACACTCACCACCAGCC	1516				
2543	TTACACGAGATGGGAAGGAC	1687	56	291	*Ava*II (+)[c]	20
474	TGCTCCGCATGTGTGTGTCA	1978				
2544	GTGAAACCCCTGGGATCTGC	1850	56	208	*Mae*II (+)	16
2545	GGCGCAACAGCATTCAGATT	2058				
2549	GCTTGTGTTATGATGGATTC	3788	51	208	*Mae*II (+)	3
390	GTTGTGTGCTCCAATTTCA	3996				
2550	GCAGGAAGCTTCAAATGTAG	4027	56	199	*Rsa*I (−)	22
2551	GAGCACTCATCAAGGTCTGT	4226				
2550	GCAGGAAGCTTCAAATGTAG	4027	56	199	*Rsa*I (+)	23
2551	GAGCACTCATCAAGGTCTGT	4226				
500	TTATACTCTAGCAGGGAATG	6468	49	215	*Mae*II (+)	15
2553	TATGACCCATAAGTGTTC	6683				

[a] The base mismatch in each primer is underlined.

[b] (+) = Restriction created by mutation; (-) = restriction site abolished by mutation. The *Rsa*I sites affected in probands 22 and 23 are different from one another.

[c] Artificially created restriction site.

coagulation (Giannelli et al. 1998). In FBN1, 41/101 reported mutations affecting EGF-like domains replace a cysteine residue (Pepe et al. 1997; Collod-Beroud et al. 1998). On the basis of the limited clinical information available, it appears that this class of mutation is typically associated with "classic MFS." In our laboratory, quantitative pulse-chase analysis of a few cysteine substitutions showed normal fibrillin synthesis and significant reduction in extracellular-matrix deposition (Aoyama et al. 1993, 1994).

During recent years, we have systematically searched for FBN1 mutations in fibroblast-derived RNA and DNA of individuals either diagnosed with or suspected of having MFS. Several methods of mutation detection were applied, including single-strand conformation analysis (SSCA) of overlapping reverse transcription–PCR (RT-PCR) products (Tynan et al. 1993) and of amplified exons (Hayward et al. 1997), heteroduplex analysis using mutation-detection enhancement (MDE) gels (Nijbroek et al. 1995), long RT-PCR to detect exon skipping (Liu et al. 1996), and denaturing high-performance liquid chromatography (DHPLC) (Liu et al. 1998). In these studies, we identified >70 unique FBN1 mutations, including nucleotide substitutions, deletions, and insertions; half of these were missense mutations.

To determine the range of molecular and clinical manifestations, the present study focuses on 25 cysteine substitutions in EGF-like FBN1 domains. Of the 19 detected in our laboratory, 6 had been reported elsewhere (Tynan et al. 1993; Liu et al. 1998). We included six cysteine substitutions identified elsewhere (Dietz et al. 1992*a,* 1992*b;* Kainulainen et al. 1994; Piersall et al. 1994). We studied the effect of these mutations on fibrillin-1 transcript stability, as well as protein synthesis and secretion, in cultured dermal fibroblasts. Results of quantitative pulse-chase analysis were used to assign probands to one of five groups based on fibrillin synthesis and extracellular-matrix deposition (Aoyama et al. 1994). Reduced fibrillin synthesis (50% of control value ± 2 SD, range of 35%–70%) characterizes groups I and II, and a normal level of synthesis (100% of control value ± 2 SD) defines groups III and IV. Deposition is proportionally reduced (50% of control value ± 2 SD) in groups I and III and is disproportionately low (<2 SD of control value) in groups II and IV. Group V samples yield synthesis and deposition values >70% of the control value and, therefore, are considered to fall within the normal range (Aoyama et al. 1994). In addition, the location of the cysteine substitution, mutant mRNA levels, protein, and clinical phenotypes were comparatively analyzed in probands and mutation-positive family members.

Subjects, Material, and Methods

Subjects and Samples

Informed consent, skin biopsies, and aortic-tissue samples were obtained in accordance with a study protocol approved by the Stanford University Panel on Human Subjects in Medical Research. Probands and available family members were examined in the Center for Marfan Syndrome and Related Connective Tissue Disorders. Additional information was obtained from fam-

ily- and medical-history questionnaires, medical records, and phone interviews.

Fibroblast cultures were established for all probands. Blood samples or buccal swabs (Cytobrush Plus Cell Collector; Medscand Medical AB) were obtained from probands and relatives. Genomic DNA and total RNA were isolated from blood leukocytes or fibroblast cultures, according to standard procedures. Buccal-swab lysates were prepared as described elsewhere (Richards et al. 1997) and were used directly in PCR.

Mutation Detection

For screening at the cDNA level, 22 PCR fragments covering the FBN1 cDNA were amplified as reported elsewhere (Tynan et al. 1993) and were analyzed by SSCA with silver staining (Bio Rad) (Orita et al. 1989). Genomic DNA was amplified with primers for individual FBN1 exons (Nijbroek et al. 1995; Liu et al. 1998). Heteroduplexes were identified either by MDE (J. T. Baker, Philipsburg, NJ) or by DHPLC, as described elsewhere (Liu et al. 1998).

DNA Sequencing

PCR products displaying abnormal migration bands during SSCA or heteroduplex analysis in MDE gels were directly sequenced by the Sequenase PCR Product Sequencing Kit (United States Biochemical). All amplicons with DHPLC peak profiles distinct from the homoduplex peaks of control samples, as well as some PCR products with a heteroduplex band on MDE gels, were purified with exonuclease and shrimp alkaline phosphatase, according to the manufacturer's recommendations (United States Biochemical), and were then sequenced with fluorescent terminators on an ABI Prism 377 Sequencer (PE Biosystems).

Allele-Specific Expression Studies

To compare the transcripts of the mutant and normal alleles, a PCR-RFLP method was devised for seven samples with cysteine substitutions. Because, in two cases, the mutations did not abolish or create a restriction site, artificial restriction sites were generated by PCR primers that introduce a base substitution close to the codon of interest (Haliassos et al. 1989; Lappalainen et al. 1995) (table 1).

Reverse transcription (RT) was performed on DNaseI-treated total RNA by means of Superscript II (Gibco BRL Products), according to the protocol recommended by PE Biosystems. RT products were denatured at 94°C for 9 min and then were PCR amplified for 20 cycles of 94°C for 30 s, annealing at each primer set's optimal temperature (see table 1) for 30 s, and 72°C for 30 s. After that, one [^{32}P]-dATP–labeled oligonucleotide of each primer pair was added, and the reaction was continued for one more cycle, followed by a final extension step of 72°C for 10 min. The PCR products were digested with the appropriate restriction enzymes (table 1) and were loaded onto an 8% polyacrylamide gel, to separate the two digested products. The signal intensities on autoradiographs were determined by densitometry.

Five probands carrying cysteine substitutions were heterozygous for the single-nucleotide polymorphism (SNP) C8931T in the 3′ UTR (Maslen et al. 1991; Hewett et al. 1994*a*) and/or T1875C in exon 15 (Hayward et al. 1994). Allele-specific mRNA levels were studied at the polymorphic sites by RT-PCR with published primers for the 3′ UTR SNP. The exon 15 SNP was amplified with the following RNA-specific primers: forward, 5′-TGCAAAGACATTAACGAGTG-3′, bridging exons 14 and 15; and reverse, 5′-CTCCGCATGTG-TGTGTCAAC-3′, bridging exons 16 and 15—for 30 cycles, under the aforementioned conditions, at an annealing temperature of 58°C. The products of the two alleles were distinguished by SSCA and silver staining, according to the protocol provided by the manufacturer (Bio Rad), and were subsequently quantified by scanning densitometry.

Quantitative Pulse-Chase Analysis

According to a previously established protocol (Brenn et al. 1996), primary fibroblast cultures from probands and control individuals were grown to confluency and were maintained for 10 d in Dulbecco's modified eagle medium (DMEM) with 15% FBS and antibiotics. Cells were subsequently seeded at high density (5×10^5 cells/35-mm dish) and were maintained for 6 d. On day 7, metabolic labeling was performed with 200 μCi of ^{35}S-L-cysteine (ICN) for 30 min. Unincorporated isotope was removed by three washes with DMEM, and the newly synthesized fibrillin was chased in DMEM for 8 and 20 h. The soluble cell-lysis fraction was collected after three washes with DMEM and lysis with 700 μl of 50 mM Tris-HCl, pH 8.0, containing a broad-spectrum protease inhibitor (CompleteTM; Boehringer Mannheim) and 1% NP40. The insoluble fraction was collected after one wash with the same NP40 buffer, by scraping the culture dish with a rubber policeman and centrifugation at 13,000 *g* for 25 min at 4°C. Trichloroacetic acid precipitation and SDS-PAGE were performed as described elsewhere (Aoyama et al. 1993). The dried gels were exposed to a phosphor screen and were visualized on a phosphorimager (Molecular Dynamics). Labeled fibrillin-1 was identified and quantitated as described elsewhere (Aoyama et al. 1994; Brenn et al. 1996). The levels of FBN1 production and deposition, as insoluble fibrillin, in the extracellular matrix were expressed as percentages of control values obtained for normal control fibroblast cultures that were studied in parallel for

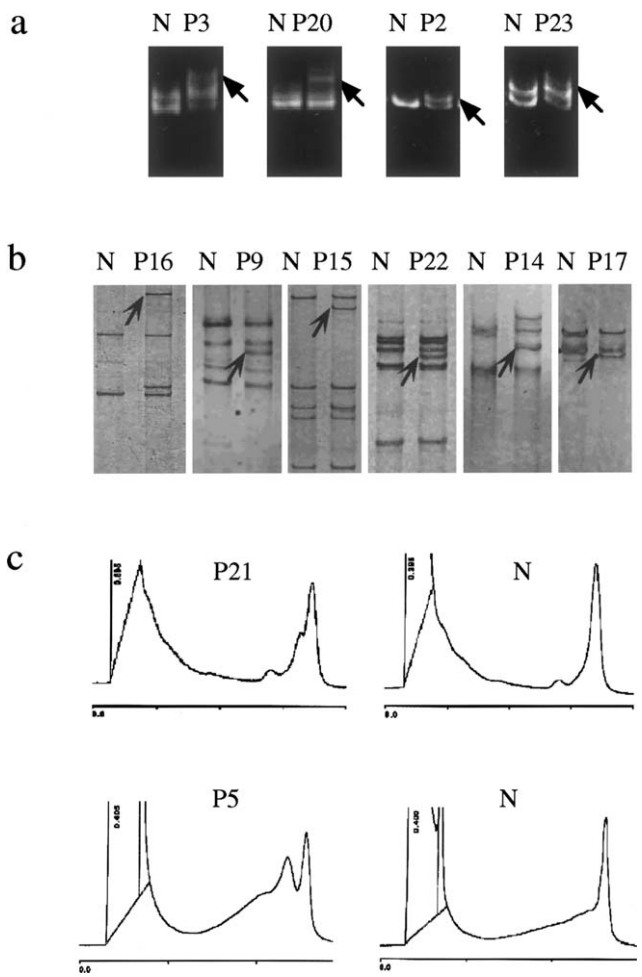

Figure 1 Mutation detection in FBN1, by three different methods. Mutation screening of amplified FBN1 exons was conducted in individuals with MFS. *a,* MDE heteroduplex analysis of individuals P3, P20, P2, and P23 and a normal control (lanes N). Additional bands are indicated by arrows. *b,* SSCA of individuals P16, P9, P15, P22, P14, and P17 and of a normal control (lanes N). Migration position of additional fragments is indicated by arrows. *c,* Altered elution profiles, by denaturing high-performance liquid chromatography of PCR products of individuals P21 and P5 and of normal controls (N). On sequencing of the PCR products with altered migration patterns or DHLPC profiles, mutations leading to cysteine substitutions were identified in each case.

each experiment. Assignments to biosynthetic groups were made as before (Aoyama et al. 1994).

Results

Identification of Cysteine Substitutions

The different mutation-detection methods resulted in the identification of 19 cysteine substitutions in EGF-like FBN1 domains. SSCA of 22 overlapping RT-PCR products yielded seven, heteroduplex analysis of all 65 amplified FBN1 exons by MDE gels revealed four, and exon amplification and DHPLC detected eight (fig. 1). Each of the abnormal fragment migration patterns and DHPLC profiles was unique; the former were not seen in 28 controls analyzed by SSCA or MDE, and the latter were not present in 95 unrelated individuals with MFS or related connective-tissue disorders who were studied by DHPLC (Liu et al. 1998).

Regarding the type of substitution, in eight instances arginine, in six tyrosine, in three phenylalanine, and in one each serine and tryptophan replaced the cysteine residue. The cysteine substitutions were distributed over most of the FBN1 gene, from exon 11 to exon 64, which encode the 6th–47th EGF-like domains (table 2). All mutations but one, C476G in exon 11, were located in EGF-like domains with consensus sequences for calcium binding. All mutations were novel, except for C1242Y, which elsewhere had been reported as C344Y by Kainulainen et al. (1994). C1242Y was found as a de novo occurrence in our patient and is only the 12th known recurring mutation in the FBN1 gene (Collod-Beroud et al. 1998).

Of the 19 cysteine substitutions identified, 13 represented de novo mutations in the probands, and 6 were inherited. Pedigree analysis identified 35 individuals at risk of carrying the mutation, and they were invited to join the study. Blood samples or buccal swabs obtained from 30 relatives of probands were genotyped by PCR amplification and direct sequencing. Nineteen additional mutation carriers were identified in five families.

Allele-Specific mRNA Levels

Allele-specific expression tests were developed for 10 representative mutant samples; in 7 of these cases the tests were based on the specific mutation (fig. 2), and in the 3 others they were based on heterozygosity for expressed SNPs. The mutations in these samples affect cysteine residues in each of the six positions of an EGF-like domain ranging from the 3d to the 34th calcium-binding domains (C1 [C1326R exon 32], C2 [C499R exon 12], C3 [C2221R exon 54], C4 [C637R exon 15], C6 [C570R exon 13], C1361Y exon 32, and C1402W exon 33). The ratio of transcripts from the mutant and normal alleles was virtually equal in all samples, regardless of the positions of the substituted cysteines (fig. 2).

C1326R, C637R, and C832Y (C5, exon 20) were analyzed for the C8931T SNP in the 3′ UTR (Maslen et al. 1991; Hewett et al. 1994*a*) and revealed equal levels of both allele-specific transcripts. C1086Y (C3, exon 26) and C1672R (C4, exon 40) were informative for the 3′-UTR SNP, as well as for the T1875C SNP in exon 15 (Hayward et al. 1994). Testing at both sites revealed equal transcript levels (data not shown). Thus, in all of

Table 2

Cysteine Substitutions in the FBN1 Gene, with Corresponding Biochemical Phenotypes, Grouped According to Replaced Cysteine within an EGF-Like Domain

EFG-Like Domain and FBN1 Exon	Nucleotide Change	Amino Acid Change	Fibrillin Synthesis (%)	Fibrillin Deposition (%)	Protein Group	Proband	Reference(s)[a]
C1:							
27	G3350A	C1117Y	98	27	IVd	1	Aoyama et al. (1993), Tynan et al. (1993)
30	G3725A	C1242Y	100	24	IV	2	
30[b]	G3725A	C1242Y	73	20	IV	4	Kainulainen et al. (1994)
32	T3976C	C1326R	122	27	IVd	3	
49	T6049C	C2017R	92	20	IVd	6	Liu et al. (1997, 1998)
64	G8057T	C2686F	91	26	IVd	5	Liu et al. (1997, 1998)
C2:							
12	T1495C	C499R	81	20	IVd	9	
25	G3116A	C1039Y	116	23	IVd	8	Liu et al. (1997, 1998)
30[b]	G3746C	C1249S	116	27	IV	7	Dietz et al. (1992*b*), Aoyama et al. (1993)
C3:							
26	G3257A	C1086Y	98	18	IV	14	
28	T3511C	C1171R	119	26	IVd	10	
40[b]	T4987C	C1663R	93	45	IIId	12	Dietz et al. (1992*b*), Aoyama et al. (1993)
54	T6661C	C2221R	102	22	IV	15	
54[b]	G6662C	C2221S	94	27	IVd	13	Dietz et al. (1992*b*), Aoyama et al. (1993)
56	G6920C	C2307S	98	22	IVd	11	Dietz et al. (1992*a*), Aoyama et al. (1993)
C4:							
15	T1909C	C637R	38	11	II	16	
40	T5014C	C1672R	38	27	II	17	
40	G5015T	C1672F	75	28	IV	18	
C5:							
11[b]	T1426G	C476G	102	28	IV	19	Piersall et al. (1994)
20	G2495A	V832Y	56	13	II	20	Liu et al. (1997, 1998)
C6:							
13	T1708C	C570R	50	28	II	21	
31	T3958A	C1320S	104	11	IV	22	Liu et al. (1997, 1998)
32	G4082A	C1361Y	87	10	IVd	24	
33	T4206G	C1402W	81	15	IV	23	
49	G6158T	C2053F	58	44	I	25	

[a] Mutations without reference(s) listed are reported here for the first time.
[b] Donated by another laboratory.

the 10 samples studied, mutant-gene transcription and mutant-mRNA stability were apparently normal.

Fibrillin-Protein Studies

Fibroblasts from 25 individuals were studied by quantitative pulse-chase analysis; they included the 19 with cysteine substitutions described here, the 1 reported individual with the recurrent C1242Y mutation (Kainulainen et al. 1994), and 5 fibroblast strains that had been analyzed elsewhere (Aoyama et al. 1993) (table 2). Eight mutant-cell cultures were studied more than once, by different researchers and at different passage levels. One example (C2017R) is shown in figure 3. In comparison with control fibroblasts, this mutation results in both normal synthesis and a marked reduction in the amount of insoluble fibrillin, a measure of matrix deposition of newly synthesized fibrillin (Brenn et al. 1996). This type of analysis yields four different patterns, depending on the amount of synthesis and insoluble fibrillin (Aoyama et al. 1994). The mutant-cell strain shown in figure 3 was also associated with a delay in intracellular transport and/or secretion of fibrillin and was designated as "group IVd." Delayed secretion was observed in 11/20 cultures that generated apparently stable mutant protein (groups III and IV) (table 2). All cysteine substitutions except for two, designated as "group I" or "group IIId," caused severe reduction (i.e., <35% of control value) of extracellular deposition of newly synthesized fibrillin molecules.

To assess whether the position of the affected cysteine within the EGF-like domain influences the protein phenotype, the 24 different substitutions were plotted onto a model EGF-like domain, together with exon numbers and corresponding protein phenotypes (fig. 4). Fibrillin synthesis was within normal limits (for groups III and IV) when residues C1, C2, and C3 of an EGF-like domain had been substituted by another amino acid. These substitutions apparently did not affect stability of the

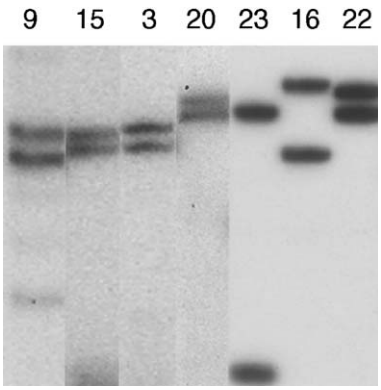

Figure 2 Allele- specific RT-PCR products derived from the normal and mutated alleles of seven individuals with cysteine substitutions (probands 9, 15, 3, 20, 23, 16, and 22), revealing equal biallelic expression.

mutant protein during the 30-min pulse time. When residues C4, C5, and C6 were replaced, fibrillin synthesis was either normal or reduced to approximately one half, the amount expected to represent the product of the normal FBN1 allele (groups I and II).

To determine whether the specific EGF-like domain or its location within the fibrillin monomer may have a differential effect on the protein phenotype, we compared data for several EGF-like domains in which more than one cysteine had been substituted (fig. 4). In the domain encoded by exon 30, two mutations affect cysteine residue C1, and one affects C2. The three fibroblast cultures displayed the same biosynthetic profile defined as group IV. In exon 40, a C3 substitution was associated with protein group IIId, and two mutations affecting C4 had different consequences. Replacement of C4 by phenylalanine resulted in a stable mutant protein (group IV), whereas arginine substitution was associated with a group II profile. Exons 54, 32, and 49 contained two mutations each. Both mutations in exon 54 affected C3 and caused protein phenotype IV, yet only substitution by serine (C2221S)—and not by arginine (C2221R)—was associated with delayed secretion. The two mutations in exon 32, affecting C1 and C6, caused delayed secretion as well (group IVd). The mutation in exon 49, affecting C1, resulted in group IVd, but when C6 was replaced, the protein was unstable and deposition was proportionately reduced (group I).

A potential positional effect was detected when we were searching for explanations for biosynthetic groups I and II, which were observed in five samples. The half-normal amount of newly synthesized fibrillin protein in the presence of normal amounts of mutant mRNA pointed to rapid degradation of presumably misfolded protein. Four of the mutants involved the EGF-like domains encoded by exons 15, 20, 40, and 49, each of which immediately precedes an 8-cysteine or hybrid domain (C637R, C832Y, C1672R, and C2053F, respectively) (fig. 5). Whereas these mutations disrupt either the C2-C4 or the C5-C6 disulfide bond, two others, causing disruption of the C1-C3 bond and also located in EGF-like domains on the amino-terminal side of 8-cysteine domains (C2017R, in exon 49, and C2307S, in exon 56), were associated with normal fibrillin production but delayed intracellular transport/secretion of full-length protein molecules (group IVd). Likewise, the IVd protein phenotype was observed for a mutation that disrupted the C2-C4 bond and that was located immediately adjacent to—but on the carboxy-terminal side of—an 8-cysteine domain (C1039Y). An observed synthesis level of 75% of the control value placed C1672F into group IV, in apparent discrepancy with the placement of C1672R in group II. This suggests that, in addition to position within the EGF domain and within the fibrillin monomer, other factors—such as conformational features due to substitutions—may influence mutant-protein stability as well.

Clinical Phenotypes

Genotype/phenotype comparisons were performed for 44 individuals including 19 with newly identified cysteine mutations and their available affected family members and six previously published cases (table 3). Al-

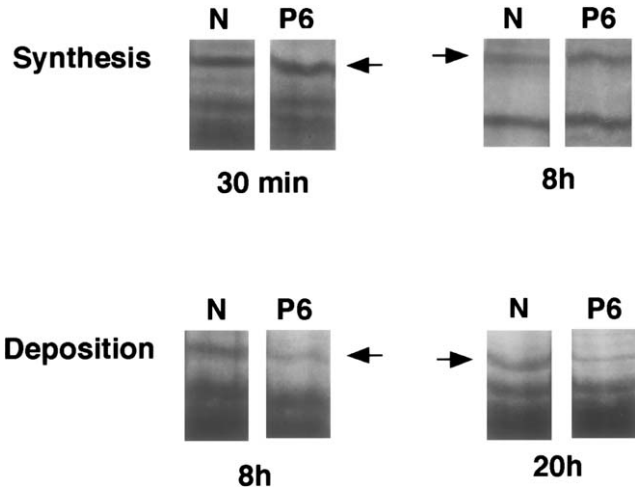

Figure 3 Abnormal deposition of labeled fibrillin (*arrows*), detected by pulse-chase analysis of cultured dermal fibroblasts. Similar amounts of fibrillin are found in the cytosol of normal control fibroblasts (lanes N) and in those of individual P6 (affected by mutation C2017R), after a 30-min pulse with ^{35}S-cysteine. A delay in intracellular transport and/or secretion is indicated by the high amount of fibrillin retained in the P6 cells after an 8-h chase period, compared with that in a control (lanes N). The incorporation of pulse-labeled fibrillin into the insoluble extracellular-matrix microfibrillar form is severely reduced in the P6 cells, to ~20% of that in normal control fibroblasts, at 8 h and 20 h of chase. This result places P6 into protein group IVd.

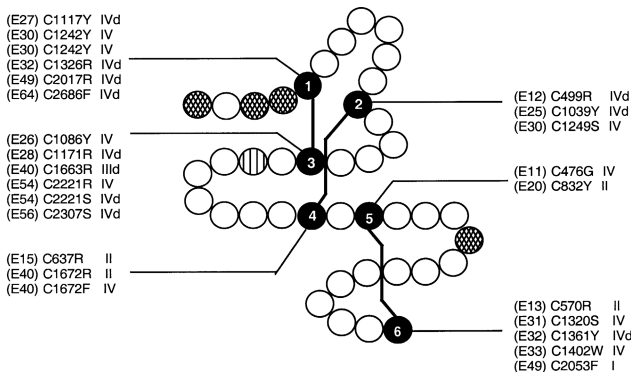

Figure 4 Location of cysteine substitutions in a prototypical EGF-like domain. Exon number (E), affected-codon number (C...X), and biosynthetic groups (I, II, IIId, IV, and IVd; for explanation, see text and the work of Aoyama et al. [1994, 1995]) are indicated for 25 individual substitutions of cysteine residues 1–6, denoted by blackened circles. Amino acids important for calcium binding are indicated by cross-hatched or vertically striped circles.

though most of the study subjects met the revised diagnostic criteria for MFS (de Paepe et al. 1996), there were several notable exceptions (see below).

A highly consistent manifestation of cysteine substitutions in EGF-like domains is ectopia lentis (EL). It was documented by dilated-eye examination in 36/42 (86%) of the individuals so examined. When the information was available, lens dislocation was always bilateral. Myopia, generally high grade, was also very common (33/41 [80%]) and was often associated with retinal detachment (9/39 [23%]). In the revised diagnostic criteria, the term "myopia" has been replaced by "increased axial length of the globe" (de Paepe et al. 1996); however, ultrasonic axial length measurements were not available for any of the patients studied. Since lengthening of the globe leads to myopia, we have used medium- to high-grade myopia in our patient evaluations. Other connective-tissue manifestations, such as pneumothorax (4/39 [10%]) and bilateral or recurrent inguinal hernias (3/40 [8%]), were less frequent.

With respect to the *position of the replaced cysteine residue within the EGF-like domain,* the available data suggested one possible correlation: scoliosis occurred with higher frequency and severity, often requiring surgical intervention, when the C1-C3 or C2-C4 disulfide bonds were disrupted. For all other features, no clear predilections were evident (table 3).

When clinical features were compared with respect to the *location of the affected exon within the FBN1 gene,* classic MFS was observed in association with mutations in exons 11, 15, 27, 28, 30–33, 40, and 54. In general, cysteine substitutions in exons 26–32 were associated with aortic dilatation diagnosed in childhood and with aortic-root replacement at age <30 years. Only one individual, patient 14 with a C3 substitution in exon 26, had "neonatal MFS"; this individual died from cardiopulmonary failure a few months after birth. Aortic-root dilatation progressed slowly when the mutation was present in exon 12 (family 9) or exon 20 (proband 19). In the latter case, the proband's child (19-01) has been treated with β-blockers since age 3 years, which may have contributed to slow progression. Family 5, with a cysteine substitution in exon 64, had predominantly cardiovascular involvement. On the other hand, the mutation in exon 13 (proband 20) caused very few skeletal features and no cardiac abnormalities. Serious cardiovascular involvement was also absent in the eight members of family 6, whose ages were 2–69 years, and in unrelated proband 25, all of whom carried a mutation in exon 49. Although all of these nine individuals had EL, thus far only one member of family 6 has been diagnosed with a dilated aortic root. Not enough skeletal system information was available to determine if this individual (6-3) would meet current clinical criteria for MFS. Possibly, none of the eight mutation-positive members of this family have clinically defined MFS; neither does proband 25.

The clinical data were also analyzed with respect to *protein group,* on the basis of the assumption that individuals with the same mutation share biochemical characteristics. There were 25 subjects in group IVd, 11 in group IV, 6 in group II, and 1 each in groups I and IIId. Retinal detachment was reported in 9/31 individuals in protein groups IV and IVd and in 0/8 individuals in groups I, II, and IIId. In addition, all recurrent and/or bilateral inguinal hernias were associated with groups IV and IVd. The frequency of other features was not obviously different.

Discussion

The first mutation of the FBN1 gene was identified in 1991, in two individuals with MFS (Dietz et al. 1991), and many more have been reported since (Hayward and Brock 1997; Pepe et al. 1997; Collod-Beroud et al. 1998); they include missense mutations, in-frame deletions and insertions, splice-site mutations leading to exon skipping, and nonsense or frameshift mutations leading to premature-termination codons. Genotype-phenotype correlations have been hampered by clinical variability within and between families. The pronounced interfamilial variability that is characteristic of MFS has been attributed to the fact that most FBN1 mutations are unique and family specific (Dietz and Pyeritz 1995). Until now, significant genotype-phenotype associations emerged only in cases of neonatal MFS. This severe, early-lethal manifestation has been correlated with mutations in exons 24–32; these exons encode the central stretch of contiguous EGF-like domains (Milewicz and Duvic 1994; Nijbroek et al. 1995; Liu et al. 1996; Lonnqvist et al. 1996). Whereas it is possible that this

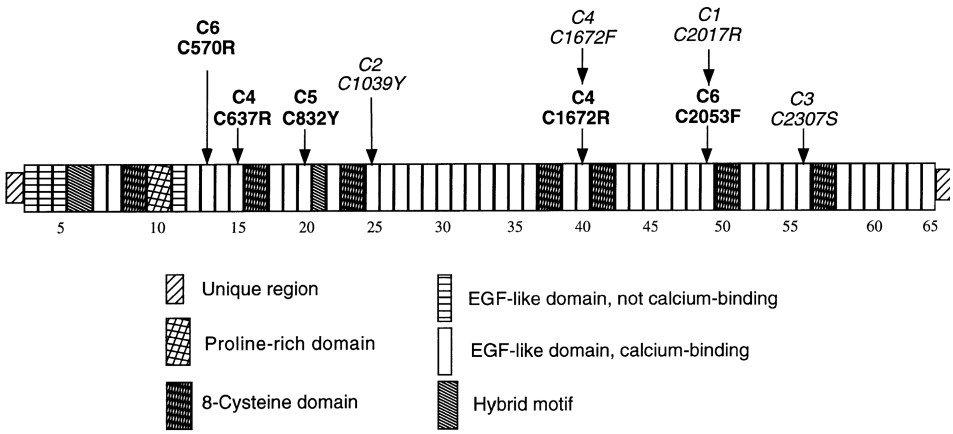

Figure 5 Schematic representation of the fibrillin-1 domain structure and location of cysteine substitutions associated with groups I, II, and IV, in relation to 8-cysteine or hybrid domains. Cysteine-residue mutations in boldface are associated with unstable mutant protein (group II or group I [C2053F]), and those in italics are associated with stable mutant protein (group IV). Disruption of the disulfide bonds between C2 and C4 or between C5 and C6, in an EGF-like domain immediately preceding either an 8-cysteine domain or a hybrid domain, appears to predispose to protein degradation.

central region is important for alignment and stability of the 10-nm microfibrils in the extracellular matrix (Lonnqvist et al. 1996), the correlation of mutations in this region with the neonatal MFS phenotype is not absolute (Wang et al. 1997). Besides location, the type of mutation also needs to be considered. Exon-skipping mutations leading to in-frame deletion of one entire domain often cause neonatally lethal MFS (Liu et al. 1996), whereas most missense mutations in this region do not (Collod-Beroud et al. 1998; present study).

Mutations of FBN1 are associated not only with MFS but also with a range of related phenotypes such as isolated ascending-aortic ectasia, EL, or marfanoid body habitus (Dietz and Pyeritz 1995). To separate MFS from these and other connective-tissue diseases, clinical criteria have been established and recently have been revised (de Paepe et al. 1996). These criteria are quite stringent and exclude many individuals and families with isolated or systemic connective-tissue manifestations. Since some of these individuals and families may still carry mutations in FBN1, FBN2, or other genes and may be at risk for potentially severe and life-threatening complications, we have proposed an expanded concept, called "fibrillinopathy," to include all disorders associated with a mutation of either FBN1 or FBN2 (Aoyama et al. 1995; Furthmayr and Francke 1997). A further subclassification could emerge that is based on systematic analyses of molecularly defined subgroups. Type of nucleotide change, type and stability of mutant mRNA, and synthesis, processing, and secretion of mutant fibrillin monomers are some of the parameters that need to be included in such studies, in order to assess molecular and clinical pathological effects of FBN1 mutations.

Here, we present the first such comprehensive analysis of cysteine substitutions in EGF-like domains of FBN1. This type of mutation is present in ~25% of all patients with MFS and, quite surprisingly, in others not meeting MFS diagnostic criteria. Our study included 25 unrelated probands and 19 mutation-positive relatives. The majority of these cysteine substitutions are novel and are reported here for the first time.

Allele-specific mRNA levels were equal in all 10 samples that were selected to represent mutations affecting each of the six cysteine residues in a distribution ranging from the 3d to the 34th of the 43 calcium-binding EGF-like domains. The finding of stable mutant transcripts in all of the samples points to a mechanism of pathogenesis in which the mutant fibrillin protein and its assembly into microfibrils is central.

Cysteine substitutions disrupt one of three disulfide bridges that covalently connect three pairs of cysteine residues that are highly conserved in EGF-like domains (Aoyama et al. 1993; Downing et al. 1996). This has a predictable detrimental effect on the domain itself, as shown in structural studies of similar domains in other proteins, but it also perturbs calcium binding even when the required consensus sequence is intact (Reinhardt et al. 1997*a*). Calcium stabilizes the conformation of the individual domain (Downing et al. 1996) and influences packing of tandemly arranged EGF-like domains within the same polypeptide chain, as well as the packing of neighboring molecules that are thought to be precisely aligned within the microfibril (Handford et al. 1990; Rao et al. 1995; Reinhardt et al. 1997*a*). Thus, one consequence could be that fibrillin structure is abnormal

beyond the mutated domain, to include neighboring domains as well.

To determine the effect that the different cysteine substitutions have on fibrillin-1 biosynthesis, processing, and matrix deposition, quantitative pulse-chase analyses were performed. The deposition of newly synthesized fibrillin in the extracellular matrix of cultured fibroblasts was severely reduced (<35%; groups II and IV [Aoyama et al. 1994] in all but 2 of the 25 samples studied. In earlier studies (Aoyama et al. 1995), assignment to these two groups has been associated with early onset of ascending-aortic dilatation and earlier surgical intervention, in comparison with individuals in groups I and III (matrix deposition of 35%–70% of control value). The consequences of the severely reduced deposition are greater than expected and are attributable to a dominant-negative effect of the mutant protein on the assembly of fibrillin synthesized from the normal allele. The quantitative pulse-chase technique is quite reliable. Consistent results were obtained either with the same fibroblast strains maintained in our laboratory in repeated experiments and from different passages or with fibroblasts carrying the same mutation that are from related or unrelated individuals. Appropriately matched controls are essential for quantitative analyses, since both the age of the donor at the time of skin biopsy and the passage number of the fibroblasts in culture clearly affect fibrillin synthesis (authors' unpublished data). For example, relatively low levels of synthesis (73% of control value) in proband 4, in comparison with that in the unrelated proband 2 with the same C1242Y mutation, is likely due to the advanced in vitro age of fibroblasts with unknown passage level received from another laboratory (Kainulainen et al. 1994).

Overall, fibrillin synthesis was within normal range for the majority (20/25) of the mutations. The newly synthesized fibrillin was apparently normal in size but was retained intracellularly for a prolonged period in a high proportion (11/20) of mutant samples. Presumably, disruption of a disulfide bond causes the fibrillin monomer to assume a protein conformation that delays intracellular processing and/or secretion from the cell. Kinetic data indicate that this delay affects both the products of the normal allele and those of the mutant allele. This finding suggests that fibrillin monomers interact with each other within cellular compartments. It is currently unknown in which form fibrillin is transported/secreted and whether the postulated dominant-negative effect on fibrillogenesis occurs intra- or extracellularly. It also remains to be determined whether the observed disruption of intracellular transport is linked to intracellular degradation of the polypeptide chain. The delayed-secretion phenotype did not correlate either with the position of the mutated cysteine within the EGF-like domain or with particular substituted amino acids (fig. 4).

Fibrillin synthesis was consistently reduced in five mutant samples (groups I and II). Allele-specific mRNA expression, however, was equal, excluding instability of the mutant transcripts as the cause. The mutations affected C4, C5, or C6 nonrandomly but not exclusively (fig. 4). When the position of the affected EGF-like domain within the molecule is taken into account (fig. 5), a possible correlation becomes apparent. In 4/5 group I or group II samples, the disruption affected the C2-C4 or C5-C6 disulfide bond in an EGF-like domain immediately preceding an 8-cysteine TGFβ binding protein–like or hybrid domain (C637R, C832Y, C1672F, and C2053F). Degradation was not apparent either when the C1-C3 bond was disrupted in such a domain (C2017R and C2307S) or when the C2-C4 bond of the EGF-like domain immediately following an 8-cysteine domain was affected (C1039Y). However, secretion was delayed (group IVd) in all three cases. This may suggest that the folding of the 8-cysteine module critically depends on proper formation of the C2-C4 and C5-C6 bonds in the preceding EGF-like domain. If misfolded, the polypeptide chain may be particularly sensitive to proteolysis, provided that sites for such an event are present (Reinhardt et al. 1997*b*). The finding that the C1672R mutation generates a more stable mutant protein in comparison with the C1672F substitution could be related to different conformations and/or protease sensitivities of the mutant products; more-detailed studies are required for resolution of the underlying structural reason. The putative truncated products of such selective cleavage are not detectable in our system but could be further pursued with other methods of analysis (Reinhardt et al. 1997*b*). Relatively stable proteolytic products might exist in sufficient concentrations in the group II samples because a group I phenotype would be expected if the mutant monomers were completely degraded (Aoyama et al. 1994).

The clinical phenotypes of our study group ranged from severe manifestations of neonatal MFS in a single individual to syndromes with relatively mild, mostly skeletal findings (proband 6 and her seven affected relatives and probands 19 and 25; table 3). The majority of individuals with cysteine substitutions had classic MFS and met diagnostic criteria on clinical grounds alone. Clinical/molecular correlations were complicated by a wide age range (2–73 years) in the individuals. In the younger-age group, clinical phenotype and symptoms may not be fully developed; in others, early medical intervention in relatives of patients either diagnosed with MFS or undergoing relevant cardiovascular surgical procedures may influence our clinical analyses, in two ways. First, in such relatives at risk, the aortic-root diameter will be more carefully monitored, which may lead to the

Table 3

Clinical Findings in Individuals with Cysteine Substitutions in EGF-Like Domains, at Age Listed

		Clinical Status[b]																						
		Skeletal[d]											Ophthalmological[e]			Cardiovascular[f]					Other[g]			
Exon	Subject (Age)[a]	DCS	PE/PC	SC	H/NP	DC	CTR	HSJ	HLJ	Wrist Sign	Thumb Sign	ARD	Myopia	EL	RD	AAA (Age [years])	AD	ARR (Age [years])	MVP	MR	PMT	Hernia	Striae	Reference[c]
Cysteine 1:																								
27	1 (30 years)		+[h]	+[i]	+	+	−	+	+			+	+	+[j]	+	9	+	26	+	+	−	−	+	
30	2 (6 years)	−	+[k]	+	+	−	−	+	+	+	+	+	+	+[j]	−	8	−	−	+	+	−	+[l]	+	
32	3 (7 years)	−	+[m]	−	+	−	−	+	+	+	+	+	+	+	−	1	−	7	+	+	+	−	−	
30	4 (20 years)		+									+	+	+[j]	NA[n]	+		19	+					1
64	5 (27 years)	−	+[m]	−	+	−	+	−	−	−	−	+	+	−	−	+	−	21	−	−	−	−	+	
	5-2 (24 years)	−	+[o]	−	−	−	+	+	+	−	−	+	−	+	−	26	−	−	−	−	−	−	+	
	5-12 (>60 years)	−	+[m]	−	+		+	−	+	−	−	−	+		−	+	−	+	+[p]		−	−	+	
	5-01 (7 years)	−	+[m]	±	+	+	−	+	+	+	+	+	−	−	−	−	−	−	+	+	−	−	−	
49	6 (39 years)	±	+[m]	+	+	+	+	+	−	−	−	−	−	+[j]	+	−	−	−	+	+	−	−	+	
	6-11 (69 years)		+	−	−	−	+	−	+			+	+	+[j]	−		−	−	−	−	−	+[l]	−	
	6-02 (2 years)	+	+[h]	+	+	−	−	+	−	−	+	+	−	+[j]	−	−	−	−	−	−	−	−	−	
	6-2 (40 years)	+	−	−	+	+	+	+	−	+	−	+	+[q]	+[j]	+	−	−	−	−	−	−	+[r]	+	
	6-03 (12 years)	−	+[m]	+	+	+	+	+	−	+	+	+	−	+[j]	−	−	−	−	+	+	−	−	+	
	6-3 (38 years)		+	−			+	+				+	+	+[j]	−	+	−	−	−	−	−	−	+	
	6-04 (6 years)		−	−	+	+	−	+				+	−	+	−		−	−			−	+[s]	−	
	6-05 (3 years)		−	−	+	+	−	+				+	−	+	−		−	−			−	−	−	
Cysteine 2:																								
30	7 (27 years)	+	−	+	−		−	−	−			+	+	+	−	+	−		+	−	−	−	−	3
25	8 (30 years)		−		+	+		+		+	±	+	+[q]	−	−	+	−	31	+	+[p]	−	−	+	
12	9 (50 years)	±	+[m]	+	+	+	+	+	+	−	−	+	+	+[j]	+	50	−	−	+	+	−	−	+	
	9-12 (73 years)		+[m]	+	+	−	−	−	−	+	−	+	+	−	+	+	−	65	−	−	−	+[t]	+	
Cysteine 3:																								
28	10 (22 years)	−	+[k]	+[i]	+	+	+	+	+	+	+	+	+[q]	+[j]	−	+	−	22	+	−	−	+[l]	+	
56	11 (53 years)	+	+[u]	+[p]				+				+	−	−		+	+	+	+					2
40	12 (30 years)	+	+	+[i]	+		−	+				+	+	+	−	+	−	+	+	−	−	+	+	3
54	13 (43 years)	+	−	+	−		−	+				+	+	+	−	+	−		+	−	−	−	+	3
26	14 (2 mo)	+	+[h]	−	+	NA	+	+	+	+	+	+	+	+	+	+		−	+	+	−	+[i]	−	
54	15 (54 years)	+	+[h]	+[i]	+	+	+	+	+	+	+	+	+[q]	+[j]	−	47	+	47	+	+	−	+[i]	+	
	15-01 (27 years)	−	+[m]	+	+	+	+	+	+	+	+	+	+[q]	+[j]	+	18	−	24	+	−	−	−	+	
Cysteine 4:																								
15	16 (13 years)	+	+[h]	+	+	−	+	−	−			+	+	+[j]	−	13	−	−	+	+	−	−	−	

40	17 (38 years)	+	+[h]	+	+	+	+	+	+	+	+	+	+	−	−	25	−	36	+	+	−	+[r]	+	
	17-2 (44 years)	+	−	+		+	−	−	+	−	−	+	+[q]	+[j]	−	37	+	43	+	+	−	−	+	
	18 (38 years)	+	+	+	+	+	+	+	+	+	+	+	+	+	−	14	−	35	+	+	+	−	+	
Cysteine 5:																								
11	19	+	+[m]		+							+		+[j]		+			+					4
20	20 (31 years)	−	−	+	−	−	−	−	−	+	−	−	+[q]	+[j]	−	34	−	−	−	−	−	−	−	
	20-01 (9 years)	+	+[m]	−	+		−	+	−			−	+[q]	+[j]	−	3	−	−	+	+	−	−	−	
Cysteine 6:																								
13	21 (24 years)	−	+[u]	−	+	−	−	+	+	+	−	−	+	+[j]	−	−	−	−	−	−	−	−	+	
31	22 (12 years)	+	+[u]	−	+	+	+	+	−	−	+	+	+[q]	+[j]	−	+	−	11	+	+	−	−	+	
33	23 (26 years)	+	+[m]	±	+	+	−	+	−	+	+	+	+	+[j]	+	25	+	25	−	+	−	−	+	
	23-01 (5 years)	−	−	−	+	−	−	+	+	−	+	+	+[q]	+[j]	−	−	−	−	−	−	−	−	−	
32	24 (28 years)	+	−	±	+	+	−	+	−	−	−	+	+	+[j]	−	24	+	28	−	+	−	−	+	
	24-11 (55 years)	−	+[m]	−	+		+	+	−	−	+	+	+	+[j]		36		54				+	−	
	24-2 (25 years)	+	+[m]	−	+		−	+	+			+		+[j]	+	26	−	−	−	−	+	−	−	
	24-02 (2 years)	+	−													−	−	−	−	−				
	24-03 (8 years)	−	+[m]	+	+	−	+	+	+	−	−	+	+[q]	+[j]	−	−	−	−	+	−	−	−	+	
49	25 (28 years)	+	−	±	−	+	+	−	−	+	−	+	+	+[j]	−	−	−	−	+	−	+	−	+	

[a] Probands are denoted by single numbers, and their relatives are denoted by hyphenated numbers; "-11" and "-12" denote the proband's father and mother; "-1"–"-3" denote relatives in the proband's generation; and "-01"–"-05" represent offspring of individuals in the proband's generation.

[b] A plus sign (+) denotes presence, a minus sign (−) denotes absence, a blank denotes that the status is unknown, and NA = not applicable.

[c] 1 = Kainulainen et al. (1994); 2 = Dietz et al. (1992*a*); 3 = Dietz et al. (1992*b*); 4 = Piersall et al. (1994).

[d] DCS = dolichostenomelia; PE/PC = pectus excavatum/pectus carinatum; SC = scoliosis; H/NP = high/narrow palate; DC = dental crowding; CTR = contractures; HSJ = hypermobile small joints; HLJ = hypermobile large joints; ARD = arachnodactyly.

[e] EL = ectopia lentis; RD = retinal detachment.

[f] AAA = ascending-aortic aneurysm; AD = aortic dissection; ARR = aortic-root replacement; MVP = mitral-valve prolapse; MR = mitral regurgitation.

[g] PMT = pneumothorax.

[h] Pectus excavatum.

[i] Severe, requiring surgical correction.

[j] Bilateral.

[k] Pectus excavatum, severe.

[l] Unilateral.

[m] Pectus carinatum.

[n] Retinal aplasia.

[o] Pectus carinatum, severe.

[p] Severe.

[q] >4 diopters.

[r] Recurrent, bilateral.

[s] Umbilical.

[t] Incisional.

[u] Thoracic asymmetry.

false impression that dilatation occurred at a younger age. Second, preventive therapy with β-blockers in symptomatic children of families with MFS is often initiated at an early age and could substantially increase the interval between diagnosis and aortic root–replacement surgery. This delay in progression would be difficult to separate from slow development of dilatation associated with particular disease-causing mutations.

The most frequent distinctive features—arachnodactyly (88%), EL (86%), high/narrow palate (85%), and hypermobile small joints (78%)—were equally frequent in probands and secondarily ascertained relatives. One of the major diagnostic criteria, EL, was also common (10/13 individuals) in previous case reports of cysteine substitutions in EGF-like domains (Hewett et al. 1994*b;* Dietz and Pyeritz 1995; Mathews et al. 1995; Ades et al. 1996; Putnam et al. 1996; Booms et al. 1997; Pepe et al. 1997). This high incidence of EL presumably reflects the generally more severe nature of the disease in the cysteine-substitution group. When these previous reports are considered together with our data, the overall frequency of EL (46/55 [84%]) is thus significantly higher in individuals with this type of mutation, compared with the estimated 60% frequency in all cases of MFS (Maumenee 1981) and with the estimated 29% frequency in all individuals with abnormal fibrillin biosynthesis (Aoyama et al. 1995). These numbers imply that the high frequency of EL associated with cysteine substitutions in EGF-like domains predicts a frequency of <60% for other types of FBN1 mutations. This prediction is confirmed by the low incidence of EL in a group with premature-termination mutations (authors' unpublished data).

With respect to the possible effects that position of the mutant EGF-like domain may have on the phenotype, mutations in exons 26–32 were associated with earlier onset of ascending-aortic aneurysms. In our study group, only 1/9 individuals with substitutions in this region had a neonatal lethal form of the disease (Kainulainen et al. 1994; Dietz and Pyeritz 1995), whereas several others represented de novo mutations with diagnostic clinical manifestations early in life. This rather severe phenotypic spectrum underscores the importance of this central region of the molecule in the lateral alignment and stacking of fibrillin monomers (Liu et al. 1996).

Attempts to further correlate the frequency or severity of other clinical manifestations with (1) involvement of specific EGF-like domains, (2) cysteine residues within domains, or (3) protein groups failed to yield significant results. A larger sample size and more-sophisticated analytical methods may detect such differences, if they in fact exist. The common endpoint of pathogenic pathways for these mutations has been defined here by the striking reduction of extracellular microfibrils, as determined by the pulse-chase assay. In most, if not all, of these cases, a severe reduction of extracellular microfibrils can also be observed by immunofluorescence studies (Brenn et al. 1996). Deposition assessment by phosphorimaging of fibrillin bands on gels represents a more reliable quantitative measure and can serve to differentiate fibrillinopathies from other connective-tissue disorders.

It is clear from our study that cysteine substitutions in EGF-like domains play a major role in the pathogenesis of fibrillinopathies and that most of them are associated with classic MFS. Genotype-phenotype relationships—even in this selected group of individuals–are complex, but our data will have additional value when the molecular configuration of microfibrils and their interactors become better understood. Results from mutation-detection and biochemical studies of the fibrillin protein will enhance diagnostic precision in the large spectrum of fibrillinopathies and could eventually lead to more-accurate prognostic predictions and treatment of connective-tissue disorders, at an earlier age.

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Electronic-Database Information

Accession numbers and URL for data in this article are as follows:

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim (for FBN1 [MIM 134757] and MFS [MIM 154700)

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