Defective secretion of recombinant fragments of fibrillin-1: implications of protein misfolding for the pathogenesis of Marfan syndrome and related disorders

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Fibrillin-1 is a large modular glycoprotein that assembles to form 10–12 nm microfibrils in the extracellular matrix. Mutations in the fibrillin-1 gene (FBN1) cause Marfan syndrome and related connective tissue disorders (fibrillinopathies) that show autosomal dominant inheritance. The pathogenic mechanism is thought to be a dominant negative effect of a mutant protein on microfibril assembly, although direct evidence is lacking. A significant group of disease-causing FBN1 mutations are cysteine substitutions within EGF domains that are predicted to cause misfolding by removal of disulphide bonds that stabilize the native domain fold. We have studied three missense mutations (C1117Y, C1129Y and G1127S) to investigate the effect of misfolding on the trafficking of fibrillin-1 from fibroblast cells. We demonstrate that both C1117Y and C1129Y, expressed as recombinant fragments of fibrillin-1, are retained and accumulate within the cell. Both undergo core glycosylation but lack the complex glycosylation observed in the secreted wild-type fragment, suggesting retention in the endoplasmic reticulum (ER). In addition, co-immunoprecipitation experiments show association with the ER chaperone calreticulin, but not calnexin, 78kDa glucose-regulated protein (Grp78/BiP) or protein disulfide isomerase. In contrast, G1127S, which causes a moderate change in the EGF domain fold, shows a pattern of glycosylation and trafficking profile indistinguishable from the wild-type fragment. Since expression of the recombinant fragments does not disrupt the secretion of endogenous fibrillin-1 by the cell, we propose that G1127S causes disease via an extracellular dominant negative effect. In contrast, the observed ER retention of C1117Y and C1129Y suggests that disease associated with these missense mutations is caused either by an intracellular dominant negative effect or haploinsufficiency.

INTRODUCTION

Mutations in the gene that encodes human fibrillin-1 (FBN1; MIM 134797) give rise to the autosomal dominant disorder Marfan syndrome (MFS; MIM 154700) and other related diseases of connective tissue collectively known as type-1 fibrillinopathies (1,2). MFS has highly variable clinical manifestations including aortic dilation and dissection, ectopia lentis and musculoskeletal abnormalities (2). Human fibrillin-1 is a 350 kDa glycoprotein and the major structural component of the 10–12 nm diameter connective tissue microfibrils (3,4). It has a modular organization typical of many extracellular proteins (Fig. 1A) and is composed mainly of epidermal growth factor (EGF)-like domains, which are widely distributed, independently folding modules found in many transmembrane and extracellular proteins (5). This domain type is characterized by six highly conserved cysteine residues that disulphide bond in a 1–3, 2–4, 5–6 arrangement and stabilize the global fold of the domain. In fibrillin-1 there are 47 EGF-like modules, 43 of which contain a calcium-binding consensus sequence and are termed calcium-binding EGF modules (cEGF) (6). The cEGF modules are often arranged as repeating tandem arrays in which the individual modules display higher calcium-binding affinities due to specific pairwise domain interactions (7). The bound calcium performs a key structural role in restricting interdomain flexibility, which may facilitate protein–protein interactions (8,9) and also protect the modules against proteolytic cleavage (10). In the central

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portion of the fibrillin-1 molecule, the longest stretch of 12 cbEGF repeats is thought to form a rigid rod-like structure, which may be important for microfibril assembly. The cbEGF domains are mostly interspersed by latent transforming growth factor β binding protein-like (TB) domains. There is also a proline-rich region, a cysteine-rich hybrid domain and N- and C-termini which undergo proteolytic processing to produce a ~320 kDa product before incorporation into the microfibrils.

The biochemical pathway of fibrillin-1 assembly into microfibrils is poorly understood and as a consequence the mechanism by which mutations in FBN1 result in disease is unclear. Although direct evidence is lacking, a dominant negative model of pathogenesis has been proposed (11) in which the mutant monomer disrupts assembly of normal fibrillin-1 into microfibrils or is itself misincorporated into the microfibril, and the level of mutant protein modulates the severity of the disease. This is probably an over-simplification of the mechanism since MFS-causing premature termination codon (PTC) mutations, that cause selective degradation of mutant mRNA, can result in severe disease (12,13). Irrespective of the mechanism involved, a critical threshold of microfibril function in the extracellular matrix appears to be required since reduced expression of normal fibrillin-1 in mice has been shown to result in a MFS-like phenotype (14,15). It seems likely that different FBN1 mutations will have a variety of pathogenic effects, but all ultimately resulting in a drop below this threshold (16).

Of the >500 published FBN1 mutations leading to the disease phenotype (17–19) many are missense mutations
affecting one of the conserved cysteine residues of the EGF or TB domains and would be predicted to cause misfolding. Disruption of the global fold of the domain might also occur by substitution of other key structural residues such as for example in the G1127S mutation studied here, which involves substitution of a highly conserved glycine residue by a serine. Despite extensive structural knowledge of EGF and TB domains, a genotype–phenotype relationship has not emerged apart from the observation that a cluster of mutations in the region corresponding to exons 24–32 encoding TB3 and cbEGF domains 11–18 are associated with the severe neonatal form of the disease (20), although mutations which cause classic MFS as well as milder phenotypes are also found in this region (21,22). Mutations in exons 24–34 of FBN2, which encodes the related protein fibrillin-2, are associated with congenital contractual arachnodactyly (23,24) emphasizing a crucial role for this central region of both fibrillin proteins. To date there have been few studies of the functional effects of missense mutations in fibrillin-1 cbEGF domains, which are predicted to cause misfolding. The present study was undertaken to investigate the effects of folding substitutions on trafficking of fibrillin-1 and provide information on the pathogenic mechanism leading to MFS. We decided to focus on cbEGF13 (Fig. 1B) since an unusually high phenotypic variability is associated with mutations that affect this domain. We have determined the consequences of three different folding substitutions for the secretion of recombinant fragments of fibrillin-1 by a human fibroblast cell line. These are C1117Y (25) and C1129Y (26) which both give rise to classic MFS, and the G1127S substitution which has been identified as a risk factor for ascending aortic aneurysm (27). The fragments harbouring these changes have been compared with the wild-type and a fragment containing a P1148A polymorphism (28,29) also in cbEGF13 (Fig. 1B).

This study has demonstrated that amino acid substitutions which impair folding of cbEGF domains can have different effects on trafficking. Evidence is presented to show that two substitutions may result in a retention of mutant fibrillin-1 within the endoplasmic reticulum (ER), while the third is secreted normally. On the basis of these findings we conclude that different pathogenic mechanisms are associated with FBN1 mutations that cause protein misfolding and contribute to the complexity of the genotype–phenotype relationship.

RESULTS

Cloning of recombinant fragments

To investigate the secretion of recombinant fibrillin-1 fragments containing different folding substitutions in cbEGF13, a cDNA fragment encoding the N-terminus to the proline-rich region was cloned into the pKG52(polyA) mammalian expression vector. A second fragment comprising wild-type cbEGF11-22, or cbEGF11-22 containing C1117Y, G1127S, C1129Y or P1148A substitutions in cbEGF13 (Fig. 1A and B), was cloned downstream of the N-terminal fragment. In this system the effect of the substitutions in cbEGF13 on trafficking was being studied in the context of the cbEGF11-22 fragment, therefore in a native-like environment and in a construct using the natural leader sequence of fibrillin-1, under the control of the HSVTK promoter. The recombinant expression construct contains the proline-rich region, which can be utilized in the detection of the recombinant fragment by an anti-Pro antibody (30) and since its molecular weight is less that of full-length fibrillin-1 it is readily distinguished from it on SDS–PAGE.

The MSU-1.1 fibroblast cell line was selected for expression of the recombinant fragments since it has previously been shown, by pulse-chase analysis, to produce fibrillin-1 in a manner similar to that of human primary dermal fibroblasts (30). Further, it can assemble microfibrils and therefore contains the necessary factors required for fibrillin-1 processing, secretion and assembly. By expressing the construct in MSU-1.1 we could also determine any dominant negative effect of recombinant expression on the processing and secretion of full-length fibrillin-1.

The NterPro-cbEGF11-22 recombinant fragment is expressed into conditioned medium

Transfection of the MSU-1.1 cell line with recombinant constructs and subsequent selection by puromycin yielded pools of clones. Western blot analysis and detection with the anti-Pro antibody of isolated single clones from such a pool obtained after transfection with the wild-type construct demonstrated the clonal variability in the level of expression, with ~30% of the total clones showing a detectable level of recombinant fragment in the medium (Fig. 2A). Therefore, in order to make a comparison between the effects of different mutations on the secretion of the fragment and eliminate integration effects, protein expression was usually assessed by the use of pools of transfected clones. To exclude the possibility that expression differences might arise due to variable levels of mRNA, despite the fact that the analyses were performed on pools of clones, mRNA derived from the wild-type, C1117Y, C1129Y and G1127S cDNA constructs was measured and, relative to the amount of endogenous fibrillin-1 mRNA, no difference in the amounts present could be detected (data not shown).

On electrophoresis of the conditioned medium from a pool of clones obtained after transfection with the NterPro-cbEGF11-22 wild-type construct on SDS–PAGE under both reducing and non-reducing conditions followed by western blot analysis, the construct was detected as a monomer and, within the limits of the detection, there was no evidence for the formation of dimers or multimers during or after secretion (Fig. 2B). The position on the gel corresponded to an approximate molecular mass of 125 kDa, somewhat higher than the predicted molecular mass of 103 kDa for this recombinant fragment, suggesting that it was glycosylated.

Wild-type, P1148A and G1127S recombinant fragments are secreted into the medium

A western blot analysis of the conditioned medium from pools of clones obtained after transfection with the NterPro-cbEGF11-22 wild-type construct and the same construct containing amino acid substitutions C1117Y, C1129Y, G1127S or P1148A is shown in Figure 3. The wild-type fragment, the P1148A polymorphism and the G1127S-containing
fragments were always detectable in the medium at comparable levels with no evidence for any proteolysis occurring for any of the secreted fragments. However, in the case of the C1117Y and C1129Y substitutions no fragment was detected in the medium on immunoblotting. All recombinant cell lines, including those containing the cysteine substitutions, showed comparable levels of endogenous fibrillin-1 secretion (Fig. 3; upper band).

C1117Y and C1129Y recombinant fragments are retained in the cell

Western blot analysis of the cell lysate fractions of the pools of clones under reducing (Fig. 4A) and non-reducing (Fig. 4B) conditions indicated that the fragments containing C1117Y and C1129Y were synthesized, but retained inside the cell. Under reducing conditions immunoblot analysis of the C1117Y-containing fragment indicated retention of a major species within the cell to high levels. The C1129Y substitution, on the other hand, was usually accompanied by a lower band, as well as other lower molecular weight immunoreactive products. The wild-type, P1148A and the G1127S-containing fragments were only just detectable in the cell lysate fractions, consistent with normal trafficking through the secretory pathway. The non-reducing gel indicated that the bulk of these retained C1117Y and C1129Y-containing fragments existed in a monomeric form although there was a small percentage of higher molecular weight material on the gel.

Secreted and retained fragments display different glycosylation patterns

A difference in the post-translational modifications of recombinant wild-type, G1127S and P1148A secreted and the same fragments detectable in the cell lysate fraction was suggested by their migratory differences on SDS–PAGE analysis. As shown for the wild-type recombinant fragment in Figure 5, the protein in the conditioned medium (lane 1) ran to a position indicative of a higher molecular weight than the corresponding fragment detected in the cell lysate fraction (lane 2). Treatment with the amidase PNGase F, which hydrolyses all types of N-glycan chains by cleaving between the innermost GlcNAc and asparagine residues of high mannose, hybrid and complex oligosaccharides from N-linked glycoproteins, resulted in a reduction in molecular weight of the fragment inside the cell (lane 3) and in the medium (lane 4). The increase in migration rate was more marked for the secreted fragment, and after PNGase F hydrolysis the recombinant fragment from both the conditioned medium and the cell lysate co-migrated. This demonstrates there is a difference in the N-linked glycosylation between the secreted fragment and that detected inside the cell.

The nature of the glycosylation was investigated further with a second glycosidase, EndoH, which cleaves the chitobiose core of high mannose and some hybrid oligosaccharides from N-linked glycoproteins, i.e. only simple sugars not the more complex glycosylation structures. In

In the absence of an endogenous fibrillin-1 band on the reducing gel (Fig. 4A), under conditions which clearly revealed the retained recombinant fragment, a visual comparison of an internal marker to ensure comparable loading of the samples was not possible. However, all cell lysate samples were obtained from cells harvested at a similar confluence and lysed in an equivalent volume of SDS sample buffer. Further, media and lysate samples from the same expression experiments were run in parallel (Figs 3 and 4A) and demonstrated a similar level of fibrillin-1 in the media samples of all clones.
contrast to its susceptibility to hydrolysis by PNGase F, the wild-type fragment in the medium was resistant to Endo H (Fig. 6A) although the protein inside the cell showed a small but discernable reduction in molecular weight (Fig. 6B). These results further define the difference in the N-linked glycosylation between the wild-type fragment in the medium and that detectable inside the cell, and suggest that the secreted wild-type fragment contains more complex modifications and hence demonstrates Endo H resistance. A similar result was found for the G1127S and P1148A secreted fragments (data not shown).

The retained C1117Y and C1129Y fragments in the cell lysates showed similar migration to the wild-type, G1127S and P1148A fragments which were only just detectable inside the cell. Treatment of the C1117Y and C1129Y retained fragments with glycosidase enzymes showed a reduction in apparent molecular weight to that of the deglycosylated form with both Endo H and PNGase F (Fig. 7). These retained fragments, as well as the low level of wild-type, G1127S and P1148A protein detected inside the cell (which is presumably in transit between the ER and Golgi) contain simple carbohydrate structures and the cysteine substitutions, or the resulting retention of the fragment, have prevented their further complex modification to become Endo H resistant.

Retained fragments associate with calreticulin

The evidence that the NterPro-cbEGF11-22 fragments containing the C1117Y and C1129Y substitutions were retained in the ER indicated possible interactions with molecular chaperones. A co-immunoprecipitation strategy was employed to investigate such interactions in cell lysate fractions of the C1117Y and C1129Y clones that retained the fragment and also with lysates of the clones that expressed the fragment into the medium. Western blot analysis of the cell extracts with anti-calreticulin antibody revealed equivalent amounts of calreticulin migrating at ~60 kDa (Fig. 8A). On immunoprecipitation of these extracts with anti-calreticulin antibody followed by western blot analysis of the immunoprecipitates and detection with the anti-Pro antibody, the NterPro-cbEGF11-22 fragments containing the C1117Y and, to a lesser extent, the C1129Y substitutions were clearly detected (Fig. 8B). Trace amounts of the fragment were also detected in the wild-type, G1127S and P1148A cell lysates but not in an untransfected MSU-1.1 control. Immunoprecipitation in the absence of anti-calreticulin antibody (Fig. 8C) eliminated non-specific binding of protein to the sepharose beads as a cause of the signal. No association was detectable on immunoprecipitation with antibodies against other candidate chaperones: calnexin (Fig. 8D), Grp78 (BiP) (Fig. 8E) or protein disulfide isomerase (PDI) (Fig. 8F). The band migrating at ~90 kDa in Figure 8D was not detected in the absence of anti-calnexin antibody and is presumably due to cross reaction of the anti-Pro antibody with calnexin. The faint band in the position of the NterPro-cbEGF11-22 fragment in Figure 8D was also detected in the MSU-1.1 control and therefore does not represent co-immunoprecipitation with this antibody.

Effect of recombinant expression on endogenous fibrillin-1

Investigation of the effect of the expression of NterPro-cbEGF11-22 fragments on the synthesis, processing and glycosylation of endogenous fibrillin-1 was carried out with

Figure 4. Comparison by western blot analysis of cell lysate fractions of the NterPro-cbEGF11-22 pools after electrophoresis on a 4–15% gradient gel under (A) reducing and (B) non-reducing conditions. The recombinant fragment was clearly detectable in the cellular fraction of the C1117Y and C1129Y pools and just detectable on the reducing gel in the wild-type, G1127S and P1148A pools of clones. Conditioned media from untransfected MSU-1.1 was run as control. Molecular masses (kDa) of reduced marker proteins are indicated and show the position of the non-reduced fragments corresponding to a lower molecular weight than that observed after reduction.

Figure 5. Treatment of both conditioned media (med) and cell lysate (cell) samples of the wild-type NterPro-cbEGF11-22 fragment with PNGase F results in their co-migration, indicating the observed migratory difference between these fragments is due to a difference in their N-linked glycosylation. Detection was by the anti-Pro antibody following western blot analysis on a reducing gel. Arrows indicate wild-type NterPro-cbEGF11-22 in the medium (upper), in the cell (middle) and the co-migrating fragments after PNGase F treatment (lower). The cell lysate fraction was concentrated before PNGase digestion to ensure the intracellular fragment was detectable by the anti-Pro antibody. Molecular masses (kDa) of marker proteins are indicated.
isolated single clones, since any effects would not be apparent in pools due to the presence of fibrillin-1 from non-expressing clones. Immunoblotting of the full-length fibrillin-1 in the conditioned medium of an isolated clone that retained the C1117Y recombinant fragment, or of a wild-type clone that secreted the fragment, did not reveal any effects on the level of expression or processing of endogenous fibrillin-1 when compared with an untransfected MSU-1.1 control (Fig. 9). In all the cell lines some profibrillin-1, but mainly fibrillin-1, was present in the medium. The ratio between the upper profibrillin-1 and predominant lower fibrillin-1 bands were similar with no detectable differences in the sensitivity to PNGase F (data not shown), indicative of no effect on glycosylation. Pulse-chase analyses of these clones also showed no detectable effect on the disappearance of profibrillin-1 from the cells or the appearance of fibrillin-1 in the medium (data not shown), all profibrillin-1 being lost from the cells within 2h, as was observed for the MSU-1.1 control in this and previous studies (30). From this we conclude that this recombinant fragment has no significant inhibitory effect on the secretion of fibrillin-1.

**DISCUSSION**

MFS is an autosomal dominant disorder with the products of both the wild-type and mutant alleles being co-expressed in cells. The molecular mechanisms which result in microfibril abnormality have proved difficult to identify and therefore no obvious basis for the varying phenotypes associated with FBN1 mutations is apparent. Pulse-chase analyses of fibrillin-1 from dermal fibroblasts of MFS patients have demonstrated a variety of defects in fibrillin-1 synthesis, secretion and extracellular matrix (ECM) deposition (31,32). However, these experiments do not differentiate between the wild-type and mutant protein, hence the consequences of different mutations for the biosynthesis and secretion of the mutant fibrillin-1 monomer and their potential to disrupt microfibril assembly are unclear.

Although the pulse-chase analyses frequently show a marked reduction in the matrix deposition suggesting a dominant negative effect of the mutant fibrillin-1 protein, they do not identify whether this dominant effect occurs intra- or extracellularly. Further, for some mutations a reduction in fibrillin-1 synthesis occurs and it is possible that in these cases a functional haploinsufficiency may result in disease.

Here we demonstrate cellular effects of the structural changes introduced by the C1117Y, C1129Y and G1127S folding substitutions by the use of recombinant fragments which can be distinguished from the wild-type full-length fibrillin-1. We show that there are differences in the intracellular trafficking of the three mutants and hypothesize that defective trafficking as a result of protein misfolding is likely to be a significant contributory factor in the pathogenesis of MFS.

A wild-type NterPro-cbEGF11-22 fragment and also NterPro-cbEGF11-22 containing a P1148A polymorphism which does not introduce any structural defect into cbEGF13 (33) and a G1127S folding substitution (34) were all secreted efficiently into the conditioned medium. Together with the pulse-chase studies of patient fibroblasts containing the G1127S substitution, which showed normal synthesis and
secretion of fibrillin-1 but reduced deposition in the ECM (27),
this finding substantiates the proposal that the mutation has an extracellular dominant negative effect either on, or after, incorporation of fibrillin-1 into the microfibril. Previous in vitro studies on the structural effect of the G1127S substitution in a cbEGF12-14 triple construct and in a cbEGF12–13 pair by protease digestion assays and two-dimensional NMR spectroscopy respectively demonstrated that localized structural changes had been introduced into the mutant domain but it was not severely misfolded (35). This localized effect of G1127S on the structure of cbEGF13 could exert its dominant negative effect by disruption of protein binding sites involved in assembly, or affect the properties of the assembled microfibril but appears unlikely to introduce a proteolytic susceptibility as has been observed for calcium-binding mutations (36,37).

In contrast, pulse-chase studies of the fibroblast cell line containing C1117Y identified normal synthesis, but a delay in secretion of fibrillin-1 and a severe reduction of the extracellular matrix deposition (38). Pulse-chase studies of the C1129Y patient cells have not been reported. In the recombinant system both the cysteine substitutions were retained inside the cell, most likely due to the presence of an unpaired cysteine (Fig. 1B), which results in greater disruption of the mutant cbEGF13 than that caused by the G1127S substitution. This indicates the ‘delay’ observed in the patient fibroblast cell lines is due to selective retention of the mutant protein within the cell. No evidence was obtained in the present study for a significant inhibitory effect of the retained recombinant fragment on the secretion of endogenous fibrillin-1. However, any dominant negative effect may involve regions not expressed in this recombinant fragment, and interaction between the full-length mutant and wild-type fibrillin-1 monomers cannot be ruled out. The ‘delayed secretion’ phenotype is however not observed for all patient fibroblasts with cysteine substitutions (32,38) and it is possible that this type of substitution may show varying degrees of retention, influenced by the degree of disruption of the domain as well as the position of the missense mutation within the fibrillin-1 monomer.

Defects other than in intracellular processing and secretion may occur as a consequence of domain misfolding and have significance for the variable phenotypes associated with MFS. An increase in susceptibility to proteolysis may result, or disruption of tertiary structure could affect presentation of a protein binding site. Enhanced protease susceptibility has been demonstrated for a cysteine substitution (39) and hence may contribute to the disease phenotype. While there is little direct evidence for the involvement of a protein binding site in the cbEGF11-22 fragment of fibrillin-1 it has been demonstrated that the versican lectin domain binds to a site on fibrillin-1 between cbEGF11 and 21 (40). Further, we have speculated that a flexible loop region of cbEGF12 may be involved in inter- or intra-molecular protein-protein interactions (41). A distortion of the potential binding interface could occur due to an increase in the intrinsic flexibility of this region as a result of misfolding and/or loss of calcium binding in cbEGF13.

On entry of proteins into the ER, control mechanisms inspect the conformation of polypeptides and ensure that only correctly folded and assembled proteins are transported further to the Golgi apparatus. The fate of misfolded proteins is as a rule retention and aggregation or eventual degradation. The G1127S-containing fragment is sufficiently well folded in the context of the NterPro-cbEGF11-22 fragment to escape quality
indicating where in the cell the transport of mutant proteins has been characterized for a number of diseases. In this study on the molecular pathology of MFS, we demonstrate that ER retention of misfolded recombinant protein occurs, primarily in the ER where a further modification of the core carbohydrate structures does not occur and the mutants fail to accumulate in the Golgi complex. Retention within an incorrect subcellular compartment as a result of misfolding of multidomain proteins is likely to be in the ER, preventing further glycosylation in the Golgi complex and targeting to the cell surface. We suggest that trafficking and secretion behaviour comparable to that of the wild-type, the cysteine substitutions appear to cause a retention of the fragment, most likely in the ER, preventing further glycosylation in the Golgi complex and targeting to the cell surface. We suggest that trafficking and secretion is therefore secreted. The eventual fates of the retained cysteine substitutions studied here may differ from each other. Immunoblot analyses of the C1117Y and C1129Y-containing fragments indicated retention within the cell to high levels possibly due to aggregation as a result of the exposure of hydrophobic regions of the domain. However, C1129Y appeared to be handled differently by the cell undergoing more rapid intracellular degradation. The significance of this observation for full-length C1129Y fibrillin-1 remains to be determined but the possibility exists that, for some substitutions, rapid intracellular degradation of the mutant fibrillin-1 occurs. Instability of some cysteine substitutions within cbEGF domains explains the reduction of fibrillin-1 synthesis to approximately 50% of normal in some patient fibroblast cell lines in spite of the presence of normal amounts of mutant mRNA (32).

The studies on the differential effects of the glycosidase enzymes PNGase F and Endo H indicate the observed accumulation of the NterPro-cbEGF11-22 mutant fragments is likely to be in the ER where a further modification of the core carbohydrate structures does not occur and the mutants fail to be targeted to the Golgi complex. Retention within an incorrect cellular compartment as a result of misfolding of multidomain proteins has been characterized for a number of diseases. In this study on the molecular pathology of MFS, we demonstrate that an ER retention of misfolded recombinant protein occurs, indicating where in the cell the transport of mutant fibrillin-1 could be delayed. However, the mechanisms by which disease-causing mutations, specifically affecting EGF-like domains, lead to abnormal protein function appear somewhat variable. In familial hypercholesterolemia arising from mutations in the low-density lipoprotein (LDL) receptor locus ~50% of characterized mutations result in ER retention (42). In contrast, missense mutations in the EGF repeats of Notch3, which give rise to CADASIL (cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy), did not affect targeting to the cell surface, but resulted in extracellular accumulation of the Notch3 ectodomain (43).

The glycoprotein quality control system in the ER involving the lectin chaperones calnexin and calreticulin has been well characterized (44). The observation that calreticulin, but not calnexin, interacts with the retained fragment suggests a distinct function for this chaperone in the folding and/or degradation of these recombinant fibrillin-1 fragments, but the chaperone system is likely to be complex. The specialized receptor-associated protein (RAP) has been defined as a chaperone for members of the LDL receptor family of proteins (45) and promotes folding and processing of a number of mutant proteins which show an ER retention (46). However, the ER chaperone Grp78 (BiP) has been implicated in the retention of misfolded LDL receptor containing two mutations including a C646Y substitution in an EGF-like repeat (47).

In summary, although protein misfolding is the underlying defect in all three of the disease-causing mutations studied here, our studies demonstrate they can have variable effects on intracellular trafficking and secretion. While the G1127S substitution is synthesized normally and displays secretion behaviour comparable to that of the wild-type, the cysteine substitutions appear to cause a retention of the fragment, most likely in the ER, preventing further glycosylation in the Golgi and targeting to the cell surface. We suggest that trafficking differences, observed in recombinant fragments, are recapitulated in full-length fibrillin-1 and contribute to the disease phenotype, resulting in a variety of pathogenic mechanisms. We propose that in the case of the G1127S substitution an extracellular dominant negative effect is the pathogenic mechanism which causes disease. The retention of the cysteine substitutions C1117Y and C1129Y suggests either, that they result in functional haploinsufficiency, or that the dominant negative effect of the mutant protein is intracellular.

**MATERIALS AND METHODS**

**DNA constructs and expression vectors**

A DNA fragment encompassing residues 134–1471 of human fibrillin-1 cDNA and encoding amino acids 1–446, numbering according to (48), which contained a SpeI site at the N-terminus and an XhoI site at the C-terminus was amplified using a forward primer, Fib IU (30) and the reverse primer 5’-TAGTAGCTCGAGATGGCAGCACCCTGGTGCGTCTC with Pfu polymerase (Stratagene) and the pCR-Script cDNA fragment I (30) as template. The SpeI–XhoI restricted PCR product was cloned into SpeI–XhoI restricted pKG52(polyA) a mammalian expression vector which contains a puromycin resistance marker (a gift from Dr K. Gould), to generate a construct containing the N-terminus to the proline-rich region of fibrillin-1.

The cbEGF11-22 fragment (nucleotides 3212–4714; amino acid residues 1027–1471) containing a 6xHis tag at the C-terminus and a SalI restriction site at both the N- and C-termini was amplified in a similar manner using the forward primer 5’-TAGTAGGTCGACCTAAAGATATCAATGAGTG
CAAGATG and the reverse primer 5'-TAGTACCGTCAGCTTTAGTATGACGTACGTACCAGACGTTGG with PC-Script cDNA fragment II/III as template (30). The SalI restricted product was cloned in frame into the XhoI restricted pKG52(polyA) vector containing the N-terminus to proline-rich region. Positive colonies were detected by PCR screening and plasmid DNA sequenced to confirm correct orientation of the insert with no errors introduced into the N-terminal fragment or the cbEGF11-22 insert during PCR.

In order to generate missense mutations C1117Y, G1127S, C1129Y or P1148A within the cbEGF11-22 fragment, the wild-type cbEGF11-22 construct was ligated into the SalI site of pBluescript II (pBS) K+ (Stratagene) and the missense mutations introduced by a PCR based site directed mutagenesis method with Pfu DNA polymerase. In each case the forward primer contained the mutated residue which is enclosed in brackets—C1117Y: forward primer, 5'-TCAGT(A)TCAGAG AGATCCTC, reverse primer, 5'-TCAATATCCATGCAGTTC; G1127S: forward primer, 5'-GAGGT(A)GTGTTGCGCCATAAC; reverse primer, 5'-GGCATAAGGAGAGATC; C1129Y: forward primer, 5'-GTGGTGGTTT(A)CCATAACAG; reverse primer, 5'-CTCGCATAGGAGAGGAGATC; P1148A: forward primer, 5'-TGTC(G)CCAACATCTCCGG; reverse primer, 5'-GCTGATGGCCAGCCG.

The mutated fragments were excised from the religated pBS vector by SalI digestion and ligated into the pKG52(polyA) mammalian expression vector which had been previously engineered to contain the N-terminal fragment of fibrillin-1. In this way other mutations in the vector which may have occurred during PCR mutagenesis were excluded. Sequencing was carried out to confirm correct orientation of the inserts with no errors introduced other than the desired mutations.

**Maintenance of cell lines and DNA transfection**

The near-diploid transformed human dermal fibroblast line MSU-1.1 (49) was maintained as described previously (30). Plasmid DNA (5 μg) of each of the mutant clones was used for transfection of 50–70% confluent cell monolayers in 75 cm² flasks by means of LipofectAMINE reagent (Invitrogen Life Technologies) according to the manufacturer’s instructions. The selection medium containing 2.5 μg/ml puromycin (Sigma) was changed every 3–4 days until individual clones of ~2–5 mm were observed (2–3 weeks). The pools of clones were washed with PBS containing 0.02% EDTA, trypsinized and transferred to 25 cm² flasks or six-well plates for protein expression or RNA extraction. Individual clones were isolated by the use of 8 × 10 mm cloning cylinders, trypsinized and transferred to single wells of 24-well tissue culture plates. Clones were amplified in selective medium prior to expression or pulse-chase experiments.

**Expression of wild-type and mutant constructs**

Pools of clones were plated at a cell density of 5 × 10⁵ to 5 × 10⁶ cells/25 cm² flask. Where pools of clones from different constructs grew at varying rates, the media and lysate samples were compared at similar cell confluency. A comparison between the wild-type and the mutant containing fragments was only considered legitimate if the pool contained sufficient clones, >50 (but usually >100)/75 cm² flask, the expression was compared at a low passage number for each pool and from at least three transfections of each construct. The NterPro-cbEGF11-22 fragments were secreted into the medium over 3–5 days. Conditioned media samples were removed at various time points, centrifuged at 12 000g for 5 min, added to 2× SDS sample buffer in the presence or absence of β-mercaptoethanol (5% v/v) and heated at 95°C for 6 min. Cell pellets were lysed in protein sample buffer and heated at 90°C. Samples were analysed by SDS–PAGE on 4–15% Tris–HCl gradient gels (Bio Rad) for the recombinant fragments or 4% gels for fibrillin-1. Proteins were transferred to nitrocellulose membranes and immunoblotted with an anti-Pro antibody (1:100) essentially as described previously (30). Anti-rabbit IgG horseradish peroxidase conjugate (1:2000) was used as secondary antibody followed by enhanced chemiluminescent detection (Amersham Biosciences).

**Pulse-chase labelling**

Pulse-chase analyses were carried out essentially as described previously for primary fibroblasts (50). Isolated clones (1 × 10⁶ cells/well) in 24-well plates were starved of L-methionine and L-cysteine for 30 min and pulsed with 50 μCi Pro-mix (Amersham Biosciences) for 30 min. Cells were then incubated in complete media and chased in the absence of label for up to 5 h.

**Glycosylation assays**

Conditioned media and cell lysates from a confluent monolayer of a transfected pool of clones in a 75 cm² flask were denatured at 100°C for 10 min and then treated with Peptide: N-Glycosidase F (PNGase F) and Endoglycosidase H (Endo H) for 1–3 h according to the manufacturer’s instructions (New England BioLabs Inc.). A time course of digestion was performed to determine optimal time of activity.

**Co-immunoprecipitation with chaperones**

A confluent monolayer of a transfected pool of clones in a 25 cm² flask was washed with PBS and lysed on ice for 10 min with 500 μl lysis buffer (50 mM Tris–HCl pH 8.0, 1% NP40, 1 mM PMSF and 1 mM NEM). The cell lysate was removed and chaperones immunoprecipitated with the rabbit polyclonal antibodies: anti-calreticulin (1:200), anti-calnexin CT (1:200) and anti-PDI (1:100) and, for the identification of Grp78 (BiP), the mouse monoclonal antibody anti-KDEL receptor antibody (10 μg/ml) at 4°C for 4 h. Immunocomplexes were recovered with Protein A-Sepharose, or Protein G-Sepharose for the anti-KDEL receptor antibody, at 4°C overnight. Antibodies were obtained from Stressgen Biotechnologies. The Sepharose beads were washed five times with PBS and immunoprecipitated proteins released by addition of protein sample buffer containing 100 mM DTT (40 μl) and boiling for 5 min before analysis on 4–15% SDS–PAGE and immunodetection with the anti-Pro antibody as described above. Western blot analysis with anti-calreticulin antibody (1:5000) was carried out as described for the anti-Pro antibody.
Transcript detection

Total RNA was isolated from 25 cm² cell cultures with the RNeasy Mini Kit (Qiagen) and analysed by RT-PCR with an Omniscript RT kit (Qiagen) and a reverse primer derived from exon 36 (cbEGF22), followed by PCR in the presence of specific primers, KG1 (30) and a primer derived from exon 14, which distinguished between recombiant construct and endogenous fibrillin-1 transcripts respectively. The expected sizes of transcript were 2.8 kb for the construct and 2.7 kb for fibrillin-1. The PCR reactions were carried out in the presence of serial dilutions of RT product and controls in the absence of reverse transcriptase were included to exclude DNA contamination.

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REFERENCES


