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Review

The molecular mechanisms of congenital hypofibrinogenaemia

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Abstract. Congenital hypofibrinogenaemia is characterized by abnormally low levels of fibrinogen and is usually caused by heterozygous mutations in the fibrinogen chain genes (α, β and γ). However, it does not usually result in a clinically significant condition unless inherited in a homozygous or compound heterozygous state, where it results in a severe bleeding disorder, afibrinogenaemia. Various protein and expression studies have improved our understanding of how mutations causing hypo- and afibrinogenaemia affect secretion of the mature fibrinogen molecule from the hepatocyte. Some mutations can perturb chain assembly as in the γ153 Cys → Arg case, while others such as the Bβ353 Leu → Arg and the Bβ414 Gly → Ser mutations allow intracellular hexamer assembly but inhibit protein secretion. An interesting group of mutations, such as γ284 Gly → Arg and γ375 Arg → Trp, not only cause hypofibrinogenaemia but are also associated with liver disease. The nonexpression of these variant chains in plasma fibrinogen is due to retention in the endoplasmic reticulum, which in turn leads to hypofibrinogenaemia.

Key words. Fibrinogen; hypofibrinogenaemia; afibrinogenaemia; mutation; expression; protein structure; protein stability.

Introduction

The fibrinogen molecule is the focal point of the coagulation cascade and plays a pivotal role in blood coagulation and fibrinolysis. Human fibrinogen is an acute phase protein with a half-life of 4 days and a plasma concentration of 1.5–4.0 g/l. It is synthesized in hepatocytes from three homologous polypeptide chains (Aα, Bβ and γ) that are assembled into a hexameric (Aα Bβ γ)$_2$ structure (reviewed in [1]). The chains are encoded by three genes (α, β and γ, respectively) located in a cluster spanning some 50 kb on the long arm of chromosome 4 at 4q28-31 [2]. Each gene is separately transcribed and translated, and the nascent chains translocated into the endoplasmic reticulum (ER), where assembly occurs in a stepwise fashion [3].
Upon vascular injury, thrombin initiates the conversion of fibrinogen to an insoluble fibrin clot by removing fibrinopeptides A and B from the N-termini of the Aα and Bβ chains, respectively. The fibrin clot is then stabilized by factor XIIIa-mediated covalent cross-links. This process stems acute blood loss, while the slower processes of fibrinolysis and tissue repair proceed. However, disruption of this balanced system can lead to haemorrhagic or thrombotic events.
Congenital hypofibrinogenaemia is associated with low functional and antigenic fibrinogen levels (less than 1.5 g/l) and can result from a variety of mutations in the fibrinogen genes. These can affect transcription, messenger RNA (mRNA) processing, translation, posttranslational processing, peptide folding and chain assembly, export from the hepatocyte or the stability of the mature protein. Heterozygosity for such mutations causes hypofibrinogenaemia but does not usually produce clinically significant bleeding. Congenital afibrinogenaemia, the complete absence of fibrinogen, is a rare autosomal recessive disorder which is due to the inheritance of these mutations in

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Table 1. Mutations that cause hypo- and afibrinogenaemia.

Mutation[1]	Name	Dose[2]	Level	Ratio	Structure affected	Ref
Aα -1138 c→t*	Hamamatsu	he	0.84		reduced transcription	[69]
Aα g. 34 ins c*		co	0		terminates in prepeptide	[8]
Aα 11 kb deletion (Δ exons 2–5)		ho	0		Δ residues 1–625	[58]
Aα IVS1+3 a→g		co	0		prepeptide	[8]
Aα g.1185 del t**		co	?		severely truncated Aα chain	[55]
Aα13 Gly→X		co	<0.01		severely truncated Aα chain	[66]
Aα20 Val→Asp	Canterbury[b]	he	1.30	0.7:1	Golgi cleavage at Arg19	[29]
Aα20 Val→X frameshift stop at 51		co	0.01		severely truncated Aα chain	[66]
Aα76 Tyr →X		ho	?		severely truncated Aα chain	[55]
Aα100 Ser→X		ho	0.04		truncates in coiled coil	[66]
Aα IVS3+1_+4del		ho	?		truncates in coiled coil	[55]
Aα110 Arg→X		ho	<0.01		truncates in coiled coil	[66]
Aα125 Lys→ Frameshift stop		co	0		truncates in coiled coil	[8]
Aα149 Arg→X	Baffin Bay	ho	0		truncates in coiled coil	[67]
Aα IVS4+1 g→t		ho	0		truncates in coiled coil	[8]
Aα219 Lys→ Frameshift stop		ho	<0.01		truncates before αC	[71]
Aα268 Arg→ Gln-Glu-Pro-X	Otago[b]	he ho	1.60 0.10	0:1	truncates in αC after 270	[64]
Aα g. 4179 del a** (frameshift mutation)		ho he	<0.01 2.0		truncates in αC after 399	[65, 55]
Aα g. 4190 del t** (frameshift mutation)		ho	0		truncates in αC after 399	[65, 55]
Aα297 Gly→X		co	0		terminates in αC after 296	[8]
Aα315 Trp→X		co	0		terminates in αC after 314	[8]
Aα328 Gln→X	Keokuk[b]	he co	2.5 0.5	0.1:1	truncates in αC after 327	[63]
Aα333 Pro→ Leu…His-X		co	0		truncates in αC after 394	[8]
Aα452 Gly→ Trp-Ser-X	Milano III[b]	he ho	2.80 2.60	0.1:1[c]	truncates in αC after 453	[72]
Aα461 Lys→X	Marburg[b]	he ho	1.90 0.60	0.1:1[c]	truncates in αC after 460	[61]
Aα476 Met→ His-Cys-Leu-Ala-X	Lincoln[b]	he	3.00	0.2:1	truncates in αC after 479	[60]
Aα495 Pro→ frameshift stop	Perth[b]	he	3.30	0.2:1	truncates in αC after 517	[62]
Bβ17 Arg→X		ho	0.01		severely truncated Bβ chain	[65]
Bβ255 Arg→His	Merivale	he	1.10	0:1	D-domain 5-stranded β sheet	[42]
Bβ IVS6+1 g→a	Avon	he	1.20	0:1	D-domain truncates after Bβ307	[47]
Bβ IVS6+13 c→t		ho he	0.03 1.61		predicted truncation after Bβ314	[50]
Bβ316 Asp →Tyr	Hamilton	he	1.10	0:1	D-domain 5-stranded β sheet	[41]
Bβ IVS7+1 g→t		he ho	1.86 0.01		predicted truncation after Bβ350	[50]
Bβ353 Leu→Arg		he ho	1.3 <0.01	N.E.	distal P region of D-domain	[44]
Bβ400 Gly→Asp		he ho	1.5 0.02	N.E.	distal P region of D-domain	[44]
Bβ414 Gly→Ser		he co	1.48 0	N.E	H-bond to 5-stranded β sheet	[43]

Table 1 (continued)

Mutation [1]	Name	Dose [2]	Level	Ratio	Structure affected	Ref
Bβ437 Trp → Gly		he ho	1.57 <0.01	N.E.	distal P region of D-domain	[73]
Bβ437 Trp → X		he ho	1.20 <0.05	N.E.	distal P region of D-domain	[49]
Bβ440 Trp → X	Mt Eden	he	0.7	0:1	distal P region of D-domain	[48]
γ g.194 del a**		ho	?			[55]
γIVS1+5 g → a		he ho	1.18 <0.01			[74]
γIVS2 –3 c → t		ho	?			[55]
γIVS2+1 g → a	Waikato	he	1.10		truncates before 1st SS ring	[54]
γIVS3+5 g → a		he ho	1.20 0		predicted truncation after γ76	[75]
γ82 Ala → Gly	Dunedin	co	0.8	0.5:1	affects centre of coiled-coil	[53]
γ153 Cys → Arg	Matsumoto IV	he	0.87	N.E.	S-S bridge in γD (γ153–γ182)	[30]
γ197 Arg → X		ho	?			[55]
γ231 Glu → X	Hakata	he ho	1.35 <0.02		truncates after γ230	[32]
γ284 Gly → Arg	Brescia	he	0.70	0:1	D-domain 5-stranded β sheet	[33]
γ345 Asn → Asp	Stuttgart	he	<0.5		distal P region of D-domain	[76]
γ371 Thr → Ile	Muncie	he	0.80	0:1	H-bond to 5-stranded β sheet	[46]
γ375 Arg → Trp	Aguadilla	he	0.60	0:1	hinge structure of strand 2	[38]

[1] Residue numbering is from the mature N-terminal. For the full nascent chain numbering, add 19 for the Aα chain, 30 for the Bβ chain and 26 for the γ chain. The character (X) is used to denote a terminal codon.
[2] he (heterozygous), ho (homozygous), co (compound heterozygote).
[a] Estimated from transfection experiments.
[b] These truncations also cause dysfibrinogenaemia.
[c] Estimated from published SDS acrylamide gels.
Ratio, ratio of variant to normal chain in plasma fibrinogen; N.E., not expressed in transfected cell supernatant.
Level, antigenic fibrinogen concentration (g/l).
* numbering is from the transcription initiation site.
** numbering is based on the genomic sequence (with the A in the initiator ATG codon counted as nucleotide 1)

a homozygous or compound heterozygous state. Surprisingly, the coagulation defect is no more severe than haemophilia. This is due, in part, to von Willebrand factor, which facilitates platelet aggregation and adhesion in the absence of fibrin [4]. Afibrinogenaemia is usually detected at birth, when it causes uncontrolled bleeding from the umbilical cord. Spontaneous intracerebral bleeding and splenic rupture can occur throughout life, while other bleeding episodes such as gum bleeding, epistaxis and gastrointestinal bleeding are common [5]. Patients respond well to fibrinogen replacement therapy, which is necessary to prevent spontaneous abortion in pregnant women with afibrinogenaemia or severe hypofibrinogenaemia.

The investigation of dysfibrinogenaemias and the association of defined mutations with specific functional defects have provided unique insights into the location of functionally important sites in the fibrinogen molecule. Many of these sites have now been shown in detail in crystal structures. Mutations responsible for hypofibrinogenaemia are also yielding new insights into amino acid residues that are involved in molecular assembly, secretion and domain stability. In the last 5 years, there has been a major increase in the number of hypo- and afibrinogenaemias that have been defined at the molecular level. Here, we consider these cases (see table 1) in relation to the insights they provide into protein processing, assembly, secretion and molecular instability. Mutations that result in afibrinogenaemia have also been included, as they usually represent the homozygous or compound heterozygous state of hypofibrinogenaemic mutations.

Afibrinogenaemic mutations have been previously numbered according to the guidelines for human gene mutation nomenclature [6, 7], which incorporate the signal peptide residues into the numbering (i.e. 19 amino acids for the Aα chain, 30 for the Bβ chain and 26 for the γ chain). Since confusion arises when this is directly translated into a protein location, mutations in this review have been numbered according to their position in the mature protein sequence, as has been used for fibrinogen variants over the last 50 years. For example, the Aα G316X and

W334X mutations reported by Neerman-Arbez et al. [8], with numbering from the start of the signal peptide, have been redesignated G297X and W315X in table 1 to prevent confusion when highlighting the affected protein structure.

Fibrinogen structure

In the original model proposed by Hall and Slayter, fibrinogen was seen as a trinodular molecule, in which two outer domains were connected to a central nodule by thin filamentous regions [9]. The regions connecting the globules were thought to consist of coiled α helices [10]. This model is still functional but has been refined over the last 40 years especially by the availability of crystal structures of individual fibrinogen fragments [11–13].

Fibrinogen is a dimeric symmetrical molecule comprising three pairs of homologous chains in which the Aα chain consists of 610 amino acids, the Bβ chain 461 and the γ chain 411 residues (fig. 1a). The three chains have significant homology, although the Aα chain is radically different in its C-terminal two-thirds. The Bβ and γ chain homology persists through their entire sequence but is more evident towards the C-terminal ends [14].

All three chains possess a homologous region of 110 amino acids with a repetitive polarity characteristic of α helices. This area, which is flanked by CPXXC motifs that form the disulphide rings, consists of a set of coiled coils with the nonpolar residues turned inward and the polar side chains extended out into the aqueous solvent [15, 16]. Beyond this coiled coil, the Aα chain contains an extended hydrophilic region termed the αC domain, which lacks a clearly defined structure and is readily removed by a wide variety of proteases [17, 18]. The two αC domains that make up the C-terminal two-thirds of the Aα chains account for ~25% of the mass of the molecule.

The central E domain contains all six N-termini, which are linked by disulphide bonds. The C-termini of the Aα chains fold back to associate with the centre of the molecule [19], while the independently folding homologous C-terminal regions of the Bβ and γ chains form the outer D domains [11, 12] (fig. 1a). Both subdomains (βD and γD) contain three distinct regions: an N-terminal region that folds into a three-stranded β sheet anchored to a short helix; a central region consisting of a five-stranded β sheet and two helices; and the C-terminal 'P' region that has little secondary structure but contains the polymerization pockets and calcium binding sites (fig. 1b).

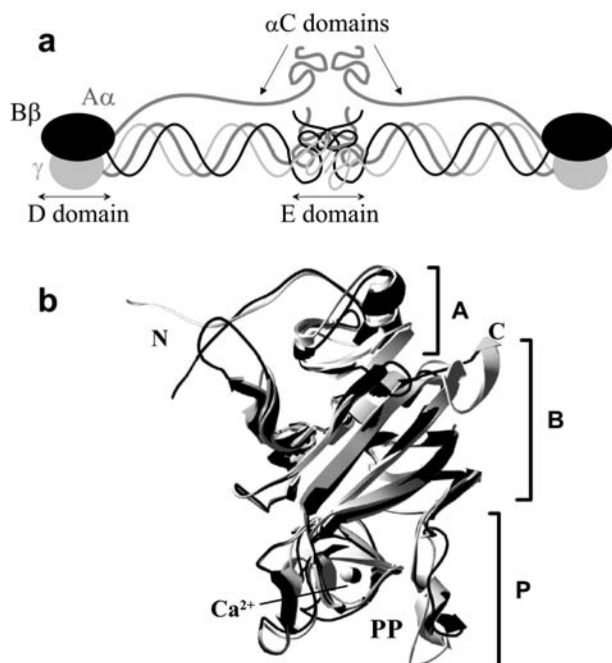

Figure 1. (*a*) Diagram of the fibrinogen molecule showing the major structural domains. The Bβ chain is represented in black, while the Aα and γ chains are shown in dark and light grey, respectively. The regions separating the outer D domains from the central E domain are termed the coiled coils. (*b*) Superimposed ribbon diagrams of the C-terminal globular domains of the Bβ and γ chains. The figure emcompasses residues Bβ198–458 (black) and γ139–397 (grey) and demonstrates the overall structural homology between the two domains. Bound calcium ions are depicted as spheres. The amino and carboxy-termini are labelled N and C, respectively. The N-terminal region, the five-stranded β-sheet and the C-terminal region of the domains are labelled A, B and P. The image was generated with Swiss-Pdb Viewer and POV-Ray from the Protein Databank file 1fzc.pdb.

Fibrinogen biosynthesis, assembly and secretion

Studies on the mechanisms of fibrinogen biosynthesis and secretion have been aided by in vitro studies using mainly HepG2 human hepatoma cells (reviewed in [20]). Fibrinogen assembly requires translation of each chain, independent translocation into the ER, interactions of the chains with chaperones that assist in the assembly and folding processes, and quality control mechanisms that distinguish properly assembled fibrinogen from aberrantly folded forms.

Assembly of the nascent chains into the final hexamer $(A\alpha\ B\beta\ \gamma)_2$ occurs rapidly in the lumen of the ER. It is a stepwise process in which single chains interact with each other to form Aα-γ and Bβ-γ complexes [21]. The two-chain complexes then acquire another chain to form $(A\alpha\ B\beta\ \gamma)_1$ half-molecules, which in a final step are linked to form the six-chain structure. Studies with recombinant systems, using deletion and substitution mutants, indicate that the coiled-coil region as well as inter- and intrachain disulphide bonds are needed to complete chain assembly [22–24]. Although the majority of the synthesized fibrinogen in HepG2 cells is secreted, a substantial pool of Aα and γ chains is retained and eventually degraded intracellularly.

Mutations affecting intracellular processing

At least two intracellular endoproteases are involved in the export and posttranslational modification of fibrinogen [25, 26]. In the ER, signal peptidase removes the prepeptide from the N-terminal of all three chains. Subsequently in the Golgi/secretory vesicles, a furin-like protease cleaves the propeptide from the C-terminal of the Aα chain [27]. This protease cleaves after dibasic residues in either an X Y Arg Arg, or better still an Arg X Y Arg configuration. Normal cleavage occurs after the -Arg-Pro-Val-Arg-sequence in the C-terminus of the Aα chain (fig. 2). Subsequent carboxypeptidase-H pruning of the P_1 Arg results in the mature protein with its C-terminal valine. However, for processing to occur, the P_1' residue following the cleavage site must not have a large alkyl side chain [28]. In fibrinogen, the exposed Aα Arg^{16}-Gly-Pro-Arg-Val^{20} sequence conforms to the dibasic motif, but the P_1' Val normally protects it from cleavage. In fibrinogen Canterbury, the valine residue at position 20 is replaced by an aspartic acid, removing this protection, and the Aα Arg^{16}-Gly-Pro-Arg-Asp^{20} sequence becomes a substrate for furin [29]. This results in the removal of the first 19 residues of the Aα chain within the Golgi/secretory vesicles.

Mutations affecting intracellular assembly

The Matsumoto IV (γ153 Cys$\rightarrow$Arg) mutation affects the proximal A region of the γD domain [30]. As indicated in crystal structures, the γ153 Cys residue forms an intrachain disulphide bond with γ182 Cys [12, 11], and the loss of this disulphide bond appears to result in abnormal folding. Transient expression of γ^{153Arg} chains with normal Aα and Bβ chains in Chinese hamster ovary (CHO) cells demonstrated that while the variant chains were synthesized, no variant fibrinogen molecules were secreted into the culture medium [30]. In addition, pulse-chase experiments showed that there was no assembly of $\alpha\gamma$ or $\beta\gamma$ intermediates or mature fibrinogen in cells expressing the variant γ chain. This suggested that the hypofibrinogenaemia is caused by disruption of the intrachain disulphide link and that this leads to defective assembly. This was confirmed by Zhang et al. [24] who demonstrated that disruption of the γ153-γ182 disulphide loop markedly inhibited formation of two-, three- and six-chain fibrinogen molecules.

The C-terminus of the γ chain has also been shown to be important for assembly and secretion of the hexamer [31]. Transfection of γ chain truncation variants into CHO cells indicated that variants with 386 residues or less were synthesized but were not assembled into intermediates and hence were not secreted. This is further illustrated by the γ231 Glu$\rightarrow$X mutation in fibrinogen Hakata [32]. Although containing an intact γ153-γ182 disulphide loop, this variant lacks the C-terminal of the γD domain and causes afibrinogenaemia when inherited in the homozygous state.

Mutations causing endoplasmic retention

Two different fibrinogen mutations are known to cause hypofibrinogenaemia through the formation of intrahepatic inclusion bodies. As with ZZ antitrypsin deficiency, the progressive accumulation of these inclusion bodies in the rough ER leads to liver damage and a decrease in the plasma levels of the protein. All seven heterozygotes for the fibrinogen Brescia γ284 Gly$\rightarrow$Arg mutation had hypofibrinogenaemia, but only two had demonstrable liver cirrhosis [33]. But again, this is similar to antitrypsin deficiency, where only 15% of ZZ homozygotes develop any significant liver damage [34].

The Gly 284 side chain is conserved not only in all known γ and Bβ chain sequences but also in all fibrinogen homologues, suggesting a structural, rather than a functional importance. The residue is located in the γD domain at the end of the most distal strand of the five-stranded β

	P_6	P_5	P_4	P_3	P_2	P_1	P'_1	P'_2	P'_3
Profibrinogen$^{606\text{-}614}$	Lys	Ser	Arg	Pro	Val	Arg	Gly	Ile	His
Fibrinogen $\alpha_E$$^{606\text{-}614*}$	Lys	Arg	Arg	Pro	Val	Arg	Asp	Cys	Asp
Proalbumin	Arg	Gly	Val	Phe	Arg	Arg	Asp	Ala	His
Albumin Blenheim*	Arg	Gly	Val	Phe	Arg	Arg	Val	Ala	His
Fibrinogen A$\alpha$$^{14\text{-}22*}$	Gly	Arg	Arg	Gly	Pro	Arg	Val	Val	Glu
Fibrinogen Canterbury	Gly	Arg	Arg	Gly	Pro	Arg	**Asp**	Val	Glu

Figure 2. Amino acid sequence of representative human proproteins processed at dibasic motifs in liver Golgi/secretory vesicles. Cleavage occurs after either XYRR or RXYR sites unless followed by a large alkyl residue. The arginine residues involved in the recognition sequence are underlined. The 1Asp$\rightarrow$Val mutation in proalbumin Blenheim prevents propeptide cleavage, while in fibrinogen Canterbury the converse Aα20 Val$\rightarrow$Asp mutation creates a new cleavage site. (*) sequences resistant to furin like proteases. The noncleavage of α_E chains is possibly due to a disulphide bond to the P_2' Cys.

sheet (fig. 3). This strand starts at Tyr 280 and extends to Ala 286 though it may be quite mobile, as residues 280–284 appear as a random coil in the double D structure [12, 35]. The β-sheet structure is reminiscent of the A sheet in antitrypsin that goes from a metastable five- to a stable six-stranded structure on cleavage within its reactive centre loop [36]. The Z mutation is at the hinge point of this loop sheet insertion, and its 342 Glu → Lys substitution provokes intermolecular loop-to-sheet insertion, leading to formation of Z fibrils and the blockage of secretion [37]. Like antitrypsin, the fibrinogen molecule is in a metastable state since its D : D polymerization sites preexist and do not require thrombin activation. The γ284 Gly → Arg mutation may perturb the β sheet array and somehow facilitate intramolecular strand insertions, leading to fibril formation and the blockage of secretion. Certainly the electron microscopic (EM) images of fibrinogen Brescia inclusions show a degree of order similar to that seen in Z antitrypsin fibrils [33].

The heterozygous γ375 Arg → Trp mutation in fibrinogen Aguadilla also causes hypofibrinogenaemia through formation of hepatic inclusion bodies and blockage of secretion [38]. The γ375 Arg residue occurs at the entrance of the polymerization pocket in a potential hinge structure in the γD domain, which connects strand 2 of the β sheet structure to the P domain of the γD module (fig. 3) [11]. The replacement of the highly polar guanidino group by a large hydrophobic tryptophan residue, in fibrinogen Aguadilla, would be expected to distort the polypeptide backbone, since the indole side chain would seek refuge in the hydrophobic interior of the domain. Based on the position of the γ375 residue and the similarity of the β sheet to that of antitrypsin and other serpins, the hypofibrinogenaemia might be a result of domain destabilization, leading to the intermolecular insertion of strand 2. Interestingly, Yakovlev et al. [39] have demonstrated that strand 2 of the γD module can be pulled out of the sheet without destroying the compact structure of the domain, lending support to our suggestion that the new Trp residue might allow intermolecular insertion of this strand.

An interesting feature of the fibrinogen Aguadilla and Brescia families is the variation in the severity of the liver disease. This is analogous to Z antitrypsin, in which the number and density of liver inclusions vary considerably between homozygous individuals. The formation of loop-sheet polymers in Z antitrypsin is dependent on the temperature and concentration of the protein [37]. Antitrypsin and fibrinogen are acute phase proteins, and hence undergo a marked increase in synthesis in association with elevated temperature (up to 41 °C) during inflammation. This will likely overwhelm the mechanisms responsible for clearing misfolded and aberrant proteins by degradation, leading to the accumulation and aggregation of the variant protein in the hepatocytes.

As four different types of fibrinogen inclusion bodies have been described [40], it is highly probable that mutations other than the γ284 Gly → Arg and γ375 Arg → Trp might also cause hypofibrinogenaemia through endoplasmic retention. For example, the fibrinogen Hamilton mutation (Bβ316 Asp → Tyr) occurs at the end of strand 3 of the five-stranded sheet of the Bβ chain, and the mechanism of hypofibrinogenaemia may therefore be similar to that seen in the Brescia case [41]. The structural importance of the Bβ316 Asp residue (which corresponds to Asp252 in the γ chain) is again highlighted by its interchain and interspecies conservation (fig. 3) [41]. Heterozygotes for the mutation have no variant Bβ chains in their plasma fibrinogen, but as no liver biopsy material was available, intracellular fibrinogen aggregation could not be confirmed.

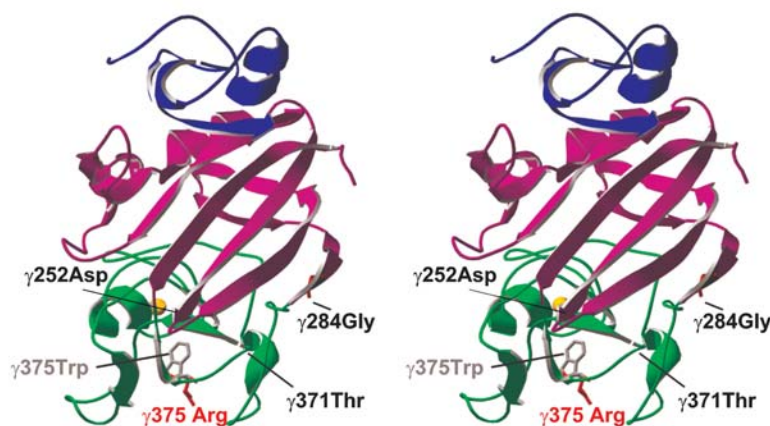

Figure 3. Stereo view of the γD domain showing the five-stranded β sheet structure (purple), the distal P region (green) and the N-terminal region (blue). The location of the Brescia γ284 Gly → Arg mutation is indicated, as is that of γ252 Asp. The latter position is equivalent to the Bβ316 site of the Hamilton Bβ316 Asp → Tyr mutation. The γ375 Arg residue, at the hinge of strand 2 is depicted as a red stick structure at the mouth of the polymerization pocket, while the mutation in fibrinogen Aguadilla (γ375 Trp) is shown in grey. The image was generated with Swiss-Pdb Viewer and POV-Ray using the protein databank file 1fib.pdb.

Mutations affecting domain stability and protein secretion

The tertiary structure and folding of the fibrinogen chains is very important for secretion, as highlighted by Bβ- and γ-chain substitutions that cause hypo- or afibrinogenaemia, such as fibrinogen Merivale (Bβ255 Arg → His) [42]. Individuals with this mutation have plasma fibrinogen levels of about 1.1 g/l with no variant Bβ^{255Arg} chains present in the circulating molecule. Arg255 is absolutely conserved, not only in all known Bβ chains, but also in all γ and extended α_E chains (a minor alternative form of the Aα chain) and in all fibrinogen related proteins such as tenascin and the *Drosophila* scabrous protein. Since sequence homology predicts similarity of tertiary structure, this level of conservation implies that Arg255 has a critical role in maintaining domain stability. Arg255 occurs at the C-terminal end of the first strand of the five-stranded β sheet that forms the major structural feature of the βD domain. It has very low surface accessibility, and its side chain makes hydrogen bond contacts with the backbone carbonyls of Pro412 and Gly414 that are located in the distal P region of the βD domain (fig. 4). The absolute conservation of Gly 414 in all fibrinogen chains and all fibrinogen-like proteins confirms the importance of the 255-414 interaction, and substitution of the guanidino with an imidazole side chain would perturb this interaction. The hypofibrinogenaemia in this case is very likely a direct result of protein instability caused by the Bβ255 Arg → His mutation. In support of this, a recent report has identified an afibrinogenaemic patient with compound heterozygous Bβ414 Gly → Ser and Bβ17 Arg → X mutations [43]. Transfection studies in COS-7 cells demonstrated that the Bβ414 Gly → Ser mutation allowed intracellular hexamer assembly but inhibited secretion of the protein into the media.

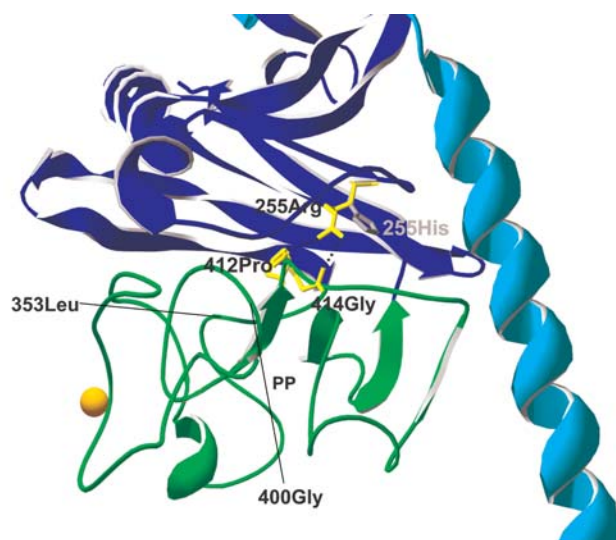

Figure 4. Part of the crystal structure of the βD domain. The β sheet subdomain (blue) is above the P subdomain (green) containing the polymerization pocket, while the coiled coil is depicted in cyan. Calcium bound to the primary site is shown as an orange sphere, and the polymerization pocket is labelled (PP). Selected residues shown in stick form include the side chain of Bβ255 Arg, and the backbone carbonyls of Bβ412 Pro and Bβ414 Gly. Hydrogen bonds formed by the Bβ255 Arg side chain are depicted as dashed lines. A putative position for the Bβ255 His side chain (fibrinogen Merivale mutation) is shown as a transparent overlay. The locations of the Bβ353 and Bβ400 residues are also indicated. The image was generated with Swiss-Pdb Viewer and POV-Ray using the protein databank file 1fzc.pdb.

Two other homozygous point mutations in the βD domain (Leu353Arg and Gly400Asp), located in the hydrophobic distal P region, have also been found to cause afibrinogenaemia [44] (fig. 4). Heterozygosity for either mutation lowers fibrinogen levels by ~50%. The Bβ353 site is conserved as a large hydrophobic residue (Leu, Phe or Met) between species, chains and fibrinogen-like proteins, whereas the Bβ400 residue has similarly conserved small side chains (Gly or Ser). Both substitutions introduce bulky charged side chains into the hydrophobic core of the βD domain where they are located. The charge and steric effects of these changes would be expected to distort the packing of the region, resulting in protein instability, as occurs in the unstable haemoglobin Hb Volga (β27 Ala → Asp) [45]. Transfection experiments in COS-1 cells expressing the variant Bβ chains together with normal Aα and γ chains showed that these mutations did not affect protein synthesis but prevented fibrinogen secretion [44].

Hypofibrinogenaemia due to the γ371 Thr → Ile mutation may also reflect impaired secretion due to domain instability [46]. The γ371 Thr residue, which is conserved among all known γ chain sequences, is located in the P region of the γD domain occurring just after a short β strand leading towards the polymerization pocket. Its hydroxyl group forms hydrogen bonds to the main chain where it bends between a pair of highly conserved Ala residues at 286 and 289 that are located at the end of the five-stranded β sheet. Interestingly, we have recently identified a γ289 Ala → Val in a family with hypofibrinogenaemia [unpublished data], highlighting the importance of the hydrogen bonding between the P region and the β sheet of the γD domain in maintaining domain stability. Ala 286 is at the end of strand 5 in the β sheet, in proximity to the fibrinogen Brescia mutation (γ284 Gly → Arg). The γ371 Thr → Ile substitution directly affects the same region, through loss of hydrogen bonding to the main chain at Ala 286, and hypofibrinogenaemia might be due to similar intracellular accumulation of abnormally conformed molecules. However, as in the Bβ316 Asp → Tyr case, no liver biopsy material was available to investigate this further.

Truncations of the Bβ chain

The importance of proper folding and domain stability is further demonstrated by truncations of the Bβ chain that

cause hypo- or afibrinogenaemia. The heterozygous IVS6 +1 g → a mutation (fibrinogen Avon) predicts read through into the intron, causing the insertion of 18 amino acids and truncation after residue 307 [47]. In this aberrant Bβ chain, the majority of the βD domain is missing (fig. 5). The heterozygous Mount Eden 440Trp → Stop mutation also causes truncation of the Bβ chain at residue 440 and results in the loss of strand 2 of the five-stranded sheet [48]. Neither fibrinogens Avon nor Mount Eden were found in purified plasma fibrinogen. These mutations probably cause misfolding of the D domain, which would result in instability and possible intracellular degradation.

Since the identification of the Avon and Mount Eden mutations, several other Bβ chain truncations that cause afibrinogenaemia have been identified. A Bβ437 Trp → X mutation that leads to the truncation of three amino acids prior to the Mount Eden mutation also causes nonexpression of the truncated Bβ chain in plasma fibrinogen [49]. Transfection studies in COS-7 cells indicated that the mutant chain is assembled into fibrinogen hexamers, but the mature molecule is not secreted; only incomplete forms containing Aα and/or γ chains were detectable in the supernatant. Two mutations (IVS6+13 c → t and IVS7+1 g → t) that would be expected to produce similarly truncated Bβ chains also cause afibrinogenaemia [50] (fig. 5). Expression of these mutations in HeLa cells revealed that IVS6+13 c → t creates a new donor splice site 11 nucleotides downstream, predicting a mutant Bβ chain of 290 normal amino acids, followed by 24 new residues and truncation after residue 314. The IVS7+1 g → t mutation predominantly creates a new splice site in exon 7, leading to a truncated Bβ chain of 350 amino acids. Neither variant was detected in the cell supernatants, demonstrating that the aberrant Bβ chains were not secreted. The observation that these C-terminal Bβ chain truncations are not expressed in plasma fibrinogen suggests that this region is required for secretion.

However, by coexpressing Bβ chain deletion mutants with normal Aα and γ chains in COS cells, Zhang et al. previously determined that the C-terminal half of the Bβ chain (208–461) is not necessary for assembly or secretion [51]. In agreement with this, the recently identified Bβ236 Tyr → X truncation (fibrinogen Lozanne), which terminates at the N-terminal of the βD domain, is incorporated into plasma fibrinogen [52]. This confirms that the βD domain may not be necessary for assembly and secretion, while the absence of other reported Bβ truncations from plasma fibrinogen indicates that incorrect folding of the domain due to premature termination may interfere with secretion. Therefore, it seems likely that the presence of a misfolded or truncated domain, rather than the absence of a properly folded one, is the cause of chain instability and non-secretion of certain truncated Bβ chains.

Mutations expressed in circulatory fibrinogen

The Dunedin mutation is present in the circulatory fibrinogen of heterozygotes, albeit at a reduced ratio (1 : 2) to

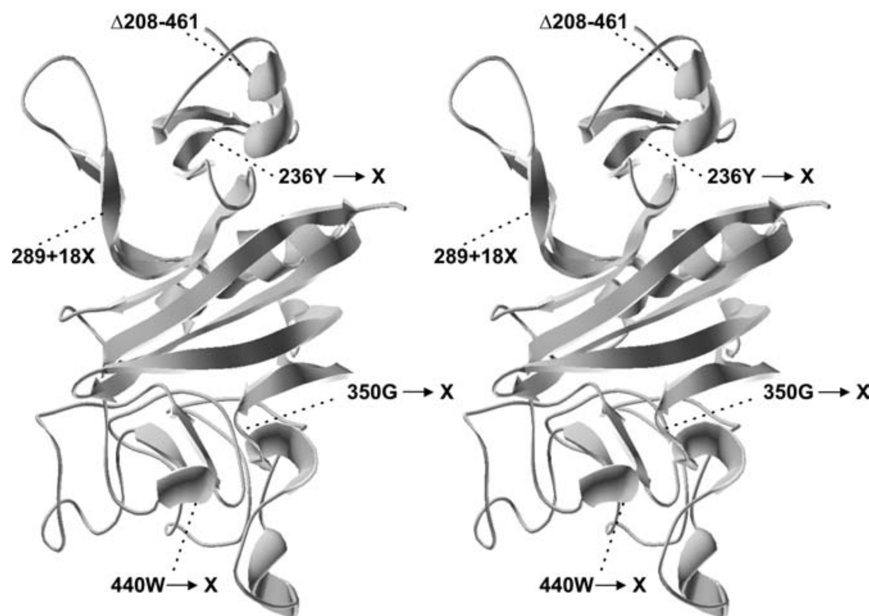

Figure 5. Stereo view of the βD domain showing the locations of various Bβ truncations. Δ208–461 corresponds to the recombinant deletion mutant used by Zhang et al. [51]. The 289+18X mutation corresponds to the predicted truncation in fibrinogen Avon (Bβ IVS6+1 g → a), while the 350G → X truncation is predicted from the Bβ IVS7+1 g → t mutation. The 236Y → X mutation represents the truncation in fibrinogen Lozanne, and 440W → X corresponds to fibrinogen Mount Eden. The image was generated with Swiss-Pdb Viewer and POV-Ray using the protein databank file 1fzc.pdb.

wild-type γ chains [53, 54]. This substitution occurs near the plasmin-sensitive region in the coiled coil connecting the E and D domains. In this triple-helix area, glycine is grossly underrepresented, accounting for only 3 of 337 residues. Destabilized coil packing along with increased proteolytic sensitivity may account for the decreased fibrinogen levels in this case.

Aα chain mutations

The most common cause of afibrinogenaemia is a (IVS4 +1 g→t) donor splice site mutation in intron 4 of the α gene [8, 55]. This nucleotide substitution results in the loss of the splice site gt sequence, leading to the activation of cryptic splice sites that result in premature truncation of the Aα chains [56]. Other splice site mutations, such as IVS3+1_+4del gtaa, cause exon skipping in transfected COS-7 cells [57]. An 11-kb deletion of exons 2–5, which removes all the coding sequence of the Aα chain, is also a frequent cause of afibrinogenaemia when inherited homozygously or together with the intron 4 splice site IVS4+1 g→t mutation [8, 55, 58].

The α gene mutations that cause afibrinogenaemia (table 1) involve either partial gene deletions, splice sites mutations, frameshift mutations or nonsense mutations; no simple amino acid substitutions have been found to cause hypo- or afibrinogenaemia. Apart from fibrinogen Hamamatsu with a lesion in the 5′ promoter region that causes hypofibrinogenaemia, all other Aα chain mutations listed in table 1 involve some form of truncation. These truncations range from molecules with only the prepeptide to the substantially complete 517-residue fibrinogen Perth and include the first reported case of afibrinogenaemia, Aα149 Arg→X [59].

Heterozygotes for the Lincoln truncation, after residue 479, do not actually have hypofibrinogenaemia, but the low ratio of variant to wild-type Aα chains (0.2:1) suggests that the Lincoln chains compete less well than wild-type Aα chains when forming complexes with other chains, or that they are degraded intracellularly [60]. Similarly, heterozygotes for the Marburg truncation, after residue 460, do not have hypofibrinogenaemia, but they also have a decreased ratio of aberrant to normal chains and homozygotes have marked hypofibrinogenaemia (0.6 g/l) [61]. These truncations suggest that the αC domain might be involved in assembly of the protein. This is confirmed by the recent reports of the fibrinogen Perth (Aα495 Pro→frameshift stop) and fibrinogen Keokuk (Aα328 Gln→stop) mutations (with Aα truncations of 15 and 46%, respectively) [62, 63]. Simple heterozygotes for fibrinogen Perth or Keokuk have normal plasma fibrinogen levels but diminished allelic expression ratios ($A\alpha^{variant}/A\alpha^{normal}$) of 0.2:1 and 0.1:1, respectively.

The compound heterozygote, with the fibrinogen Keokuk truncation and the Aα IVS4+1 g→t splice site mutation, had low plasma fibrinogen levels of 0.5 g/l. Similarly, the homozygote for the shorter truncation at 270 (fibrinogen Otago) had very low levels of 0.1 g/l [64], and her heterozygous son was in the low normal range with a plasma fibrinogen concentration of 1.6 g/l. However, the son had no detectable variant chains in his plasma, again suggesting that the variant chain is either degraded or outcompeted by wild-type Aα chains during assembly. This suggests that generally, the shorter the variant Aα chain, the less it is found in circulating fibrinogen, especially in the absence of normal chains.

Interestingly, the afibrinogenaemia causing g.4179delA and g.4190delT deletions [55, 65] predict frameshift mutations after residues 291 and 295, respectively, leading to incorporation of 108 and 104 new amino acids into the chain before premature termination after residue 399. These novel leucine- and valine-rich stretches probably disturb chain folding and prevent assembly and/or secretion of the fibrinogen molecule. The remaining homozygous mutations that cause truncation before the C-terminal end of the coiled coil totally prevent the expression of fibrinogen in plasma, indicating that the formation of the coiled coil is a minimal requirement for normal assembly and secretion [8, 66, 67].

Discussion

During the last 5 years, the molecular basis of hypofibrinogenaemia has been extensively characterized and has improved our knowledge of the structural features required for normal synthesis and secretion of fibrinogen. It is now clear that congenital hypo- and afibrinogenaemia are caused by defects in the fibrinogen gene cluster. Although advances in DNA analysis have made mutation detection easier, it is not always clear whether the identified mutation causes the presenting phenotype. Family studies showing segregation of the mutation with the phenotype, together with allele frequency analysis showing exclusion from the general population, and structural correlations are prerequisites for establishing cause and effect.

In certain cases, this may not be sufficient, and additional protein and/or coexpression studies should be performed to determine the amount of variant chain present in plasma fibrinogen or culture media fibrinogen. The need for such analyses is highlighted by the characterization of fibrinogen Merivale [42]. In this case, a total of seven sequence variations were identified, all of them in the β gene [42]. Five of these were previously identified polymorphisms, while two novel point mutations were detected (Bβ255 Arg→His and Bβ148 Lys→Asn). Linkage studies indicated that the Bβ255 Arg→His mutation segregated with hypofibrinogenaemia and was on a different allele to the Bβ148 mutation. However, neither mutation was found in

182 unrelated healthy individuals, and therefore the Bβ148 Lys$\rightarrow$Asn could not be dismissed as polymorphic. Protein analysis was subsequently undertaken, and the results clearly demonstrated that the variant Bβ^{255His} chains were not present in plasma fibrinogen, but the variant Bβ^{148Asn} chains were incorporated in plasma fibrinogen of heterozygotes for the Bβ148 Lys$\rightarrow$Asn mutation.

When there is a well-established mechanism for diminished expression such as the introduction of a stop codon or a change in the 3′ (gt) or 5′ (ag) mRNA splice sites, additional investigations may not be necessary. However, when a single amino acid substitution is the putative cause of hypofibrinogenaemia, further protein or expression studies are required. In these cases, fibrinogen chain composition can be examined directly by SDS-polyacrylamide gel electrophoresis (PAGE), reverse phase high-pressure liquid chromatography (HPLC), isoelectric focussing or by mass spectrometry. Additionally, mass spectrometry peptide mapping can be employed if other analyses are inconclusive, as the exact m/z of both the variant and normal peptides can be predicted, permitting a definitive conclusion concerning expression level [41, 42].

It is well established that the Bβ chains are rate limiting during production of the mature fibrinogen molecule [3, 68], and this is supported by the observation that all heterozygous Bβ mutations that affect chain synthesis result in hypofibrinogenaemia. Redman et al. [20] demonstrated that HepG2 cells, expressing fibrinogen, contained a steady pool of surplus Aα and γ chains, indicating that neither of these chains is limiting in fibrinogen biosynthesis. However, all of the heterozygous γ chain mutations in table 1 cause hypofibrinogenaemia, and it seems that the γ chain may not be present in excess in vivo. There does, however, appear to be an excess pool of Aα chains, as heterozygotes have normal plasma fibrinogen levels except for fibrinogen Hamamatsu (table 1). Additionally, in heterozygous individuals with Aα truncations, such as fibrinogen Lincoln, Marburg, Perth, Keokuk and Otago, the variant Aα chains are expressed at very low levels in circulating fibrinogen as compared with normal Aα chains [60–64]. This suggests that any aberrant Aα chains produced would be outcompeted by the surplus of normal chains, resulting in low or no expression of these variants in the circulating fibrinogen of heterozygotes. In fibrinogen Hamamatsu, which is associated with hypofibrinogenaemia, the mutant –1138 T allele was shown to decrease α gene transcription by 35% compared with the wild-type –1138 C allele [69]. However, this heterozygous mutation would not necessarily cause hypofibrinogenaemia even if it were to affect both alleles (i.e. trans acting). In fact, the authors who reported this mutation do not exclude the possibility that the affected individual might have a large heterozygous deletion in one of the fibrinogen genes [69].

While heterozygosity for a particular mutation reduces fibrinogen levels, it does not usually produce a clinically significant condition unless inherited in a homozygous or compound heterozygous state. There are, however, exceptions; heterozygosity for the Brescia or the Aguadilla mutations can lead to liver disease. Gene deletions, premature stop codons and splice site mutations might be expected to have a lesser impact on fibrinogen levels than amino acid substitutions. Even total loss of one gene should only decrease chain availability by 50%. However, because of the dimeric nature of fibrinogen, the random incorporation of a variant chain into nascent hexamers in the ER would mean that 75% of the mature fibrinogen molecules would contain at least one variant chain. In fibrinogen Brescia, carriers of the γ284 Gly$\rightarrow$Arg mutation had one-third the fibrinogen concentration of their non-affected relatives (average fibrinogen levels of 0.7 and 2.4 g/l, respectively).

Caution is needed when assessing how a DNA mutation might induce hypofibrinogenaemia. Researchers should be aware some nonsense mutations may primarily affect mRNA stability rather than protein stability [70], while some point mutations might introduce novel cleavage sequences for intracellular as well as extracellular proteolysis. In recent years, expression of recombinant variants has become a more prominent technique for investigating hypofibrinogenaemias. However, recombinant variants cannot fully replace protein analyses of naturally occurring variants, which permit a better understanding of which molecules enter the circulation and the physiological consequences of the mutation. As more of the mechanisms that underpin hypofibrinogenaemia are elucidated, we can expect to gain a clearer understanding of how the six chains of fibrinogen are assembled and secreted into circulation and how mutations disrupt this process.

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