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Quality control of fibrinogen secretion in the molecular pathogenesis of congenital afibrinogenemia

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Congenital afibrinogenemia is a rare bleeding disorder characterized by the absence in circulation of fibrinogen, a hexamer composed of two sets of three polypeptides (Aα, Bβ and γ). Each polypeptide is encoded by a distinct gene, *FGA, FGB* and *FGG*, all three clustered in a region of 50 kb on 4q31. A subset of afibrinogenemia mutations has been shown to specifically impair fibrinogen secretion, but the underlying molecular mechanisms remained to be elucidated. Here, we show that truncation of the seven most C-terminal residues (R455–Q461) of the Bβ chain specifically inhibits fibrinogen secretion. Expression of additional mutants and structural modelling suggests that neither the last six residues nor R455 is crucial *per se* for secretion, but prevent protein misfolding by protecting hydrophobic residues in the βC core. Immunofluorescence and immuno-electron microscopy studies indicate that secretion-impaired mutants are retained in a pre-Golgi compartment. In addition, expression of Bβ, γ and angiopoietin-2 chimeric molecules demonstrated that the βC domain prevents the secretion of single chains and complexes, whereas the γC domain allows their secretion. Our data provide new insight into the mechanisms accounting for the quality control of fibrinogen secretion and confirm that mutant fibrinogen retention is one of the pathological mechanisms responsible for congenital afibrinogenemia.

INTRODUCTION

Congenital afibrinogenemia (OMIM 202400) is a rare autosomal recessive disorder characterized by the complete absence in circulation of fibrinogen, the precursor of fibrin, which is the major protein of blood clots. Affected patients suffer from various haemorrhagic manifestations during life: umbilical cord haemorrhage is often the first sign of the disorder; gum bleeding, epistaxis, menorrhagia, muscle haematoma and haemarthrosis occur with varying intensity and spontaneous intracerebral bleeding or splenic rupture can occur throughout life (1,2).

Fibrinogen is synthesized predominantly in hepatocytes from two sets of three homologous polypeptide chains (Aα, Bβ and γ), which assemble to form a 340 kDa hexameric molecule $[A\alpha B\beta\gamma]_2$, held together by 29 disulphide bonds. The hexamer is characterized by a symmetrical structure, with a central E domain connected by three-stranded coiled-coils to two peripheral D domains. The D domains consist of the globular C-terminus of the Bβ (βC) and γ chains (γC) and of a portion of the coiled-coils (3). Both βC and γC are members of the FReD (fibrinogen-related domain) family (4). Each polypeptide is encoded by distinct genes, *FGA*, *FGB* and *FGG*, clustered in a region of 50 kb on human chromosome 4 (4q31). Since our identification of the first causative mutation for congenital afibrinogenemia (5), more than 40 mutations causing the disorder have been described, all located in the fibrinogen gene cluster. The

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majority of these mutations are found in *FGA* and are null, i.e. large deletions, frameshift, early-truncating nonsense or splice-site mutations (6,7).

Mechanisms of fibrinogen assembly have been well studied. During translocation of the single chains into the endoplasmic reticulum (ER), a signal peptide is co-translationally cleaved from each chain, and the ^{364}Bβ and ^{52}γ asparagine residues are N-glycosylated. Assembly proceeds in the ER with the formation of an [Aαγ] or [Bβγ] intermediate. The addition of either a Bβ or an Aα chain gives rise to a [AαBβγ] half-molecule, which dimerizes to form the functional hexamer (8). The protein undergoes several post-translational modifications in the Golgi complex, including maturation of N-linked oligosaccharides, phosphorylation, hydroxylation, sulfation (9). In contrast, fibrinogen secretion is not well understood. As for other secreted proteins, fibrinogen secretion is likely to be submitted to a quality control, but so far, mechanisms controlling fibrinogen secretion are unknown, although N-glycosylation and disulphide-bond formation, which both occur during fibrinogen synthesis (10–12), are known to play a crucial role in the proper folding of membrane and secretory proteins in the ER.

Recently, the identification of several mutations has provided insight into the domains putatively involved in the control of secretion. The L353R, G400D (13), G414S (14), W437X (15) and W437G (16) mutations, numbered according to the mature protein, all localized in the Bβ chain, were identified in afibrinogenemic patients and studied in transfected COS cells. In this model, expression of the mutant *FGB* cDNA, in combination with normal *FGA* and *FGG*, demonstrated that all five mutations had no effect on the intracellular assembly of the hexamer, but inhibited its secretion into the medium. Three other mutations in the βC domain, R255H (17), D316Y (18) and W440X (19), were identified in heterozygosity in patients with reduced fibrinogen levels. In these cases, the mutant chain was not present in the circulating fibrinogen. Altogether, these data strongly suggested that the βC domain plays an important role in the control of fibrinogen secretion.

Okumura *et al*. (20) previously used serial C-terminal truncations of the γ chain to study the role of this chain in fibrinogen assembly and secretion. These authors demonstrated that truncation of 25 residues from the C-terminus completely abolished fibrinogen assembly. In this study, we used a similar approach to investigate which residues of the βC sequence are essential for hexamer secretion. We show that removal of seven or more residues from the C-terminus of the Bβ chain leads to impairment of fibrinogen secretion. Additional mutation analyses indicate that the six-amino acid tail and the R455 residue may be important for secretion, by preventing the exposure of hydrophobic amino acids located within the βC domain. Using immunofluorescence and immuno-electron microscopy, we demonstrate that secretion-impaired mutants are retained in a pre-Golgi compartment. Finally, we generated and expressed chimeric chains combining the N- and the C-termini of Bβ and γ chains or angiopoietin-2 (Ang2). Our results show that presence of a βC domain is sufficient to inhibit the secretion of chimeric chains or complexes, with the exception of the native fibrinogen hexamer. In contrast, all γC-containing chains or complexes are secreted effectively.

RESULTS

Deletion of at least seven amino acids from the Bβ C-terminus leads to inhibition of fibrinogen secretion

We previously identified a mutation in the fibrinogen Bβ chain (W437X) that, in transfected COS cells, specifically abolished fibrinogen secretion into the medium (15). This mutation resulted in the loss of 25 residues from the Bβ C-terminus. To determine how far in the primary sequence the amino acids are essential for secretion, we generated *FGB* cDNA mutants with serial truncations of the Bβ C-terminus: W440X (corresponding to the mutation identified in 19), Y445X, K449X, K453X and F457X (Fig. 1A). When these constructs were co-transfected in COS-7 cells with normal *FGA* and *FGG* cDNAs, each Bβ chain variant was expressed (Fig. 1B, lysate, red) and did not impair fibrinogen assembly, because the hexamer was detectable in the cell lysate (Fig 1B, lysate, non-red). There is an apparent decrease in the ratio of beta chain to gamma chain ratio as the extent of truncation increases (Fig. 1B, lysate, red), suggesting greater instability of these truncated chains. This is supported by the non-reducing gel which shows a decrease in assembled hexamers and [AαBβγ] intermediates and a corresponding increase in [Aαγ] intermediate.

On reduction of hexamers or assembled intermediates, Aα, Bβ and γ chains (Fig. 1B, media, red) and hexamers (Fig. 1B, media, non-red) were observed in the medium of cells transfected with the wild-type (WT) *FGB* and the F457X mutant. In contrast, when cells were transfected with the W440X, Y445X, K449X, and K453X variants, no hexamers or Bβ chains could be detected in the medium, in which only decreased amounts of Aα and γ chains/complexes were observed.

To refine this analysis, we co-expressed *FGB* constructs carrying I454X, R455X or P456X mutations with normal *FGA* and *FGG* (Fig. 1A). P456X did not abolish fibrinogen assembly and secretion, whereas both I454X and R455X impaired secretion (Fig. 1C). These experiments demonstrate that the last six amino acids, from P456 to Q461, are dispensable for fibrinogen secretion and that deletion of any additional residue impairs this secretion.

Presence of either the six-residue tail or the R455 is required for fibrinogen secretion

To determine whether the nature of the six-residue tail was important, we substituted it with six alanines. The resulting P456_Q461$(A)_6$ mutant (Fig. 2A) did not affect assembly or secretion of fibrinogen (Fig. 2B), indicating that neither the presence nor the nature of the C-terminal tail are crucial for these two processes. To assess the intrinsic importance of R455, we replaced it with an alanine. The R455A mutant (Fig. 2A) was released as a hexamer (Fig. 2B), demonstrating that the nature of amino acid 455 was again not critical for fibrinogen secretion. In contrast, we found that a Bβ mutant carrying the R455A substitution in combination with the truncation of the six most C-terminal amino acids (R455A + P456X, Fig. 2A) was not secreted (Fig. 2B), showing that R455 becomes critical for secretion in the absence of the C-terminal tail.

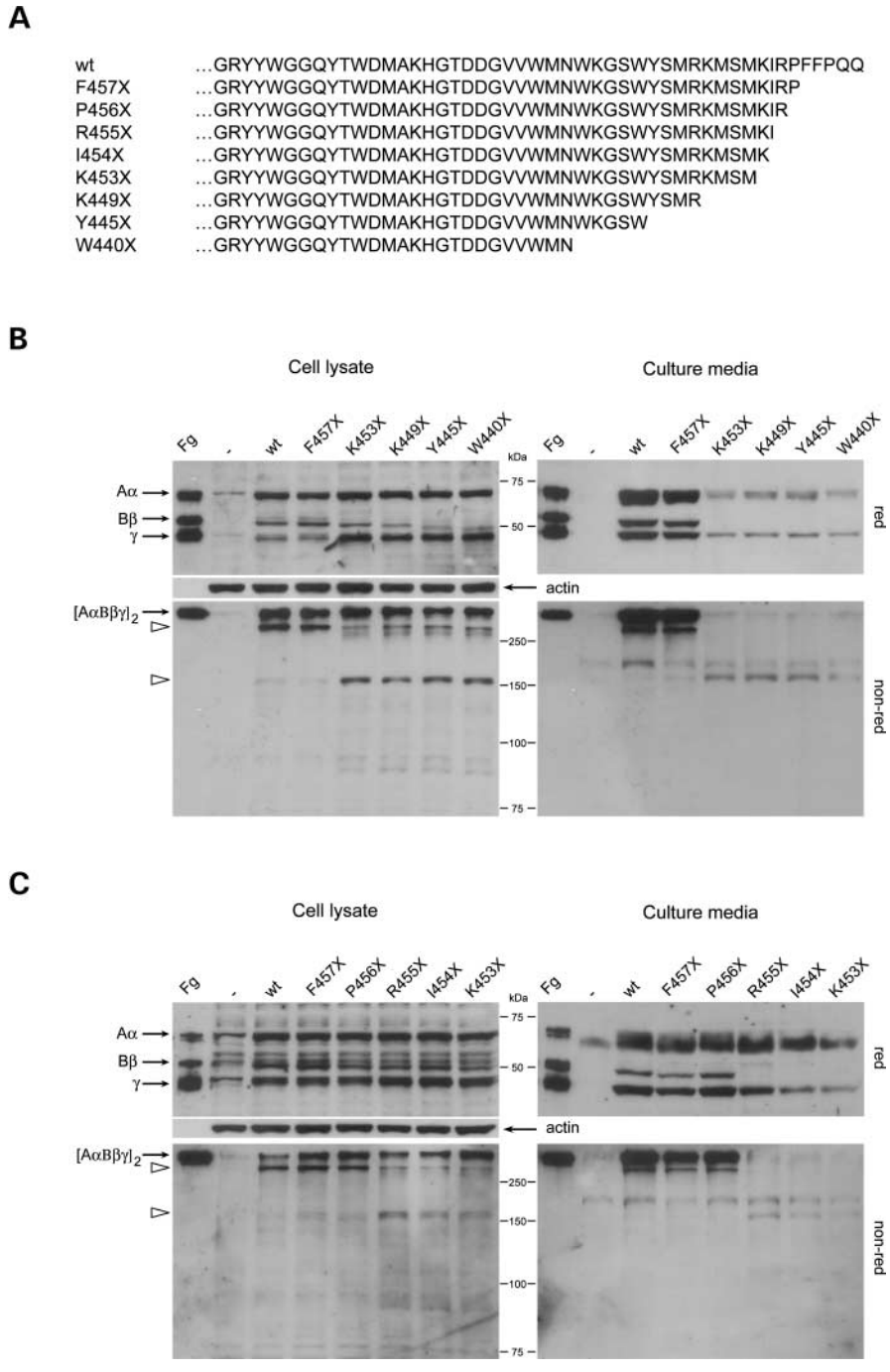

Figure 1. Serial truncations demonstrate that loss of seven or more residues from the Bβ C-terminus inhibits fibrinogen secretion. (**A**) Sequences of normal and mutated Bβ chains are shown from residue G414. (**B** and **C**) Western blots of cell lysates and conditioned medium. COS-7 cells were co-transfected either with the empty vector (−), normal *FGA*, *FGG* and *FGB* cDNAs (WT) or with the *FGA*, *FGG* and the indicated *FGB* mutant. Purified fibrinogen (Fg) was loaded as a control. Samples were analysed by immunoblotting using anti-fibrinogen antibodies under reducing (red) or non-reducing (non-red) conditions. The positions of Aα, Bβ and γ chains and of the $[A\alpha B\beta\gamma]_2$ hexamer are indicated. Open arrowheads indicate fibrinogen intermediates, presumably the $[A\alpha B\beta\gamma]$ half-molecules and $[A\alpha\gamma]$ molecules (8). Loading control was performed with anti-actin antibodies on reduced cell extracts. NB: the background signal for cells transfected with the empty vector (cell lysate, red) is more intense in 1C than in 1B.

Secretion-impaired fibrinogen mutants are retained in a pre-Golgi compartment

To determine where the secretion-impaired mutants were retained, COS-7 cells were transiently co-transfected with either WT *FGA*, *FGG*, *FGB* or normal *FGA*, *FGG* and one of the three *FGB* secretion-impaired mutants, R455X, W437X (15) and G414S (14), and immunostained for fibrinogen. As shown by confocal microscopy (Fig. 3A), both WT and mutant fibrinogen molecules were localized in a

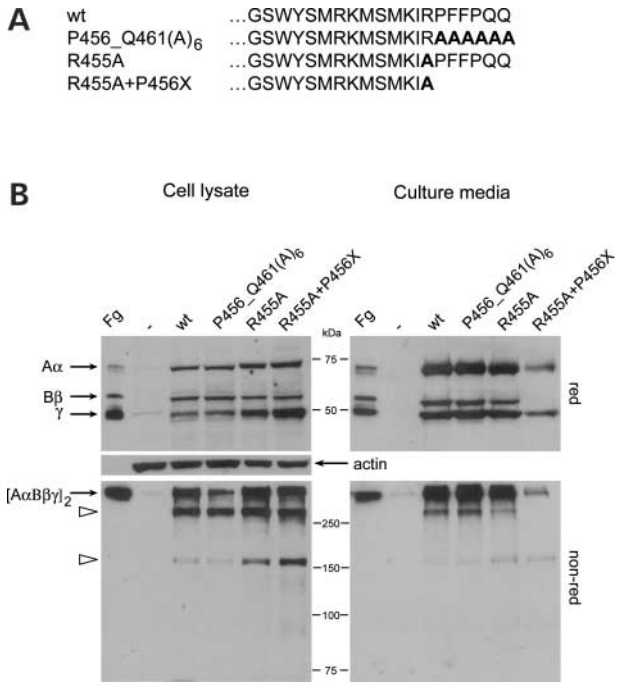

Figure 2. Expression of Bβ mutants show that the nature of the six-residue tail and that of the R455 is not critical for fibrinogen secretion. (**A**) Sequences of normal and mutated Bβ chains shown from residue G442. Amino acid substitutions are highlighted in bold. (**B**) Western blots on cell lysates or conditioned medium were performed as described in Figure 1B and C.

perinuclear network pattern typical of ER. In addition, WT fibrinogen co-localized with giantin, a Golgi marker, resulting in a yellow merge signal. In contrast, none of the secretion-impaired mutants co-localized with the Golgi marker.

Immuno-electron microsocopy revealed WT fibrinogen in both the lamellae and the vesicles of the Golgi apparatus, as well as in the cisternae of rough ER (Fig. 3Ba and b). In contrast, the R455X mutated form of the molecule was not immunodetected in the Golgi apparatus, but was abundant in the ER (Fig. 3Bc and d). These experiments show that the three secretion-impaired fibrinogen mutants we analysed are retained in a pre-Golgi compartment.

Presence of a βC domain causes retention of chimeric chains or complexes, whereas a γC domain allows their secretion

The βC and γC globular domains of the fibrinogen molecule are 42% identical at the amino acid level (60% similar) and are founding members of the FReD family (4). FReDs are found in the C-terminus of proteins such as the fibrinogen α_E variant (21), angiopoietins (22–24), tenascins (25), ficolins (26), fibroleukin (27) or *Drosophila melanogaster* scabrous (28). Analysis of phylogenetic trees suggests a common ancestor for the various FReDs (29), and shows their high conservation from invertebrates to human, which underlines their importance. So far, however, none of the FReDs has been assigned a specific function, even though, to our knowledge, all FReD-containing proteins multimerize and are secreted.

To investigate the role of the Bβ and γ FReDs in fibrinogen secretion, we constructed chimeras (Fig. 4A) with the γ N-terminus fused with the βC (γ–Bβ mutant) domain, and the Bβ N-terminus fused with γC (Bβ–γ mutant). These chimeras were transfected in COS cells either alone or in combination with either Aα and Bβ or Aα and γ chains. Both Bβ–γ and, in reduced amounts, γ–Bβ chimeras are expressed in the cells (Fig. 4B, lysate, red). The much fainter band of γ–Bβ may result from decreased stability. Both chimeras were able to form a complex detected at similar intensity, with an electrophoretic mobility comparable to that of the fibrinogen hexamer (Fig. 4B, lysate, non-red). This complex was found only when the γ–Bβ chimera was co-transfected with Aα and Bβ chains or when the Bβ–γ chimera was co-expressed with Aα and γ chains. These combinations are those in which the coiled-coil regions of all three chains, known to be essential for fibrinogen hexamer assembly (30), are preserved. In the medium, neither the γ–Bβ chimera nor the hexameric [AαBβγ–Bβ] complex were detected, suggesting that the fibrinogen molecule requires the presence of a γC domain to be secreted. In contrast, the Bβ–γ chain and the [AαγBβ–γ] complex were both secreted in the medium, indicating that the Bβ N-terminus does not limit secretion.

To further investigate the roles of the Bβ and γ FReDs, we generated four other chimeras combining Bβ or γ fragments and parts of Ang2, another FReD-containing protein (Fig. 4A). The structure of Ang2 is very similar to that of the Bβ and γ chains, as it contains a coiled-coil region, and the Ang2 FReD shares >50% homology with the Bβ and γ FReDs. Furthermore, the Ang2 FReD was recently crystallized and its structure was shown to be superimposable with the γ FReD (31). All the chimeras we generated were expressed in COS cells (Fig. 4C, lysate), with a higher expression or stability of Ang2–γ. Both Ang2–Bβ and Ang2–γ chimeras were found to dimerize and further multimerize. In the medium, Ang2–γ was abundant, Bβ–Ang2 and γ–Ang2 were easily and barely detected, respectively, whereas Ang2–Bβ was undetectable (Fig. 4C, media).

DISCUSSION

Congenital afibrinogenemia is mainly caused by null mutations, i.e. large deletions, frameshift, early-truncating nonsense or splice-site mutations (6,7). However, several causative missense mutations have been recently described. Among these, four mutations in *FGB* were shown to specifically impair fibrinogen secretion (13,14,16) and in all likelihood two others act in the same way (17,18). All six mutations are localized in the βC domain (Fig. 5A) and are predicted to destabilize its structure. In addition, two nonsense mutations, W437X (15) and W440X (19), in this region were also reported, removing 25 and 22 residues from the Bβ C-terminus, respectively. In this study, we investigated which residues in the βC sequence are essential for hexamer secretion. Using a COS cell model, we showed that removal or substitution of the last six C-terminal amino acids, as long as R455 is present, does not affect fibrinogen assembly and secretion. In the βC domain, which features a central

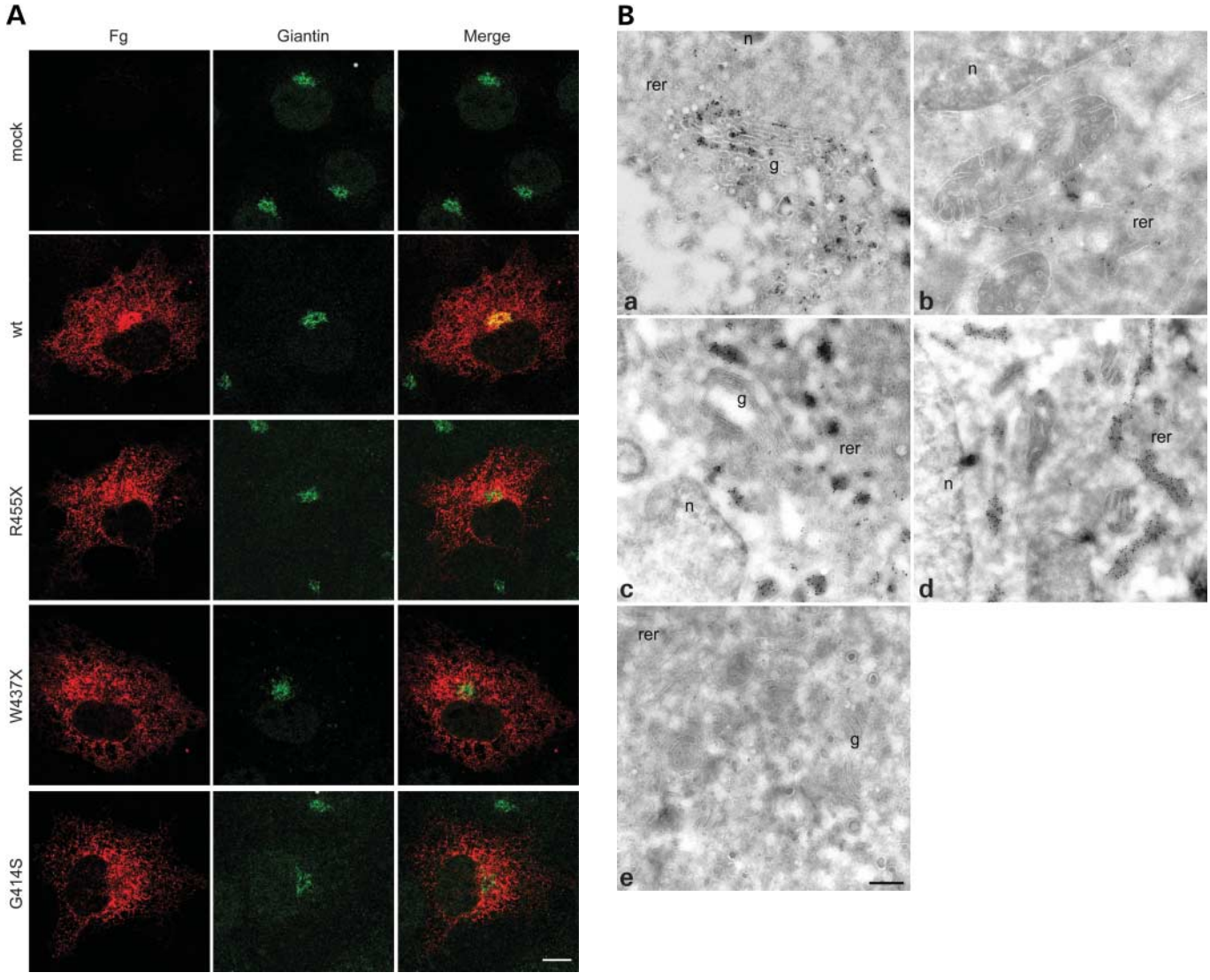

Figure 3. Secretion-impaired fibrinogen mutants are not found in the Golgi. (**A**) COS-7 cells co-transfected with either an empty vector or normal *FGA*, *FGG*, *FGB* or *FGA*, *FGG* and a mutant *FGB* (R455X, W437X or G414S), were fixed, double-labelled with anti-fibrinogen (red) and anti-giantin (green) antibodies and visualized by confocal microscopy. The images are representative of three independent experiments. Bar, 10 μm. (**B**) Immunolabelling of thin cryosections of transfected COS-7 cells revealed WT fibrinogen in the Golgi apparatus (g) and rough ER (rer, a and b). In contrast, the R455X mutant was not detected in the Golgi (c), but was abundant in the ER (c and d). Omission of the antibody against fibrinogen suppressed the aforementioned labelling (e), showing its specificity. n, nucleus; bar, 300 nm.

five-stranded β-sheet (Fig. 5A), the last five residues form a highly flexible tail, and P456 ensures the transition between the central β-sheet and the tail. The R455 position is stabilized by six hydrogen bonds with three neighbours: F457, E309 and G247 (Fig. 5B). Substitution of R455 by alanine disrupts at least the hydrogen bonds formed by the arginine side chain, i.e. one with E309 and two with G247. Under this condition, secretion of fibrinogen was unaffected, indicating that these bonds are not critical for this process. In the PDB structure 1FZA (3), R455, together with D241, E245, E309, E313, H325 and K453, forms a closed sphere of mainly charged amino acids, which presumably hinder solvent access to W249, and thus to the hydrophobic core (Fig. 5C and D). We postulate that the R455A substitution is of no consequence for aqueous access to the core when the flexible C-terminal tail is present. On mutation of R455 to alanine (Fig. 5E), one building block of this protective barrier is replaced by a small, hydrophobic residue. Because hydrophobic amino acids in water minimize their surface by aggregating together, we further hypothesize that the flexible tail collapses toward A455 and that the terminal glutamines form the interface to the solvent. In the absence of the tail, the R455A substitution exposes the predominantly hydrophobic core to solvent, which most likely leads to destabilization of the globular βC domain (Fig. 5F). Accordingly, removal of the C-terminal tail as well as R455 would further expose the core (Fig. 5G). These data, together with the reported mutations in the βC region, strongly suggest that fibrinogen secretion requires a proper folding of the βC domain and is sensitive to subtle variations in the distribution of hydrophobic and hydrophilic residues.

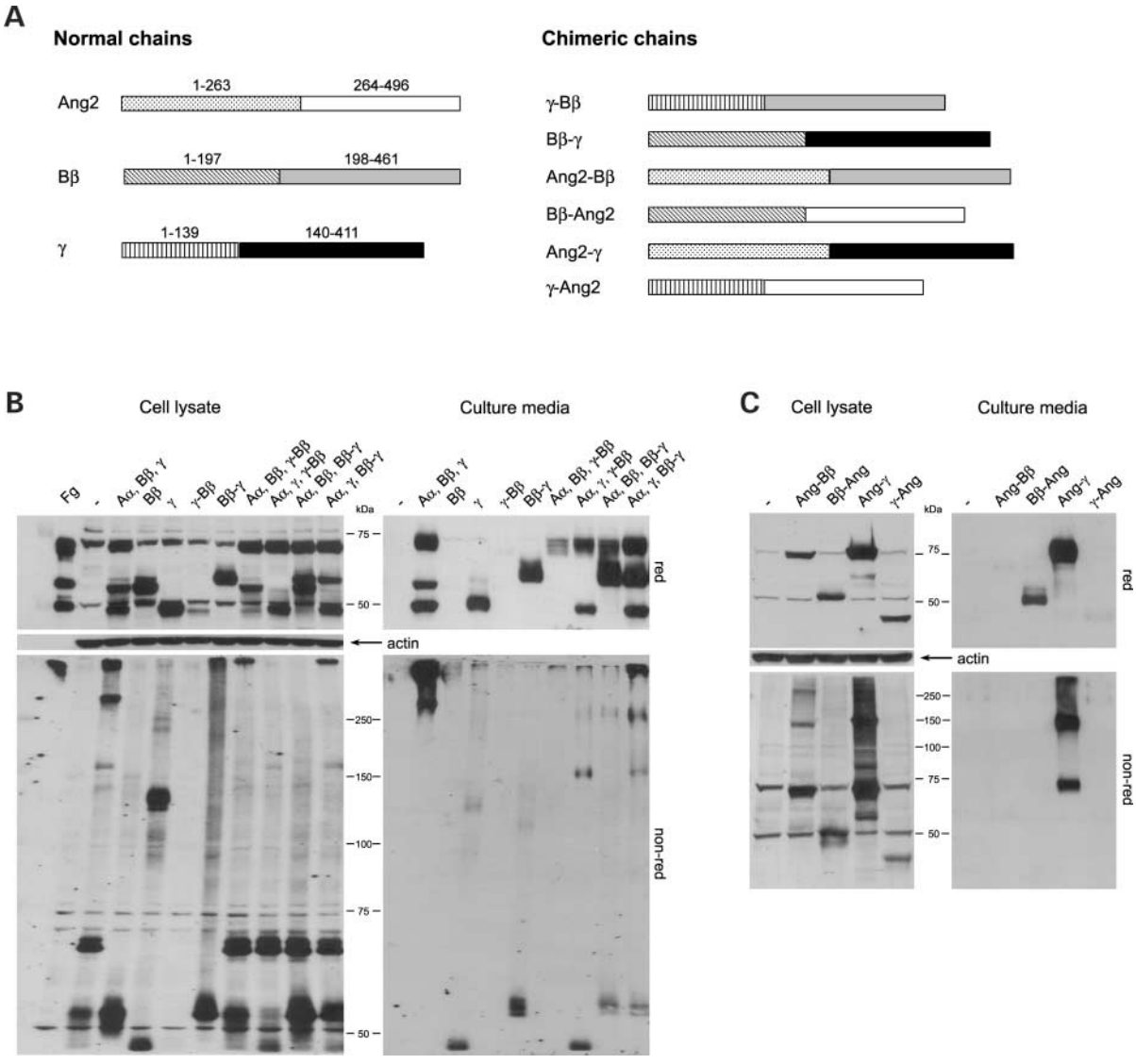

Figure 4. Expression of chimeric chains reveals a limiting role of the βC domain, not of the Bβ N-terminus, and a positive role of the γC domain in secretion. (**A**) Schematic representation of normal and chimeric chains combining Bβ, γ, or Ang2 half-proteins. Each segment is labelled by the number of the first and last forming residue. (**B** and **C**) Western blots on cell lysates or conditioned medium were performed under the conditions indicated as in Figure 1. COS-7 cells were transfected with Aα, Bβ, γ, Ang2 and/or chimeric cDNAs.

Previous studies in COS or HepG2 cells have attempted to follow the transit of WT or mutant fibrinogen molecules along the secretory pathway, using endoglycosidase H (endo H) treatment (32–36). Endo H cleaves the high mannose form of N-linked glycoproteins, found in the ER and the *cis*-Golgi. In the *medial*-Golgi, proteins acquire a complex oligosaccharide form, rendering them endo H-resistant. These studies failed to detect endo H-resistant fibrinogen molecules in cells, although one would expect to find some fibrinogen in the Golgi. This may be explained by the fact that intracellular fibrinogen molecules are predominantly in the ER, as observed in our study (Fig. 3A) and that endo H assays are not sensitive enough to detect the few high mannose-containing fibrinogen forms. Only secreted fibrinogen molecules in cell media were found to be endo H resistant. Here, by using immuno-electron microsocopy, we were able to detect WT fibrinogen in the ER and the Golgi and demonstrate that the three secretion-impaired mutants we analysed, R455X, W437X (15) and G414S (14), failed to be targeted to the Golgi compartment. Misfolding of many membrane or secreted proteins, such as CFTR or α1-antitrypsin, leads to their retention in the ER and to human disease (reviewed in 37). Our results suggest that fibrinogen secretion-impaired mutations lead to similar retention, thereby causing the disease.

In an effort to specifically characterize the role of the Bβ and γ FreDs on fibrinogen secretion, we studied chimeric chains combining the N- and the C-termini of Bβ and γ chains or Ang2. As summarized in Table 1, all chains (Bβ, γ–Bβ, Ang2–Bβ) and complexes [AαBβγ–Bβ] containing

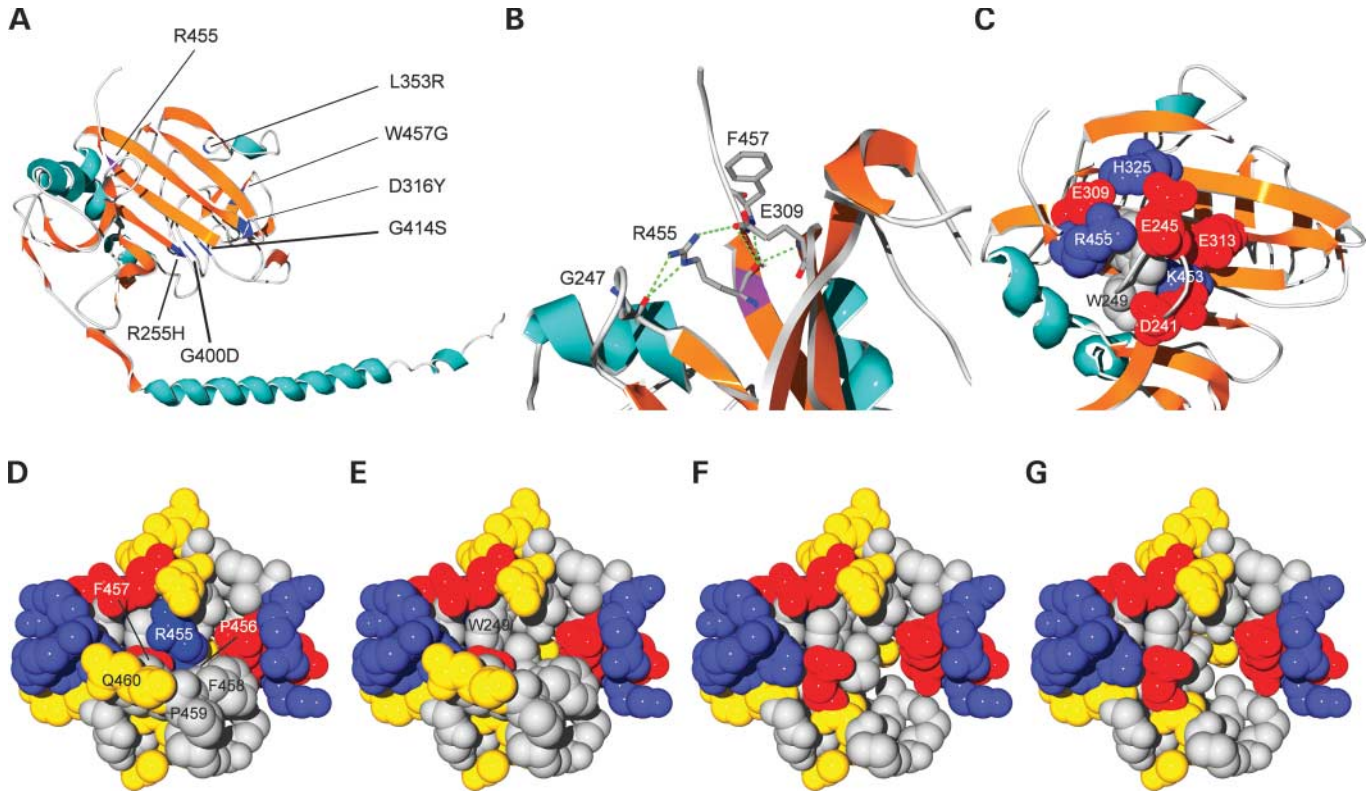

Figure 5. Structural analysis of the βC domain suggests that the six-residue tail and R455 prevent exposure of the βC core. (**A**) Localization of R455 (pink) and of the missense mutations so far reported in Bβ (blue). (**B**) Zoom on the R455 residue and its hydrogen bonds (green) shared with neighbouring amino acids. (**C**) View on the charged sphere surrounding the non-polar W249. (**D**–**G**) Amino acids within a 10 Å radius of R455 in the (D) WT, (E) R455A, (F) R455A + P456X and (G) R455X context. The secondary structures are highlighted: helix in green, β-strand in orange. For atom representation, acid residues were coloured in red, basic in blue, polar in yellow and non-polar in grey. Figures were prepared using Swiss-PdbViewer and POV-Ray from the PDB file 1FZA (3).

Table 1. Secretion of Ang2, fibrinogen and chimeric proteins

Protein	Secretion
Aα	+
Bβ	−
γ	+++
$[A\alpha B\beta\gamma]_2$	+++
γ–Bβ	−
Bβ–γ	+++
[AαBβγ–Bβ]	−
[AαBβ–γ]	++
Ang2–Bβ	−
Bβ–Ang2	++
Ang2–γ	+++
γ–Ang2	+

Chains and complexes were detected in culture medium with anti-fibrinogen antibodies. −, undetected; +, barely detected; ++, detected; +++, highly detected.

the βC domain failed to be secreted, with the sole exception of the native fibrinogen hexamer, suggesting that the Bβ FReD requires a suitable protein environment to be secreted. In contrast, chimeric chains or complexes with Bβ N-terminus fused to γ or Ang2 FReD were secreted, demonstrating that the Bβ N-terminus does not limit secretion. Finally, γC-containing chains (γ, Bβ–γ, Ang2–γ) and complexes [AαγBβ–γ] were abundantly secreted, whereas γC-lacking fibrinogen molecules [AαBβγ–Bβ] were retained within cells, revealing a positive, if not essential role of αC in secretion. These results are consistent with those of a study on chimeras combining the N-terminus of the *Drosophila* FReD-containing scabrous protein and human fibrinogen C-terminus (38). In this case, scabrous-α_E and -γ were secreted, whereas scabrous-Bβ was arrested in the secretory pathway. Moreover, several studies on a variety of cells (including transfected COS, CHO and BHK cells or Hep3B and HepG2 hepatoma cells) have shown that single Aα and γ chains and [Aαγ] intermediates can be found in culture medium (8,15,33,39). In all the cases, Bβ chains were detected in conditioned media only when incorporated in the full hexamer. Our present results extend these observations by indicating that the Bβ chain cannot be secreted on its own, unlike Aα and γ chains, because of the limiting action of the βC domain.

In summary, our data show that the βC domain, not the Bβ N-terminus, limits secretion because it can be secreted only if properly folded and incorporated in a suitable protein environment, provided by the two other fibrinogen chains. In contrast, the γ chain, specifically the γC domain, favours secretion. Obviously, these conclusions, which derive from experiments on COS-7 cells, may not be immediately extended to the endogenous mechanisms of *in vivo* fibrinogen secretion by hepatocytes. However, they are likely to summarize the

Table 2. Oligonucleotide primers used for site-directed mutagenesis.

Template	Mutation	Oligonucleotide sequence (5′ → 3′)
Bβ	R440X	GTAGTATGGATGAAT**TAG**AAGGGGTCATGG
Bβ	Y445X	GGAAGGGGTCATGG**TAA**TCAATGAGGAAG
Bβ	K449X	GGTACTCAATGAGG**TAG**ATGAGTATGAAGATC
Bβ	K453X	GGAAGATGAGTATG**TAG**ATCAGGCCCTTC
Bβ	I454X	GATGAGTATGAAG**TAG**AGGCCCTTCTTCCC
Bβ	R455X	GAGTATGAAGATC**TGA**CCCTTCTTCCCAC
Bβ	P456X	GTATGAAGATCAGG**TAG**TTCTTCCCACAGC
Bβ	F457X	GAAGATCAGGCCC**TAG**TTCCCACAGCAATAG
Bβ	R455A	GAGTATGAAGATC**GCG**CCCTTCTTCCCAC
Bβ	R455A + P456X	GTATGAAGATCGCG**TAG**TTCTTCCCACAGC
Bβ	P456_F458$(A)_3$	GAGTATGAAGATCAGG**GCCGCCGCC**CCACAGCAATAGAAGG
Bβ + P456_F458$(A)_3$	P456_Q461$(A)_6$	GATCAGGGCCGCCGCC**GCAGCGGCA**TAGAAGGGCAATTCTG
Bβ	*Bsp* EI site	GGAATATTGTCGCACCCCA**TCCGGA**GTCAGTTGCAATATTCC
γ	*Bsp* EI site	CAGTGCCAGGAACCT**TCCGGA**GACACGGTGCAAATC
Ang2	*Bsp* EI site	CTGACTATGATG**TCCGGA**TCAAACTCAGCTAAG
γ-Bβ	C135S	GCTTGAAGCACAG**AGC**CAGGAACCTTCC
Bβ-γ	C193S	CTCAAATGGAATAT**AGT**CGCACCCCATCC

List of forward primers used in this study for site-directed mutagenesis. Mutated codons are shown in bold. W437X and G414S mutations were generated as previously described (14,15).

central features of this control, as findings consistent with ours have been made in various cell models or organisms.

Previous co-expression studies in COS-1 cells have shown that Bβ chains deleted of their C-terminal half (amino acids 208–461) could be secreted on their own and assembled with Aα and γ chains to form a secreted hexamer (40). Together with our data, this observation favours a model in which molecular chaperones recognize and retain, in a pre-Golgi compartment, the fibrinogen molecules containing Bβ chains with misfolded βC domains. Hence, Bβ chains that lack the globular region may escape the quality control machinery. Moreover, it is conceivable that some proteins bind to the γC domain to favour its own secretion and that of the fibrinogen hexamer. In this regard, two secretion-impaired mutations recently identified in the γ chain, G284R (32,41) and W227C (42), might interfere with the positive role of γC on secretion demonstrated here.

Interestingly, four chemically induced mutations (two of these truncations in the FReD, two others uncharacterized) in the scabrous protein have been found to alter the intracellular location of the molecule (43). The WT protein was found in intracellular vesicles that underwent exocytosis, whereas the mutants were retained within the cell in a perinuclear location, possibly corresponding to the ER. All these data concur to indicate that structural defects in the FReDs limit the secretion, and especially exit from the ER, of several proteins, in particular, fibrinogen.

In conclusion, our data provide new insight into the mechanisms accounting for the quality control of fibrinogen secretion and demonstrate that mutant fibrinogen retention is one of the pathological mechanisms responsible for congenital afibrinogenemia.

MATERIAL AND METHODS

Plasmid constructs and site-directed mutagenesis

Human *Ang2* cDNA was amplified by PCR from cDNAs of HUVEC cells, using forward 5′-GCTGCTGGTTTATTACT GAAGAA-3′ and reverse 5′-TCAGGTGGACTGGGATGTT TAG-3′ oligonucleotides, and cloned into the pcDNA3.1/V5-His-TOPO expression vector (Invitrogen), as were *FGA*, *FGB* and *FGG* cDNAs (15). Insertion of mutations was performed using the QuickChange Mutagenesis Kit (Stratagene), with primers listed in Table 2. Throughout the text, mutations and amino acids are numbered from the mature protein, i.e. without the peptide signal. Addition of 19, 30 and 26 residues corresponding to the signal peptides will yield the corresponding amino acid number in the complete Aα, Bβ and γ chains, respectively. For chimera generation, a *Bsp* EI restriction site was inserted in *FGB*, *FGG* and *Ang2* cDNAs. Then, for a given X–Y chimera, the Y cDNA N-terminal part was removed with *Kpn* I/*Bsp* EI digestion and gel purification. The X cDNA N-terminal fragment was excised with *Kpn* I/*Bsp* EI restriction and inserted in-frame into the digested Y construct. All constructs had intact FReD domains (amino acids 207–457, 149–389 and 280–494 for Bβ, γ and Ang2, respectively; http://www.sanger.ac.uk/Software/Pfam/) and fibrinogen intra-disulphide bonds and disrupted fibrinogen C-terminal inter-disulphide bonds. The remaining free cysteines in γ-Bβ (γ C135) and Bβ–γ (Bβ C193) were mutagenized into serine residues. All PCR-based amplified and mutated cDNAs were validated by sequencing.

Cell culture and transfection

COS-7 cells were grown in a humidified incubator at 37°C and 5% CO_2 in DMEM supplemented with 10% fetal serum and penicillin/streptomycin. Cells were transiently transfected with FuGENE6 reagent (Roche) according to the manufacturer's instructions.

Western blot analysis

Cells and culture media were harvested, and western blots were performed as previously described with minor modifications

(44). One day after transfection, cells were washed twice with PBS and incubated for 24 h in serum-free medium. Conditioned medium was concentrated using Amicon Ultra, 4 5 kDa (Millipore). Western blot analysis was performed using polyclonal rabbit anti-human fibrinogen antibodies (DakoCytomation) or mouse anti-actin IgG (Chemicon).

Immunofluorescence

Twenty-four hours after transfection, cells were fixed with 4% PFA, permeabilized with 0.1% Triton X-100, blocked with 1% bovine serum albumin (BSA), incubated for 1 h with primary antibodies (anti-fibrinogen, 1:2000; mouse anti-giantin, 1:400, Calbiochem) in 0.1% BSA and then for 45 min with goat anti-rabbit and anti-mouse IgG conjugated to Alexa Fluor 568 and 488 (1:500, Molecular Probes), respectively. Images were captured at room temperature with a LSM510 laser scanning confocal microscope (Carl Zeiss), using a Plan-Apochromat $63\times/1.4$ oil objective. Three independent experiments were performed.

Immuno-electron microscopy

Transfected cells were fixed for 5 min at room temperature in either 4% PFA or 4% PFA plus 0.1% glutaraldehyde, followed by a 60 min fixation in 4% PFA (all fixatives diluted in 0.1 M phosphate buffer, pH 7.4). Cells were washed in 0.1 M phosphate buffer, embedded in 12% gelatin and cooled on ice. Small blocs of cells were infused with 2.3 M sucrose, frozen in liquid nitrogen and sectioned with an EMFCS cryoultramicrotome (Leica). Ultrathin sections were mounted on parlodion-coated copper grids. The sections were processed as in previously described protocols (45,46) which, in these experiments, included a 1 h exposure to anti-fibrinogen antibodies (1:100), and a 20 min exposure to 10 nm gold-conjugated goat anti-rabbit IgG (1:10, BBInternational). Cryosections were screened and photographed in a CM10 electron microscope (Philips). Negative controls, run by exposing the sections to only the gold-conjugated goat antibodies, resulted in a minimal, inconsistent staining of the cells (Fig. 3Be). About 20 transfected cells, immunostained above the minimal background labelling, were examined in each group.

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