Manipulating the quality control pathway in transfected cells: low temperature allows rescue of secretion-defective fibrinogen mutants

Dung Vu,[1] Corinne Di Sanza,[1] and Marguerite Neerman-Arbez[1,2]

[1]Department of Genetic Medicine and Development, University Medical Center, Geneva; [2]Division of Angiology and Hemostasis, University Hospital, Geneva, Switzerland

ABSTRACT

Background
Congenital afibrinogenemia is characterized by the absence of fibrinogen, a hexamer composed of two copies of three polypeptides, Aα, Bβ and γ. The disease is caused by mutations in one of the three fibrinogen-encoding genes, *FGA*, *FGB* and *FGG*. Among these, several mutations have been reported to specifically impair fibrinogen secretion. We previously showed that secretion-defective fibrinogen mutants are retained in a pre-Golgi compartment and demonstrated the importance of the homologous βC and γC domains in secretion. Here our aim was to restore the secretion of these mutants and study the properties of the rescued mutant molecules.

Design and Methods
COS-7 cells were transfected and incubated with chemical chaperones or at low temperature. Clotting assays and plasmin digestion studies were performed to characterize secreted fibrinogen molecules.

Results
The secretion defect of two missense mutants but not that of late-truncating mutants could be partially corrected by incubating cells at 27°C. By contrast, exposure of cells to chemical chaperones i.e. 4-phenylbutyrate, dimethyl sulfoxide or trimethylamine N-oxide had no effect. The mutants rescued at 27°C were incorporated into fibrin clots and formed factor XIII-mediated γ-γ dimers in contrast to the dysfibrinogenemia Vlissingen/Frankfurt IV mutant, a negative control for these assays. However, plasmin digestion analyses revealed aberrant patterns for the mutants compared to normal fibrinogen.

Conclusions
Low temperature can restore the secretion of a subset of mutant fibrinogen molecules demonstrating that therapeutic manipulation of the quality control pathway is feasible for afibrinogenemia even though functional assays suggested a non-native conformation for the mutant molecules analyzed.

Key words: fibrinogen, afibrinogenemia, secretion, chemical chaperones, protein quality control, fibrinogen-related domain

Citation: Vu D, Di Sanza C, and Neerman-Arbez M. Manipulating the quality control pathway in transfected cells: low temperature allows rescue of secretion-defective fibrinogen mutants Haematologica 2008 Feb; 93(2):224-231 DOI: 10.3324/haematol.11868

Acknowledgments: we are grateful to Prof. Martin Fussenegger for the generous gift of the pTRIDENT2 vector, Prof. David H. Farrell for helpful technical advice and Prof. Philippe de Moerloose for fruitful discussions and the supply of human plasma samples.

Funding: this study was supported by grants #631-066023 and #PP00B-110429 from the Swiss National Science Foundation.

Manuscript received June 21, 2007. Manuscript accepted November 28, 2007.

Correspondence: Marguerite Neerman-Arbez, 1 rue Michel Servet, CH-1211 Geneva, Switzerland. E-mail: marguerite.arbez@medecine.unige.ch

Introduction

Congenital afibrinogenemia (OMIM #202400) is a rare bleeding disorder characterized by the complete absence of fibrinogen, the precursor of fibrin, which is the major protein component of blood clots. Affected patients suffer from various hemorrhagic manifestations, bleeding from the umbilical cord often being the first sign of the disorder.[1] Other symptoms manifest with variable severity and include oral, musculoskeletal and intracerebral bleeding, hemarthrosis, epistaxis, menorrhagia and recurrent early abortions. Patients may, however, also experience thrombotic events. Current therapy for afibrinogenemia is based on fibrinogen replacement, usually in the form of cryoprecipitate or fibrinogen concentrates. Afibrinogenemia is caused by mutations, in homozygosity or compound heterozygosity, in one of the three fibrinogen-encoding genes, i.e. *FGA, FGB* and *FGG*, clustered on human chromosome 4 (4q32.1). Heterozygosity for any of these mutations can cause hypofibrinogenemia, characterized by plasma fibrinogen levels below 1.5 g/L, which are found mostly in asymptomatic patients.

Circulating fibrinogen is mainly synthesized in hepatocytes. It is a symmetrical molecule composed of two sets of three polypeptide chains, Aα, Bβ, and γ, which assemble to form a 340-kDa hexamer $[A\alpha B\beta\gamma]_2$, held together by 29 disulfide bonds. The N-terminal ends of all six chains are folded in the central domain and connected to the two peripheral globular domains by two three-stranded coiled coils. Plasmin proteolysis of fibrinogen occurs in the coiled-coil connectors, notably generating a small fragment E, corresponding to the central domain, and two fragments D, corresponding to the lateral regions, formed by the globular C-termini of the Bβ (βC) and γ chains (γC) and by a portion of the coiled-coils. The βC and γC nodules are homologous and are members of the FReD (fibrinogen-related domain) family.[2] Since our identification of the first causative mutation for congenital afibrinogenemia,[3] more than 70 mutations causing afibrinogenemia or hypofibrinogenemia have been described, all located in the fibrinogen gene cluster.[4,5] The majority of these mutations are null, i.e. large deletions, frameshift, early-truncating nonsense, or splice-site mutations. Several causative missense or late-truncating nonsense mutations in the βC domain or γC domain have been shown to specifically impair fibrinogen secretion. All are localized in the βC domain: p.L383R (or p.L353R according to the mature form of the chain), p.G430D (p.G400D),[6] p.G444S (p.G414S),[7] p.G464D (p.G434D),[8] p.W467X (p.W437X),[9] p.W467G (p.W437G),[10] p.W470X (p.W440X);[11] or in the γC domain: p.W253C (p.W227C),[12] p.G310R (p.G284R).[13] Indeed, fully assembled hexamers were detected intracellularly in co-transfected COS-1 or COS-7 cells, but were not observed in culture media.

Although mutagenesis of specific residues had shown that the disruption of several inter-[14] or intra-chain[15] disulfide bonds specifically impaired secretion, the molecular mechanisms underlying the process of fibrinogen secretion were not very well characterized until recently. In a transfected COS-7 cell model, we demonstrated that deletion of the last six residues from the Bβ C-terminus had no effect on fibrinogen assembly or secretion, but that removal of seven amino acids from residue R485 (or R455) or more (up to 25 amino acids) abolished secretion. Expression of chimeric proteins combining the FReD of fibrinogen Bβ, γ, and angiopoietin-2 with the N-terminus of one or the other protein demonstrated that the γC domain plays a limiting role in secretion of fibrinogen molecules while the βC domain appears to favor their secretion. Finally, immunofluorescence and immuno-electron microscopy studies indicated that secretion-defective mutants are retained in a pre-Golgi compartment.[11] Therapeutic approaches to rescue the secretion of mutant molecules in protein misfolding diseases based on pharmacological chaperones are promising.[16-18] In this context, chemical chaperones and temperature lowering are often used *in vitro* as a first step to investigate the feasibility of such a therapy. Here, we investigate whether this approach is applicable to a selection of secretion-impaired fibrinogen mutants identified in patients with afibrinogenemia or hypofibrinogenemia.

Design and Methods

Plasmid construct and site-directed mutagenesis

In order to increase the efficiency of transfection in COS-7 cells, we cloned the human *FGB*, *FGA* and *FGG* cDNA (5'→3') in the tricistronic mammalian expression pTRIDENT2 plasmid. Expression of the three cistrons, separated by two internal ribosome entry site (IRES) sequences, is driven by a single SV40 promoter.[19] To allow cloning in this vector, site-directed mutagenesis was performed on the *FGA*-containing pcDNA3.1/V5-His TOPO vector construct[9] in order to insert a *Sac*II restriction site upstream of the cDNA region, using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) with the forward oligonucleotide (the restriction site is underlined): 5'-GCTTGGTACCGCGGTCGGATCCAC-TAG-3'. The *FGB*, *FGA* and *FGG* cDNA were excised from the pcDNA3.1/V5-His TOPO plasmid constructs by digestion with *Sac*I/*Pst*I/*Avr*II (*Avr*II was used in order to eliminate a contaminating fragment with the same size as the fragment to be excised), *Sac*II, and *Spe*I/*Xho*I respectively. The *FGB* fragment was ligated into the pTRIDENT2 vector previously cut with *Sac*I/*Pst*I. The resulting construct was linearized with *Swa*I restriction enzyme and ligated with the *Spe*I/*Xho*I-digested and blunted *FGG* fragment. Finally, the construct was linearized with *Sac*II and ligated with the *Sac*II-cut *FGA* fragment. The final construct containing the three human fibrinogen cDNA was verified by restriction analyses and sequencing of the three inserts.

The *FGB* p.W467X (p.W437X), FGB p.G444S (p.G414S), FGG p.W253C (p.W227C) or FGG p.N345_D346del

(p.N319_D320del) mutation was introduced in the tricistronic fibrinogen-containing plasmid with the forward oligonucleotides as follows (introduced mutations are underlined): 5'-GATGGTGTAGTATAGATGAATTGGAAGGGG-3' for *FGB* p.W467X (p.W437X), 5'-GCAGCCAATCCAAACAGCAGATACTACTGGGG-3' for *FGB* p.G444S (p.G414S), 5'-GCACAACAGAATTTTGTCTGGGAAATGAGAAG-3' for *FGG* p.W253C (p.W227C), and 5'-CCTGGGACAATGACAAGTTTGAAGGCAAC-3' for *FGG* p.N345_D346del (p.N319_D320del) (6-nucleotide deletion between the two underlined nucleotides). The resulting constructs were checked by sequencing all three fibrinogen cDNA.

Higher fibrinogen expression was obtained with single transfection of tricistronic constructs in comparison with triple co-transfections, but with similar results as regards protein assembly and secretion (*data not shown*). In this study single transfections with tricistronic constructs were performed for expression of the γ p.N345_D346del (p.N319_D320del) mutant and all clotting and plasmin digestion assays while triple co-transfections were performed for cell treatment with chemical chaperones or low temperature incubation (see online supplement for further details).

Results

Rescue of secretion-defective mutants

To investigate whether the secretion of the fibrinogen *FGB* p.G444S (p.G414S),[7] *FGB* p.W467X (p.W437X)[9] and *FGG* p.W253C (p.W227C)[12] mutants can be rescued, we co-transfected COS-7 cells with empty vectors, the three normal fibrinogen cDNA constructs, or the mutant *FGB* G444S, *FGB* W467X, *FGG* W253C cDNA (residues numbered from the initiator codon) combined with the other two corresponding normal cDNA. Cells were exposed to one of the three common chemical chaperones with concentrations found to be successful in other studies (10 mM 4-phenylbutyrate (4-PBA), 2% dimethylsulfoxide (DMSO), and 100 mM trimethylamine N-oxide (TMAO).[18,23,24] Cells were also incubated at a temperature of 27°C. Two days after transfection, cell extracts and culture media were harvested and analyzed by western blotting.

Figure 1 shows that in all conditions wild-type fibrinogen chains were expressed and assembled into hexameric molecules which were secreted into the culture medium. In non-treated cells, the three mutants Bβ p.G444S (p.G414S), Bβ p.W467X (p.W437X) and γ p.W253C (p.W227C) were

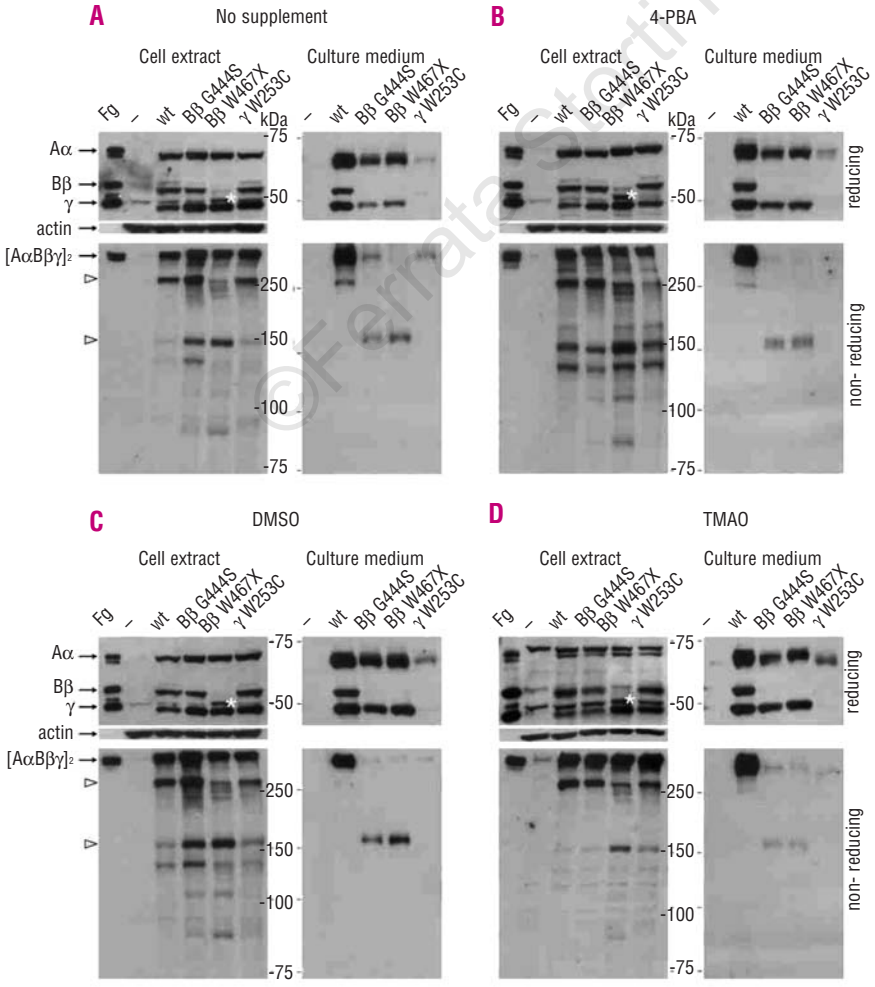

Figure 1. Treatment of transfected COS-7 cells with chemical chaperones. COS-7 cells were co-transfected with mock (-), normal (wt) or mutant fibrinogen, and exposed either to (A) no compound, or to the chemical chaperones (B) 4-PBA, (C) DMSO or (D) TMAO. Proteins were harvested and separated on 9% or 7% SDS-PAGE under reducing or non-reducing conditions, respectively, and immunoblotted with anti-fibrinogen antibodies. The positions of Aα, Bβ, γ chains and hexamers are shown. Open arrowheads indicate fibrinogen intermediates, presumably [Aα, Bβγ] half-molecules and [Aα, γ] complexes.[42] The loading control was obtained using anti-actin antibodies. Fg: purified human plasma fibrinogen. Asterisks indicate truncated Bβ p.W467X (p.W437X) chains.

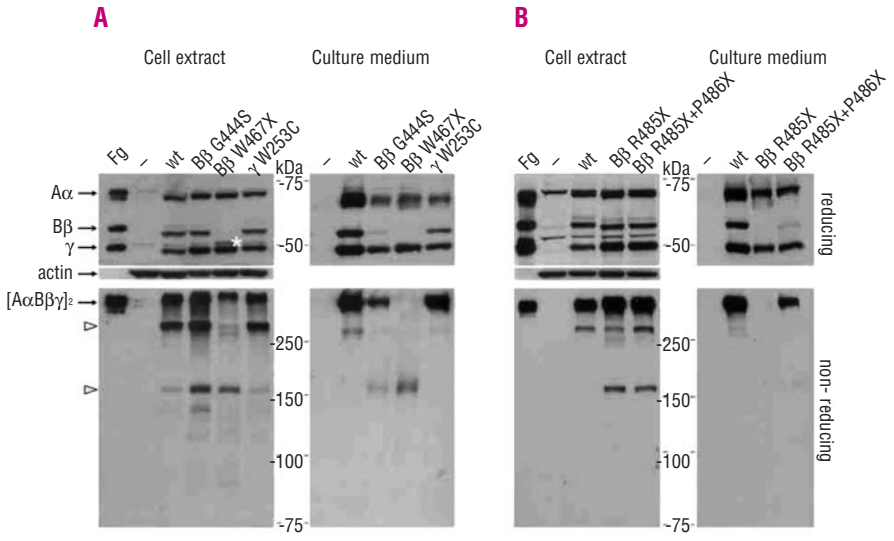

Figure 2. Rescue at 27°C of secretion-defective fibrinogen mutants, either (A) naturally occurring in patients, or (B) experimentally designed.[11] Samples were resolved on 9% or 7% SDS-PAGE under reducing or non-reducing conditions, respectively, and immunoblotted with anti-fibrinogen antibodies. The positions of Aβ, Bβ, γ chains and hexamers are shown. Open arrowheads indicate fibrinogen intermediates, presumably [AαBβγ] half-molecules and [Aαγ] complexes.[42] Loading was controlled by immunoblotting using anti-actin antibodies. Fg: purified human plasma fibrinogen. An asterisk indicates truncated Bβ p.W467X (p.W437X) chains.

also expressed and incorporated into hexamers (Figure 1A, cell extract), but these were not secreted (Figure 1A, culture medium), as previously shown.[7,9,12] Upon exposure of cells to the chemical chaperones 4-PBA, DMSO and TMAO, expression and assembly of the mutants was unaltered (Figure 1B-D, cell extract), but secretion was not restored (Figure 1B-D, culture medium). By contrast, when cells were incubated at 27°C, the two missense mutants i.e. Bβ p.G444S (p.G414S) and γ p.W253C (p.W227C), but not the nonsense mutant Bβ p.W467X (p.W437X), were secreted, albeit to a lesser extent than wild-type fibrinogen (Figure 2A culture medium). We also investigated whether two secretion-defective mutants in *FGB* designed for our previous study[11] were secreted at low temperature: interestingly, the secretion of the Bβ p.R485X (p.R455X) mutant was not restored, while that of the Bβ p.R485A+P486X (p.R455A+P456X) mutant, differing from the former only by an additional alanine in the C-terminus, was partially restored (Figure 2B, culture medium).

Secretion of the dysfibrinogenemia Vlissingen/Frankfurt IV/Otsu mutant

As a negative control for the functional analyses that we wished to perform on the rescued mutant molecules i.e. polymerization and conformation, we used the dysfibrinogenemia γ mutant Vlissingen/Frankfurt IV/Otsu. This mutant was identified in heterozygosity in two unrelated families and is characterized by the deletion of two residues, p.N345_D346del (p.N319_D320del), in the γ chain.[25-27] Studies with purified patient plasma comprising a mixture of normal and mutant fibrinogen showed impaired calcium binding, fibrin polymerization, factor XIII (FXIII)-catalyzed cross-linking and platelet adhesion.[25,26,28,29] Other studies with pure recombinant γ p.N345_D346del (p.N319_D320del) fibrinogen expressed from CHO cells confirmed these observations, suggesting that the two-residue deletion affects the structural integrity of the C-terminal domain of γ chains.[28,30] Here we expressed the γ

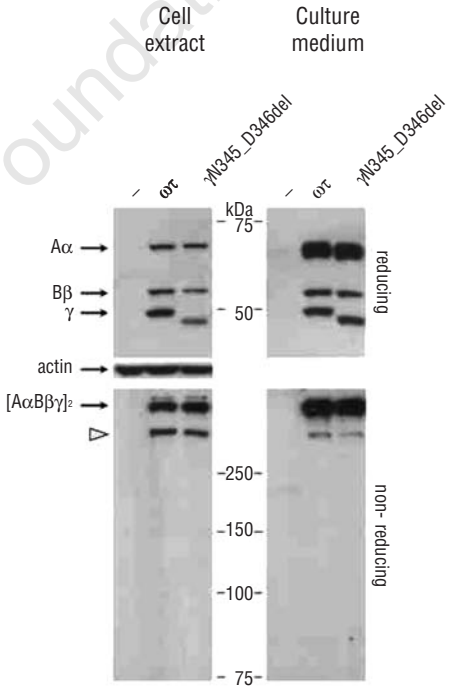

Figure 3. Expression, assembly and secretion of the γ p.N345_D346del (p.N319_D320del) fibrinogen mutant. COS-7 cells were transfected with empty vectors (-), normal (wt) or γ p.N345_D346del (p.N319_D320del) mutant fibrinogen construct. Proteins of cell lysates and media were harvested and separated on 9% or 7% SDS-PAGE under reducing or non-reducing conditions. Immunoblots were carried out with anti-fibrinogen antibodies, and loading was controlled with anti-actin antibodies. The positions of Aα, Bβ, γ chains and hexamers are shown. The open arrowhead indicates probable [AβBβγ] half-molecules.[42]

p.N345_D346del (p.N319_D320del) mutant in COS-7 cells. Western blot analysis shows that it is assembled into hexamers and secreted to the same extent as normal fibrinogen at 37°C (Figure 3) and 27°C (*data not shown*), demonstrating the suitability of this mutant as a negative control for the functional studies described below.

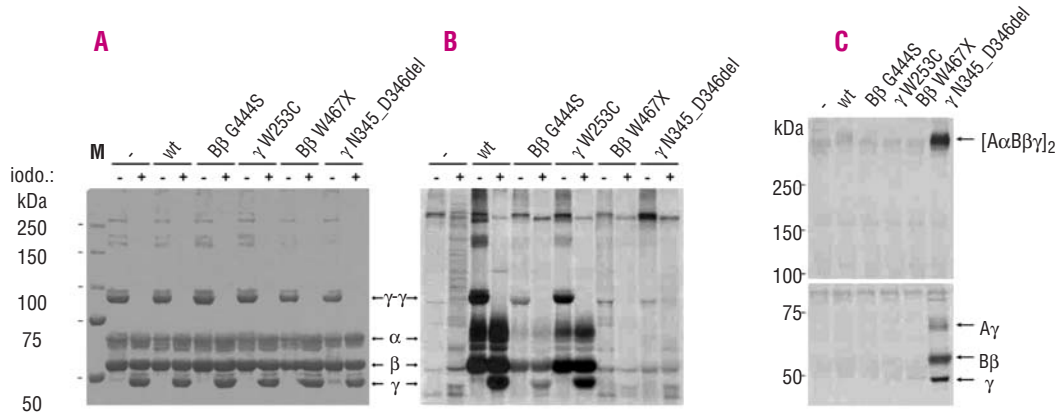

Figure 4. Clotting assays. Cells were transfected with mock (-), normal fibrinogen (wt), or mutant cDNA constructs, radiolabeled and incubated at 27°C to allow secretion of the Bβ p.G444S (p.G414S) and γ p.W253C (p.W227C) variants. Plasma was added to the harvested media and clotting assays were performed for 30 min at 37°C. Clots were resolved by 7% SDS-PAGE under reducing conditions and visualized by (A) Coomassie blue staining and (B) autoradiography. (C) Immunoprecipitation of fibrin(ogen) molecules remaining in the clot supernatant. Samples separated under non-reducing conditions on 5% SDS-PAGE (A and B) or reducing conditions on 9% SDS-PAGE (C) were visualized by autoradiography. M indicates protein marker; - and + indicate absence or presence of the FXIII inhibitor iodoacetamide during clot formation. The positions of α, β, γ chains, γ-γ dimers and hexamers are indicated.

The rescued mutants can be incorporated into a clot and form γ-γ dimers

We wished to determine whether the two missense fibrinogen mutants Bβ p.G444S (p.G414S) and γ p.W253C (p.W227C) that are secreted at low temperature might be functional i.e. whether they are able to incorporate into a fibrin clot and form cross-links. To address this question, standard clinical tests (e.g. the Clauss method) or *in vitro* assays[30,31] which rely on high levels of secreted fibrinogen, cannot be applied to our transfected cell model, where we estimate that only approximately 50 ng/mL of recombinant fibrinogen is secreted into the culture medium. We, therefore, adapted a protocol that measures the incorporation of small amounts of radiolabeled secreted fibrinogen into a clot formed with normal plasma.[20-22] Normal or mutant recombinant fibrinogen was expressed in transfected COS-7 cells at 27°C, labeled with [^{35}S]-methionine/cysteine and incorporation into clots formed by normal human plasma assayed after incubation of samples for 30 min or 3 h at 37°C or 27°C (the results were the same for all conditions). Centrifuged clots were collected and cross-linked fibrinogen molecules were visualized by autoradiography after SDS-PAGE. The specificity of FXIII-catalyzed cross-linking was assessed by performing control experiments in the presence of iodoacetamide, a FXIII inhibitor. Any fibrinogen remaining in the supernatant after clot centrifugation was immunoprecipitated to estimate the amount of unincorporated molecules.

Equal amounts of normal plasma were added to all samples, as can be seen on the Coomassie-stained gel (Figure 4A). In the absence of iodoacetamide, clots were formed in all conditions as shown by the absence of γ monomers due to their cross-linking into γ-γ dimers. In the presence of iodoacetamide, no γ-γ dimers were detected, demonstrating that their formation specifically results from covalent FXIII-mediated cross-linking. Radiolabeled fibrinogen molecules secreted by transfected COS-7 cells which were integrated into the clot were visualized by autoradiography (Figure 4B). As expected, no radiolabeled fibrinogen was observed in the mock condition, while a large amount of clottable fibrinogen was produced from COS-7 cells transfected with normal constructs. Apparently all wild-type molecules were incorporated in the clot, since no fibrinogen was detectable in the supernatant (Figure 4C). This suggests that the COS-7 cell system allows expression and secretion of recombinant fibrinogen proteins that are functional. Similarly, both temperature-sensitive Bβ p.G444S (p.G414S) and γ p.W253C (p.W227C) mutants could incorporate into the clot and undergo covalent FXIII-catalyzed cross-linking to form γ-γ dimers (Figure 4B). While the intensity of their corresponding bands was lower than for normal fibrinogen this was not because of lesser efficiency of incorporation into clots, since no residual mutant fibrinogen molecules were observed in supernatants (Figure 4C). The difference in intensity most likely results from the fact that fewer mutant molecules than wild-type molecules are secreted at 27°C (Figure 2A). As expected, in the case of the Bβ p.W467X (p.W437X) mutant, for which secretion was not restored by temperature lowering (Figure 2A), radiolabeled fibrinogen was not found in either the clot or the supernatant (Figure 4B-C). Finally, the γ p.N345_D346del (p.N319_D320del) mutant used as a negative control did not enter into the clot (Figure 4B) but remained in the supernatant in a non-crosslinked form (Figure 4C), in accordance with previous analyses for this mutant.[25,26,28-30]

The rescued fibrinogen mutants show aberrant plasmin digestion profiles

Plasmin sequentially cleaves fibrinogen into four major groups of fragments named X, Y, D and E,[32,33] a cleavage which depends on the overall three-dimensional structure of fibrinogen.[34] We analyzed the kinetics of plasmin digests of wild-type, Bβ p.G444S (p.G414S), γ p.W253C

(p.W227C) or γ p.N345_D346del (p.N319_D320del) mutant fibrinogen molecules secreted by COS-7 cells at permissive temperature. As a control, human purified plasma fibrinogen was digested in parallel. Plasmin digestions were performed at 37°C (Figure 5) or at 27°C (*data not shown*) with similar results. Western blots in non-reducing conditions (Figure 5A) showed a standard pattern of plasmin digests for purified fibrinogen,[33] with the progressive degradation of full hexamers (t=0) into fragments X, Y, D (detected in our assays from 5 min of incubation with plasmin) and E (from 30 min). After 30 minutes, fragments D and E were predominant. Plasmin digestion of wild-type fibrinogen secreted by COS-7 was indistinguishable from that of plasma fibrinogen, demonstrating that recombinant fibrinogen produced by COS-7 cells has the same susceptibility to plasmin digestion as plasma fibrinogen. The γ p.N345_D346del (p.N319_D320del) mutant showed similar degradation products at 5 minutes of plasmin incubation while after 30 minutes additional bands below fragment D were detected and at 6 hours, partial degradation of fragments D1 into fragments D2 and D3 was clearly observed (Figure 5A) as previously reported.[20,25,30] As before, the signals obtained for the Bβ p.G444S (p.G414S) and γ p.W253C (p.W227C) mutants were weaker because of the smaller amount of molecules secreted at 27°C compared to wild-type or γ p.N345_D346del (p.N319_D320del) mutants. The Bβ G444S mutant showed aberrant degradation products after 5 minutes, migrating at around 70-75 kDa (Figure 5A). The γ p.W253C (p.W227C) mutant had a normal digestion pattern up to 30 minutes of incubation but at 6 hours, one aberrant doublet at approximately 60-65 kDa predominated.

Calcium[35] and the GPRP peptide[36] are known to protect the degradation of fibrinogen fragments D1 into D2 and D3. Assessing the protection from plasmin degradation with one or the other compound indicates whether fibrinogen molecules can bind calcium or have functional "a" polymerization sites, respectively. Indeed, the GPRP peptide mimics the N-terminal "A" site of α chains which are exposed after cleavage of fibrinopeptide A by thrombin. The "A" site interacts with the "a" site present in the outer γD domain of another molecule, allowing the alignment of fibrin monomers into two-stranded, half-staggered protofibrils.[37]

Normal recombinant fibrinogen secreted by COS-7 cells was protected to the same extent as plasma fibrinogen while the γ p.N345_D346del (p.N319_D320del) mutant showed reduced protection against plasmin digestion in

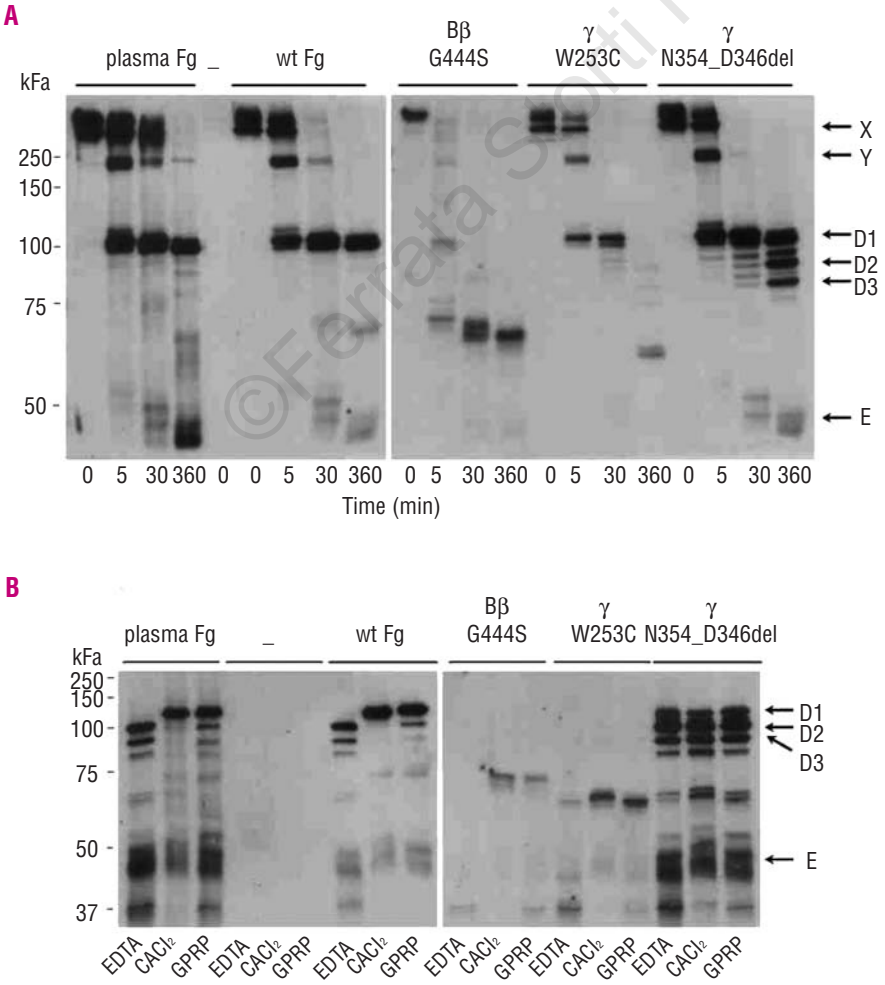

Figure 5. Plasmin digestion of fibrinogen. (A) Kinetics of plasmin digestion at 37°C of plasma fibrinogen or culture medium from non-transfected (–) or transfected COS-7 cells in the presence of approximately 0.9 mM $CaCl_2$ (due to the 1.8 mM $CaCl_2$ in DMEM) (B) Plasmin protection assay of plasma fibrinogen or culture medium from non-transfected (–) or transfected COS-7 cells in the presence of 10 mM EDTA, 5 mM $CaCl_2$, or 4 mM GPRP+10 mM EDTA. Positions of fragments X, Y, D1, D2, D3 and E are indicated.

the presence of calcium or GPRP, as shown by the increases of D2 and D3 in these conditions (Figure 5B). Smaller unidentified bands were also detected. Surprisingly, when EDTA was added, some protection remained, contrasting with the results for normal plasma and recombinant fibrinogen, as well as previous studies on the γ p.N345_D346del (p.N319_D320del) mutant.[26,30] However, partial protection was also observed for plasma fibrinogen of mice heterozygous for the mutation.[38] In the case of the Bβ p.G444S (p.G414S) and γ p.W253C (p.W227C) mutants, a clearly aberrant pattern of plasmin cleavage was observed. No fragments D could be found in either case. Only smaller bands were present suggesting further digestion beyond fragments D, although altered migration of mutant molecules during SDS-PAGE cannot be ruled out.

Discussion

Chemical chaperones and low growth temperature are often used as a first step to evaluate the feasibility of treatments using pharmacological chaperones for secretion-impaired mutations. In this context, the examples of the CFTR ΔF508 or α_1-antitrypsin Z variants responsible for cystic fibrosis and α_1-antitrypsin deficiency, respectively, are promising.[16–18] In our transfected cell model we found that none of the chemical chaperones used (4-PBA, DMSO and TMAO) restored the secretion of the fibrinogen mutants studied. By contrast, low-temperature incubation (27°C) allowed partial secretion of the Bβ p.G444S (p.G414S) and γ p.W253C (p.W227C) mutants. Low temperature may act kinetically by slowing down the folding process thus allowing a higher amount of mutant proteins to adopt a suitable conformation and reach their final destination.[17] Interestingly, in this condition, the two nonsense mutants Bβ p.W467X (p.W437X) and Bβ p.R485X (p.W455X) were not secreted. This suggests a more severe conformational defect for the nonsense mutants than for the missense mutations analyzed here. Indeed, C-terminal deletions of Bβ chains are thought to destabilize the βC domain, allowing solvent access which may induce an aberrant βC conformation that is too severe to be rescued.[11] Interestingly, a small alanine residue (Bβ R485A+P486X or R455A+P456X) placed at the C-terminal entry site of the globular domain appears to be sufficient to partially restore secretion at low temperature. Fibrinogen molecules that contain the two temperature-sensitive mutants Bβ p.G444S (p.G414S) and γ p.W253C (p.W227C) fibrinogen seem to be at least partially functional since they can enter a clot and form cross-linked γ-γ dimers, implying fibrinopeptide cleavage by thrombin and proper half-staggered alignment in protofibrils for FXIII-catalyzed cross-linking. We cannot exclude that the mutants are only able to associate with normal plasma fibrinogen molecules (provided in excess in our assay) which would not be possible in the case of a patient homozygous for the mutation. However, we were at least able to demonstrate that these mutants differ from the dysfibrinogen γ p.N345_D346del (p.N319_D320del) mutant, which is unable to polymerize or cross-link, either in pure form or in combination with normal plasma fibrinogen.

Previous studies reported a secretion defect associated with the γ p.N345_D346del (p.N319_D320del) mutant we used here as a control. Indeed, the Otsu patient heterozygous for the γ p.N345_D346del (p.N319_D320del) mutation had intermediate plasma fibrinogen levels of 1.7 g/L, just below the normal range and reduced levels of variant γ chains compared to normal ones.[25] Furthermore, mice carrying the same mutation had approximately half (heterozygous animals) and one-eighth (homozygous animals) of the plasma fibrinogen levels of wild-type animals.[39] In contrast, normal fibrinogen levels and equal amounts of normal and mutant γ chains were found in the plasma of the Vlissingen patient heterozygous for the mutation,[26] and no hypodysfibrinogenemia was reported for the other affected members of the family.[27] In our *in vitro* system, the mutant does not seem to have a defect in secretion whereas in a similar study in CHO cells, a slight reduction in secretion was observed.[40] This discrepancy may be attributable to a greater permissiveness of COS-7 cells in comparison with CHO cells regarding secretion of mutant fibrinogen molecules, as we had previously found for assembly of truncated γ chains into hexamers.[12] In this regard, half-molecules, [Aαγ] complexes and free Aα or γ chains in the culture medium of COS-7 cells (Figures 1, 2) were also observed in that of hepatoma cell lines such as Hep3B and HepG2 cells, transfected BHK, CHO and COS-1 cells[14,21,41,42] and even in the plasma of afibrinogenemic patients.[43,44] It is difficult to determine which cell model reflects the *in vivo* situation best, since these differences are also found in patients, possibly reflecting polymorphic differences in modifier genes, which remain to be identified. However, the fact that normal recombinant fibrinogen secreted by COS-7 cells is clottable with a susceptibility to plasmin degradation indistinguishable from plasma fibrinogen indicates that the COS-7 model, like the CHO model,[31] is suitable for these studies.

In conclusion, we found that lowering the incubation temperature can restore the secretion of mutant fibrinogen molecules in transfected COS-7 cells, demonstrating that therapeutic manipulation of the quality control pathway is feasible for afibrinogenemia even though functional assays suggested a non-native conformation for the mutant molecules analyzed here.

Authorship and Disclosures

DV designed and performed the research, analyzed the data and wrote the paper; CDS performed research; MNA designed research, analyzed data and wrote the paper. The authors reported no potential conflicts of interest.

References

1. Lak M, Keihani M, Elahi F, Peyvandi F, Mannucci PM. Bleeding and thrombosis in 55 patients with inherited afibrinogenaemia. Br J Haematol 1999;107:204-6.
2. Doolittle RF. A detailed consideration of a principal domain of vertebrate fibrinogen and its relatives. Protein Sci 1992; 1:1563-77.
3. Neerman-Arbez M, Honsberger A, Antonarakis SE, Morris MA. Deletion of the fibrinogen alpha-chain gene (FGA) causes congenital afibrinogenemia. J Clin Invest 1999;103:215-8.
4. Neerman-Arbez M, de Moerloose P. Mutations in the fibrinogen gene cluster accounting for congenital afibrinogenemia: an update and report of 10 novel mutations. Hum Mutat 2007;28: 540-53.
5. Vu D, Neerman-Arbez M. Molecular mechanisms accounting for fibrinogen deficiency: from large deletions to intracellular retention of misfolded proteins. J Thromb Haemost 2007;5[Suppl 1]:125-31.
6. Duga S, Asselta R, Santagostino E, Zeinali S, Simonic T, Malcovati M, et al. Missense mutations in the human beta fibrinogen gene cause congenital afibrinogenemia by impairing fibrinogen secretion. Blood 2000;95:1336-41.
7. Vu D, Bolton-Maggs PH, Parr JR, Morris MA, De Moerloose P, Neerman-Arbez M. Congenital afibrinogenemia: identification and expression of a missense mutation in FGB impairing fibrinogen secretion. Blood 2003;102:4413-5.
8. Monaldini L, Asselta R, Duga S, Peyvandi F, Ghosh K, Malcovati M, et al. Fibrinogen Mumbai: intracellular retention due to a novel G434D mutation in the Bbeta-chain gene. Haematologica 2006;91:628-33.
9. Neerman-Arbez M, Vu D, Abu-Libdeh B, Bouchardy I, Morris MA. Prenatal diagnosis for congenital afibrinogenemia caused by a novel nonsense mutation in the FGB gene in a Palestinian family. Blood 2003;101:3492-4.
10. Spena S, Asselta R, Duga S, Malcovati M, Peyvandi F, Mannucci PM, et al. Congenital afibrinogenemia: intracellular retention of fibrinogen due to a novel W437G mutation in the fibrinogen Bbeta-chain gene. Biochim Biophys Acta 2003;1639:87-94.
11. Vu D, Di Sanza C, Caille D, de Moerloose P, Scheib H, Meda P, et al. Quality control of fibrinogen secretion in the molecular pathogenesis of congenital afibrinogenemia. Hum Mol Genet 2005;14:3271-80.
12. Vu D, de Moerloose P, Batorova A, Lazur J, Palumbo L, Neerman-Arbez M. Hypofibrinogenaemia caused by a novel FGG missense mutation (W253C) in the gamma chain globular domain impairing fibrinogen secretion. J Med Genet 2005;42:e57.
13. Duga S, Braidotti P, Asselta R, Maggioni M, Santagostino E, Pellegrini C, et al. Liver histology of an afibrinogenemic patient with the Bbeta-L353R mutation showing no evidence of hepatic endoplasmic reticulum storage disease (ERSD); comparative study in COS-1 cells of the intracellular processing of the Bbeta-L353R fibrinogen vs. the ERSD-associated gamma-G284R mutant. J Thromb Haemost 2005; 3:724-32.
14. Zhang JZ, Redman CM. Role of interchain disulfide bonds on the assembly and secretion of human fibrinogen. J Biol Chem 1994;269:652-8.
15. Zhang JZ, Redman C. Fibrinogen assembly and secretion. Role of intrachain disulfide loops. J Biol Chem 1996;271:30083-8.
16. Welch WJ. Role of quality control pathways in human diseases involving protein misfolding. Semin Cell Dev Biol 2004;15:31-8.
17. Bernier V, Lagace M, Bichet DG, Bouvier M. Pharmacological chaperones: potential treatment for conformational diseases. Trends Endocrinol Metab 2004;15:222-8.
18. Perlmutter DH. Chemical chaperones: a pharmacological strategy for disorders of protein folding and trafficking. Pediatr Res 2002;52:832-6.
19. Fussenegger M, Mazur X, Bailey JE. pTRIDENT, a novel vector family for tricistronic gene expression in mammalian cells. Biotechnol Bioeng 1998; 57:1-10.
20. Farrell DH, Huang S, Davie EW. Processing of the carboxyl 15-amino acid extension in the alpha-chain of fibrinogen. J Biol Chem 1993;268:10351-5.
21. Hartwig R, Danishefsky KJ. Studies on the assembly and secretion of fibrinogen. J Biol Chem 1991;266:6578-85.
22. Farrell DH, Mulvihill ER, Huang SM, Chung DW, Davie EW. Recombinant human fibrinogen and sulfation of the gamma' chain. Biochemistry 1991;30: 9414-20.
23. Ohashi T, Uchida K, Uchida S, Sasaki S, Nihei H. Intracellular mislocalization of mutant podocin and correction by chemical chaperones. Histochem Cell Biol 2003;119:257-64.
24. Burrows JA, Willis LK, Perlmutter DH. Chemical chaperones mediate increased secretion of mutant alpha 1-antitrypsin (alpha 1-AT) Z: a potential pharmacological strategy for prevention of liver injury and emphysema in alpha 1-AT deficiency. Proc Natl Acad Sci USA 2000;97:1796-801.
25. Terasawa F, Hogan KA, Kani S, Hirose M, Eguchi Y, Noda Y, et al. Fibrinogen Otsu I:A gammaAsn319,Asp320 deletion dysfibrinogen identified in an asymptomatic pregnant woman. Thromb Haemost 2003;90:757-8.
26. Koopman J, Haverkate F, Briet E, Lord ST. A congenitally abnormal fibrinogen (Vlissingen) with a 6-base deletion in the gamma-chain gene, causing defective calcium binding and impaired fibrin polymerization. J Biol Chem 1991;266: 13456-61.
27. Haverkate F, Samama M. Familial dysfibrinogenemia and thrombophilia. Report on a study of the SSC Subcommittee on Fibrinogen. Thromb Haemost 1995;73:151-61.
28. Hogan KA, Lord ST, Okumura N, Terasawa F, Galanakis DK, Scharrer I, et al. A functional assay suggests that heterodimers exist in two C-terminal gamma-chain dysfibrinogens: Matsumoto I and Vlissingen/Frankfurt IV. Thromb Haemost 2000;83:592-7.
29. Terasawa F, Kani S, Hongo M, Okumura N. In vitro fibrin clot formation and fibrinolysis using heterozygous plasma fibrinogen from gammaAsn319, Asp320 deletion dysfibrinogen, Otsu I. Thromb Res 2006;118:651-61.
30. Hogan KA, Gorkun OV, Lounes KC, Coates AI, Weisel JW, Hantgan RR, et al. Recombinant fibrinogen Vlissingen/Frankfurt IV. The deletion of residues 319 and 320 from the gamma chain of fibrinogen alters calcium binding, fibrin polymerization, cross-linking, and platelet aggregation. J Biol Chem 2000; 275:17778-85.
31. Gorkun OV, Veklich YI, Weisel JW, Lord ST. The conversion of fibrinogen to fibrin: recombinant fibrinogen typifies plasma fibrinogen. Blood 1997;89: 4407-14.
32. Marder VJ, Shulman NR, Carroll WR. High molecular weight derivatives of human fibrinogen produced by plasmin. I. Physicochemical and immunological characterization. J Biol Chem 1969;244: 2111-9.
33. Takagi T, Doolittle RF. Amino acid sequence studies on plasmin-derived fragments of human fibrinogen: amino-terminal sequences of intermediate and terminal fragments. Biochemistry 1975; 14:940-6.
34. Lucas MA, Fretto LJ, McKee PA. The relationship of fibrinogen structure to plasminogen activation and plasmin activity during fibrinolysis. Ann N Y Acad Sci 1983;408:71-91.
35. Haverkate F, Timan G. Protective effect of calcium in the plasmin degradation of fibrinogen and fibrin fragments D. Thromb Res 1977;10:803-12.
36. Yamazumi K, Doolittle RF. The synthetic peptide Gly-Pro-Arg-Pro-amide limits the plasmic digestion of fibrinogen in the same fashion as calcium ion. Protein Sci 1992;1:1719-20.
37. Mosesson MW. Fibrinogen and fibrin structure and functions. J Thromb Haemost 2005;3:1894-904.
38. Hogan KA, Maeda N, Kluckman KD, Lord ST. Synthesis of a mouse model of the dysfibrinogen Vlissingen/Frankfurt IV. Ann N Y Acad Sci 2001;936:117-21.
39. Hogan KA, Merenbloom BK, Kim HS, Lord ST. Neonatal bleeding and decreased plasma fibrinogen levels in mice modeled after the dysfibrinogen Vlissingen/Frankfurt IV. J Thromb Haemost 2004;2:1484-7.
40. Kani S, Terasawa F, Lord ST, Tozuka M, Ota H, Okumura N, et al. In vitro expression demonstrates impaired secretion of the gammaAsn319, Asp320 deletion variant fibrinogen. Thromb Haemost 2005;94:53-9.
41. Binnie CG, Hettasch JM, Strickland E, Lord ST. Characterization of purified recombinant fibrinogen: partial phosphorylation of fibrinopeptide A. Biochemistry 1993;32:107-13.
42. Huang S, Mulvihill ER, Farrell DH, Chung DW, Davie EW. Biosynthesis of human fibrinogen. Subunit interactions and potential intermediates in the assembly. J Biol Chem 1993; 268:8919-26.
43. Iida H, Ishii E, Nakahara M, Urata M, Wakiyama M, Kurihara M, et al. A case of congenital afibrinogenemia: fibrinogen Hakata, a novel nonsense mutation of the fibrinogen gamma-chain gene. Thromb Haemost 2000;84:49-53.
44. Plate M, Asselta R, Peyvandi F, Tenchini ML, Duga S. Molecular characterization of the first missense mutation in the fibrinogen Aalpha-chain gene identified in a compound heterozygous afibrinogenemic patient. Biochim Biophys Acta 2007;1772:781-7.