Fibrinogen Milano XII: a dysfunctional variant containing 2 amino acid substitutions, Aα R16C and γ G165R

Bettina Bolliger-Stucki, Susan T. Lord, and Miha Furlan

Introduction

Fibrinogen, a soluble plasma glycoprotein of 340 kd, is made up of 2 copies of 3 different polypeptide chains (Aα, Bβ2, γγ), linked together with 29 interchain and intrachain disulfide bridges. The molecule is organized in a dimeric fashion consisting of a central E domain containing the amino termini of all 6 polypeptide chains and 2 outer D domains. In the final stage of blood coagulation, thrombin cleaves the fibrinopeptides A and B in a sequential manner from the amino termini of the Aα- and Bβ-chains. Cleavage occurs between residues R16 (single-letter amino acid abbreviations) and G17 of the Aα-chain and residues R14 and G15 of the Bβ-chain, exposing the A and B polymerization sites. Resultant monomers join together to form 2-stranded, half-staggered prototibrils. It has been shown that prototibrils result from longitudinal D-D interactions and noncovalent contacts between the A and the a sites. The a site is formed by residues 329, 330, 340, and 364 in the carboxy-terminal part of the γ-chain.1,2 Subsequently, the growing fibrils aggregate in a lateral fashion to form fibers that increase progressively in thickness and develop branch points. The evolving network is finally stabilized with covalent bonds by the activated factor XIII, resulting in a clot resistant to mechanical disruption.

Dysfibrinogenemia is a heritable disorder characterized by structural mutations in any of the 3 polypeptide chains of fibrinogen. A repertoire of 191 individual cases in which the structural defects have been elucidated are listed at http://www.geht.org/pages/database_ang.html (accessed January 2001). Regarding hemostasis, most of the affected patients are asymptomatic, but some suffer from bleeding, thrombosis, or both.3 To date, only one compound heterozygote with 2 mutations in fibrinogen has been described.4 The patient had hypofibrinogenemia but had no polymerization defect.

We report a dysfunctional fibrinogen variant with 2 single heterozygous amino acid substitutions, one at position Aα R16C and the other at position γ G165R. The defect in the Aα-chain is located in the thrombin cleavage site and impairs the exposure of the A site. The amino acid substitution in the γ-chain is novel. Although it is sequentially and structurally remote from all known functional sites, we found that it affects the proper folding of the carboxy-terminal part of the γ-chain. From both in vivo and in vitro data, it is evident that these 2 amino acid substitutions result in a severe polymerization defect.

Materials and methods

Routine coagulation tests

Routine coagulation tests were performed with citrated plasma samples. Thrombin time and reptilase time were assayed by conventional methods. Fibrinogen concentrations in plasma were determined by the functional clotting rate method5 and by electroimmunoassay.6

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Purification of fibrinogen

Fibrinogen Milano XII was isolated from citrated plasma of the propositus by affinity chromatography using fibrin-monomer-Sepharose CL-2B. Because the propositus is a heterozygous carrier of abnormal fibrinogen, the purified fibrinogen preparation contained normal and abnormal α- and γ-chains. Normal fibrinogen was isolated from a plasma pool of healthy donors. The purified protein was extensively dialyzed against 0.05 M triethanolamine-HCl, pH 7.4, 0.1 M NaCl (TEA-buffer).

Coagulation profiles of purified fibrinogen

Fibrin polymerization was evaluated turbidimetrically.5 Fibrinogen (540 µL, 0.55 mg/mL) was preincubated with 30 µL 20 mM CaCl2 or 20 mM ethylenediaminetetraacetic acid (EDTA) (final concentration 1 mM) in polystyrene cuvettes for 5 minutes at 37°C. After the addition of 30 µL 10 U/mL bovine thrombin (final concentration, 0.5 U/mL; Diagnostec AG, Liestal, Switzerland), the increase in turbidity at 350 nm was measured in a spectrophotometer at 37°C. Each experiment was performed twice.

DNA analysis

Genomic DNA was isolated as previously described.8 The entire coding region of all 3 fibrinogen genes was amplified by polymerase chain reaction (PCR), and the PCR products were purified using the QiAquick PCR purification kit (Qiagen, Valencia, CA). Coding and complementary strands of the purified products were sequenced (Automated DNA Sequencing Facility, University of North Carolina at Chapel Hill) using the same primers as for PCR.

Kinetics of fibrinopeptide release

Fibrinogen solutions were diluted with TEA-buffer to a final concentration of 0.2 mg/mL. Human α-thrombin (Enzyme Research Laboratory, South Bend, IN) was added to a final concentration of 0.005 U/mL, and the individual reactions were stopped at designated time points by boiling the incubation mixtures for 15 minutes. To measure the total amount of fibrinopeptide A and B released at an infinity time point, the fibrinogen was incubated with 10 U/mL thrombin for 240 minutes. After centrifugation, the supernatants were immediately analyzed by reverse-phase high-performance liquid chromatography (HPLC) using the Shimadzu HPLC-System (Shimadzu, Columbia, MD) with a Discovery C18 250-mm, 5-µm column (Supelco, Bellefonte, PA). The column was equilibrated with buffer A (25 mM NaH2PO4/Na2HPO4, pH 6.0), and the autosampler loaded 200 µL each sample on the column. Peptides were eluted with a linear gradient from 15% to 36% buffer B (25 mM NaH2PO4/Na2HPO4, pH 6.0, with 50% acetonitrile) and monitored by absorbance reading at 210 nm. Fibrinopeptide peak areas from 2 experiments were determined by the accompanying software class 5 VP (Shimadzu). Fibrinopeptide release curves were constructed by plotting the percentage release, assuming the total FpA and FpB, and stained with Coomassie blue.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immunoblot analysis of purified fibrinogen

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) of nonreduced normal and variant fibrinogen was performed on a 3% to 12% polyacrylamide gel under nonreducing and reducing conditions, respectively. Gels were loaded with 20 µg initial fibrinogen preparation and stained with Coomassie blue. The experiment was repeated in the presence of the peptide GPRP (0, 2, and 5 mM) with 1 mM EDTA added to all reactions.13 Immunoblot analysis was performed with nonreduced fibrinogen degradation products. Electrophoresed proteins were transferred onto nitrocellulose sheets and incubated at room temperature with the following antibodies: polyclonal rabbit anti-human fibrinogen (A0080; Dako); polyclonal rabbit anti-human fibrinogen γ-chain prepared by Hazeltown Research (Denver, PA) using γ-chain purified from inclusion bodies expressed in Escherichia coli as the antigen12; monoclonal anti–human serum albumin (clone HAS-11, A6684; Sigma, St. Louis, MO); monoclonal antibody E2F8E5 to fragment E, recognizing the sequence GHRPLDK (β-15–21) (Immunotech, Marseilles, France); peroxidase-conjugated anti-mouse or anti–rabbit IgG (Calbiochem, La Jolla, CA). Bound antibodies were visualized by enhanced chemiluminescence Western blotting detection reagents (Amersham Pharmacia Biotech, Piscataway, NJ). Human serum albumin (A9511; Sigma) was used as a control.

Purification of the fibrinogen degradation fragment D3

The fragment D3 was isolated as described earlier,14 with a slight modification. Briefly, 2 mL normal or variant fibrinogen (3 mg/mL) were proteolytically degraded for 4 hours at 37°C by the addition of 1 U/mL plasminogen (Kabi, Stockholm, Sweden) and 200 U/mL streptokinase (Behring, Marburg, Germany) in the presence 10 mM EDTA. The incubation mixture was dialyzed against the starting buffer (0.01 M TEA, 1 mM CaCl2, pH 7.4) and loaded on a Lysine-Sepharose 4B (Amersham Pharmacia Biotech) column (15 mL) equilibrated with starting buffer. The column was successively rinsed with 20 mL starting buffer, 20 mL elution buffer 1 (0.03 M TEA, 1 mM CaCl2, pH 7.4), and 20 mL elution buffer 2 (0.05 M TEA, 0.1 M NaCl, 1 mM CaCl2, pH 7.4). Fractions of 1 mL were collected and analyzed by SDS-PAGE on 8% polyacrylamide gels. Fragment D3–containing fractions were pooled and dialyzed either against TEA-buffer and frozen or against water and lyophilized.

Digestion of purified fragment D3 with chymotrypsin

Digestion was performed according to Medved et al.15 Lyophilized fragment D3 (500 µg) was dissolved in 100 µL 0.1 M phosphate buffer pH 7.0, α-Chymotrypsin (36 U/mg; type VII, TLCK-treated, bovine pancreas; Sigma) was added in an enzyme-substrate ratio of 1:5.0. The reaction was stopped at 0, 8, and 23 hours by boiling the samples. Fractions were separated by SDS-PAGE on 10% polyacrylamide gel16 and stained with Coomassie blue.

Circular dichroism analysis of purified fragment D3

Purified fragment D3 was dialyzed against 10 mM phosphate buffer pH 7.4. The concentration of the dialyzed samples was determined at 280 nm in the presence of 1 M urea (E1 cm 1% 200.0.15 Far UV-circular dichroism (CD) spectra from 260 to 190 nm were recorded on a Pirtar-180 Circular Dichroism Spectrophotometer (Applied Photophysics, Surrey, United Kingdom). Before taking the CD spectra, the absorbance spectra of the samples were recorded from 320 to 180 nm to verify and adjust the protein concentration to an OD of approximately 0.8. Then a 0.07 mg/mL solution (400 µL) of purified fragment D3 was measured in a quartz cuvette with 0.1-cm path length at room temperature. The CD spectrum of the buffer solution was subtracted from each sample spectrum. CD data were converted to mean residual ellipticity, assuming a mean residue molecular
weight of 115.16,17 Protein secondary structures were estimated from CD spectra using Continll18 and Selcon319 software from the CDPro software package (http://lamar.colostate. edu/~sreeram/CDPro/).

Results

Case report and routine coagulation tests

The propositus of dysfibrinogen Milano XII was born in 1929 in Italy and had no reported history of bleeding or thrombosis. Unfortunately, no other family members were available for testing. Routine coagulation tests with plasma samples from the propositus revealed a significantly prolonged thrombin time; furthermore, her plasma was not clottable with reptilase (Table 1). Immunologically determined fibrinogen levels (Laurell) were within normal range, but fibrinogen concentrations measured by a functional method (Clauss) were dramatically lower.

Coagulation profiles of purified fibrinogen

Turbidity curves (Figure 1) represent the kinetics of fibrin formation after the addition of thrombin to purified normal or variant fibrinogen in the presence of 1 mM calcium ions or 1 mM EDTA. In the presence of calcium, the variant showed a prolonged lag time and strongly reduced final turbidity compared to normal fibrinogen. In the presence of EDTA, only a minute turbidity increase occurred over the recording period.

DNA analysis

Sequence analysis of the entire coding region of the 3 fibrinogen genes revealed 2 point mutations (data not shown). The first mutation was exon 6 of the γ-chain, a substitution of base 4682 guanine to adenine, changing the amino acid glycine to arginine at position 165 of the γ-chain. The second mutation was detected in exon 2 of the αα-chain. A single base change at position 1202 from cytosine to thymine led to the replacement A16C.

Kinetics of fibrinopeptide release

We examined the rate of thrombin-catalyzed fibrinopeptide release by measuring the peak areas of FpA and FpB as detected by reverse-phase HPLC. After a 240-minute incubation, only approximately 50% of the FpA was released from fibrinogen Milano XII (Figure 2). Increasing the amount of thrombin, the time of incubation, or both did not change this percentage. The release of FpB from fibrinogen Milano XII was delayed but approached 100% with a higher thrombin concentration (data not shown).

SDS-PAGE and immunoblot analysis of fibrinogen degradation products

It is known that the extent of plasmin degradation of fibrinogen is sensitive to calcium bound to the high-affinity site and to the peptide GPRP bound to the a polymerization site. Both sites are located in the D domain, and normal binding of Ca2+ or the peptide GPRP protects the fibrinogenolysis fragment D1 against further degradation to D2 and D3. Plasmin digests of normal and Milano XII fibrinogens produced fragment D1 in the presence of calcium and fragment D3 in the presence of EDTA (Figure 4A), indicating complete protection of the substrates by bound calcium.
albumin-linked bands in panel B with their counterparts in panel A. Ca^{2+} fragments D2 and D3 because of only partial protection of D1 by antibodies against polyclonal antibodies against fibrinogen and min digests comparing the patterns obtained with 4 antibodies: albumin, we performed immunoblot analysis of nonreduced plasmanormal in fibrinogen Milano XII. high-affinity calcium-binding site and the a polymerization site are (data not shown). These degradation patterns indicate that both the m and 5 mM GPRP and saw complete protection in both samples digested the normal and variant fibrinogens in the presence of 2 additional band of apparently higher molecular weight, denoted D1* and D3* in Figure 4A. We compared the plasmin digestion of fibrinogen Milano XII to the digestion of fibrinogen St Gallen I (¢ G292V), a variant that is abnormally digested to give the fragments D2 and D3 because of only partial protection of D1 by Ca^{2+}. As shown in Figure 4A, fragment D3* was not the same as fragment D2 and thus did not arise from the incomplete conversion of D1 in D3. When the plasmin digests were examined under reducing conditions, normal and variant chain remnants were indistinguishable (Figure 4B). This indicates that the unusual D1* and D3* fragments arose from disulfide-linked molecules. We also digested the normal and variant fibrinogens in the presence of 2 mM and 5 mM GPRP and saw complete protection in both samples (data not shown). These degradation patterns indicate that both the high-affinity calcium-binding site and the a polymerization site are normal in fibrinogen Milano XII.

To identify the fragment of fibrinogen Milano XII that bound albumin, we performed immunoblot analysis of nonreduced plasmin digests comparing the patterns obtained with 4 antibodies: polyclonal antibodies against fibrinogen and γ-chain and monoclonal antibodies against β-chain 15-21 (E2F8E5) and human serum albumin (HAS-11). The antifibrinogen blot (Figure 5A) showed 2 bands of 90 kd and 100 kd in the fibrinogen Milano XII samples (lanes M) that were also faintly apparent in the Coomassie-stained gels (Figure 4A, EDTA, lane M), and it showed the bands D1* and D3*. The anti–γ-chain antibody reacted with D1* and D3* but not with the 90- and 100-kd bands or with fragment E (Figure 5B). Because the resolution in these blots was insufficient to separate D1* and D3* species, the bands are not distinct from D1 and D3, appearing rather as single broad bands. The 90-kd and 100-kd bands reacted with antifibrinogen, anti–β-chain and antialbumin antibodies (Figure 5A,C,D), indicating that albumin is linked to fragment E. As expected for fragment E, the same bands were seen in the presence of calcium or EDTA, though the 90-kd bands were distorted by the D1 fragment in the presence of calcium. We were surprised to find that E2F8E5 reacted with the plasmin degradation fragments because it has been reported that Bβ1-42 is removed by plasmin cleavage early in the reaction. In control experiments, we found that the E2F8E5 reaction depended on the reaction conditions. We saw only weak reactivity with the 90-kd, 100-kd, and normal E fragments with 10-fold higher concentrations of plasmin-ogen and streptokinase, as expected for the subsequent removal of Bβ1-42 (data not shown). We also showed that E2F8E5 did not cross-react with human albumin (data not shown). Taken together, these results indicate that albumin is bound to the E-domain of fibrinogen Milano XII likely through the neo-cysteine at position 16 in the Aα-chain.

Purification of the fibrinogen degradation fragment D3

Using a Lysine Sepharose 4B column and a strategy of different buffers with increasing salt concentration, fragment D3 was eluted in an early peak and was separated from fragment E and plasmin. The D3 and D3* fraction from fibrinogen Milano XII eluted concomitantly and could not be separated from each other (data not shown).

Digestion of purified fragment D3 with chymotrypsin

Fragment D3 (M, 82 kd) can be further digested by chymotrypsin to a fragment called TSD (thermodynamic stable domain). In bovine fibrinogen, a chymotrypsin cleavage site had been identified between γ 151 and γ 152 leading to a digestion fragment of 63 kd. Because of the similarity of bovine and human fibrinogen, it is likely that the cleavage site for chymotrypsin in human fibrinogen may be situated close to that found in bovine fibrinogen. Incubation of normal and variant fragment D3 with chymotrypsin led to a 63-kd fragment, and the double band associated with fibrinogen Milano XII disappeared (data not shown). Therefore, removal of the mutation site at position 165 led to a chymotryptic fragment of the same size as the fragment derived from normal fibrinogen.
spectra is the result of an individual plasmin digestion and subsequent purification of fibrinogen.

Figure 6. Far UV-CD spectra of purified fragments D3 of normal and variant circulated at a normal level in the plasma of the propositus. The of the mutations. Both defects were present in the fibrinogen that no other family members were available to study the transmission studies led to a diagnosis of dysfibrinogenemia. Unfortunately, ered in a woman of Italian origin, born in 1929. Routine coagula-
g conformational changes in human fibrinogen after different in vitro phosphorylation procedures. 27 Kirschbaum et al 17 applied this method to visualize the difference between urea-denatured and native D fragments in a platelet-binding study. Furthermore, CD spectroscopy has been a useful tool for detecting structural changes caused by single amino acid substitutions in different enzymes. 36,39 We observed a reduction in the mean residual ellipticity (Figure 6) and a significant decrease in the -helical content between normal and variant fragment D3. These results support our suggestion of a conformational abnormality in the D-domain of fibrinogen Milano XII.

Far UV-CD analysis of purified fragment D3

Figure 6 shows far UV-CD spectra of purified fragments D3 of normal and variant fibrinogen. A slight reduction of the mean residual ellipticity was calculated with fragment D3 derived from fibrinogen Milano XII. The curve representing the normal fragment D3 showed a minimum at 210 nm reflecting the -helical structure in the protein. This feature was less prominent in the CD-spectra of variant fragment D3. The -helical content in the variant fragment D3 (28.2% ± 0.9%) was significantly different from normal D3 (31.8% ± 0.7%; P = .005).

Discussion

We describe a dysfunctional fibrinogen variant with 2 mutations, one in the -chain at position 16 (R16C) and the other in the -chain at position 165 (G165R). This dysfibrinogen was discovered in a woman of Italian origin, born in 1929. Routine coagulation studies led to a diagnosis of dysfibrinogenemia. Unfortunately, no other family members were available to study the transmission of the mutations. Both defects were present in the fibrinogen that circulated at a normal level in the plasma of the propositus. The mutation Aα R16C inhibited the release of fibrinopeptide A by thrombin. HPLC analysis of fibrinopeptide release revealed that only 50% of fibrinopeptide A was cleaved from the purified fibrinogen Milano XII (Figure 2). This result confirmed the heterozygosity found in DNA analysis. We found that small amounts of abnormal fibrinogen circulate as disulfide-linked complexes with albumin. The disulfide bonds likely form between the neo-cysteine introduced by the mutation Aα R16C and the free sulphydryl group (C34) present in albumin. 26 Covalent binding of albumin to Aα C16 was previously described in fibrinogen Bern V containing the same mutation (Aα R16C). 27

The mutation in the γ-chain (G165R) caused abnormal electrophoretic migration on SDS-PAGE of the unreduced variant fibrinogen and of some of its nonreduced degradation fragments. The relative migration rate of a protein in a polyacrylamide gel containing SDS depends on its molecular size, shape, 28 and binding affinity for SDS. 29 The latter factor was proposed to be responsible for shifts in electrophoretic mobility of the fibrinogen γ-chain that have been observed in several fibrinogen mutants 30-35 because of the introduction of a more basic or more hydrophobic amino acid. 36 Fibrinogen Milano XII showed abnormal migration under nonreducing conditions but normal migration under reducing conditions. We hypothesize that the amino acid exchange from glycine to arginine at position 165 affects the folding pattern of the carboxy-terminal globular domain of the γ-chain. The abnormal shape of the fragment, held together by disulfide bridges, may thus affect the electrophoretic migration of nonreduced fragments but is compatible with the normalization of electrophoretic behavior after the reduction of disulfide bonds. To investigate tentative structural differences, we analyzed purified D3 fragments derived from normal fibrinogen and fibrinogen Milano XII, the latter a mixture of D3 and D3*, using CD spectroscopy. CD analysis has been applied to study the conformational differences among fibrinogen, fragment D, and fragment E 36 and between the thermodynamically stable domains 1 and 2 of bovine fibrinogen. 23 It has also been used to estimate the conformational changes in human fibrinogen. 25

Figure 6. Far UV-CD spectra of purified fragments D3 of normal and variant fibrinogen. Each curve represents the mean of 3 individual spectra. Each of these 3 spectra is the result of an individual plasmin digestion and subsequent purification of fragment D3. Normal fibrinogen, ---; variant, · · ·. The standard deviation is given at selected wavelengths.
The amino acid substitution Aα R16C is common and accounts for 18% of reported dysfunctional fibrinogen variants. This mutation leads to abnormal coagulation test results in plasma and to impaired polymerization of purified fibrinogen. Delayed clotting of fibrinogen Milano XII is in agreement with reports on functional behavior of other Aα R16C variants. 12 Because the polymerization defect attributed to the Aα-chain mutation is so dramatic, it may obscure any additional disturbance of fibrin clot formation resulting from the G165R mutation. Our plasmid digestion experiments in the presence of GPRF suggest that the α sites in the carboxy-terminal domains of fibrinogen Milano XII are intact.

Therefore, if the mutation in the γ-chain has an effect on polymerization, it is unlikely to have been caused by impaired A-a interactions.

Fibrinogen Milano XII is the first dysfunctional fibrinogen variant with 2 amino acid substitutions associated with a strong polymerization defect. Brennan et al. 11 reported earlier a fibrinogen variant with 2 mutations in the coding region of fibrinogen. Both structural defects were detectable in the circulating fibrinogen but did not affect fibrin polymerization. Neither the substitution γ G165R nor the unusual migration of plasmic fragments D on SDS-PAGE has previously been reported. The mutation is located in a loop of the γ-chain formed by a cysteine bridge between residues 153 and 182 in the globular domain of the γ-chain (Figure 7). It affects the structure of the D-domain and may contribute to the observed polymerization defect. We intend to study the functional properties of the homozygous G165R substitution using a recombinant fibrinogen variant with the same mutation.

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References


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