Analysis of Engineered Fibrinogen Variants Suggests That an Additional Site Mediates Platelet Aggregation and That "B−b" Interactions Have a Role in Protofibril Formation†

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ABSTRACT: The C-terminal domain of the fibrinogen γ-chain includes multiple functional sites that have been defined in high-resolution structures and biochemical assays. Calcium binds to this domain through the side chains of γD318 and γD320 and the backbone carbonyls of γF322 and γG324. We have examined variant fibrinogens with alanine at position γ318 and/or γ320 and found that calcium binding, fibrin polymerization, and fibrinogen-mediated platelet aggregation, but not FXIIIa-catalyzed cross-linking, were abnormal. When measured by turbidity, thrombin-catalyzed polymerization was severely reduced, and batroxobin-catalyzed polymerization was completely obliterated. Moreover, thrombin-catalyzed polymerization was abolished by the peptide GHRP, which binds to the polymerization site in the β-chain but does not inhibit polymerization of normal fibrinogen. ADP-induced platelet aggregation was also severely impaired. In contrast, as measured by SDS−PAGE, FXIIIa introduced cross-links between γ-chains for all three variants, as expected if the γ-chain C-terminal sites were normal. In addition, binding of the monoclonal antibody 4A5, which recognizes the C-terminal residues, was not different from normal. These data suggest two specific conclusions: (1) a site in the γ-module other than the C-terminus is critical for platelet aggregation and (2) "B−b" interactions have a role in protofibril formation.

Fibrinogen is a trinodular molecule consisting of six polypeptide chains, $(A\alpha,B\beta,\gamma)_2$. The central nodule, called E, contains the N-termini of the six chains. The two outer nodules, called D, contain the C-termini of the Bβ- and γ-chains, which fold independently into globular domains, called the β- and γ-modules. Soluble fibrinogen is converted to a fibrin clot when thrombin cleaves fibrinopeptides A and B (FpA, FpB)[1] from the N-termini of the Aα- and Bβ-chains, respectively. The newly exposed N-termini of the α- and β-chains constitute the polymerization sites "A" and "B" that are complementary to the intrinsic polymerization sites "a" and "b" found in the γ- and β-modules, respectively (*1*). Following the release of FpA, the fibrin monomers interact with each other through sites "A" and "a" to form double-stranded protofibrils. The protofibrils grow until they reach a critical length at which they aggregate laterally into thicker and branched fibrin fibers (*2*). FpB is cleaved during the assembly of protofibrils, and the unmasked site "B" interacts with its complementary site "b". The "B−b" interaction has been found to enhance the rate of lateral aggregation (*3*). Nevertheless, the precise role of the "B" site remains controversial, since the release of FpA alone with a specific enzyme, such as batroxobin, leads to clot formation, indicating that the "A−a" interaction alone could support fibrin polymerization (*4*). Recently, Yang et al. have proposed a model of polymerization where the primary driving force for lateral aggregation is a set of interactions between γ-modules on different protofibrils (*5*). In this model, a second, perhaps subsidiary, interaction between β-modules on different protofibrils follows the release of FpB, as the "B−b" interaction within one protofibril alters the orientation of the β-module permitting interactions between protofibrils. Thus, the interactions mediated by the release of FpB strengthen those mediated by the release of FpA, consistent with the observation that batroxobin-catalyzed fibrin clots are similar to but weaker than thrombin-catalyzed clots (*6*).

Calcium ions are important for multiple functions of fibrinogen. Calcium promotes fibrin polymerization, is essential for Factor XIII (FXIII) activation, and modulates the specificity of plasmin-catalyzed fibrinolysis (*7*−*9*). Fibrinogen binds three calcium ions per molecule with an affinity of 10−50 μM, one in each outer nodule and the third possibly in the central nodule (*10*, *11*). Crystal structure studies located the known sites in the γ-modules; each site

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[1] Abbreviations: γD318A, γD320A, γD318+320A, and γD364A, Asp substituted with Ala at position 318, 320, both 318 and 320, or 364 in the γ-chain; FpA, fibrinopeptide A; FpB, fibrinpeptide B; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; CHO, Chinese hamster ovary; ADP, adenosine diphosphate; GPRP, Gly-Pro-Arg-Pro-amide; GHRP, Gly-His-Arg-Pro-amide; EDTA, ethylenediaminetetraacetic acid; HPLC, high-performance liquid chromatography; TEM, transmission electronic microscopy; FXIII, coagulation factor XIII.

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involves the side chain of residues γD318 and γD320 and the main chain carbonyl oxygens of γF322 and γG324, and two molecules of water (*12*). Since this calcium-binding site is adjacent to the "a" polymerization site, one could hypothesize that these sites are interdependent. In fact, at least two dysfibrinogens, Bastia and Vlissingen, with changes in this calcium-binding site have defective fibrin polymerization (*13, 14*). Nevertheless, studies with the peptide GPRP, which mimics the polymerization site "A", have shown that calcium ions do not influence GPRP binding (*15*). Moreover, plasmin specificity studies have demonstrated that GPRP binds to fibrinogen in the presence of EDTA, which removes calcium from this site (*16*). Finally, Côté et al., using recombinant γ-module variants, showed that mutations in the "a" polymerization site did not alter calcium binding to this nearby site (*17*). They concluded that the two sites function independently.

Previously, we examined the significance of this calcium-binding site using γΔ319,320 fibrinogen, a recombinant protein analogous to the dysfibrinogen Vlissingen (*18*). This variant, which lacks the residues γAsn319 and γAsp320, did not bind calcium. Moreover, the functions associated with the γ-module were impaired. We did not detect either FXIII-catalyzed cross-linking to form γ−γ dimers or fibrinogen-mediated platelet aggregation. As both these activities require sites at the very end of the γ-chain (397−411), we concluded that the two amino acid deletion caused a substantive conformational change in the γ-module. Thus, although γΔ319,320 fibrinogen did not polymerize, we were not able to associate this loss specifically with a change in the calcium-binding site.

To further probe this potential interrelation, we synthesized three fibrinogen variants, γD318A, γD320A, and γD318+320A, that alter the residues whose side chains contribute to calcium binding. Our data showed that a single substitution in the calcium-binding site greatly impaired fibrin polymerization and platelet aggregation, but did not significantly alter FXIIIa-catalyzed γ−γ dimer formation. Moreover, our data suggest that "B−b" interactions promote protofibril assembly.

EXPERIMENTAL PROCEDURES

All chemicals were of reagent grade and, unless specified, were purchased from Sigma (St. Louis, MO). Human thrombin was purchased from Enzyme Research Laboratories, Inc. (South Bend, IN), and batroxobin was from Calbiochem-Novabiochem Corp. (San Diego, CA). FXIII was a generous gift from Dr. Kevin Siebenlist, Sinai Samaritan Medical Center (Milwaukee, WI). Plasminogen was purified from human plasma by the method previously described (*19*). Streptokinase was obtained from American Diagnostica (Greenwich, CT). The Transformer site-directed mutagenesis kit was purchased from Clontech Laboratories (Palo Alto, CA); Sepharose 4B, Superose 6B, Sepharose CL-2B, and ECL Western blotting detection reagents were from Amersham-Pharmacia-Biotech (Piscataway, NJ). IF-1 monoclonal antibody was purchased from Iatron Corp. (Tokyo, Japan); monoclonal antibody 4A5 (Mab 4A5) that recognizes γ-chain fragment γ401−411 was a generous gift from Dr. Gary Matsueda, Bristol-Meyers Squibb Pharmaceuticals (Princeton, NJ); horseradish peroxidase-conjugated rabbit antibody to fibrinogen was from Dako Corp. (Carpinteria, CA); polyclonal rabbit anti-human fibrinogen γ-chain was prepared by Hazelton Research Products (Denver, PA) using γ-chain purified from inclusion bodies expressed in *E. coli* as the antigen (*20*); and goat anti-rabbit IgG conjugated to horseradish peroxidase was from Calbiochem. TMB microwell peroxidase substrate was from Kirkegaard & Perry Laboratories (Gaithersburg, MD). GPRP and GHRP peptides were purchased from the Protein Chemistry Laboratory at the University of North Carolina (Chapel Hill, NC). ADP was purchased from BioData Corp. (Horsham, PA).

Fibrinogen Variant Expression. The expression vector pMLP-γ containing the entire human γ-chain cDNA was previously described (*21*). The aspartic acids γD318, γD320, and γD318+320 were substituted with alanine by oligonucleotide-directed mutagenesis, using the Transformer site-directed mutagenesis kit based on the method described by Deng et al. (*22*). The primers used to introduce the substitutions were as follows: γD318A, 5′CCTGGGACAATGCCAATGATAAGTTTGAAGGC3′; γD320A, 5′CCTGGGACAATGACAATGCTAAGTTTGAAGGC3′; and γD318+320A, 5′CCTGGGACAATGCCAATGCTAAGTTTGAAGGC3′. For each variant, the entire coding region of the mutated vector was sequenced to confirm that only the expected change was present. Chinese hamster ovary cells expressing the human Aα and Bβ polypeptide chains were cotransfected with each mutated pMLP-γ vector and the selection vector pMSV-His. Individual clones, secreting high amounts of fibrinogen as determined by an enzyme-linked immunosorbent assay, were grown in roller bottles for large-scale protein synthesis in serum-free medium supplemented with aprotinin. The medium was harvested periodically and stored at −70 °C.

Purification of Recombinant Fibrinogen. The normal and the variant fibrinogens were purified by a two-step method as reported previously (*23*). Briefly, the protein was concentrated by ammonium sulfate precipitation, and then purified by immunoaffinity chromatography using IF-1 monoclonal antibody conjugated to Sepharose 4B. The purified proteins were extensively dialyzed against HEPES buffer (20 mM HEPES, pH 7.4, 150 mM NaCl) and stored at −70 °C. The purity and the proper assembly of the fibrinogen variants were tested by SDS−PAGE under reducing and nonreducing conditions according to the method of Laemmli (*24*).

Plasmin Protection Assay. Plasmin was generated by activation of 100 μg/mL plasminogen by 100 units/mL streptokinase. Normal recombinant or variant fibrinogens (0.2 mg/mL) in HEPES buffer containing 5 mM $CaCl_2$, 5 mM EDTA, or 2 mM peptide (GPRP or GHRP supplemented with 1 mM EDTA) were incubated with 10 μg/mL plasmin for 4 h at 37 °C. The reactions were stopped by heating at 100 °C for 5 min, and the plasmin digests were analyzed on 7.5% gels that were stained with Coomassie brilliant blue.

Fibrinopeptide Release. Thrombin-catalyzed FpA and FpB release was performed essentially as described by Mullin et al. (*25*). Briefly, fibrinogen at 0.1 mg/mL in HEPES buffer was incubated with 0.005 NIH unit/mL thrombin at room temperature. At specified time points, the reaction was stopped by heating at 100 °C for 5 min, the sample was centrifuged, and the supernatant was analyzed with a reverse-phase HPLC. To verify that batroxobin releases FpA in the

conditions used to study fibrin polymerization, fibrinogen at 0.2 mg/mL was incubated with 0.1 unit/mL batroxobin at room temperature for 1 h.

Turbidity Measurements. Polymerization, at ambient temperature, was monitored continuously at 350 nm in a Biospec 1601 spectrophotometer (Shimadzu Corp., Tokyo, Japan). The reaction was initiated by adding 10 μL of thrombin (1 NIH unit/mL) or 10 μL of batroxobin (1 unit/mL) to 100 μL of fibrinogen solution (0.2 mg/mL) in HEPES buffer in the presence or absence of 5 mM $CaCl_2$. For polymerization in the presence of GHRP peptide, fibrinogen solutions (0.2 mg/mL) were preincubated with 2 mM GHRP for 30 min at room temperature prior to adding thrombin. The results were analyzed by considering two parameters: lag time and V_{max}. Lag time was measured as the time elapsed until an increase in turbidity was seen, and V_{max} was calculated as the slope of the steepest part of the polymerization curve.

Size-Exclusion Chromatography. Fibrin polymerization reactions were initiated by adding 10 μL of thrombin (1 NIH unit/mL) or batroxobin (1 unit/mL) to 100 μL of fibrinogen (0.2 mg/mL) with or without 2 mM GHRP. The mixtures were injected into the chromatography column at 2, 40, and 80 min from the start of polymerization of normal fibrinogen, γD320A, and γD318A, respectively. These times correspond to the length of the lag period except for normal fibrinogen, which was incubated only for 2 min to avoid gelation of the solution before loading it onto the column. We used an HR16/50 column packed with Superose 6B resin, according to the manufacturer's instructions. The column was equilibrated with HEPES buffer, and the products eluted at a flow rate of 1 mL/min.

Transmission Electronic Microscopy (TEM). The samples were prepared similarly to the gel filtration experiments. At the specified time points, the samples were diluted 1:10 v/v in 50 mM ammonium formate, pH 7.4, containing 70% glycerol, and sprayed onto freshly cleaved mica. The samples were dried at room temperature and rotary-shadowed with tungsten in a Denton Vacuum DV-502 as previously described (*18*). The shadowed protein films were picked up on 400 mesh copper grids and viewed on an LEO EM 910 transmission electron microscope.

FXIII-Catalyzed Cross-Linking of Fibrin. The normal and the variant fibrinogens (0.2 mg/mL in HEPES buffer containing 2 mM $CaCl_2$) were clotted at room temperature with 0.1 NIH unit/mL thrombin and 2 μg/mL FXIII. After 4 h incubation, either the reaction was stopped by adding an equal volume of Laemmli sample buffer containing 10% β-mercaptoethanol or the clots were collected by centrifugation at 10 000 rpm for 30 min, and the pellets were dissolved in Laemmli sample buffer. Samples were heated at 100 °C for 5 min, prior to electrophoresis in a 7.5% gel, and transfer onto a nitrocellulose membrane. The membrane was blocked overnight at 4 °C with 20 mM Tris-HCl, pH 7.4, 150 mM NaCl (TBS) containing 5% nonfat milk, and then incubated with a 1:3000 dilution of rabbit polyclonal antiserum to γ-chain for 2 h in TBST (TBS supplemented with 0.05% Tween 20) containing 1% bovine serum albumin (BSA). The membrane was then washed 3 times with TBST and incubated with a 1:5000 dilution of goat anti-rabbit IgG conjugated to horseradish peroxidase for 1 h in TBST with 1% BSA. The blot was washed 3 times and developed with ECL Western blotting detection reagents.

ADP-Induced Aggregation of Isolated Platelets. Blood was drawn into citrate from healthy donors, and the platelets were isolated as described previously (*26*). Briefly, platelet-rich plasma (PRP) was obtained by centrifuging blood at 800 rpm for 15 min. After adding 20 nM prostaglandin I_2, the PRP was centrifuged again at 1400 rpm for 15 min, and the platelets were resuspended in Tyrode's buffer (20 mM HEPES, pH 7.2, 135 mM NaCl, 2.7 mM KCl, and 12 mM $NaHCO_3$) supplemented with 5.5 mM glucose and 2% BSA. Thereafter, the platelets were loaded onto a Sepharose CL-2B column, eluted, and supplemented with 1 mM $CaCl_2$ and 2 mM $MgCl_2$. For platelet aggregation experiments, 2×10^8 platelets/mL were preincubated at 37 °C for 2 min with 300 nM fibrinogen, prior to activation with 10 μM ADP. Platelet aggregation was monitored in a multichannel platelet aggregometer (BioData Corp., Horsham, PA). Normal and variant fibrinogens were assayed 2 times with three different platelet donors.

Accessibility of the αIIbβ3 Ligand γ401−411. The monoclonal antibody 4A5 (100 μL/well of 1 μg/mL) in phosphate-buffered saline (PBS) was immobilized on a micro-well plate at 4 °C for 16 h. After washing the plates twice with PBS, nonspecific binding sites were saturated with 100 μL of 1% BSA in PBS for 1 h at 37 °C. Normal and variant fibrinogens (concentration ranging from 4 ng/mL to 10 μg/mL) in PBST (PBS containing 0.05% Tween 20) were incubated with the immobilized antibody for 90 min at 37 °C. The bound fibrinogen was measured using a rabbit polyclonal antibody to fibrinogen conjugated to peroxidase (1:1000 in PBST). The reaction was developed with TMB substrate, stopped by adding phosphoric acid, and read at 405 nm. The data presented represent the mean of one experiment performed in triplicate.

RESULTS

Characterization of the Fibrinogen Variants. We synthesized three variant fibrinogens, γD318A, γD320A, and γD318A+320A, and purified them from cell culture medium by immunoaffinity chromatography, as described under Experimental Procedures. We characterized the proteins by SDS−PAGE under nonreduced conditions, and found that all three variants were pure and assembled into 340 kDa molecules, similar to normal fibrinogen (data not shown). Under reduced conditions, we found the expected bands comparable to the three normal polypeptide chains, although all three variant γ-chains migrated further than normal γ-chain (*27*). This result was not surprising, as similar changes in electrophoretic mobility have been reported for a number of dysfibrinogens with small changes in this C-terminal part of the γ-chain (*28*).

Plasmin Protection Assay. To determine whether these substitutions altered the calcium-binding site or the "a" polymerization site, we assessed binding of both calcium and the peptide GPRP using plasmin protection assays. As shown in Figure 1, plasmin cleaved normal fibrinogen into fragments D1, D2, and D3, which were readily separated by SDS−PAGE. In the presence of 5 mM $CaCl_2$ or 2 mM GPRP supplemented with 1 mM EDTA, essentially only fragment D1 was seen. In the presence of 5 mM EDTA, only the smaller fragments D2 or D3 were evident. Thus, with normal fibrinogen, calcium and GPRP protected the D1 fragment

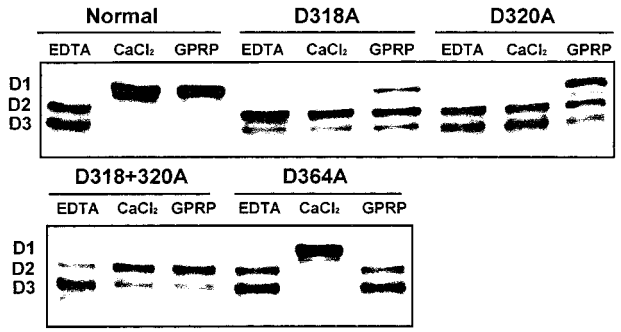

FIGURE 1: Plasmin protection assay. Normal or variant fibrinogens (0.2 mg/mL) were preincubated with either 5 mM EDTA, 5 mM $CaCl_2$, or 2 mM GPRP and digested with 10 μg/mL streptokinase-activated plasminogen for 4 h at 37 °C. The plasmin digests were analyzed on 7.5% SDS−PAGE. The fibrinogen degradation products are indicated on the left.

from further cleavage to D2 and D3. As expected, changes in the "a" site, such as in fibrinogen γD364A, specifically altered GPRP binding and conserved the ability to bind calcium (*29*). In contrast, fragments D2 and D3 were the dominant products of plasmin digests with all three variant fibrinogens under all conditions, as shown in Figure 1. Fragment D1 was still evident in digests of both γD318A and γD320A fibrinogens in the presence of calcium or GPRP, demonstrating partial protection from further cleavage as expected for reduced binding of these ligands. For the doubly substituted variant γD318+320A fibrinogen, fragment D1 was scarcely evident in the presence of either calcium or GPRP, indicating complete loss of protection. We concluded that the Ala substitutions impaired both calcium binding to the altered calcium-binding site and GPRP peptide binding to the adjacent polymerization "a" site.

Fibrinopeptide Release. We followed thrombin-catalyzed release of FpA and FpB by measuring the fraction of released peptides following their separation on HPLC. As shown in Figure 2, the rate of FpA release from each of the three variants was the same as from normal fibrinogen. In contrast, FpB release was markedly delayed relative to normal. We ascribed the delay in the FpB release to impaired polymerization, as it has been reported that protofibril formation enhances FpB release (*30*). We also measured fibrinopeptide release from γD318A and γD320A using the reaction conditions of the turbidity experiments depicted in Figure 3, and found that FpB was completely released by the end of the lag periods (data not shown). In addition, we demonstrated that batroxobin-catalyzed release of FpA from γD318A and γD320A fibrinogens was not different from normal and was complete after an hour incubation (data not shown).

Turbidity Measurements. We monitored polymerization as the change in turbidity at 350 nm, measuring the lag time, which reflects protofibril formation, and the V_{max}, which reflects lateral aggregation. We examined thrombin-catalyzed polymerization at two concentrations of calcium, 5 mM and without added calcium, and found that the polymerization was severely impaired. Representative data are shown in Figure 3A; average data are given in Table 1. For both fibrinogens with single substitutions, γD318A and γD320A, polymerization at either calcium concentration was very slow, with markedly prolonged lag times and decreased V_{max} values, as compared to normal fibrinogen. Comparing the two variant fibrinogens, the data show that polymerization of γD318A was slower than γD320A. These polymerization data are consistent with the results of the plasmin protection assays. As shown in Figure 1, in the presence of GPRP, plasmin digests of both variants showed partial protection, indicating the "a" site is impaired but not lost. Further, digests of the γD320A variant were more like normal than the γD318A variant, consistent with the turbidity data that show polymerization of γD320A was less impaired than polymerization of γD318A. Moreover, the two substitutions seem to have an additive effect, as polymerization of the double variant, γD318+320A fibrinogen, was more impaired with no increase in turbidity after 4 h. We concluded that the substitutions γD318A, γD320A, and γD318+320A markedly impaired thrombin-catalyzed fibrin polymerization, consistent with the loss of GPRP binding.

We also examined batroxobin-catalyzed polymerization, as shown in Figure 3B. Surprisingly, with all three variants, we saw no increase in turbidity, under conditions where FpA was completely released after 1 h. The discrepancy between the thrombin and batroxobin data indicated that polymerization of γD318A and γD320A fibrinogens required the release of FpB. To test this possibility, we examined thrombin-catalyzed polymerization in the presence of the peptide GHRP, which mimics the "B" site and binds to the

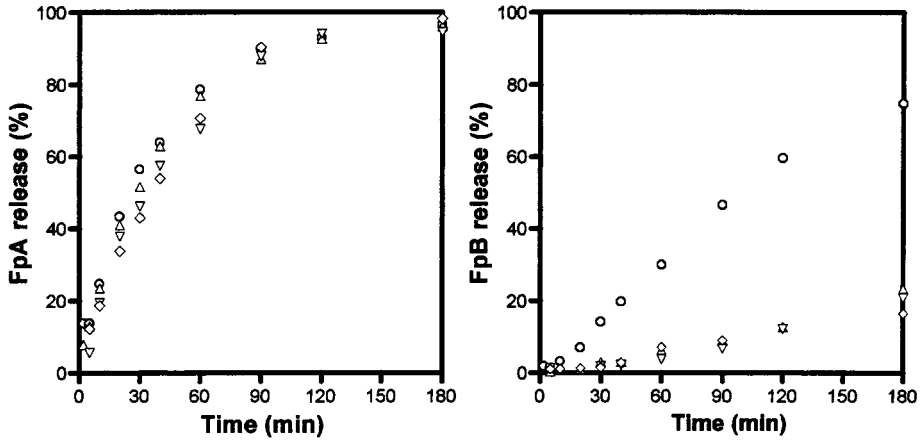

FIGURE 2: Kinetic release of FpA and FpB. Thrombin-catalyzed release of FpA (left panel) and FpB (right panel) of normal (circles), γD318A (triangles), γD320A (inverted triangles), and γD318+320A fibrinogen (diamonds). The reactions were initiated by adding 0.005 NIH unit/mL thrombin to 0.1 mg/mL fibrinogen, stopped at the indicated times points, and analyzed by HPLC as described under Experimental Procedures.

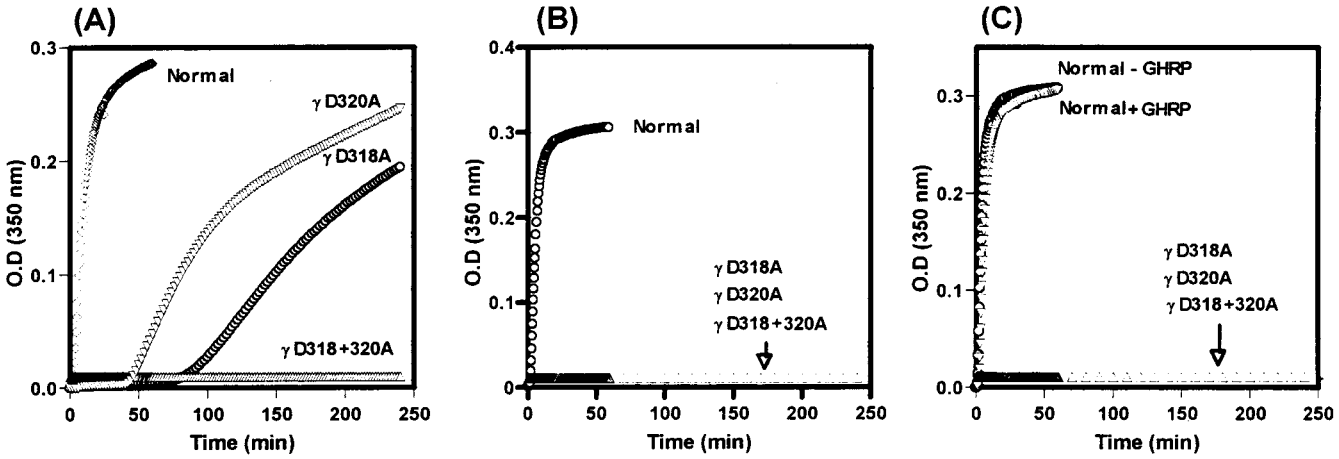

FIGURE 3: Turbidity measurement of fibrin polymerization. The polymerization reactions were initiated by adding 0.1 NIH unit/mL thrombin (panel A) or 0.1 unit/mL batroxobin (panel B) to normal or variant fibrinogens (0.2 mg/mL). The optical density at 350 nm was recorded continuously. In panel C, the fibrinogens were preincubated with 2 mM GHRP peptide for 30 min, prior to adding thrombin (0.1 NIH unit/mL). The data presented represent the average of the curves.

Table 1: Polymerization Parameters of Normal and Variant Fibrinogens

	lag period ($\times 10^3$ s)	V_{max} ($\times 10^{-5}$ s^{-1})
normal[a]	0.22 ± 0.01	25.9 ± 4.9
γD318A		
−$CaCl_2$	4.9 ± 0.4	1.83 ± 0.08
+$CaCl_2$	3.9 ± 0.2	1.57 ± 0.34
γD320A		
−$CaCl_2$	2.4 ± 0.1	3.01 ± 0.6
+$CaCl_2$	2.1 ± 0.5	2.68 ± 1.47

[a] Polymerization experiments were done in the absence of added calcium.

"b" polymerization site (*1*). As shown in Figure 3C, the addition of 2 mM GHRP completely abolished the thrombin-catalyzed polymerization of γD318A and γD320A fibrinogens, whereas polymerization of normal fibrinogen was not changed by the addition of GHRP. Considering the data in Figure 3B,C, we concluded that polymerization of γD318A and γD320A fibrinogens proceeds through a mechanism that involves "B−b" interactions.

Gel Filtration. To further characterize fibrin polymerization of γD318A and γD320A fibrinogens, we examined the early polymerization products by gel filtration. We did not examine γD318+320A fibrinogen, as this variant did not show an increase in turbidity. Thrombin or batroxobin was added to normal and variant fibrinogens, and at time points near the end of the lag period, the reaction mixtures were immediately loaded onto a Superose 6B column, which was able to separate monomers from polymers with no indication of the polymer size. Chromatograms of control reactions in the absence of enzyme showed one major peak corresponding to monomeric fibrinogen molecules (Figure 4A−C). After a 2 min incubation of normal fibrinogen with thrombin, the elution profile showed two peaks corresponding to fibrin monomers and polymers (Figure 4D). Under similar conditions, two peaks were also seen with both γD318A and γD320A fibrinogens after significantly longer incubation times corresponding to the lag periods, 80 and 40 min, respectively (Figure 4E,F). Similarly, after a 2 min incubation of normal fibrinogen with batroxobin, the elution profile showed both monomer and polymer peaks (Figure 4G). In contrast, only one peak corresponding to fibrinogen monomers was evident in chromatograms of either variant following incubation with batroxobin for 80 or 40 min, respectively (Figure 4H,I). We also examined thrombin-catalyzed polymer formation in the presence of 2 mM GHRP. With normal fibrinogen, the elution profile in the presence or absence of GHRP was not changed (Figure 4J). In contrast, in the presence of GHRP, only the peak corresponding to fibrin monomers was seen following incubation of γD318A and γD320A fibrinogen with thrombin for 80 and 40 min, respectively (Figure 4K,L). These data confirmed the inference of the turbidity data, that the variants γD318A and γD320A must release both FpA and FpB to assemble polymers. Further, the gel filtration data suggest that "B−

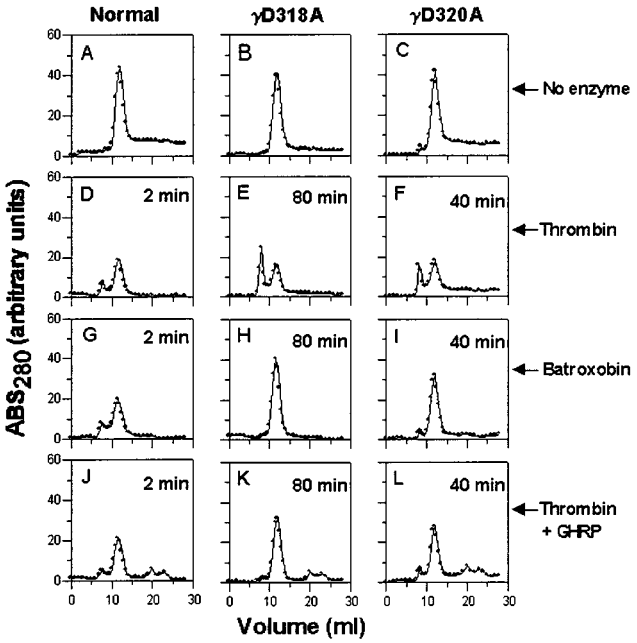

FIGURE 4: Analysis of fibrin polymers with gel filtration. Thrombin or batroxobin (0.1 unit/mL) was added to normal or variant fibrinogens (0.2 mg/mL) in the presence or absence of 2 mM GHRP, and the at times specified, the mixtures were loaded onto the column. The eluted products were monitored by measuring the absorbance at 280 nm. Reprentative chromatograms are shown indicating the fibrinogens and the enzymes on the top and the right sides, respectively, in addition to the enzyme incubation time for each sample.

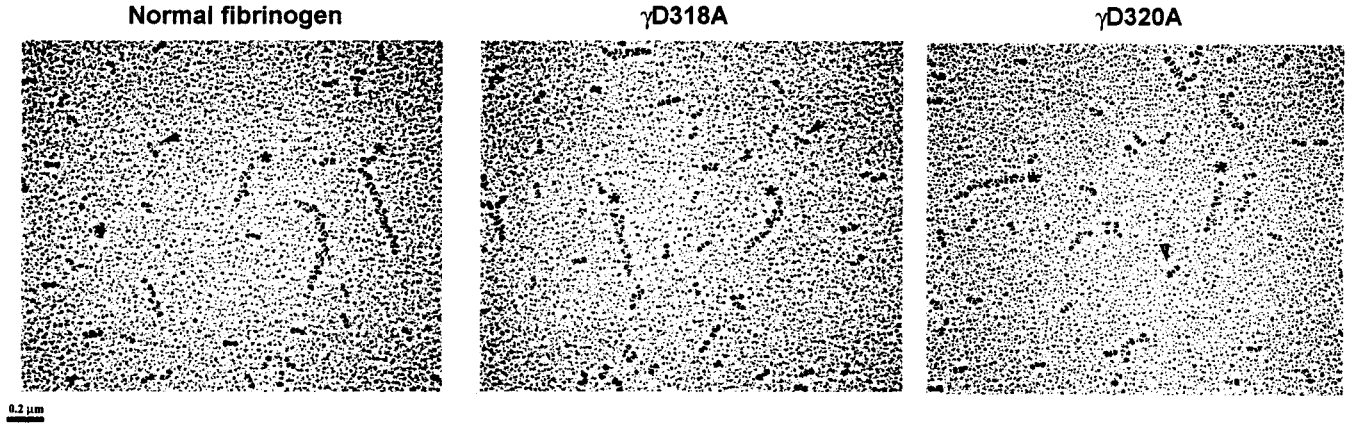

FIGURE 5: Transmission electron microscopy. Thrombin was mixed with fibrinogen and incubated for 2 min (normal), 40 min (γD320A), and 80 min (γD318A). The corresponding sample was immediately diluted and sprayed onto mica. The mica samples were stained as indicated under Experimental Procedures. Scale bar represents 0.2 μM. Arrows indicate single fibrin monomers and stars elongated protofibrils.

b" interactions are critical early in polymerization, prior to the formation of fibrin fibers that are detectable by turbidity measurements.

Transmission Electronic Microscopy. We examined the structures of the thrombin-generated polymers by TEM. The samples were prepared as for gel filtration, such that we examined the structures present near the end of the lag period. Representative TEM pictures are shown in Figure 5. Consistent with the chromatography data, we saw both monomers and polymers with all three fibrinogen variants. Moreover, the structures of the polymers formed by γD318A and γD320A fibrinogens were comparable to those seen with normal fibrinogen. We concluded that both variant fibrinogens formed protofibrils whose structures were comparable to normal protofibrils, although the kinetics of polymerization were substantially slower.

FXIII-Catalyzed γ-Chain Cross-Linking. We performed FXIII-catalyzed cross-linking of fibrin to determine whether the γ-chain cross-linking sites were functional in the variant fibrinogens. As shown in Figure 6A, activated FXIII introduced cross-links between γ-chains of all three variant fibrinogens. As judged from these gels, the normal γ-chain monomer was completely transformed to $\gamma-\gamma$ dimer, while differing amounts of γ-chain monomer remained in the variant fibrinogens. In another set of experiments performed in similar conditions, the insoluble clots were removed by certrifugation and analyzed. As shown in Figure 6B, only $\gamma-\gamma$ dimers were detected in normal, γD318A, or γD320A clots. No $\gamma-\gamma$ dimers were seen in fibrinogen γD318+320A, presumably because this variant did not form a stable clot. We ascribed the delay in $\gamma-\gamma$ dimer formation for the variant fibrinogens to the impaired fibrin polymerization, as it is known that the rate of FXIIIa-catalyzed cross-linking of the γ-chains depends on the rate of protofibril formation (*31*, *32*). In control experiments, we found no $\gamma-\gamma$ dimer in reactions of normal fibrinogen incubated for 4 h with preactivated FXIII (data not shown), consistent with the conclusion that we measured cross-links formed between assembled monomers.

Platelet Aggregation. Because $\gamma\Delta$319,320 fibrinogen, which also eliminated the calcium-binding site, did not support platelet aggregation, we conducted platelet aggregation assays with all three variants. Representative experiments, shown in Figure 7, demonstrated that platelet

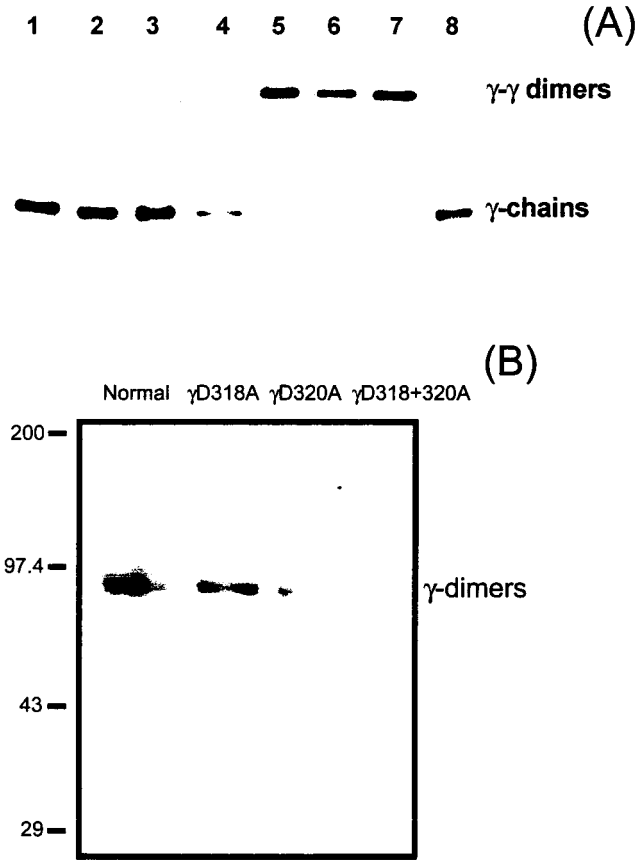

FIGURE 6: FXIII-catalyzed γ-chain cross-linking. The reaction consisted of adding 2 μg/mL FXIII and 0.1 NIH unit/mL thrombin to 0.2 mg/mL fibrinogen in HEPES buffer containing 2 mM $CaCl_2$. The γ-dimer formation in the whole sample is shown in panel A; normal fibrinogen (lane 5), γD318A (lane 6), γD320A (lane 7), and γD318+320A (lane 8), and the corresponding non-cross-linked normal and variant fibrinogens, are indicated in lanes 1, 2, 3, and 4, respectively. The γ-dimer formation in the fraction containing only the insoluble clot is shown in panel B; the normal and variant fibrinogens are indicated on the top of the blot.

aggregation was impaired. When gel-filtered platelets were mixed with normal fibrinogen and aggregation was induced by the addition of 10 μM ADP, we saw the expected dramatic rise in light transmission due to the formation of aggregates. When platelets were mixed with either γD318A or γD320A fibrinogen, we saw a substantially reduced rise in light transmission, and the extent of aggregation with γD320A

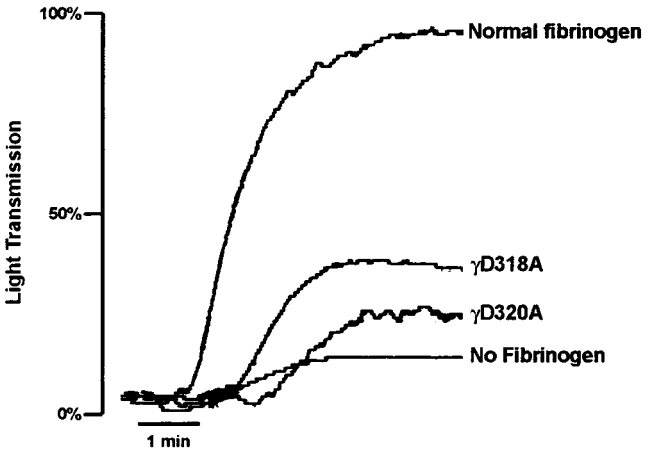

FIGURE 7: ADP-induced platelet aggregation. 2×10^8 platelets were mixed with 300 nM fibrinogen and preincubated at 37 °C for 2 min prior to adding 10 μM ADP. Platelet aggregation of stirred solutions was monitored as an increase in light transmission.

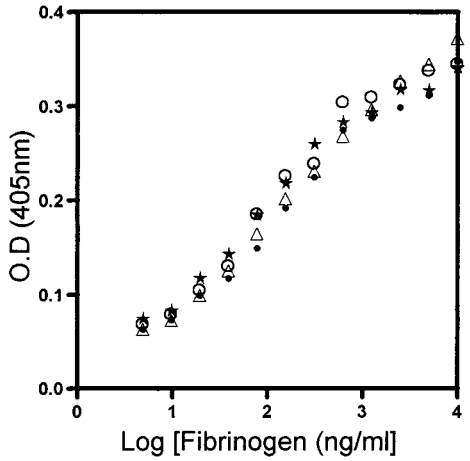

FIGURE 8: Binding of fibrinogen to Mab 4A5. The accessibility of the αIIbβ3 binding site (γ400−411) in solution was examined by using the monoclonal antibody 4A5. The normal (open circles), γD318A (triangles), γD320A (closed circles), and γD318+320A (stars) fibrinogens captured by immobilized 4A5 Mab were quantified using a rabbit peroxidase-conjugated polyclonal antibody.

fibrinogen was always less than with γD318A fibrinogen. In the presence of γD318+320A fibrinogen, we saw only a slight increase in light transmission, and this increase was not different from that observed when platelets were incubated without added fibrinogen (data not shown). These data suggest that the H12 site, which is essential for fibrinogen-mediated platelet aggregation (*26*, *33*), may be altered by the Ala substitutions in the calcium-binding site.

Binding of Fibrinogen to Mab 4A5. To test whether the γ401−411 site was present and accessible in these three variants, we measured binding of the variant fibrinogens in solution to immobilized Mab 4A5. This antibody recognizes residues γ401−411, but not the recombinant fibrinogen whose γ-chain ends with residue γ407 (*26*, *34*). We coated microtiter plate wells with 4A5 monoclonal antibody and measured binding with a colorimetric substrate and peroxidase-conjugated polyclonal antibody to fibrinogen. As shown in Figure 8, the binding of normal fibrinogen to Mab 4A5 was concentration dependent, and the binding profiles of all three variant fibrinogens were indistinguishable from normal. No fibrinogen was detected when normal or variant fibrinogens were added to albumin-coated wells, or in the absence of fibrinogen. These results demonstrated that residues γ401−411 were present and equally accessible in all fibrinogens tested, whether normal or variant.

DISCUSSION

Taken together, the data reported here indicate that the structural integrity of the high-affinity calcium-binding site, located in the γ-module, is essential for fibrinogen-mediated platelet aggregation and fibrin polymerization, but not for FXIIIa cross-linking. As discussed below, our results provide data to support two specific conclusions: (1) a site in the γ-module other than H12 is critical for platelet aggregation and (2) "B−b" interactions have a role in protofibril formation. Moreover, because our studies show that a single site is important for both platelet aggregation and fibrin polymerization, they suggest that fibrinogen has a role in regulating these two events. As one site is critical for both functions, it appears that the two events are conflicting. This conflict has been directly demonstrated as GPRP binding to normal fibrinogen inhibited platelet aggregation (*35*). In vivo, platelet aggregation and fibrin polymerization are nearly coincident in both time and space, yet some clots are platelet rich while others are predominantly fibrin. We believe the data presented here indicate that fibrinogen may regulate the in vivo clot structure.

Earlier investigations have shown that the γ-chain alone is sufficient (*36*−*39*), and the C-terminal residues γ407−411 are necessary for platelet aggregation (*26*). The current studies showed normal binding of Mab 4A5, demonstrating that γ407−411 was present and accessible in the variants γD318A, γD320A, and γD318+320A. Our studies also showed that FXIIIa-catalyzed cross-linking of the single substituted variants was normal, suggesting that the C-terminal domain in these variants can support normal function and thus has a conformation analogous to normal fibrinogen. Nevertheless, we found that none of the variants supported normal platelet aggregation. We conclude that an additional site, which was altered in these variants, is critical for platelet aggregation. This site may act independently from the C-terminal site, or may form a composite site with the C-terminal site. Novel fibrinogen sites that participate in binding to αIIbβ3, the platelet receptor that mediates aggregation, were first suggested by Parise et al. (*40*). They examined αIIbβ3 binding to fibrinogen immobilized on Sepharose and found receptor binding was not inhibited by either RGD-containing peptides or H12, a peptide analogous to γ400−411. We also examined platelet binding to immobilized fibrinogen and found that platelet binding to immobilized fibrinogens γD318A, γD320A, and γD318+320A was significantly lower than to immobilized normal fibrinogen (*41*). We conclude that a novel site, which was altered in our variants, functions either alone or in conjuction with the known C-terminal γ-chain site to mediate platelet aggregation.

Thrombin-catalyzed polymerization was severely impaired with all three variant fibrinogens. This result was expected following the plasmin cleavage experiments (Figure 1) that showed the disruption of "A−a" interactions. This result was also consistent with previous work that demonstrated polymerization was impaired for dysfibrinogens with substitutions in this calcium-binding site (*13*, *14*). Unexpectedly, we

found batroxobin-catalyzed polymerization was undetectable in multiple assays. We conclude that "A−a" interactions, which support normal protofibril formation, are either not present in these variants or weakened such that they are unable to sustain protofibril formation alone. Rather, "B−b" interactions, which become available following release of FpB, support the minimal thrombin-catalyzed polymerization found with the single substituted fibrinogens, γD318A and γD320A. This unusual role for "B−b" interactions was apparent in our chromatography studies that showed GHRP inhibited thrombin-induced polymer formation (Figure 4K,L) of these variants. We considered the possibility that GHRP could bind to the polymerization site "a" (*1*), but we believe this is unlikely because GHRP did not alter plasmin cleavage patterns for fibrinogens γD318A and γD320A (data not shown).

The electron microscopy data specifically showed that in the two single substituted variants the "B−b" interactions support protofibril formation. This is in agreement with the data reported previously by J. Weisel et al., which showed that at low temperature (14 °C) the exclusive release of FpB could lead to fibrin formation (*6*). The idea that "B−b" interactions can support protofibril formation is seen in the modified model of polymerization proposed by Yang et al. (*5*). The detailed interactions of this model were derived from the crystal packing interactions found in structures of cross-linked D−D fibrinogen fragments. By comparing the structure formed in the presence of peptides GPRP and GHRP to that formed without peptides, the authors found the intermolecular contacts were different. Based on these differences, they proposed a model that included specific interactions that are critical to lateral aggregation. An unanticipated aspect of this model was that the "B−b" interactions are juxtaposed to the "A−a" interactions, with both pairs occurring between the same fibrin molecules. With this model, one could anticipate that either "A−a" or "B−b" interactions will support protofibril formation. It is well-known that "A−a" interactions alone can support protofibril formation, as is seen with batroxobin, and there are reports that "B−b" interactions can mediate polymerization, though only at low temperatures (*42*). The juxtaposition of the N-termini of the α- and β-chains during polymerization had been suggested earlier by Moskowitz et al., who proposed that fragment E binds to cross-linked fragment D−D through a composite site containing residues α17−19 and β20−49. They specifically excluded the "B" site (β15−18), because GHRP did not dissociate the D−DE complex (*43*). We believe these apparent contradictions are simply resolved by considering the different affinities for "A−a" and "B−b" interactions. Thus, with normal fibrinogen, the early release of FpA leads to the formation of strong "A−a" interactions, which are sufficient for relatively normal polymerization. After release of FpB, the formation of "B−b" interactions reinforces the strand associations in protofibrils, producing a stronger clot (*6*). When "A−a" interactions are weak or disrupted, as with the fibrinogen variants examined here, then the "B−b" interactions are able to support protofibril formation. An important implication of this line of reasoning is that the interactions that mediate lateral aggregation occur only between the outer D nodules where novel binding sites are exposed following protofibril formation.

Our data also showed that the structure of this region is critical for normal function. Although these aspartic acid to alanine substitutions altered the side chains known to bind calcium and not residues in the "a" site, binding of both calcium and the GPRP peptide was impaired. This indicates that the structure of the "a" site was altered in these variants. We know, in normal fibrinogen, that the "a" site can function in the absence of bound calcium, because GPRP can bind to normal fibrinogen in the presence of EDTA, as shown by the plasmin protection assay. This indicates that the substitutions γD318A, γD320A, and γD318+320A not only prevented calcium binding, but also induced changes that hindered GPRP binding. To reconcile these conflicting results, we considered the nature of the aspartic acids per se. Without bound calcium, the orientations of the side chains of γD318 and γD320 in normal fibrinogen are likely altered to minimize the repulsive forces between like charged residues; the altered orientation may stabilize the structure in a conformation that permits GRPR binding. In contrast, with alanine at one or both of these positions, the repulsive forces are not present, such that the orientation of the altered site hinders GPRP binding.

Although we suggest that conformational changes occur with these substitutions, we believe these changes are relatively local. Previously, we have shown the loss of all the hemostatic functions in fibrinogen γΔ319,320, the recombinant equivalent of fibrinogen Vlissingen. From these experiments, we concluded that the overall conformation of the C-terminal γ-domain (γ143−411) was extensively altered by this two-residue deletion (*18*). In contrast, the variants γD318A and γD320A were able to form protofibrils and FXIIIa-catalyzed γ-dimers, indicating that the conformation of this domain was more like normal. From the current experiments, we propose that the single aspartic acid to alanine substitutions induced changes in the two juxtaposed loop structures that form the calcium-binding site and the "a"-binding site, while the remainder of the domain retained a relatively normal structure.

In summary, analysis of three variant fibrinogens with substitutions designed to abrogate a calcium-binding site has shown that one part of the C-terminal γ-chain is critical for both platelet aggregation and fibrin polymerization. The results suggest that a site other than H12 is necessary for platelet aggregation, that "B−b" interactions occur in parallel with "A−a" interactions during fibrin polymerization, and that fibrinogen may have a regulatory role in clot organization. We plan to examine this last possibility using these variant fibrinogens to form clots in a reconstituted system with platelets and coagulation factors.

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