Type: Original Article
Running head: B:b binding in variant fibrinogen at γ364Asp
Section: Coagulation

B:b interactions are essential for polymerization of variant fibrinogens with impaired holes "a"

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Parts of these results were presented at the XXIst Congress of the International Society on Thrombosis and Haemostasis; Journal Thrombosis Haemostasis 2007; 5, Supplement 2, P-W-389.

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Word count: summary, 240; manuscript, 4949.

Summary

Background: Fibrin polymerization is mediated by interactions between knobs "A" and "B" exposed by thrombin cleavage, and holes "a" and "b" always present in fibrinogen. The role of A:a interactions is well-established, but the roles of knob:hole interactions A:b, B:b, or B:a remain ambiguous. *Objectives:* To determine whether A:b or B:b interactions have a role in thrombin-catalyzed polymerization, we examined a series of fibrinogen variants with substitutions altering holes "a": γ364Ala, γ364His, or γ364Val. *Methods:* We examined thrombin- and reptilase-catalyzed fibrinopeptide release by HPLC, fibrin clot formation by turbidity, fibrin clot structure by scanning electron microscopy (SEM) and FXIIIa-catalyzed crosslinking by SDS-PAGE. *Results:* Thrombin-catalyzed fibrinopeptide A release was normal, but fibrinopeptide B release was delayed for all variants. The variant fibrinogens all showed markedly impaired thrombin-catalyzed polymerization; polymerization of γ364Val and γ364His were more delayed than γ364Ala. There was absolutely no polymerization of any variant with reptilase, which exposed only knobs "A". SEM showed that the variant clots formed after 24 hours had uniform, ordered fibers that were thicker than normal. Polymerization of the variant fibrinogens was inhibited dose-dependently by the addition of either GPRP or GHRP, peptides that specifically block holes "a" and "b", respectively. Factor XIIIa-catalyzed cross-linking between γ-chains was markedly delayed for all the variants.

Conclusion: These results demonstrate B:b interactions are critical for polymerization of variant fibrinogens with impaired holes "a". Based on these data, we propose a model wherein B:b interactions participate in protofibril formation.

Keywords: fibrin polymerization, holes "a", holes "b", B:b interactions, knob "B", dysfibrinogen,

Introduction

Fibrinogen is a 340-kDa plasma glycoprotein consisting of two copies of three polypeptide chains, Aα, Bβ and γ [1]. The six chains are arranged into three globular nodules connected by linear segments (Figure 1). The central E-nodule contains the N-termini of all chains. Coiled-coils of the three chains form the two connectors. The distal D-nodules contain the independently-folded C-termini of the β- and γ-chains, called the βC- and the γC-modules. The C-termini of the α-chains extend briefly through the D-nodule and fold back into the coiled-coil; thereafter, the structure of this domain is uncertain [1].

During coagulation, thrombin cleaves fibrinogen, releasing fibrinopeptide A (FpA) and fibrinopeptide B (FpB) from the N-termini of the Aα- and Bβ-chains, respectively, converting fibrinogen to fibrin monomers [1]. Fibrin monomers polymerize spontaneously through a two-step process: the formation of half-staggered, double-stranded protofibrils and the lateral aggregation of protofibrils into fibers. The interactions that promote protofibril formation are well-established. The release of FpA exposes a new N-terminal segment, called knob "A", that binds to the hole "a" in the γC-module of another fibrin(ogen) molecule (Figure 1). These A:a interactions mediate the formation of protofibrils. The interactions that promote lateral aggregation are unclear, but the release of FpB enhances the extent of lateral aggregation [2]. Thus, exposure of the knobs "B" and subsequent B:b interactions may contribute to this phase of polymerization.

Three decades ago, the synthetic peptide Gly-Pro-Arg-Pro (GPRP), which mimics knob "A", was shown to bind to fibrinogen and inhibit thrombin-catalyzed fibrin polymerization. In contrast, the peptide Gly-His-Arg-Pro (GHRP), which mimics knob "B", binds to fibrinogen but does not inhibit thrombin-catalyzed fibrin polymerization [3]. However, the knob "B" peptide GHR does inhibit polymerization catalyzed by venzyme, a snake venom enzyme that removes predominantly FpB [4]. Crystallographic studies have provided high-resolution structures of the D-nodule in the presence of these synthetic peptides. These studies identified the molecular details of the A:a and B:b interactions, and showed that the two interactions share many features. Indeed, the crystallography studies also showed that each of these peptides was able to bind in both sites [5-8], indicating thrombin-catalyzed fibrin polymerization might be mediated by A:b, B:b, or B:a interactions in addition to the A:a interactions known to participate in protofibril formation.

In previous work we have used recombinant variant fibrinogens to show that the residue γ364D, located at the base of the hole "a", is critical to normal thrombin-catalyzed polymerization [9]. Nevertheless, the following fundamental question remains to be answered: what interactions mediate thrombin-catalyzed polymerization of variant fibrinogens with impaired holes "a"? Here we describe analysis of three variant fibrinogens: γ364A, γ364H (analogous to dysfibrinogen Matsumoto I [10]), and γ364V (analogous to dysfibrinogen Melun [11]). Our data indicate that thrombin-catalyzed polymerization of these variant fibrinogens is contingent upon B:b knob-hole interactions.

Methods

Preparation of recombinant variant fibrinogens. The fibrinogen γ-chain expression vector, pMLP-γ, was altered by oligonucleotide-directed mutagenesis using a Transformer Site-Directed Mutagenesis kit (CLONTECH Laboratories, Palo Alto, CA) and 5'-phosphorylated primers for mutagenesis (the altered base is underlined; 5'-AATGGTTATGTTAATGGCAT) for γ364V, and selection (5'-TCTAGGGCCCAGGCTTGTTTGC) [9]. The resultant expression vector, γ364V, was transfected into Chinese hamster ovary (CHO) cells that expressed normal human fibrinogen Aα- and Bβ-chains, and the stable transfectants were selected as described [9]. CHO cell lines producing γ364H-, γ364A- and normal (γ364D)-fibrinogen have been described [9]. Fibrinogen was purified from the harvested culture medium by ammonium sulfate precipitation followed by immunoaffinity chromatography utilizing a calcium-dependent monoclonal antibody (IF-1, Iatron Laboratories, Tokyo) [9]. Eluted fibrinogen was pooled and dialyzed against 20 mM N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid] (HEPES), pH 7.4, 0.12 M NaCl (HBS) [9]. The fibrinogen concentration was determined from the $\Delta A_{280\text{-}320}$, assuming a 1 mg/mL solution has a Δabsorbance of 1.51.

Kinetics of FpA and FpB release by thrombin or reptilase. Fibrinopeptide release was monitored by HPLC, as described [12]. Fibrinogen (0.15 mg/ml) in HBS was added to human α-thrombin (0.01 U/ml) (Enzyme Research Laboratories, South Bend, IN; 3265 U/mg) or reptilase-R (0.01 U/ml) (snake venom from *Bothrops atrox*, Pentapharm Ltd., Basel, Switzerland; 70 U/mg) and incubated at 25˚C. FpA data were fitted to a simple first-order, while FpB data were fitted to a standard equation describing two first-order processes, and the curves were plotted using DeltaGraph (DeltaPoint Inc., Monterey, CA). To calculate the percent fibrinopeptide release, the amount of FpA or FpB released at 2 h of incubation with a 120-fold concentration of thrombin or a 12-fold concentration of reptilase at 37˚C was taken as 100%. The specificity constant, k_{cat}/K_m, was determined as described [9].

Thrombin or reptilase-catalyzed fibrin polymerization. Polymerization at ambient temperature was monitored at 350 nm using a UV-110-02 spectrophotometer (Shimadzu Corp., Tokyo, Japan). Briefly, fibrinogen (0.45 mg/ml) in HBS supplemented with various concentrations of $CaCl_2$ was mixed with human α-thrombin (0.05 U/ml) or reptilase-R (0.05 U/ml). The reactions were performed in triplicate and lag time, maximum slope, and final optical density were obtained from the turbidity curves, as described elsewhere [12].

Clottability of the fibrinogens was determined in triplicate, by mixing thrombin (0.05 U/ml) and fibrinogen (0.45 mg/ml) in HBS containing 1 mM $CaCl_2$ and incubating for 24 h at 37 °C. Clottability was calculated as described [9].

Inhibition of polymerization by the synthetic peptides, GPRP (acetate salt, purity >97%; SIGMA-ALDRICH, St. Louis, MO), GHRP (acetate salt, purity 95.01%; OPERON Biotechnologies, Tokyo, Japan), or GGG (MP Biomedicals Inc., Solon OH), was determined by adding each peptide to the fibrinogen solution prior to mixing with thrombin.

Factor XIIIa-catalyzed cross-linking of fibrin or fibrinogen. FXIII (Enzyme Research Laboratories, South Bend, IN) was activated with human α-thrombin for 60 min at 37 °C in HBS with 5 mM $CaCl_2$ [13]. To examine the cross-linking of fibrin, fibrinogen (0.47 mg/ml) was incubated at 37 °C in HBS with 5 mM $CaCl_2$ with a mixture of FXIIIa (3.3 U/ml) and human α-thrombin (0.07 U/ml). To examine the cross-linking of fibrinogen or

reptilase-catalyzed fibrin, hirudin (10 U/ml) was added to thrombin-activated FXIIIa prior to incubation with fibrinogen or with fibrinogen that had been incubated with reptilase (0.05 U/ml). The reactions were stopped by the addition of an equal volume of sodium dodecyl sulfate (SDS)-sample buffer with 2-mercaptoethanol followed by incubation at 100 ºC. Samples equivalent to 2.5 μg of fibrinogen were separated by 8 % SDS-polyacrylamide gel electrophoresis (PAGE) and stained with Coomassie Brilliant Blue R-250. Densitometric analyses of stained gels were performed using the Rapid Electrophoresis System (Helena Lab, Saitama, Japan) and γ-γ/Bβ ratios were calculated.

Scanning electron microscopy. Samples for scanning electron microscopy were prepared as described [11]. Briefly, fibrin clots were formed from fibrinogen (0.4 mg/ml) and thrombin (0.1 U/ml), incubated at 37 °C for 24 h. Images were recorded at 3000x or 20000x magnification. Fiber diameters were measured using a vernier caliper on a 300% enlargement from a photograph made at 20000x magnification.

Statistical analysis. The statistical significance of differences between normal control and variant fibrinogen was determined using unpaired *t*-tests. A difference was considered significant when the *p*-value was <0.05.

Results

Fibrinopeptide release.

We monitored thrombin- and reptilase-catalyzed fibrinopeptide release by HPLC as described in methods; the data are shown in Figure 2. The k_{cat}/K_m values (unit: $\times 10^6$M/sec) obtained with thrombin for FpA release from the three variant fibrinogens, γ364H (6.6 ± 0.2), γ364V (6.6 ± 0.2), and γ364A (7.2 ± 0.2), were not significantly different from that for normal-fibrinogen (7.3 ± 0.2). In contrast, the k_{cat}/K_m values obtained for FpB release from the variant fibrinogens, γ364A (0.79 ± 0.02, *p<0.01*), γ364H (0.86 ± 0.03, *p<0.01*), and γ364V (0.75 ± 0.01, *p<0.001*), were significantly less than normal fibrinogen (3.6 ± 0.1). Reptilase-catalyzed FpA release was more rapid than thrombin-catalyzed, using equivalent units as defined by the vendors. The rate of reptilase-catalyzed FpA release from the three variants was not different from normal. Importantly, no FpB release was observed from any of the fibrinogens even at a 12-fold higher concentration of reptilase.

Polymerization.

Polymerization of normal and variant fibrinogens was measured by turbidity. Representative curves are shown in Figure 3 and averaged data are shown in Table 1, column 1 mM Ca. When we examined reactions for 3 h (Figure 3B), polymerization of γ364A-fibrinogen was markedly impaired such that the lag period (41 ± 3 min) was longer and the maximum slope (26 ± 2 $\times 10^{-5}$/sec) was lower compared to normal fibrinogen (lag period: 2.4 ± 0.1 min, maximum slope: 397 ± 21 $\times 10^{-5}$/sec; Figure 3A). No change in turbidity was apparent for the other variants at 3 h. Remarkably, when turbidity was monitored for longer times (Figure 3B), changes became apparent for both γ364H- and γ364V-fibrinogens, with lag times of 208 ± 15 and 169 ± 13 min, respectively. In contrast, we saw no change in turbidity with any of the variants (Figure 3D) when we examined reptilase-catalyzed polymerization under conditions where normal fibrinogen reached a maximal turbidity at 30 min (Figure 3C). No turbidity was evident even for reactions as long as for 24 h (data not shown). As reptilase removed only FpA, we conclude that the turbidity seen with thrombin required the release of FpB.

To further examine these marked differences in the thrombin-catalyzed polymerization, we measured clottability after 24 h. As is typical, the clottability was 97 ± 2% for normal fibrinogen. For γ364A-, γ364H-, and γ364V-fibrinogens, the corresponding values were 90 ± 3% ($p>0.05$), 49 ± 6% ($p<0.01$), and 47 ± 5% ($p<0.001$), respectively. To determine whether these insoluble clots had an organized structure, we examined them by scanning electron microscopy; representative images are shown in Figure 4. Surprisingly, in all cases the images showed organized clots with uniform fibers. All the variant clots appeared different from normal, with thicker fibers and larger pores. Measurement of the fiber diameters showed the variant fibers were thicker: 288 ± 60 nm for γ364A (n = 27, $p<0.001$), 214 ± 35 nm for γ364H (n = 30, $p<0.001$), and 247 ± 80 nm for γ364V (n = 36, $p<0.001$) compared to 106 ± 27 nm for normal (n = 44) fibrinogen. In the microscopy images the variant fibers appeared more twisted and perhaps more translucent than those in the normal clot (Figure 4E-H).

Because normal polymerization varies with the calcium concentration and because the release of FpB has been linked to changes in calcium binding during polymerization [14], we examined thrombin-catalyzed polymerization at four calcium concentrations. The data are summarized in Table 1. For normal fibrinogen the lag period was shorter and the maximum slope steeper in the presence of 1 mM calcium relative to no added calcium, as previously reported [15]. In contrast, comparing polymerization at 1 mM calcium to that with no added calcium, there was no difference in maximum slope for γ364A, and no difference in either lag time or maximum slope for γ364H and γ364V. These data indicate polymerization of the variants proceeds by a different path from normal, a path that is partially or completely independent from modulation by < 1 mM calcium. We also examined the calcium dependence of reptilase-catalyzed polymerization, and observed no turbidity signal for any of the variant fibrinogens at any of the four calcium concentrations (data not shown).

Inhibition of thrombin-catalyzed fibrin polymerization by GPRP or GHRP. To examine the participation of B:b and/or B:a interactions in polymerization of the variant fibrinogens, we assessed polymerization in the presence of peptides that mimic the knobs "A" and "B", GPRP and GHRP, respectively. We followed turbidity with time (curves not shown) and determined the percent absorbance at 30 min for normal fibrinogen, at 3 h for γ364A and 10 h for γ364V and γ364H (Figure 5). Because polymerization of the variants was optimal at 0.1 mM calcium, the peptide inhibition studies were performed in HBS with 0.1 mM $CaCl_2$. In the presence of GPRP, polymerization was inhibited to the same extent for all four fibrinogens; 1 mM peptide was sufficient to completely inhibit polymerization (Figure 5). In contrast, the addition of 1 mM GHRP to normal fibrinogen led to only a 5% decrease in the final absorbance (Figure 5A), whereas, with the variant fibrinogens 0.5 mM GHRP completely or almost completely (95%) inhibited polymerization (Figure 5B, 5C, and 5D). This inhibition by GHRP indicates the variant fibrinogen polymers were all dissociated when peptide was bound in hole "b". Using GGG as a control peptide, we saw no inhibition of polymerization under any of these conditions (data not shown).

FXIIIa-catalyzed cross-linking. Brummel *et al.* have shown that FXIIIa-catalyzed γ-γ dimers form early in polymerization, likely as soon as protofibrils are present [16]. We therefore analyzed by SDS-PAGE (Figure 6) the kinetics of γ-γ dimer formation to further characterize this early phase of polymerization of the variants. With normal fibrinogen, the γ-γ dimer band was weakly evident after 2 min and increased in intensity with longer incubation (Figure 6A).

With γ364A, the γ-γ dimer band was clearly evident after 20 min (Figure 6B), while with γ364H and γ364V the γ-γ dimer band was clearly evident after 60 min (Figure 6C and 6D). These data indicate that polymer formation was delayed for all three variants, and that polymerization of γ364A, proceeded more rapidly than polymerization of γ364H and γ364V. We also performed experiments in the presence of FXIIIa and reptilase (Figure 6F-J). With normal fibrinogen, the γ-γ dimer was again weakly evident at 2 min and increased in intensity with longer incubation (Figure 6F). In contrast, the variant fibrinogens showed only weak γ-γ dimer bands after 60 min of incubation, and the intensity of this band changed minimally with time. These data were almost the same as those for γ-γ dimer formation from fibrinogen (data not shown), indicating that the bands observed with the variants and reptilase did not depend on polymer formation.

Discussion

Our previous work with γ364A- and γ364H-fibrinogens showed impaired thrombin-catalyzed polymerization [9]. The analyses presented herein of these variants and γ364V-fibrinogen provide insight into the molecular basis for the altered function. Our new data are all consistent with the conclusion that holes "a" are lost or severely impaired in these variant fibrinogens. As a result, A:a interactions are lost and protofibril formation is delayed. Further, our data suggest that B:b interactions are necessary for fibrin fiber formation when A:a interactions are compromised.

Our studies with reptilase showed normal release of FpA from all three variants, but polymerization was not evident by either turbidity or γ-γ dimer formation. These data indicate that A:a interactions are compromised and that A:b interactions alone cannot mediate polymerization of these variants. Our studies with thrombin showed normal release of FpA, but markedly longer lag times by turbidity. We interpret these findings using the kinetic model developed by Weisel and Nagaswami. They modeled the development of turbidity with five steps: the release of FpA, the initiation of protofibrils from monomers, the elongation of protofibrils, the aggregation of two protofibrils to initiate a fiber, and the growth in fiber diameter. This model shows an increased lag time correlates with a decreased rate of either FpA release or protofibril initiation [17]. Thus, the data indicate that the rate of protofibril initiation is markedly slower with the variants. We also observed a delayed release of FpB, consistent with previous work that suggests FpB release occurs more rapidly from protofibrils [18]. Considering these results together, we conclude that A:a interactions are lost and as a result protofibril formation is delayed.

Nevertheless, organized clots were formed, as shown in SEM images. Fibers formed from the variants were thicker than normal. The thicker fiber is consistent with the kinetic model, which would indeed predict that a longer lag time is associated with an increased fiber diameter [17]. Still, we were surprised by this finding because the clottability of the variants was lower than normal, particularly for γ364H and γ364V. If less protein was incorporated into the clots, then we would expect a lower final turbidity and thinner fibers. That is, we equate 50% clottable variant fibrin with half the concentration of 100% clottable normal fibrinogen; at half the normal fibrinogen concentration, the kinetic model predicts lower final turbidity and clots with thinner fibers [17]. This inconsistency suggests that the variants polymerize by a different kinetic path from normal fibrinogen. A different pathway is also consistent with the altered calcium modulation seen with the variants.

We found thrombin-catalyzed polymerization was inhibited by the peptide that mimics the knob "B", GHRP, indicating B:b interactions are required for polymerization of the variant fibrinogens. The knob "A" mimic, GPRP, also inhibited polymerization, but this result must be considered in light of the known lack of specificity of this peptide. Previous experiments have shown that in the presence of mM calcium GPRP binds not only to holes "a" but also to holes "b" [19], which could account for the inhibition observed here. Further, recent X-ray analysis of fragment D isolated from γ364A showed GPRP was bound in both holes "a" and "b". (SR Bowley *et al.*, unpublished observations). Thus, the observed inhibition was likely due to GPRP binding in holes "b". We also note that the delayed lag times correlate with the release of FpB. Although we did not measure fibrinopeptide release for extended periods, the data presented show a substantial fraction of FpB release occurred prior to the end of the lag time measured by turbidity. This suggests knobs "B" have a role in variant fibrin polymerization, through either B:a or B:b interactions. As our reptilase experiments showed A:a interactions are not sufficient to support polymerization, we believe that B:a interactions similarly would not support polymerization and thus, we conclude that B:b interactions are an essential aspect of the polymerization of these variant fibrinogens.

Our interpretation of these experiments is summarized in Figure 7. With normal fibrinogen FpA is released immediately upon addition of thrombin, leading to the rapid formation of protofibrils through A:a interactions. Protofibrils are the substrate FXIIIa-catalyzed formation of γ-γ dimers and for thrombin-catalyzed release of FpB. Subsequently, protofibrils associate into the branched fibers of a normal clot. With the variant fibrinogens FpA is also released immediately upon addition of thrombin, but because A:a interactions are missing or unstable, protofibrils do not form. Consequently, thrombin-catalyzed release of FpB occurs slowly. Protofibrils then form through either B:b interactions alone (γ364H and γ364V) or B:b interactions with weakened A:a interactions (γ364A). These unusual protofibrils are substrates for FXIIIa, so γ-γ dimers are formed, but only after FpB release. The unusual protofibrils aggregate slowly into clots with thicker than normal fibers and larger than normal pores.

Our conclusion, that B:b interactions mediate protofibril formation in variants with incompetent holes "a", suggests that B:b interactions may participate in normal protofibril formation. Recent experiments using surface plasmon resonance (SPR) are consistent with this suggestion. The SPR data showed both A:a and B:b interactions participate in binding the central nodule of fibrin monomer (desAB-NDSK) to fibrinogen (CB Geer et al., unpublished observation). These data showed B:b interactions can occur alongside A:a interactions, as between strands within a protofibril. Considering our data together with the SPR results, we propose a model of polymerization where B:b interactions participate in normal protofibril formation. A similar model was first proposed more than 20 years ago by John Weisel. Based on his electron microscopy data, Weisel concluded that both A:a and B:b interactions support protofibril formation [20]. This earlier model differs from our model in the location of the holes "b", which we place in the βC nodule identified in crystallographic studies. Nevertheless, both models assert that B:b interactions occur within protofibrils and promote later aggregation by enhancing the formation of protofibrils rather than by direct interactions between protofibrils.

Acknowledgements
We gratefully acknowledge Dr. Oleg V. Gorkun (Department of Pathology and Laboratory Medicine, University of North Carolina at Chapel Hill) for his generous help with our analysis of the fibrinopeptide release data and the preparation of Figure 2. We also thank Carri Geer

(Department of Chemistry, University of North Carolina at Chapel Hill) for her thoughtful review of the manuscript. This work was supported in part by the Hokuto Foundation for Bioscience (NO; 2006).

Contribution of authors

N Okumura, K Yamauchi, H Ota: made the design of the study.
N Okumura, F Terasawa, A Haneishi, N Fujihara, M Hirota-Kawadobora: performed experiments.
N Okumura, F Terasawa, A Haneishi: made figures.
N Okumura, ST Lord: wrote the manuscript.

Disclosure of Conflict of Interests

None of the authors had any conflicts of interest with regards to this work.

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Table 1. Thrombin- or reptilase-catalyzed polymerization of normal and variant fibrinogens.

	Calcium concentration (mM)			
	0	0.1	1.0	10
Thrombin-catalyzed polymerization				
Normal fibrinogen				
lag period (min)	3.5 ± 0.2†	2.5 ± 0.1	2.4 ± 0.1	3.1 ± 0.2‡
maximum slope ($\times 10^{-5}$/sec)	321 ± 17‡	407 ± 23	397 ± 21	314 ± 15‡
γ364A				
lag period (min)	55 ± 3†	38 ± 3	41 ± 3	78 ± 5*
maximum slope ($\times 10^{-5}$/sec)	20 ± 3	32 ± 2¶	26 ± 2	15 ± 2†
γ364H				
lag period (min)	176 ± 11	185 ± 12	208 ± 15	390 ± 17*
maximum slope ($\times 10^{-5}$/sec)	4.5 ± 0.7	5.4 ± 0.8	3.7 ± 0.6	1.9 ± 0.3‡
γ364V				
lag period (min)	142 ± 10	142 ± 12	169 ± 13	352 ± 18*
maximum slope ($\times 10^{-5}$/sec)	7.0 ± 0.9	7.3 ± 0.8	5.8 ± 0.7	2.2 ± 0.4†
Reptilase-catalyzed polymerization				
Normal fibrinogen				
lag period (min)	3.6 ± 0.2*	2.8 ± 0.2‡	2.1 ± 0.1	2.5± 0.2
maximum slope ($\times 10^{-5}$/sec)	298 ± 18†	355 ± 25	397 ± 22	309 ± 20‡

Polymerization of fibrinogen (0.45 mg/ml) was initiated with thrombin (0.05 U/ml) or reptilase (0.05 U/ml). Reactions, at 37 °C in HBS with the indicated calcium concentrations , were followed by turbidity at 350nm. Values for lag period and maximum slope were determined as described in methods. * $p<.001$; † $p<.01$; ‡ $p<.02$; ¶$p<.05$ significantly different from 1 mM calcium for each fibrinogen.

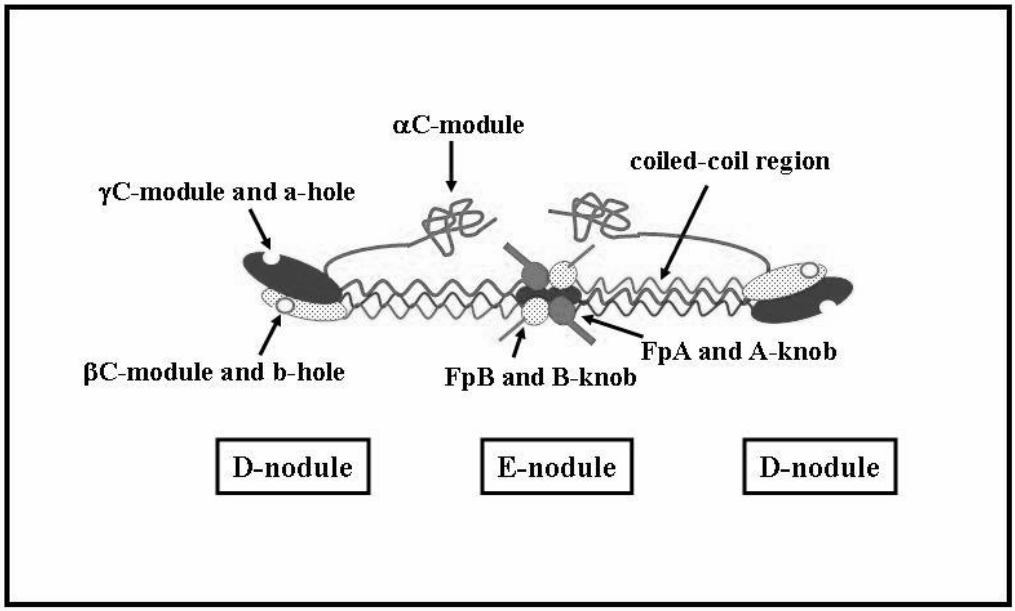


Figure 1. Schematic structure of fibrinogen. Thrombin removes fibrinopeptides A (FpA) and B (FpB) from the central E nodule of fibrinogen, exposing knob "A" and "B", respectively, of fibrin. These knobs fit into holes "a" and "b", which are located in the γC- and βC-modules, respectively.

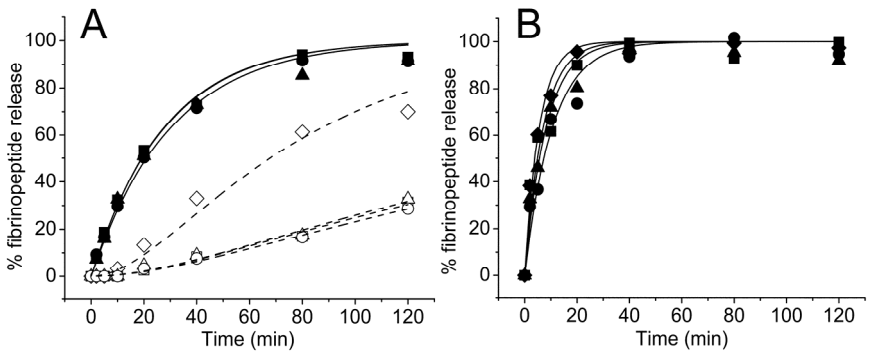


Figure 2. Thrombin- and reptilase-catalyzed fibrinopeptide release. Representative fibrinopeptide release curves with Panel A: thrombin (0.01 U/ml) and Panel B: reptilase (0.01 U/ml) and normal (◆,◇), γ364A- (■, □), γ364H- (▲, Δ), and γ364V- (●, ○) fibrinogens (0.15 mg/ml). FpA data were fit to a first-order rate equation (solid symbols and solid lines); FpB data were fit to a first order equation assuming FpB release follows FpA release (open symbols and broken lines). Reactions were performed at ambient temperature in HBS with 1 mM $CaCl_2$ as described in Materials and Methods.

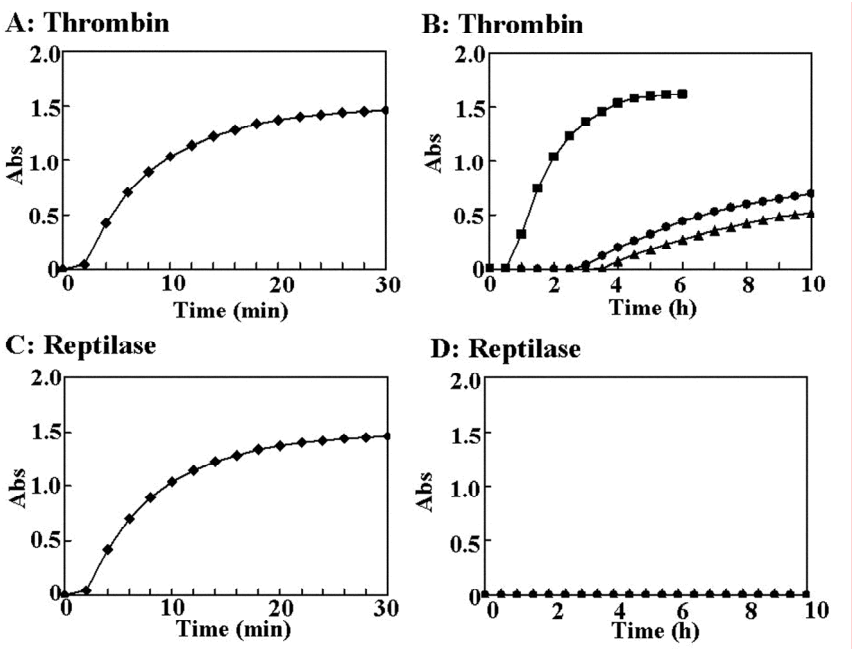


Figure 3. Thrombin- or reptilase-catalyzed polymerization. Polymerization of fibrinogen (0.45 mg/ml) was initiated with thrombin (0.05 U/ml) (Panels A and B) or reptilase (0.05 U/ml) (Panel C and D) in the presence of 1 mM Ca ion. Representative turbidity curves from triplicate experiment are shown for normal (♦) fibrinogen (Panels A and C), and for γ364A (■), γ364H (▲), and γ364V (●) fibrinogens (Panels B and D). Note the time scale is in min for panels A and C and in hours for panels B and D.

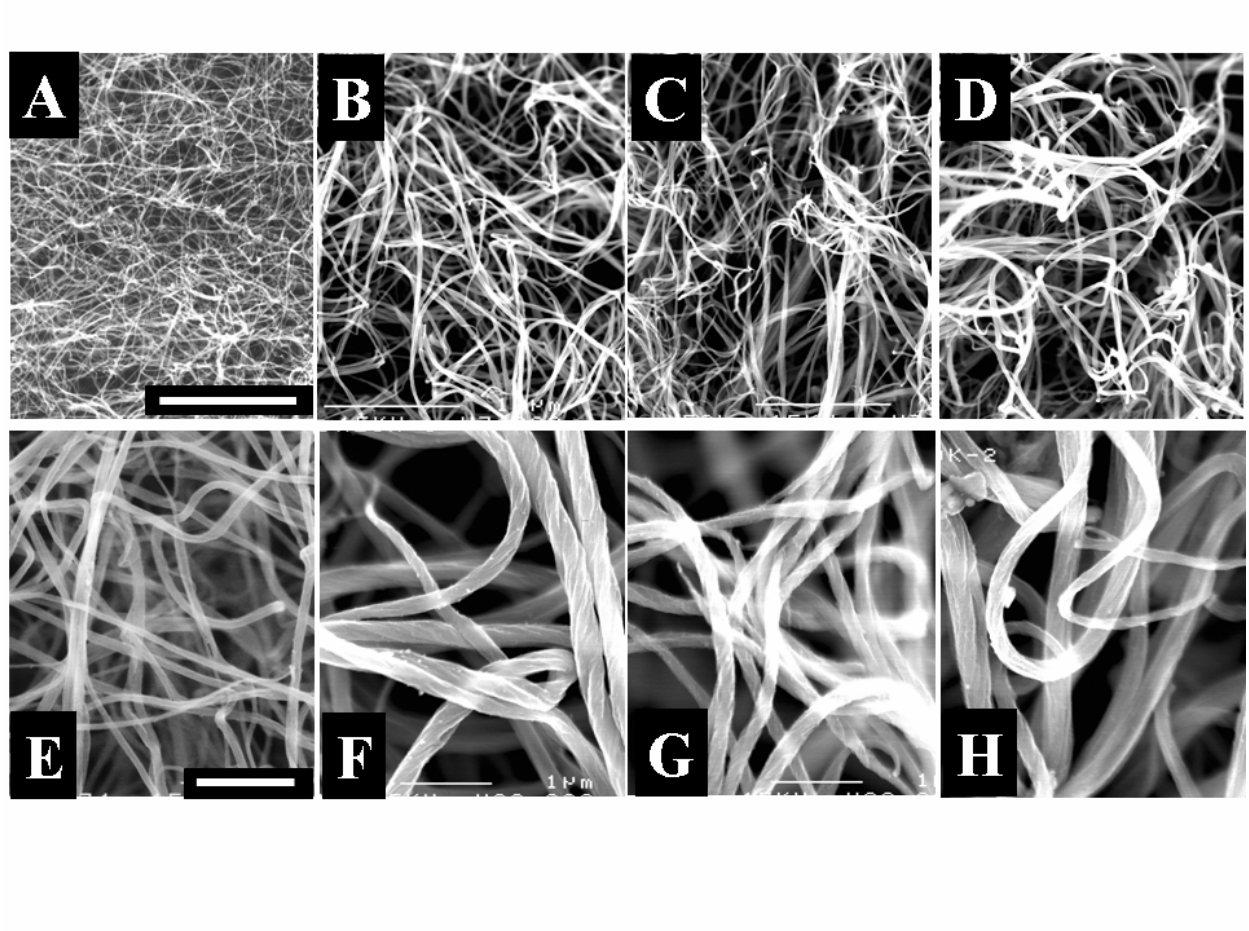
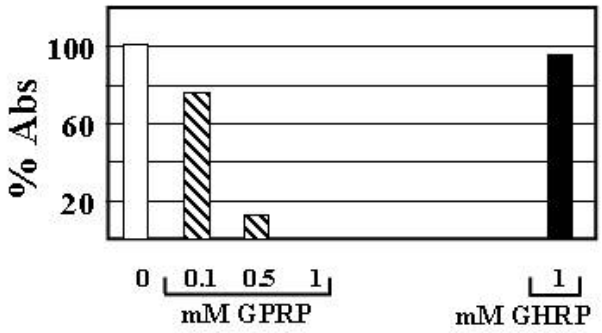
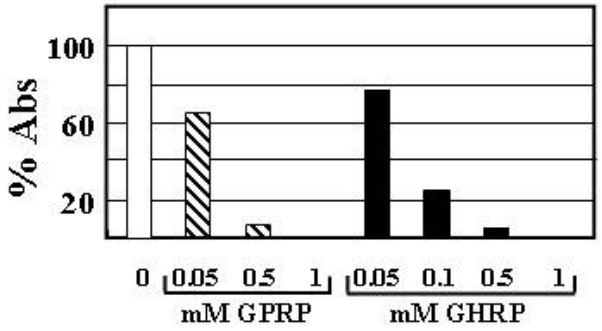


Figure 4. Scanning electron microscopy of fibrin clots. Fibrinogen (0.4 mg/ml) was incubated with thrombin (0.1 U/mL) for 24 h at 37 °C in HBS with 1 mM $CaCl_2$. Images were recorded at 3000× (A-D) or 20000× magnification (E-H). Photomicrographs of fibrin clots made from normal (A, E), γ364A- (B, F), γ364H- (C, G), and γ364V- (D, H) fibrinogen. The thick white bar represents 10 μm in A and 1.0 μm in E.

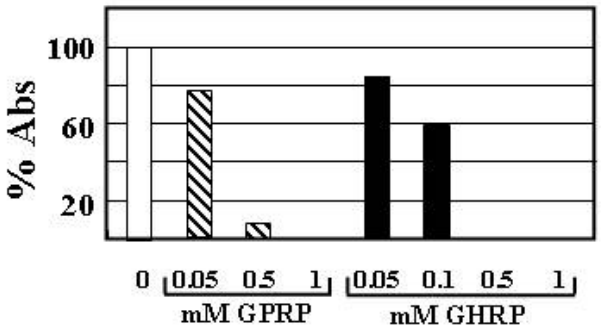
A: normal fibrinogen



B: γ364A fibrinogen



C: γ364H fibrinogen



D: γ364V fibrinogen

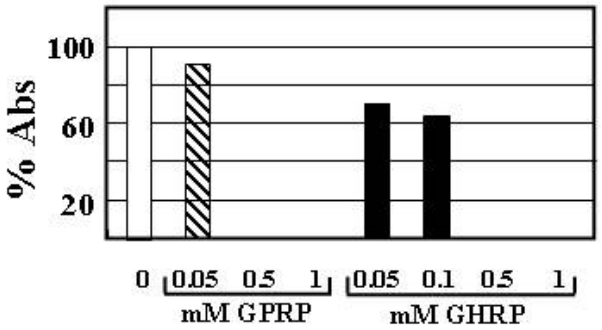


Figure 5. Inhibition of fibrin polymerization by GPRP or GHRP. Thrombin-catalyzed polymerization of fibrinogen (0.45 mg/ml = 1.32 μM) was performed in the absence or presence of GPRP (0.05 -1 mM) or GHRP (0.05 -1 mM), as described in Materials and Methods. The per cent absorbance = [absorbance with peptide/absorbance without peptide] x 100% was determined at 30 min for normal (A), 3 h for γ364A- (B), and 10 h for γ364H- (C), and γ364V-fibrinogen (D). Open bars: without peptide, slashed bars: with GPRP, and black bars: with GHRP.

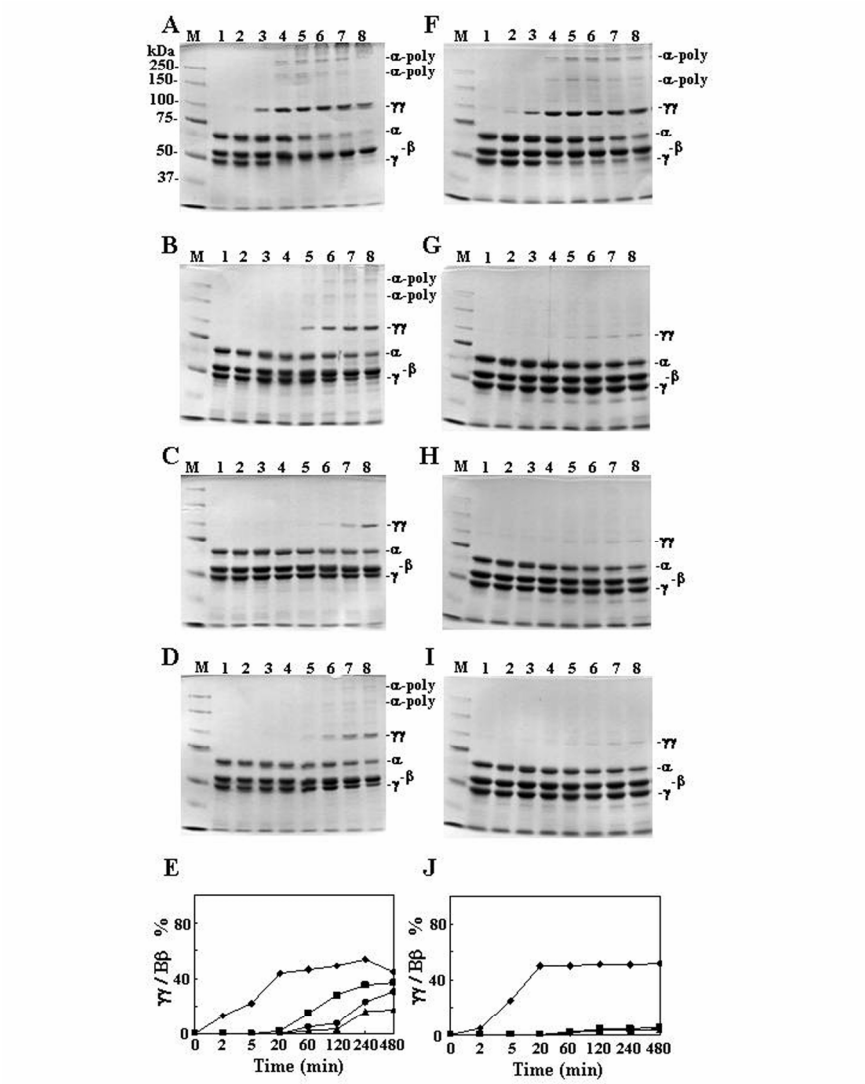


Figure 6. FXIIIa-catalyzed cross-linking of fibrin. Cross-linking by FXIIIa was examined by 8% SDS-PAGE under reducing conditions. Fibrinogen was incubated with FXIIIa and thrombin (Panel A-D) or with FXIIIa and reptilase (Panels F-I), as described in Materials and Methods. The fibrin chains (α, β, γ, cross-linked γ-γ dimer, and cross-linked α-chain polymers) are indicated on the right side of the gels, and molecular weight markers in kDa are on the left side of panel A. Gels with normal fibrinogen are shown in panels A and F; with γ364A in panels B and G; with γ364H in panels C and H; with γ364V in panels D and I. Samples were incubated for 0 min (lane 1), 2 min (lane 2), 5 min (lane 3), 20 mine (lane 4), 1 hr

(lane 5), 2h (lane 6), 4 h (lane 7) and 8 h (lane 8). The ratios of γ-γ dimer/Bβ were determined by densitometry and plotted in panel E for thrombin and panel J for reptilase; normal (♦), γ364A (■), γ364H (▲), and γ364V (●).

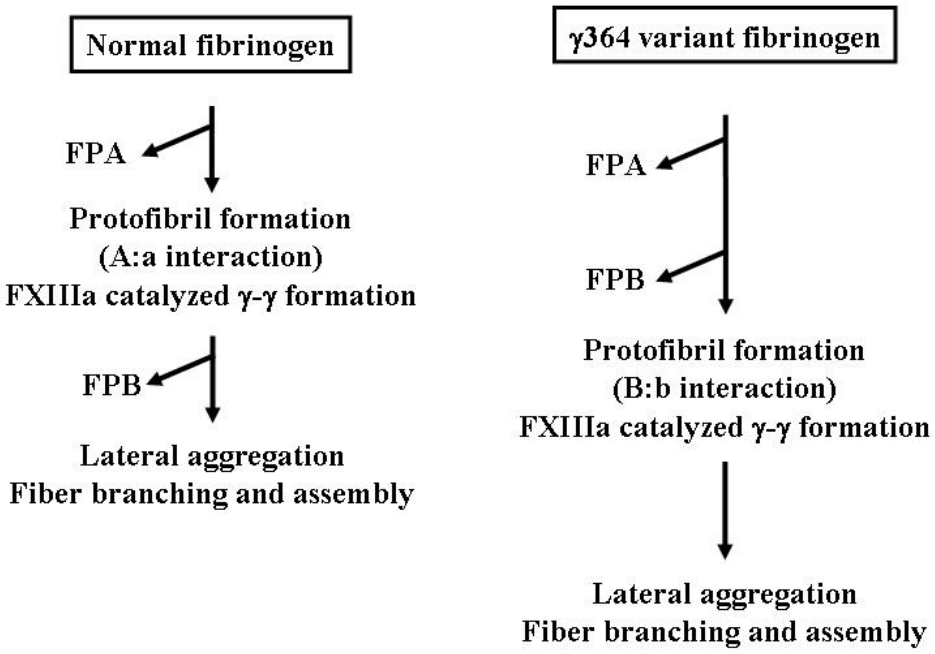


Figure 7: Schematic comparing polymerization of normal (left) and γ364 variant (right) fibrinogens. Under conditions where the time for normal polymerization was minutes, the time for variant polymerization was hours. The details are described in the text.